FUNCTIONAL CHARACTERIZATION OF THE T CELLS AND ANTIGEN PRESENTING CELLS OF *SALMONELLA*-INFECTED MICE

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ABSTRACT

This work was carried out to further characterize the cell-mediated immune response to *Salmonella* infection in mice, by comparing the antigen (Ag) presenting cell (APC) activity of peritoneal cells (PCs) obtained from infected mice (IPCs) with that of PCs from normal mice (NPCs) and analysing the T cell subsets induced by primary and secondary *Salmonella* infections.

To evaluate the effect of intraperitoneal (ip) immunization on the APC activity, IPCs harvested from mice 1-3 days after ip injection of 10^5 *Salmonella enteritidis* 11RX (11RX; D 1-3 IPCs) and NPCs were used to stimulate primary allogeneic responses and secondary *Salmonella*-specific responses *in vitro*. Intraperitoneal immunization with live 11RX (L11RX) clearly had dramatic effects on the PC population, increasing the PC yields and altering the cell profiles, mainly due to a large influx of inflammatory neutrophils. A distinct change in the appearance of macrophages was also noted. A population of "inhibitory" adherent cells were detected when large numbers of D 3 IPCs were used as APCs, which prevented the stimulation of both the allo-Ag and *Salmonella* Ag-specific T cell responses. This inhibitory effect did not appear to be due to the release of prostaglandins, as indomethacin was routinely added to all cultures, nor to the presence of large numbers of neutrophils. However, the inhibition of proliferation of L11RX-primed T cells could be, at least partially, explained by an increase in the degradative capacity of the macrophages and the neutrophils in the D 3 IPC suspension. This was suggested from experiments using large amounts of various Ags in culture, as well as *in vivo* and *in vitro* Ag-pulsed APCs and treatment of Ag-pulsed APCs with paraformaldehyde. Cytospin smears of the *in vivo* Ag-pulsed APCs, showed that *in vivo* Ag-pulsed NPCs had more cells containing bacteria, with more bacteria per cell than *in vivo* Ag-pulsed D 3 IPCs. Interestingly, *in vivo* pulsing with Ag also ensured that the larger numbers of D 3 IPCs, which were normally inhibitory became stimulatory in the allogeneic system, further emphasizing the fact that

the inhibitory effect was not *Salmonella*-specific and only partially due to increased degradation of Ags.

Using NPCs or D 3 IPCs as the APC source, only L3T4$^+$ T cells in L11RX-primed T cell populations were induced to proliferate in response to Ags plus APCs or to *in vivo* and *in vitro* Ag-pulsed APCs. Similarly, NPCs and D 3 IPCs induced the same pattern of proliferation in response to allo-Ags with most of the proliferation being due to Lyt2.2$^+$ T cells, although some proliferation of L3T4$^+$ T cells was also detected. In addition, both APC populations induced considerable numbers of allo-Ag-specific Lyt2.2$^+$ cytotoxic T cells (CTLs). Therefore, other than the obvious phenotypic changes and the appearance of "inhibitory" adherent cells, ip infection with L11RX did not modify the APC activity of the PCs, as assessed by the magnitude of the T cell responses and the T cell subset(s) they induced.

A comparison was also made of the ability of primed T cells obtained from mice immunized with L11RX and mice which were given a secondary ip challenge of L11RX or live *Salmonella typhimurium* C5 (LC5) to proliferate and release IL 2 *in vitro*, to mediate cytotoxicity *in vitro* and to transfer DTH reactivity to *Salmonella* Ags to normal mice. Secondary immunization with either L11RX or LC5 did not alter the ability of PCs or spleen cells (SCs) to release IL 2 or proliferate in response to formalin killed 11RX (F11RX). Even though the magnitude of the proliferative responses and the amounts of IL 2 released by all T cells obtained from the peritoneal cavities were similar, most of the proliferative response of cells obtained from mice given only a primary or both a primary and a secondary ip challenge of L11RX was due to L3T4$^+$ T cells, whilst both L3T4$^+$ and Lyt2.2$^+$ T cells proliferated in suspensions obtained from mice receiving a secondary challenge of LC5. IL 2 production by T cells obtained from the spleens of primary and secondary immunized mice was not detected and proliferative responses of all these populations to F11RX were very inconsistent. However, when splenic T cells from secondary infected mice did respond, both the L3T4$^+$ and Lyt2.2$^+$ T cell subsets were induced to proliferate.

The ability of T cells obtained from the peritoneal cavities or spleens of

primary and secondary immunized mice to mediate DTH reactivity to a variety of *Salmonella* Ags was very similar and involved L3T4$^+$ effector T cells. Lyt2.2$^+$ DTH effector T cells were detected when P815 cells which had been infected with LC5 were used as the Ag source to stimulate T cells from the peritoneal cavities of mice immunized with L11RX (but not early after infection) and T cells obtained from the peritoneal cavities and spleens of mice given a secondary challenge of LC5.

Because suitable Ag-pulsed targets were not available until late in this study, lectin-mediated cytotoxicity assays were used to detect cytotoxic activity induced by immunizing with L11RX. This analysis revealed that the peak of lectin-mediated cytotoxic activity of the T cells present in the peritoneal cavity occurred by the fifth day of infection with L11RX and was mediated by Lyt2.2$^+$ T cells. Secondary infection with either L11RX or LC5 resulted in a longer lasting increase in the cytotoxicity to levels which were comparable to that of the IPCs obtained from mice infected with L11RX 5 days earlier. P815 cells infected with L11RX were used to detect *Salmonella*-specific CTLs and it was demonstrated that *Salmonella*-specific cytotoxic activity accounted for up to 50% of the cytotoxic activity detected by the lectin-mediated assay. On the contrary, T cells obtained from the spleens of primary and secondary immunized mice contained no detectable cytotoxic activity, unless they were cultured *in vitro*. ConA cultured SCs (from normal and immunized mice) contained Lyt2.2$^+$ CTLs with comparable levels of lectin-mediated cytotoxicity, however *Salmonella*-specific Lyt2.2$^+$ CTLs (accounting for approximately 50% of the lectin-mediated activity) were only present in the ISC population obtained from mice with a secondary infection of LC5.

In conclusion, it was established that *Salmonella* infection induced Ag-specific L3T4$^+$ T cells and Lyt2.2$^+$ T cells in the peritoneal cavity and spleen, with the activity of the Lyt2.2$^+$ T cell subpopulation being increased during secondary *Salmonella* infections. However, ip immunization with L11RX did not induce any detectable change in the APC function which could account for the induction of particular T cell subsets and/or for the induction a cell-mediated immune response.

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This thesis contains no material which has been accepted for the award of any other degree in any University and to the best of my knowledge and belief, this thesis contains no material previously published by another person, accept where due reference is made in the text. I consent to this thesis being made available for photocopying and loan, if accepted for the award of the degree.

Melissa J. Pope

LIST OF ABBREVIATIONS

"L11RX P815" : L11RX invaded P815 cells

"LC5 P815" : LC5 invaded P815 cells

[^{3}H]-TdR : tritiated thymidine

11RX : *Salmonella enteritidis* 11RX

11RX flag : 11RX flagellin

2° : secondary

α : anti (when describing Abs, eg. α-Thy1.2 : anti-Thy1.2)

Ab : antibody

Abs : antibodies

Ag(s) : antigen(s)

allo-Ag(s) : allogeneic antigen(s)

APC(s) : antigen presenting cell(s)

BCG : Bacillus Calmette-Guérin

BSA : bovine serum albumin

C : complement

C5 : *Salmonella typhimurium* C5

cDNA : complementary DNA

CMI : cell-mediated immunity/immune

ConA : concanavalin A

conc^n : concentration

cpm : counts per minute

CSIF : cytokine synthesis inhibitory factor

CTL(s) : cytotoxic T cell(s)

D 1 IPC(s) : day 1 immune peritoneal cell(s)

D 14 IPC(s) : day 14 immune peritoneal cell(s)

D 14 ISC(s) : day 14 immune spleen cell(s)

D 2 IPC(s) : day 2 immune peritoneal cell(s)

D 21 ISC(s) : day 21 immune spleen cell(s)

D 3 IPC(s) : day 3 immune peritoneal cell(s)

D 56 IPC(s) : day 56 immune peritoneal cell(s)

DAG : diacylglycerol

DC(s) : dendritic cell(s)

dil^n : dilution

DNA : deoxyribonucleic acid

DTH : delayed type hypersensitivity

ER : endoplasmic reticulum

F1 : (BALB/c x C57BL/6) F1

F11RX : formalin killed 11RX

FCS : foetal calf serum

FITC : fluorescein isothiocynate

GM CSF : granulocyte-macrophage colony stimulating factor

HBSS : hanks' balanced salt solution

HLA : human leukocyte antigen

IBP(s) : intracellular bacterial parasite(s)

ICAM-1 : intercellular adhesion molecule-1

ICAM-2 : intercellular adhesion molecule-2

IFN-γ : interferon-γ

Ig : immunoglobulin

IL 1 : interleukin 1

IL 10 : interleukin 10

IL 2 : interleukin 2

IL 3 : interleukin 3

IL 4 : interleukin 4

IL 5 : interleukin 5

IL 6 : interleukin 6

IP_3 : 1, 4, 5-triphosphate

IPC(s) : immune peritoneal cell(s)

ISC(s) : immune spleen cell(s)

kDa : kilodalton

L11RX : live 11RX

L11RX 2° IPCs : L11RX secondary immune peritoneal cells

L11RX 2° ISCs : L11RX secondary immune spleen cells

L11RX 2° NW IPCs : L11RX secondary nylon wool fractionated immune peritoneal cells

L11RX 2° NW ISCs : L11RX secondary nylon wool fractionated immune spleen cells

LAK(s) : lymphokine activated killer cell(s)

LC5 : live C5

LC5 2° IPCs : LC5 secondary immune peritoneal cells

LC5 2° ISCs : LC5 secondary immune spleen cells

LC5 2° NW IPCs : LC5 secondary nylon wool fractionated immune peritoneal cells

LC5 2° NW ISCs : LC5 secondary nylon wool fractionated immune spleen cells

LFA-1 : Leukocyte Functional Antigen-1

LFA-3 : Leukocyte Functional Antigen-3

LK(s) : lymphokines

Lm : *Listeria monocytogenes*

LPS : lipopolysaccharide

LT : lymphotoxin

M. bovis : *Mycabacterium bovis*

M. leprae : *Mycobacterium leprae*

M. lepraemurium : *Mycobacterium lepraemurium*

M. microti : *Mycobacterium microti*

M. tuberculosis : *Mycobacterium tuberculosis*

MAF : macrophage activating factor

MHC : major histocompatibility complex

MIF : macrophage inhibitory factor

MLNC(s) : mesenteric lymph node cell(s)

MLR : mixed leukocyte reaction

MoAbs : monoclonal antibodies

mRNA : messenger RNA

NK(s) : natural killer cell(s)

No. : number

NPC(s) : normal peritoneal cell(s)

NRS : normal rabbit serum

NSC(s) : normal spleen cell(s)

NW : nylon wool

NW IPC(s) : nylon wool fractionated immune peritoneal cell(s)

NW ISC(s) : nylon wool fractionated immune spleen cell(s)

OD : optical density

OVA : ovalbumin

P IPC(s) : plastic nonadherent immune peritoneal cell(s)

P ISC(s) : plastic nonadherent immune spleen cell(s)

P/B/A : PBS, BSA, Azide solution containing 10% NRS

PBLs : peripheral blood lymphocytes

PBS : phosphate buffered saline

PC(s) : peritoneal cell(s)

PFA : paraformaldehyde

PIP_2 : phosphatidyl inositol 4, 5-biphosphate

PKC : protein kinase C

PPD : protein purified derivative

^{r} : resistant

RNA : ribonucleic acid

rpm : revolutions per minute

^{s} : sensitive

S. enteritidis : *Salmonella enteritidis*

S. typhimurium : *Salmonella typhimurium*

S11RX : soluble 11RX antigen

SA5 : *Salmonella enteritidis* SA5

SA9 : *Salmonella enteritidis* SA9

SC(s) : spleen cell(s)

SCAF : suppressor cell activating factor

SDS : sodium dodecyl sulphate

sem : standard error of the mean

SHAM : sheep anti-mouse immunoglobulin

SHAM-FITC : FITC-conjugated SHAM

TCR : T cell receptor

TNF : tumour necrosis factor

Tris : Tris (hydroxymethyl) aminomethane

U IPC(s) : unfractionated immune peritoneal cell(s)

U ISC(s) : unfractionated immune spleen cell(s)

UV : ultraviolet

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CHAPTER 1

CELLULAR REQUIREMENTS FOR INDUCTION OF T CELLS INVOLVED IN IMMUNITY TO INTRACELLULAR BACTERIAL PARASITES

1.1 Immunity to intracellular bacterial parasites

1.1.1 Introduction

Although it was known for for quite some time before the 1940's that antibodies (Abs) did not provide as effective immunity to intracellular bacterial parasites (IBPs) as to extracellular bacterial parasites, it was only in 1942 that Lurie reported the importance of cell-mediated immunity (CMI) in resistance to the IBP *Mycobacterium tuberculosis* (*M. tuberculosis*). Over many years numerous groups have studied the immune responses to infection with various IBPs and the involvement of CMI in protection against *Listeria* and *Salmonella*, has also been established (Mackaness, 1962; Collins, 1969a and 1969b). As with *Mycobacteria* (Lurie, 1942), it was established that resistance to *Listeria* infection could be transferred with lymphoid cells but not with serum, indicating the role of CMI (Mackaness, 1971). However, the importance of the humoral immune response appears to vary between IBPs.

1.1.2 Humoral immunity to IBPs

As a result of infection with IBPs like *Mycobacteria*, *Listeria* or *Salmonellae*, specific Abs are induced, but they alone cannot provide immunity (Lurie, 1942; Collins, 1969a and 1969b; Mackaness, 1971). The role Abs play in immunity has been investigated and it has been shown that they play a major role in immunity to some, but not all IBPs. Although infections with *Listeria* induce specific Abs, they are not

required for resistance to infection against homologous challenge (Miki and Mackaness, 1964; North, 1975), whereas, expression of CMI to *Salmonellae* may be ineffecient when Abs are limiting (Davies and Kotlarski, 1976). Work done by Cooper *et al.* in 1983 provided a possible explanation. They demonstrated that the binding of *Listeria monocytogenes* (*Lm*) to macrophages *in vitro* and the subsequent killing of the organisms by activated macrophages, did not require Abs.

In contrast, *Salomonella*-specific Abs are required for effective immunity to *Salmonella.* In 1964, Jenkin *et al.* demonstrated that specific Abs had an important role in determining the resistance of mice to *Salmonella typhimurium* C5 (C5). Further investigation revealed that infection of mice with *Salmonella enteritidis* 11RX (11RX) provided immunity to the normally virulent C5 and such immunity required the presence of the specific Abs (Rowley *et al.*, 1968). In 1970, Collins proposed that Ab production in response to immunization with killed *Salmonella* vaccine played a major part in survival following oral challenge with virulent *Salmonellae*, by reducing the size of the inoculum and prolonging dissemination of organisms to the spleen and liver. In addition, Davies and Kotlarski (1976) demonstrated the need for Abs, in conjunction with the CMI response, for the clearance of large doses of C5 in mice. Preimmunization of mice with alcohol killed C5 followed by infection with 11RX resulted in increased ability to clear high challenge doses of C5 ensuring the survival of the mice. Both CMI and Abs were essential for expression of immunity, as mice immunized with killed vaccines or live 11RX alone were as susceptible as unimmunized animals (Collins, 1969a and 1969b; Davies and Kotlarski, 1976).

1.1.3 T cells are necessary for effective immunity to IBPs

In addition to the role(s) played by specific Abs, CMI responses to IBPs were found to be necessary for immunity and actually require the use of live vaccines for induction. Examination of the lymphoid populations capable of transferring resistance to *Listeria* revealed the involvement of T cells (Blanden and Langman, 1972; Lane and Unanue, 1972). Immunity and/or delayed type hypersensitivity (DTH) to listerial

antigens (Ags) were similarly shown to be T cell dependent (North, 1973). Similarly, Davies and Kotlarski (1976) demonstrated that *in vivo* depletion of T cells resulted in decreased clearance of avirulent 11RX and lack of protection against C5, normally provided by immunization with live 11RX. More recently, *in vivo* T cell depletion has been found to decrease the control of mycobacterial growth in mice (Brett and Butler, 1986). Hence, it is obvious that T cells play an important part in immunity to IBPs.

1.2 T cell characteristics

1.2.1 Requirements for T cell activation

Several pieces of evidence suggest that interactions between at least two T cell subsets and between T cells and other accessory cells, such as B cells, macrophages or dendritic cells, are necessary for activation of T cells. In 1976, Doherty *et al.* reported that activation of unprimed T cells requires presentation of Ags in association with major histocompatibility complex (MHC) coded Class I or Class II molecules on the surface of Ag presenting cells (APCs). In addition, numerous workers including Rosenthal and Shevach (1973) and Smith (1980) have shown that stimulation of T cells, expressing the $L3T4^+$ phenotype, to proliferate and release IL 2 requires Ag presented by accessory cells. In 1980, Larsson *et al.* found that the lectin dependent production of a T cell growth factor, which is involved in the maintenance of T cell proliferation, involves two cell types, the $Thy1^+$ T cells and MHC Class II^+ ($I\text{-}A^+$), $Thy1^-$ macrophages. Corley (1982) reported that in the presence of specific Ag the interaction between T cells and macrophages was essential for the release of a soluble factor(s) by the macrophages which promoted the growth of IL 2 producing T cells. Further studies revealed the existence of at least two secondary nonspecific signals, IL 1 and IL 6, necessary for T cell stimulation (Oppenheim *et al.*, 1986; Hurme, 1987; Unanue and Allen, 1987; Kawakami *et al.*, 1989). Similarly, the induction of expression of IL 2 receptors on $L3T4^+$ T cells has been shown to require the interaction between T cells and accessory cells (Malek *et al.*, 1985). Several pieces of evidence have shown different accessory cell

requirements for the induction of T cell proliferation and IL 2 secretion than for the activation of specific T helper cells which provide help for B cells (Ramila and Erb, 1983; Ramila *et al.*, 1983; Erb *et al.*, 1985; Shigeta *et al.*, 1986). The T cell-accessory cell contact required for T cell activation is facilitated by adhesion between a variety of cell surface molecules on the T cells and their respective ligand on the accessory cell surface (reviewed by Springer *et al.*, 1987).

The Ag-specific T cell receptor (TCR) complex on the T cell binds to the Ag-MHC complex, providing the first signal for activation and the secondary nonspecific signal(s) are supplied by the APCs, initiating a cascade of complex intracellular metabolic events ultimately responsible for the activation of genes encoding various cytokines and their receptors which are essential for T cell activation and subsequent clonal expansion (reviewed by Altman *et al.*, 1990). The first signal alone, provided by the binding of Ag-MHC complexes to TCR complexes, is not sufficient for the activation of T cells. A second signal, delivered by IL 1 (and possibly other cytokines like IL 6), is necessary to induce responsiveness to the cytokine IL 2 (Meuer and Meyer zum Büschenfelde, 1986). For T cells to utilise IL 1 they must express IL 1 receptors on their cell surface. In 1987, using human peripheral blood lymphocytes (PBLs), Shirakawa *et al.* demonstrated that IL 1 receptors are expressed in the early stage of T cell activation as the result of monocyte-T cell interactions and requires the accessory function of HLA DR^+ (MHC Class II^+ equivalent) monocytes. Furthermore, in 1989 Weaver *et al.* identified two pathways of T cell-mediated induction of expression of membrane associated IL 1 by Ag presenting macrophages. One pathway was lymphokine (LK)-independent and mediated by cell contact, the other was dependent on a T cell derived LK belonging to the tumour necrosis factor (TNF) family.

The major role of IL 1 appears to be to promote the synthesis and expression of high affinity IL 2 receptors on the T cell surface and the production of IL 2 molecules (Kaye *et al.*, 1984; Meuer and Meyer zum Büschenfelde, 1986). In 1986, Shirakawa *et al.* demonstrated that IL 1 exerts its activity at the level of gene transcription, inducing genes for the IL 2 receptor α chain in appropriate IL 1 responsive

cell types. Furthermore, Hagiwara *et al.* (1987) found that IL 1 upregulated mRNA levels of LKs, such as IL 2 and other molecules associated with T cell activation in a T cell lymphoma, supporting the theory that this cytokine may behave as a co-stimulator of T cell activation. Production of the IL 2 receptor α chain is required to form high affinity IL 2 receptors by association with the constitiutively expressed β chain and expression of the IL 2 receptor α chain is used routinely as a marker of T cell activation (reviewed by Greene *et al.*, 1989). A complex mechanism of action of IL 1 has been postulated possibly involving several secondary messengers required for induction of specific gene transcription and maybe other intracellular activities (reviewed by Mizel, 1990; O'Neill *et al.*, 1990). Furthermore, in 1990 Fischer *et al.* demonstrated that the optimal production of interferon-γ (IFN-γ) by T cells is dependent on the "second signal" monokine, IL 1, but not on IL 6.

To summarize, considerable evidence exists for the involvement of IL 1 in T cell activation, although there are some exceptions. It now appears that both IL 1 and IL 6 (also produced by APCs) may be required to provide the "second signal" essential for T cell activation (reviewed by Wong and Clark, 1988). IL 6 has been reported to act in synergy with IL 1 (Ceuppens *et al.*, 1988; Holsti and Raulet, 1989; Kawakami *et al.*, 1989; Mizutani *et al.*, 1989) and IL 2 (Garman *et al.*, 1987; Tosato and Pike, 1988) in the mitogen induced proliferation of T cells. Furthermore, Houssiau *et al.* (1989) proposed that IL 6 induces T cells to pass from the G_0 to the G_1 phase of the cell cycle, rendering them more sensitive to the action of IL 1 and resulting in IL 2 production and T cell proliferation.

1.2.2 T cell surface molecules involved in T cell activation

1.2.2.1 T cell receptors (TCRs)

TCRs exist in two forms, consisting of heterodimeric complexes of either α and β chains (TCR αβ) or γ and δ chains (TCR γδ) (Marrack and Kappler, 1986; Allison and Lanier, 1987; Davis and Bjorkman, 1988). The majority of T cells express

TCR αβ and either CD4 or CD8 molecules, whilst the TCR γδ are found predominantly on $CD4^-$, $CD8^-$ T cells. TCR chains are encoded by four genes α, β, γ and δ, located at three loci, α/δ, β and γ (reviewed by Allison and Lanier, 1987), and are rearranged using a mechanism similar to immunoglobulin (Ig) gene rearrangement (eg. Hedrick *et al.* 1984a and 1984b; Yanagi *et al.*, 1984). Structurally, TCR αβ and TCR γδ are very similar, consisting of two extracellular Ig-like domains, each containing Ig-like variable and constant regions. A short connecting peptide region, important in the formation of the heterodimer, separates the constant region from the lipid bilayer, joining it to the transmembrane domain and the cytoplasmic tail (reviewed by Allison and Lanier, 1987). Particular residues in the transmembrane region of the α and β chains are highly conserved and are believed to have a functional role, perhaps interacting with similarly conserved residues in the transmembrane regions of the peptide chains of the CD3 complex. The TCRs are noncovalently associated with the CD3 complex (Samelson *et al.*, 1985; Oettgen *et al.*, 1986), which together form the TCR complexes required for recognition of Ags. TCR αβ complexes bind antigenic peptides presented in association with either Class I or Class II MHC molecules, as demonstrated by the finding that transfection of T cell lines with the TCR α and β chain genes conferred Ag specificity (Dembic *et al.*, 1986; Saito *et al.*, 1987). Much less is known about the antigenic determinants bound by TCR γδ complexes (Allison and Havran, 1991).

1.2.2.2 CD3 complexes

CD3 complexes are essential for the expression of functional TCR complexes on T cells. In the mouse, the CD3 complex consists of at least five glycoprotein chains, γ, δ, ε, ζ and η (reviewed by Manolios, 1992). The γ, δ and ε chains are structurally related, consisting of an extracellular Ig-like constant domain, a transmembrane region and a cytoplasmic tail. The presence and location of negatively charged residues in the transmembrane region suggest that they play a functional role. It is postulated that these regions of the CD3 complex, in conjunction with the highly conserved basic residues in the TCR transmembrane region are critical for cell surface

expression of functional TCR complexes. It is proposed that the CD3 complex assists the asssembly of the TCR and its transport to the cell membrane and stabilizes the complex via the formation of salt bridges with the lipid bilayer. The other less well characterized ζ and η chains each possess an external, transmembrane and a very long cytoplasmic region. Unlike the short cytoplasmic tails of the TCR, functional roles for the cytoplasmic domains of all CD3 chains have been implied, most probably via interactions with cytoplasmic components involved in signal transduction. Evidence that the cytoplasmic chains of CD3 are involved in signal transduction was provided by the discovery that within minutes of occupancy of the TCR complexes with Ag-MHC complexes or mitogen, phosphorylation of CD3 γ cytoplasmic chain occurs (Cantrell *et al.*, 1987; Friedrich and Gullberg, 1988). Phosphorylation of the γ, and to a lesser extent ε and δ chains, occurs as a result of a reaction catalysed by protein kinase C (PKC), via diacylglycerol (DAG) produced by the phosphatidyl inositol 4, 5-biphosphate (PIP_2) pathway. Phosphorylation of the less well characterized ζ chain results from tyrosine kinase activity, the definite nature of which is still uncertain (Samelson *et al.*, 1986; Patel *et al.*, 1987). Possible candidates for this tyrosine kinase activity have come to light since the discovery of high levels of expression of the $p56^{lck}$ tyrosine kinase, a member of the src family, and its association with the CD4 and CD8 molecules in T cells (Rudd *et al.*, 1988; Viellette *et al.*, 1988) and the association of the CD3 complex with another member of the src family of protein tyrosine kinases, *fyn* (Samelson *et al.*, 1990). This has important implications in TCR complex function as both tyrosine kinases and PKC have been associated with T cell mitogenesis (reviewed by Alexander and Cantrell, 1989).

1.2.2.3 The CD2 molecule

CD2 is a single glycosolated polypeptide chain expressed on the majority of T cells and most natural killer (NK) cells (Howard *et al.*, 1981; Kamoun *et al.*, 1981; van Wauwe *et al.*, 1981; Krensky *et al.*, 1983; Martin *et al.*, 1983). It consists of external, Ig-like transmembrane and cytoplasmic domains (Sewell *et al.*, 1986; Sayre

et al., 1987; Seed and Aruffo, 1987). The natural ligand for CD2 is the Leukocyte Functional Antigen-3 (LFA-3), a glycoprotein present on most nucleated cells (Hünig, 1985; Shaw *et al.*, 1986; Hünig *et al.*, 1987). The strong interaction between CD2 and LFA-3 is postulated to enhance contact between T cells and accessory or target cells. In addition, it is postulated that CD2 magnifies the proliferative signal primarily induced as a result of Ag binding to the TCR complex. At least some of the amplification is achieved by an increase in CD2 surface expression after signalling via the TCR (Springer *et al.*, 1987). Initially, a role for CD2 in T cell activation was indicated by the findings that particular pairs of anti(α)-CD2 monoclonal antibodies (MoAbs) induced T cell proliferation and effector functions (Meuer *et al.*, 1984; Brottier *et al.*, 1985; Yang *et al.*, 1986). T cell activation via this CD2 pathway, like the TCR complex pathway, involves elevated intracellular Ca^{++} levels and production of inositol triphosphates (reviewed by Alcover *et al.*, 1987). Furthermore, Ag independent T cell activation via CD2 interacting with LFA-3 has been reported (Tiefenthaler *et al.*, 1987; Bierer *et al.*, 1988), supporting the theory that this interaction amplifies the preliminary signals delivered as a result of Ag binding. Extensive *in vitro* evidence is available which demonstrates that the Ag independent CD2 pathway and the Ag dependent TCR complex pathway are interrelated (Krensky *et al.*, 1983; Meuer *et al.*, 1984; Brottier *et al.*, 1985; Yang *et al.*, 1986; Breitmeyer *et al.*, 1987; Moretta *et al.*, 1987; Pantaleo *et al.*, 1987; Bierer *et al.*, 1988).

The structure of CD2 and its large cytoplasmic domain favour the possibility that it is involved in signal transduction. In support, CD2 mutant molecules with truncated cytoplasmic domains were expressed in a murine T cell hybridoma and such cells showed variable responsiveness to pairs of α–CD2 MoAbs which normally induced signal transduction (Chang *et al.*, 1989; Moingeon *et al.*, 1989). Hence, the cytoplasmic tail of the CD2 molecules may possess a number of functional zones and when some of these are deleted, a deficiency in signal transduction results. Furthermore, for maximal response to Ag, signal transduction by CD2 is necessary, as demonstrated by the finding that cells possessing the CD2 with a mutated cytoplasmic region were unable to respond to the antigenic stimulus, but could still bind to LFA-3 (Bierer *et al.*, 1989).

1.2.2.4 Leucocyte functional antigen-1 (LFA-1) and intercellular adhesion molecules-1 and -2 (ICAM-1 and -2)

In addition to the adhesive interaction between LFA-3 and CD2, the binding of LFA-1 to ICAM-1/2 may also be important in the initial cell-cell contact required for T cell activation (Dustin *et al.*, 1986; Rothlein *et al.*, 1986). The adhesive interaction between these two molecules can be bidirectional because both LFA-1 and ICAM-1/2 are expressed by T cells and some APC populations (Kurzinger *et al.*, 1981; Krensky *et al.*, 1983; Dustin *et al.*, 1986). The adhesion pathway for LFA-1/ICAM-1/2 interactions is Mg^{++} and temperature dependent. Following TCR mediated signalling it has been proposed that an increase in the avidity of LFA-1 for ICAM-1/2, movement of LFA-1 to the zone of contact between the cells and association of LFA-1 with cytoskeletal components, stabilize the cellular interactions (reviewed by Male *et al.*, 1991).

1.2.2.5 CD4/L3T4 and CD8/Lyt2 molecules

The CD4 molecule is a single 55kDa glycosylated polypeptide chain, comprising an extracellular region which is subdivided into four domains, an hydrophobic transmembrane region and a basic cytoplasmic domain (Littman, 1987). The CD4 molecule is expressed on the main portion of mature T cells not expressing the CD8 molecule. CD8 molecules are structurally different from the CD4 molecules, and are made up of two distinct glycoprotein chains, α and β (Lyt2 and Lyt3 respectively), which usually associate in $\alpha\beta$ complexes. The α and β chains are similarly structured members of the Ig superfamily, having an external variable-like region, an hydrophobic transmembrane region and a highly basic cytoplasmic tail. The external domain is linked to the transmembrane segment via a proline rich region (Littman, 1987). CD4 and CD8 molecules on T cells bind to monomorphic determinants of the Class II (Greenstein *et al.*, 1984; Doyle and Strominger, 1987) and I MHC molecules (Ratnofsky *et al.*, 1987; Norment *et al.*, 1988; Rosenstein *et al.*, 1989), respectively, thereby enhancing the avidity of the T cell-APC interactions.

Several groups have demonstrated the importance of the CD4/8-MHC interaction(s), especially under suboptimal conditions for T cell stimulation, such as when Ag is limiting, or the affinity of the TCR complex for the Ag is low and it is believed that the adhesive interaction between CD4 or CD8 molecules and the respective MHC molecules enhances T cell-APC interactions which may be essential for T cell activation (Biddison *et al.*, 1982; MacDonald *et al.*, 1982; Marrack *et al.*, 1983; Wilde *et al.*, 1983; Moretta *et al.*, 1984; Greenstein *et al.*, 1985; Shimonkevitz *et al.*, 1985). Antigenic stimulation of T cells has been reported to induce phosphorylation of CD4 and CD8 molecules (Acres *et al.*, 1986). It has been postulated that the binding of CD8 or CD4 molecules to the Class I or Class II MHC molecules induces intracellular signals involved in the regulation of signals transduced by the TCR complex (reviewed by Bierer *et al.*, 1989). Another possible regulatory role for CD4 and CD8 molecules, was proposed when it was found that CD4 and CD8 associate with the $p56^{lck}$ tyrosine kinase on the inner aspect of the plasma membrane (Rudd *et al.*, 1988; Viellette *et al.*, 1988). The $p56^{lck}$ is expressed at high levels only in mature resting T cells (Marth *et al.*, 1985) and fifty to eighty percent of $p56^{lck}$ in murine T cells is associated with the CD4 molecules (Veillette *et al.*, 1988). During T cell activation, $p56^{lck}$ is regulated at both the mRNA and protein levels (Marth *et al.*, 1987), suggesting that it may be involved in functions unique to T cells. Furthermore, Rudd *et al.* (1988) and Viellette *et al.* (1988) reported that $p56^{lck}$ was specifically down-modulated by crosslinking of CD4 or CD8 with Abs, and the majority of the $p56^{lck}$ co-immunoprecipitated with CD4 or CD8 molecules. The TCR complexes on T cells interact with antigenic peptides in association with Class I or Class II MHC molecules presented by APC and CD4 or CD8 molecules bind to the invariable parts of the Class II or Class I MHC determinants, therefore bringing the CD4 and CD8 molecules to the immediate vicinity of the TCR-CD3 complexes (Saizawa *et al.*, 1987; Rivas *et al.*, 1988). Recent studies by Chalupny *et al.* (1991) demonstrated that the presence of a functional $p56^{lck}$ binding site on the CD8 molecule is essential for the early signalling events transduced by CD8, including increased $[Ca^{2+}]_i$ and protein tyrosine phosphorylation. Similarly, Glaichenhaus *et al.* (1991) found that T cell

activation occurred only when CD4 was associated with p56^{lck}.

Therefore, p56^{lck} bound to the CD4 or CD8 molecules, is brought closer to the TCR complex favouring the possibility that p56^{lck} is responsible for phosphorylation of the CD3 ζ chain, as proposed by Marth *et al.* (1985) and Voronova and Sefton (1986). This was further supported by the finding that CD4 and CD8 molecules interact with TCR complexes during T cell activation (Kupfer and Singer, 1988), as a consequence of CD8 molecules binding to Class I MHC products and CD4 molecules binding to Class II MHC products (Flomenberg *et al.*, 1983; Fazekas de St. Groth *et al.*, 1986; Aparicio *et al.*, 1987; Jones *et al.*, 1987). Furthermore, crosslinking of CD4 was found to induce p56^{lck} activation and elevated CD3 ζ chain phosphorylation (reviewed by Alexander and Cantrell, 1989). Consequently, Mustelin and Altman (1989) postulated that CD4 and CD8 molecules may have an important role in controlling the phosphorylation of the CD3 ζ chain by preventing p56^{lck} from phosphorylating it in a resting T cell and, conversely, by bringing p56^{lck} to the immediate vicinity of the TCR-CD3 complex and the ζ chain when Ag is presented to the T cell on the appropriate MHC molecule.

1.2.2.6 CD45 molecules

The CD45 group of molecules are single chain glycopeptides also referred to as leucocyte common antigens, and are expressed in a variety of forms (eg. Woollett *et al.*, 1985). The various forms of the CD45 molecules have different molecular weights and possess both common and restricted epitopes (reviewed by Thomas, 1989). MoAbs directed against different forms of CD45 molecules have been identified and have allowed the detection of several different molecular forms called CD45R, CD45RA and CD45RO. Expression of the various isoforms of these molecules is controlled in a cell type-specific manner and a complex pattern of expression has been detected on mature T cells, with at least four different molecular weight forms detected. The form being expressed on a particular T cell is dependent on the subset and differentiation state of the T cell (reviewed by Sanders *et al.*, 1988; Thomas, 1989). A short stretch of amino acids

at the N-terminal end of the mature protein is common to all forms and is encoded by a discrete exon within the gene encoding the CD45 protein. However, generation of the various isoforms occurs via differential splicing of three particular exons on the CD45 gene (reviewed by Male *et al.*, 1991). During T cell activation the pattern of expression of these molecules has been reported to vary.

These molecules have an extracellular segment which is divided into three domains, a transmembrane region and an extremely large globular cytoplasmic domain (reviewed by Thomas, 1989). This group of molecules has been implicated in a variety of functional activities, including a regulatory role. Apart from the size of the cytoplasmic domain of CD45 molecules, suggesting possible interactions with intracellular molecules involved with signal transduction, these molecules possess phosphotyrosine phosphatase activity (Tonks *et al.*, 1988). Ledbetter *et al.* (1988) reported that CD45 modifies signal transduction via the CD2, CD3 and CD4 molecules. That CD45 molecules may have a regulatory role was also inferred from experiments which demonstrated that mutant murine T cell clones lacking CD45 molecules failed to proliferate in response to Ag or cross-linked CD3. Effective proliferative responses were restored in CD45 revertants, emphasizing the important role these molecules may play in assisting activation of T cells in response to antigenic stimuli. Consequently CD45 molecules can have considerable influence over intracellular signalling by altering the phosphorylation state of regulatory proteins (reviewed by Male *et al.*, 1991).

1.2.3 Summary of the mechanism of T cell activation

For activation of T cells metabolically active APCs are required to provide Ag presented in association with Class I and/or II MHC molecules and secondary soluble signals, called LKs or cytokines. This triggers a complex series of metabolic events in the T cells, culminating in initiating both the effector functions of the cell and the proliferation required to expand the specific T cell pool (reviewed by Altman *et al.*, 1990). Not unlike activation pathways in other mammalian cells, the complex series of metabolic events which occur include changes in the concentration of intracellular free

Ca^{++}, pH, protein phosphorylation, gene transcription, mRNA processing and protein synthesis.

The initial T cell-APC contact, via TCR αβ binding to specific antigenic peptide-MHC complexes, is essential for subsequent T cell activation and is enhanced by the interaction of the various molecules on T cells with their respective ligands on the APCs. As stated above, the main contributors increasing the avidity of the cell-cell contact are the LFA-1/ICAM-1, LFA-1/ICAM-2 and the CD2/LFA-3 interactions. The CD4/MHC Class II or CD8/MHC Class I interactions also contribute to the T cell-APC adhesion. Interactions between the CD4 or CD8 molecules and the MHC Class II or Class I molecules may, however, have a more important role in signalling. Further stabilization of the cellular interaction results from the increased avidity of LFA-1 for its ICAM-1 (or ICAM-2) following occupancy of the TCR complex, movement of the LFA-1 molecules to the cell contact zone and association of the LFA-1 with cytoskeletal components, thereby maximizing the chance for T cell triggering. Following these initial cellular interactions a variety of metabolic events occur which ultimately result in the activation of T cell proliferation, resulting in expansion of the cell pool, and induction of T cell effector functions.

The numerous, complex events taking place after occupancy of the TCR complex are not completely understood, but the initial signal is believed to be mediated by the CD3 molecule of the TCR complex (reviewed by Alcover *et al.*, 1987). Adding to the complexity of T cell activation, contributions from the CD2 activation pathway and signals mediated by CD4 or CD8 molecules also occur. The initial signal received by the cell is believed to activate a cascade of hydrolysis, phosphorylation and dephosphorylation events of memebrane bound molecules (Altman *et al.*, 1990). Phospholipase C, known to bind CD3, hydrolyses membrane bound PIP_2 generating secondary molecules, one of which, 1, 4, 5-triphosphate (IP_3), influences the intracellular Ca^{++} levels and the other, DAG, the activation state of PKC. Phosphorylation of the IP_3 provides another molecule involved in regulating membrane Ca^{++} channels, contributing to the increased levels of intracellular Ca^{++}. The importance of elevated internal Ca^{++}

levels in T cell activation have been reported although the specific role is unclear, as is the significance of the efflux of Mg^{++} from the cells.

DAG, which is the second messenger generated by hydrolysis of PIP_2, can also be generated by hydrolysis of other phospholipids and is known to activate PKC. Activation of the Ca^{++} dependent enzyme PKC provides an avenue for modulation of a vast array of proteins via phosphorylation. Synergistic activity between PKC and calcium ionophores, which increase intracellular Ca^{++} levels, have been reported indicating the importance of both PKC and increased Ca^{++} levels in T cell activation. The importance of PKC in T cell activation is supported by the finding that PKC is involved in the induction of expression of genes required for T cell activation, including IL 2 and IL 2 receptor genes. It is proposed that expression of these genes resulting in production of IL 2 and expression of IL 2 receptors is directed by a cascade of PKC mediated phosphorylation signals (Dröge, 1986).

As mentioned previously, it has been postulated that another kinase involved in the phosphorylation events occurring during T cell activation is the T cell specific $p56^{lck}$ tyrosine kinase. On interaction of TCR complexes with Ag-MHC complexes residues on the cytoplasmic tails of several cell surface molecules become phosphorylated, including CD3, CD2, CD4, CD8 and CD45. Phosphorylation of all these molecules occurs as a result of PKC activity, which may be involved in regulating the surface expression of these molecules (Cantrell *et al.*, 1985; Krangel, 1987). However, phosphorylation of a tyrosine residue on one of the CD3 chains is carried out by a tyrosine kinase, possibly the $p56^{lck}$ tyrosine kinase. This was supported by the finding that $p56^{lck}$ associates with CD4 and CD8 molecules (Rudd *et al.*, 1988; Viellette *et al.*, 1988), which are known to bind to MHC molecules, to which the TCR complex is bound, thereby bringing the tyrosine kinase in closer proximity to the CD3 molecule. Further implications for the role of the $p56^{lck}$ in T cell activation came from the findings that activation of human T cells to produce IL 2 correlated with alterations in the amount of $p56^{lck}$ mRNA and protein in the cells (Marth *et al.*, 1987).

Additional enzymic activity is provided by the CD45 molecule, which

possesses phosphotyrosine phosphatase activity within its large cytoplasmic tail region (Tonks *et al.*, 1988). A possible role for this phosphatase in the dephosphorylation and subsequent activation of the $p56^{lck}$ tyrosine kinase was postulated when CD45 mutants showed increased phosphorylation of $p56^{lck}$. At this level there appears to be little evidence to suggest that there are different signals for the different T cell subsets, since CD45 molecules are present on both subsets (Thomas, 1989) and $p56^{lck}$ can associate with both CD4 and CD8 molecules (Rudd *et al.*, 1988; Veillette *et al.*, 1988). Hence, following interactions of TCR complexes with their ligands, a complex network of enzymatically catalysed metabolic events occur, resulting in phosphorylation of regulatory proteins ultimately influencing the activity of a variety of genes involved in mediating T cell responses, such as genes for cytokines and their receptors crucial to the subsequent activities of the cell.

1.2.4 T cell subsets in CMI

T cells are divided into subsets on the basis of expression of the CD4 and CD8 molecules. In general, $CD4^+$ T cells respond to Ag presented in association with Class II MHC molecules and are described as Class II MHC restricted, whilst $CD8^+$ T cells respond to Ag presented in the context of Class I MHC molecules and are Class I MHC restricted (Swain, 1983). This division was initially believed to also represent a functional grouping, where $CD4^+$ T cells provided "help", in the form of LKs such as IL 2, for the activation of effector $CD8^+$ T cells and the LKs necessary for proliferation and differentiation of B cells (revieved by Fitch, 1986). However, functional overlap between the subsets has been reported, where both $CD4^+$ and $CD8^+$ T cell subsets possess cytotoxic activity and the ability to proliferate and release IL 2 *in vitro* (MacDonald *et al.*, 1980; Andrus, *et al.*, 1981; Tite and Janeway, 1984; Heeg *et al.*, 1987a and 1987b). For instance, in 1987 Kaufmann *et al.* reported the detection of *Listeria*-specific Class II MHC restricted $L3T4^+/CD4^+$ cytotoxic T cells (CTLs). Several groups have reported that $CD4^+$ clones also express specific cytolytic effector function upon activation (Lukacher *et al.*, 1985; Tite *et al.*, 1985; Ju *et al.*, 1986 and 1990;

Nakamura *et al.*, 1986). Analyses of the characteristics of virus-specific human CTLs have revealed that both Class I restricted CD8$^+$ CTLs (reviewed by Biddison, 1982) and Class II restricted CD4$^+$ CTLs exist (Jacobson *et al.*, 1984; Kaplan *et al.*, 1984; Yasukawa and Zarling, 1984; Gomard *et al.*, 1986; Torpey *et al.*, 1989). However, the frequency of CTL precursors in the Class II MHC-specific CD4$^+$ T cell population had been reported to be less than that of the Class I MHC-specific CD8$^+$ T cells (MacDonald, *et al.*, 1980; Beretta *et al.*, 1986).

In view of the often reported finding that CD4$^+$ T cells act mainly as helpers to deliver IL 2 to CD8$^+$ precursor T cells (or a series of signals to induce B cells to develop into plasma cells), it was somewhat surprising to find that the precursor frequencies of IL 2 producing CD4$^+$ T cells and CD8$^+$ T cells reactive to mutant Class II and Class I MHC allo-Ag, respectively, were similar (Heeg *et al.*, 1987a and 1987b). Several reports have indicated that Lyt2$^+$/CD8$^+$ T cells could proliferate and release IL 2 *in vitro* in response to mitogenic (Guerne *et al.* 1983; Andrus *et al.*, 1984; Moretta, 1985; Granelli-Piperno *et al.*, 1986) and antigenic (von Boehmer *et al.*, 1984; Mizuochi *et al.*, 1985 and 1986; Sprent and Schaefer, 1985; Sprent *et al.*, 1986; Heeg *et al.*, 1987a) stimulation. In addition, cytotoxic Lyt2$^+$ T cell clones were also reported to be able to release IL 2 in response to Ag (Roopenian *et al.*, 1983; Andrus *et al.*, 1984). These findings suggested that in addition to CD4$^+$ T cell dependent activation of CD8$^+$ T cells (Cantor and Boyse, 1975; Bach *et al.*, 1977; Wagner and Röllinghoff, 1978), CD8$^+$ T cells can potentially function independently of the T cells belonging to the CD4$^+$ subset. Several groups have observed the autonomous activities of CD8$^+$ T cells (eg. von Boehmer *et al.*, 1984; Mizuochi *et al.*, 1985 and 1986; Sprent and Schaefer, 1985 and 1986). In 1985, Sprent and Schaefer demonstrated that when purified, unprimed Lyt2$^+$ T cells were exposed *in vitro* to APCs with H-2 differences in the absence of L3T4$^+$ T cells, high cell-mediated lympholysis and strong mixed lymphocyte reactions were induced. By employing the mutant mouse strains, bm1 and bm12, which differ from the C57BL/6 (B6) strain only at Class I or Class II loci, respectively, they found that B6 Lyt2$^+$ T cells responded selectively to allo-Class I differences (bm1), whereas B6

L3T4$^+$ T cells responded only to the Class II differences (bm12). In the case of the Lyt2$^+$ T cells, inclusion of α-L3T4 MoAb in the cultures did not reduce the responses observed. This, in conjunction with data provided by Singer *et al.* (1984), suggests that the *in vitro* primary allogeneic response of Lyt2$^+$ T cells can occur in the absence of L3T4$^+$ T cells. Further work by Sprent *et al.* in 1986 revealed that, independent of L3T4$^+$ T cells, Lyt2$^+$ T cells can mediate alloaggressive functions *in vivo*. Similarly, in 1988, Heeg *et al.* reported that *in vivo* depletion of L3T4$^+$ T cells by injection of α-L3T4 MoAbs abolished reactivity toward Class II MHC allo-Ags *in vivo* and *in vitro*, but not toward Class I MHC allo-Ags. Upon *in vivo* priming of L3T4$^+$ T cell-depleted mice with Class I MHC allo-Ags, the frequencies of Class I MHC-specific precursors of IL 2 producing and cytolytic Lyt2$^+$ T cells increased up to twenty fold. Clearly, grouping of T cells into the CD4$^+$ and CD8$^+$ subsets does not reflect a functional dichotomy, as both helper T cell (IL 2 production) and CTL function can be executed by L3T4$^+$ as well by Lyt2$^+$ T cells.

1.2.4.1 Subpopulations of CD4$^+$ T cells

CD4$^+$ T cells have been further subdivided into two populations called Th1 and Th2. Using CD4$^+$ T cell clones, Mosmann *et al.* (1986) demonstrated the existence of two separate CD4$^+$ T cell populations, Th1 and Th2, divided on the basis of their LK profiles and functional capacities. Th1 CD4$^+$ T cell clones are characterized by their ability to produce the LKs IL 2, IFN-γ and TNF-β and their ability to induce DTH responses (Cher and Mosmann, 1987). On the other hand, Th2 T cell clones are characterized by their ability to produce IL 4, IL 5, IL 6 and IL 10 and can induce IgE synthesis which mediates immediate type hypersensitivity (Kim *et al.*, 1985; Coffman and Carty, 1986; Mosmann *et al.*, 1986; Cher and Mosmann, 1987; Fiorentino *et al.*, 1989). Hence, Th1 T cell clones are preferentially involved in cell-mediated responses, such as DTH reactivity and activation of macrophages (Cher and Mosmann, 1987; Stout and Bottomly, 1989), whilst the Th2 clones are more efficient at, and sometimes exclusively responsible for, inducing humoral responses (Killar *et al.*, 1987; Boom *et al.*,

1988). The possibilty of Th1/Th2 cross-regulation has been inferred from several pieces of data. IFN-γ produced by Th1 cells was found to inhibit proliferation of Th2 clones *in vitro* (Fernandez-Botran *et al.*, 1988; Gajewski and Fitch, 1988). In 1986, Horowitz *et al.* detected an activity produced by Th2 cells which inhibits Th1 proliferation. Furthermore, Fiorentino *et al.* (1989) identified a cytokine produced by Th2 clones that inhibits synthesis of several cytokines by Th1 clones. In 1990, Swain *et al.* reported that IL 4, a cytokine secreted by Th2 clones, can markedly affect the development of Th1 and/or Th2 clones in culture. The presence of IL 4 directs the preferential development of IL 4/IL 5 secreting Th2-like effector cells, suppressing the development of IL 2 and IFN-γ-secreting effector cells.

The *in vivo* relevance of the *in vitro* distinction between Th1 and Th2 $CD4^+$ T cells is not as clear cut, as $CD4^+$ T cell clones isolated from immunized mice or human peripheral blood release a combination of the Th1 and Th2 LKs, IL 2, IL 4 and IFN–γ (Maggi *et al.*, 1988; Paliard *et al.*, 1988; Street *et al.*, 1990). However, Street *et al.* (1990) reported that prior immunization of the mice from which responder cells were derived strongly affected the type of Th clone obtained, whereas the source of stimulator cells had much less effect, suggesting the commitment of Th cells to the Th1 or Th2 phenotypes occurred mainly *in vivo*. On the basis of their own data and the findings of others, Street *et al.* proposed a simple model of Th cell differentiation, which is outlined in Fig. 1.1. They have postulated that a Th cell precursor population exists, which can differentiate into either the Th1 and Th2 subset, depending on the signals received from Ag and/or APCs and that intermediate form(s) that exhibit an "atypical" LK profile exist during the transformation into one or other of the subsets. Data provided by Swain *et al.* (1988) and Street *et al.* (1990), implied the existence of a Th precursor cell (Thp cell) which secretes only IL 2 after activation. The presence of the ThO cell, which produces IL 2, IFN-γ, IL 4, IL 5, granulocyte-macrophage colony stimulating factor (GM CSF) and IL 3, has been inferred from cloning experiments showing that some short term clones *in vitro* demonstrate this phenotype (Street *et al.*, 1990). The proposed model suggests that the ThO cell can differentiate into either Th1

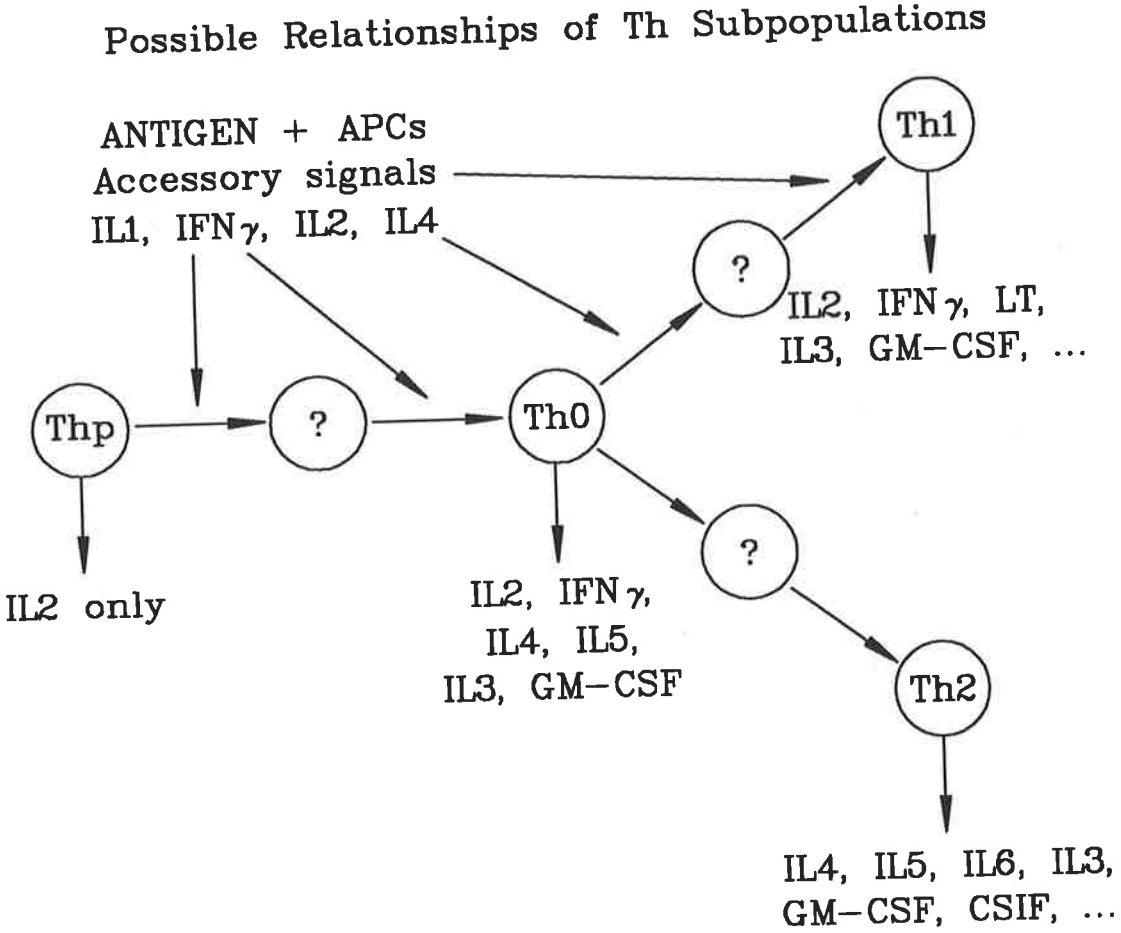


Figure 1.1 Model of Th cell differentiation adapted from Street et al. (1990).

[LT, lymphotoxin; CSIF, cytokine synthesis inhibitory factor]

or Th2 phenotypes, depending on the Ag/APC signals that it receives. However, it is possible that some Th cells are precommitted to differentiate into either Th1 or Th2 phenotypes before exposure to Ag.

There is evidence supporting the possibility that differential activation of T cells that produce distinct cytokines occurs as a result of stimulation by Ag presented by different APCs. In 1990, Simon *et al.* reported that low dose ultraviolet B-irradiated Langerhans cells preferentially activate $CD4^+$ Th2 cells. Lichtman *et al.* (1988) and Greenbaum *et al.* (1988) found that for optimal proliferative responses to a variety of stimuli, Th2 clones require the addition of IL 1, whereas Th1 clones do not. Furthermore, Th2 clones were reported to proliferate suboptimally in response to Ag presented by B cells unless IL 1 is added, whilst proliferation in response to Ag presented by macrophages does not require additional IL 1 (Chang *et al.*, 1990). In contrast, Th1 clones responded to Ag presented by both B cells and macrophages, without the requirement for IL 1. This can be explained at least partially by the fact that Th2 cells, in contrast to Th1 cells, express IL 1 receptors (reviewed by Bottomly, 1988). Data provided by Fiorentino *et al.* in 1989, suggested that IL 10 produced by Th2 clones may suppress Th1 function indirectly, via APC. More detailed analysis by Fiorentino *et al.* in 1991 revealed that IL 10 impairs the ability of splenic and peritoneal APCs to stimulate cytokine production by Th1 clones, but not Th2 clones. Obviously, stimulation of T cells which secrete different amounts or types of cytokines have important implications on the particular type of reponse that could be induced.

Identification of cytotoxic $CD4^+$ T cells introduced the possibility that these cells represent a distinct population of cells or may be included in either the Th1 and Th2 cell groups. Some virus-specific $CD4^+$ CTLs have been shown to be multifunctional in that they display both cytotoxic and specific proliferative responses including helper activity, as demonstrated by their ability to produce IL 2 (Yasukawa and Zarling, 1984) and humoral factors which help autologous B cells to produce virus-specific Abs (Yasukawa *et al.*, 1988). Many $CD4^+$ clones exhibit cytotoxic activity upon activation with mitogens, Ags or MoAbs. Tite *et al.* (1985) reported that

after stimulation with Ag or mitogen, some Th2 clones which secreted low levels of TNF and undetectable amounts of IFN-γ, were noncytolytic. Consequently, they proposed that TNF and IFN-γ are the soluble mediators for target cell lysis. However, many targets killed by $CD4^+$ CTLs are resistant to high concentrations of IFN-γ and TNF, suggesting the induction of a TNF/IFN-γ independent cytolytic mechanism (Ju *et al.*, 1989). Analysis of the cytotoxic activity of activated Th1 and Th2 clones revealed that all Th1 clones tested were strongly cytolytic, whilst Th2 clones were either noncytolytic or weakly cytolytic (Ju *et al.*, 1990). Supporting their earlier data (Ju *et al.*, 1989), MoAb blocking experiments revealed the existence of two distinct TNF-mediated cytolytic mechanisms among the CD4 T cell subsets - one IFN-γ-dependent and the other IFN–γ-independent. Further evidence that $CD4^+$ CTLs belong to the Th1 cell group, was that cytotoxic $CD4^+$ T cells produced IFN-γ and lymphotoxin/TNF, while $CD4^+$ T cells that "helped" specific Ab responses produced IL 4, but not TNF or IFN-γ, which coincides with the Th1 and Th2 subsets respectively (reviewed by Bottomly, 1988).

Studies carried out in the human, rat and mouse have shown that $CD4^+$ T cells can also be subdivided on the basis of expression of restricted populations of CD45 molecules (T200 or leukocyte common antigen), referred to generically as CD45R (reviewed by Bottomly, 1988). There is evidence to suggest that the level of expression of CD45R molecules distinguishes naive and memory $CD4^+$ T cells (Ericsson *et al.*, 1990). They reported that $CD45R^-$ $CD4^+$ T cells and $CD45R^+$ $CD4^+$ T cells have the characteristics of memory and naive cells, respectively. Clement *et al.* (1988) provided evidence supporting this possibility, by demonstrating that after polyclonal activation $CD45R^+$ $CD4^+$ T cells acquire both the phenotypic and functional characteristics of $CD45R^-$ $CD4^+$ T cells. $CD4^+$ T cells expressing little or no CD45R (CD45-low) provide help to B cells, whereas $CD4^+$ T cells expressing high levels of the CD45R (CD45-high) do not provide B cell help but release IL 2. Further analysis demonstrated that in addition to helping B cell responses, CD45-low cells produce IL 4 protein and IL 4 mRNA, but secrete no IL 2 and produce no mRNA for IFN-γ or IL 2. In

contrast, CD45-high cells produce mRNA encoding IFN-γ and IL 2, secretes IL 2 and provide no B cell help (reviewed by Bottomly, 1988). Hence, $CD4^+$ T cells can be subdivided into the two functional groups Th1 and Th2, which are distinguished on the basis of LK production and on the expression of a restricted form of CD45R.

1.2.4.2 Evidence for $CD8^+$ T cell subsets

Little data for functional grouping of $CD8^+$ T cells into Th1 and Th2 equivalents is available. In 1987, Fichtner and colleagues reported that $CD8^+$ T cells could be divided on the basis of expression of the cell surface marker Ly6C and more recent investigations have revealed that $CD8^+$ T cells can be divided into four different subpopulations based on the level of expression of Ly6C. $Ly6C^{neg}$, $Ly6C^{low}$, $Ly6C^{int}$ and $Ly6C^{high}$ $CD8^+$ T cells showed specific differences in their responses to mitogenic and alloantigenic stimulation (Kung *et al.*, 1991). Highly purified populations of these cell subsets were stimulated with α-CD3 MoAbs or allogeneic stimulator cells to induce proliferation and IL 2 production. Greatest responses were obtained with $Ly6C^{neg}/Ly6C^{low}$ $CD8^+$ T cells, moderate responses with $Ly6C^{int}$ T cells, and $Ly6C^{high}$ T cells responded minimally. Similarly, allogeneic stimulation induced a strong cytotoxic response from $Ly6C^{neg/low}$ T cells but not $Ly6C^{high}$ T cells. Addition of excess IL 2 enhanced the proliferation and cytotoxic activity induced by α-CD3 in all $CD8^+$ T cell populations, whereas in the presence of excess IL 2 allo-Ag elicited an approximately five-fold higher response by $Ly6C^{high}$ than $Ly6C^{neg/low}$ $CD8^+$ T cells. Furthermore, coculturing experiments demonstrated that help provided by $Ly6C^{neg/low}$ T cells can be utilized by both $Ly6C^{neg/low}$ and $Ly6C^{high}$ T cells.

With the knowledge that naive and memory $CD4^+$ T cells can be distinguished on the basis of expression of the CD45R molecule (Powrie and Mason, 1988; Sanders *et al.*, 1988), Ericsson *et al.* (1990) investigated whether such dichotomy also applies to $CD8^+$ T cells. After immunization with either tumour cells or allogeneic spleen cells, tumour-selective cytolytic $CD8^+$ T cells express the CD45R molecule, while allo-Ag-specific cytolytic $CD8^+$ T cells are found in both the $CD45R^+$ and $CD45R^-$

populations. Hence, these very recent, preliminary experiments suggest that it may be possible to subdivide $CD8^+$ T cells into functional groups on the basis of cell surface expression of cell surface markers.

1.2.5 Role of T cell subsets in immune responses to IBPs

1.2.5.1 Introduction

To successfully detect specific primed T cells of a particular subset(s), the appropriate assay systems must be employed. Over the years a number of detection systems have been established including, *in vitro* antigenic or mitogenic stimulation of T cells to proliferate and release various LKs, the *in vitro* ^{51}Cr release assay for the detection of CTLs and the adoptive transfer of resistance or DTH reactivity to normal, unimmunized recipients. To induce either T cell subset to proliferate, it is obvious that unless the appropriate Ag and/or APC populations are used, the observed responses can be biased toward a particular T cell subset (Ramila *et al.*, 1985; Townsend *et al.*, 1986). Similarly, the form of Ag used to elicit DTH reactivity is equally important, to allow the detection of all cells in the population capable of mediating DTH reactivity (Leung and Ada, 1980; Ertl, 1981; Attridge and Kotlarski, 1985b; Chen-Woan *et al.*, 1985; Kaufmann *et al.*, 1985). Furthermore, to ascertain the presence of Ag-specific CTLs of the $CD4^+$ or $CD8^+$ phenotype, target cells expressing the relevant antigenic determinants and Class II or I MHC molecules, respectively are required (Kaufmann *et al.*, 1986 and 1987). Not surprisingly, in the absence of suitable assay systems the observed results can be distorted, implicating only one of the T cell subsets, when they both may play a role. This probably provides the explanation for discrepancies reported between different groups studying the T cells involved in immunity to IBPs.

1.2.5.2 T cells induced by infection with *Listeria*

Immune responses to *Listeria* have been extensively studied. Earlier work carried out to determine the H-2 restriction of the immune response induced by

listerial infection was contradictory. First reports on the H-2 restriction of the immunity to *Lm* claimed that the response was H-2I (Class II) resticted (Zinkernagel *et al.*, 1977). Similarly, Farr *et al.* (1979) reported that H-2I region homology between the macrophages and T cells, was required for expression of cytocidal activity by macrophages. Using *Listeria*-specific T cell lines, Kaufmann and Hahn (1982) found that the expression of DTH and immunity to *Lm* was dependent on Class II restricted *Listeria*-specific T cells. *Listeria*-specific Class II MHC restricted T cells capable of killing *Listeria*-infected target cells have also been also detected (Kaufmann *et al.*, 1987).

However, contradicting these reports, both Lyt1$^+$ and Lyt123$^+$ T cells were reported to be involved in the immune response to *Lm* (Kaufmann *et al.*, 1979 and 1982). This supported the data of Cheers and Sandrin (1983), which demonstrated that passive transfer of immunity to *Listeria* was Class I MHC restricted. The importance of CD8$^+$ T cells in protective immunity to *Listeria* had also been suggested by other workers (Czuprynski and Brown, 1987). Studies on the T cell subsets involved in the immune response to *Listeria* infection in rats suggested cooperation between Class I and Class II MHC restricted T cell subsets is necessary for effective resistance to infection (Chen-Woan *et al.*, 1985). Treatment of T cells from immunized animals with various MoAbs and complement prior to transfer to unimmunized recipients demonstrated that the DTH effectors were W3/25$^+$ T cells (CD4$^+$ equivalent) and the mediators of protective immunity were OX8$^+$ T cells (CD8$^+$ equivalent). The T cells capable of conferring protection could not mediate DTH reactivity, but the DTH effectors were able to enhance the protective capacity of the OX8$^+$ protective T cells, suggesting cooperation between the two T cell subsets could occur.

Further analysis of the cells mediating DTH reactivity and immunity to *Listeria* in mice has also provided information for a cooperative role between L3T4$^+$ and Lyt2$^+$ T cells (Kaufmann *et al.*, 1985). Cooperation betweeen Ag-specific L3T4$^+$ T cells and Lyt2$^+$ T cells was necessary for effective clearance of *Listeria*, with the Lyt2$^+$ T cells being ultimately responsible for clearance of a secondary listerial infection (Kaufmann *et al.*, 1985; Bishop and Hinrichs, 1987; Mielke *et al.*, 1988). The discovery of

Listeria-specific Lyt2$^+$ CTLs reinforced the evidence that listerial infection induces Lyt2$^+$ T cells (Kaufmann *et al.*, 1986).

In 1989, Orme demonstrated that cyclophosphamide-sensitive Lyt2$^+$/CD8$^+$ T cells were responsible for the passsive transfer of immunity to *Lm*. In contrast, cyclophosphamide-resistant L3T4$^+$/CD4$^+$ T cells mediated immunological memory to *Lm*. Similarly, different T cell subsets were found to be responsible for expression of systemic protection and DTH to *Lm*. Pretreatment of ConA stimulated *Listeria*-specific spleen cells with α-CD8 MoAb and complement abrogated the ability to transfer systemic immunity to *Listeria*, but had no influence on the ability to transfer DTH reactivity (Baldridge *et al.*, 1990). Conversely, treatment with α-CD4 and complement abolished the ability to transfer DTH reactivity with little impact on the capacity to transfer resistance. *In vivo* depletion of either T cell subset confirmed these observations; cells responsible for mediating resistance were cyclophosphamide-sensitive whilst those mediating DTH were cyclophosphamide-resistant. These data provide substantial evidence that both L3T4$^+$ T cells and Lyt2$^+$ T cells are induced and are required for immunity to listerial infection.

Further analysis of the T cell response to infection with *Lm* in mice revealed another factor contributing to the type of immune response induced. Virulent strains of *Lm* are known to secrete an exotoxin called listeriolysin O (Njoku-Obi *et al.*, 1963; Groves and Welshimer, 1977), which has been identified as a major virulence factor (Skalka *et al.*, 1982; Gaillard *et al.*, 1986). Several reports indicate that listeriolysin O mediates bacterial virulence by enhancing bacterial multiplication within macrophages (Gaillard *et al.*, 1986; Portnoy *et al.*, 1988; Camilli *et al.*, 1989; Cossart *et al.*, 1989) and by instigating inhibition of Ag processing and presentation (Cluff and Ziegler, 1987; Cluff *et al.*, 1990). Expression of listeriolysin O has been found to play a major role in immunity to *Lm*, as immunization with either heat killed or listeriolysin O non-producing strains of *Lm* does not protect mice from subsequent challenge with virulent, listeriolysin O producing strains (Wirsing von Koenig *et al.*, 1982; Berche *et al.*, 1987a and 1987b). In 1991, Safely *et al.* reported a correlation between listeriolysin O

production by infecting bacteria and generation of protective immunity and that production of listeriolysin O was necessary to induce Ag-specific T cells, as assessed by IL 2 production *in vitro*. Mutants expressing inactive listeriolysin O were reported to be unable to grow intracellularly (Gaillard *et al.*, 1987; Portnoy *et al.*, 1988), possibly explaining their inability to induce protection. An explanation for the effectiveness of listeriolysin O producing strains as vaccines was provided by Berche *et al.* (1987a) and Tilney and Portnoy (1989), who reported that listeriolysin O producing *Lm* organisms escaped from the endosomal compartment and entered the cytosol where they underwent bacterial replication, whilst non-producing strains remained within the phagolysosome and were unable to divide. This localization of *Lm* into the cytosolic compartment provides a mechanism for these bacteria to induce $CD8^+$ T cells, as it has been reported previously that $CD8^+$ T cell responses occur preferentially to Ags derived from the endogenous (cytoplasmic) compartment (Morrison *et al.*, 1986; Moore *et al.*, 1988; see Sections 1.4.1 and 1.4.4.1). This was confirmed by Brunt *et al.* (1990) who found that only those *Lm* organisms which expressed listeriolysin O entered the Class I Ag processing pathway, as determined by the ability of infected macrophages to induce proliferation and IFN-γ release from $CD8^+$ T cell clones *in vitro*. Therefore, these data provide an explanation for the involvement of both $CD4^+$ and $CD8^+$ T cells in immunity to *Listeria*.

1.2.5.3 T cells induced by mycobacterial infection

Pioneering work by Lurie (1942) demonstrated the importance of CMI in resistance to infection with *Mycobacteria* and was supported by the finding that cells obtained from guinea pigs immune to mycobacterial infection could passively transfer DTH to the tuberculin protein to normal recipients (Chase, 1945). Confirmation that acquired immunity to tuberculosis infection is cell-mediated was provided by North (1973) and Lefford (1975). Lefford demonstrated that T cells from spleens of BCG-immune donors could adoptively transfer protection to sublethally irradiated recipients. Protection, determined by the numbers of infectious organisms which could

be recovered from the spleen two weeks after challenge, was conferred equally against BCG reinfection and intravenous challenge with *M. tuberculosis*. In 1983, Orme and Collins reported that spleen cells obtained from mice at the height of the primary immune response to intravenous infection with *M. tuberculosis*, were able to transfer protection to *M. tuberculosis*-infected T cell-deficient recipients. Furthermore, a population of splenic T cells taken from naturally susceptible (C57BL/6) mice, but not resistant (A/J) mice, infected with *M. avium* conferred significant protection against a subsequent challenge inoculum of *M. avium* to syngeneic recipient mice (Stokes and Collins, 1990). Replication of *M. avium* in donor mice, which did not occur in the resistant (A/J) mice, was necessary for the development of protective T cells. In addition, BCG, which replicates in A/J mice, stimulated a population of splenic T cells which protected recipient mice from subsequent infection with *M. tuberculosis*. Having established that T cells play an important role in immunity to *Mycobacteria*, attempts to type the T cell(s) involved were made and conflicting data have been obtained.

In 1985, Denis *et al.* demonstrated that four to six weeks after infection with a low dose of *M. bovis* BCG, Lyt1$^+$ T cells were generated in the spleens of BCG-susceptible (*Bcg*^{s}) mice, but not of BCG-resistant (*Bcg*^{r}) mice. Concomitantly, there was a marked decrease of Lyt123$^+$ cells in the spleens of *Bcg*^{s} mice. Detailed analysis of the T cell subsets required for resistance of *Bcg*^{s} mice (C57BL/6) to infection with *M. bovis* BCG revealed that the major effectors in controlling bacterial division were L3T4$^+$ T cells (Pedrazzini *et al.*, 1987). *In vivo* depletion of L3T4$^+$ T cells by ip administration of α-L3T4 MoAb caused a dramatic increase in the number of viable *Mycobacteria* recovered, whilst depletion of Lyt2$^+$ T cells *in vivo* had virtually no effect on the recovery of bacteria. This was supported by adoptive transfer experiments, where treatment of immune spleen cells with MoAb and complement demonstrated that L3T4$^+$ T cells were mediating resistance to the mycobacterial infection. Whereas, similar depletion of L3T4$^+$ and Lyt2$^+$ T cells from thymectomized mice indicated that both L3T4$^+$ and Lyt2$^+$ T cells participate in resistance against tuberculosis (Müller *et al.*, 1987). Furthermore, L3T4$^+$ MHC Class II restricted BCG-specific T cell lines and

clones have been shown to release macrophage activating factors (MAF or IFN-γ) upon antigenic stimulation *in vitro* and to inhibit mycobacterial growth in infected macrophages both *in vitro* and *in vivo* (Rook *et al.*, 1985; Pedrazzini and Louis, 1986). Kumararatne *et al.* (1990) reported that *Mycobacteria*-specific human T cell lines kill target cells pulsed with mycobacterial Ags, to a greater extent than unpulsed cells or cells pulsed with irrelevant Ags. This cytotoxic activity was blocked by MoAb to Class II MHC Ag, confirming the induction of Class II MHC restricted L3T4$^+$ T cells on infection with *Mycobacteria.*

Conversely, in 1984 Orme and Collins reported that adoptive transfer experiments provided evidence that Lyt2$^+$/CD8$^+$ T cells from immune mice are able to protect normal animals against an infectious challenge with *M. tuberculosis.* In addition, *M. leprae*-specific Lyt2$^+$ CTLs have been detected in the draining lymph nodes of intradermally infected mice (Chiplunkar *et al.*, 1986). Further evidence for the involvement of Lyt2$^+$/CD8$^+$ T cells in immunity to *Mycobacteria* was provided by Roch and Bach (1990), when they examined several immunological parameters of mice with varying susceptibilty to mycobacterial infection after subcutaneous infection with either *M. lepraemurium* or *M. bovis* BCG. All BCG-infected mice controlled the infection and developed early and large granulomas, whereas *M. lepraemurium*-infected mice exhibited major strain variations in their resistance to the infection and in granuloma formation. Analysis of the draining lymph nodes revealed that BCG and *M. lepraemurium* infections both induced a decrease in the percentage of B cells and a decrease in the CD4/CD8 ratio in the three mouse strains used. Stimulation with the *Mycobacteria* and IL 2 induced an increase in the number of CD8$^+$ T cells in the draining lymph nodes of all three mouse strains. However, this increase was most pronounced in the *M. lepraemurium*-resistant mice. Consequently, Roch and Bach (1990) proposed that the ability of the *M. lepraemurium*-resistant mice to generate an early and persistent CD8$^+$ T cell response to *Mycobacteria* may contribute to their resistance to *M. lepraemurium.*

Thus, evidently both CD4$^+$ and CD8$^+$ T cells appear to be involved in the immune responses generated after mycobacterial infection. The rate of progression of a

mycobacterial infection has been reported to vary depending on the route and dose of infection (eg.Turcotte, 1980; Patel, 1981) and on the genetic constitution of the host (Brown *et al.*, 1982; Curtis *et al.*, 1982). Similarly, the development of acquired immunity seems to differ between mice, as resistant mice infected with *M. bovis* BCG take longer to develop acquired resistance to *M. tuberculosis* (Orme *et al.*, 1985) than susceptible mice (Orme and Collins, 1984). *In vitro* responsiveness of spleen cells from *M. lepraemurium*-infected mice also varies, depending on the genetic constitution of the mice (Brett, 1984). Therefore, it does not seem unreasonable to suggest that the induction of $CD4^+$ and/or $CD8^+$ T cells as a result of mycobacterial infection may also be influenced by the genetic constitution of the host, as evidence for the involvement of $CD8^+$ T cells was greatest in the mycobacteria-resistant mouse strain, whilst mycobacterial infection of susceptible mouse strains appeared to induce mainly $CD4^+$ T cells.

1.2.5.4 T cells induced by *Salmonella* infection

The involvement of T cells in resistance to *Salmonellae* infection became evident in studies carried out by Davies and Kotlarski (1976), where they reported that *in vivo* depletion of T cells by thymectomy resulted in decreased clearance of the vaccine strain 11RX and lack of protection against C5. The phenotype of the T cells required for immunity was not established, but adoptive transfer experiments with cells from mice previously immunized with live 11RX have shown that the cells mediating DTH reactivity induced by an Ag extract of 11RX were $Lyt1^+$ T cells and that the response was H-2I (Class II) restricted (Attridge and Kotlarski, 1985b). These nonadherent $Lyt1^+$ T cells in the presence of specific Ag and adherent accessory cells were induced to release IL 2 and MAF *in vitro* (Attridge and Kotlarski, 1985a). Again, the interaction between the T cells and accessory cells was restricted by the H-2 I-A locus. Hence, it is possible that on exposure to *Salmonella*, T cells are induced to proliferate and to release IL 2 and MAF which activates macrophages and maintain proliferation of T cells required for clearance of the bacteria. Similarly, *Salmonella*-specific T cell lines were

established by Paul *et al.* (1985) which were capable of Ag-specific proliferation *in vitro* and transferring effective protection against infection.

Recently, the phenotype of the T cells mediating protection against *Salmonella* has been investigated. Nauciel (1990) demonstrated that *in vivo* depletion of $CD4^+$ T cells by infusion of α-CD4 MoAb, abrogated the ability to clear an infection of a temperature sensitive mutant of *Salmonella typhimurium*. Similarly, *in vitro* treatment of immune T cells with α-CD4 and complement removed their capacity to adoptively transfer immunity, whilst α-CD8 and complement had little effect. Resistance to reinfection was equally reduced after treatment with α-CD4. Hence, this preliminary investigation suggests that $CD4^+$ T cells and not $CD8^+$ T cells are responsible for mediating resistance to *Salmonellae*.

At the commencement of my work, work carried out by other members of our laboratory provided indirect evidence for the induction of $Lyt2^+/CD8^+$ T cells after infection with 11RX (unpublished data). Nylon wool (NW) nonadherent peritoneal cells (PCs) harvested from mice immunized with 11RX, were able to kill lectin-treated target cells, as measured in the standard lectin-mediated cytotoxicity assay. The cells responsible for this activty were identified as $Lyt2.2^+$, $Thy1.2^+$ cells. No cytotoxic activity was detected in the cells from normal, unimmunized mice or mice immunized with formalin killed 11RX (F11RX). Even though the Ag specificity of these $Lyt2^+$ CTLs was not established, the data suggested that *Salmonella*-specific $Lyt2^+$ T cells may be induced as a result of infection with live 11RX. Therefore, the relevance of these cells to *Salmonella*-specific immunity required further investigation.

1.2.5.5 Summary

The extensive studies carried out on the T cells induced after infection with IBPs, especially *Listeria* and *Mycobacteria*, have demonstrated the involvement of both $CD4^+$ and $CD8^+$ T cells, possibly in a cooperative manner. Considerably fewer studies of the T cell responses to *Salmonellae* infections have uncovered a major contribution by the $CD4^+$ T cells. It is possible that the lack of a detectable

listeriolysin O-equivalent in *Salmonella* may explain the induction of $CD4^+$ T cells only, as the *Salmonella* organisms may remain confined to the endosomes, hence accessing the class II Ag processing pathway, but not the Class I Ag processing pathway. However, no listeriolysin O-equivalent has been identified in *Mycobacteria* either, yet both $CD4^+$ and $CD8^+$ T cells are induced following mycobacterial infection, suggesting at least some Ags of *Mycobacteria* may be able to enter the Class I Ag processing pathway via an alternative route. Consequently, additional study of the T cells induced after infection with *Salmonella* is warranted to establish whether *Salmonella*-specific $CD8^+$ T cells can be detected.

1.3 The role of macrophages in resistance to IBPs

1.3.1 Introduction

It is now clearly established that Ag-specific T cells play an important role as effector cells in immunity to at least some IBPs. It is also well established that macrophages play a significant part in the initial control of the infection. Activation of macrophages is accompanied by increased cytocidal activity and by the synthesis, expression and/or secretion of enzymes, some complement proteins, membrane proteins such as the MHC Class II molecules and soluble products like IL 1 (Unanue, 1981; Adams and Hamilton, 1984). Activation of macrophages which can be detected shortly after IBP infection, enhances their ability to control bacterial multiplication and therefore, clear the infection. Such control of bacterial infections shortly after challenge is under the control of particular genetic loci and since the genetic status of animals has been reported to have considerable influence over the immunity to IBPs, it is not surprising that it was previously believed that activated macrophages induced early in the immune response provided the only major effector population responsible for clearance of the bacteria.

1.3.2 Genetic control of resistance to *Listeria*

Resistance to various IBPs is determined by a single gene locus (eg. Plant and Glynn, 1979). Early resistance to *Lm* infection was found to be controlled by the *Lr* locus in mice (Cheers *et al.*, 1980) and it is believed that such resistance is due to nonspecific effector cells (Skamene, 1983). The accumulation of inflammatory macrophages in the peritoneal exudates early after ip challenge with *Listeria* has been associated with the *Lr* locus controlled resistance (Stevenson *et al.*, 1980; Czuprynski *et al.*, 1985).

Goossens *et al.* (1988) concluded that the early resistance to listerial infection which is under the control of the *Lr* locus is ultimately due to the rapid influx of inflammatory macrophages, possibly recruited by *Listeria* reactive T cells which released LKs able to recruit bone marrow derived monocytes to the site of infection, thereby resulting in the control of bacterial growth in the initial stages of the infection. The more rapid influx of *Listeria* reactive T cells to the site of infection in *Listeria* resistant mice than in *Listeria* susceptible mice supported this hypothesis.

1.3.3 Genetic control of resistance to *Mycobacteria*

The early response of mice to infection with low dose of *M. bovis* BCG is under the control of the *Bcg* gene (Forget, *et al.*, 1981; Gros *et al.*, 1981). Similarly, resistance to *M. lepraemurium* varies depending on the genetic constitution of the mice (Brown *et al.*, 1982; Curtis *et al.*, 1982). The genes determining resistance to *Mycobacteria* have been shown to control the innate capacity of macrophages in the spleen and liver to allow multiplication of intracellular bacteria during the early phase of the host response to BCG infection (Gros *et al.*, 1981). The growth rates of *M. avium* within normal, resident macrophages account for the differences in the growth pattern of *M. avium* in susceptible and resistant mice (Stokes *et al.*, 1986; Stokes and Collins, 1988).

Since products of the *Bcg* gene control the resultant bacterial load in infected mice, they also have considerable influence on the immune response induced in the later phase of the infection. For example, Pelletier *et al.* (1982) discovered that there

were noticeable differences between the development of various immunological parameters after infection with low doses of *M. bovis* BCG. The finding that a low dose of *M. bovis* BCG induced Lyt1$^+$ T cells and an increase of phagocytic cells in the spleens of BCG-susceptible and not BCG-resistant mice, illustrates the influence that the bacterial load has on the ensuing immune response induced (Denis *et al.*, 1985). Even more convincing is the demonstration that splenic T cells from susceptible mice infected with *M. avium*, but not similarly challenged resistant ones, were able to transfer protection to normal syngeneic recipient mice, to subsequent challenge with *M. avium* (Stokes and Collins, 1990). Evidently, control of mycobacterial growth by the *Bcg* gene in the early phase of infection has marked influence on the T cells induced in the later stages of the infection; persistence of the IBP is essential to provide immunity to reinfection.

1.3.4 Macrophages in immunity to *Salmonella* infection

Considerably less work has been carried out on the detailed analysis of the response of lymphocytes to *Salmonella* infections, although, it is known that CMI, in conjunction with an humoral immune response, provides effective resistance to *Salmonella*, with the CMI playing a critical role. Data available suggest that the mechanism is likely to be similar to that for *Lm* and *Mycobacteria* since it has been shown that early in the infection resistance is under the control of the *Ity* gene which controls the growth rate of *Salmonella typhimurium* in macrophages (O'Brien, 1986). Resistance to reinfection and infection with heterologous bacteria is present within a few days of infection of effective, live vaccines able to persist in the tissues (Blanden *et al.*, 1966; Nauciel *et al.*, 1985). This nonspecific form of immunity has been attributed to activated macrophages (Collins, 1974), whilst the cells responsible for initiating bacterial clearance in immunized animals long after primary immunization are reported to be T cells (Collins, 1979; North, 1981).

1.3.5 Suppressive effects of macrophages following infection with IBPs

In addition to the role macrophages play in the immunity generated

following infection with IBPs, they have also been reported to behave in a suppressive manner, diminishing T cell responsiveness. Mice systemically infected with large doses of *M. lepraemurium* show diminished responsiveness to specific Ags and to nonspecific mitogens (eg. Navalkar *et al.*, 1980) as the infection progresses. *In vitro* responses such as IL 2 production (Hoffenbach *et al.*, 1983a), MLRs (Hoffenbach *et al.*, 1983b) and T cell-dependent Ab responses to sheep erythrocytes (Watson *et al.*, 1975; Bullock *et al.*, 1978) are also reduced some time after infection. Klimpel and Henney (1978) demonstrated that the development of unresponsiveness after BCG infection was paralleled by the onset of suppressive activity detectable *in vitro*. Such unresponsiveness was not induced by heat killed BCG. The suppressive activity was associated with adherent, phagocytic, Thy1$^-$ spleen cells which could be removed by passage on a NW column, suggesting the development of a suppressive macrophage-like population in the spleen of BCG-infected mice. Analysis of the responsiveness of splenic T cells from *M. lepraemurium*-infected resistant and susceptible mice strains, revealed that decreased responsiveness to mitogens and specific Ags appeared earlier and was more profound in the susceptible strain than in the resistant strain (Brett, 1984). This nonspecific immunodepressive activity was associated with NW adherent spleen cells.

Similarly, macrophages from mice infected with *Listeria* inhibit the *in vitro* proliferation of *Listeria*-immune T cells stimulated by normal macrophages and Ag (Kaufmann *et al.*, 1982). A decrease in the proliferative response of spleen cells from *Brucella abortus*-infected mice was also reported to be paralleled by an increase in the proportion of macrophage-like cells and could be overcome by increasing the amount of mitogen in culture (Riglar and Cheers, 1980). Tewari *et al.* in 1982 reported that on infection with *Histoplasma capsalatum* the *in vitro* response of such primed lymphocytes to mitogen was suppressed early in the infection.

In 1986, Deschenes *et al.* demonstrated that spleen cells from mice infected with live *Salmonella typhimurium* had reduced responsiveness to T (and B) cell mitogens and that this activity was at least partially mediated by adherent cells. Similarly, the responsiveness of cells from mice infected with live 11RX have been found

to be unresponsive in *in vitro* proliferative assays unless the adherent cells are removed by passage on NW columns (Kotlarski *et al.*, 1989). Unfractionated spleen cells obtained from mice following mycobacterial infection have been reported to show diminished responsiveness to antigenic and/or mitogenic stimuli *in vitro* (Orbach-Arbouys and Poupon, 1978; Brett, 1984). Both soluble and particulate mycobacterial Ags have been reported to induce suppression, the extent of which was inversely proportional to the proliferative response of T cells to Ag (Kaplan *et al.*, 1987). Clearly, the effect of macrophages on the T cell responsiveness of cell suspensions obtained from mice infected with IBPs must be taken into consideration when analysing the functional capacities of T cells obtained from IBP-infected mice.

1.3.6 The role of activated macrophages in the immunity to IBPs

As previously mentioned, it was initially thought that activated macrophages were the major effector population required for resistance to infection with IBPs. Extensive studies by many groups have revealed that macrophages are involved in the cellular respsonse to IBPs. For example, in 1957 Elberg *et al.* found that the bactericidal activity of macrophages is enhanced on infection and this increase in activity is nonspecific and can be expressed against various IBPs. It has also been shown that *in vivo* depletion of macrophages in recipient mice abolished the ability to transfer immunity with immune T cells, suggesting interactions between the macrophages and the T cells were necessary for clearance of the infection (Mackaness, 1969; North, 1973). It is also well established that macrophages activated by infectious and certain noninfectious agents have been shown to have increased immunologically nonspecific tumouricidal and microbicidal activity (Hibbs *et al.*, 1972; Cleveland *et al.*, 1974; Meltzer *et al.*, 1975; Meltzer and Oppenheim, 1977; Ashley and Kotlarski, 1982; La Posta *et al.*, 1982). Cheers *et al.* (1978) reported that as a result of the bacterial infection macrophages become activated, assessed as enhanced bactericidal activity. It was proposed that these cells were one of two major effector populations required for the expression of CMI and that macrophage-T cell interactions were essential for the

activation of macrophages. Substantial evidence is now available demonstrating that T cells are essential for activation of macrophages (discussed in more detail in the following section).

In addition to tumouricidal activity, PCs from mice immunized with BCG have enhanced chemotactic activity and enhanced ability to induce effector T cells (Meltzer and Oppenheim, 1977; Britz *et al.*, 1982). Furthermore, Denis *et al.* (1985) reported that following challenge with a low dose of BCG, specific $Lyt1^+$ T cells were detected in the spleens of mice in parallel with an increase in the proportion of phagocytic cells. Supporting this, PCs from 11RX-infected mice were shown to kill tumour cells *in vitro* and *in vivo* in a nonspecific fashion (Ashley and Kotlarski, 1982) and La Posta *et al.* (1982) demonstrated that most of this cytotoxic activity could be attributed to activated macrophages. Detailed analysis of the macrophages activated by *Salmonella* infection, demonstrated the existence of macrophage subpopulations defined on the basis of their tumoricidal and bactericidal activities and the persistence of these activities after immunization (Hopper and Cahill, 1983). Further evidence for the role of activated macrophages in immunity to *Salmonella* was provided by Killar and Eisenstein in 1985. They reported that adoptive transfer of immunity to *Salmonella typhimurium* could be achieved using cells from immunized mice and that the protective capacity of these cells resided in the adherent portion of the population and were therefore macrophages. This was supported by observations of Hopper and Cahill (1983) who discovered that the development of immunity induced by 11RX was dependent on the relative proportions of effector T cells and macrophages. The nonspecific immunity mediated by activated macrophages was found to be transient and the long term Ag-specific immunity has been shown to be due to the persistence of memory T cells able to activate macrophages after re-exposure to Ags (Collins, 1979; North, 1981).

The ratio of macrophages to T cells was also shown to be critical for establishing immunity to *Listeria* (Jungi, 1980). Kaufmann and Hahn (1982) and Havell *et al.* (1982) concluded that activated T cells proliferate and release LKs required for expression of CMI to *Lm*, such as MAF which activates macrophages at the site of

infection (North, 1981). Subsequently, it was shown that they also release the IL 2 required for expansion of the specific T cell pool (Unanue, 1984). At least one of the factors required for macrophage activation which is released by activated T cells has been identified as IFN-γ (Schultz and Kleinschmidt, 1983). T cells harvested from mice immunized with 11RX have also been shown to release MAF after stimulation with specific Ag and accessory cells *in vitro* (Attridge and Kotlarski, 1985a). Similarly, *Mycobacteria*-specific T cells released MAF and IFN-γ on stimulation with a mitogen or specific Ag *in vitro* (Brett, 1984; Brett and Butler, 1986). In support, *Listeria*-specific T cell clones or the supernatants produced by stimulating these clones with specific Ag, afforded protection to normal mice against infection with *Listeria* (Magee and Wing, 1988). Neutralization of IFN-γ in the supernatants with MoAb to IFN-γ abolished the ability to transfer resistance, implicating IFN-γ as at least one of the mechanisms responsible for T cell-mediated resistance to *Listeria*. Consequently, IFN-γ is a likely candidiate for mediating the activation of macrophages following infection with IBPs.

1.3.7 Functional activation of macrophages by interferon-γ

Macrophage activation following infection with IBPs is essential for the killing of intracellular parasites which otherwise persist and multiply within the normal macrophages. Considerable evidence exists supporting the possibility that IFN-γ induces activation of macrophages involved in the early nonspecific immunity observed following infection with IBPs. Pace *et al.* (1983 and 1985) demonstrated that the tumouricidal activity of macrophages was induced by treatment with IFN-γ, and could be neutralized by an α-IFN-γ MoAb (Spitalny and Havell, 1984). Similarly, IFN-γ has been shown to induce bactericidal activity in macrophages.

In vitro experiments studying the survival of the intracellular parasite *Leishmania* within macrophages indicated that IFN-γ enhanced the macrophage killing of these organisms (eg. Nacy *et al.*, 1981; Murray *et al.*, 1982, 1983 and 1987). Administration of α-IFN-γ MoAb *in vivo*, abrogated resistance to *Leishmania major* confirming the importance of IFN-γ activity at least in the initial stages of an infection

(Belosevic *et al.*, 1989). It has also been demonstrated that infusion of α-IFN-γ markedly reduces murine resistance to infection with *Listeria* (Buchmeier and Schreiber, 1985), whereas administration of recombinant IFN-γ provides significant protection against *Listeria* challenge (Kiderlen *et al.*, 1984). *In vitro* studies demonstrated the production of MAF/IFN-γ and macrophage inhibitory factor (MIF) by *Listeria*-specific T cells and T cell clones in response to stimulation with specific Ag (Sperling *et al.*, 1984). MIF appears to play an essential role *in vivo* because it influences inflammatory responses which recruit macrophages to the site of infection and it follows that production of both these LKs at the site of infection would have great influence over the ensuing clearance of the bacteria.

More detailed analysis of the role of IFN-γ in immunity to primary and secondary *Listeria* infections was provided by Nakane *et al.* (1989). They reported that intravenous administration of α-IFN-γ MoAb suppressed resistance to *Listeria* when given at day 0 or 1 of infection and inhibited the production of another cytokine, TNF, suggesting IFN-γ may also be involved in the upregulation of TNF production. TNF has also been shown to be important in the immune response to *Listeria* (Havell, 1988; Nakane *et al.*, 1988) and also has a synergistic effect with IFN-γ on various biological activities of macrophages. In secondary listerial infection, suppression of resistance only occurred when α-IFN-γ and α-TNF MoAbs were given together, indicating the importance of both in the clearance of a secondary infection (Chang and Lee, 1986; Esparza *et al.*, 1987).

One mode of action of IFN-γ on bacterial multiplication within macrophages was proposed by Portnoy *et al.* (1989). They found that treatment of macrophages with IFN-γ did not initially alter bacterial killing, but restricted bacterial multiplication within macrophages, in contrast to the considerable bacterial division detected in nonactivated macrophages. Examination by electron microscopy revealed that IFN-γ was preventing access of *Listeria* to the macrophage cytoplasm, an event which has been recently shown to be essential for cell-cell spread of the bacteria (Tilney and Portnoy, 1989). This supported earlier reports that *in vivo* induction of peritoneal

macrophages with IFN-γ did not enhance the intracellular killing of *Lm* (or *Salmonella typhimurium*) *in vitro* (van Dissel *et al.*, 1987).

In 1987, Flesch and Kaufmann confirmed the work done by Rook *et al.* (1986a and 1986b) who observed inhibition of mycobacterial growth by IFN-γ activated human monocytes. They found varying susceptibility to killing by IFN-γ activated macrophages among different strains of *M.tuberculosis*, suggesting that IFN-γ activated macrophages have an important role in the acquisition of resistance to tuberculosis. Evasion of this mechanism may enhance virulence.

1.3.8 Activation of macrophages by interleukin 6 (IL 6)

IFN-γ is not the only LK able to enhance macrophage activity. More recently, IL 6 has been shown to activate the anti-mycobacterial activity of bone marrow derived macrophages *in vitro* (Flesch and Kaufmann, 1990). IL 6 is produced by a wide range of cells, including macrophages, T cells, fibroblasts and endothelial cells (Kishimoto and Hirano, 1988; O'Garra *et al.*, 1988) and acts on a variety of cells. A role for IL 6 in conjunction with IL 1, in augmenting Ag presentation has also been proposed (reviewed by Wong and Clark, 1988). As infection of macrophages with *Mycobacteria* induced IL 6 secretion by macrophages, Flesch and Kaufmann (1990) proposed that a T cell independent autocrine activation system may exist, where infection of macrophages induces production of IL 6, which in turn activates the infected macrophages to kill the bacteria. IL 6 preferentially activates infected macrophages, whilst IFN-γ acts on noninfected macrophages. Consequently, IL 6 and IFN–γ may both be necessary for the optimum protection at the site of infection (Flesch and Kaufmann, 1990).

1.3.9 Regulation of the expression of MHC products on macrophages

1.3.9.1 Introduction

One of the characteristics accompanying macrophage activation is the

increased expression of MHC molecules on the surface of these cells. A variety of stimuli have been reported to induce the enhanced expression of MHC molecules (particularly Class II) on macrophages. These include IBPs, bacterial lipopolysaccharide (LPS) and IFN–γ. Modulation of the cell surface expression of MHC molecules may have significant affects on the Ag presenting ability of these cells and possibly on the types of T cells induced. Upregulation of the expression of Class I or Class II MHC molecules could result in preferential induction of either $Lyt2^+/CD8^+$ T cells or $L3T4^+/CD4^+$ T cells, respectively. However, virtually all work has concentrated on the modulation of Class II MHC expression, as at the time of most these studies, it was generally accepted that Ag presentation to T cells required MHC Class II^+ accessory cells, implicating $L3T4^+/CD4^+$ T cells as the major T cells induced.

1.3.9.2 Structure of Class I MHC molecules

Studies by Bjorkman *et al.* (1987) on the HLA A2 molecule provided information on the detailed structure of the Class I MHC molecules. Class I MHC molecules are made up of a glycoprotein heavy chain and a non-covalently bound light chain, β_2-microglobulin. The heavy chain has an extracellular region containing Ag peptide binding sites, an hydrophobically charged transmembrane region and an hydrophilic cytoplasmic region. The hydrophobic transmembrane region is believed to form an α-helical conformation transversing the membrane. Among the hydrophobic residues is a cluster of highly positively charged residues positioned such that they can interact with negatively charged inner membrane phospholipid headgroups, thereby assisting in anchoring the molecule to the membrane. It is proposed that phosphorylation of serine residues on the cytoplasmic region has a role in signal transmission between the MHC molecule and some intracytoplasmic mediators.

The extacellular region has three domains, α_1, α_2 and α_3, each consisting of an anti-parallel β-pleated sheet arrangement. The β_2-microglobulin, also with the anti-parallel β-pleated sheet arrangement, interacts with the α_3 domain. The anti-parallel β-pleated sheets of the α_1 and α_2 domains are spanned by an α-helical region. The

domains are arranged such that a β-sheet topped by α-helical regions forms for each domain. The large groove found between the α-helices of the α_1 and α_2 domains provides the putative binding site for the processed Ag peptides.

Association with the β_2-microglobulin and peptide is necessary for the stabilization and subsequent expression of the Class I molecules on the cell surface (Townsend *et al.*, 1989 and 1990; Schumacher *et al.*, 1990). The finding that additional β_2-microglobulin molecules enhanced the binding of peptide to both soluble Class I MHC molecules and Class I molecules on cells, suggested that β_2-microglobulin may have a role in augmenting the formation of Ag-Class I MHC complexes necessary for T cell activation (Kozlowski *et al.*, 1991). Similarly, in 1990 Rock *et al.* reported that peptide pulsing of H-2K^{b+} cells was enhanced by β_2-micrgolobulin. Therefore, the presence of β_2-microglobulin stabilizes the Class I MHC structure, optimizing the binding of antigenic peptides. This is supported by the finding that cells from mice with non-functional β_2-microglobulin genes express little if any Class I MHC products on the cell surface (Williams *et al.*, 1989; Zijlstra *et al.*, 1990).

1.3.9.3 Structure of Class II MHC molecules

Class II MHC molecules are heterodimers consisting of heavy (α) and light (β) glycoprotein chains. Like the Class I MHC molecules, the external structure consists of four domains with each chain of the Class II MHC molecules providing two of these - the so called α_1 and α_2 and the β_1 and β_2 domains. Each chain also has a transmembrane region and a small cytoplasmic domain and a short hydrophylic region which links the extracellular regions to the transmembrane region. In the transmembrane region, as in the Class I molecule, the α and β chains form α-helices and possess a cluster of positively charged residues to assist in anchoring the Class II molecule to the cell membrane.

During assembly of the Class II MHC in the endoplasmic reticulum (ER), the αβ-heterodimeric complex transiently associates with another chain, the invariant chain, forming a heterotrimeric complex (Machamer and Cresswell, 1984; Cresswell

et al., 1987). The proposed structure of the Class II MHC molecule has been based on the studies of the structure of the Class I MHC molecule, assuming the four domains of the Class II MHC molecule associate in a similar manner to the Class I heavy and β_2-microglobulin chains. Such a model uncovered an arrangement which has a cleft between the C-terminal α-helices of the α_1 and β_1 domains, the bottom of which is created by the N-terminal β-strands of the domains, this cleft probably creating the Ag binding site for the Class II MHC molecule.

It has been postulated that association of the αβ heterodimer with the invariant chain in the ER regulates binding of endogenous peptides to the Class II MHC molecules in the ER, and/or targeting the Class II MHC molecules to the endocytic pathway (Koch *et al.*, 1989; Long, 1989). The basic difference between the types of peptides Class I and II MHC molecules will bind, has been proposed as being due to the transient association of the Class II molecules with the invariant chain (Cresswell *et al.*, 1987; Hämmerling and Moreno, 1990). The association of the invariant chain with the αβ heterodimers prevents the binding of endogenous peptides to the Class II molecules, as dissociation of the invariant chain reveals the peptide binding sites on Class II MHC molecules (Roche and Cresswell, 1990; Teyton *et al.*, 1990). Further support for the importance of the invariant chain in the peptide binding was provided by Peterson and Miller (1990) when they found that Class II molecules formed in the absence of the invariant chain possess differently folded peptide binding domains, thereby, influencing the binding ability of some peptides (Stockinger *et al.*, 1989). Dissociation and degradation of the invariant chain from the heterodimers occurs in an acidic cellular compartment (Nowell and Quaranta, 1985; Nguyen *et al.*, 1989). Exogenous Ags are also degraded in acidic endosomal compartments prior to presentation in association with Class II MHC molecules (eg. McCoy *et al.*, 1989). Hence, exposure of the peptide binding sites of Class II MHC molecules in an acidic cellular compartment would ensure that binding of exogenous antigenic peptides would occur and result in subsequent expression of these complexes on the cell surface. Supporting this, Guagliardi *et al.* (1990) found that within two minutes after endocytosis of internalized surface Ig labelled

with gold particles, gold-labelled Igs were detected inside endocytic compartments. These compartments contained all the requirements for Ag processing and presentation, including proteolytic enzymes, Class II MHC molecules and invariant chains. Hence, the Ag "import" and the Class II MHC molecule "export" pathways unite in a peripheral endocytic compartment.

Production and cell surface expression of functional Class II MHC molecules does not appear absolutely dependent on association with the invariant chain, however, it does appear to influence transport of the Class II MHC molecules from the ER and their subsequent association with peptides. Evidence exists for the involvement of the invariant chain in the intracellular transport of Class II molecules (Claesson-Welsh and Peterson, 1985; Miller and Germain, 1986). Contradicting these reports, Sekaly *et al.* (1986) found that in transfected cells not expressing the invariant chains, Class II molecules were still transported from the ER to the cell membrane and these cells were capable of presenting Ag (Sekaly *et al.*, 1986; Peterson and Miller, 1990). Recently, however, detailed analysis of the invariant chains has revealed three structural motifs of the invariant chain which have all been shown to influence intracellular transport of the Class II molecules (Lotteau *et al.*, 1990). By analysis of transfected invariant chain deletion mutants, the signal responsible for directing the complex to the endocytic intracellular compartment was located to the cytoplasmic domain of the invaraint chain (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990). Consequently, although association with the invariant chain is not critical for expression of functional Class II molecules, it does have significant influence over the intracellular location of the Class II and the peptides which these molecules will bind. Association with the invariant chain prevents Class II MHC molecules from binding endogenous Ag, and ensures that once these molecules are transported to the endocytic compartment they will be exposed to a vast array of exogenous antigenic peptides. Recent studies by Roche *et al.* (1991) have demonstrated that the Class II "αβ-invariant chain" complex is a nine subunit transmembrane protein that contains three αβ dimers associated with an invariant chain trimer. They have proposed that the organization of the α- and β-subunits into this

multimeric conformation may have a role in the ability of the invariant chain to inhibit the binding of peptides to Class II molecules and that formation of the nine-chain complex could induce structural changes which ensure the release of the "Class II-invariant chain" complexes for transport to the endosomes.

1.3.9.4 Expression of MHC molecules after exposure to IFN-γ

Although macrophages, like most other cells express Class I MHC molecules, expression of Class II MHC molecules by these cells can be a transient event (Beller and Unanue, 1981). IFN-γ has been found to increase the expression of Class II MHC molecules on a wide range of cell types, including macrophages, endothelial cells, tumour cells and Langerhans cells (Wong *et al.*, 1983), although expression of Class II MHC molecules reportedly differs between macrophage populations (eg. Schwartz *et al.*, 1976; Beller *et al.*, 1980). For instance, Cowing and her colleagues reported that a macrophage population lacking Class II MHC molecules predominated in the peritoneum, whereas MHC Class II^+ macrophages were the major type found in the spleen (Cowing *et al.*, 1978).

Because the importance of MHC products in the induction of immune responses is well documented, it has been postulated that IFN-γ may have an immunoregulatory role via modulation of the expression of Class II MHC molecules on macrophages and other cell types. Substantial evidence is available indicating that modulation of Class II MHC expression on macrophages influences their ability to stimulate T cells (Beller and Unanue, 1982). After activation of T cells *in vivo* the number and percentage of Class II^+ macrophages increases (Beller *et al.*, 1980). Such a response involves an influx of phagocytes, induction of Class II MHC expression (Scher *et al.*, 1982) and is modulated by IFN-γ (Steeg *et al.*, 1982; King and Jones, 1983; Virgin *et al.*, 1985). There are numerous reports that the expression of Class II MHC products is one of the requirements necessary for cells to act as APCs to stimulate T cells. For example, Beller (1984) found that maintenance of macrophages *in vitro* resulted in loss of the ability to activate *Listeria*-specific T cells in parallel with a loss of expression of

Class II molecules. Subsequent incubation of the macrophages with IFN-γ, increased the expression of Class II MHC and restored their Ag presenting ability. Similarly treatment of the macrophage cell line P388D1 with IFN-γ rendered these cells capable of presenting specific Ag to T cells, as assessed by IL 2 release (Zlontik *et al.*, 1983). The mechanism by which IFN-γ induces MHC Class II gene expression reportedly involves either the Ca^{++} dependent pathway (Koide *et al.*, 1988) or the PKC dependent pathway (Fan *et al.*, 1988; Koide *et al.*, 1988), although it has been reported that the induction of expression of Class II MHC genes by IFN–γ appeared independent of the PKC pathway, possibly involving a Ca^{++} dependent process (Celada and Maki, 1991).

1.3.9.5 Modulation of macrophage Class II MHC expression by LPS

Another noninfectious agent which influences the expression of Class II molecules on macrophages is LPS. LPS has wide ranging effects on macrophage function (Morrison and Ryan, 1979), including considerable influence on the expression of MHC products on these cells. In a number of *in vitro* studies, LPS has been recorded as inhibiting the expression of MHC coded molecules. LPS has been shown to inhibit expression of Class II-like molecules by human monocytes and to reduce their Ag presenting ability (Yem and Parmely, 1981). Similarly, LPS inhibited the murine macrophage Class II MHC expression induced by IFN-γ *in vitro* (Steeg *et al.*, 1982).

However, Beller and Unanue, (1980) demonstrated little or no alteration in the expression of Class II MHC molecules on peritoneal exudate macrophages 3-4 days after ip injection of LPS and Ziegler *et al.* (1984) reported that peritoneal macrophages harvested from mice at least 1 week after intraperitoneal administration of LPS had a considerable increase the expression of Class II (I-A, I-E) and Class I (H-2K) MHC coded products and enhanced Ag presenting ability. In contrast to induction of murine macrophage Class II expression by IFN-γ, which is T cell dependent (Beller *et al.*, 1980; Scher *et al.*, 1980; Unanue, 1984), at least one mode of LPS induced Class II MHC expression is reported to be T cell independent. Peritoneal macrophages from athymic nude mice showed increased Class II MHC product expression 1 week after stimulation

with LPS ip (Wentworth and Ziegler, 1987), a treatment which did not induce mature T cell function. Therefore, it is possible that there is at least one T cell independent mechanism able to influence the induction of $L3T4^+/CD4^+$ T cells.

Evidence for a T cell dependent and possibly cooperative role for IFN-γ and LPS in immunoregulation was provided by Le *et al.* in 1986. They reported that LPS induced production of IFN-γ by human peripheral blood mononuclear cells. T cells produced IFN-γ only in the presence of monocytes and IL 2. Furthermore, they found that LPS induced IL 1 production by the monocytes and elevated expression of IL 2 receptors on the T cells, and proposed that this accounted for the monocyte and IL 2 dependent production of IFN-γ by the T cells. Hence, *in vivo* stimulation with LPS may have the additional affect of inducing IFN-γ production by T cells, which together induce increased expression of Class II MHC molecules. Therefore, on infection with IBPs any LPS of the bacteria may play a direct role in the initial enhancement of Class II MHC expression on macrophages prior to activation of T cells to release other factors, like IFN-γ, which can further enhance the expression of MHC products and also activate these cells.

1.3.9.6 Macrophage Class II MHC expression after infection with IBPs

Not surprisingly, infectious agents like IBPs have been found to induce the expression of Class II MHC molecules on macrophages. In 1980, Beller *et al.* discovered that ip infection of mice with *Listeria* caused a considerable increase in Class II^+ peritoneal macrophages, particularly after secondary challenge of immune mice with heat-killed *Listeria*. Confirmation that induction of Class II MHC expression by macrophages following injection of *Listeria* was T cell dependent, was provided by Lu *et al.* (1981). Similarly, infection of mice with *Mycobacteria* has been reported to cause an increase in macrophage Class II MHC (I-A and I-E) expression (Kaye and Feldmann, 1986). However, this response to *Mycobacteria* was longer lasting than that to *Listeria* and not dependent on the viability of the organisms. The increased expression of Class II MHC molecules on macrophages correlated with their enhanced ability to stimulate T cell

proliferation *in vitro* in response to specific Ag. Interestingly, the majority of cells showing increased levels of expression of Class II products did not contain intracellular organisms. Further examination revealed that live *Mycobacteria* and to a lesser extent killed bacteria, suppressed IFN-γ induced Class II MHC expression by macrophages in a dose dependent manner (Kaye *et al.*, 1986). Therefore, infections with at least some IBPs have significant effects on Class II MHC expression by macrophages and may play an important role in the modulation of the T cell responses to these bacteria.

There is also evidence for IBPs inducing the expression of Class II MHC products by macrophages in a T cell independent manner. In 1987, Wentworth and Ziegler discovered that ip infection of athymic nude mice with live *Lm* resulted in a dramatic increase in the expression of Class II MHC molecules on peritoneal macrophages, and showed that the development of mature T cell function was not responsible for the induction of Class II expression. Similarly in mice with the severe combined immunodeficiency (*scid*) mutation, which possess no detectable T cell functions (Bosma *et al.*, 1983; Dorshkind *et al.*, 1984), infection with *Listeria* induced Class II MHC expression and a slight increase in bactericidal activity in macrophages (Bancroft *et al.*, 1986). Pretreatment of *scid* mice with an α-IFN-γ MoAb abolished all *Listeria* induced Class II MHC expression *in vivo*, demonstrating that IFN-γ was the mediator inducing Class II Ag expression (Bancroft *et al.*, 1987). *In vivo* α-IFN-γ treatment also resulted in an increase in the bacterial growth. Therefore, production of IFN-γ in a T cell independent manner appears to be important in the induction of Class II^+ macrophages and activation of macrophages, enhancing the resistance to *Listeria*. It is yet to be established whether in mice with a normal complement of T cells, the T cell independent and T cell dependent mechanisms both contribute to modulation of macrophage Class II MHC expression and whether this is actually necessary for induction of an effective immune response to IBP infection.

1.4 Antigen processing and presentation

1.4.1 Introduction

The manner of Ag presentation and the type of Ag being presented have substantial influence over the type of immune response induced, as outlined in Section 1.2.5.1. An explanation for the detection of mainly Class II restricted T cell responses in the early work, is that mainly exogenous Ags were employed, thereby biasing the responses which could be induced. This view is supported by the data provided by Ramila *et al.* (1985) and confirmed by the more recent reports that endogenously derived Ags and IBPs can be presented in association with Class I MHC molecules to induce Class I restricted $CD8^+$ T cell responses.

Class I and Class II MHC molecules expressed on the surface of APCs bind peptides which can be presented to T cells. T cell receptors have been reported to bind processed Ag presented in association with either Class II or Class I MHC products (Unanue, 1984; Townsend and Bodmer, 1989, respectively). Earlier work demonstrated the importance of the products encoded by the H2-I region of the MHC (Class II MHC molecules) in T cell priming *in vivo* (Rosenthal and Shevach, 1973) and *in vitro* the interaction between macrophages and primed T cells was initially shown to be H-2I (Class II) restricted (Erb and Feldman, 1975), requiring Ag to be presented in association with Class II MHC molecules (Kappler and Marrack, 1976; Schwartz, 1985a and 1985b). In 1981, Germain reported that T cell activation requires Ag presentation by metabolically active APCs, which provide the Ag in association with the Class II MHC molecules and a second, nonspecific signal. This is consistent with earlier suggestions of a "two signal" model of *in vitro* lymphocyte activation (eg. Lafferty and Cunningham, 1975) and provided a satisfactory explanation for the need for "processed" Ag on APCs, which could also deliver the second signal, to stimulate T cells.

As stated before, the division of T cells into subsets on the basis of expression of various surface markers, also represents a division based on the context in which they recognize Ag. $CD4^+$/$L3T4^+$ T cells bind Ag presented in association with

Class II MHC molecules (eg. Unanue and Allen, 1987), whilst Ag presented bound to Class I MHC molecules is recognized by $CD8^+/Lyt2^+$ T cells (Townsend and Bodmer, 1989). It has also been established that the Ag peptides binding to Class I MHC molecules are largely of endogenous origin (Townsend *et al.*, 1989), while Class II MHC molecules preferentially bind exogenously derived Ag peptides (Yewdell and Bennink, 1990).

Using the appropriate Ags has enabled the detection of Class I restricted T cell responses. Class I restricted "recognition" of viral proteins on the surface of virally infected APCs by T cells has been reported (Morein *et al.*, 1979; Parham, 1984; Morrison *et al.*, 1986). In 1986, Townsend and his colleagues showed that T cells could recognize antigenic peptides presented by APCs in association with Class I MHC molecules. Similarly, APCs transfected with the ovalbumin (OVA) gene and consequently endogenously producing OVA, induced OVA-specific, Class I restricted CTLs *in vivo* (Moore *et al.*, 1988). Predigested OVA peptides were also found to stimulate specific CTLs *in vivo* and *in vitro* (Carbone *et al.*, 1988; Moore *et al.*, 1988).

1.4.2 Separate pathways for Class I or Class II presentation of Ags

With the knowledge that antigenic peptides can be presented in association with either Class I or Class II MHC molecules, it was of interest to determine whether the acquisition of the antigenic peptides by the MHC molceules occurred via the same or separate pathways. It is possible that both Class I and Class II Ag processing pathways are required to ensure the "presentation" of both endogenous and exogenous antigenic peptides. Considerable data has been provided showing that processing and presentation of the Ag on the surface of the APC in association with Class II MHC molecules are required for effective stimulation of T cells (Grey and Chestnut, 1985). For example, evidence exists for the recognition of linear sequential determinants on the primary protein structure of the Ag, protein fragments or denatured Ag by Class II restricted T cells (Ellner *et al.*, 1977; Shimonkevitz *et al.*, 1984; Streicher *et al.*, 1984), suggesting that for recognition of Ag by H-2I (Class II) restricted T cells, accessory cells must take

up, sequester and re-express the antigenic peptides in association with the Class II molecules on the cell membrane. Whole body UV irradiation of mice was found to result in a defect in the ability of adherent spleen cells to induce T cell activation (Letvin *et al.*, 1980). This functional defect correlated with changes in the ability of irradiated animals to mount DTH responses and primary *in vitro* plaque forming cell (PFC) responses to T cell dependent Ags. Similarly, Weinberger *et al.* in 1981 reported that Ag processing was needed to induce helper T cells for a CTL response, and showed that UV irradiated splenic adherent cells could not stimulate such a response. Further support for the presentation of Ag by metabolically active APC capable of processing the Ag was provided by Falo *et al.* (1985). In 1983, Shimonkevitz *et al.* reported that enzymatically degraded proteins could be presented by metabolically inactive cells expressing the appropriate Class II MHC molecules.

Work carried out on the affect of the lysosomotropic drugs, chloroquine and ammonium chloride, on APC function revealed that they interfere with the generation of mature surface Class II MHC molecules (Nowell and Quaranta, 1985). Because these drugs raise the pH of intracellular compartments and interfere with the degradation of endocytosed material (eg. Ziegler and Unanue, 1982), this suggested that the pathway for processing Ag and Class II molecule biosynthesis are similar, or that the reduction in Ag presenting ability simply represented a decrease in Class II MHC Ag required for effective presentation of foreign Ag. Confirming the importance of the endocytic pathway for presentation of Ag in association with Class II MHC molecules, McCoy *et al.* (1989) reported that cell mutants which fail to acidify endosomes were deficient in their capacity to process and present Ag. In addition, Ag processing was blocked by treatment with drugs, such as leupeptin, which are known to inhibit cysteine acid proteases (Streicher *et al.*, 1984; Buus and Werdelin, 1986). Endosomes and lysosomes had been previously identified as the two cellular compartments possessing acidic pH value and protease activity (Helenius *et al.*, 1983; Seglen, 1983; Diment and Stahl, 1985), supporting the idea that processing of Ag for presentation with Class II MHC occurs in an endocytic compartment.

There is substantial evidence that processing of exogenous Ag in an endocytic pathway is required for subsequent presentation of the antigenic determinants in association with Class II MHC molecules on the APC surface. Endogenously synthesized proteins, however, can also be presented by Class II MHC molecules. These include a variety of viral and histocompatibility Ags (Jacobson *et al.*, 1989; Chen *et al.*, 1990; Jaraquemada *et al.*, 1990; Nuchtern *et al.*, 1990). Further examination of the presentation of endogenous Ags in association with Class II MHC molecules revealed the existence of at least two distinct processing pathways. Class II presentation of certain endogenous Ags, which were sensitive to chloroquine, was less sensitive to treatment with Brefeldin A, a compound known to inhibit Class I presentation. Class II presentation also required a greater Ag load than was needed for presentation by Class I molecules (Jaraquemada *et al.*, 1990). Together, these results implied the existence of another Ag processing pathway besides the endocytic pathway.

The "classical" evidence for the existence of a separate processing pathway for Class I presentation was provided by Townsend *et al.* (1986). They were able to show that T cells could recognize antigenic peptides presented by APCs in association with Class I MHC molecules. Further evidence for the presentation of endogenously derived Ag in association with Class I MHC molecules was provided by Morrison *et al.* (1988) and Townsend and Bodmer (1989). In 1986, Morrison *et al.* had reported that Class II, but not Class I, restricted presentation of influenza Ag was inhibited by chloroquine treatment, whilst treatment of the cells with the protein synthesis inhibitor emetine, completely abrogated Class I restricted presentation, but not Class II presentation. Similarly, presentation of cytoplasmic Ag in association with Class I MHC molecules was inhibited by Brefeldin A, which interfers with normal vesicular traffic between the ER and the golgi (Nuchtern *et al.*, 1989; Yewdell and Bennink, 1989). Clearly, in addition to the endocytic pathway followed by Class II presented Ags, there is a separate cytoplasmic pathway which Class I presented Ags appear to follow. This is further supported by work presented by Neefjes *et al.* in 1990, which demonstrated that Class I MHC molecules do not encounter the endocytic compartment during export from

the ER to the cell surface. It is believed that the difference in the manner of acquisition of antigenic peptides by the Class I and Class II MHC molecules may reside in their structural assembly and subsequent transport to the cellular membrane.

Furthermore, evidence that Ag processing is required for the induction of an immune response to *Listeria* was provided by Ziegler and Unanue (1982), as the ability of macrophages to present specific Ag to Class II restricted T cells was inhibited by treatment with chloroquine or ammonium chloride. Intracellular processing of *Lm* was reported to be sensitive to the lysosomotropic drugs and that such processing after internalization of the IBPs was essential for providing the immunogenic molecule to be recognized by the T cells (Allen and Unanue, 1984). Hence, impaired degradation within endosomes of macrophages correlated with a reduction in the Ag presenting ability of these cells and was interpreted as suggesting that fragmentation or processing of the Ag is required for presentation of the appropriate antigenic determinants by the APC.

1.4.3 Cellular heterogeneity of APC populations

APC populations encompass a very heterogeneous group of cells, including macrophages (Beller and Unanue, 1981), dendritic cells (DCs) (Steinman *et al.*, 1986), B cells (eg. Chestnut and Grey, 1986), endothelial cells (Pober *et al.*. 1986) and Langerhans cells (Streilein and Bergstresser, 1984; Stingl *et al.* 1989). Considerable evidence is available demonstrating the capacity of macrophages and DCs in presenting Ags to T cells, however the efficiency of these cells to behave as APCs is dependent on the assay system used (Unanue, 1984; Ramila *et al.*, 1985; Macatonia *et al.*, 1989). Although all APCs express Class I MHC products, the expression Class II MHC products by these cells is regarded as highly significant since upregulation of the expression of Class II MHC molecules has been associated with increased Ag presenting ability. However, as described earlier, whether Ag is presented in association with Class I or Class II MHC products appears to be largely determined by the origin of the Ag (*ie.* endogenous or exogenous Ag).

1.4.3.1 Dendritic cells as APCs

DCs have been reported to be potent stimulators of *in vitro* primary allogeneic T cell responses and secondary Ag-specific T cell responses to small soluble peptide Ags. Class II$^+$ DCs can present soluble Ag to T cells and this response is under *Ir* gene control (Sunshine *et al.*, 1980). Strong evidence for the role of DCs in the induction of primary MLR was provided by Steinman *et al.* (1986). They proposed that Class II$^+$ DCs were exclusively controlling the induction of primary MLR, as treatment with α-DC MoAb, 33D1 and complement killed only 1% of the population yet reduced the Ag presenting ability by approximately 80%. In addition, DCs have been reported to be extremely potent stimulators of both CD4$^+$ and CD8$^+$ T cells (Inaba *et al.*, 1987).

In 1986, Kapsenberg *et al.* discovered that DCs alone could not present OVA to primed helper T cells to activate them to induce B cells to secrete Ab and required the presence of macrophages. This is the type of evidence which has indicated that DCs are not the only APCs and that the APC population may represent a heterogeneous group of cells. Guidos *et al.* (1984) found that splenic DCs were less effective at processing and presenting *Corynebacterium parvum* than macrophages, whilst both presented a soluble Ag equally well. Consequently, it has been been suggested that DCs have an important role in presenting small soluble Ags and macrophages have a major role in the processing and presentation of particulate or large soluble Ags to T cells.

DCs have also been shown to be extremely successful in stimulating Class I MHC restricted CD8$^+$ T cells. DCs effectively stimulated an allogeneic CD8$^+$ CTL response, in the absence of CD4$^+$ T cell help, whilst macrophages did not activate allogeneic CTLs (Inaba *et al.*, 1987). Similarly, purified populations of DCs induced allogeneic and male Ag-specific Class I MHC restricted CD8$^+$ CTL responses, almost entirely independent of CD4$^+$ T cells (Boog *et al.*, 1988). The Ag presenting function was found to reside in the DC subpopulation expressing the J11d$^-$ phenotype (Sprent and Schaefer, 1989). Ramila *et al.* (1985), showed that although DCs and macrophages could both stimulate Ag-specific T cell proliferation, only macrophages

could activate specific helper T cells that helped B cells to produce specific Ab.

1.4.3.2 Macrophages as APCs

It was initially believed that macrophages had a purely phagocytic role, but it has since been shown that they possess the capacity to process and present Ag to T cells (Möller, 1978; Unanue, 1981 and 1984). Critical to the Ag presenting capacity of macrophages, is the presentation of foreign Ags in the context of MHC molecules, providing the first specific signal (Schwartz, 1985a and 1985b) and to provide secondary nonspecific signals, such as IL 1 and IL 6 (as described earlier).

MHC Class II^+ macrophages have been widely reported as able to present Ags to Class II MHC restricted T cells (eg. Minami *et al.*, 1980; Sunshine *et al.*, 1982; Guidos *et al.*, 1984). For instance, in 1977, Yamashita and Shevach reported that Class II^+ porcine macrophages possessed Ag presenting ability. Class II MHC^+ murine peritoneal exudate macrophages were demonstrated to induce an *in vitro* primary MLR, the magnitude of which reflected the expression of Class II MHC molecules on these cells (Beller and Unanue, 1981). In addition to stimulating allogeneic responses, Class II^+ macrophages have been shown to present specific Ags to primed T cells *in vitro*, stimulating T cell proliferation (Kaye and Feldmann, 1986).

In contrast to reports from Inaba *et al.* (1987), who found that DCs but not macrophages stimulated considerable allo-Ag specific $CD8^+$ T cell responses, preliminary evidence for the activation of Class I restricted $CD8^+$ T cells by MHC Class II^- macrophages has been provided by Sprent and Schaefer (1988). They showed that depletion of $J11d^+$, $Thy1^+$ and MHC Class II^+ cells from spleen cell suspensions provided a population of APCs which were extremely strong stimulators $CD8^+$ T cells. Characterization of these APCs revealed that at least some of the cells were macrophage-like. Thioglycollate induced peritoneal exudate cells have also been reported to contain APC with potent stimulatory activity for $CD8^+$ T cells, again residing in the $J11d^-$, MHC Class II^- subset of cells (Sprent and Schaefer, 1989 and 1990). Approximately 99.5% of these cells were typical esterase positive macrophages and at

low doses showed comparable APC potency to DCs.

An explanation for the discrepancy with Inaba's (Inaba *et al.*, 1987) work may lie in the "type" of macrophage used. *In vitro* cultured exudate cells or fresh peritoneal macrophages lack the ability to stimulate $CD8^+$ T cells, whilst induced peritoneal exudate cells have potent APC function for $CD8^+$ T cells. A noticeable difference is that induced exudate cells are of recent bone marrow origin, whereas the majority of resident peritoneal cells are long lived. Accordingly, Sprent and Schaefer (1989 and 1990) proposed that the Ag presenting function of macrophages for $CD8^+$ T cells may reside in the "young" subpopulation of these cells.

1.4.3.3 Other APCs

As stated above, in addition to macrophages and DCs, a wide variety of cell types have been shown to present Ags to primed T cells. The significance of these various cell types as APCs appears to depend on the type of response being induced, the type of Ag used to elicit the response and also the anatomical location of the cells. Consequently, although these cells possess the ability to stimulate T cells, their relevance in inducing T cells required for immunity to bacterial infections may be questioned since most have only been shown to present Ag to primed T cells.

(i) APCs in the skin

It is likely that the type of cells initially presenting Ag to the immune system plays an important role in determining the strength, type and duration of the immune response being induced (Britz *et al.*, 1982). Exposure of the skin of mice to low amounts of UV irradiation results in the inability to sensitize these mice with contact sensitizing reagents applied to the site of irradiation, although activation of suppressor T cells does occur (Granstein *et al.*, 1984). Hence, the murine epidermis has APCs which are resistant to, or are modulated by UV irradiation and are able induce suppressor T cells. Similarly, effects of UV exposure have been reported with human skin (Baadsgaard *et al.*, 1990). UV exposed epidermal APC activated the suppressor-inducer

$CD4^+$ T cell subset, $CD45RA^+$, known to assist in the activation of $CD8^+$ suppressor T cells. Removal of either T cell subset abolished the suppressive activity.

Based mainly on *in vivo* studies where it was found that Langerhans cells are important in the induction of immunity to cutaneous Ags, it is generally accepted that Langerhans cells are the main APCs in mammalian skin (Streilein and Bergstresser, 1984; Stingl *et al.*, 1989). Studies of the Ag presenting capabilities of Langerhans cells *in vitro* have demonstrated that after 2-3 days *in vitro* cultured Langerhans cells show greater ability to induce MLR than uncultured or fresh Langerhans cells (Shimada *et al.*, 1987). This increase in Ag presenting capacity correlated with an increase in Class II MHC expression on the surface of cultured Langerhans cells.

Further work revealed that there were distinct functional differences between fresh and cultured Langerhans cells with respect to their ablility to present either foreign Ags or allo-Ags to T cells (Streilein and Grammer, 1989). They found that only cultured cells were able to induce autologous T cell proliferation and that cultured cells were more effective in stimulating allogeneic T cell proliferation, whilst fresh Langerhans cells were more efficient at processing and presentation of OVA to an OVA-specific T cell hybridoma.

(ii) B cells as APCs

Under more restricted conditions, B cells have been shown to act as APCs both *in vitro* and *in vivo* (eg. Schwartz *et al.*, 1980; Chestnut and Grey, 1986). In 1982, Glimcher *et al.* found that a B cell lymphoma could present allo-Ags and a specific protein Ag to T cells, in an MHC restricted manner. Diminished Ag presenting ability of a B cell lymphoma expressing truncated Class II MHC molecules was reported by Nabavi *et al.* (1989), indicating the importance of Class II MHC Ag expression for the stimulation of at least some T cell responses.

The role for B cells in an APC capacity *in vivo* was indicated by Janeway *et al.* (1987), when they showed that the APCs responsible for initiating a T cell proliferative response in peripheral lymph nodes were B cells. In support of B cells

having a role as APCs *in vivo*, injection of pure B cell populations into B cell depleted or irradiated mice restored T cell priming in the lymph nodes of these mice (Ron and Sprent, 1987). However, using a sensitive T helper assay *in vitro*, they detected low but significant T cell function in B cell depleted lymph nodes, suggesting all APC activity had not been removed and that the T cells were primed. They proposed that non-B cell APCs may have initiated the T cell responses but that B cells were responsible for expansion of the T cell populations.

Further *in vitro* studies reinforced the role of B cells as APCs. Gosselin *et al.* (1988) reported that resting B cells were capable of presenting rabbit anti-mouse Ig to Ag-specific T cell lines. Whilst, in 1988 Grammer and her colleagues found that activated, but not resting, B cells were able to stimulate the generation of secondary Herpes simplex virus-specific CTLs. Therefore, it seems obvious that B cells must be able to present Ag to Th cells, at least, to ensure that they receive the secondary signals they require to be activated or to differentiate.

(iii) Other types of APCs

A variety of non-lymphoid cells have also been reported to have APC function. These include murine thyroid-derived epithelial cell lines (Stein and Stadecker, 1987; Gaulton *et al.*, 1989), astrocytes (Fontana *et al.*, 1984), human melanoma cell lines (Alexander *et al.*, 1989) and fibroblasts (Umetsu *et al.*, 1985). The murine mastocytoma, P815, which expresses only Class I MHC molecules, has been shown to have strong APC function assayed by stimulation of Class I restricted allogeneic $CD8^+$ T cell proliferation and CTL induction (Sprent and Schaefer, 1986).

There is even evidence for Ag presentation by human T cells. Activation of human T cells *in vitro* (eg. Ko *et al.*, 1979) or *in vivo* (Yu *et al.*, 1980) induces the expression of Class II MHC molecules on these cells. This is also a characterisitic of human T cell clones which have been shown to be able to present specific peptide Ags in a dose dependent, Class II MHC restricted manner, as assessed by the T cell proliferation induced (Hewitt and Feldmann, 1989).

1.4.3.4 Summary

A variety of cells are capable of behaving as APCs in particular assay systems. Virtually any of the cell types included in the role of APC have been reported to stimulate either Class I or Class II MHC molecule-specific primary, MLR responses. However, limitations in their Ag presenting abilities become apparent when their ability to present large, particulate Ags is examined. DCs and macrophages seem to be good candidates as APCs for these Ags, although the efficiency of either population as APCs depends on the assay system used (Unanue, 1984; Ramila *et al.*, 1985; Macatonia *et al.*, 1989). DCs are potent stimulators of primary allogeneic responses and can present small soluble Ags to primed T cells, but are less effective than macrophages at processing and presenting large, particulate Ags, such as whole bacteria. Consequently, macrophages are the most likely candidates for the APCs involved in the presentation of bacterial Ags to T cells which results in induction of immunity to IBPs. Macrophages express both Class I and Class II MHC molecules and have the ability to process large, particulate Ags for presentation in association with both these types of MHC products (Möller, 1978; Unanue, 1981 and 1984). The marked effect on function which IBP infection has on macrophages, makes it tempting to infer that these cells play a dual, important, immunomodulatory role by direct control of bacterial multiplication and/or indirectly by modulation of T cell responses.

1.4.4 Antigen processing pathways

1.4.4.1 Class I antigen presentation pathway

Having established that antigenic peptides presented by APCs in association with Class I and Class II MHC molecules are processed via two distinct pathways, it is important to determine what dictates this difference. Endogenously derived Ag is presented in association with Class I MHC molecules (eg. Moore *et al.*, 1988; Yewdell *et al.*, 1988). Peptides bind to Class I MHC molecules in the ER, thereby assisting in the assembly of stable complexes (Townsend *et al.*, 1989), and binding of

peptides to Class I molecules is augmented by the presence of the β_2-microglobulin. These interactions ensure the formation of stable antigenic peptide-Class I complexes (Townsend *et al.*, 1989 and 1990; Rock, *et al.* 1990; Schumacher *et al.*, 1990; Kozlowski *et al.*, 1991). In 1985, Townsend *et al.* postulated that the transport of antigenic peptides from the various cellular compartments to the ER involved a specific transport mechanism. Consistent with this suggestion was the characterization of "regulatory" genes within the MHC which influence the assembly and transport of Class I MHC molecules to the cell surface (Salter and Cresswell, 1986; Powis *et al.*, 1991a). Several groups have provided further support for the existence of a transport system, by defining genes within the MHC homologous to a family of ATP-driven membrane transporters (reviewed by Parham, 1991). Several groups have suggested that the products of two MHC-encoded ATP-binding cassette transporter genes function to deliver peptides, degraded in the cytoplasm by cytoplasmic proteases (Dice, 1990), across the membrane of the ER (Deverson *et al.*, 1990; Spies *et al.*, 1990; Trowsdale *et al.*, 1990). The data of Spies and DeMars (1991), has provided strong evidence for a transporter system, as transfection with one of the putative peptide transporter genes cures some, but not all, cells which are defective in the expression of Class I MHC molecules. A recent report from Powis *et al.* (1991b) demonstrates that the complete phenotype of the mouse mutant cell line RMA-S, in which lack of surface expression of stable Class I molecules correlates with an inability to present viral peptides originating in the cytosol (Townsend *et al.*, 1989 and 1990; Ljunggren *et al.*, 1990; Schumacher *et al.*, 1990 and 1991; Elliot *et al.*, 1991), is repaired by the cDNA of the other transporter gene and they have proposed that for efficient transport of peptides across the ER membrane that the two transporter polypeptides may form a heterodimer. The antigenic peptide-Class I MHC complexes are then transported directly to the cell surface for presentation to the immune system, bypassing the endocytic compartments (Neefjes *et al.*, 1990).

1.4.4.2 Class II antigen presenting pathway

In contrast to the processing pathway for Ags presented in association with

Class I MHC molecules, Class II MHC molecules bind antigenic peptides in an endocytic compartment in the cell periphery, prior to expression on the cell surface. As already mentioned above, assembled Class II MHC dimers associate with the invariant chain in the ER (Machamer and Creswell, 1984) and from here they are transported to acidic endocytic compartments in the cell periphery containing processed Ag (Guagliardi *et al.*, 1990; Neefjes *et al.*, 1990). Association with the invariant chain may influence the intracellular transport of Class II molecules (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990) and prevents the binding of endogenous Ag. This has been proposed as the basis for the difference between the manner of peptide acquisition by Class I and II molecules (Creswell *et al.*, 1987; Hämmerling and Moreno, 1990). Once in the endocytic compartment, the invariant chain dissociates and is degraded (Nowell and Quaranta, 1985; Nguyen *et al.*, 1989), thus revealing the peptide binding site of the Class II molecules (Roche and Creswell, 1990; Teyton *et al.*, 1990), which allows the binding of a wide range of degraded (antigenic) peptides which will be subsequently expressed on the cell surface. This was supported by the findings of Guagliardi *et al.* (1990) who demonstrated that the pathways for Ag uptake and Class II molecule export merged in a peripheral endocytic compartment. Similarly, the fact that targeting the exogenous Ag to the endocytic pathway enhanced the presentation of processed Ag to T cells reinforced this theory (Lanzavecchia, 1985; Pierce *et al.*, 1988; Snider and Segal, 1989).

1.5 Summary

The type of Ag and the manner of Ag processing and presentation by APCs have considerable influence on the T cell subset(s) induced and determine whether T cell-mediated immunity is induced. The understanding of the cellular events which occur during immune responses induced by *Salmonella* infection is incomplete. Analysis of the T cell subsets induced by this infection may provide insights into the relative functional roles they play in immunity to *Salmonella*. Of equal interest is the Ag presenting and processing capacity of APC populations from *Salmonella* immunized

mice, as immunization with live *Salmonella* may modulate the function of normal APCs, enabling them to induce CMI. Furthermore, the capacity of APCs to induce particular T cell subsets may also be affected by infection with live *Salmonella*. Examination of these parameters would assist in clarifying the cellular interactions occurring in the immune response induced as a result of infection with *Salmonella*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

(BALB/c x C57BL/6) F1 (F1), BALB/c, C57BL/6 and CBA mice and semi lop-eared rabbits were obtained from the Central Animal House, University of Adelaide. Mice 8-10 weeks of age, were sex matched in all experiments and normal rabbit serum was used as a source of complement (C).

2.2 Tissue culture media and reagents

To harvest and prepare most cell suspensions, Hanks' balanced salt solution (HBSS) supplemented with 100 μg/ml Streptomycin and 5 μg/ml Penicillin was used. HBSS not supplemented with Streptomycin and Penicillin was also used, where indicated.

For the majority of the cell culture work the culture medium used was RPMI 1640 (Gibco) with 2 mM glutamine, 5 μg/ml Indomethacin, 0.1 mM β mercaptoethanol, 10% heat inactivated foetal calf serum (FCS, Flow Laboratories), 100 μg/ml Streptomycin and 5 μg/ml Penicillin, and this is referred to as culture medium. Culture medium not supplemented with Penicillin and Streptomycin was also employed for certain experiments and 40 μg/ml Gentamycin (Garamycin, Fauldings) was sometimes added in preference to the other antibiotics.

Eagles' Minimal Essential Medium (EMEM, Gibco) containing 0.1 mM β Mercaptoethanol, 26 mM $NaHCO_3$ and 2 mM glutamine was used to prepare ^{3}H-Thymidine ([^{3}H]-TdR).

Dulbecco's modified Eagles' medium (DMEM, Flow Laboratories) was supplemented with 2 mM glutamine, 26 mM $NaHCO_3$, 5 μg/ml Penicillin,

100 μg/ml Streptomycin and 10% FCS (Flow Laboratories) and used for the *in vitro* culture of the P388D1 cell line.

For preparation of phosphate buffered saline (PBS) pH 7.4, NaCl (0.139 M), KCl (0.0027 M), Na_2HPO_4 (0.008 M) and KH_2PO_4 (0.0015 M) were dissolved in milli-Q water, the pH adjusted and the solution subsequently sterilized by autoclaving.

PBS/bovine serum albumin (BSA, 0.1%)/azide (0.1%) solution was prepared by dissolving 1 g BSA (Cytosystems) and 1 g azide in 1 L PBS and supplemented with 10% heat inactivated (20 minutes at 56°C) normal rabbit serum. This solution will be referred to as P/B/A and was used for the preparation of cell suspensions and Ab dilutions for immunofluorescent labelling.

1% and 0.5% paraformaldehyde (PFA) solutions were prepared by dissolving the PFA in sterile PBS, in a steaming waterbath. The solutions were sterilized through a 0.22 μM millipore filter unit (Millipore) and kept at 4°C. The 1% PFA solution was used for resuspending immunofluorescently labelled cells to run on the FACScan (Becton Dickenson) and the 0.5% PFA solution for the fixation of APC populations, before or after Ag pulsing.

Metrizamide solutions (18% and 22% Metrizamide, Nyegaard) were prepared by dissolving centrifugation grade, Metrizamide powder in sterile HBSS. Discontinuous Metrizamide gradients were used for the removal of neutrophils from cell suspensions.

The Mitomycin C (Sigma) stock solution was made to 1 mg/ml in sterile saline and stored in the dark at 4°C. To metabolically inactivate various cell suspensions, Mitomycin C was used at a final concentration of 10 μg/ml.

Giemsa buffer was prepared by dissolving 1 g of a mixture of Na_2HPO_4 (5.447 g) and KH_2PO_4 (4.75 g) in 2 L of milli Q water (pH 7.0).

2.3 Bacteria and bacterial antigens (Ags)

Overnight cultures of *Salmonella enteritidis* 11RX (11RX) or *Salmonella typhimurium* C5 (C5) were prepared by growing single colonies (picked from nutrient agar plates) overnight at 37°C with shaking in 10 ml of nutrient broth. The overnight cultures were diluted 1/10 in nutrient broth and grown for a further 2 1/2 or 3 hours at 37°C with shaking, giving approximately $5x10^8$ or $2x10^9$ live bacteria/ml respectively.

Formalin killed 11RX (F11RX) was prepared as described by Kotlarski *et al.* (1989). F11RX was used in the *in vitro* functional assays and also for some of the *in vivo* assays.

Soluble 11RX Ag (S11RX) was prepared as decribed by Ashley and Kotlarski (1982) and was used in some of the *in vitro* proliferative assays and as an eliciting Ag in some of the DTH studies.

11RX flagellin was prepared from motile 11RX organisms selected by Craigy tube culture. Motile organisms were grown in 10 x 50 ml broths, which after overnight incubation (standing) were used to inoculate 10 x 1 L volumes of nutrient broth in 5 L conical flasks, which were incubated with continuous shaking for a further 3 hours at 37°C and another 1 hour as standing cultures. The bacteria were then harvested by centrifugation at 4K for 15 minutes, washed once in 250 ml sterile saline by centrifugation at 6K for 15 minutes and resuspended in a final volume of 250 ml saline. The flagella were sheared from the bacteria using the ultra-taurax for 1 minute at full speed and the supernatants harvested after centrifugation of this suspension at 8K for 20 minutes were centrifuged again for 1 hour at 36K in an ultra-centrifuge and the pellet resuspended in 25 ml saline (referred to as "crude" 11RX flagellin). To depolymerize the flagellin, 1.25 ml of 1 M HCl (1 in 20 N final) was added to this suspension and incubated for 1 hour at 37°C, with shaking every 20 minutes before centrifugation for 1 hour at 36K and collecting the supernatant. (To neutralize the HCl, 1.25 ml of 1 M NaOH and 100 µl of 0.5 M phosphate buffer (pH 7.4) were added.) Repolymerization of the flagellin was achieved by adding an equal volume of saturated

ammonium sulphate and incubating at 4°C for 2 hours, when the precipitated proteins were harvested by centrifugation at 12K for 20 minutes and resuspended in 25 ml saline. To remove the ammonium sulphate, the suspension was dialysed against 5 L of saline at 4°C overnight and the protein concentration determined using Folin's method and by measuring the OD_{280}. The purity of this material was confirmed by running the sample on a 16% polyacrylamide slab gel with molecular weight markers (Low Molecular Weight Calibration Kit, Pharmacia) (as described by Lugtenberg *et al.*, 1975; see Fig. 2.1).

Lipopolysaccharide (LPS) was prepared from *Salmonella enteritidis* 11RX using the phenol/water extraction method described by Westphal and Jann (1965).

Salmonella enteritidis SA9 (SA9) an 11RX strain which expresses the *E. coli* K99 Ag was constructed by Dr. Stephen Attridge. Briefly, the 11RX strain was first made thymine dependent by selection on trimethoprim, before a plasmid containing the *E coli* gene for thymidylate synthetase and the structural gene for K99 (described by Morona *et al.*, in preparation) was mobilized into this derivative strain using the standard transformation method. [*Salmonella enteritidis* SA5 (SA5) was similarly constructed by transforming the thymine-dependent 11RX strain with a plasmid expressing the gene for thymidylate synthetase, but not the structural gene for K99 (Morona *et al.*, in preparation).] Using an inhibition of ELISA assay, the expression of the K99 protein on the surface of the bacteria was confirmed, with 40 μg of fimbrial Ag being obtained per 10^{10} SA9 organisms (<0.1 μg per 10^{10} SA5 organisms). Log phase cultures of the SA9 grown in CBT broth were used for immunization of F1 mice by ip injection of 10^5 live organisms.

Preparation of K99: A 10 ml CBT broth was inoculated with a single colonly of SA9 grown on CBT agar and incubated for approximately 8 hours at 37°C with shaking before using this broth culture to seed four 30 cm CBT agar plates (containing 250 ml of agar) which were then incubated overnight at 37°C. Bacteria were harvested using 15 ml of PBS (pH 7.4) by gently scraping the agar surface and all suspensions were pooled and washed once by centrifugation at 6K for 10 minutes. The

FIGURE 2.1 Analysis of the purity of 11RX flagellin

The purity of the 11RX flagellin preparation was determined by running an 11RX flagellin sample (and the appropriate controls) on a 16% polyacrylamide slab gel and a photograph of a representative gel is provided. The lanes contained:

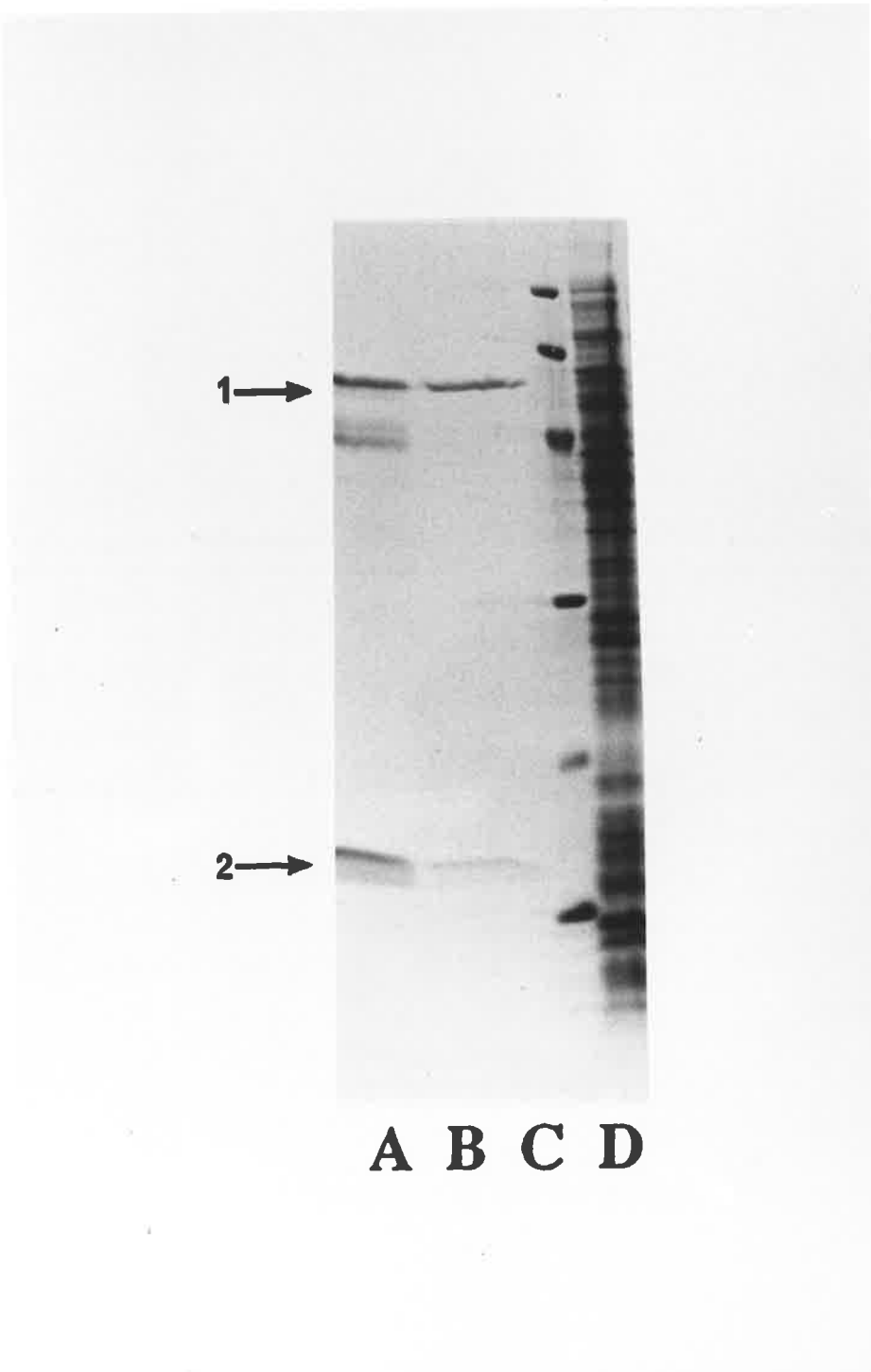
A: "crude" 11RX flagellin

B: 11RX flagellin

C: molecular weight markers (including 94, 67, 43, 30, 20 and 14.4 kDa molecular weight subunits)

D: whole 11RX organisms

"Arrow 1" indicates the 11RX flagellin proteins and "Arrow 2" shows the contaminating low molecular weight proteins.


1
2
A B C D

bacteria were resuspended in 60 ml PBS before being incubated at 65°C for 30 minutes and centrifuged at 7K for 15 minutes. The supernatant was recovered, azide added (at a final dilution of 1 in 5000) and stood overnight at 4°C, after which the aggregated K99 was harvested by centrifugation at 12000 g for 20 minutes at 4°C, resupended in 5 ml PBS and saline pH 7.4 ($P0_4$ 0.01 M) and dialysed against water overnight. K99 was further purified by polyacrylamide gel electrophoresis as described by Johnstone and Thorpe (1982), where 1 ml of this dialysed preparation in 1 ml of 2 times Lugtenberg buffer was run on a 16% linear acrylamide gel and the 17 KD band was carefully cut out of the gel. A slight modification of the procedure used to elute the protein from the acrylamide was used. This involved the addition of 10 ml 0.1 M TRIS/HCl pH 7.1 and 0.1% SDS to the sample, incubation at 37°C overnight and centrifugation at 2500 rpm for 5 minutes before harvesting the supernatant. The K99 was precipitated by adding 20 parts of "precipitin mix" (containing acetone, acetic acid and triethylamine at a ratio of 90:5:5) to 3 parts of the sample and incubating at -20°C for at least 1 hour. The precipitated proteins were harvested by centrifugation at 2500 rpm for 10 minutes, the pellet drained well and dried at 37°C, resolubilized in saline and the amount of protein determined using Folin's method.

2.4 CBT broth and CBT agar

CBT broth and CBT agar were prepared by supplementing 450 ml of milli Q water and 450 ml 1.5% water agar (respectively) with 50 ml of minimal salts [K_2HPO_4 70 g/L, KH_2PO_4 30 g/L, $(NH_4)_2SO_4$ 10 g/L, pH 7.5], 12.5 ml 20% Casamino acids, 5 ml 1% $MgSO_4$, 5 ml tryptophan, 5 ml 50% glucose and 0.5 ml vitamin B1.

2.5 Cell lines

The murine mastocytoma, P815, which expresses class I MHC molecules of the H-2^d haplotype and the T cell lymphoma, EL4, which expresses class I MHC molceules of the H-2^b haplotype, were maintained *in vitro* by continuous passage in RPMI 1640 containing 10% FCS, 5 µg/ml Penicillin and 100 µg/ml Streptomycin, in

25 cm^3 or 75 cm^3 tissue culture flasks (Corning 25100-25 and 25110-75, respectively). The murine macrophage cell line, P388D1, which expresses both class I and class II MHC molecules of the H-2^d haplotype, was maintained *in vitro* by continuous passage in DMEM supplemented with 10% FCS, 5 μg/ml Penicillin and 100 μg/ml Streptomycin, in 75 cm^3 tissue culture flasks (Corning 25110-75).

2.6 Immunization schedules

Mice were immunized with log phase cultures of L11RX, and where indicated live SA9, by intraperitoneal (ip) injection of 10^5 organisms in 200 μl of cold, sterile saline. At various times after the initial challenge cells were harvested from these primed animals and their functions examined. In addition, mice were challenged ip with a secondary dose of either $3x10^4$ C5 3 weeks or $8x10^6$ 11RX 6 weeks after the primary infection with L11RX. Cells from mice receiving secondary challenge with *Salmonella* were also examined.

2.7 Preparation of cell suspensions

Peritoneal cell (PC) suspensions were obtained by washing out the peritoneal cavity of each mouse with 5 ml of HBSS and the washouts from similar mice were pooled, centrifuged at approximately 1500 rpm for 5 minutes in a bench centrifuge (Econospin, Sorvall Instruments), washed once in HBSS, counted and adjusted to the required concentration in culture medium.

Spleen cell (SC) suspensions were prepared as described by Attridge and Kotlarski (1984) and again were washed, counted and adjusted to the appropriate concentration in culture medium.

Mesenteric lymph node cell (MLNC) suspensions were prepared in the same way as SCs, washed, counted and adjusted to the appropriate concentration in culture medium.

2.8 Preparation of adherent monolayers of PC suspensions

To obtain adherent monolayers of PCs, 100 µl aliquots of various PC populations at the appropriate concentration in culture medium were dispensed into the wells of 96-well flat-bottomed trays (Falcon, 3072), each set up in quadruplicate. The trays were incubated for 1 hour at 37°C in an atmosphere of 5-10% CO_2 in air, after which the trays were shaken briefly (Titertek Trayshaker, Flow Laboratories) prior to removing the nonadherent cells. To the remaining adherent cells, 100 µl of warm culture medium were added/well. The nonadherent cells were removed in one of two ways. Firstly, when only the adherent cells of the population were required, the nonadherent cells were carefully removed by suction through a suction line. Alternatively, when both the adherent and nonadherent cells of the population were required, 8 replicate wells were initially set up and the 100 µl of culture medium containing nonadherent cells were removed from half of them with a Gilson and transferred to four empty wells, providing the nonadherent subpopulation of these cells.

2.9 Preparation of nonadherent lymphoid cell suspensions

2.9.1 Adherence to plastic

PC suspensions at a concentration of no more than 10^7 cells/ml in 5 ml of culture medium (ensuring no more than a monolayer of cells would form on each dish) were incubated in sterile 9 cm Costar dishes for 1 hour at 37°C in an atmosphere of 5-10% CO_2 in air. Following the incubation, nonadherent cells were resuspended by gentle shaking of the dish, then transferred to 50 ml sterile, polypropylene centrifuge tubes (Disposable Products, Lab Supply) with a pasteur pipette and the dish was carefully washed with warm culture medium to remove any remaining nonadherent cells. Similar cell suspensions were pooled, centrifuged in a MSE bench centrifuge, resuspended, counted and diluted to the required concentration in culture medium.

2.9.2 Adherence to nylon wool columns

Removal of adherent cells by passage on a nylon wool (NW; Pacific Diagnostics) column was carried out as described by Attridge and Kotlarski (1985a). Briefly, no more than 10^8 cells in 2.5 ml of warm culture medium were carefully loaded onto each NW column. After 1 hour incubation at 37°C in an atmosphere of 5-10% CO_2 in air, the nonadherent cells were harvested by washing through 10-12 ml of warm culture medium into a sterile centrifuge tube (Disposable Products, Lab Supply). After fractionation, like populations were pooled, the cells were washed, counted and adjusted to the appropriate concentration in culture medium.

2.10 Monoclonal antibodies

The monoclonal antibodies (MoAbs) used in this study include: MK-D6, α-Ia^d; 34-4-20S, α-H-$2D^d$; B8-24-3, α-H-$2K^b$; 30-H12, α-Thy1.2; GK1.5, α-L3T4; HO 2.2, α-Lyt2.2; YTS169.4, α-Lyt2. YTS169.4 ascites fluid and tissue culture supernatant were kindly donated by Dr. Tony Simmons, Herpes Research Laboratory, Institute of Medical and Veterinary Science, Adelaide, South Australia. 30-H12, GK1.5 and HO-2.2 were generously donated by Dr. Lindsay Dent, Flinders Medical Centre, Adelaide, South Australia. MK-D6, 34-4-20S and B8-24-3 were purchased from ATCC. MoAbs conjugated to fluorescein isothiocynate (FITC) were purchased from PharMingen and included FITC rat α-mouse Ly-2 (CD8a), FITC rat α-mouse L3T4 (CD4), FITC α-mouse I-A^d and FITC α-mouse H-$2D^d$.

2.11 Monoclonal antibody and complement treatment of cell suspensions

2.11.1 One step treatment of bulk cultures

Cell suspensions at 10^7 cells/ml were placed into 10 ml sterile, conical polystyrene tubes (Disposable Products, Lab Supply) and spun at 1500 rpm for 5 minutes in a bench centrifuge (Econospin, Sorvall Instruments). The supernatants were removed

and the cells resuspended to 10^7 cells/ml in culture medium containing the appropriately diluted MoAb (1 in 10 final dilution) and complement (C; 1 in 20 final dilution). [Normal rabbit serum was used as the source of C for all these studies.] The suspensions were incubated for one hour at 37°C, with shaking every 20 minutes, before the cells were washed with warm culture medium and resuspended to the required concentration in culture medium, assuming no cell loss had occurred.

2.11.2 Treatment in tissue culture trays

Replicate cell suspensions (20-24 wells) were set up and cultured in 96-well flat-bottomed trays as described in Sections 2.16.1 and 2.16.2 and appropriate dilutions of the various MoAbs (1 in 10 final dilution) and C (1 in 20 final dilution), C alone or an equal volume of culture medium, were each added to four replicate wells. The trays were carefully shaken (Titretek Trayshaker, Flow Laboratories) and incubated for 1 hour at 37°C in an atmosphere of 5-10% CO_2 before measuring the amount of [^{3}H]-TdR incorporated during a subsequent 4 hour incubation at 37°C.

2.12 Immunofluorescent labelling of cell suspensions

2.12.1 Indirect immunofluorescence

Cell suspensions were diluted to 10^7 cells/ml in P/B/A and 50 µl aliquots dispensed into conical plastic tubes (Disposable Products, Lab Supply). To each suspension, 50 µl of the appropriate primary MoAb (neat) was added and the suspensions incubated for 1 hour at 4°C (shaking every 20 minutes). At the end of the incubation, the cells were washed twice in 0.5 ml fresh P/B/A, by centrifuging for 5 minutes at 1500 rpm in a bench centrifuge (Econospin, Sorvall Instruments). The cells were resuspended in 50 µl of P/B/A and 50 µl of a 1/10 dilution of SHAM-FITC (Silenus) was added to each suspension and the cells reincubated for 45 minutes at 4°C in the dark (shaking every 15 minutes) before they were washed twice with 0.5 ml fresh P/B/A and resuspended in 1 ml cold 1% PFA solution for analysis on the FACScan (Becton Dickenson).

2.12.2 Direct immunofluorescence

Cell suspensions were adjusted to 10^7 cells/ml in P/B/A and 50 µl aliquots were dispensed into conical plastic tubes (Disposable Products, Lab Supply). 50 µl of 1/100 dilutions of the FITC conjugated MoAbs (FITC-MoAbs, PharMingen) were added, the cells mixed and incubated for 1 hour at 4°C in the dark, with mixing every 20 minutes. The cells were thoroughly washed in 0.5 ml volumes of fresh P/B/A, then resuspended in 1 ml cold, sterile 1% PFA and analysed on the FACScan (Becton Dickenson).

2.13 Fixation of PC populations using paraformaldehyde

PCs were resuspended in 0.5% paraformaldehyde (PFA) solution in 10 ml sterile centrifuge tubes (Disposable Products, Lab Supply) and incubated at 37°C for 4 minutes before an equal volume of culture medium containing 10% FCS (Flow Laboratories) was added and the cells were centrifuged at 1500 rpm for 5 minutes in a bench centrifuge (Econospin, Sorvall Instruments). Each suspension was washed three times in culture medium, resuspended in fresh culture medium and allowed to stand for a further 1 hour at 37°C. Finally, the cells were recounted and adjusted to the appropriate concentration in culture medium.

2.14 Mitomycin C treatment

Cell suspensions at 10^7 cells/ml in culture medium were incubated in the presence 10 µg/ml of Mitomycin C (Sigma) for 1 hour at 37°C, with shaking every 20 minutes. At the end of the one hour incubation, the cell suspensions were washed three times in warm culture medium, counted and adjusted to the appropriate concentration.

2.15 Metrizamide density gradient centrifugation

Removal of polymorphonuclear cells (neutrophils) was achieved by centrifugation of PC populations on Metrizamide (Nyegaard) density gradients. To

create the density gradient, 2 ml of an 18% Metrizamide solution (in sterile HBSS) was carefully layered onto 2 ml of a 22% Metrizamide solution (in sterile HBSS) in a sterile, conical polystyrene tube (Disposable Products, Lab Supply). The PC suspension (containing $2x10^7$ cells) was carefully layered onto the gradient in 2 ml of culture medium and the tube was then centrifuged at 1200 g for 45 minutes in a MSE bench centrifuge. The cells located at the first interface were harvested using a sterile pasteur pipette, transferred to a fresh centrifuge tube (Disposable Products, Lab Supply), washed once, counted and readjusted to the appropriate concentration in culture medium.

2.16 *In vitro* proliferation assays

2.16.1 Using *Salmonella* Ags

Primed T cells (10^5 or $2x10^5$ cells/well) were mixed with accessory cells and specific *Salmonella* Ags [F11RX, S11RX or 11RX flagellin], K99 Ag or the nonspecific T cell mitogen Concanavalin A (ConA, Pharmacia) as a positive control, or with Ag-pulsed accessory cells, in 96-well flat-bottomed trays (Falcon 3072) [and sometimes 96-well round-bottomed trays (Linbro 76-042-05)]. Cell suspensions were set up in quadruplicate with each stimulus and cultured for 3 days at 37°C in a sealed box, in a gas phase of 10% CO_2, 7% O_2 and 83% N_2. On the third day of incubation, the amount of proliferation was measured by adding 50 µl of EMEM containing 1 µCi of ^{3}H-Thymidine ([^{3}H]-TdR, Amersham) to each well and incubating for another 4 hours at 37°C in an atmosphere of 5-10% CO_2 in air. The cells were then harvested onto glass fibre filter discs (Titertek) using a Flow cell harvester (Flow Laboratories) and the amount of radioactivity incorporated by the cells was measured with the aid of a Beta counter (Beckman) using standard procedures. Results are expressed as the cpm (mean ± sem) for each quadruplicate set of cultures. To determine the phenotype(s) of proliferating cells, 20-24 replicate wells were set up in the 96-well trays and treated with MoAbs and C as described in Section 2.11.2.

2.16.2 Using allo-Ags

NW nonadherent MLNCs obtained from BALB/c (H-2^d), C57BL/6 (H-2^b) or CBA (H-2^k) mice were cultured at $2x10^5$ cells/well in quadruplicate with varying numbers of PCs obtained from (BALB/c x C57BL/6) F1 mice (H-$2^{b/d}$) for 5 days at 37°C in 96-well flat-bottomed trays (Falcon 3072) or 24-well flat-bottomed trays (Corning 25820), in a sealed box in a gas phase of 10% CO_2, 7% O_2 and 83% N_2. When MLNCs from CBA mice were used, the F1 PCs were pretreated with Mitomycin C (Sigma) as described in Section 2.14. On the fifth day, the amount of proliferation was determined by adding 50 µl of EMEM containing 1 µCi of [^{3}H]-TdR to each well of a 96-well tray and incubating for a further 4 hours at 37°C in an atmosphere of 5-10% CO_2 in air before harvesting the cells using the Flow cell harvester (Flow Laboratories) onto glass fibre filter discs (Titretek) and measuring the amount of radioactivity incorporated with the aid of a Beta counter (Beckman). Results are expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate set of cultures. Cells cultured in the 24-well trays were harvested, counted and the presence of allo-Ag-specific cytotoxic cells measured using the standard ^{51}Cr release assay, described in Section 2.20.3. The phenotype(s) of proliferating cells was determined as described in Section 2.11.2.

2.17 IL 2 assay

2.17.1 Preparation of samples

Culture conditions employed for the IL 2 assays were similar to those described by Attridge and Kotlarski (1985a). $2x10^6$ primed T cells were cultured at 37°C overnight in the presence of 10 µg/ml F11RX in duplicate 1 ml volumes in 24-well flat-bottomed trays (Corning 25820), to produce culture supernatants. The cell free supernatants were harvested with a sterile pasteur pipette, like samples pooled into sterile 5 ml plastic vials (Disposable Products, Lab Supply) and stored at -20°C until assayed.

2.17.2 IL 2 maintenance assay

This assay measures the ability of the test supernatants to maintain the proliferation of 3 day ConA stimulated T blasts. ConA blasts were prepared by culturing 1 ml volumes normal SCs (10^6 cells/ml) with 1 μg/ml ConA in 24-well flat-bottomed trays (Corning 25820) for 3 days at 37°C in a sealed box, in a gas mix of 10% CO_2, 7% O_2 and 83% N_2. The test supernatants were assayed for the presence of IL 2 activity in duplicate, using 6 two-fold dilutions in culture medium in 96-well flat-bottomed trays (Falcon 3072) , as described by Attridge and Kotlarski (1984). To the diluted samples, 100 μl volumes containing $2.5x10^4$ washed ConA blasts were added/well and incubated overnight at 37°C in an atmosphere of 5-10% CO_2. T cell proliferation was determined by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture at 37°C in the usual way and the results expressed as the units of IL 2 released, calculated as previously described (Attridge and Kotlarski, 1984).

2.18 Nonspecific expansion and/or activation of T cells *in vitro*

T cell populations obtained from PC or SC suspensions harvested from mice immunized ip with *Salmonella*, were cultured at $2x10^6$ cells/ml with 1 μg/ml ConA (Pharmacia) or 10 units/ml recombinant IL 2 (IL 2; Boehringer Mannheim) in 25 cm^3 tissue culture flasks (Corning 25100-25) for 3 days in an atmosphere of 5-10% CO_2. After culture, the numbers of viable cells were determined and the cells resuspended to the concentration(s) required for use in particular assays to evaluate their functional status.

2.19 Delayed type hypersensitivity (DTH)

2.19.1 Transfer of DTH using primed lymphoid cells

PC and SC populations obtained from mice previously immunized with *Salmonella* were used to transfer DTH reactivity locally to normal, unimmunized mice. The T cell suspensions were partially purified by fractionation on plastic petri dishes or

NW columns or further purified by treating NW fractionated cells with α-Ia and C and $1x10^6$ cells were mixed with Ags and 50 µl of each mixture were injected subcutaneously (sc) into the left hind footpads of groups of at least 3 normal mice. Control suspensions of cells alone and Ag alone were also injected sc in 50 µl into the left hind footpads of groups of 3 normal mice. The right and left hind footpads measured 24 hours (and sometimes 48 hours) later using Micrometer dial guage calipers (Model 130, Mercer, England) and the results expressed as the percent increase in footpad size (mean ± sem), calculated with respect to the size of the uninjected right footpad for each group.

2.19.2 Induction of DTH in L11RX immunized mice

Groups of 4-5 normal mice or mice which had been immunized ip with L11RX 2-3 weeks earlier were injected sc in the left hind footpads with 50 µl of culture medium containing either 10 µg S11RX or 10 µg 11RX flagellin and the size of both the right and left footpads measured 24 and 48 hours later using Micrometer dial guage calipers (Model 130, Mercer, England). The percent increase in footpad size was determined as described in the previous section and expressed as the percent footpad swelling (mean ± sem) for each group of mice used.

2.20 *In vitro* cytotoxicity assay

2.20.1 Target cells

Cells employed as target cells in the *in vitro* cytotoxicity assay were: P815, a murine mastocytoma cell line expressing class I MHC Ag of the H-2^d haplotype; EL4, a murine T cell lymphoma expressing class I MHC of the H-2^b haplotype; P388D1, a murine macrophage cell line expressing class I and II MHC of the H-2^d haplotype; and 3 day ConA cultured SC blasts of the H-$2^{d/b}$ haplotype (which were obtained when SCs harvested from normal F1 mice (NSCs) were cultured for 3 days at 10^6 cells/ml with 1 µg/ml ConA in 1 ml volumes in 24-well flat-bottomed trays (Corning 25820), in a sealed box, in a gas mix of 10% CO_2, 7% O_2 and 83% N_2).

2.20.2 Target cell labelling with ^{51}Cr

Target cell suspensions were adjusted to $5x10^6$ cells/ml in culture medium and 0.5 ml aliquots added to sterile, siliconized glass centrifuge tubes. To each suspension, 50 µCi of $Na_2{}^{51}CrO_4$ (Amersham) was added, the suspensions mixed and incubated for 1 hour at 37°C, with shaking every 20 minutes. After the incubation, additional fresh culture medium was added and the suspensions were underlaid with approximately 1.5 ml prewarmed FCS (Flow Laboratories) and centrifuged for 3 minutes at 1500 rpm (Econospin, Sorvall Instruments). The pelleted cells were resuspended in 5 ml fresh culture medium and reincubated for a further 1 hour at 37°C, with shaking every 20 minutes. The suspensions were again underlaid with FCS (Flow Laboratories), centrifuged (Econospin, Sorvall Instruments), the cells resuspended in fresh culture medium, counted and adjusted to $2x10^5$ cells/ml.

2.20.3 *In vitro* ^{51}Cr release assay

The *in vitro* cytotoxicity assay was employed to detect the presence of cytotoxic T cells (CTLs) in various effector cell populations. The effector populations used included *in vivo* primed T cells that were obtained by NW fractionating PC and SC populations obtained from *Salmonella* immunized mice, or *in vitro* stimulated T cell blasts, obtained by culturing *Salmonella*-primed (and unprimed) T cells with ConA or IL 2 (Section 2.18), or *in vitro* allo-Ag primed T cells harvested from 5 day MLR cultures described in Section 2.16.2. The effector cells were harvested, counted and adjusted to $5x10^6$, 10^6 and $2x10^5$ cells/ml in culture medium and 100 µl of each suspension were added in quadruplicate to the wells of a 96-well round-bottomed tray (Linbro 76-042-05), to which 100 µl containing $2x10^4$ ^{51}Cr-labelled target cells were added and the tray incubated for 4 hours at 37°C in an atmosphere of 5-10% CO_2. At the end of the 4 hours, 100 µl of the supernatant from each well were harvested and each transferred to a plastic tube (Disposable Products, Lab Supply) and the amount of radioactivity released into the supernatant was measured (cpm) using a Gamma counter (Packard Auto-Gamma 5650).

Three controls were also included in each assay,

1: 100 µl of ^{51}Cr-labelled target cells with 100 µl of culture medium were added in quadruplicate to the trays to determine the amount of spontaneous release of ^{51}Cr into the supernatants during the 4 hour incubation at 37°C, (spontaneous release values of approximately 5-10% were usually observed).

2: four 100 µl aliquots of ^{51}Cr-labelled target cells were dispensed directly into four plastic tubes and the amount of radioactivity measured to define the total cpm of ^{51}Cr incorporated into the cells.

3: duplicate 1 ml volumes of the ^{51}Cr-labelled target cells were placed into two plastic tubes and two drops of 1% SDS were added to lyse the cells during a subsequent 1 hour incubation at 37°C before centrifuging for 5 minutes at 1500 rpm, splitting the supernatants in half and measuring the amounts of ^{51}Cr present in each, to indicate the total amount of ^{51}Cr that could be released and the amount which remained in cell debris after this treatment. A total release value of 85% was routinely observed and the percent Various concentrations of cytotoxicity was calculated for the experimental cultures using the following equation:

$$\% \text{ Cytotoxicity} = 100 \times \frac{\text{experimental release cpm - spontaneous release cpm}}{\text{total cpm - spontaneous release cpm}}$$

Both lectin-dependent and Ag-specific cytotoxicity assays were used. In lectin-mediated assay, a portion of the ^{51}Cr-labelled target cells was pretreated with the lectin ConA (Pharmacia) during the last 20 minutes of the labelling procedure and the remainder left untreated. To detect *Salmonella* Ag-specific CTLs, target cells were pulsed with killed or live *Salmonella* prior to labelling with ^{51}Cr (Amerham) and incubated with the effector population(s) in the normal way, whilst the specificity of the allo-Ag primed CTLs was determined using target cells which expressed the appropriate MHC coded products.

2.21 Ag pulsing of various cell populations

2.21.1 Pulsing cell lines with killed *Salmonella* Ags

Adherent monolayers of P388D1 in 75 cm^3 tissue culture flasks (Corning 25110-75) were pulsed with 20 µg/ml F11RX overnight at 37°C. The adherent cells were dislodged using a sterile pasteur pipette, transferred to centrifuge tubes, centrifuged for 5 minutes at 1500 rpm (Econospin, Sorvall Instruments). The number of viable cells were counted, adjusted to $5x10^6$ cells/ml in culture medium and labelled with ^{51}Cr (Amersham) following the standard procedure.

To pulse the nonadherent P815, 1 ml containing 10^6 P815 cells was mixed with 200 µl of 10^8 F11RX in a centrifuge tube (Disposable Products, Lab Supply) and centrifuged at 3700 rpm for 10 minutes (Econospin, Sorvall Instruments). The "pelleted cells/bacteria mix" was incubated for 30 minutes at 37°C before resuspending the cells, pooling similar populations and washing thoroughly with culture medium to remove extracellular Ags. The cells were resuspended in culture medium, transferred to sterile 25 cm^3 tissue culture flasks (Corning 25100-25), incubated overnight at 37°C, after which the number of viable cells recovered was determined and the cell concentration adjusted to $5x10^6$ cells/ml for labelling with ^{51}Cr (Amersham).

2.21.2 Pulsing cell lines with live *Salmonella*

To pulse with live *Salmonella*, both adherent and nonadherent cells were adjusted to 10^6 cells/ml in antibiotic free culture medium and 1 ml of cells was mixed with 200 µl of 10^8 bacteria in a 10 ml centrifuge tube (Disposable Products, Lab Supply). [An overnight culture of bacteria was diluted 1/10 in nutrient broth and subcultured for another 2 1/2 hours at 37°C, to obtain log phase bacterial suspensions at $5x10^8$ bacteria/ml. 1 ml aliquots of bacteria were pelleted in an Eppendorf centrifuge and resuspended to $5x10^8$ bacteria/ml in antibiotic free culture medium.] The cell/bacteria mixes were centrifuged for 10 minutes at 3700 rpm (Econospin, Sorvall Instruments), incubated for 30 minutes in a 37°C waterbath, resuspended and like

suspensions pooled. The cells were washed thoroughly in culture medium without antibiotics, to remove any contaminating extracellular bacteria, resuspended to approximately 10^6 cells/ml in culture medium supplemented with 40 μg/ml Gentamycin (Garamycin, Fauldings) and transferred to sterile 25 cm^3 tissue cultutre flasks (Corning 25100-25), in which they were cultured overnight at 37°C. After overnight culture, the cells were washed and adjusted to the required concentration after determining the number of viable cells present.

2.21.3 Ag pulsing of PC populations

2.21.3.1 *In vitro* Ag pulsing

In vitro Ag pulsing was carried out in two ways; in sterile, conical polystyrene tubes (Disposable Products, Lab Supply) or in the 96-well flat-bottomed trays (Falcon 3072). In the tubes, PC suspensions of $5x10^6$ cells/ml in warm culture medium were incubated in the presence or absence of 20 or 100 μg/ml F11RX for 2-3 hours at 37°C, with shaking. After the incubation, the cells were washed 2-3 times, counted and adjusted to the required concentration in culture medium.

In the trays, replicate 100 μl volumes of PC suspensions at the appropriate concentration in warm culture medium containing 10 μg/ml F11RX, were placed into the wells of a 96-well flat-bottomed tray and incubated for 2 hours at 37°C. At the end of the incubation period, the trays were shaken briefly (Titretek Trayshaker, Flow Laboratories) and the medium, containing free Ag and nonadherent cells, was sucked off using a suction line and the Ag-pulsed adherent cell monolayers were washed twice with 100 μl aliquots of fresh, warm culture medium.

2.21.3.2 *In vivo* Ag pulsing

To pulse PCs with Ags *in vivo*, 10 or 250 μg of F11RX or 10^7 L11RX organisms were injected ip into mice and fifteen minutes later the mice were sacrificed and the PCs harvested, as previously described, with PC suspensions from similar mice

being pooled. The PC suspensions were then prepared following the normal procedure described in Section 2.6.

2.22 Preparation of Cytospin cell smears

PC suspensions were diluted to $5x10^5$ cells/ml in FCS, 100 µl loaded into the holders already secured in the Cytospin centrifuge (Shandon Southern) with the slides and centrifuged for 8-12 minutes at setting 60. The slides were then removed, air dried and the smears were fixed in methanol and stained with a 1/25 dilution of Giemsa stain (BDH, Lab Supply) for 10-25 minutes and quickly destained in Giemsa buffer.

2.23 Estimation of protein concentration using Folin's method

A protein standard, BSA, was diluted in water to concentrations ranging from 0.1 mg/ml to 1 mg/ml and duplicate 100 µl aliquots of each dilution, the test sample and the diluent were each added to separate glass test tubes. 2.5 ml of a mixture containing 50 parts of Na_2CO_3 in 0.1M NaOH, 1 part 0.5% $CuSO_4.5H_2O$ and 1% sodium tartrate, were added to each tube and the contents mixed well before being left at room temperature for 15 minutes. 250 µl of Folin and Ciocalteus phenol reagent which had been diluted 1 in 3 in water were added to each tube, the tubes mixed once more and 30 minutes later the absorbance at 650 nm was measured for each sample. To determine the amount of protein present, the absorbance of the test sample was compared to that of the standards.

CHAPTER 3

CHARACTERIZATION OF PRIMED POPULATIONS OF RESPONDING T CELLS

3.1 Introduction

To study the T cells and APCs induced following infection of mice with *Salmonella*, appropriate assay systems had to be established to evaluate their functions. Parameters commonly used to assess the functional capacity of primed T cells include their ability to mediate resistance to bacterial infection, DTH reactivity to specific Ags, lysis of specific target cells and ability to proliferate and release LKs *in vitro* in response to antigenic stimuli (see Chapter 1, Section 1.2.5). The function of APCs is usually investigated by assaying their ability to induce purified suspensions of T cells to release LKs and/or to proliferate when these cell populations are cultured *in vitro* together with Ags or mitogens (Chapter 1).

Obviously, induction of primary Ag-specific T cell responses would provide the most accurate measure of the functional capabilities of particular APC populations. However, the standard assay systems which have been developed use primed T cells as the responding populations, because most unprimed T cells are not readily stimulated by Ags *in vitro*. In other words, the requirements for initial stimulation of T cells appear to be more stringent than those needed for secondary stimulation of primed T cells and may reflect the involvement of various ill-defined secondary signals in T cell priming. *In vitro* responses of T cells to allo-Ags provide the most obvious exceptions to this "rule" - primary responses to these Ags are easily induced *in vitro* and are known as MLRs (e.g. Wagner *et al.*, 1972; MacDonald *et al.*, 1973). While the induction of a primary MLR may provide some information about MHC

molecules and the secondary signals that different APC populations used for stimulation can deliver (Sunshine *et al.*, 1982), more information about the Ag processing and presentation capacity of APCs would be gained by examining their ability to present other foreign Ags to syngeneic, unprimed T cells.

It is interesting to note that such an approach has been used successfully by a number of groups. For example, in 1988, Carbone *et al.* were able to elicit strong, Ag-specific, MHC restricted CTL responses by culturing normal spleen cells *in vitro* for 5 days together with defined antigenic peptide fragments of purified proteins. Use of peptide fragments probably ensured that a relatively high density of *identical* antigenic determinants could be presented on APCs. The importance of this for induction of primary T cell responses *in vitro* is indicated by the work of others who have succeeded in inducing strong primary T cell responses *in vitro* using various well defined Ags (eg. Bevan, 1984; Owen *et al.*, 1990). It follows that failure to induce primary T cell responses *in vitro* with more complex Ags may reflect the inability of APCs to process such Ags and/or to present a relatively high density of any individual antigenic determinant on their surface. [Whether this means that the "wrong" APCs are being studied *in vitro* or that tissue culture conditions alter APC function has not been addressed.] The possibility that the functional T cell subsets stimulated is determined by the particular secondary signals that an APC can deliver adds another level of complication to the *in vitro* study of primary T cell responses.

At the commencement of these studies the *Salmonella* Ags available were formalin killed preparations of 11RX and C5 (F11RX and FC5), a soluble extract of 11RX (S11RX), 11RX flagellin (11RX flag) and live 11RX and C5 organisms (L11RX and LC5). With the exception of flagellin, they are all complex Ags and therefore unlikely to elicit *in vitro* responses from normal, unprimed T cells. Hence, *in vivo* primed T cells were used as the responding populations in studies designed to compare the functional activity of APC populations harvested from mice immunized with L11RX with those obtained from normal, unimmunized mice. Development of an efficient method of removing APCs from primed T cell populations was essential, to ensure that

Ag presentation occurred via the APCs added to T cell cultures and not by APCs contaminating the T cell populations.

It was evident that large numbers of mice would have to be used to provide the lymphoid cells needed. Accordingly, initial studies were designed to define the most appropriate approach which would guarantee a reliable source of purified T cells primed with *Salmonella* Ags while keeping the numbers of animals needed to a minimum. This Chapter describes these investigations, the methods used to remove APCs from these populations and a preliminary characterization of the primed T cells.

3.2 Results

3.2.1 IPCs as a source of primed T cells

3.2.1.1 Initial characterization of PCs

PCs obtained from mice immunized with L11RX ip (IPCs) have been found to contain primed T cells which can mediate DTH reactivity and can be induced to proliferate and release LKs in response to specific Ags *in vitro* (Attridge and Kotlarski, 1985a and 1985b). A more detailed analysis of IPCs obtained at various times following infection with L11RX was carried out to establish the quality of this population as a reliable source of primed T cells and part of this study has already been published (Kotlarski *et al.*, 1989).

At particular timepoints after infection the IPCs were harvested and the yields from each mouse were recorded. Fig. 3.1 shows the PC yields obtained per mouse (average of at least 5 mice) and illustrates the initial increase in yield with time after infection. Between days 8-15 a maximum of approximately $4\text{-}5\text{x}10^7$ cells/mouse were harvested. After this the yields gradually decreased and by day 28 were comparable to those harvested from normal, unimmunized mice ($5\text{x}10^6$ cells/mouse). Hence, between days 8-15 after immunization fewer mice were required to obtain a standard number of IPCs than the number needed earlier or later after immunization.

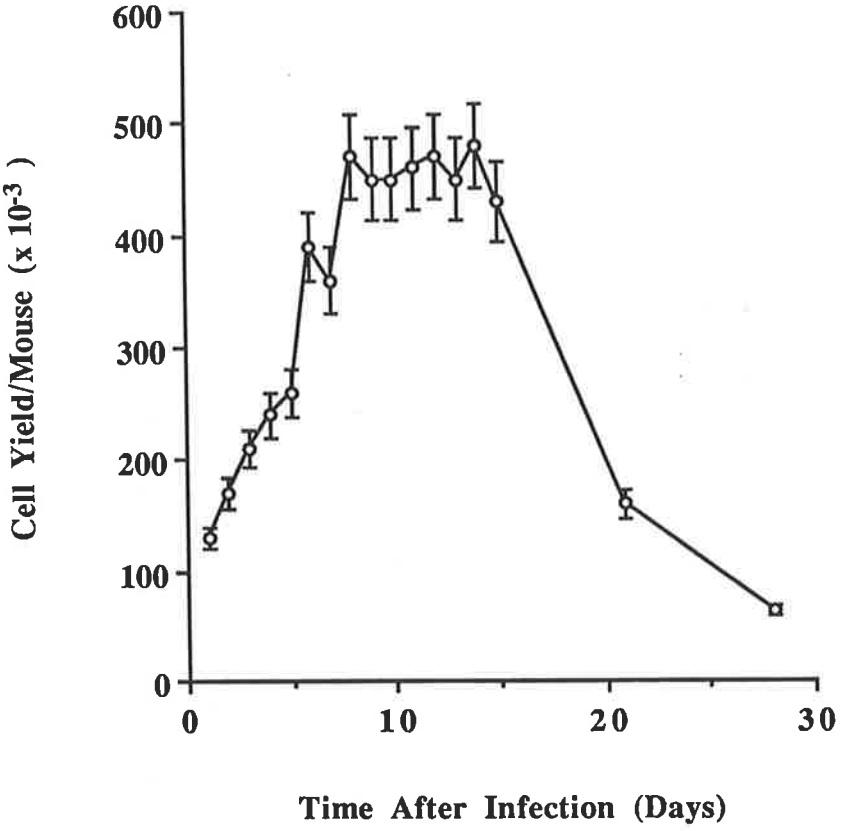


FIGURE 3.1 PC yields after immunization with L11RX

The PCs were harvested from 5 animals at various timepoints after ip immunization with 10^5 L11RX, the numbers recovered were recorded from each animal and the data are presented as the cell yield per mouse (mean ± sem) at each timepoint.

Cytospin smears of at least three samples of IPCs harvested at all the time points were prepared, stained with Giemsa, viewed under oil immersion microscopy and photographed. The various cell types present in each smear were identified and their numbers recorded. The results obtained with smears from mice 14 and 56 days after infection with L11RX (D 14 and 56 IPCs) and PCs from normal mice (NPCs) are presented in Table 3.1 and serve to illustrate the considerable heterogeneity of the D 14 IPCs compared to NPCs and D 56 IPCs. Following infection with L11RX there was a vast increase in the proportion of neutrophils and a relative decrease in the percentage of lymphocytes in PC suspensions, presumably reflecting an ongoing acute inflammatory response. By day 56 the IPCs were more homogeneous, containing virtually no neutrophils and approximately equal numbers of macrophages and lymphocytes (Fig. 3.2 [A-C]). [It is important to note that the fractionated, nonadherent IPCs which could be induced to proliferate and release LKs *in vitro* (Attridge and Kotlarski, 1985a and 1985b) contained few macrophages and neutrophils (Table 3.3 and Fig. 3.3)].

It is now well established that to stimulate T cells of IBP-infected animals to proliferate *in vitro* adherent cells must be removed prior to culture (Orbach-Arbouys and Poupon, 1978; Riglar and Cheers, 1980; Brett, 1984; Deschennes *et al.*, 1986; Kotlarski *et al.*, 1989). Table 3.2 shows data confirming these observations with IPCs of L11RX immunized mice. Unfractionated IPCs (U IPCs) were unable to proliferate in response to doses of various *in vitro* stimuli which induced significant proliferative responses by NW nonadherent IPCs (NW IPCs). This was shown by culturing U IPCs and NW IPCs *in vitro* for 3 days with a range of doses of a variety of stimuli, in the presence and absence of NPCs as a source of additional APCs. On the third day of culture proliferation was measured by the amount of [^{3}H]-TdR incorporated by the cells over a 4 hour period at 37°C. Clearly, U IPCs could not be stimulated by either Ag or T or B cell mitogenic stimuli, even in the presence of additional APCs, whereas NW IPCs proliferated in response to all the stimuli used. Addition of NPCs enhanced the proliferative responses to Ag, indicating that NW fractionation produced

TABLE 3.1 Cell profiles of PCs obtained from normal F1 mice and F1 mice immunized with L11RX

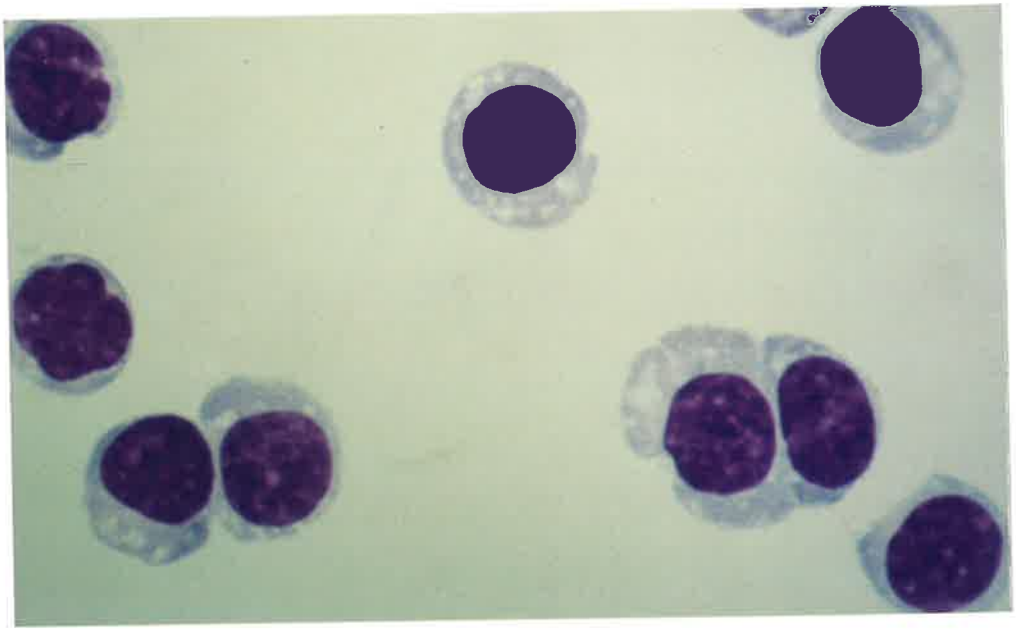
	Percent (mean ± sem) of each cell type*:				
Population:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
NPCs	34 ± 1.1	63 ± 1.4	1 ± 0.2	1 ± 0	1 ± 0.3
D 14 IPCs	33 ± 1.5	23 ± 0.8	43 ± 2.5	1 ± 0.1	0 ± 0
D 56 IPCs	46 ± 2	49 ± 1.9	4 ± 0.7	1 ± 0.4	0 ± 0

* Cytospin smears of NPCs, D 14 IPCs and D 56 IPCs were prepared from 3-5 animals, stained with Giemsa and examined using oil immersion microscopy. A total of at least 200 cells (per smear) were counted, the proportions of the various cell types identified recorded and the percent (mean ± sem) represented by each cell type was calculated.

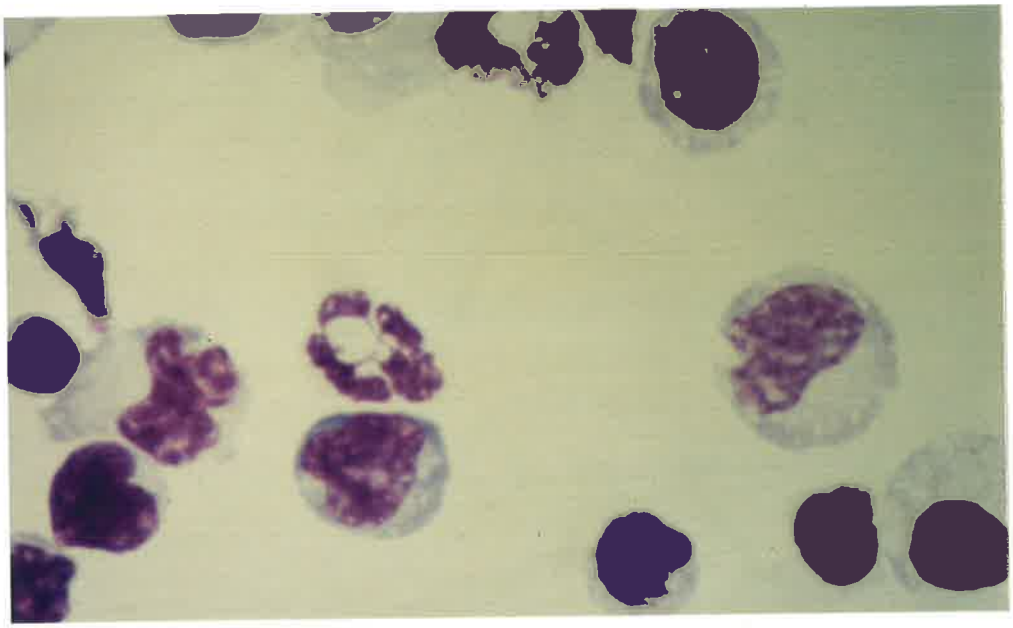
FIGURE 3.2 Cell profiles of PCs from normal mice and mice ip immunized with L11RX

Cytospin smears of NPCs, D 14 IPCs and D 56 IPCs were stained with Giemsa stain and studied under oil immersion microscopy. Representative populations were photographed at 1000 times magnification and examples are presented showing typical populations of NPCs (A), D 14 IPCs (B) and D 56 IPCs (C).

A



B



C

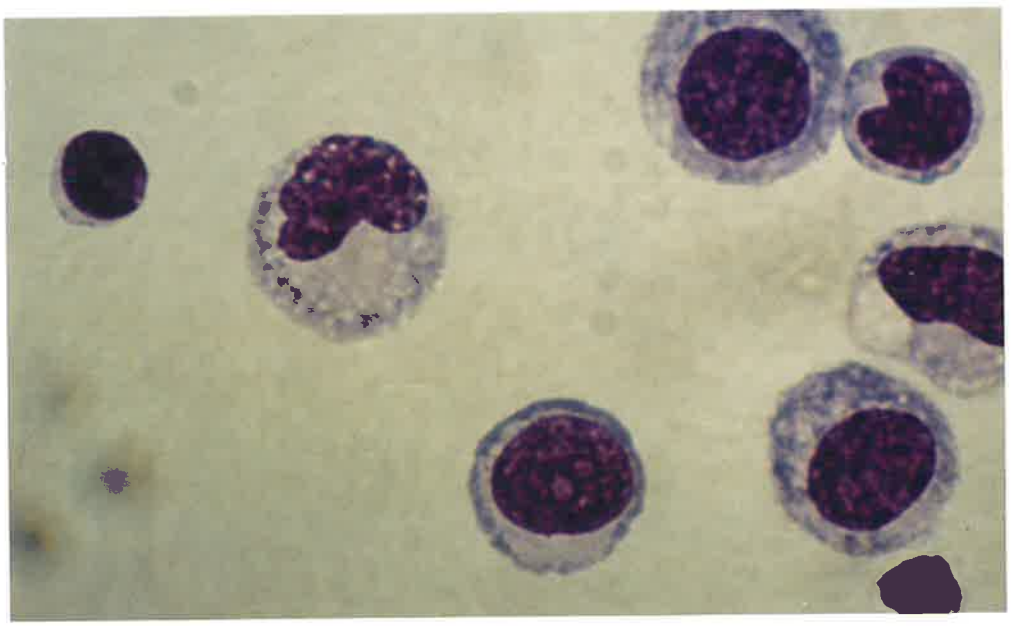


TABLE 3.2 Proliferative responses by unfractionated versus NW fractionated IPCs

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with various stimuli ± 2x10^4 NPCs:			
		U IPCs		NW IPCs	
Stimulus	Stimulus dose (µg/ml)	- NPCs	+ NPCs	- NPCs	+ NPCs
F11RX	1	194 ± 25	178 ± 36	136 318 ± 3969	164 505 ± 4805
	10	953 ± 167	721 ± 68	89 652 ± 3550	134 730 ± 7853
	100	8227 ± 470	23 357 ± 1195	35 368 ± 4433	62 164 ± 7719
S11RX	2	248 ± 38	205 ± 23	27 828 ± 388	42 083 ± 3078
	20	342 ± 94	210 ± 27	44 002 ± 3048	69 535 ± 1796
	100	2225 ± 389	259 ± 50	4637 ± 334	18 907 ± 3474
ConA	0.3	508 ± 139	210 ± 14	25 114 ± 2272	124 219 ± 1757
	1	227 ± 25	170 ± 25	127 893 ± 3513	120 706 ± 15 099
	3	179 ± 28	182 ± 23	154 582 ± 4757	160 977 ± 4097
11RX LPS	10	296 ±44	ND#	20 021 ± 329	ND#

* U IPCs and NW IPCs were used as a source of primed T cells and 10^5 cells of each suspension cultured for 3 days with varying concentrations of F11RX, S11RX, ConA and 11RX LPS, in the presence or absence of NPCs (each combination assayed in quadruplicate). On the third day of culture, proliferation was determined by measuring the amount of [^{3}H]-TdR taken up by these cells and the results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each quadruplicate set.

ND : not done

IPCs were harvested from mice immunized with 11RX 14 days earlier.

IPC suspensions which contained limiting numbers of APCs. Not surprisingly, examination of Cytospin smears of the NW IPCs revealed that they were more homogeneous than the U IPCs, containing mainly lymphocytes (Table 3.3 and Fig. 3.3)

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It was interesting to observe that, despite differences in their ability to respond to Ags and mitogen in the proliferative assays, both U IPCs and NW IPCs released comparable levels of IL 2. To induce IL 2 release, $2x10^6$ U IPCs and NW IPCs were mixed with 10 μg/ml F11RX and cultured for 20 hours at 37°C. The test supernatants were harvested from these cultures and assayed for the presence of IL 2 which was detected by the ability of the test supernatants to maintain the proliferation of day 3 ConA induced SC blasts overnight at 37°C. Proliferation of the ConA blasts was assessed by measuring the amount of [^{3}H]-TdR incorporated by the cells during the final 4 hours of culture at 37°C and the amounts of IL 2 released was calculated in the normal manner. Table 3.4 shows an example of the levels of IL 2 released by U IPCs and NW IPCs after overnight stimulation with F11RX and illustrates the fact that U IPCs were consistently found to release greater amounts of IL 2 than NW IPCs, even when additional APCs were added to the fractionated cell suspensions. Hence, the inability to respond to various stimuli *in vitro* only applies to proliferative responses of these cells.

3.2.1.2 Removal of neutrophils using Metrizamide density gradient centrifugation

Since NW fractionation of IPCs yielded populations of nonadherent cells which were responsive to Ags and T cell mitogens, it was likely that either the macrophages or neutrophils present in U IPC suspensions (or both cell of these types) prevented cell proliferation. The possibility that neutrophils were the cells mainly or entirely responsible for this inhibition was investigated by determining the effect of selectively removing virtually all neutrophils from the U IPC population with Metrizamide gradients instead of NW, which removes both macrophages and neutrophils

The technique described by Lopez *et al.* (1983) was adapted for this purpose. Aliquots of $2x10^7$ PCs were loaded onto hypertonic Metrizamide gradients,

TABLE 3.3 Differential cell counts of U IPCs and NW IPCs

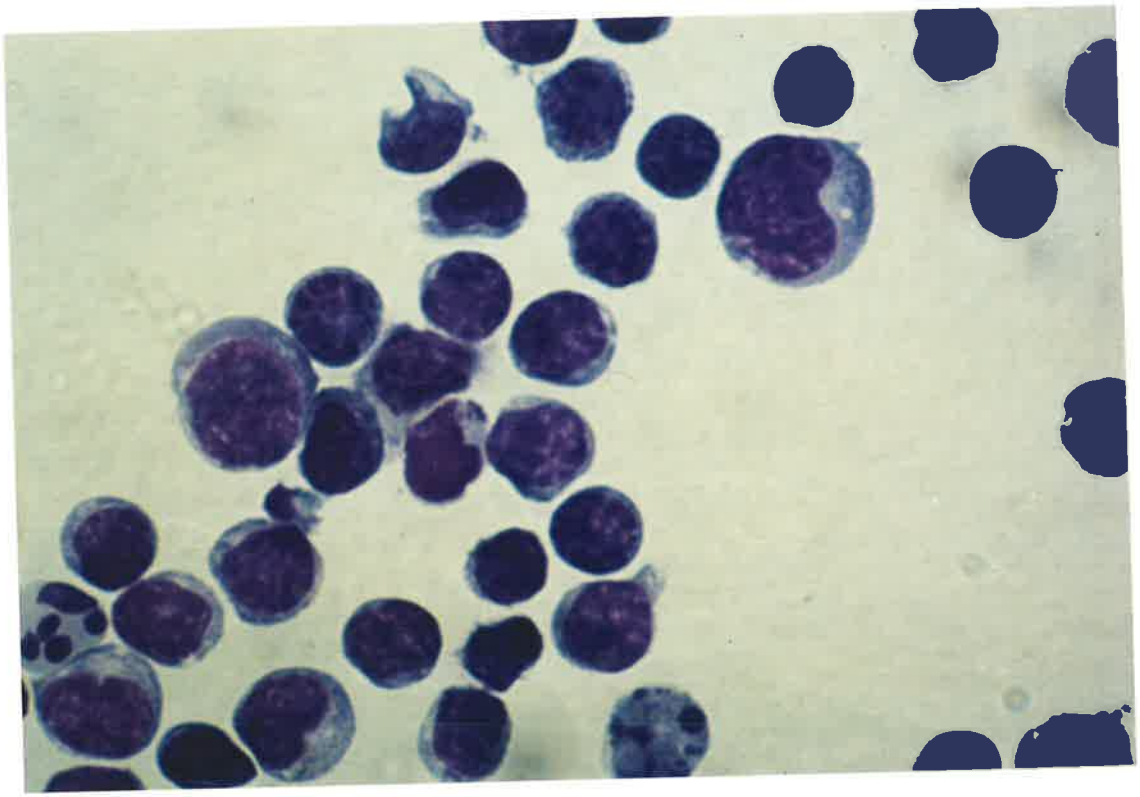
	Percent (mean ± sem) of each cell type*:				
Population:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
U IPCs	33 ± 1.2	23 ± 0.9	43 ± 2.3	1 ± 0.1	0 ± 0
NW IPCs	15 ± 0.5	81 ± 2.1	4 ± 0.3	0 ± 0	0 ± 0

* Cytospin smears of U IPCs and NW IPCs were prepared, stained with Giemsa stain and viewed under oil immersion. A total of approximately 200 cells (per smear) were counted and from the numbers of the particular cell types identified, the percent (mean ± sem) represented by each type was determined and typical data are presented.

FIGURE 3.3 Examination of the homogeneity of NW IPCs

IPCs harvested from mice 14 days after ip injection of L11RX were fractionated on NW columns, Cytospin smears prepared, stained and viewed using oil immersion microscopy. Photographs taken at 1000 times (A) and 400 times (B) magnification illustrate the characteristic, more homogeneous population obtained following NW fractionation.

A



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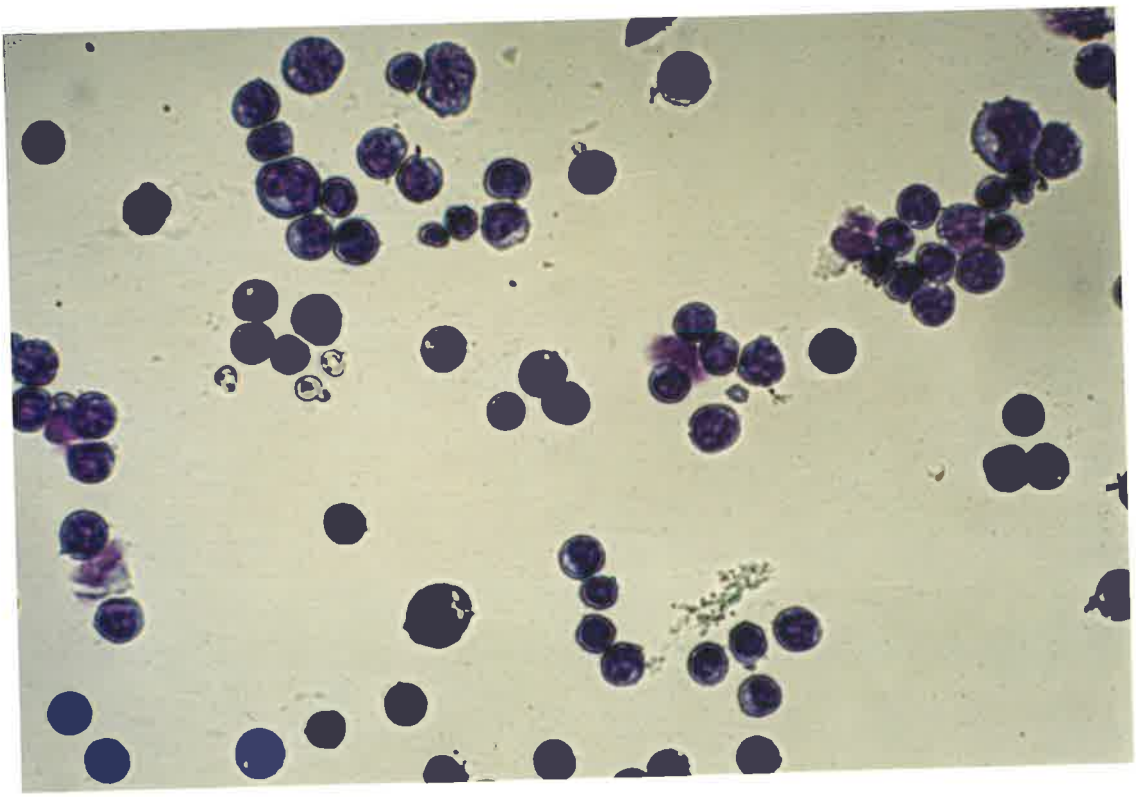


TABLE 3.4 IL 2 released by unfractionated and NW fractionated D 14 IPCs

	Units of IL 2 released by primed T cells* after overnight culture with:	
Primed T cell source:	F11RX	F11RX + NPCs
U IPCs	84	72
NW IPCs	30	50

* $2x10^6$ U IPCs or NW IPCs were mixed with F11RX in the presence or absence of additional $2x10^5$ NPCs and incubated in 1 ml volumes at 37°C in an atmosphere of 5% CO_2 overnight. Cell free supernatants were then harvested and like samples pooled. The presence of IL 2 in the supernatants was determined by their ability to maintain the proliferative responses of 3 day ConA blasts and the titres were calculated as described in the Materials and Methods.

consisting of an 18% Metrizamide top layer and a 22% Metrizamide bottom layer and after centrifugation for 45 minutes at 1200 g the cells remaining at the first, low density interface were harvested. To determine the efficiency of removal of the neutrophils, Cytospin smears of these suspensions were made, stained with Giemsa and viewed under oil immersion microscopy. Differential cell counts were made and expressed as the percentages of the total population. They established that this procedure reduced neutrophil contamination by approximately 95% (Table 3.5 and Fig. 3.4). However, examination of the cells harvested from the second interface of the Metrizamide gradient and the cell pellet revealed that some loss of lymphocytes and macrophages from the first layer had occured, although these losses were not as large as those regularly obtained using NW fractionation, where most of the macrophages and a significant portion of lymphocytes were removed (data not shown).

The *in vitro* responsiveness of the cells harvested from the first interface of the Metrizamide gradient was determined by culturing these cells with 1 μg/ml F11RX or ConA in the presence or absence of NPCs, for 3 days at 37°C. Following the 3 days incubation, the amount of proliferation was measured by the amount of [^{3}H]-TdR incorporated during the final 4 hour period at 37°C. Table 3.6 contains representative data from several experiments which demonstrated that the removal of the majority of neutrophils had no effect on unresponsiveness of U IPCs. Even in the absence of neutrophils no proliferative responses could be induced, suggesting that the most likely candidates as cells with inhibitory activity were (activated) macrophages.

Similar conclusions have been made using *Listeria* immune PCs. Removal of adherent cells from such populations was reported to be essential for induction of proliferation *in vitro* in response to listerial Ags (Jungi, 1980). The macrophages present in unfractionated PC suspensions were deemed to be responsible for this inhibition, although no direct evidence for their involvement was provided and the mechanism responsible for inhibition was not defined. The possibility that macrophages were responsible for the rapid and complete degradation of Ag was dismissed because increasing the concentration of listerial Ags had no effect on the responsiveness of

TABLE 3.5 Efficiency of removal of neutrophils by Metrizamide density gradient centrifugation

	Percent (mean ± sem) of each cell type*:				
Population:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
U IPCs	33 ± 0.7	23 ± 1.1	43 ± 1.4	1 ± 0.3	0 ± 0
Metrizamide U IPCs#	67 ± 2.3	30 ± 1.5	3 ± 0.5	0 ± 0	0 ± 0

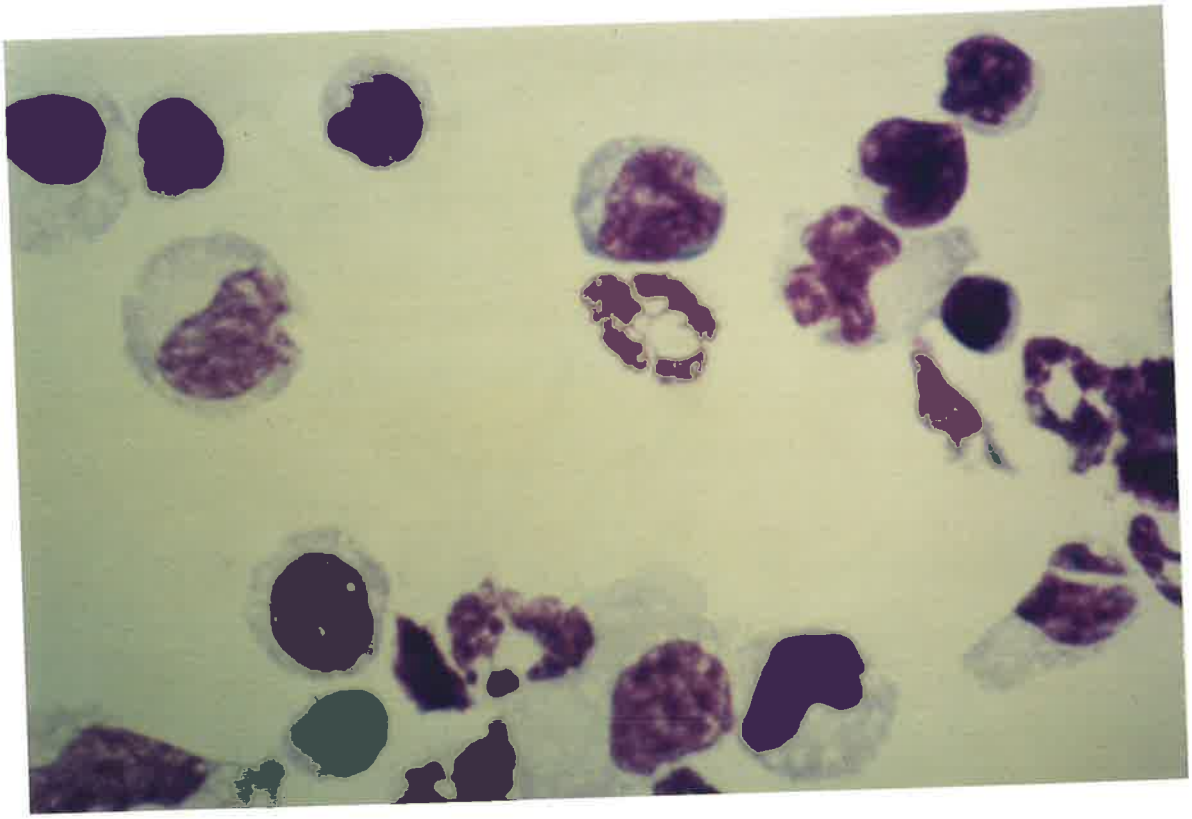
D 14 IPCs were fractionated on a discontinuous Metrizamide density gradient and the cells at the first, low density interface were harvested and are referred to as Metrizamide U IPCs.

* Cytospin smears of four preparations of U IPCs and Metrizamide U IPCs were prepared, stained with Giemsa and studied using oil immersion microscopy. Approximately 200 cells were counted (per smear) and the percent (mean ± sem) of each cell type identified was calculated for each smear.

FIGURE 3.4 Effect of Metrizamide density gradient centrifugation on the cell prolfiles of D 14 IPCs

D 14 IPCs were fractionated on a Metrizamide density gradient (Metrizamide fractionated U IPCs), or left untreated (U IPCs) and Cytospin smears of each population were prepared and stained with Giemsa. Photographs taken at 1000 times magnification show the typical populations present in U IPCs (A) and Metrizamide fractionated U IPCs (B).

A



B

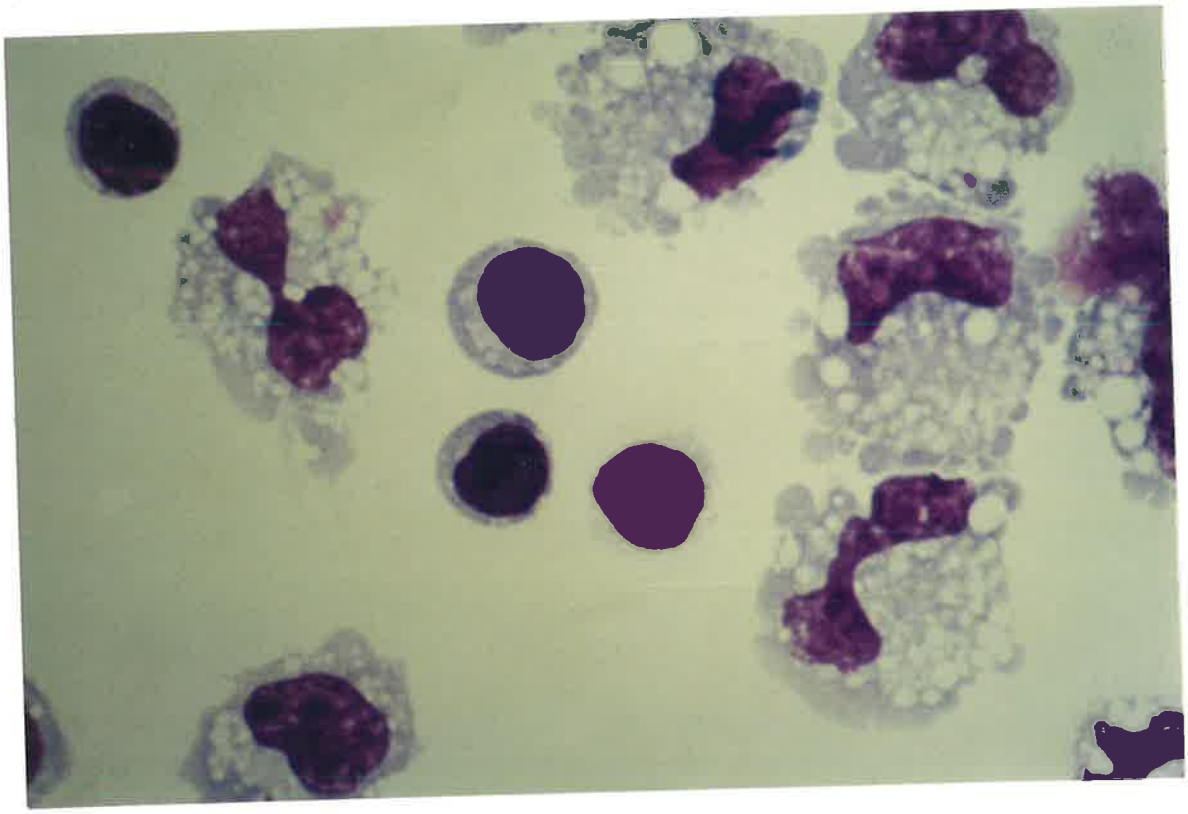


TABLE 3.6 Effect of fractionation on Metrizamide density gradients on the *in vitro* proliferative responses of unfractionated D 14 IPCs

		[^{3}H]-TdR uptake (cpm ± sem) by U IPCs* after 3 days of culture with F11RX ± 2x10^4 NPCs	
Cells Cultured:	F11RX (µg/ml)	- NPCs	+ NPCs
Untreated U IPCs	1	186 ± 14	302 ± 54
	1	3549 ± 564	4245 ± 628
	100	7296 ± 841	30 842 ± 17820
Metrizamide* U IPCs	1	3872 ± 125	2368 ± 302
	10	11 879 ± 935	19495 ± 1047
	100	1025 ± 103	3979 ± 999

* Unfractionated D 14 IPCs (U IPCs) were fractionated or not on a discontinuous Metrizamide density gradient (referred to as: Metrizamide U IPCs and Untreated U IPCs, respectively) and their ability to proliferate was tested. Each suspension (10^5 cells) was cultured in quadruplicate with varying concentrations of F11RX for 3 days at 37°C, before assessing the proliferation by measuring the amount of [^{3}H]-TdR incorporated in the final 4 hours of culture. Results are expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each quadruplicate set.

unfractionated immune PCs.

3.2.1.3 Summary

It was possible to induce NW IPCs, but not U IPCs, to proliferate *in vitro* in response to *Salmonella* Ags and the T cell mitogen ConA. APC function in the NW fractionated IPC populations was somewhat limiting and could be enhanced by the addition of adherent NPCs. In contrast, U IPCs could be induced to release somewhat larger amounts of IL 2 than NW IPCs. The significance of the latter finding has not been examined, but could indicate that NW fractionation removes some of the T cells able to release IL 2.

3.2.2 ISCs as a source of immune T cells

Another potential source of L11RX primed T cells was SCs prepared from mice previously immunized with L11RX (ISCs). Since appproximately $2x10^8$ cells could be obtained from the spleens of immunized mice taken within a few weeks of infection, such an approach appeared to offer the obvious advantage of reducing the numbers of animals needed to obtain large numbers of primed T cells. Accordingly, the ability of ISCs to proliferate in response to F11RX and ConA and to release IL 2 after stimulation with F11RX were examined with the same assay systems used for IPCs. The observations made from this series of experiments revealed an obvious and sometimes confusing difference between the responses of IPCs and ISCs. Table 3.7 provides data showing the proliferative responses of U ISCs in response to ConA (1 μg/ml). NW fractionation of ISCs reduced proliferative responses to ConA but these could be restored by addition of NPCs (data not shown). However, the proliferative responses of both NW ISCs and U ISCs to F11RX were very inconsistent, showing quite high responses in some assays and little or no responsiveness in others (data not shown). The low responses observed on numerous occasions were not due to the responses having peaked earlier than day 3 of culture, because on microscopic examination of the cultures it was obvious that no proliferation had been induced. The finding that F11RX did not induce

TABLE 3.7 Lectin-induced proliferation of SCs obtained from normal and L11RX immunized mice

	[^{3}H]-TdR uptake (cpm ± sem) by SCs* after 3 days of culture with 1 μg/ml ConA
NSCs	53 178 ± 519
D 14 ISCs	56 281 ± 899
D 21 ISCs	47 013 ± 6566

* Mixtures of 10^5 unfractionated NSCs, D 14 ISCs or D 21 ISCs and ConA were cultured (in quadruplicate) for 3 days at 37°C and the proliferative responses were measured by the uptake of [^{3}H]-TdR during the final 4 hours of incubation. Presented are typical results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate culture.

IL 2 release from either U ISCs or NW ISCs, even when cell suspensions which were able to proliferate in response to *Salmonella* Ags were used (data not shown), was consistent with an earlier observation that, although ISCs released MAF, no IL 2 release could be detected (Attridge and Kotlarski, 1985a).

In conclusion, this data indicated that ISCs were unlikely to provide a reliable source of Ag-primed T cells, even though it had been shown previously that *Salmonella*-specific T cells capable of mediating DTH and activating macrophages were present in the spleen following ip or iv immunization with L11RX (Ashley *et al.*,1974; Attridge and Kotlarski, 1985b). Thus, it was decided that IPCs taken from mice shortly after a primary ip infection with L11RX would be the more useful source of primed T cells, especially since two *in vitro* parameters of T cell stimulation could be measured with these cells. The inevitable consequence of this was that quite large numbers of mice would be needed in these studies. In an attempt to boost cell yields/mouse (and, possibly, the responsiveness of the T cells), L11RX immunized mice were challenged with a second dose of live *Salmonella.*

3.2.3 Secondary immunization with live *S. enteritidis* 11RX

3.2.3.1 Establishing the challenge inoculum

To determine the maximum nonlethal dose of L11RX which could be administered, a range of doses were given to mice 6 weeks after the primary ip immunization with 10^5 L11RX, when virtually all the primary infection had been cleared (data not shown). This series of experiments revealed that a second challenge dose of $8x10^6$ L11RX could be administered quite safely. A detailed characterization of the peritoneal and spleen cells (PCs and SCs) from these mice was then carried out. The PCs and SCs were harvested from mice 1, 2 and 3 weeks after receiving a second dose of $8x10^6$ L11RX, administered 6 weeks after the primary immunization, and the numbers of viable bacteria and total cells recovered were recorded. Not surprisingly, the ISC suspensions contained higher numbers of cells than cell suspensions prepared from the

spleens of normal or primed animals, yielding approximately $3x10^8$ cells/spleen. The IPC yields were also elevated compared to those obtained from mice only receiving the single dose of L11RX 6 weeks earlier. As early as one day after the second challenge the IPC yield had increased approximately 3-fold, yielding around $1.5x10^7$ cells from each mouse. The yields gradually increased to a peak of approximately $3.2x10^7$ by the seventh day after challenge, after which the yields began to decrease (Fig. 3.5). On analysis of the number of bacteria recovered from the peritoneal cavities and spleens of these mice it was observed that the numbers of viable bacteria decreased with time after challenge, with complete clearance by the third week (Table 3.8). The functional capabilities of these cells were then studied.

3.2.3.2 *In vitro* proliferative responses of ISCs and IPCs

IPCs and ISCs were harvested from mice 1, 2 and 3 weeks after the secondary challenge, aliquots of these suspensions were fractionated on NW columns and both fractionated and unfractionated suspensions of IPCs and ISCs were cultured with F11RX and ConA in the presence and absence of NPCs as additional APCs. Three days later the amount of proliferation was determined by measuring the level of [^{3}H]-TdR incorporated by pulse-labelling during the last 4 hours of incubation. The proliferative responses obtained were very similar at the three timepoints examined. The U ISCs, NW ISCs and NW IPCs of these mice usually responded well to ConA, although the response by NW fractionated cells was somewhat variable (Table 3.9). This variation could be attributed to inconsistencies in the removal of APCs by adherence to NW (encountered reasonably often) because responses were always restored by addition of NPCs. In contrast, the responses of U ISCs and NW ISCs to F11RX were unusually inconsistent, and similar to those observed with ISCs from mice given only a single dose of L11RX. [Microscopic examination of the cultures again suggested that the lower responses were not due to the peak of proliferation occurring earlier than the third day of culture.] As expected, F11RX induced significant proliferation of NW IPCs which was increased by the addition of $2x10^4$ NPCs; and U IPCs did not proliferate in response to (data not shown)

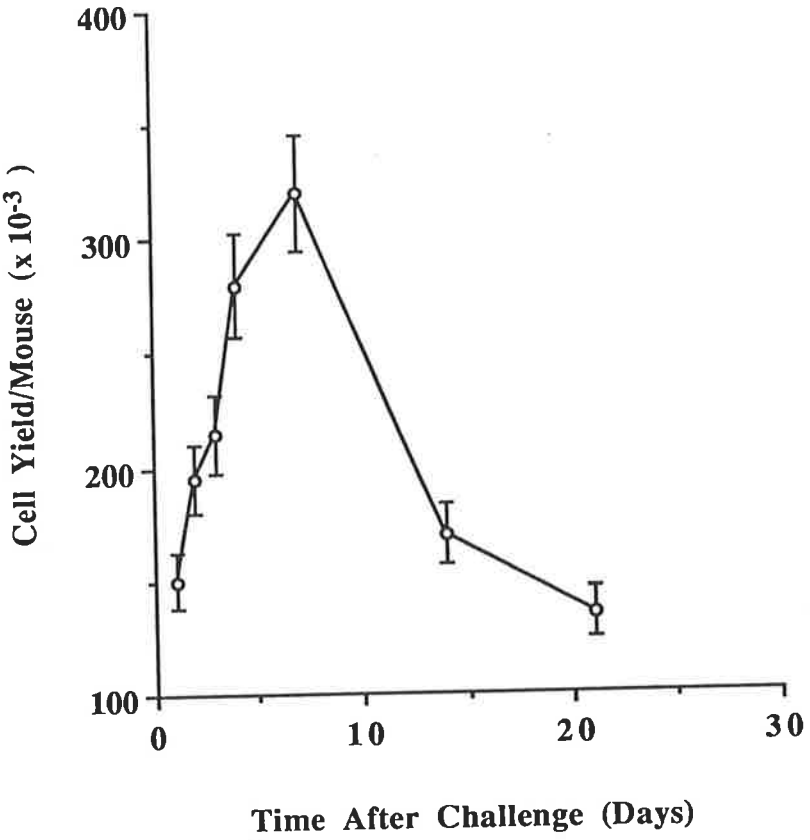


FIGURE 3.5 PC yields following a secondary ip challenge of L11RX

PCs were harvested from mice at various times after a second ip dose of $8x10^6$ L11RX and the numbers of viable cells recovered from individual animals were counted. For each timepoint, the average cell yields per mouse (mean ± sem) from at least five mice are provided.

TABLE 3.8 Recovery of bacteria from the spleens and peritoneal cavities of mice given a secondary ip challenge of $8x10^6$ L11RX

	Total number of bacteria recovered* from mice receiving a second ip dose of $8x10^6$ L11RX:		
	Time after challenge (Days):		
Source:	7	14	21
Peritoneal cavity	225 ± 17	0 ± 0	0 ± 0
Spleen	1667 ± 98	233 ± 27	0 ± 0

* The peritoneal cavities of five mice with a secondary infection of L11RX were washed out with 5 ml antibiotic free HBSS. Half of each spleen from these mice was also removed and homogenized in 5 ml of sterile saline. Duplicate 100 µl aliquots of the neat and diluted samples of the washouts and spleen cell suspensions were plated onto nutrient agar plates, the numbers of colonies after overnight growth at 37°C were recorded and the total numbers of bacteria recovered were calculated for each suspension. The results are expressed as the total numbers of bacteria recovered per mouse (mean ± sem), for similar groups of mice.

TABLE 3.9 Proliferative responses of primed T cells obtained from mice 14 days after a secondary ip challenge of L11RX

	[^{3}H]-TdR uptake (cpm ± sem) by L11RX secondary primed T cells* after 3 days of culture with:	
T cell source:	1 µg/ml F11RX	1 µg/ml ConA
U IPCs	417 ± 192	266 ± 82
NW IPCs	123 908 ± 1336	175 039 ± 14 816
U ISCs	NR#	324 113 ± 14 238
NW ISCs	NR#	298 548 ± 10 573

NR: No results are presented because the amount of [^{3}H]-TdR incorporated in repeat experiments was highly variable, ranging from 2372 ± 167 to 176 398 ± 2094.

* Primed T cells were obtained from the spleens and peritoneal cavities of mice 14 days after they were given a second ip dose of $8x10^6$ L11RX and the PCs and SCs were fractionated on NW (NW IPCs and NW ISCs), or left untreated (U IPCs and U ISCs). Mixtures of each cell suspension ($2x10^5$ cells/well) with F11RX or ConA were cultured (in quadruplicate) for 3 days at 37°C, before the proliferation was measured by the amount of [^{3}H]-TdR incorporated (cpm) during the last 4 hours of culture. Typical data are presented with the results expressed as the cpm (mean ± sem) for each replicate set.

either F11RX or ConA. In other words, all responses detected were comparable to those observed with cells from mice receiving only a primary dose of L11RX 14 days earlier.

3.2.3.3 IL 2 release by IPCs and ISCs

The ability of these IPCs and ISCs to release IL 2 in response to 10 µg/ml of F11RX *in vitro* was also measured using the standard IL 2 maintenance assay. The patterns of IL 2 release detected were similar to those observed using IPCs and ISCs from mice receiving only one dose of L11RX (Table 3.10). Both U IPCs and NW IPCs released considerable amounts of IL 2 and there were no significant differences in the amounts released at each timepoint examined, whilst IL 2 secretion by ISCs was undetectable. Hence, secondary immunization of mice with L11RX did not alter the ability of ISCs obtained from these mice to release IL 2 *in vitro*, nor did it appear to enhance the responses of IPCs.

3.2.3.4 Transfer of DTH Reactivity

Another parameter often used to detect the induction of a cell-mediated immune response is the ability to transfer DTH reactivity. Attridge and Kotlarski (1985b) reported that IPCs and ISCs from mice immunized ip or iv with L11RX could transfer DTH reactivity to *Salmonella* Ags to normal mice. To determine whether a second dose of L11RX affected the ability of these cells to transfer DTH, NW IPCs and NW ISCs from mice given a second dose of L11RX 1, 2 or 3 weeks earlier (L11RX 2° NW IPCs and ISCs) were used to transfer DTH reactivity to normal mice.

Mixtures of 10^6 NW IPCs or NW ISCs and 2.5 µg of F11RX in 50 µl were injected sc into the left hind footpads of normal mice. Footpad swelling was measured at 24 and 48 hours as described by Attridge and Kotlarski (1985b). In accordance with the *in vitro* experiments, the ability of the NW IPCs or NW ISCs to transfer DTH to F11RX was not enhanced by the secondary challenge, when compared with cells from mice given only a primary dose of L11RX (Fig. 3.6).

TABLE 3.10 IL 2 released by primed T cells harvested from mice with a secondary infection of L11RX

	Units of IL 2 released by L11RX secondary primed T cells* after overnight culture with 10 µg/ml F11RX		
	Time after challenge (Days):		
T cell source:	7	14	21
U IPCs	109	204	255
U ISCs	<4	<4	<4

* Mixtures of $2x10^6$ U IPCs or U ISCs (harvested from mice at various times after a secondary ip challenge of $8x10^6$ L11RX) and F11RX in 1 ml volumes, were cultured overnight at 37°C and the supernatants harvested. The presence of IL 2 was determined by the ability of these supernatants to maintain the proliferation of 3 day ConA blasts. The titres of IL 2 were calculated for each suspension as described in the Materials and Methods and characteristic data are presented showing the units of IL 2 detected.

FIGURE 3.6 Transfer of DTH reactivity using L11RX 2° NW IPCs and ISCs

Mixtures of 10^6 day 14 L11RX 2° NW IPCs or ISCs and 2.5 μg F11RX were each injected sc into the left hind footpads of groups of 3 normal, unimmunized F1 mice (control suspensions of cells alone and Ag alone were also included). The sizes of the right and left footpads were measured 24 and 48 hours later and the percent increase in footpad size was determined in the normal way for each mouse; data presented show the percent footpad swelling (mean ± sem) for each group and are characteristic of the results obtained on several occasions. Groups of mice recieved:

A: NW IPCs + F11RX

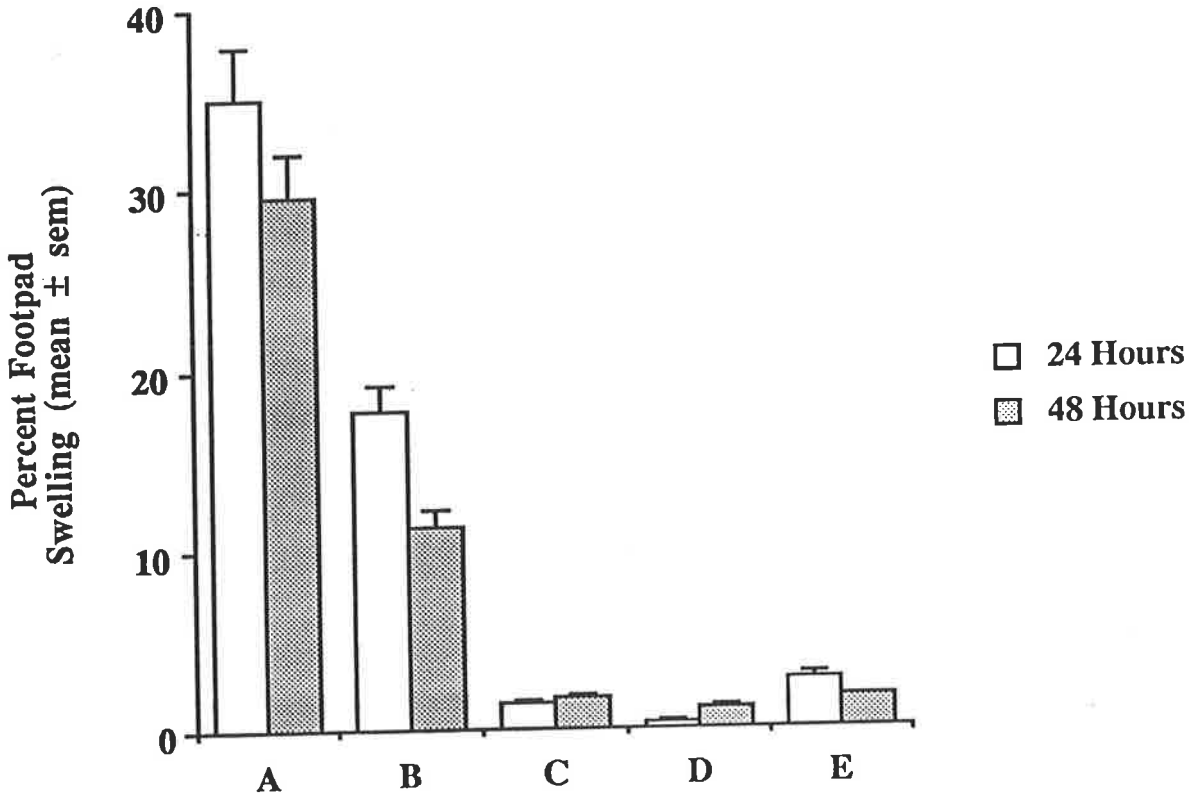
B: NW ISCs + F11RX

C: NW IPCs alone

D: NW ISCs alone

E: F11RX alone

DTH responses (at 24 hours) with 10^6 IPCs and ISCs from 11RX primed animals ranged from 30-40% and 15-22%, respectively (data not shown).


Percent Footpad
Swelling (mean ± sem)
40
30
20
10
0
A
B
C
D
E
24 Hours
48 Hours

3.2.3.5 Summary

By the criteria used to assess T cell function, a secondary dose of $8x10^6$ L11RX did not induce a more effective population of T cells than the population induced by just one ip dose of L11RX. A possible explanation for these results was that the second dose of L11RX was killed so quickly that it was handled more like a dose of killed 11RX, reported to induce only an humoral immune response and not a cell-mediated one (e.g. Rowley *et al.*, 1968; Collins, 1970). This was supported by the finding that the enhanced PC yields obtained from these mice coincided with the recovery of live organisms - that once the bacteria were cleared, the PC yields decreased.

Hence, in an attempt to provide a longer lasting live inoculum, which may be more affective in boosting a CMI response, it was decided to examine the effectiveness of the normally highly virulent LC5 as the secondary challenge. This approach was possible because it is well established that infection with L11RX provides effective T cell-mediated immunity to challenge with LC5 and that LC5 persist in such mice for a longer time than L11RX (Davies and Kotlarski, 1974 and 1976).

3.2.4 Secondary immunization with live *S. typhimurium* C5

3.2.4.1 Numbers of IPCs, ISCs and bacteria recovered

If secondary infections were able to induce a more effective responding population of T cells, practical considerations demanded that this had to be achieved in no more than 6 weeks. Therefore, it was decided to challenge mice with virulent LC5 organisms 3 weeks after the primary infection, when mice were still in an "active" state (Phase 1) of immunity. Mice used in these experiments were immunized ip with the standard dose of 10^5 L11RX. A range of doses of LC5 were used to determine the maximum nonlethal dose which could be administered ip 3 weeks after a primary immunization with L11RX and it became evident that a maximum of $3x10^4$ LC5 could be used without killing the recipients.

The fate of the LC5 organisms was followed to establish the rate of

clearance of bacteria from the peritoneal cavity and spleen. Up to 3 weeks after the challenge significant numbers of viable C5 could still be recovered from these two sites, demonstrating the increased persistence of the LC5 in comparison to a secondary dose of L11RX (Table 3.11). The cell yields recovered from the spleens and peritoneal cavities were also recorded. Approximately $3x10^8$ cells were harvested from each spleen at the various timepoints. After challenge with LC5 the PC yields gradually increased to approximately $4.2x10^7$ cells by day 7 and, unlike the yields from mice with a secondary L11RX infection, this level was maintained up to day 21 and correlated with the maintenance of C5 infection (Fig. 3.7).

3.2.4.2 Proliferative responses of ISCs and IPCs

NW fractionation of IPCs and ISCs from L11RX-immunized mice challenged with LC5 established that the secondary challenge did not result in any increase in nonadherent cell yields. Nevertheless, both fractionated and unfractionated cells were assessed for their ability to proliferate in response to F11RX and ConA, using the established procedure of measuring proliferation after 3 days of culture by the amount of [^{3}H]-TdR taken up during a 4 hour pulse with radioactive thymidine. Three repeat experiments using cells harvested 1, 2 and 3 weeks after C5 challenge established that the proliferative responses of these cells did not alter with time after challenge and that they were no more responsive than the IPCs or ISCs harvested after a primary dose of L11RX, that NW fractionation of ISCs again yielded cells which were somewhat variable in response and that U IPCs still did not proliferate in response to F11RX or ConA. The results of one of these experiments is presented in Table 3.12.

3.2.4.3 IL 2 release

IPCs and ISCs obtained from mice challenged with LC5 3 weeks after immunization with L11RX were also tested for their ability to release IL 2 in response to *in vitro* culture with F11RX, using the standard IL 2 maintenance assay to detect IL2. This experiment was repeated three times, using cells harvested 1, 2 and 3 weeks after

TABLE 3.11 Recovery of C5 from the peritoneal cavities and spleens of L11RX immunized mice given a secondary ip challenge of $3x10^4$ LC5

	Total number of C5 recovered* from mice receiving a secondary ip challenge of $3x10^4$ LC5:		
	Time after challenge (Days):		
Source:	7	14	21
Peritoneal cavity	2010 ± 65	1586 ± 101	2207 ± 127
Spleen	6143 ± 209	6400 ± 315	8550 ± 623

* At various times after challenging L11RX immunized mice with a secondary dose of LC5, the mice were sacrificed and their peritoneal cavities washed out with 5 ml antibiotic free HBSS. The spleens were also removed from these mice and half of each was homogenized in 5 ml of sterile saline. Duplicate 100 µl aliquots of neat and diluted samples of both the washouts and spleen homogenates were plated onto nutrient agar plates and the number of C5 colonies were recorded after overnight incubation at 37°C. Typical data are presented showing the total numbers of C5 recovered (mean ± sem) from the spleen and peritoneal cavity of each group of similar mice.

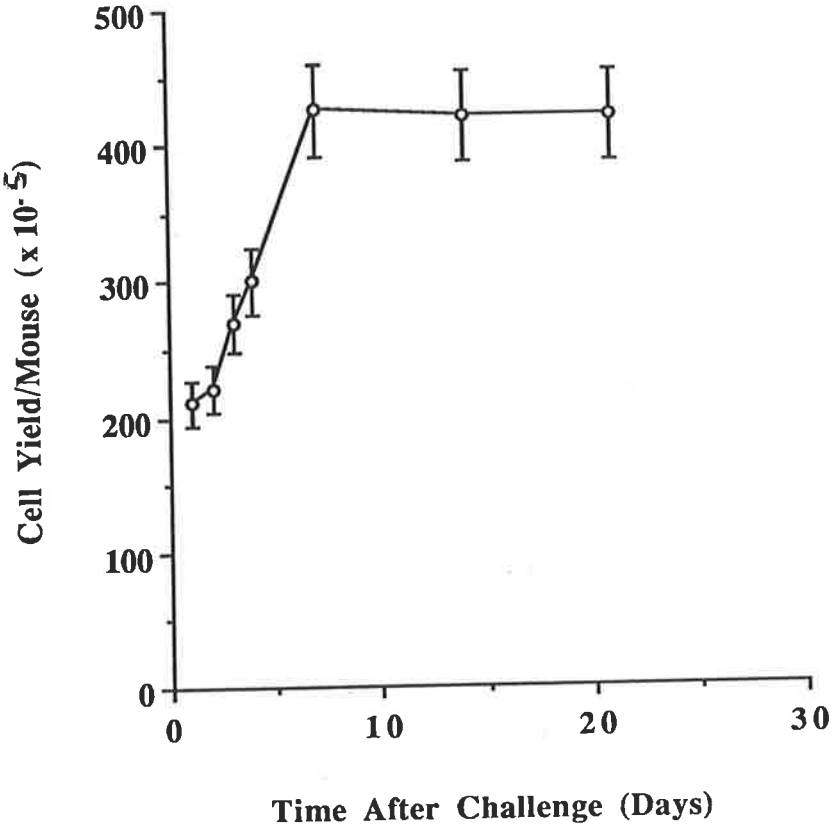


FIGURE 3.7 PC yields following secondary ip challenge with LC5

PCs were obtained from mice at a number of timepoints after a secondary ip challenge of $3x10^4$ LC5 and the yields of viable cells were recorded. At least 5 mice were examined at each timepoint and the cell yields from individual animals (mean ± sem) are presented.

TABLE 3.12 Proliferation of T cells obtained from mice with a secondary ip infection of C5

	[^{3}H]-TdR uptake (cpm ± sem) by LC5 secondary primed T cells* after 3 days of culture with:	
T cell source:	1 µg/ml F11RX	1 µg/ml ConA
U IPCs	207 ± 55	212 ± 42
NW IPCs	265 452 ± 9440	212 612 ± 5301
U ISCs	NR#	309 827 ± 9043
NW ISCs	NR#	242 298 ± 3978

NR: No results are presented due to the inconsistencies in the responses observed in repeat experiments, with the amount of [^{3}H]-TdR incorporated ranging from 5097 ± 109 to 298 376 ± 9823 cpm.

* Primed T cells were obtained from the peritoneal cavities and spleens of mice given a secondary ip challenge of $3x10^4$ LC5 14 days earlier and fractionated on NW (NW IPCs and NW ISCs), or left untreated (U IPCs and U ISCs). Each cell suspension was cultured (in quadruplicate) with F11RX or ConA ($2x10^5$ cells/well) for 3 days at 37°C, before measuring the amount of proliferation by the [^{3}H]-TdR incorporated (cpm) during the last 4 hours of the incubation. Data from one of several reproducible experiments are presented, with the results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each quadruplicate set.

challenge with LC5. Consistent with the results previously obtained with IPCs and ISCs of mice given one or two doses of L11RX, quite large amounts of IL 2 were released by IPCs whilst no IL 2 release was detected from ISCs and this appeared not to change significantly with time after challenge with LC5. The results of one experiment are shown in Table 3.13.

3.2.4.4 Transfer of DTH reactivity

NW IPCs and NW ISCs harvested from mice at different timepoints after secondary infection with LC5 (LC5 2° NW IPCs and ISCs) were also used to transfer DTH to normal mice. Four separate experiments were carried out with cells harversted 1, 2 or 3 weeks after infection, using an identical experimental design. Mixtures of 10^6 NW IPCs or NW ISCs and 2.5 μg F11RX in 50 μl were injected sc into the left hind footpad of groups of 3 mice and the amount of footpad swelling was measured 24 hours later, in the usual way. The responses elicited were expressed as an average of the percentage change in size of the left hind footpad when compared to the right, control hind footpad. DTH was transferred with both cell types and the levels of reactivity measured were very similar to those obtained with NW fractionated cells of mice injected once or twice with L11RX. Data obtained from one experiment using cells harvested 2 weeks after LC5 challenge are shown in Fig. 3.8 and are representative of the data obtained for all this series of experiments.

3.2.4.5 Summary

Secondary immunization with live *Salmonella* did not enhance the *in vitro* or *in vivo* responsiveness of IPCs or ISCs to *Salmonella* Ags nor provide higher yields of unfractionated or nonadherent IPCs than those obtained from mice given a primary dose of 10^5 L11RX 14 days earlier (D 14 IPCs). Hence, for convenience, D 14 IPCs were routinely used to provide the T cells needed to assess the *in vitro* activity of APCs.

TABLE 3.13 IL 2 released by primed T cells harvested from L11RX immunized mice given a secondary ip challenge of $3x10^4$ LC5

	Units of IL 2 released by LC5 secondary primed T cells* after overnight culture with 10 µg/ml F11RX		
	Time after challenge with C5 (Days)#:		
T cell source:	7	14	21
U IPCs	96	250	151
U ISCs	<4	<4	<4

Three weeks after a primary ip immunization with 10^5 L11RX, mice were challenged ip with $3x10^4$ LC5. The PCs and SCs were harvested from these mice 7, 14 and 21 days after receiving the secondary challenge and examined for their ability to release IL 2.

* $2x10^6$ LC5 2° U ISCs and U IPCs (used as a source of primed T cells) were cultured in a 1 ml volume with F11RX overnight, and the presence of IL 2 in the cell free supernatants of these cultures was studied by testing their capacity to maintain the proliferation of 3 day ConA blasts. The arbitrary units of IL 2 released were calculated for each suspension as described in the Materials and Methods and a summary of one set of results is presented.

FIGURE 3.8 Transfer of DTH reactivity with LC5 2° NW IPCs and ISCs

Mixtures of 10^6 LC5 2° NW IPCs or ISCs and 2.5 μg F11RX were transferred in 50 μl volumes to the left hind footpads of groups of 3 normal F1 mice and the amount of footpad swelling measured 24 and 48 hours later. [The normal controls were also included.] The percent (mean ± sem) of footpad swelling was calculated for each group in the usual manner and typical results are shown for mice receiving:

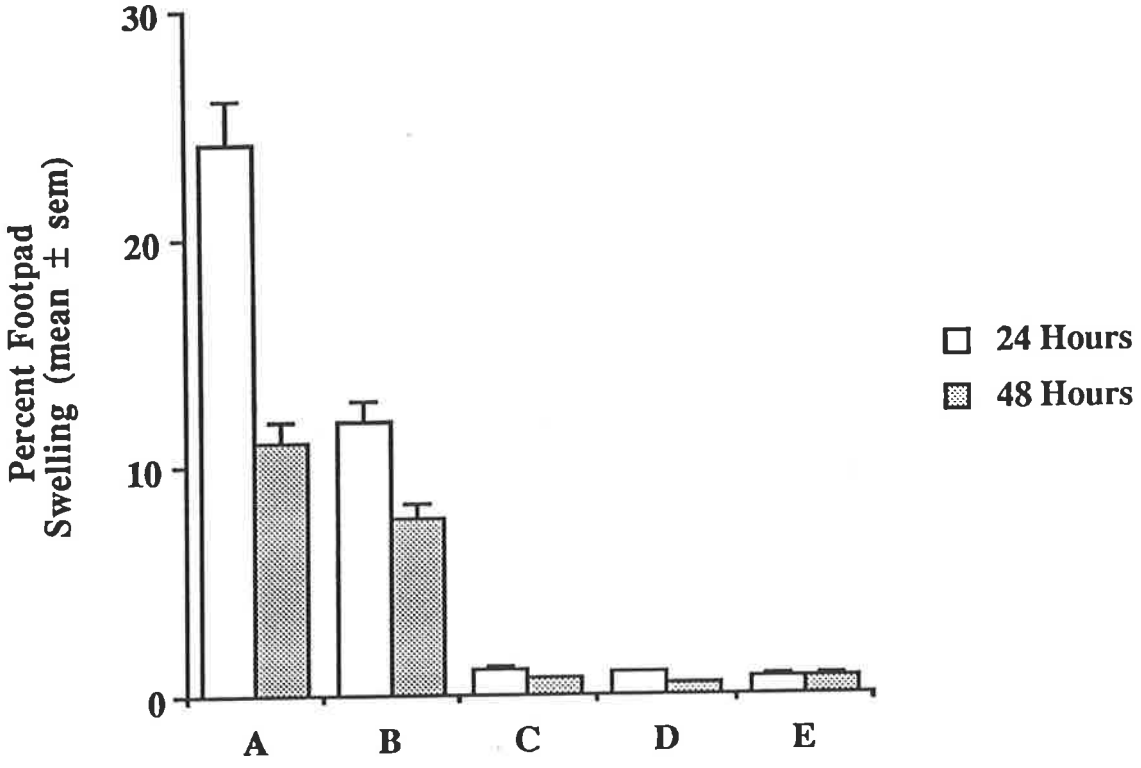
A: NW IPCs + F11RX

B: NW ISCs + F11RX

C: NW IPCs alone

D: NW ISCs alone

E: F11RX alone


Percent Footpad
Swelling (mean ± sem)
30
20
10
0
A
B
C
D
E
24 Hours
48 Hours

3.2.5 Removal of APCs from D 14 IPC suspensions

To use T cells of D 14 IPCs for assessing APC activity it was necessary to develop a protocol for removing APC function from these cell suspensions. The experiments already described had established that NW fractionation yielded T cell preparations with limited APC activity, but clearly further purification was still required. Besides adherence, the other characteristic of many APCs is their ability to express Class II MHC (Ia) molecules. Consequently, treatment of the plastic or NW nonadherent cells with an α-Ia MoAb and C is commomly used to deplete the APC function of lymphoid cell suspensions. This approach was also used to treat nonadherent IPC populations, which were then tested for their ability to respond to F11RX and ConA.

NW or plastic fractionated IPCs were incubated with α-Ia (1 in 10 final dilution) and C (1 in 20 final dilution) for one hour at 37°C. The cells were then washed and adjusted to the required concentration and cultured with 1 μg/ml F11RX or ConA for 3 days at 37°C when the amount to T cell proliferation was measured using the standard established procedures. Although some variation was obtained from experiment to experiment, differences in the efficiency of removal of APC activity were observed with the treatments used (Table 3.14). Clearly, removal of adherent cells by passage on NW for one hour at 37°C, followed by α-Ia^d + C treatment provided a reasonably efficient removal of APCs from D 14 IPCs, as assessed by the ability of NPCs to restore their responsiveness to both F11RX and ConA (supported by Vordermeier and Kotlarski, 1990).

This was confirmed by comparing the ability of F11RX to induce proliferation of NW IPCs treated with α-Ia^d + C when cultured together in flat- and round-bottomed wells. Various numbers of these cells, ranging from 10^4-$1x10^5$ cells/well (in quadruplicate) were incubated with F11RX in flat- and round-bottomed trays for 3 days at 37°C before measuring the proliferative responses induced. Control, replicate cultures which contained $2x10^4$ NPCs/well to provide an additional source of APCs were also included. The data obtained (Table 3.15) indicated that, as might be expected for the dose range of IPCs used, APC function was more

TABLE 3.14 Purification of primed T cells

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with:			
	1 μg/ml F11RX ± 2x10^4 NPCs		1 μg/ml ConA ± 2x10^4 NPCs	
Fractionation#	-	+	-	+
Plastic	125 958 ± 8225	142 467 ± 10 230	148 061 ± 15 630	114 828 ± 8559
Plastic, α-Ia + C	75 871 ± 3133	107 355 ± 4889	74 313 ± 3257	129 629 ± 3497
NW	101 568 ± 10 985	103 450 ± 6439	86 408 ± 5547	84 146 ± 3478
NW, α-Ia + C	1469 ± 234	45 837 ± 4276	11 398 ± 2984	114 464 ± 4230

D 14 IPCs were fractionated in various ways in an attempt to purify the T cells and remove the bulk of the APC activity. Initially, a large proportion of the adherent cells were removed by incubating the cells on plastic petri dishes or NW columns for 1 hour at 37°C. The nonadherent cells were harvested and subsequently treated or not with α-Ia and C for 1 hour at 37°C.

* 10^5 fractionated IPCs were mixed with F11RX or ConA in the presence or absence of NPCs and incubated in a 96-well flat-bottomed tray at 37°C for 3 days (each "mix" was set up in quadruplicate). The proliferative responses were assessed by measuring the amount of [^{3}H]-TdR (cpm) taken up by the cells during the final 4 hours of culture and the results expressed as the cpm (mean ± sem) for each quadruplicate set.

TABLE 3.15 Proliferation of purified, primed T cells in round- and flat-bottomed trays

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with F11RX ± 2x10^4 NPCs in:			
	Flat-bottomed trays		Round-bottomed trays	
No. cells/well	- NPCs	+ NPCs	- NPCs	+ NPCs
10^5	2717 ± 1355	149 096 ± 6703	29 827 ± 1910	85 886 ± 269
5x10^4	1737 ± 231	54 608 ± 2978	17 521 ± 24	52 052 ± 536
2x10^4	444 ± 91	61 484 ± 6093	8085 ± 909	40 191 ± 3118
10^4	202 ± 62	11497 ± 2586	592 ± 98	41 887 ± 1344

* Varying numbers of NW, α-Ia + C IPCs were mixed with F11RX (1 μg/ml) and cultured in the presence and absence of NPCs (in quadruplicate) in either a 96-well flat-bottomed or round-bottomed tray for 3 days, before proliferation was measured by the amount of [^{3}H]-TdR incorporated (cpm) during the last 4 hours of incubation. Results are expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each quadruplicate set.

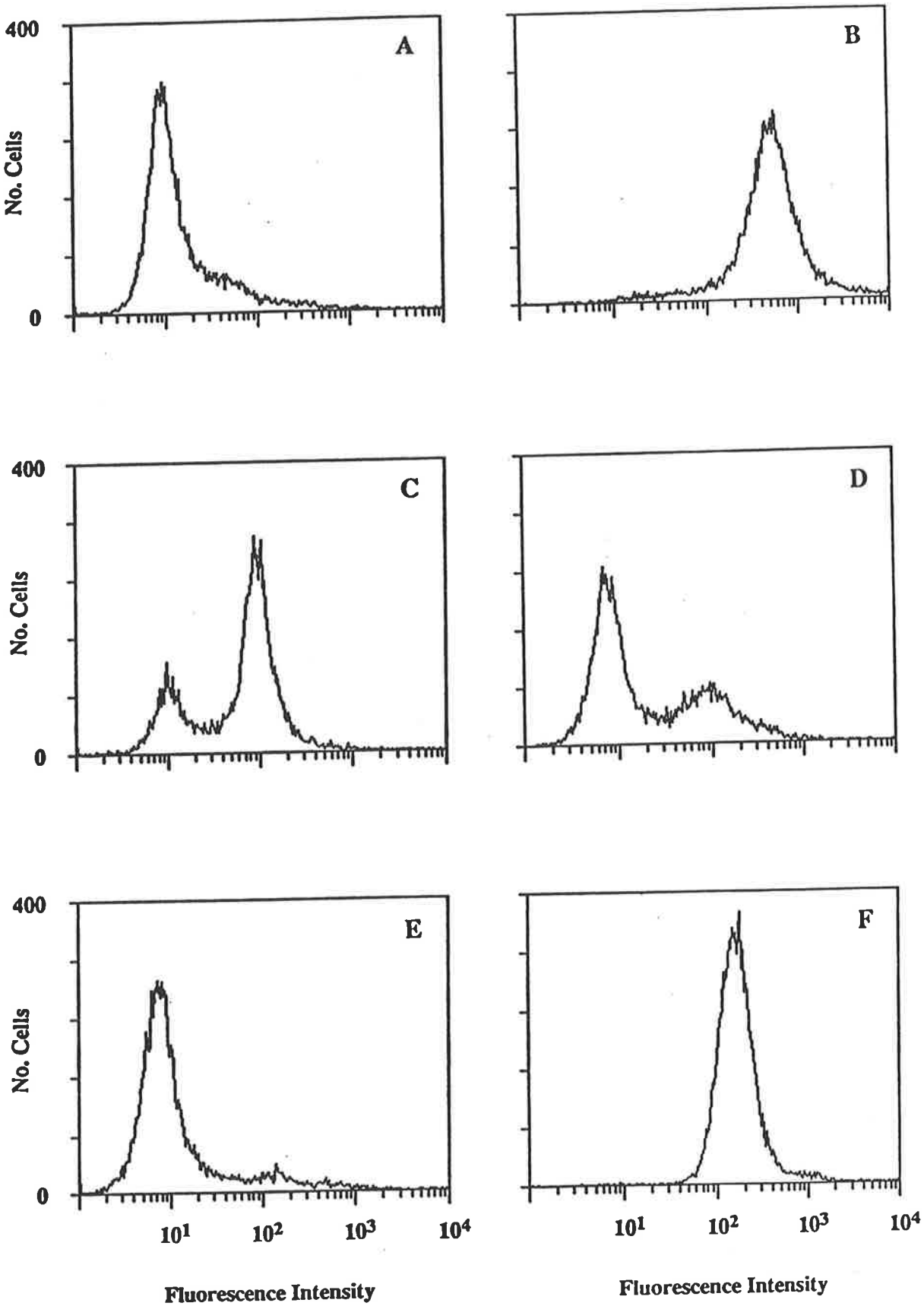
limiting in flat-bottomed than round-bottomed wells. At the lowest cell dose used, no proliferation was induced in either set of cultures unless APCs were added [as NPCs], but with doses ranging from $2x10^4$-$1x10^5$ cells/well significant responses were detected in round-bottomed wells, even in the absence of NPCs. This indicated that some APCs were still contaminating the T cell population. Presumably, APC activity, although limiting, was expressed more efficiently when IPCs were cultured in round-bottomed wells because the chances of the required cell-cell contacts being made were greater. Similar contacts in flat-bottomed wells evidently required larger numbers of cells or additional NPCs. However, the response observed when 10^5 T cells cultured in the round-bottomed trays (in the presence of NPCs) was smaller than that by the same number of T cells (when APCs were present) cultured in the flat-bottomed trays and was probably due to the peak of proliferation occurring earlier (in the round-bottomed trays) than the third day of culture, as a result of the increased cell-cell contact in the rounded wells.

3.2.6 Analysis of APC depleted IPCs following immunofluorescent labelling

NW fractionated and α-Ia^d + C treated IPCs were labelled with immunofluorescence using the indirect method to further analyse the cell composition of this suspension and to determine which T cell types were present. This experiment was done on three separate occasions and the results obtained were quite reproducible. To label the cells they were incubated with α-Thy1.2, α-L3T4, α-Lyt2.2, α-Ia^d or α-H-2^d MoAbs, followed by incubation with the SHAM-FITC (as described in the Materials and Methods). Fig. 3.9 [A-F] illustrates that most of the fractionated IPCs were $Thy1.2^+$ cells of the $L3T4^+$ phenotype, that detectable numbers of $Lyt2.2^+$ T cells were also present and that very few contaminating Ia^+ cells remained in the population. Hence, NW fractionation followed by treatment with α-Ia^d + C appeared to provide a satisfactory protocol for the preparation of reasonably pure populations of T cells, which could be used for analysing the Ag presenting capacity of various APC populations. From now on these cells will be referred to as purified, primed T cells.

FIGURE 3.9 FACScan analysis of purified, primed T cells

NW, α-Ia + C treated IPCs were indirectly labelled with immunofluorescence, using α-Thy1.2 (B), α-L3T4 (C), α-Lyt2.2 (D), α-Ia (E) and α-H-2K (F) as the primary Abs. Cells incubated with P/B/A for the first hour were also included as a negative control (A). All groups were then incubated with the SHAM-FITC secondary Ab for 1 hour ar 4°C in the dark and the fluorescence intensities of these populations obtained by analysis on the FACScan are presented in Fig. 3.9 [A-F].


400
No. Cells
0
A
B
C
D
E
F
10^1
10^2
10^3
10^4
Fluorescence Intensity
Fluorescence Intensity

3.2.7 Phenotype of the T cells proliferating in response to F11RX

Two different approaches were used in these studies. The first involved Ag-activated T cells and was reproduced on several occasions. Purified, primed T cells were cultured with F11RX and NPCs for 3 days at 37°C. On the third day of culture, C (1/20 final dilution) + MoAbs (1/10 final dilution) specific for markers expressed on all T cells or particular T cell subsets were added to four replicate cultures at 37°C one hour prior to pulse-labelling with [^{3}H]-TdR, using the standard method. Representative data from one such experiment (Table 3.16) indicate that the cells responsible for the majority, if not all of the proliferation in response to F11RX and NPCs expressed the phenotype Thy1.2$^+$, L3T4$^+$. [Control suspensions incubated with the various MoAbs alone showed similar responses to untreated cultures and cultures incubated with C alone (data not shown).] Additional results, included in the paper by Kotlarski *et al.* (1989), also showed that the bulk of this response was class II MHC (I-A) restricted. Although treatment with α-Thy1.2 and C almost completely abolished the proliferative response, treatment with α-L3T4 and C (or α-L3T4, α-Lyt2.2 and C) did not reduce the response by the same amount, suggesting that a double negative T cell population (possibly γδ T cells) may have been induced to proliferate in these cultures. Possible explanations for this will be discussed in Chapter 7.

The second approach was to test the effect of adding α-Ia MoAbs to cultures of these cells and F11RX. This experiment was repeated twice and was carried out using the following design. A range of doses of NPCs were cultured with purified, primed T cells for 3 days in the presence of increasing amounts of F11RX, with and without the addition of α-Ia. All cultures were then pulse-labelled with [^{3}H]-TdR and the proliferation induced was measured in the usual way. The results were quite reproducible and only set are are shown (Table 3.17). In the absence of α-Ia, the optimal response was observed with 1 μg/ml F11RX in the presence of $2x10^4$ NPCs, with reduced responses to higher and lower doses of F11RX and NPCs. Addition of α-Ia at a final dilution of 1/10, completely blocked all of these responses apart from the response of IPCs cultured with 100 μg/ml F11RX in the presence of $2x10^5$ NPCs. Furthermore, a

TABLE 3.16 Phenotype of the cells proliferating in the L11RX purified, primed T cell suspension

Treatment#	[^{3}H]-TdR uptake (cpm ± sem) by purified, primed T cells* after 3 days of culture with 2x10^4 NPCs and 1 μg/ml F11RX:
None	52 015 ± 3117
C alone	48 183 ± 1377
α-Thy1.2 + C	1526 ± 139
α-L3T4 + C	16 678 ± 1277
α-Lyt2.2 + C	47 990 ± 1019
α-L3T4, α-Lyt2.2 + C	11 804 ± 1151

* NW, α-Ia + C IPCs (10^5) were cultured with NPCs and F11RX for 3 days, before measuring the amount of proliferation by the [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture.

Prior to pulsing with the [^{3}H]-TdR, diluted mixtures of the MoAbs (1/10 final) and C (1/20 final) or C alone (1/20 final) were added to 4 replicate cultures and the tray incubated for 1 hour at 37°C. Presented are the results of a representative experiment showing the cpm (mean ± sem) for each replicate set.

TABLE 3.17 Effect of α-Ia in culture on the proliferation of L11RX primed T cells

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with NPCs and F11RX in the presence or absence of α-Ia MoAb:			
		F11RX (μg/ml):			
α-Ia in culture	No. NPCs (/well)	0.1	1	10	100
None	$2x10^3$	3525 ± 382	41 621 ± 3993	12 778 ± 1399	4974 ± 825
	$2x10^4$	57 189 ± 2787	126 081 ± 4281	87 261 ± 4434	27 937 ± 629
	$2x10^5$	231 ± 48	369 ±39	17 852 ± 832	66 310 ± 794
1/10 dil^n	$2x10^3$	315 ± 62	586 ± 108	773 ± 126	617 ± 71
	$2x10^4$	556 ± 19	3187 ± 217	2496 ± 264	1738 ± 185
	$2x10^5$	451 ± 38	648 ± 76	4629 ± 156	56 898 ± 2441
1/100 dil^n	$2x10^3$	1789 ± 465	7053 ± 523	3736 ± 337	4880 ± 313
	$2x10^4$	19 620 ± 1259	62 592 ± 2712	29 785 ± 1598	21 197 ± 3987
	$2x10^5$	538 ± 2	19 111 ± 2688	60 981 ± 2584	98 190 ± 2657

* $2x10^5$ NW, α-Ia + C IPCs were mixed with varying amounts of F11RX and NPCs and loaded into a 96-well flat-bottomed tray. Appropriately diluted α-Ia MoAb or culture medium, were added to each of these mixtures in quadruplicate and cultured for 3 days, before the amount of proliferation was determined by measuring the incorporation of [^{3}H]-TdR (cpm) and results expressed as the cpm (mean ± sem) for each quadruplicate set.

1/100 dilution of the α-Ia did block the response induced by F11RX in the presence of $2x10^3$ NPCs, whilst the responses induced by $2x10^4$ NPCs were only partially blocked by a 1/100 dilution of the α-Ia and the responses induced by $2x10^5$ NPCs in the presence of 1, 10 and 100 μg/ml of F11RX were actually enhanced. This finding was consistent with the observations of Matis *et al.* (1983) that the amounts of antigenic determinants and MHC molecules available for presentation to T cells determine the magnitude of the response induced (this will be discussed in more detail in Chapter 7).

3.2.8 Effect of depleting Lyt 2^+ T cells on proliferative responses of IPCs

Because the T cells induced to proliferate were of the $L3T4^+$ phenotype, it was possible to carry out experiments to determine whether the inability of U IPCs to proliferate was caused by the presence of Ag-specific, suppressor T cells. There have been several reports which demonstrated the induction of $Lyt2^+/CD8^+$ suppressor T cells following infection with *Mycobacteria* (eg. Collins and Watson, 1979; Turcotte, 1981). In addition, stimulation of peripheral blood adherent cells with *Mycobacteria* causes the production of a suppressor cell activating factor which activates $CD8^+$ suppressor T cells that inhibit lymphocyte proliferation and LK production (Wadee *et al.*, 1980 and 1983; Wadee and Rabson, 1981). More recently, Sussman and Wadee (1991) also demonstrated that following activation with mycobacterial components *in vitro* $CD8^+$ T cells released a suppressor factor which inhibited lymphocyte blastogenesis. Therefore, it was possible that a similar mechanism was acting in the *Salmonella* system and removal of the $Lyt2.2^+$ T cells prior to *in vitro* culture, would clarify this point.

Accordingly, U IPCs were prepared from mice 14 days after L11RX immunization and were treated with C and MoAbs specific for various T cell markers prior to culture with F11RX or ConA. Treatment with α-Thy1.2 or α-L3T4 and C was carried out simply as a control, since cells expressing both these markers are normally responsible for the proliferation induced by F11RX (and ConA). After three days, the amount of proliferation was measured by the uptake of $[^3H]$-TdR in the usual way. Table 3.18 clearly illustrates that removal of various T cell populations had no effect on

TABLE 3.18 Investigation into the presence of Lyt2.2+ "suppressor" T cells in U IPC suspensions

	[^{3}H]-TdR uptake (cpm ± sem) by U IPCs* after 3 days of culture with:			
	1 µg/ml F11RX ± NPCs		1 µg/ml ConA ± NPCs	
Pretreatment#	-	+	-	+
None	323 ± 21	298 ± 12	267 ± 34	245 ± 42
C alone	265 ± 13	272 ± 24	301 ± 22	343 ± 33
α-Thy1.2 + C	210 ± 34	299 ± 11	245 ± 54	280 ± 64
α-L3T4 + C	314 ± 53	356 ± 41	272 ± 23	298 ± 14
α-Lyt2.2 + C	297 ± 27	243 ± 19	313 ± 28	310 ± 30

* Mixtures of $2x10^5$ U IPCs and F11RX or ConA in the presence and absence of $2x10^4$ NPCs were incubated in a 96-well flat-bottomed tray for 3 days at 37°C. On the third day the proliferation was measured by the amount of [^{3}H]-TdR incorporated (cpm).

Prior to culture, appropriately diluted mixtures of MoAbs (1/10 final) and C (1/20 final), or C alone (1/20 final), were added to aliquots of the U IPC suspensions at 10^7 cells/ml, mixed thoroughly and incubated for 1 hour at 37°C. Each suspension was cultured in quadruplicate and representative data are provided showing results expressed as the cpm (mean ± sem) for replicate set.

the proliferative response of U IPCs. Therefore, the inability of U IPCs to proliferate in response to antigenic or mitogenic stimuli *in vitro* was not likely to be due to the presence of "classical" $Lyt2^+$ suppressor T cells. Similar results were obtained with U IPCs from mice receiving two doses of L11RX and mice receiving a dose of L11RX followed by challenge with LC5. Pretreatment with C and MoAbs specific for the Lyt 2 marker of T cells did not render these cells responsive to either F11RX or ConA (Tables 3.19 and 3.20).

3.2.9 Immunogenicity of 11RX flagellin

A preparation of flagellin extracted from 11RX organisms and purified using standard methods was tested for its ability to stimulate L11RX primed T cells. This was done to assess the usefulness of this protein for detailed studies of the Ag processing and presentation by various APCs, where a well defined Ag would be very useful.

Accordingly, purified, primed T cells were cultured with a range of doses of 11RX flagellin in the presence and absence of NPCs and the amount of proliferation induced was measured on the third day of culture (Table 3.21). Little or no proliferation was detected in the absence of NPCs even when F11RX was used as Ag. However, in the presence of NPCs a significant response to F11RX was observed as expected, whilst little response to the flagellin preparation was observed unless 100 μg/ml was used, a dose so high that it seemed likely that the proliferation had been induced by a minor contaminant in the flagellin preparation. The rationale for this interpretation is as follows. A concentration of 10 μg/ml of S11RX induces maximum proliferation of primed T cells and the immunogenic component of this preparation resides in the low molecular weight fraction (16-18 kDa; Vordermeier and Kotlarski, 1990), which represents only a minor proportion of the S11RX preparation. It follows, that a concentration less than 10 μg/ml of a purified protein, like 11RX flagellin, should induce maximum T cell responses. Accordingly, the responses observed with 100 μg/ml of 11RX flagellin were attributed to small amounts of contaminating low molecular weight

TABLE 3.19 Examination of the presence of the Lyt2.2+ "suppressor" T cells in the IPC suspensions obtained from mice with secondary L11RX infection

	[^{3}H]-TdR uptake (cpm ± sem) by L11RX secondary U IPCs* after 3 days of culture with:	
Treatment#	1 µg/ml F11RX	1 µg/ml ConA
None	417 ± 192	266 ± 82
C alone	207 ± 32	205 ± 46
α-Thy1.2 + C	238 ± 80	153 ± 46
α-L3T4 + C	273 ± 87	261 ± 58
α-Lyt2.2 + C	214 ± 42	154 ± 34

* $2x10^5$ U IPCs were mixed with ConA or F11RX in 96-well flat-bottomed trays and cultured for 3 days at 37°C, before measuring the proliferation by the uptake of [^{3}H]-TdR in the final 4 hours of culture.

Before culture, combinations of the T cell specific MoAbs and C, or C alone, were added to aliquots of U IPC suspensions (10^7 cells/ml) and incubated for 1 hour at 37°C. Results expressed as the cpm (mean ± sem) were calculated for each group of treated cells and a representative set of data are provided.

TABLE 3.20 Investigations into the existence of Lyt2.2+ "suppressor" T cells in IPCs harvested from mice with a secondary C5 infection

	[^{3}H]-TdR uptake (cpm ± sem) by LC5 secondary U IPCs* after 3 days of culture with:	
Treatment#	1 µg/ml F11RX	1 µg/ml ConA
None	207 ± 55	212 ± 42
C alone	164 ± 25	201 ± 26
α-Thy1.2 + C	154 ± 31	195 ± 40
α-L3T4 + C	171 ± 34	208 ± 59
α-Lyt2.2 + C	147 ± 24	157 ± 20

* $2x10^5$ LC5 2° U IPCs were mixed with F11RX or ConA in a 96-well flat-bottomed tray (in quadruplicate) and incubated for 3 days at 37°C, before the proliferation was measured by the uptake of [^{3}H]-TdR (cpm) during the final 4 hours of culture.

Prior to being cultured, mixtures of T cell-specific MoAbs (1/10 final) and C (1/20 final), or C alone (1/20 final), were added to aliquots of the U IPC suspensions (10^7 cells/ml), the tubes shaken and incubated for 1 hour at 37°C. Typical results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate set are presented.

TABLE 3.21 Immunogenicity of 11RX flagellin for L11RX primed T cells

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *Salmonellae* Ags ± 2x10 ^{4} NPCs:	
Ag	Ag conc^n	- NPCs	+ NPCs
11RX flag#	0.1	1135 ± 115	6558 ± 2519
	1	606 ± 37	3567 ± 127
	10	1054 ± 201	8523 ± 836
	100	5716 ± 416	58 537 ± 4035
F11RX	1	16 453 ± 3728	83 781 ± 3479

* 2x10^5 purified, primed T cells were cultured in quadruplicate with F11RX or 11RX flag (#: 11RX flagellin) in the presence and absence of NPCs for 3 days at 37°C before the proliferative response was determined by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture. For each cell-Ag mixture the cpm (mean ± sem) was calculated and data illustrating a characteristic response are presented.

proteins present in the 11RX flagellin preparation (see Fig. 2.1). The IL 2 released by IPCs stimulated with flagellin was also measured using the standard technique. Neither U IPCs nor NW IPCs released any detectable levels of IL 2 in response to 11RX flagellin (data not shown).

Another parameter used to assess the immunogenicity of the preparation was its ability to elicit DTH reactivity in immunized mice. 10 μg of 11RX flagellin or S11RX was injected sc into the left hind footpad of groups of five normal mice and five mice which had been immunized with L11RX 2-3 weeks earlier. The hind footpads were measured 24 and 48 hours later and the percentage footpad swelling was determined as previously described. Fig. 3.10 presents the amount of swelling elicited by these Ags. S11RX elicited significant DTH reactivity in the immunized mice, with a peak at 48 hours, whilst the flagellin elicited much less swelling, further emphasizing the fact that 11RX flagellin preparation was not immunogenic enough to be useful for further studies. Since no other well defined Ags of 11RX were available, the studies which could be done to analyse the APC function of PCs of 11RX immunized animals were severely restricted. Another Ag which was initially considered for use in these studies was S11RX, but was rejected for two main reasons. First, an unusual "inhibitory" effect of S11RX, manifested as abolition of a proliferative response of normal SCs cultured with ConA and a standard dose (10-20 μg/ml) of S11RX, has been detected quite consistently (Kotlarski *et al.* unpublished), and second because the major T cell stimulatory activity of S11RX was associated with a minor proportion of the total protein present in the S11RX preparation (Vordermeier and Kotlarski, 1990). Obviously, a purified preparation of the peptides present in the 16-18kDa region of S11RX would have been useful, but the quantities of this Ag needed were not available before these studies were completed. Accordingly, whole killed (and live) organisms were used as the stimulating Ags.

3.3 Summary and conclusions

These studies established that ip immunization of mice with L11RX

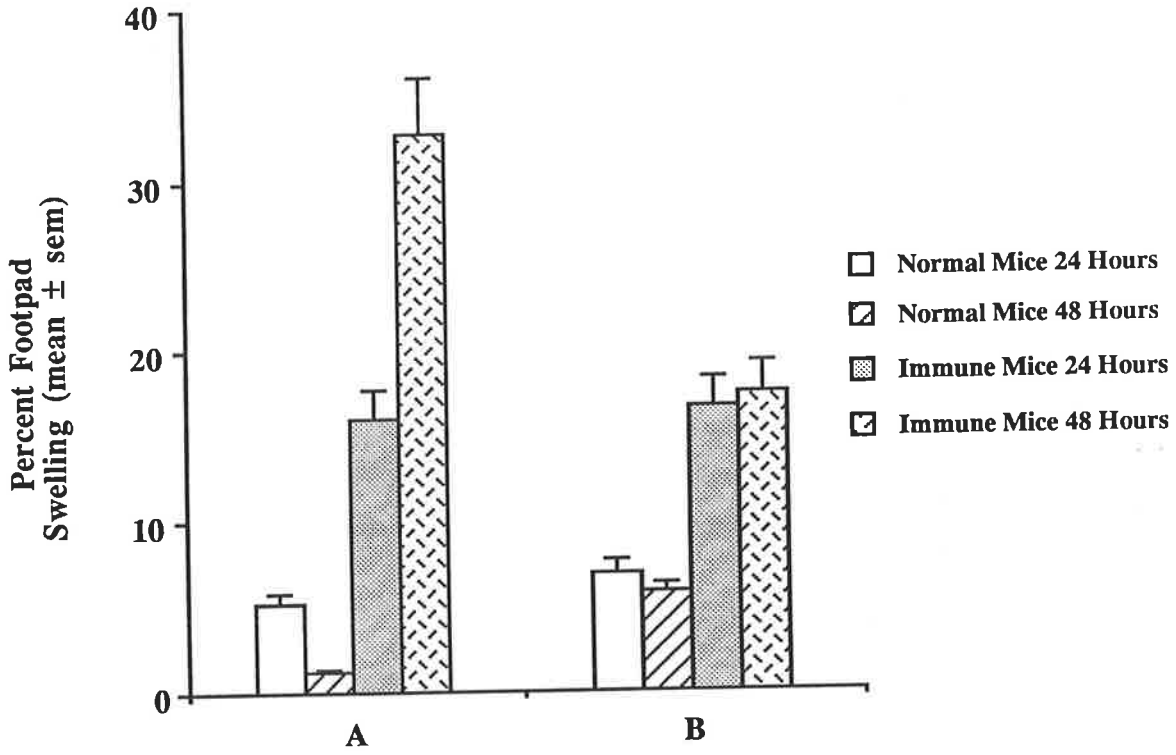


FIGURE 3.10 DTH reactivity induced by 11RX flagellin

Groups of five normal mice and mice ip immunized with 10^5 L11RX 2-3 weeks earlier were injected sc with 10 μg of S11RX (A) or 11RX flagellin (B) in the left hind footpads and the right and left footpads were measured 24 and 48 hours later. The percent footpad swelling (mean ± sem) was calculated for each group, with respect to the size of the right, uninjected footpad and representative data from one of four experiments are provided.

induced *Salmonella*-specific T cells in the peritoneal cavity and in the spleen. However, on further analysis it became evident that spleen cells did not provide the best source of primed T cells, because they could not be induced to release detectable amounts of IL 2 and their proliferative response to F11RX was highly variable. The effect of secondary infection with *Salmonella* was investigated and revealed no enhancement of the responsiveness by T cells recovered from the peritoneal cavity or spleen. However, secondary infection did induce an increase in IPC yields to levels similar to those induced early after primary infection. Increased IPC yields were maintained following secondary infection with LC5 but not L11RX and correlated with the persistence of bacteria. It was concluded that D 14 IPCs would provide a reliable source of primed T cells.

A protocol for preparing T cells with little or no APC function was also developed. NW fractionated, α-Ia and C treated IPCs showed minimal proliferative responses to F11RX or ConA in the absence of NPCs and consisted of mainly T cells, as indicated by the FACScan analysis. Furthermore, since both $L3T4^+$ and $Lyt2.2^+$ T cells were present, this population was likely to contain cells specific for *Salmonella* Ags presented in association with both Class I and Class II MHC products, if such Ag presentation did occur *in vivo*. In other words, these purified suspensions of D 14 IPCs seemed satisfactory for assessing differences in the Ag presenting capacity of various APC populations.

CHAPTER 4

ACTIVITY OF APCs FROM NORMAL MICE AND MICE IMMUNIZED WITH L11RX

4.1 Introduction

Immunization of mice with L11RX has been shown to induce both cellular and humoral immune responses (Rowley *et al.*, 1968), whilst immunization with F11RX induces only an humoral immune response (Collins, 1970). Obvious explanations for this difference include the possibilty that antigenic determinants crucial for the induction of CMI may be damaged during the preparation of the killed bacterial vaccine or that APCs "handle" or process live IBPs differently from killed ones. Until recently the explanation favoured by most was that the MHC coded molecules involved in the presentation of the bacterial Ags to the immune system determined the type of response elicited. This interpretation was based on the generally held view that Ag presentation in association with Class I MHC products favoured induction of T cells able to mediate CMI responses (Te cells), whereas presentation of Ag complexed to Class II MHC products resulted in induction of Th cells (reviewed by Fitch, 1986). This view is no longer tenable and alternative explanations for differences in the immunogenicity of IBPs must be considered. An attractive alternative is the possibility that live bacteria modulate APC function in such a way that they are able to stimulate cellular as well as humoral immune responses. In support of this possibility, it has been reported that BCG infection does modulate APC activity of mouse PCs, as determined by their enhanced ability to induce Te cells (Meltzer and Oppenheim, 1977; Britz *et al.*, 1982).

To investigate whether infection with L11RX did induce modulation of APC function, PCs were harvested from mice immunized ip with 10^5 L11RX 1-3 days

earlier (D 1-3 IPCs) and their APC activity was compared with that of PCs obtained from normal mice (NPCs). This period after immunization was chosen for study because Ashley and Kotlarski (1982) reported that CMI to *Salmonella* Ags was detectable 2-3 days after ip infection with L11RX.

4.1.1 Outline of experimental approaches used

The Ag presenting capacity of these PC suspensions was studied by analysing their ability to stimulate L11RX primed T cells to proliferate *in vitro* in response to specific Ags and to induce primary allogeneic responses *in vitro*. Purified, L11RX primed T cells, obtained by treating NW D 14 IPCs with α-Ia and C, were cultured for 3 days with the various APCs in the presence of specific Ags (in quadruplicate), before determining the proliferation induced by measuring the amount of [^{3}H]-TdR taken up during the final 4 hours of culture. The cpm (mean ± sem) of [^{3}H]-TdR incorporated by the cells was calculated for each replicate set. To study the ability of all four PC populations to induce a primary MLR, each F1 PC population was incubated in quadruplicate for 5 days (unless stated otherwise) at 37°C with an allogeneic T cell population, obtained by NW fractionating the MLNCs harvested from normal BALB/c, C57BL/6 or CBA mice. Again the proliferation induced was determined by measuring the [^{3}H]-TdR taken up by each population during the last 4 hours of culture and results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated. Measurement of cytotoxicity generated by culture of allogeneic populations was accomplished by incubating these "effector" cells with ^{51}Cr-labelled target cells (at ratios of 25:1, 5:1 and 1:1, in quadruplicate) for 4 hours at 37°C, before measuring the amount of ^{51}Cr released into the supernatants. Results are expressed as the percent cytotoxicity (mean ± sem) for each quadruplicate set.

To characterize the phenotype of the responding lymphoid cells, MoAb and C depletion techniques were employed. To define the cells induced to proliferate, T cell-specific MoAbs (1 in 10 final dilution) and C (1 in 20 final dilution), or C (1 in 20 final dilution) alone were added to 4 replicate cultures which were incubated for 1 hour at

37°C before being pulsed with [^{3}H]-TdR. To characterize cytotoxic cells, MLR blasts, generated in 1 ml bulk cultures in 24-well trays, were adjusted to 10^7 cells/ml and incubated for 1 hour at 37°C with the MoAbs (1 in 10 final dilution) and C (1 in 20 final dilution), or C alone (1 in 20 final dilution), before incubating them with ^{51}Cr-labelled P815. These standard procedures were employed to assess the APC activity of the four PC suspensions studied and any exceptions are noted in the text. Each type of experiment was carried out at least three times and data shown are representative of the results obtained.

4.2 Results

4.2.1 Cell profiles of NPCs and day 1-3 IPCs

(BALB/c x C57BL/6) F1 mice were injected ip with approximately 10^5 L11RX and the cellular composition of PCs harvested 1, 2 or 3 days later (D 1, 2 or 3 IPCs) was compared with NPCs suspensions. The most obvious effect of ip infection with L11RX was the marked increase in PCs that could be recovered from the peritoneal cavity (Table 4.1).

To define the cell types present, Cytospin smears of all four types of PC suspensions were made, stained with Giemsa stain and viewed by oil immersion microscopy. At least 250 cells/smear were counted and categorized and the numbers of the various cell types present were expressed as a percentage of the total population (Table 4.2). Infection with L11RX induced very marked changes in the PC profiles. Even as early as 1 day after challenge, the cells present in the peritoneal cavity were dramatically different to those characteristic of NPC suspensions. An influx of neutrophils was observed, with a resultant large decrease in the relative numbers of lymphocytes while the relative numbers of macrophages remained fairly constant in all four populations. Photographs of the cell smears were taken to show representative populations of the cells present and to illustrate the increased heterogeneity of the D 1, 2 and 3 IPCs (Fig. 4.1 [A-D]). A considerable increase in the size of macrophages,

TABLE 4.1 Total cells recovered from the peritoneal cavities of normal or L11RX immunized mice

	PC yields/mouse (mean ± sem)*
NPCs	$5.5 \times 10^6 \pm 4.3 \times 10^5$
D 1 IPCs	$1.3 \times 10^7 \pm 2.0 \times 10^6$
D 2 IPCs	$1.7 \times 10^7 \pm 2.9 \times 10^6$
D 3 IPCs	$2.1 \times 10^7 \pm 2.6 \times 10^6$

* The peritoneal cavities of normal and L11RX immunized mice were washed out with approximately 5 ml HBSS and like suspensions pooled. The average numbers of viable cells recovered from groups of at least fifteen mice are provided.

TABLE 4.2 Cell profiles of PCs recovered from normal mice and mice injected with L11RX 1, 2 or 3 days earlier

	Percentage of each cell type*:				
Population examined:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
NPCs	33	62	1	1	3
D 1 IPCs	47	20	33	0	0
D 2 IPCs	42	19	39	0	0
D 3 IPCs	36	19	44	0	1

* At least 250 cells (per smear) were examined and the relative proportions of the various cell types were recorded as the average percentage of the total population. These data represent the averages of 2 or 3 smears for each PC suspension.

A

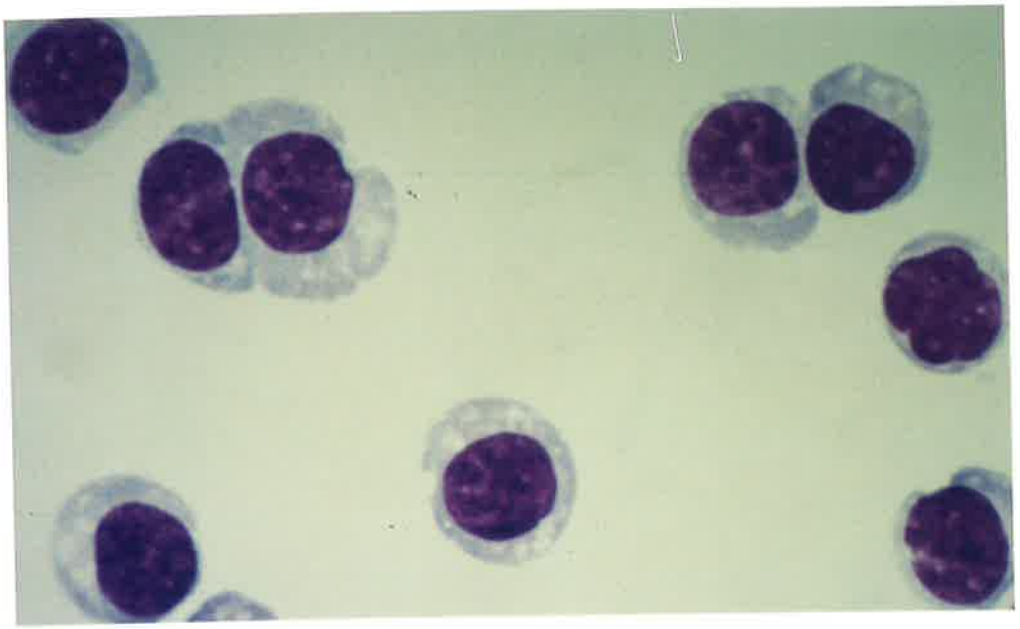
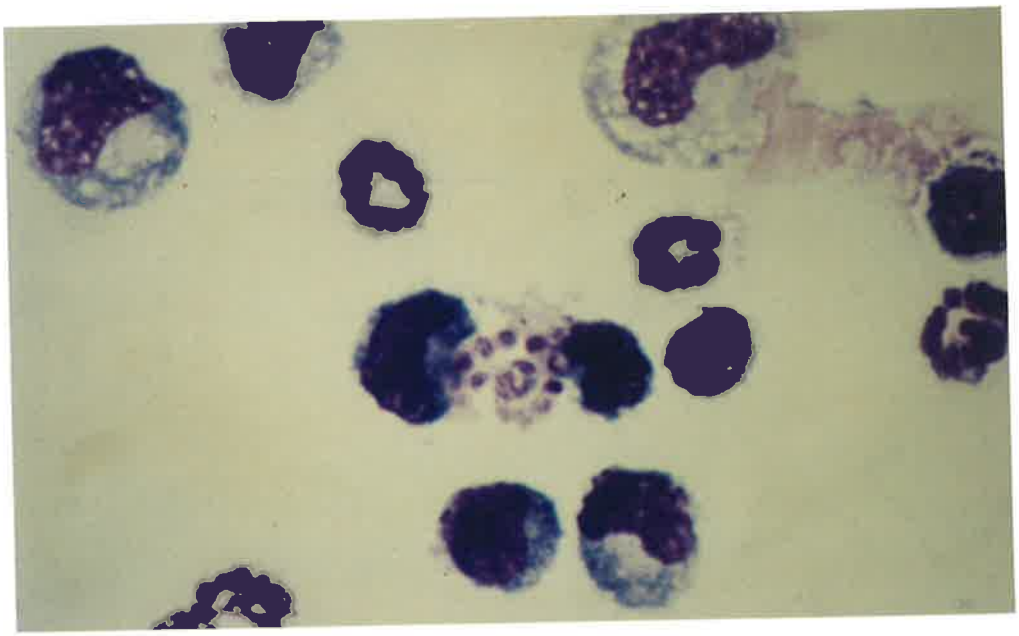


FIGURE 4.1 Cells present in various PC populations

Cytospin smears of NPCs, D 1, 2 and 3 IPCs were fixed and stained with Giemsa stain in the usual manner and photographs of representative populations were taken at 1000 times magnification ; NPCs (A), D 1 IPCs (B), D 2 IPCs (C) and D 3 IPCs (D).

B



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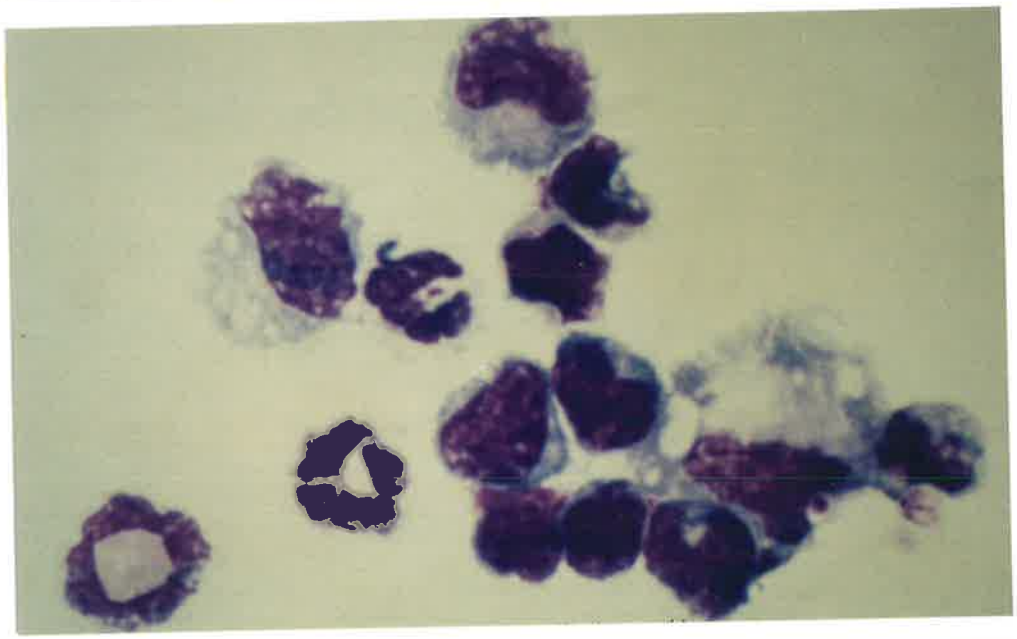
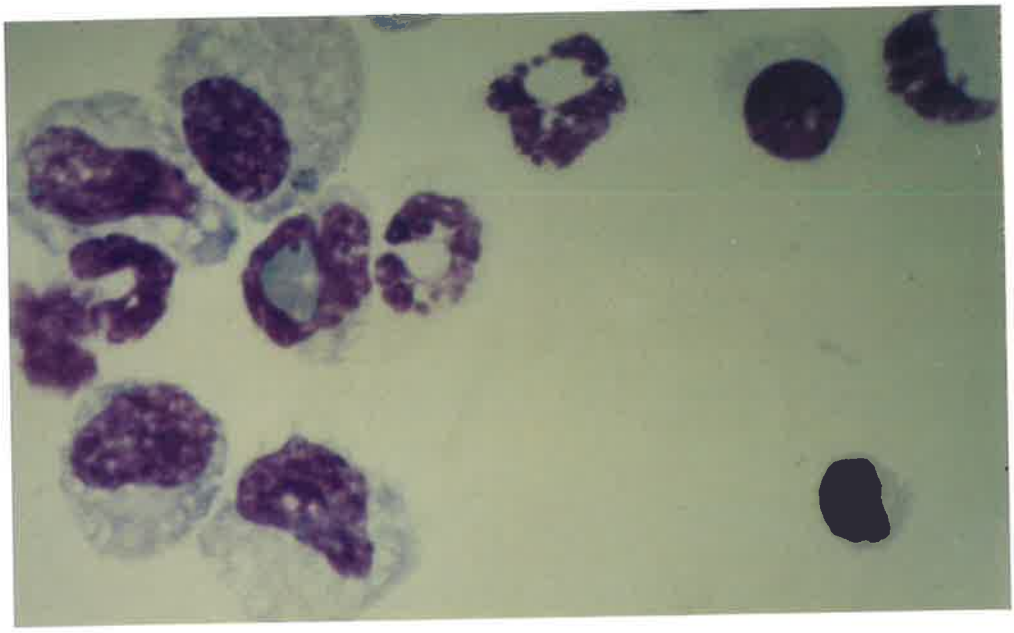


D



with their cytoplasm becoming foamy in appearance, was the other marked change induced by L11RX infection, suggesting that these macrophages had become activated.

Whether these changes in cell profiles were associated with any major modification in their ability to stimulate T cells remained to be established and studies addressing this issue constitute the remainder of this Chapter.

4.2.2 Stimulation of primed T cells

4.2.2.1 Comparison of NPCs and D 1, 2 or 3 IPCs as APCs

Preliminary experiments were carried out to assess the ability of NPCs and D 1, 2 and 3 IPCs to induce primed T cells to proliferate *in vitro* in response to 1 μg/ml F11RX. Two concentrations of the four types of PCs ($2x10^4$ and $1x10^5$/well) were cultured with 10^5 purified, primed T cells in the presence of 1 μg/ml F11RX for 3 days at 37°C and the amounts of proliferation induced by these APCs was determined by pulsing the cells with [^{3}H]-TdR as described.

Significant proliferative responses were induced in the presence of all four PC populations, and the number of PCs added determined the magnitude of the responses obtained (Table 4.3). NPCs stimulated marked responses at both cell concentrations used. D 1 and 2 IPCs induced responses which usually were similar to those induced by NPCs, although 10^5 D 2 IPCs sometimes did not induce responses which were as high as those induced in the presence of $2x10^4$ cells, with some variability between experiments. In contrast, $2x10^4$ D 3 IPCs consistently induced good responses, whilst 10^5 failed to do so. Hence, NPCs, D 1 and D 2 IPCs appeared to have very similar APC capabilities, whilst D 3 IPCs were nonstimulatory or inhibitory in larger numbers. Consequently, more detailed analysis of the Ag presenting capacity of D 3 IPCs was carried out, using the NPCs as control APCs. There are a number of possible explanations for the lack of response observed in the presence of larger numbers of D 3 IPCs. One obvious alternative is that insufficient Ag was being presented to the T cells, as a consequence of increased Ag degradation by APCs in the D 3 IPC suspensions. Another is that the lack

See Addendum Comment 1

TABLE 4.3 APC activity of NPCs and D 1-3 IPCs

	[^{3}H]-TdR uptake by primed T cells* after 3 days of culture with 1 μg/ml F11RX in the presence of various APC populations:	
	No. APCs (/well)	
APC type	$2x10^4$	10^5
NPCs	169 445 ± 3083	155 036 ± 7051
D 1 IPCs	127 533 ± 392	105 273 ± 140
D 2 IPCs	141 131 ± 256	94 496 ± 3648
D 3 IPCs	100 924 ± 199	1774 ± 66

* Mixtures of 10^5 purified, primed T cells, F11RX and the various APC populations were incubated for 3 days, after which proliferation was measured in the usual manner and the cpm of [^{3}H]-TdR incorporated (mean ± sem) are provided. Each APC suspension and the primed T cell suspension were incubated in culture medium, in the presence and absence of F11RX. The cpm of ^{3}H-thymidine incorporated by these control cultures were : ranging from 200 to 1100 cpm, for all APC suspensions cultured in culture medium ± F11RX; 361 ± 25, for purified, primed T cells in culture medium; 5001 ± 308, for purified, primed T cells cultured with F11RX.

of response was due to inhibition of T cell proliferation by activated macrophages and/or the large numbers of neutrophils present in the peritoneal cavity following L11RX infection. These possibilities were addressed in the following sections.

4.2.2.2 Adherent and nonadherent PCs as APCs

In an attempt to identify the cells which were responsible for the nonstimulatory/inhibitory effect observed with D 3 IPCs, the total PC population and the adherent and nonadherent subpopulations were examined for their ability to stimulate proliferation of L11RX-primed T cells in five separate experiments. Various numbers of NPCs and D 3 IPCs ranging from $4x10^3$-$1x10^5$, were added to the wells of a 96-well flat-bottomed tray (100 µl/well, 8 wells with each concentration) and incubated for at least 1 hour at 37°C. After the incubation the trays were shaken and the 100 µl of medium (containing nonadherent cells which represented approximately 50% of the total population) were removed from half of the wells originally set up and transferred individually to an unused set of wells. Fresh culture medium (100 µl/well) was added to the adherent cells left behind. The other half of the PC cultures were left untouched (ie. these wells contained unfractionated PCs). Purified, primed T cells (10^5/well) and F11RX (0.2 µg/well) were then added in 100 µl/well and the trays were incubated for 3 days at 37°C before measuring the amount of proliferation induced.

Table 4.4 presents results representative of several repeat experiments which provided very similar data. The results indicate that all the concentrations of unfractionated, adherent and nonadherent subpopulations of NPCs (plus F11RX) used induced significant proliferative responses by the purified, primed T cells. Although varying from experiment to experiment, the nonadherent cells usually induced slightly smaller responses than the other two populations of NPCs suggesting that fewer "classical" APCs were present in this population. The variation in responses was probabaly due to slight, but significant, variations in the separation of the adherent and nonadherent subpopulations and was more noticeable with the NPCs. Presumably this occurred because NPCs contain fewer adherent cells, resulting in lower contamination of

TABLE 4.4 Proliferation of primed T cells induced by unfractionated populations and the adherent and nonadherent subpopulations of NPCs and D 3 IPCs

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with 1 μg/ml F11RX in the presence of:		
APCs	No. APCs (per well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	10^5	201 478 ± 11 368	191 803 ± 9115	60 482 ± 6810
	$2x10^4$	206 948 ± 10 297	200 744 ± 3796	41 501 ± 8251
	$4x10^3$	87 819 ± 9004	81 792 ± 3754	22 046 ± 2433
D 3 IPCs	10^5	236 ± 40	270 ± 60	131 839 ± 3642
	$2x10^4$	207 553 ± 9887	172 735 ± 4605	38 839 ± 1893
	$4x10^3$	125 392 ± 4032	120 482 ± 4519	22 030 ± 3089

* Mixtures of 10^5 primed, purified T cells and the various APC suspensions were cultured in quadruplicate for 3 days at 37°C before determining the amount of proliferation by measuring the amount of [^{3}H]-TdR incorporated during the final 4 hours of culture. Data from one of four repeat experiments are presented, the results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated. Minimal amounts of radioactive thymidine were incorporated by control suspensions: primed T cells in culture medium, 1007 ± 32; primed T cells + F11RX, 1562 ± 25; primed T cells + APCs, ranged from 107-376 cpm; APCs in culture medium ± F11RX, ranged from 85-208 cpm.

the nonadherent population with "APC-like" cells. In contrast, no T cell proliferation was induced in the presence of the largest numbers of unfractionated and adherent D 3 IPCs. However, all concentrations of the nonadherent D 3 IPCs and lower doses of the unfractionated and adherent D 3 IPCs induced significant responses which were quite similar to those induced by the NPCs. This suggested that the lack/inhibition of T cell proliferation observed was due to a subpopulation of adherent cells which were induced by the third day of infection with L11RX. It appeared that the inhibitory subpopulation was a minor one because no inhibitory effect was observed when the IPC numbers used were reduced five-fold.

Unfractionated NPCs and D 3 IPCs and adherent and nonadherent subpopulations of these cells were also compared for their ability to induce IL 2 release from primed T cells. L11RX primed T cells were cultured with the various APCs in the presence of F11RX for 20 hours and the amount of IL 2 released into the culture supernatants was measured using the standard IL 2 maintenance assay. Because the results of all four of the experiments were very similar, Table 4.5 shows only one set of data. It was interesting to find that NPCs and D 3 IPCs had very similar APC activities; they induced the release of virtually identical amounts of IL 2, with the nonadherent subpopulations of these cells being less effective than the adherent subpopulations.

In summary, the only difference between the NPCs and IPCs obtained shortly after immunization with L11RX that was detected in this series of experiments was the inability of purified, primed T cells to proliferate in the presence of large numbers of D 3 IPCs despite the fact that these PCs were shown to be able to act as APCs, as assessed by the release of IL 2 from a comparable primed T cell population. In other words, the inability of primed T cells to proliferate in the presence of D 3 IPCs was not due to a lack of IL 2 production and was mediated by the adherent subpopulation of D 3 IPCs. The following section presents further information about the APC function of these cells which were pulsed *in vivo* with *Salmonella* Ags and then examined for their ability to induce proliferation when cultured with purified, primed T cells.

TABLE 4.5 IL 2 released by primed T cells stimulated with F11RX and NPCs or D 3 IPCs

	Units of IL 2 released by primed T cells* after stimulation with F11RX and APCs#:		
APC source	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	59	40	19
D 3 IPCs	57	33	21

$4x10^5$ unfractionated NPCs and D 3 IPCs, and the adherent and nonadherent subpopulations of these cells, were used as APCs to stimulate IL 2 release in the presence of F11RX.

* Duplicate 1 ml volumes of $2x10^6$ purified, primed T cells, 10 µg/ml F11RX and the various APC suspensions were cultured for 20 hours at 37°C in an atmosphere of 5% CO_2. The cell free supernatants were harvested, like samples pooled and the presence of IL 2 determined by the routine assay for IL 2 using ConA blasts as the indicator cells. The units of IL 2 were calculated for each sample, as described in Materials and Methods.

4.2.2.3 *In vivo* pulsing with F11RX

(i) Introduction

Earlier preliminary work in our laboratory had shown that NPCs harvested from mice injected ip with 250 μg of F11RX 15 minutes earlier were able to induce primed T cells to proliferate without the addition of any more *Salmonella* Ags to culture medium (Attridge and Kotlarski, unpublished). Several experiments were carried out to confirm these observations, they were all successful in showing that *in vivo* pulsed NPCs induced significant proliferation of purified, primed T cells at both the cell concentrations tested. The addition of extra Ag to the cultures had little or no affect on proliferation (data not shown). This approach was adopted for the further investigation of the APC activity of PCs harvested from mice immunized with L11RX 3 days earlier.

(ii) PC profiles

Before examining the stimulatory capacity of PCs pulsed with *Salmonella* Ags *in vivo*, the types of cells present in these suspensions were defined. Groups of 3 normal mice and mice immunized with L11RX 3 days previously were injected ip with 250 μg F11RX fifteen minutes before they were sacrificed and their PC suspensions harvested. Cytospin smears of the PC suspensions were made, stained with Giemsa stain and viewed under oil immersion to categorize the cell types present and to detect bacteria present. Table 4.6 shows the PC profiles of these Ag-pulsed NPCs and D 3 IPCs .

In vivo pulsing had little influence on the proportions of the various types of cells detected in either PC suspension. Photographs taken of the Cytospin smears illustrated the relative proportions of cell types seen and confirmed that considerable numbers of bacteria were present in the macrophage-like cells of both NPCs and D 3 IPCs (Fig. 4.2 [A and B]). It did appear that a larger proportion of the macrophage-like cells recovered from the normal mice had engulfed more bacteria than those recovered from mice immunized 3 days earlier, and that the normal cells contained larger numbers of bacteria per cell as well.

See Addendum Comment 2

TABLE 4.6 Cell profiles of *in vivo* Ag-pulsed and non-pulsed PCs

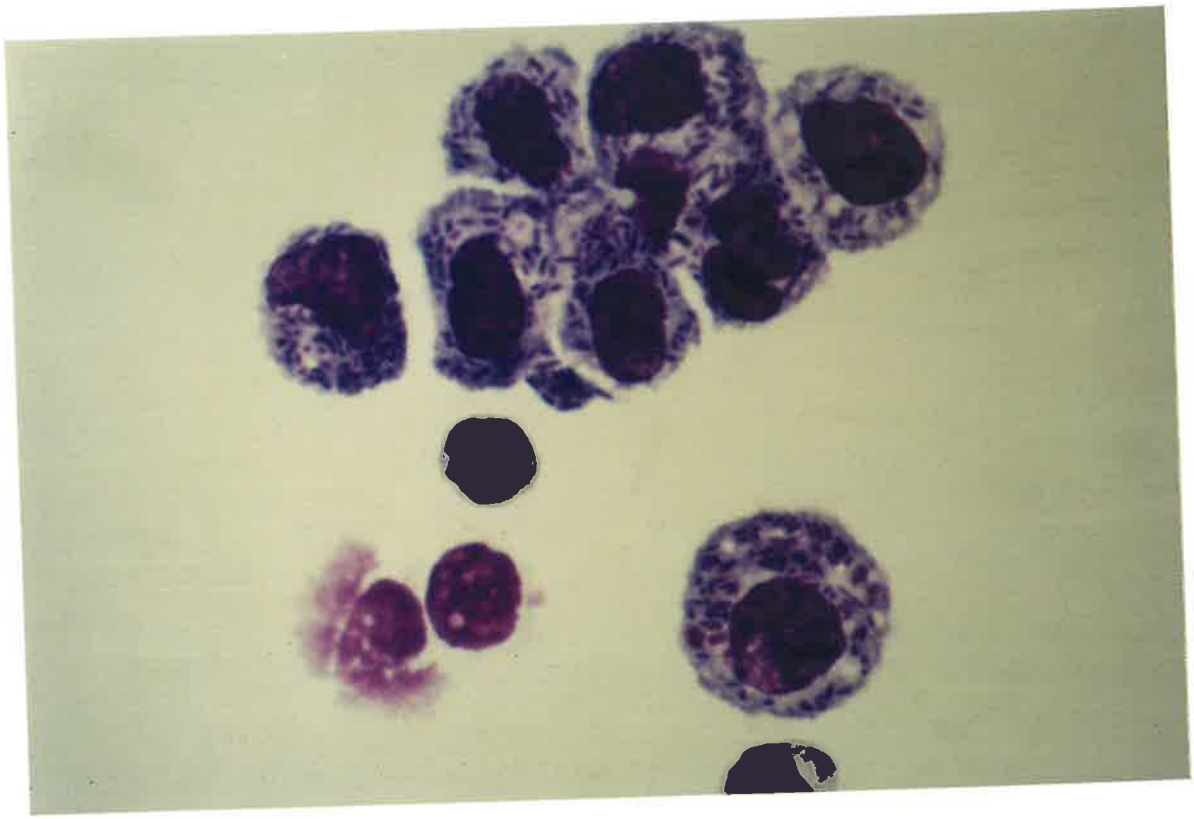
	Percent of each cell type*:				
Population:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
NPCs	33	62	1	1	3
In vivo pulsed NPCs	37	63	0	0	0
D 3 IPCs	36	19	44	0	1
In vivo pulsed D 3 IPCs	40	20	40	0	0

* Cytospin smears were prepared of PCs obtained from normal mice and mice immunized with L11RX 3 days earlier (NPCs and D 3 IPCs) and from normal and immunized mice which had been injected ip with 250 µg F11RX 15 minutes earlier (*in vivo* pulsed NPCs and *in vivo* pulsed D 3 IPCs). The smears were fixed, stained with Giemsa stain and viewed using oil immersion microscopy. At least 300 cells were counted (per smear), the cells categorized and the numbers of all cell types present were recorded and expressed as the percent of the total population.

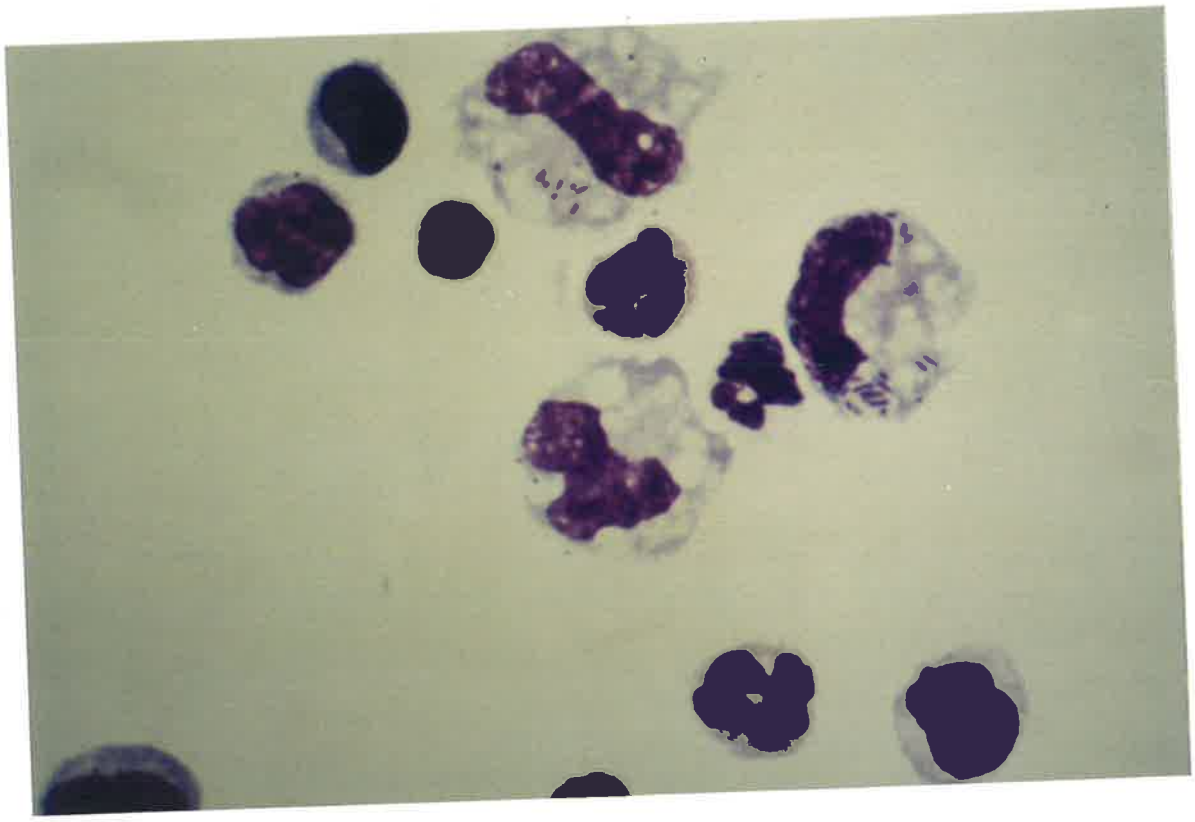
FIGURE 4.2 Analysis of *in vivo* F11RX pulsed NPCs and D 3 IPCs

Normal mice and mice immunized with 10^5 L11RX 3 days earlier were injected with 250 μg F11RX ip and 15 minutes later the mice were sacrificed and their PCs harvested in the usual manner. Cytospin smears of both *in vivo* pulsed NPCs and D 3 IPCs were made, stained with Giemsa and photographs were taken at 1000 times magnification ; *in vivo* Ag-pulsed NPCs [A] and *in vivo* Ag-pulsed D 3 IPCs [B].

A



B



(iii) ***APC activity of in vivo F11RX pulsed PCs***

Groups of four normal mice or mice immunized with L11RX 3 days previously were ip injected with 250 μg of F11RX. Fifteen minutes later these mice were sacrificed and their PCs were harvested, counted and adjusted to the required cell concentrations. The APC activity of these cells was assayed as usual, by culturing them for 3 days with purified, primed T cells before pulse-labelling with [^{3}H]-TdR during the final 4 hours of culture at 37°C and measuring the uptake of radioactivity. Both *in vivo* pulsed populations of PCs induced proliferation of the primed T cells at the two concentrations used (Table 4.7). [Control suspensions of APCs or primed T cells in culture medium alone incorporated minimal amounts of [^{3}H]-TdR (data not shown).] In other words, *in vivo* pulsing with F11RX removed the inhibitory effect observed with large numbers of D 3 IPCs.

Although these experiments did not reveal the reason why *in vivo* pulsing with F11RX removed the inhibitory activity from D 3 IPCs, several explanations can be provided. For example, *in vivo* pulsing with Ag may have resulted in the selective adherence of the cells responsible for inhibition, thus ensuring that they would not be recovered when the PCs were harvested. This possibility was consistent with the finding that the numbers of PCs recovered from *in vivo* Ag pulsed mice were slightly reduced when compared to cell recoveries from non-pulsed mice. It was likely that such an adherent, inhibitory population represented only a minor proportion of the total population because *in vivo* pulsing with Ag had virtually no influence on the PC profile observed. Alternatively, if the lack/inhibition of response by purified, primed T cells was caused by rapid removal of Ag due to increased degradation by activated macrophages and/or the large numbers of neutrophils which were present in the unfractionated D 3 IPCs, it follows that *in vivo* pulsing of these cells provided sufficient Ag to stimulate T cell proliferation, despite any degradation that did occur. In any case, an analysis of the effect of removal of neutrophils from D 3 IPCs of APC function seemed warranted.

TABLE 4.7 Proliferation of purified, primed T cells stimulated by *in vivo* Ag-pulsed APCs

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with various APCs#:	
	No. APCs (per well)	
APCs#	$2x10^4$	10^5
In vivo Ag-pulsed NPCs	224 367 ± 4297	118 428 ± 10 948
NPCs + F11RX	145 789 ± 8916	206 861 ± 10 596
In vivo Ag-pulsed D 3 IPCs	199 805 ± 15 246	209 948 ± 8127
D 3 IPCs + F11RX	196 783 ± 9311	1327 ± 107

PCs were harvested from normal mice and mice immunized with L11RX 3 days earlier (NPCs and D 3 IPCs) and from normal and immunized mice 15 minutes after they had been injected ip with 250 µg F11RX (*in vivo* Ag-pulsed NPCs and D 3 IPCs). Two doses of NPCs and D 3 IPCs + F11RX (1 µg/ml), or the *in vivo* Ag-pulsed APCs were used to stimulate primed T cells.

* Quadruplicate cultures of 10^5 purified, primed T cells and the various APC suspensions were incubated for 3 days at 37°C before the amount proliferation was determined by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture. Representative data are shown, with the results expressed as the cpm (mean ± sem) of radioactive thymidine incorporated for each replicate set.

4.2.2.4 Removal of neutrophils on Metrizamide density gradients

(i) ***Initial characterization***

Purification of cell populations using density gradients is widely practiced. For example, Lopez *et al.* (1983) have reported that Metrizamide density gradients can be used to remove neutrophils from heterogeneous PC populations. Following centrifugation of PCs at 1200 g for 45 minutes on discontinuous Metrizamide gradients, mononuclear cells were located at the first interface and neutrophils at the second.

The same approach was used to fractionate NPCs and D 3 IPCs (see Materials and Methods section). Cytospin smears made of the cells harvested from the first interface were stained with Giemsa, examined for contaminating neutrophils and photographed. The results obtained (Table 4.8 and Fig. 4.3 [A-D]) illustrate the success of this procedure. As expected, the composition of NPC populations was virtually unchanged, whereas most of the neutrophils normally present in the D 3 IPC suspension had been removed. The removal of neutrophils from D 3 IPCs was reproducible and was consistently effective in reducing their numbers by 85-95%.

(ii) ***Stimulation of primed T cells by PCs fractionated on Metrizamide density gradients***

The total, adherent and nonadherent subpopulations of NPCs and D 3 IPCs were used in these studies. Varying numbers of Metrizamide fractionated and unfractionated suspensions of these cells were cultured *in vitro* with primed T cells and 1 μg/ml F11RX and the proliferation induced was measured. Clearly, removal of neutrophils had little, if any effect on the APC function of D 3 IPCs (Table 4.9 A). Removal of neutrophils from the total and adherent subpopulations of D 3 IPCs did not enhance the ability of large numbers of these cells to induce T cell proliferation. The slight increase in responses induced by $5x10^4$ cells fractionated on the Metrizamide gradients probably reflected the relative increase in APC numbers in the total and adherent populations. The reduced levels of [^{3}H]-TdR incorporation obtained in the

TABLE 4.8 Cell profiles of unfractionated and Metrizamide fractionated NPCs and D 3 IPCs

	Percent of each cell type#:				
Population:	Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cells
NPCs	33	62	1	1	3
Metrizamide NPCs*	34	66	0	0	0
D 3 IPCs	36	19	44	0	1
Metrizamide D 3 IPCs*	62	31	6	1	0

* NPCs and D 3 IPCs were fractionated on a Metrizamide density gradient by centrifugation at 1200 g for 45 minutes, before the cells at the first interface were harvested to provide the Metrizamide fractionated NPCs and D 3 IPCs (Metrizamide NPCs and D 3 IPCs, respectively).

Cytospin smears were prepared of the Metrizamide fractionated cells and unfractionated NPCs and D 3 IPCs, stained with Giemsa stain and viewed under oil immersion microsocopy. At least 250 cells were counted per smear, the cells categorized and the data presented are typical of several repeat experiments, with the results expressed as the percent of the total cell population represented by each cell type.

A

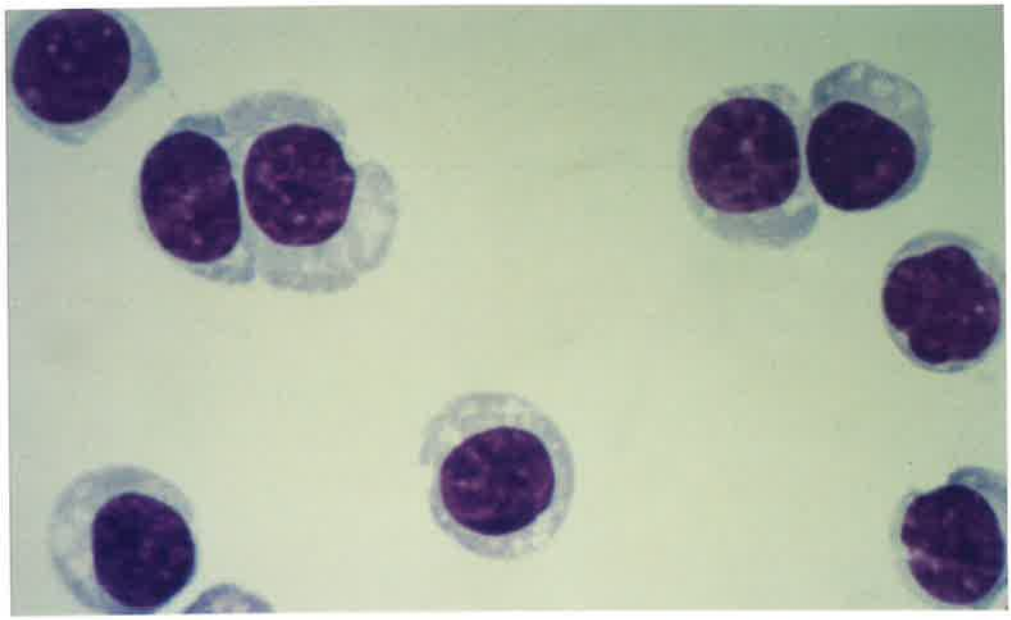
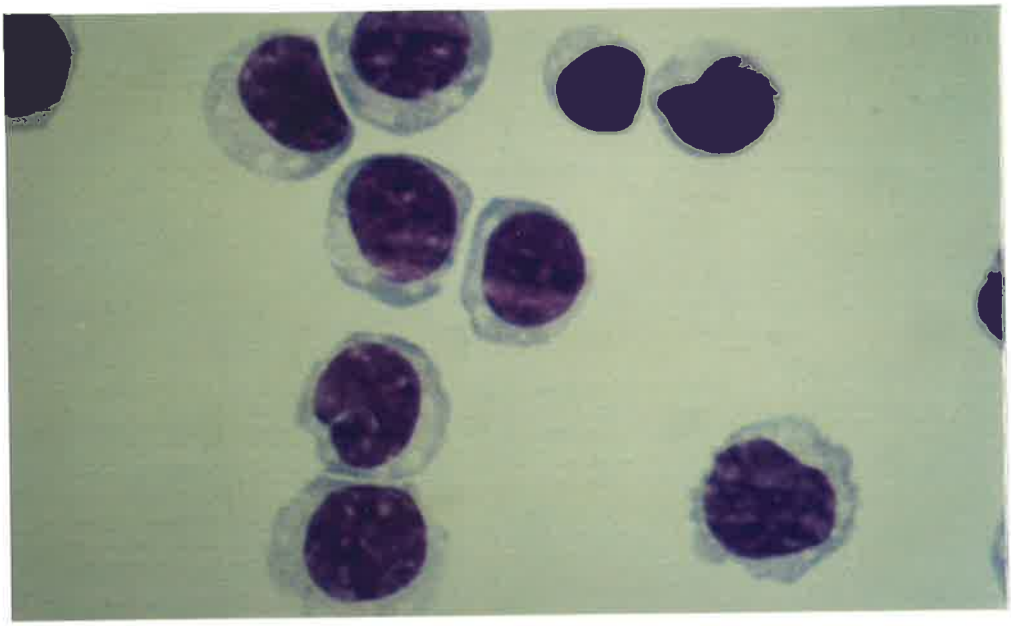


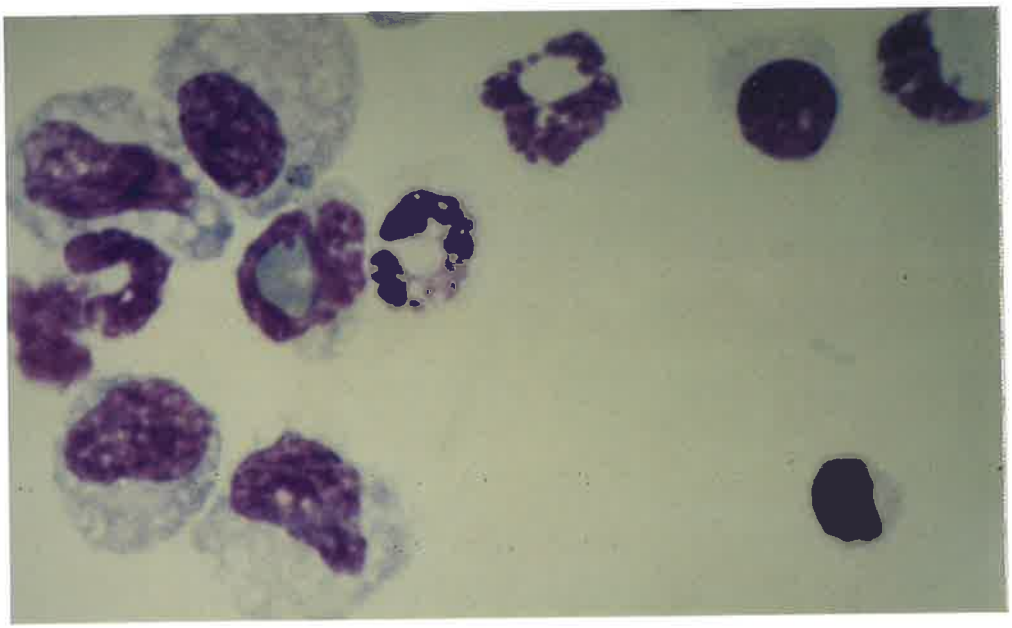
FIGURE 4.3 The effect of fractionation on Metrizamide density gradients on the cell types present in the NPC and D 3 IPC populations

NPCs and D 3 IPCs were fractionated by centrifugation for 45 minutes at 1200 g on Metrizamide density gradients. Cytospin smears of fractionated and unfractionated NPC and D 3 IPC suspensions were prepared, stained with Giemsa and viewed using oil immersion microscopy. Photographs were taken at 1000 times magnification and show unfractionated NPCs [A], Metrizamide fractionated NPCs [B], unfractionated D 3 IPCs [C] and Metrizamide fractionated D 3 IPCs [D].

B



C



D

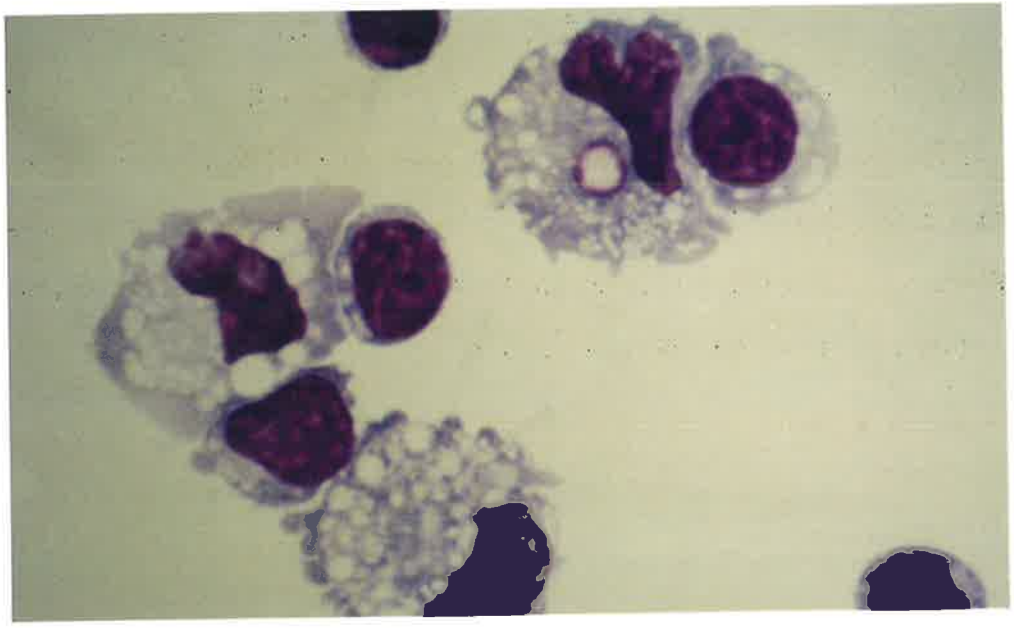


TABLE 4.9 A Proliferation of primed T cells induced by D 3 IPCs and Metrizamide fractionated D 3 IPCs

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with 1 µg/ml F11RX in the presence of:		
APCs	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
Untreated D 3 IPCs	10^5	13 519 ± 1562	1268 ± 520	208 465 ± 5937
	$5x10^4$	17 359 ± 1679	27 715 ± 8166	169 783 ± 8427
	$2x10^4$	183 758 ± 16239	174 892 ± 16 944	132 472 ± 4036
	10^4	251 257 ± 3812	239 863 ± 7149	115 003 ± 5787
Metrizamide D 3 IPCs#	10^5	2604 ± 47	1859 ± 296	197 173 ± 13 475
	$5x10^4$	65 365 ± 10 868	37 003 ± 9890	161 780 ± 14 519
	$2x10^4$	187 365 ± 8533	158 009 ± 6638	93 929 ± 7061
	10^4	178 047 ± 12 138	188 260 ± 7123	63 451 ± 5356

PCs were obtained from mice immunized ip with L11RX 3 days earlier (D 3 IPCs) and half were fractionated on a Metrizamide density gradient by centrifugation at 1200 g for 45 minutes and the cells located at the first interface were harveted to provide the Metrizamide fractionated suspension (Metrizamide D 3 IPCs). The unfractionated populations and the adherent and nonadherent subpopulations of untreated D 3 IPCs and Metrizamide D 3 IPCs were used as the APCs for this study.

* Quadruplicate mixtures of 10^5 purified, primed T cells and a range of concentrations of the various APC suspensions were cultured for 3 days at 37°C before determining the proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) by the cells during the final 4 hours of culture at 37°C. A representative set of results are presented, showing results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated by the replicate cultures.

presence of 10^5 unfractionated and, to a lesser extent, 10^5 adherent NPCs are misleading (Table 4.9 B) because they suggest that a larger response has been induced by 10^5 nonadherent NPC subpopulation. Microscopic examination of the cultures containing 10^5 unfractionated or adherent NPCs indicated that T cell proliferation had "peaked" earlier, because all these cultures contained many more cells than were originally added to the cultures. In addititon, this set of results further illustrates the variation which was often observed with these cultures, although overall the responses induced in the presence of the nonadherent NPC subpopulation were lower than those induced when either the unfractionated or adherent populations were used.

Because the presence of neutrophils did not account for the inability of large numbers of D 3 IPCs to present Ag to primed T cells, it seemed more likely that this effect was macrophage mediated. The next set of experiments were designed to distinguish between the possibility that the inhibitory effect was exerted directly on the T cells was mediated indirectly, by reducing the amount of Ag(s) available for presentation to the primed T cells.

4.2.2.5 The effect of increasing Ag dose on the stimulation of proliferation by NPCs and D 3 IPCs

The Ag presenting capacity of total and the adherent and nonadherent subpopulations of NPCs and D 3 IPCs, with and without fractionation on Metrizamide density gradients, were compared using the standard proliferation assays involving purified, primed T cells and 1-100 μg/ml F11RX as Ag. Table 4.10 A shows data representative of several experiments using NPCs and D 3 IPCs not fractionated on Metrizamide gradients. When $2x10^4$ NPCs or D 3 IPCs and 1 or 10 μg/ml F11RX were used, the responses induced were very similar in magnitude and increasing the concentration of F11RX to 100 μg/ml reduced the responses induced by up to 75% for both types of PCs. [This was confirmed by microscopic examination which revealed small numbers of cells/well on day 3 of culture.] This reduction in response was not seen in the presence of 10^5 NPCs - the proliferation induced in the presence of total,

TABLE 4.9 B Proliferation of primed T cells induced by NPCs and Metrizamide fractionated NPCs

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with 1 µg/ml F11RX in the presence of:		
APCs used	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
Untreated NPCs	10^5	17 049 ± 4849	87 424 ± 9474	158 674 ± 9760
	$5x10^4$	302 350 ± 7836	320 340 ± 8797	171 635 ± 7292
	$2x10^4$	269 224 ± 14 004	261 890 ± 9058	133 806 ± 7783
	10^4	222 744 ± 11 776	220 040 ± 6877	118 728 ± 8641
Metrizamide NPCs#	10^5	213 690 ± 2432	168 470 ± 4938	71 044 ± 15 258
	$5x10^4$	271 253 ± 12 183	262 203 ± 12 094	143 346 ± 6823
	$2x10^4$	205 038 ± 14 030	217 085 ± 5203	131 458 ± 6303
	10^4	167 677 ± 3275	178 521 ± 4678	120 950 ± 7691

PCs were harvested from normal mice (NPCs) and half were fractionated on a Metrizamide density gradient by centrifugation at 1200 g for 45 minutes, when the cells at the first interface were harvested providing the fractionated population (Metrizamide NPCs).

* 10^5 purified, primed T cells were mixed (in quadruplicate) with a range of doses of the unfractionated populations and adherent and nonadherent subpopulations of the untreated and Metrizamide NPCs and cultured for 3 days at 37°C before determining the proliferation induced by measuring the amount of [^{3}H]-TdR taken up (cpm) by the cells during the final 4 hours of culture. Representative data are presented showing the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate set.

TABLE 4.10 A Proliferation of primed T cells induced by various APCs and a range of doses of F11RX

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with various APCs and F11RX:		
APCs	No. APCs (/well)	F11RX (µg/ml)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	$2x10^4$	1	277 375 ± 18 161	269 600 ± 4078	120 201 ± 9144
		10	216 631 ± 9213	217 930 ± 4311	136 756 ± 11 149
		100	161 901 ± 3966	131 275 ± 5918	55 290 ± 4865
	10^5	1	194 671 ± 2623	183 675 ± 3750	196 568 ± 10 392
		10	167 552 ± 8636	185 078 ± 5584	211 087 ± 9860
		100	158 166 ± 11 537	193 975 ± 8351	109 767 ± 13 462
D 3 IPCs	$2x10^4$	1	218 373 ± 5546	196 897 ± 9497	142 916 ± 14 457
		10	188 733 ± 7484	150 134 ± 17 991	88 992 ± 167
		100	34 517 ± 12 051	24 821 ± 3980	24 427 ± 5181
	10^5	1	2250 ± 335	2195 ± 620	189 806 ± 12 653
		10	3484 ± 439	1345 ± 108	186 117 ± 3875
		100	32 550 ± 1189	24 011 ± 1787	49 800 ± 2020

* Quadruplicate cultures of 10^5 purified, primed T cells and the various APC suspensions (including the unfractionated populations and adherent and nonadherent subpopulations of NPCs and D 3 IPCs) in the presence of 1, 10 or 100 μg/ml F11RX, were incubated for 3 days at 37°C before establishing the proliferation induced, by measuring the amount of [^{3}H]-TdR taken up (cpm) by the cells during the last 4 hours of culture at 37°C. A characteristic set of results are presented, expressed as the cpm (mean ± sem) for each replicate set.

adherent or nonadherent NPCs was approximately the same for all F11RX concentrations studied, with one exception; the response to 100 μg/ml F11RX in the presence of nonadherent NPCs showed a reduction of approximately 50%. Interestingly, in this particular experiment the responses of T cells cultured with 10^5 nonadherent NPCs and 1-10 μg/ml F11RX were almost identical to those induced by 10^5 adherent or unfractionated NPCs (unlike the responses shown in Tables 4.4 and 4.9 B), again illustrating the variations frequently observed using these cells as APCs. The proliferation induced in the presence of 10^5 D 3 IPCs was quite different. Consistent with the results reported previously, addition of 10^5 nonadherent D 3 IPCs induced proliferative responses similar to those observed with nonadherent NPCs and this applied to all three F11RX concentations used. Similarly, 1 or 10 μg/ml F11RX induced no proliferation when 10^5 total D 3 IPCs and 10^5 cells of the adherent subset were used and only marginal responses were detected with 100 μg/ml F11RX. In other words, increasing the Ag concentration up to 100-fold did not completely overcome the lack of repsonse induced by large numbers of D 3 IPCs. See Addendum Comment 1

Table 4.10 B illustrates the effect of removing neutrophils on the ability of the NPCs and D 3 IPCs to stimulate T cell proliferation in the presence of varying amounts of F11RX. The data obtained suggested that the neutrophils may contribute to the enhanced degradation of Ag because, in the presence of 100 μg/ml F11RX, 10^5 total and adherent subpopulations of Metrizamide fractionated D 3 IPCs induced significantly higher responses than those induced by unfractionated D 3 IPCs. Removal of neutrophils had no detectable effect on any of the other responses.

In conclusion, the large numbers of neutrophils present in the D 3 IPC suspensions were not *directly* responsible for the lack of response when 10^5 D 3 IPCs were used as APCs in the T cell proliferation assays with 1 μg/ml F11RX. The major factor contributing to the inability of large numbers of D 3 IPCs to stimulate T cells was the presence of an "inhibitory" adherent cell population, and the mechanism of inhibition could be partially explained by increased degradation of Ag by adherent macrophages, but the neutrophils normally present also played a contributing role in Ag degradation.

TABLE 4.10 B Proliferation of primed T cells stimulated by Metrizamide fractionated APCs and F11RX

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with various APCs and a range of doses of F11RX:		
APCs	No. APCs (/well)	F11RX (µg/ml)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
Met. NPCs#	$2x10^4$	1	279 358 ± 10 886	256 556 ± 5846	199 989 ± 7606
		10	192 231 ± 13 206	180 838 ± 1039	136 836 ± 15 897
		100	152 424 ± 3925	147 510 ± 27 810	65 747 ± 18 888
	10^5	1	301 250 ± 5460	306 385 ± 15 296	259 250 ± 4903
		10	262 400 ± 2206	246 306 ± 7622	202 870 ± 889
		100	240 573 ± 1335	238 320 ± 6687	166 383 ± 15 472
Met. D 3 IPCs#	$2x10^4$	1	297 650 ± 8683	271 444 ± 7545	156 389 ± 11 386
		10	124 832 ± 5416	125 386 ± 1375	85 703 ± 3519
		100	74 966 ± 4864	58 076 ± 5523	24 883 ± 2253
	10^5	1	6315 ± 1449	5248 ± 1053	273 687 ± 4370
		10	12 834 ± 1336	22 699 ± 3764	237 873 ± 6395
		100	123 529 ± 8942	125 894 ± 3736	73 999 ± 8933

NPCs and D 3 IPCs were fractionated on Metrizamide density gradients by centrifugation at 1200 g for 45 minutes and the cells located at the first interface were harvested to provide the Metrizamide fractionated NPC and D 3 IPC suspensions (Met. NPCs and Met. D 3 IPCs). Two doses of the unfractionated/total populations and the adherent and nonadherent subpopulations of these suspensions were used as the APCs for this study.

* 10^5 purified, primed T cells were mixed in quadruplicate with each of the APC suspensions and the various concentrations of F11RX, and cultured at 37°C for 3 days before determining the proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) by the cells during the last 4 hours of culture. Presented are a typical set of results expressed as the cpm (mean ± sem) for each quadruplicate set.

These results are consistent with previous reports that infection with various IBPs, including *Salmonella*, enhances the metabolic activity of macrophages. Since macrophages become activated following infection with L11RX (Ashley and Kotlarski, 1982; La Posta *et al.*, 1982), it seems reasonable to expect that these cells can degrade Ag more efficiently than NPCs. However, a result which was not predicted was the finding that increasing the concentration of F11RX to 100 μg/ml induced less proliferation than 1 or 10 μg/ml F11RX when NPCs were used as APCs. The following experiments were carried out to determine whether such large amounts of excess Ag were somehow inhibiting the T cells if present during *in vitro* culture.

4.2.2.6 Pulsing of APCs with F11RX *in vitro*

NPCs and D 3 IPCs ($5x10^6$ cells/ml) were incubated in the presence of 20-100 μg/ml F11RX for 2-3 hours in a shaking waterbath at 37°C before thoroughly washing them and resuspending them to 10^6/ml and $2x10^5$/ml. Purified, primed T cells ($2x10^6$/ml) were mixed with these Ag-pulsed APCs and four 200 μl aliquots of each mixture were cultured for 3 days at 37°C before measuring the uptake of [^{3}H]-TdR during the final 4 hours of culture at 37°C.

Initial experiments indicated that pulsing with 20 μg/ml F11RX for 2 hours was sufficient to produce APCs able to induce proliferation of purified, primed T cells in the absence of additional Ag during culture (Table 4.11). Proliferation induced by $2x10^4$ *in vitro* Ag-pulsed NPCs was greater than that induced by the $2x10^4$ Ag-pulsed D 3 IPCs when 20 or 100 μg/ml F11RX were used for a 2 hour pulse. However, in the presence of 10^5 Ag-pulsed cells the responses by NPCs and D 3 IPCs were very similar, especially when the PCs were pulsed with 100 μg/ml F11RX for 3 hours, when Ag-pulsed D 3 IPCs induced larger responses than Ag-pulsed NPCs. Obviously, providing more Ag to the APCs and then washing away any excess Ag not taken up by the APCs, ensured greater stimulation of the T cells than providing the same levels of Ag in culture. It is important to note that it is possible that some adherent cells may have been lost during this 2-3 hour incubation (even though the cells were shaken

TABLE 4.11 Proliferative responses induced by *in vitro* Ag-pulsed APCs

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *in vitro* Ag-pulsed APCs#	
			No. APCs (/well):	
APCs	Ag dose (μg/ml)	Pulsing time (hours)	$2x10^4$	10^5
NPCs	20	2	87 904 ± 2820	136 066 ± 7387
	100	2	206 873 ± 10 388	269 317 ± 4105
	100	3	235 255 ± 3525	182 531 ± 12 790
D 3 IPCs	20	2	49 152 ± 2469	143 470 ± 3178
	100	2	122 050 ± 1617	247 706 ± 2537
	100	3	303 875 ± 11 623	271 437 ± 18 439

Quadruplicate sets of $2x10^4$ and 10^5 NPCs and D 3 IPCs with 20 or 100 μg/ml F11RX, were added to the wells of a 96-well flat-bottomed tray and incubated for 2 or 3 hours at 37°C in an atmosphere of 5% CO_2. After this, the trays were gently shaken, the nonadherent cells and any free Ag removed and the adherent monolayers washed with fresh culture medium.

* 10^5 purified, primed T cells were added to the Ag-pulsed monolayers and incubated for 3 days at 37°C before determining the amount of proliferation induced by measuring the amount of [^{3}H]-TdR uptake (cpm) by the cells during the final 4 hours of culture at 37°C. Presented are representative results expressed as the cpm (mean ± sem) of radioactive thymidine incorporated for each replicate set.

continuously) and could explain why larger numbers of D 3 IPCs were stimulatory (this was supported by a reduction in cell numbers after pulsing with Ag).

Previous workers have established that for Ag presentation to occur, Ag must be taken up by APCs and processed/degraded to provide the antigenic determinants which are expressed on the APC surface in association with MHC molecules (Rosenthal and Shevach, 1973; Doherty *et al.*, 1976). Clearly, more Ag was required to induce the same amount of T cell stimulation by the *in vitro* Ag-pulsed D 3 IPCs as that induced by Ag-pulsed NPCs, supporting the view that D 3 IPCs had enhanced degradative capacity and therefore required more Ag to provide sufficient antigenic determinants to induce T cell responses of similar magnitude. More information on the degradative capacity of these *in vitro* Ag-pulsed cells was obtained by assessing the effect of paraformaldehyde (PFA) fixation on their APC activity.

4.2.2.7 Examination of APC activity of cells after PFA treatment

(i) L11RX-primed T cell responses

Presentation of Ags has been shown to require metabolically active APCs (reviewed by Grey and Chestnut, 1985). Preliminary experiments established that treatment of PCs with 0.5% PFA resulted in the inactivation of APC function, as demonstrated by the inability of such cells to induce T cell proliferation (reported by Vordermeier *et al.*, 1990). Consequently, treatment of APC populations with PFA prior to or after exposure to Ags allowed further analysis of the Ag processing/degradative capacity of NPCs and D 3 IPCs.

In the first series of experiments, *in vitro* incubation of $5x10^6$ PCs/ml with 20 µg/ml F11RX for 2 hours was the procedure used for Ag pulsing. Aliquots of unpulsed and Ag-pulsed NPCs and D 3 IPCs were fixed with 0.5% PFA (as described in the Materials and Methods section) and the remainder left unfixed. Following thorough washing, the ability of all these PC suspensions to stimulate T cell proliferation was assessed by culturing them with purified, primed T cells. Table 4.12 illustrates that PFA

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TABLE 4.12 The effect of PFA fixation on the APC activity of 20 µg/ml F11RX pulsed APCs

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with:			
			NPCs ± F11RX#		D 3 IPCs ± F11RX#	
No. APCs (/well)	F11RX pulsed^	PFA treated>	-	+	-	+
$2x10^4$	-	-	2369 ± 522	30 420 ± 2058	889 ± 207	23 821 ± 1078
	+	-	87 904 ± 2820	99 560 ± 4667	49 152 ± 2469	47 734 ± 1363
	-	+	1015 ± 199	984 ± 79	369 ± 12	1165 ± 2
	+	+	617 ± 68	1551 ± 257	958 ± 2	1892 ± 298
10^5	-	-	5849 ± 239	122 428 ± 1214	1812 ± 169	8749 ± 158
	+	-	136 066 ± 7387	160 464 ± 803	143 470 ± 3178	83 588 ± 1281
	-	+	633 ± 126	710 ± 132	428 ± 16	2383 ± 269
	+	+	5266 ± 446	2419 ± 172	9539 ± 1797	8582 ± 216

^ NPCs and D 3 IPCs ($5x10^6$ cells/ml) were incubated in culture medium containing 20 µg/ml F11RX or culture medium alone for 2 hours in a shaking 37°C waterbath. After this, the cells were washed with fresh culture medium, counted and adjusted to the required concentrations for use as the APCs in this assay.

> Aliquots of the F11RX pulsed and non-pulsed APCs were treated with 0.5% PFA solution for 4 minutes at 37°C. An equal volume of culture medium + 10% FCS was then added to each suspension and the cells were washed 3 times by centrifugation and allowed to stand for a further 1 hour at 37°C. Following this, the cells were washed for a final time, counted and adjusted to the required concentration.

The various APC suspensions of the NPC and D 3 IPC populations were added to 4 replicate wells of a 96-well flat-bottomed tray in the presence and absence of 1 µg/ml F11RX.

* 10^5 primed, purified T cells were added to the various populations of APCs (in the presence and absence of additional F11RX) and incubated at 37°C for 3 days prior to determining the proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the last 4 hours of culture at 37°C. A characteristic set of data are presented, showing the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate set.

fixation did inhibit the APC function of both NPCs and D 3 IPCs. Both cell populations were unable to induce proliferation of T cells when F11RX was added during culture, whereas a response was induced with unfixed cells. Exposure of either cell type to 20 μg/ml F11RX for 2 hours did not result in sufficient Ag uptake and/or processing to induce significant T cell proliferation, unless 10^5 D 3 IPCs were used.

Consequently, these experiments were repeated using 100 μg/ml F11RX for 2 or 3 hours for *in vitro* Ag-pulsing the NPCs and D 3 IPCs. The results obtained (Tables 4.13 [A and B]) showed that both types of Ag-pulsed PCs stimulated significant T cell proliferation and that the 3 hour Ag-pulse rendered them more stimulatory than the 2 hour pulse, particularly when the lower number of D 3 IPCs was used. An important point to note is that some T cell proliferation was induced when 10^5 "control" D 3 IPCs, which had been incubated for 2-3 hours at 37°C in culture medium without Ag, were used. Although the response detected was lower than the responses detected using the same number of NPCs or fewer D 3 IPCs, these data supported the possibility that adherent cells were lost during the 2-3 hour incubation required for Ag pulsing. Fixation without Ag pulsing again abolished the APC function of the PC populations but PFA fixation of Ag-pulsed cells did not entirely abolish their APC function. It was interesting to note that the responses induced by Ag-pulsed and fixed D 3 IPCs were greater than those induced by Ag-pulsed and fixed NPCs, supporting the conclusion that D 3 IPCs could degrade/process the F11RX Ag more rapidly then NPCs. This conclusion was further supported by work carried out in our laboratory which demonstrated that addition of IL 1 to these cultures did not enhance the responses observed, suggesting that the reduction in the proliferation induced by PFA-treated APCs was not simply due to the failure to provide important secondary signals, such as IL 1 (Vordermeier *et al.*, 1990).

(ii) Primed MLR blast responses

Fixation of cells with PFA may alter the MHC molecules on the cell surface and therefore affect the ability of the cells to present Ag to T cells. In other words, the reductions in stimulation of T cells by the PFA treated APCs may have been

TABLE 4.13 A The effect of PFA fixation on the APC activity of PCs pulsed with 100 µg/ml F11RX for 2 hours

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with:			
			NPCs ± F11RX#		D 3 IPCs ± F11RX#	
No. APCs (/well)	F11RX pulsed^	PFA treated>	-	+	-	+
2x10^4	-	-	671 ± 1	46 763 ± 1636	1219 ± 105	164 818 ± 7242
	+	-	206 873 ± 10 388	166 201 ± 5462	122 050 ± 1617	80 005 ± 976
	-	+	497 ± 75	4505 ± 138	733 ± 88	16 423 ± 1799
	+	+	7489 ± 694	13 890 ± 737	43 295 ± 7465	32 339 ± 4199
10^5	-	-	2119 ± 198	163 695 ± 11 589	1316 ± 111	56 023 ± 4587
	+	-	269 317 ± 4105	223 975 ± 9119	247 706 ± 2537	178 656 ± 5372
	-	+	608 ± 56	11 447 ± 1191	485 ± 65	8143 ± 20
	+	+	26 108 ± 562	19 107 ± 1703	32 983 ± 1800	31 911 ± 1210

TABLE 4.13 B The effect of PFA fixation on the APC ability of PCs pulsed with 100 µg/ml for 3 hours

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with:			
			NPCs ± F11RX#		D 3 IPCs ± F11RX#	
No. APCs (/well)	F11RX pulsed^	PFA treated>	-	+	-	+
$2x10^4$	-	-	5425 ± 1333	126 708 ± 1408	1885 ± 220	212 859 ± 15 012
	+	-	235 255 ± 3525	198 131 ± 6855	303 875 ± 11 623	257 880 ± 6487
	-	+	727 ± 163	7890 ± 300	2600 ± 232	6766 ± 306
	+	+	26 288 ± 4136	11 314 ± 387	44 311 ± 1382	24 840 ± 2702
10^5	-	-	2983 ± 137	125 214 ± 3849	2253 ± 27	38 556 ± 2757
	+	-	182 531 ± 12 790	196 404 ± 9182	271 437 ± 18 439	234 700 ± 14 463
	-	+	850 ± 92	11 107 ± 1080	1527 ± 110	26 710 ± 2562
	+	+	71 711 ± 3872	68 264 ± 3296	107 002 ± 2495	102 109 ± 2651

^ NPCs and D 3 IPCs ($5x10^6$ cells/ml) were incubated in culture medium containing 100 µg/ml F11RX or culture medium alone for 2 (Table 4.13 A) or 3 (Table 4.13 B) hours in a shaking 37°C waterbath. After this, the cells were washed with fresh culture medium, counted and adjusted to the required concentrations for use as the APCs in this assay.

Footnotes >, # and * are identical to those listed for Table 4.12.

caused by alterations to the MHC molecules or possibly even toxicity of PFA-treated cells, rather than inhibition of metabolic/processing activity. For this reason the ability of PFA treated PCs to restimulate allo-Ag-specific, primed T cells was compared with the activity of unfixed cells.

NW nonadherent mesenteric lymph node cells (NW MLNCs) harvested from C57BL/6 mice (H-2^b haplotype) were cultured with NPCs derived from (C57BL/6 x BALB/C) F1 mice (H-$2^{b/d}$ haplotype) for 9 days at 37°C. On the ninth day of culture the MLR blasts were harvested, washed, readjusted to the required concentration and aliquots of these cells were mixed with three different concentrations of PFA-fixed and unfixed F1 NPCs and D 3 IPCs and cultured for a further 3 days. A dose of $2x10^4$ unfixed NPCs and D 3 IPCs induced similar levels of proliferation, which were reduced when $4x10^3$ PCs were used (Table 4.14). However, somewhat surprisingly, although 10^5 unfixed NPCs were more stimulatory than $2x10^4$ unfixed NPCs, 10^5 unfixed D 3 IPCs exhibited similar nonstimulatory activity to that observed with the *Salmonella*-primed T cells. This suggested that lack of stimulation by the D 3 IPCs could not be entirely explained by enhanced Ag degradation, since it could also be observed in a situation where "processing" of exogenous Ag was not required. However, a possible alternative is that it may reflect differences in the cell metabolism, resulting in changes in the association of "self peptides" with self-MHC molecules, which may alter the amounts of effective allo-Ag detected in MLR cultures. It is possible that the lack of response to the PFA-fixed cells is simply due to the toxicity of PFA still present in the fixed cell suspensions, however the steps taken in the fixation procedure have been reported to minimize this effect. Furthermore, since responses were induced in the *Salmonella*-primed T cells by Ag-pulsed and fixed cells and could be increased when cells were pulsed with more Ag for a longer period of time, suggested that the toxic effect of residual PFA could not entirely account for these observations.

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(iii) ***Summary***

It was concluded that ip immunization with L11RX induced considerable

TABLE 4.14 *In vitro* secondary MLR induced by PFA fixed *vs* unfixed PCs

		[^{3}H]-TdR uptake (cpm ± sem) by 9 day C57BL/6 MLR blasts* restimulated with F1 NPCs or D3 IPCs:	
No. APCs (/well)	PFA treated#	NPCs	D3 IPCs
10^5	-	217 350 ± 6944	13 655 ± 44027
	+	4166 ± 297	19 423 ± 2170
$2x10^4$	-	140 004 ± 1268	143 019 ± 1842
	+	361 ± 40	2065 ± 84
$4x10^3$	-	99 132 ± 1577	21 278 ± 4865
	+	133 ± 46	236 ± 22

PCs were harvested from normal mice (NPCs) and mice immunized with L11RX 3 days earlier (D 3 IPCs), counted and adjusted to the required concentrations. Half of each of these populations were treated with PFA (the others remaining untreated) by incubating the cells in a solution of 0.5% PFA at 37°C for 4 minutes. The PFA was inactivated by the addition of an equal volume of culture medium + 10% FCS, the cells were washed thoroughly and reincubated for a further 1 hour at 37°C. Finally, the cells were washed, counted and adjusted to the appropriate concentrations.

* 9 day MLR blasts generated by culturing $2x10^6$ C57BL/6 NW MLNCs with $4x10^5$ F1 NPCs for 9 days at 37°C, were washed, counted and adjusted to 10^6 cells/ml. Four replicate 200 µl aliquots of 10^5 MLR blasts and each concentration of the APC populations were cultured for a further 3 days at 37°C before pulsing the cultures with [^{3}H]-TdR in the final 4 hours of culture and measuring the amounts of [^{3}H]-TdR incorporated (cpm). Typical data are presented showing the cpm (mean ± sem) of [^{3}H]-TdR taken up by each replicate culture.

changes in the PC profiles, cell yields and the functional capacity of PCs harvested from such mice. Alterations in their functional capacity were detected (using relatively large numbers of these cells) by their inability to induce proliferative alloresponses and *Salmonella* Ag-specific responses in cultures of F11RX and purified, primed T cells. Their inability to induce T cell proliferation may be explained (at least partly) by their enhanced metabolic/degradative ability. Attempts to further characterize changes in PCs after infection with L11RX using surface markers and FACScan analysis has been carried out and will be reported in a later section.

4.2.2.8 Phenotype of the T cells induced to proliferate in response to *Salmonella* Ags presented by NPCs or D 3 IPCs *in vitro*

(i) T cell subsets responding to Salmonella Ags

Since it has been concluded that the Te cells responsible for mediating immunity to other IBPs belong to the $Lyt2^+$ phenotype, it was of interest to determine whether there is a difference in the phenotype of the T cells induced *in vitro* by NPCs and D 3 IPCs in response to various *Salmonella* Ags. The purified, primed T cells appeared to be a reasonable source of T cells to use for these studies because it had been demonstrated that such preparations contained cells of both the $L3T4^+$ and $Lyt2.2^+$ phenotypes (Fig. 3.9 [A-F]).

Initial experiments were carried out to determine whether the phenotype(s) of the T cells stimulated by NPCs (+ F11RX) was the same as those stimulated by D 3 IPCs (+ F11RX). Twenty four wells of a 96-well tray containing $2x10^4$ NPCs/well and another 24 containing $2x10^4$ D 3 IPCs/well were incubated in the presence of 1 μg/ml F11RX and 10^5 purified, primed T cells for 3 days at 37°C when proliferation was assessed using the standard procedure used to measure [^{3}H]-TdR uptake. One hour prior to addition of [^{3}H]-TdR, various combinations of MoAbs and C at the appropriate dilutions were added to the cultures to determine the phenotype(s) of the proliferating cells. Control suspensions incubated with MoAbs alone showed almost identical

responses to those cultured with culture medium or C alone (data not shown).

Table 4.15 contains data illustrating the effect of these treatments on the proliferative responses induced. Clearly, the cells responsible for the majority of the proliferation induced by NPCs and F11RX were T cells of the L3T4$^+$ phenotype, because both α-Thy1.2 + C and α-L3T4 + C reduced the amount of proliferation quite markedly, although not completely. Similarly, the cells proliferating in response to F11RX + D 3 IPCs were mainly of the L3T4$^+$, Thy1.2$^+$ phenotype. Incomplete removal of activity was routinely encountered when proliferating populations of purified, primed T cells were treated in this way. A possible explanation for the incomplete depletion of the proliferative responses induced was simply that in this system the amount of MoAbs and C were insufficient to abrogate such large responses, since even α-Thy1.2 and C treatment, which was virtually 100% effective in other systems such as DTH transfers and *in vitro* cytotoxicity assays (which involved treatment of "bulk" cultures of cells using the procedure described in Section 2.11.1), did not abolish the response entirely. [However, the numbers of mice required to obtain sufficient cells for such "bulk" cultures were too great for this procedure to be employed for all proliferation assays. Consequently, cells were treated in the tissue culture trays and the trends of the effect of the MoAb and C treatments on the proliferative responses were recorded.] The finding that treatment with α-Thy1.2 and C reduced the proliferation to a greater extent than treatment with α-L3T4 and C suggested that a Thy1.2$^+$, L3T4$^-$, Lyt2.2$^-$ population may have also been induced to proliferate in these cultures and possible explanations will be discussed in Chapter 7. Hence, this approach did not eliminate the possibility that there may be a difference between the ability of NPCs and D 3 IPCs to present Ags and indicated that alternative assay systems had to be employed to determine whether L11RX did induce any differences in APC function.

(ii) T cell subsets proliferating in response to in vitro pulsed APCs

APC populations were pulsed *in vitro* with F11RX by adding 2x10^4 NPCs or D 3 IPCs to a series of wells and incubating them in the presence of 20 μg/ml F11RX

TABLE 4.15 Phenotype of the T cells proliferating in response to stimulation with F11RX and various APCs

	[^{3}H]–TdR uptake (cpm ± sem) by primed T cells* after culture for 3 days with F11RX and:	
Treatment#	NPCs	D 3 IPCs
None	236 683 ± 13 493	245 992 ± 13 006
C alone	236 805 ± 6908	230 146 ± 13 267
α-Thy1.2 + C	53 378 ± 1281	52 884 ± 4801
α-L3T4 + C	92 433 ± 5619	98 585 ± 3514
α-Lyt2.2 + C	209 101 ± 11 095	210 360 ± 1164
α-L3T4, α-Lyt2.2 + C	87 892 ± 11 944	115 190 ± 3862

* Twenty four replicate cultures of 10^5 purified, primed T cells with either $2x10^4$ NPCs or $2x10^4$ D 3 IPCs were incubated in a 96-well flat-bottomed tray for 3 days at 37°C before determining the proliferation induced by measuring the amount of [^{3}H]-TdR taken up (cpm) by the cells during the final 4 hours of culture.

Prior to pulsing with the [^{3}H]-TdR, MoAbs specific for T cell markers (1 in 10 final dilution) and C (1 in 20 final dilution), C (1 in 20 final dilution) alone or culture medium were added to 4 replicate cultures of each suspension, the trays shaken and incubated for 1 hour at 37°C. Representative results expressed as the cpm (mean ± sem) for each replicate set are presented.

for 2 hours at 37°C before shaking the trays and removing the nonadherent cells and free Ag. The cell monolayers were washed a couple of times in fresh culture medium before adding the standard numbers of purified, primed T cells to each well in 200 µl of culture medium and incubating the trays for 3 days at 37°C. On the third day the various MoAbs and C were added to the trays, as previously described, and the trays incubated for an hour at 37°C prior to pulsing with [^{3}H]-TdR for a further 4 hours.

The results presented in Table 4.16 demonstrate that the bulk of the cells proliferating in response to the F11RX pulsed NPCs or D 3 IPCs also expressed the L3T4$^+$, Thy1.2$^+$ phenotype. A slight reduction in the proliferation following treatment with α-Lyt2.2 and C was observed on a few occasions, suggesting that Lyt2.2$^+$ T cells had been induced to proliferate. However, when both α-L3T4, α-Lyt2.2 and C were used little difference between this and treatment with only α-L3T4 and C was observed. [Again treatment with the various MoAbs alone had no effect on the proliferative responses.] Therefore, using F11RX in the form of Ag-pulsed cells did not appear to alter the type of T cell induced, again indicating that no significant differences between NPCs and D 3 IPCs could be detected. Obviously, a likely explanation is that the antigenic peptides derived from F11RX do not enter the appropriate processing pathway, irrespective of the cell type used to present them to the T cells. Observations consistent with this conclusion were provided using the *in vivo* Ag-pulsing protocol.

(iii) T cell responses to APCs pulsed with F11RX in vivo

The phenotype of the T cells induced to respond to *in vivo* Ag-pulsed PCs which were harvested from normal mice and mice immunized with L11RX 3 days earlier was determined using the experimental design used in the previous two sets of experiments. PCs were *in vivo* pulsed with F11RX as previously described, then harvested and 2x10^4 APCs were added to the standard number purified, primed T cells and cultured for 3 days at 37°C before establishing the phenotype of the proliferating cells.

Data presented in Table 4.17 indicate that pulsing with F11RX *in vivo* did

TABLE 4.16 Phenotype of the T cells proliferating in response to *in vitro* Ag-pulsed APCs

	[^{3}H]–TdR uptake (cpm ± sem) by primed T cells* after culture for 3 days with *in vitro* F11RX pulsed APCs^:	
Treatment#	NPCs	D 3 IPCs
None	183 567 ± 4052	182 264 ± 35 034
C alone	199 044 ± 14 150	133 376 ± 8885
α-Thy1.2 + C	24 309 ± 1405	13 177 ± 1172
α-L3T4 + C	60 149 ± 8184	68 196 ± 5839
α-Lyt2.2 + C	131 125 ± 3975	106 884 ± 6565
α-L3T4, α-Lyt2.2 + C	53 647 ± 2382	61 413 ± 3460

^ 100 µl aliquots containing $2x10^4$ NPCs or D 3 IPCs and 10 µg/ml F11RX were each added to twenty four replicate wells of a 96-well flat-bottomed tray, incubated for 2 hours at 37°C before removing the nonadherent cells and any free Ag and washing the adherent monolayers with fresh culture medium.

* 10^5 purified, primed T cells were added to these Ag-pulsed APC populations and cultured at 37°C for 3 days before pulsing the cells with [^{3}H]-TdR for the final 4 hours of culture and proliferation was determined by the amount of [^{3}H]-TdR taken up (cpm) by these cells during this time.

Before pulsing with radioactive thymidine, mixtures of appropriately diluted MoAbs (1 in 10 final) and C (1 in 20 final), C alone (1 in 20 final) or culture medium alone were added to 4 replicate cultures and incubated for 1 hour at 37°C. A characteristic set of data are presented, showing the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each replicate set.

TABLE 4.17 Phenotype of the T cells proliferating in response to *in vivo* F11RX-pulsed APCs

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *in vivo* F11RX pulsed APCs#	
	In vivo pulsed APCs:	
Treatment^:	NPCs	D 3 IPCs
C alone	122 404 ± 3680	115 561 ± 3800
α-Thy1.2 + C	69 081 ± 3301	36 086 ± 1935
α-L3T4 + C	93 144 ± 1116	75 466 ± 3067
α-Lyt2.2 + C	134 169 ± 4506	137 801 ± 5969
α-L3T4, α-Lyt2.2 + C	72 381 ± 9960	61 614 ± 2742

Normal mice and mice immunized ip with L11RX 3 days earlier were injected ip with 250 μg of F11RX and 15 minutes later the mice were sacrificed and the PCs harvested. These *in vivo* Ag-pulsed PCs were counted and adjusted to $2x10^5$ cells/ml for use as the APCs in these studies.

* Mixtures of 10^5 purified, primed T cells and $2x10^4$ Ag-pulsed NPCs or D 3 IPCs were each dispensed into 24 wells of a 96-well flat-bottomed tray and incubated for 3 days at 37°C before determining the proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture.

^ Before adding the [^{3}H]-TdR, C (1 in 20 final dilution) and MoAbs specific for T cell markers (1 in 20 final dilution), C alone (1 in 20 final dilution) or culture medium alone were added to 4 replicate cultures of each mixture. Results from one of several repeat experiments are presented, showing the cpm (mean ± sem) of [^{3}H]-TdR incoporated for each replicate group.

not effect the resultant responses induced by either APC population; as the majority of the proliferating cells again expressed the L3T4$^+$, Thy1.2$^+$ phenotype, indicating that exposure of the APCs to F11RX *in vivo* presumably again did not allow access to the Class I presentation pathway. Since live *Listeria* organisms had been shown to be presented in the context of Class I and II MHC molecules (Jungi *et al.*, 1982a and 1982b), L11RX was used as the Ag in the next set of experiments.

(iv) ***In vivo pulsing with L11RX***

Normal and day 3 immune mice were injected ip with approximately 10^7 L11RX (equivalent to 10 μg of bacteria). After 15 minutes the mice were sacrificed and the PC suspensions harvested to provide the APC populations used in these experiments. The PCs were washed, counted and resuspended to allow addition of $2x10^4$ APCs to 10^5 purified, primed T cells/well and these suspensions were cultured in culture medium containing gentamycin, to control the growth of extracellular bacteria, for 3 days at 37°C. On the third day of culture the phenotypes of the cells proliferating in response to these stimuli were examined using the standard technique.

In vivo pulsing with L11RX provided PC populations capable of inducing T cells to proliferate *in vitro*, although the levels of proliferation induced were lower than in the previous sets of experiments (Table 4.17 *vs* Tables 4.18 A and B). This reflected the lower Ag dose used for Ag-pulsing, as indicated by the response induced by control PCs pulsed with equivalent numbers of killed bacteria (10 μg F11RX). It was somewhat disappointing to find that the T cells which proliferated in response to both L11RX-pulsed PC suspensions expressed the L3T4$^+$, Thy1.2$^+$ phenotype. Again the MoAb and C depletions were incomplete, suggesting either that this method was not optimal for removal of all proliferating cells (as described earlier) and/or that a double negative T cell population was also proliferating in these cultures (the possibility of which will be discussed in the final Chapter).

In conclusion, it was not possible to induce proliferation of Class I MHC restricted Lyt2.2$^+$ T cells using either NPCs or D 3 IPCs as APCs to present F11RX or

Normal mice and mice immunized ip with L11RX 3 days earlier were injected ip with 10 μg F11RX or 10^7 L11RX and 15 minutes later the PCs were harvested, counted and adjusted to $2x10^5$ cells/ml.

* Mixtures of 10^5 purified, primed T cells and either *in vivo* Ag-pulsed NPCs (Table 4.18 A) or D 3 IPCs (Table 4.18 B) were each loaded into 24 wells of a 96-well flat-bottomed tray and incubated at 37°C for 3 days before determining the amount of proliferation induced by measuring the amount of [^{3}H]-TdR taken up (cpm) during the last 4 hours of the incubation.

> Before adding the [^{3}H]-TdR, mixtures of appropriately diluted MoAbs (1/10 final) and C (1/20 final), C alone (1/20 final) or culture medium alone were added to quadruplicate cultures of each T cell-APC mixture, and incubated for 1 hour at 37°C. The data presented are representative of several experiments and are expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each quadruplicate set.

TABLE 4.18 A Phenotype of the proliferating T cells induced by *in vivo* Ag-pulsed NPCs

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *in vivo* Ag pulsed NPCs#:	
	NPCs from mice pulsed with:	
Treatment>	10 µg F11RX	10^7 L11RX
C alone	45 822 ± 2084	36 139 ± 1001
α-Thy1.2 + C	16 530 ± 2492	2 801 ± 1025
α-L3T4 + C	27 317 ± 2440	6 311 ± 1033
α-Lyt2.2 + C	40 993 ± 2028	31 556 ± 1226
a-L3T4, α-Lyt2.2 + C	22 744 ± 880	5 427 ± 1209

TABLE 4.18 B Phenotype of the proliferating T cells induced by *in vivo* Ag-pulsed D 3 IPCs

	[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *in vivo* Ag pulsed D3 IPCs#:	
	D3 IPCs from mice pulsed with:	
Treatment>:	10 µg F11RX	10^7 L11RX
C alone	96 068 ± 7631	29 528 ± 1053
α-Thy1.2 + C	19 811 ± 308	2087 ± 190
α-L3T4 + C	36 541 ± 1407	10 380 ± 1039
α-Lyt2.2 + C	100 289 ± 3467	27 717 ± 1163
α-L3T4, α-Lyt2.2 + C	39 779 ± 1262	8720 ± 609

L11RX to purified, primed T cells. Only L3T4$^+$ T cells were induced to proliferate. This could be interpreted to indicate that infection with L11RX does not induce modulation of APC activity which results in the *in vitro* induction of primed T cells of the Lyt2.2$^+$ phenotype and/or that Ag-specific Lyt2.2$^+$ T cells are not induced by *Salmonella* infection. However, it was also possible that the assay system employed to study APC activity was not optimal, because the Ags used or the Ag-pulsing protocols failed to ensure that Ag was presented in the form required for stimulation of *Salmonella*-specific Lyt2.2$^+$ T cells. However, the Ags used were the only preparations available at the time these studies were carried out. As discussed in the Chapter 1, it has now become evident that presentation of Ag by APCs can be manipulated to ensure presentation with either Class I or Class II MHC products. The characterization of *Salmonella* Ag preparations, to provide a well defined, low molecular weight Ag, which can be introduced to or directly access the Class I processing pathway, would obviously be very useful for extending these studies. It may result in the detection of Lyt2.2$^+$ T cells proliferating in response to such Ags and/or the recognition of differences in the APC function of NPCs and IPCs harvested shortly after infection with *Salmonella*.

4.2.2.9 Presentation of K99 to primed T cells

(i) Introduction

As stated above, use of a well defined Ag which contains fewer antigenic determinants than the more complex formalin killed or live bacterial Ag preparations, may provide more information on the Ag processing and presenting capacities of APCs. In the absence of a well defined *Salmonella* Ag, a "model system", using a hybrid *Salmonella enteritidis* 11RX strain expressing a well defined Ag of *Escherichia coli* (K99) was used to further examine the Ag presenting capacity of NPCs and D 3 IPCs. An 11RX strain expressing the K99 pilin (SA9) was constructed by Dr. Stephen Attridge and used to immunize mice following the standard procedure established for immunization with L11RX. Since L11RX induces a CMI response, it was expected that

SA9 would be able to induce a similar CMI response and that the K99 pilin expressed by 11RX might also be able to induce Ag-specific Te cells which could be analysed *in vitro* using K99 protein purified from SA9.

(ii) K99 in culture

Mice were immunized with 10^5 live SA9 ip and 14 days later the PCs were harvested and purified, primed T cells (SA9 IPCs) were prepared using the standard technique established with IPCs of 11RX immunized mice. SA9 IPCs were cultured with NPCs or D 3 IPCs in the presence of varying concentrations of K99 for 3 days at 37°C. Each combination was assayed in quadruplicate and on the third day of culture the amount of proliferation was measured by the level of [^{3}H]-TdR incorporated over a 4 hour period. Table 4.19 presents data representative of several experiments, illustrating that, although the proliferative responses induced by K99 were considerably lower than those induced by F11RX, they were quite significant when sufficient K99 was added. [The proliferative response of SA9 IPCs to K99 was K99-specific, because when purified, primed T cells were obtained from mice immunized with a hybrid 11RX strain which did not express K99 (SA5) no proliferation could be induced in response to NPCs + purified K99 (data not shown)].

(iii) In vitro pulsing with K99

A range of doses of NPCs and D 3 IPCs were mixed with 20 and 50 µg/ml K99, or 20 µg/ml F11RX and 100 µl volumes of each of these mixtures were added to two sets of four wells of a flat-bottomed tray and incubated for 2 or 4 hours at 37°C. After the incubation the trays were shaken, the nonadherent cells removed and the adherent monolayers washed twice with fresh culture medium. The standard number of SA9 IPCs were added to these K99-pulsed APCs and cultured for 3 days at 37°C before pulsing with [^{3}H]-TdR during the final 4 hours of incubation.

Table 4.20 illustrates that all the Ag-pulsed NPCs induced proliferation of SA9 IPCs. Increasing the dose of K99 and the length of time for Ag-pulsing

TABLE 4.19 Presentation of the K99 Ag to primed T cells by NPCs and D 3 IPCs

		[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with specific Ags and $2x10^4$:	
Ag	Ag conc^n (μg/ml)	NPCs	D 3 IPCs
K99	1	15 203 ± 1794	12 189 ± 1479
	5	18 203 ± 2068	16 409 ± 2434
	50	49 616 ± 2907	37 071 ± 4591
F11RX	1	213 917 ± 13 196	214 680 ± 2036

* Purified, primed T cells were prepared using the standard techniques from the PC suspensions harvested from mice which had been immunized ip with 10^5 live SA9 organisms 14 days earlier. Quadruplicate mixtures of 10^5 primed, purified T cells, Ag and either NPCs or D 3 IPCs as APCs, were cultured for 3 days at 37°C before being pulse-labelled with [^{3}H]-TdR during the final 4 hours of culture. Proliferation was deterimined by the amount of [^{3}H]-TdR incorporated (cpm) by the cells and the results expressed as the cpm (mean ± sem) for each replicate set of cultures. Minimal amounts of [^{3}H]-TdR were taken up by control suspensions: primed T cells in culture medium, 293 ± 12; primed T cells + F11RX, 1089 ± 64; primed T cells + 50 μg/ml K99, 473 ± 21; primed T cells + NPCs, 123 ± 10; primed T cells + D 3 IPCs, 87 ± 12; APCs alone or with either Ag, 134-250 cpm.

TABLE 4.20 Proliferation induced by K99-pulsed APCs

			[^{3}H]-TdR uptake (cpm ± sem) by primed T cells* after 3 days of culture with *in vitro* Ag-pulsed# APCs:		
			No. APCs (/well):		
APCs	Pulsed with:	Pulsing time (hours):	10^5	$5x10^4$	$2x10^4$
NPCs	20 µg/ml K99	2	44 503 ± 3619	70 885 ± 4751	25 747 ± 718
		4	45 566 ± 10 269	66 396 ± 6454	41 895 ± 4113
	50 µg/ml K99	2	53 590 ± 5259	92 548 ± 5845	28 363 ± 2659
		4	73 103 ± 8463	126 816 ± 3508	50 560 ± 5489
	20 µg/ml F11RX	2	269 525 ± 4252	222 640 ± 11 810	159 029 ± 9765
D 3 IPCs	20 µg/ml K99	2	1623 ± 109	5183 ± 666	7241 ± 743
		4	1529 ± 83	9272 ± 1435	8346 ± 981
	50 µg/ml K99	2	2979 ± 334	10 370 ± 380	22 566 ± 1809
		4	1665 ± 194	10 178 ± 837	31 632 ± 8346
	20 µg/ml F11RX	2	13 240 ± 3427	181 346 ± 6150	182 490 ± 8183

Mixtures of the various concentrations of NPCs and D 3 IPCs with the appropriate dilutions of the Ags were each loaded in quadruplicate 100 µl volumes into the wells of a 96-well flat-bottomed tray and incubated for 2 or 4 hours, as indicated. Following the incubation, the trays were shaken gently, the nonadherent cells and free Ag removed and the adherent monolayers were washed with fresh culture medium.

* 10^5 SA9 purified, primed T cells were added to the various APC populations and the trays incubated at 37°C for 3 days before determining the proliferation induced by measuring the amount of [^{3}H]-TdR taken up (cpm) by the cells during the last 4 hours of culture at 37°C. A representative set of results are presented showing the cpm (mean ± sem) of [^{3}H]-TdR incorporated by each replicate set. Negligible amounts of [^{3}H]-TdR were again incoporated by control suspensions: all APC suspensions in the range of 120-381 cpm; primed T cells + 10 µg/ml K99, 1026 ± 36; primed T cells + 10 µg/ml F11RX, 2590 ± 126.

corresponded to an increase in the proliferation induced, with the optimal responses occurring in the presence of $5x10^4$ Ag-pulsed NPCs. In contrast, 10^5 K99-pulsed D 3 IPCs were nonstimulatory at both doses of K99 used and $5x10^4$ K99-pulsed D 3 IPCs induced only marginal responses which were only slightly greater when the dose of K99 and the length of time used for Ag-pulsing was increased. As previously observed with 11RX primed T cells, F11RX-pulsed APCs stimulated quite large responses. Hence, both K99-pulsed NPCs and D 3 IPCs could stimulate SA9 IPCs, but more Ag was required to pulse D 3 IPCs to observe significant responses. This was consistent with the earlier conclusion that the D 3 IPCs may be degrading the Ag faster than the NPCs, presumably reflecting the increased metabolic activity of these cells. The phenotype of the proliferating cells was again $L3T4^+$ in both cases (data not shown).

4.2.2.10 Summary

Intraperitoneal immunization of mice with L11RX had considerable effects on the PC population. Infection with L11RX resulted in a large influx of inflammatory neutrophils into the peritoneal cavity and an increase in overall yield of PCs which could be harvested from infected mice. Analysis of the Ag presenting capacity of these cells revealed that L11RX infection rendered the PCs nonstimulatory when used in large numbers. This nonstimulatory effect was mediated by a minor population of adherent cells, which were probably macrophages, with some contribution from the neutrophils. At least some of this effect could be explained by an increase in the capacity of the D 3 IPCs to degrade Ags. No other differences were detected by the assays used. In response to NPCs or D 3 IPCs + Ags in culture or *in vitro* or *in vivo* Ag-pulsed NPCs or D 3 IPCs, the T cells induced expressed the $L3T4^+$, $Thy1.2^+$ phenotype. Since *Salmonella*-specific primary T cell responses could not be induced with the Ag preparations available, the assay which could be used to compare the APC function of normal and immune PCs was their ability to induce a primary MLR. An analysis of the induction of a primary MLR may provide valuable information about the stimulatory capacity of these APCs with respect to the magnitude of responses and the

Addendum
nment 1

T cell subsets induced to respond.

4.2.3 Analysis of primary MLRs stimulated by NPCs and D 3 IPCs

4.2.3.1 FACScan analysis of NPCs and D 3 IPCs

Before studying the ability of NPCs and D 3 IPCs to induce primary MLRs, the levels of expression of MHC molecules by these cells were examined. NPCs and D 3 IPCs were labelled using immunoflurescent reagents and analysed on the FACScan. PCs were incubated with FITC-conjugated MoAbs directed against Class I and Class II MHC molecules, washed, fixed and analysed on the FACScan. Preliminary experiments had demonstrated the need to use the direct immunofluorescent labelling technique as these heterogeneous PC populations showed considerable background staining with the developing SHAM-FITC used in the indirect labelling method (data not shown).

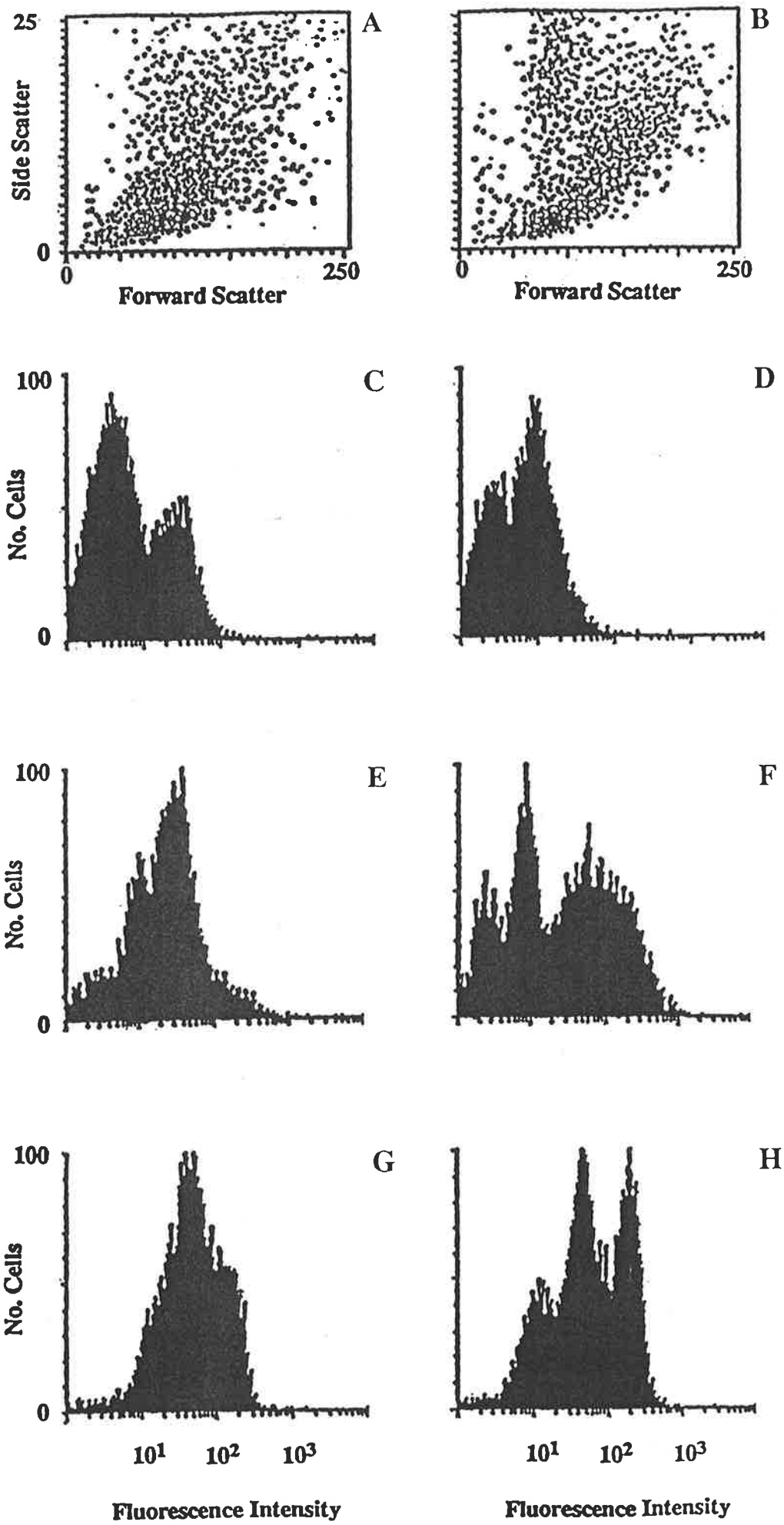
Fig. 4.4 [A-H] illustrate the data generated by FACSCan analysis of these populations. The dot plot diagrams in Fig. 4.4 [A and B] illustrate the heterogeneity of the NPCs and D 3 IPCs respectively. Both populations are virtually all Class I MHC$^+$ (Fig. 4.4 [G and H]), with the D 3 IPCs appearing more heterogeneous in their levels of expression of these molecules and significant proportions of each express the Class II MHC molecules (Fig. 4.4 [E and F]). Close examination of these data suggested that D 3 IPCs contained fewer Class II$^+$ cells, but with slightly greater fluorescence. Hence, 3 days after *Salmonella* infection only marginal, if any, alteration in the expression of MHC molecules had occurred. With this in mind, the ability of NPCs and D 3 IPCs to induce primary MLRs was compared.

4.2.3.2 Proliferation induced in a primary MLR

Initial experiments were carried out using MLNCs obtained from BALB/c or C57BL/6 mice as the responding populations to determine which were more suitable for these studies. The MLNCs were passaged on NW columns (NW MLNCs) to provide

FIGURE 4.4 FACScan analysis of NPCs and D 3 IPCs

NPCs and D 3 IPCs were directly labelled with FITC conjugated MoAbs for 1 hour on ice and examined by FACScan analysis. Cells were incubated with P/B/A [A-D], FITC conjugated α-I-A [E and F] or α-H-2D [G and H]. Both the forward scatter *vs* side scatter diagrams [A and B] and the fluorescence intensities of these cells [C-H] are shown.


A
B
25
Side Scatter
0
0
250
Forward Scatter
0
250
Forward Scatter
C
D
100
No. Cells
0
E
F
100
No. Cells
0
G
H
100
No. Cells
0
10^1
10^2
10^3
Fluorescence Intensity
10^1
10^2
10^3
Fluorescence Intensity

semi-purified T cell preparations. The stimulator cells were PCs obtained from normal, unimmunized (BALB/c x C57BL/6) F1 mice (NPCs) or PCs from F1 mice immunized with 10^5 L11RX ip 3 days prior to use (D 3 IPCs). The capacity of the unfractionated populations and the adherent and nonadherent subpopulations of these PCs to stimulate proliferation in response to allo-Ags, was examined to establish whether the adherent cell population of D 3 IPCs was also unable to induce proliferation of unprimed allogeneic responder cells, as had been observed with secondary MLR and *Salmonella*-specific responses. Total, adherent and nonadherent populations of F1 NPCs and D 3 IPCs were obtained as previously described and a range of cell numbers from $2x10^4$ to $2x10^5$ were examined for their stimulatory capacity, each cell population being assayed in quadruplicate. $2x10^5$ NW fractionated MLNCs from BALB/c or C57BL/6 mice were cultured with the various APC populations for 5 days at 37°C before the amount of proliferation was determined by measuring the amount of [^{3}H]-TdR taken up during the final 4 hours of culture at 37°C.

Both D 3 IPCs and NPCs induced considerable proliferation of BALB/c and C57BL/6 NW MLNCs, the actual level being determined by the number of APCs used (Tables 4.21 [A and B]). Consistent with earlier observations, $1x10^5$ and $2x10^5$ total or adherent D 3 IPCs induced little or no proliferation of either responding population and removal of the adherent cells rendered the remaining nonadherent cells highly stimulatory for both responder cell types used. In contrast, NPCs induced considerable responses at all doses used, despite the fact that less [^{3}H]-TdR incorporation was usually observed with the largest dose of NPCs. Microscopic examination of these cultures showed that considerable increase in cell numbers had occurred, indicating that proliferation had peaked prior to the time of pulsing. It was also consistently observed that fewer nonadherent APC populations induced smaller responses in both systems, especially when D 3 IPCs were used, indicating that this subpopulation contained fewer "APC-like" cells necessary for activation of T cell proliferation. It is important to note that although this was a consistent observation, the degree to which these responses were reduced (as a result of decreasing the numbers of "nonadherent APCs") varied between

TABLE 4.21 A Proliferation of BALB/c MLNCs stimulated by (BALB/c x C57BL/6) F1 APCs

		[^{3}H]-TdR uptake (cpm ± sem) by NW BALB/c MLNCs after 5 days of culture with F1 APCs*:		
APCs	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	$2x10^5$	29 587 ± 8219	40 889 ± 11 570	194 715 ± 12 755
	10^5	200 676 ± 55 339	141 867 ± 7485	131 629 ± 3581
	$4x10^4$	248 660 ± 13 123	162 627 ± 14 881	33 175 ± 5739
	$2x10^4$	137 550 ± 24 232	70 497 ± 17 203	12 060 ± 3835
D 3 IPCs	$2x10^5$	785 ± 29	760 ± 47	268 137 ± 16 170
	10^5	1496 ± 173	5301 ± 259	215 820 ± 10 161
	$4x10^4$	72 516 ± 6205	105 495 ± 8603	69 358 ± 7639
	$2x10^4$	80 928 ± 23 880	39 965 ± 8485	14 540 ± 3520

* $2x10^5$ NW BALB/c MLNCs were mixed with varying numbers of the unfractionated populations and adherent and nonadherent subpopulations of either F1 NPCs or D 3 IPCs, and each combination was loaded into 4 replicate wells of a 96-well flat-bottomed tray. The tray(s) was incubated at 37°C for 5 days before the amount of proliferation was determined by measuring the [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture. A typical result is presented, showing the cpm (mean ± sem) for each group of cells. Small amounts of [^{3}H]-TdR were taken up by control suspensions: MLNCs + culture medium, 376 ± 14; all APCs + culture medium, in the range of 100 to 356 cpm.

The data presented in this Table and in Table 23 were obtained using the same group of immunized mice.

TABLE 4.21 B Proliferation of C57BL/6 MLNCs stimulated by (BALB/c x C57BL/6) F1 APCs

		[^{3}H]-TdR uptake (cpm ± sem) by NW C57BL/6 MLNCs after 5 days of culture with F1 APCs*:		
APCs	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	$2x10^5$	19 603 ± 2685	18 642 ± 2251	238 674 ± 21 358
	10^5	201 924 ± 11 113	223 613 ± 10 887	188 456 ± 10 390
	$4x10^4$	243 605 ± 14 826	281 204 ± 18 117	71 707 ± 13 988
	$2x10^4$	277 314 ± 23 245	198 948 ± 6828	11 005 ± 3406
D 3 IPCs	$2x10^5$	18 678 ± 4283	6325 ± 819	262 723 ± 4713
	10^5	44 289 ± 11 859	75 712 ± 15 266	145 042 ± 20 297
	$4x10^4$	267 903 ± 28 821	311 650 ± 10 683	19 267 ± 6759
	$2x10^4$	228 315 ± 13 452	234 523 ± 16 113	5267 ± 1643

* $2x10^5$ NW C57BL/6 MLNCs were cultured in quadruplicate with varying numbers of the unfractionated population and adherent and nonadherent subpopulations of both F1 NPCs and D 3 IPCs for 5 days at 37°C, prior to establishing the proliferative responses induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the last 4 hours of culture. The results from one of several repeat experiments are presented, with the results expressed as the cpm (mean ± sem) for each group of 4 replicate cultures. Again minimal uptake of [^{3}H]-TdR by control suspensions occurred: NW MLNCs + culture medium, 213 ± 25; all APC suspensions, in the range of 87 to 186 cpm.

experiments.

These data are similar to the secondary *Salmonella* Ag-specific responses already presented and reinforce the earlier conclusion that this nonstimulatory effect observed when using large numbers of D 3 IPCs is at least partially nonspecific, as it was detected using *Salmonella* Ags, K99 and also allo-Ags. Further examination of the cells responsible for this effect was carried out by studying the effect that fractionation on Metrizamide density gradients had on the ability of D 3 IPCs to induce proliferation of allogeneic lymphoid cells. The following series of experiments were carried out to identify the cells responsible for the nonstimulatory effect and since BALB/c and C57BL/6 MLNCs responded similarly, only the responses of BALB/c NW MLNCs will be presented.

4.2.3.3 Stimulation of MLRs by Metrizamide fractionated NPCs and D 3 IPCs

NPCs and D 3 IPCs were loaded onto discontinuous Metrizamide gradients, and centrifuged at 1200 g for 45 minutes before harvesting the cells at the first interface. As described in Section 4.2.2.4 *(i)* this procedure had virtually no effect on the proportions of cell types present in the NPCs, whilst removing almost all neutrophils from the D 3 IPCs. These cells were adjusted to the required concentrations and the total suspensions and the adherent and nonadherent subpopulations of these cells were cultured with NW MLNCs for 5 days before measuring proliferation by the uptake of $[^3H]$-TdR, in the standard way.

Removal of neutrophils by fractionation on Metrizamide gradients had no effect on the ability of D 3 IPCs to induce a MLR (Table 4.22 A) and the responses induced by NPCs were also unchanged, as expected (Table 4.22 B). This was consistent with the results of the secondary *Salmonella*-specific responses mounted by primed T cells, indicating that the presence of large numbers of neutrophils was not responsible for the inability of these cells to induce T cell proliferation in response to allo-Ags.

One treatment which ensured that the D 3 IPCs were stimulatory when

TABLE 4.22 A The effect of fractionation of D 3 IPCs on Metrizamide density gradients on their ability to induce allogeneic proliferation

		[^{3}H]-TdR uptake (cpm ± sem) by NW BALB/c MLNCs# after 5 days of culture with F1 D 3 IPCs:		
APCs used	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
Untreated D 3 IPCs	$2x10^5$	17 243 ± 1325	58 250 ± 7283	295 449 ± 24 967
	10^5	261 895 ± 31 366	180 972 ± 3411	182 747 ± 16 182
	$5x10^4$	207 623 ± 27 116	151 517 ± 14 818	56 700 ± 4250
Metrizamide D 3 IPCs*	$2x10^5$	16 133 ± 1911	41 824 ± 4000	413 458 ± 7598
	10^5	310 365 ± 33 456	361 622 ± 2137	304 590 ± 26 959
	$5x10^4$	324 427 ± 33 751	130 301 ± 22 455	51 557 ± 6389

* F1 D 3 IPCs were harvested, half were used in this form (untreated D 3 IPCs) and to provide the adherent and nonadherent subpopulations. The remainder were fractionated by centrifugation on a Metrizamide density gradient at 1200 g for 45 minutes. The cells localized at the first interface were harvested, washed and used as unfractionated Metrizamide D 3 IPCs and to provide the adherent and nonadherent subpopulations of Metrizamide D 3 IPCs.

$2x10^5$ NW BALB/c MLNCs were cultured with the unfractionated populations and the adherent and nonadherent subpopualtions of both untreated and Metrizamide D 3 IPCs for 5 days at 37°C prior to determining the proliferation induced by measuring the amount of [^{3}H]-TdR taken up (cpm) during the last 4 hours of culture at 37°C. Typical results are presented expressed as the cpm (mean ± sem) for each group of replicate cultures. Again the background levels of [^{3}H]-TdR incorporated was no more than 245 cpm for MLNCs or any of the APC suspensions alone.

TABLE 4.22 B The effect of fractionation of NPCs on Metrizamide density gradients on their ability to induce allogeneic proliferation

		[^{3}H]-TdR uptake (cpm ± sem) by NW BALB/c MLNCs# after 5 days of culture with F1 NPCs:		
APCs used	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
Untreated NPCs	$2x10^5$	377 167 ± 9090	356 275 ± 5446	394 500 ± 8253
	10^5	292 342 ± 3802	280 869 ± 8767	177 244 ± 22 316
	$5x10^4$	404 375 ± 4810	374 102 ± 21 772	66 866 ± 3778
Metrizamide NPCs*	$2x10^5$	329 258 ± 12 549	364 325 ± 13 984	194 367 ± 13 308
	10^5	404 517 ± 16 938	403 575 ± 8829	182 244 ± 31 507
	$5x10^4$	327 046 ± 4730	242 252 ± 30 366	101 982 ± 8251

* F1 NPCs were either fractionated on a Metrizamide density gradient by centrifugation at 1200 g for 45 minutes and the cells localized at the first interface were harvested (Metrizamide NPCs), or left untreated (Untreated NPCs). Unfractionated populations and adherent and nonadherent subpopulations of both of these suspensions were prepared in the usual way and used as the APCs.

$2x10^5$ NW BALB/c MLNCs were cutlured in quadruplicate with each of the APC suspensions for 5 days at 37°C before the amount of proliferation induced was determined by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture at 37°C. A characteristic set of results are presented expressed as the cpm (mean ± sem) of [^{3}H]-TdR taken up by quadruplicate set of cells. No more than 300 cpm of [^{3}H]-TdR was incorporated by control suspensions of either the MLNCs or APCs alone.

tested for their ability to induce secondary *Salmonella*-specific responses was *in vivo* Ag-pulsing and the capacity of the APCs pulsed with F11RX *in vivo* to induce allogeneic lymphoid cells to proliferate was investigated.

4.2.3.4 MLRs stimulated by *in vivo* F11RX pulsed APCs

Normal F1 mice and F1 mice immunized ip with L11RX 3 days earlier were pulsed with 250 µg of F11RX ip. After 15 minutes the mice were sacrificed and the PC suspensions harvested, counted and adjusted to the required concentrations. A range of cells from $4x10^4$ to $2x10^5$/well were used. The total populations and the adherent and nonadherent subpopulations of PCs at the various concentrations were cultured with $2x10^5$ NW MLNCs obtained from BALB/c mice, with each combination assayed in quadruplicate. On the fifth day the proliferation was measured by the amount of [^{3}H]-TdR incorporated during the 4 hours of incubation at 37°C.

Table 4.23 illustrates that *in vivo* pulsed NPCs were again stimulatory at all cell concentrations and that pulsing D 3 IPCs with F11RX *in vivo* reduced their inhibitory activity, although $2x10^5$ total and adherent cells usually induced only very small allogeneic responses, with some variation from experiment to experiment. This implied that *in vivo* pulsing with F11RX failed to remove all the cells responsible for preventing optimal stimulation of allogeneic responses, whilst successfully removing those inhibiting stimulation of *Salmonella*-specific responses.

Addendum
nment 1

Possible explanations for the "inhibitory" effector function of the adherent cells present in the D 3 IPCs could be that they release inhibitory or suppressive factors. Macrophages are known to release prostaglandins during *in vitro* culture which are known to have suppressive effects on T cells (Chouaib *et al.*, 1985). However, this was not a likely explanation for the results presented here as indomethacin, which counteracts prostaglandin activity (Webb and Nowowejski, 1977), was routinely included in the culture medium.

Alternatively, lack of stimulation could be attributed to a change in the level of MHC products expressed on the D 3 IPCs. There is evidence to suggest that as a

TABLE 4.23 Allogeneic proliferation induced by *in vivo* Ag-pulsed F1 APCs

		[^{3}H]-TdR uptake (cpm ± sem) by NW BALB/c MLNCs# after 5 days of culture with *in vivo* F11RX pulsed F1 APCs*:		
In vivo pulsed APCs	No. APCs (/well)	Unfractionated APCs	Adherent APCs	Nonadherent APCs
NPCs	$2x10^5$	70 804 ± 6408	360 144 ± 17 254	302 991 ± 35 502
	10^5	361 536 ± 17 865	331 171 ± 4707	237 636 ± 15 727
	$4x10^4$	193 543 ± 20 558	218 480 ± 4772	83 384 ± 6361
D 3 IPCs	$2x10^5$	6933 ± 1506	9637 ± 1035	190 037 ± 20 479
	10^5	176 423 ± 22 957	184 156 ± 19 435	241 613 ± 14 901
	$4x10^4$	234 337 ± 29 021	161 512 ± 25 850	113 147 ± 8935

* Normal mice and mice immunized ip with 10^5 L11RX 3 days earlier were injected ip with 250 μg F11RX and 15 minutes later the mice were sacrificed and the PCs harvested. Various numbers of the unfractionated populations and the adherent and nonadherent subpopulations of these *in vivo* Ag-pulsed PCs were examined for their ability to induce allogeneic proliferation.

Quadruplicate mixtures of $2x10^5$ NW BALB/c MLNCs and the various F1 APC populations were incubated at 37°C for 5 days before determining the amount of proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) by these cells during the final 4 hours of culture. Results presented here are representative of several individual experiments, showing the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each replicate set. Control suspensions containing the NW MLNCs or APCs alone in culture medium incorporated little [^{3}H]-TdR (approximately 300 cpm as a maximum) and the NW MLNCs cultured with 1 μg/ml F11RX incorporated only 1394 ± 108 of [^{3}H]-TdR.

The data presented in this Table and in Table 21.A were obtained using the same group of immunized mice.

consequence of infection with IBPs, modulation of the expression of MHC molecules does occur. It has also been reported that the density of antigenic determinants on the surface of APCs, which in this case are the MHC molecules, greatly affects the level of response induced (Matis *et al.*, 1983). Hence, changes in the level of expression of MHC molecules, as a result of infection with L11RX, could effect the magnitude of responses induced to either allo-Ags or specific Ags, or both. Although the FACS analysis of NPCs and D 3 IPCs indicated that ip infection with L11RX induced no drastic change in the level of expression of Class I or Class II MHC molecules, more subtle changes, due to differences in "processing" of the self-peptides which associate with these MHC molecules, may be responsible for the differences observed.

Since a difference in the subset(s) of T cells induced to proliferate may reflect a modulation of function and/or a change in the level of expression of MHC molecules, the phenotypes of the T cells induced by NPCs and D 3 IPCs were determined. These experiments were carried out in parallel with the studies on the cytotoxic responses induced by allo-Ags [and will be discussed in the following sections] and required the use of C57BL/6 MLNCs as the responder populations.

4.2.3.5 Identification of the T cell subsets proliferating in response to the allo-Ags presented by NPCs and D 3 IPCs

$4x10^4$ F1 NPCs and D 3 IPCs were cultured with $2x10^5$ NW MLNCs from C57BL/6 mice for 5 days at 37°C in 20 replicate cultures in 96-well flat-bottomed trays. On the fifth day MoAbs and C were used (at 1/10 and 1/20 final dilution, respectively) to identify the phenoytpe(s) of the T cells induced to proliferate, using the procedure previously described. After treatment, the amount of proliferation was determined using the standard procedure.

Table 4.24 shows data repesentative of several experiments. From previous reports, it was not surprising to find that both $L3T4^+$ and $Lyt2.2^+$ T cells proliferated in response to allo-Ags presented by NPCs and D 3 IPCs, but it was somewhat unexpected that the majority of the T cells responding to both types of PCs

TABLE 4.24 Phenotype of the T cells proliferating in response to allo-Ags on F1 NPCs and D 3 IPCs

	[^{3}H]-TdR uptake (cpm ± sem) by C57BL/6 MLNCs# after 5 days of culture with F1 APCs	
	APCs:	
Treatment*	NPCs	D 3 IPCs
None	57 095 ± 267	61 048 ± 258
C alone	52 558 ± 1366	65 602 ± 452
α-Thy1.2 + C	2019 ± 119	1342 ± 145
α-L3T4 + C	47 856 ± 2857	54 353 ± 2127
α-Lyt2.2 + C	5683 ± 233	12 893 ± 554

Mixtures of $2x10^5$ NW C57BL/6 MLNCs and $4x10^4$ F1 NPCs or D 3 IPCs (20 wells of each) were cultured for 5 days at 37°C prior to determining the proliferation induced by the amount of [^{3}H]-TdR taken up (cpm) during the last 4 hours of culture.

* Before pulsing with the [^{3}H]-TdR, mixtures of C (1 in 20 final dilution) and MoAbs specific for T cell markers (1 in 10 final dilution), C alone (1 in 20 final dilution), or culture medium were added to 4 replicate cultures of each and incubated for 1 hour at 37°C. Typical data from one of several repeat experiments are presented, with the results expressed as the cpm (mean ± sem) of [^{3}H]-TdR incorporated for each group of treated cells.

expressed the Lyt2.2$^+$ phenotype. This is contrary to many reports which have shown a major involvement of the L3T4$^+$ T cells in the proliferative response. These results were confirmed by the FACScan analysis of the MLR blasts induced by NPCs and D 3 IPCs which had been immunofluorescently labelled using the indirect method (Fig. 4.5 [A-H]). These cells were virtually 100% Thy1.2$^+$, and approximately 85% were Lyt2.2$^+$ cells.

To define the phenotype(s) of the cells involved in the initiation of the response, NW MLNCs were prepared and half of them were treated with various MoAbs specific for T cell markers and C in a one step procedure, as indicated in Table 4.25. The untreated and MoAb + C treated NW MLNCs were cultured with NPCs and D 3 IPCs for 5 days at 37°C before measuring the proliferation in the usual way. The results are shown in Table 4.25 and indicate that the stimulation of MLR responses required both L3T4$^+$ and Lyt2.2$^+$ T cells, although removal of L3T4$^+$ T cells reduced the proliferative responses only slightly (though consistently), whilst removal of Lyt2.2$^+$ T cells reduced the proliferation induced by either NPCs or D 3 IPCs by approximately 80%. Pretreatment with a mixture of α-L3T4, α-Lyt2.2 and C resulted in very little response, and was similar to α-Thy1.2 and C treatment. Therefore, although the majority of the cells induced to proliferate were Lyt2.2$^+$ T cells, a minor involvement of L3T4$^+$ T cells was necessary, presumably to provide some form of "help" for the Lyt2.2$^+$ T cells.

Addendum
nment 4

Again, there appears no difference in the ability of NPCs and D 3 IPCs to act as APCs because they both induced comparable levels of proliferation by allogeneic T cells, most of which were Lyt2.2$^+$ T cells. However, as well as stimulating cells to proliferate, allo-Ags are also able to induce CTLs. Consequently, the ability of F1 NPCs and D 3 IPCs to induce cytotoxic activity in suspensions of allogeneic lymph node cells was investigated.

4.2.4 *In vitro* cytotoxicity of C57BL/6 MLNCs induced by F1 PCs

4.2.4.1 Detection of cytotoxicity

Initial experiments were carried out to establish the peak of cytotoxic

FIGURE 4.5 FACScan analysis of NPC and D 3 IPC stimulated MLR blasts

MLR blasts generated by culturing 10^6 C57BL/6 NW MLNCs with $2x10^5$ F1 NPCs or D 3 IPCs for 5 days at 37°C, were labelled with immunofluorescence using the indirect method. The information obtained following FACScan analysis of the blast populations is presented, illustrating the fluorescence intensities of NPC or D 3 IPC stimulated MLR blasts after incubation with P/B/A [A and B] or α-Thy1.2 [C and D], α-L3T4 [E and F] and α-Lyt2.2 [G and H].

NPC stimulated MLR blasts - left panels; D3 IPC stimulated blasts - right panels

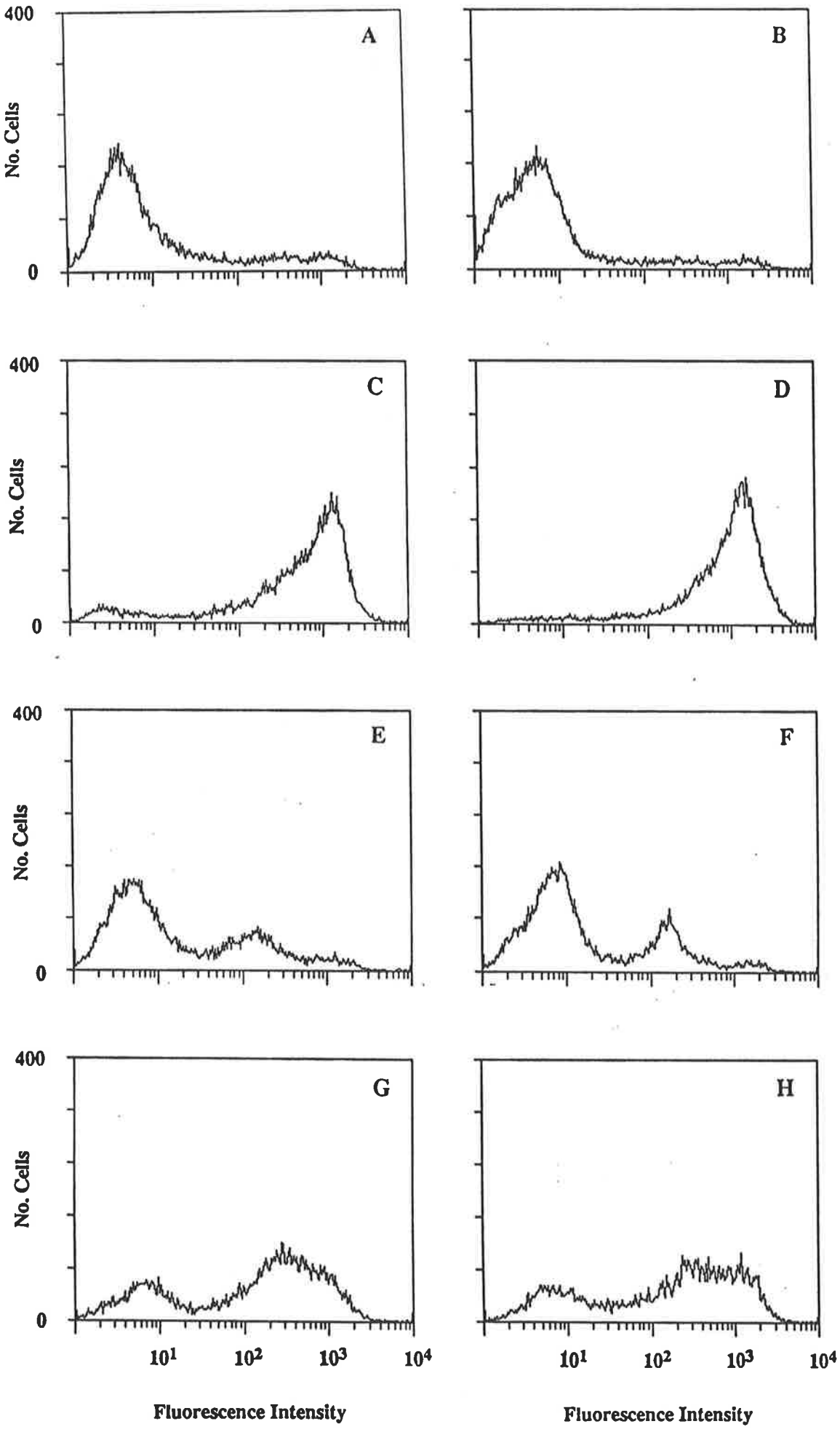

400
No. Cells
0
A
B
C
D
E
F
G
H
10^1
10^2
10^3
10^4
Fluorescence Intensity
Fluorescence Intensity

TABLE 4.25 Characterization of the T cell subset(s) required for induction of allogeneic proliferation in response to F1 APCs

	[^{3}H]-TdR uptake (cpm ± sem) by C57BL/6 MLNCs* after 5 days of culture with F1 APCs	
	APCs:	
Pretreatment*	NPCs	D 3 IPCs
None	95 248 ± 15 734	75 434 ± 5319
C alone	100 770 ± 6539	72 552 ± 9012
α-Thy1.2 + C	826 ± 104	925 ± 15
α-L3T4 + C	92 964 ± 2844	63 259 ± 3226
α-Lyt2.2 + C	23 830 ± 5268	11 148 ± 2003
α-L3T4, α-Lyt2.2 + C	3074 ± 277	2485 ± 844

* Aliquots of NW C57BL/6 MLNCs were either incubated for 1 hour at 37°C with the various MoAbs (1/10 final dilution) and C (1/20 final dilution) or C alone (1/20 final dilution), or left untreated prior to using them as the responding cells in the standard MLR assay. Quadruplicate mixtures of $2x10^5$ treated or untreated NW MLNCs and $4x10^4$ F1 NPCs or D 3 IPCs, were incubated for 5 days at 37°C before measuring the proliferative responses induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) by the cells during the final 4 hours of culture. Results from one of three identical experiments are presented, expressed as the cpm (mean ± sem) of [^{3}H]-TdR taken up by each quadruplicate set of cultures.

response induced by F1 NPCs and D 3 IPCs. The target cells chosen for these experiments were the murine mastocytoma cell line P815 which was maintained *in vitro* and had been found previously to provide very reliable target cells. As P815 expresses MHC molecules of the H-2^d haplotype, the MLNCs used in these experiments were obtained from the C57BL/6 mice of the H-2^b haplotype, ensuring that any response that was induced by the H-$2^{b/d}$ F1 cells was H-2^d-specific. Mixtures of 10^6 NW MLNCs, harvested from C57BL/6 mice, and $2x10^5$ F1 NPCs or D 3 IPCs were cultured in 1 ml volumes in 24-well tray for up to 6 days at 37°C. On days 3, 4, 5 and 6 of culture, the cytotoxic activity of the populations was measured using the standard ^{51}Cr release assay. The MLR blasts induced were counted, adjusted to appropriate concentrations and incubated with ^{51}Cr-labelled P815 cells for 4 hours at 37°C. After this incubation, 100 µl of each supernatant were harvested, transferred to separate tubes and the amount of radioactivity present measured in a gamma counter.

The data in Fig. 4.6 [A and B] demonstrate the levels of cytotoxic activity generated in response to both APC populations at different times after culture. Significant levels of cytotoxicity were induced by both NPCs and D 3 IPCs with the peak of cytotoxic activity occurring after 5 days of culture. Although exhibiting similar responses by day 5 of culture, in the experiment presented here the response induced by D 3 IPCs by the fourth day was considerably lower than that induced by NPCs and may reflect a difference in the kinetics of the responses induced by these populations, however it is important to note that this pattern was not consistently observed. For more detailed analysis of cytotoxic activity induced, 5 day cultured MLR blasts were used and the Ag specificity of these responses was investigated.

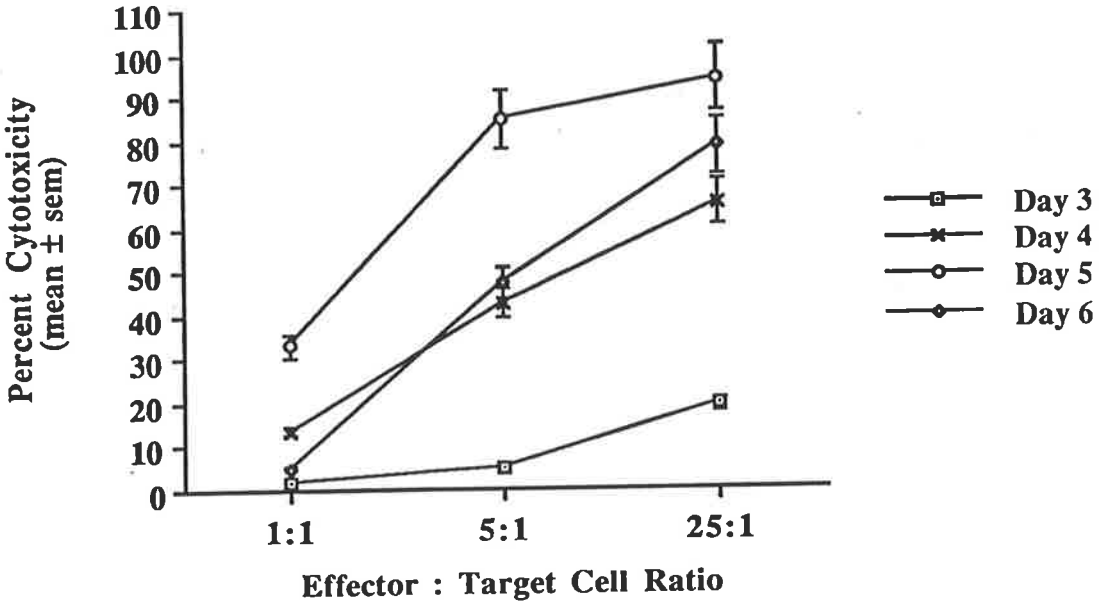
4.2.4.2 Determination of the Ag specificity of the CTLs

The specificity of cytotoxic activity of 5 day cultured MLR blasts stimulated by allo-Ags of either F1 NPCs or D 3 IPCs was demonstrated by incubating these cells for 4 hours with either ^{51}Cr-labelled P815 or ^{51}Cr-labelled EL4, before measuring the amount of radioactivity released, as previously described. Fig. 4.7 shows

FIGURE 4.6 Kinetics of cytotoxic activity induced by allo-Ags on NPCs and D 3 IPCs

Mixtures of 10^6 C57BL/6 NW MLNCs and $2x10^5$ F1 PCs were incubated in 1 ml cultures in 24-well flat-bottomed trays for 3-6 days at 37°C. To assay cytotoxicity, the MLR blasts were harvested and adjusted to three standard concentrations, added to 4 replicate wells of a 96-well round-bottomed tray and mixed with ^{51}Cr-labelled P815 at ratios of 25:1, 5:1 and 1:1 and incubated for 4 hours at 37°C. Following the incubation, 100 µl of each supernatant was harvested and the amount of ^{51}Cr present in each was measured and the percent cytotoxicity (mean ± sem) was calculated for each quadruplicate set. The results shown are representative of five similar experiments and indicate the cytotoxicity induced by either F1 NPCs [A] or F1 D 3 IPCs [B] after 3, 4, 5 and 6 days of culture.

A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(mean ± sem)
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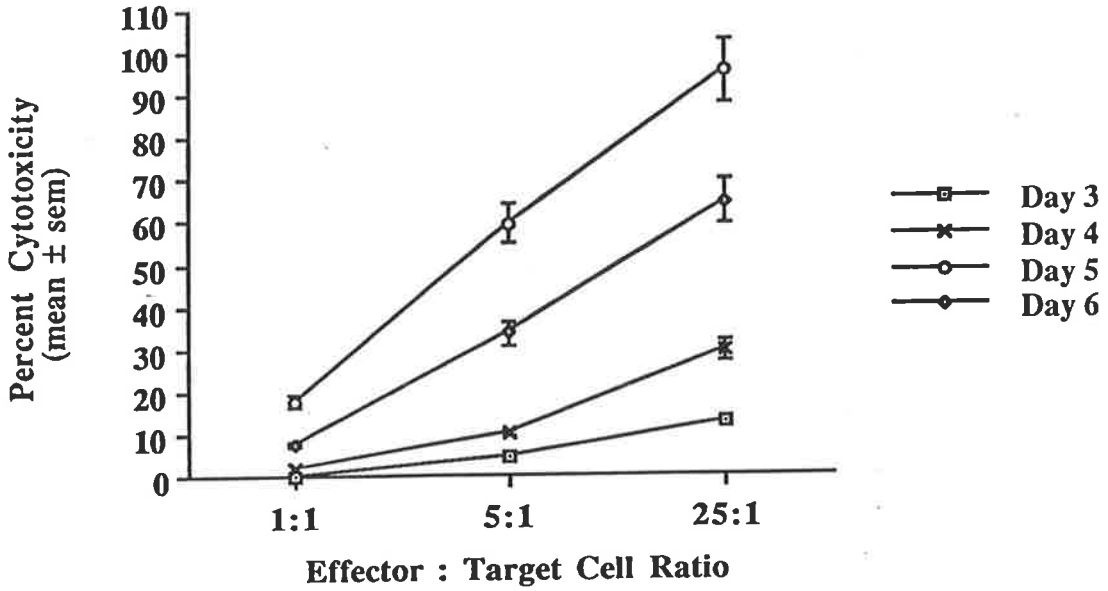
100
90
80
70
60
50
40
30
20
10
0
1:1
5:1
25:1
Effector : Target Cell Ratio
Day 3
Day 4
Day 5
Day 6

B


Percent Cytotoxicity
(mean ± sem)
110
100
90
80
70
60
50
40
30
20
10
0
1:1
5:1
25:1
Effector : Target Cell Ratio
Day 3
Day 4
Day 5
Day 6

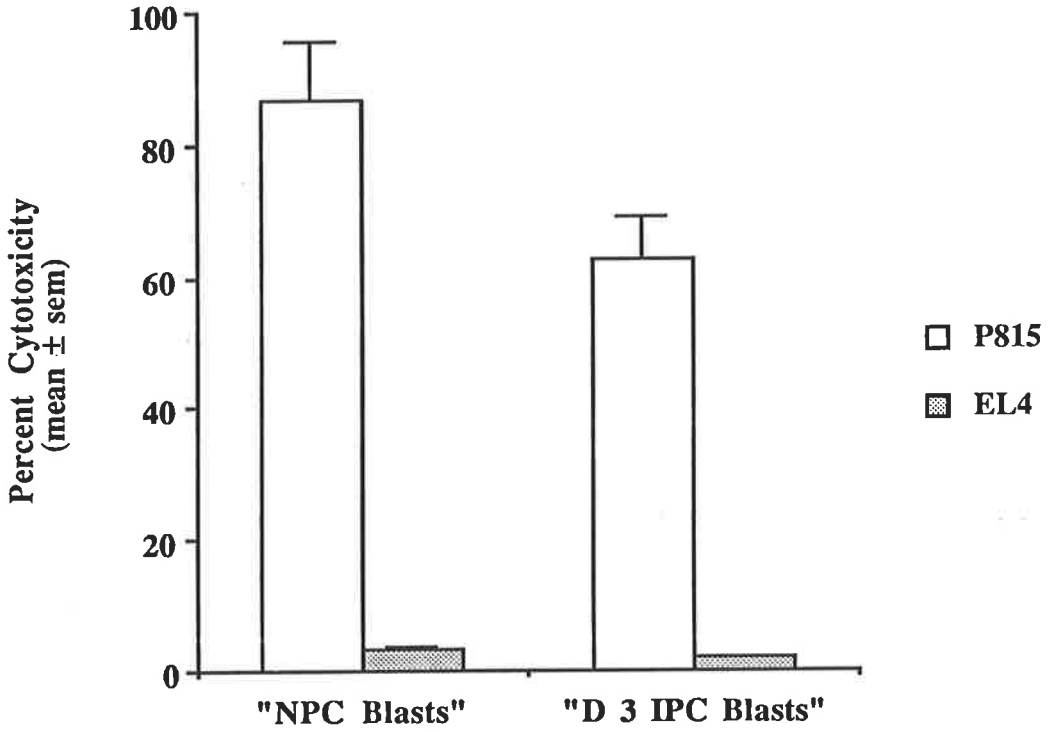


FIGURE 4.7 Analysis of the specificity of the cytotoxicity induced in the MLR

C57BL/6 NW MLNCs (10^6 cells) were cultured with $2x10^5$ F1 NPCs or D 3 IPCs for 5 days, when $5x10^5$ MLR blasts were mixed (in quadruplicate 200 µl volumes) with $2x10^4$ ^{51}Cr-labelled P815 or ^{51}Cr-labelled EL4 and incubated at 37°C for 4 hours. The amounts of ^{51}Cr released were measured and the cytotoxicity calculated using standard techniques.

the amount of cytotoxic activity of NPC or D 3 IPC stimulated MLR blasts against ^{51}Cr-labelled P815 and ^{51}Cr-labelled EL4. As expected, neither NPC nor D 3 IPC stimulated MLR blasts showed cytotoxic activity against ^{51}Cr-labelled EL4, whilst considerable lysis of ^{51}Cr-labelled P815 occurred in the presence of both blast populations. This is because EL4 is a T cell lymphoma which expresses only Class I MHC molecules of the H-2^{b} haplotype and C57BL/6 MLR blasts generated against the F1 cells should be anti-H-2^{d} and therefore, not lyse the ^{51}Cr-labelled EL4. Clearly, F1 NPCs and D 3 IPCs induced significant allo-Ag-specific cytotoxic responses after 5 days of culture with C57BL/6 NW MLNCs.

4.2.4.3 Phenotype of the CTLs

(i) In vitro lysis of ^{51}Cr-labelled P815

Five day C57BL/6 MLR blasts stimulated by F1 NPCs or D 3 IPCs were incubated with the MoAbs, α-Thy1.2, α-L3T4 or α-Lyt2.2 and C for 1 hour at 37°C, before being adjusted to the required concentration. Either untreated or the various MoAb and C treated MLR blasts were incubated in quadruplicate with ^{51}Cr-labelled P815 (at a ratio of 25:1) for 4 hours at 37°C and the amount of cytotoxicity determined as previously described.

Fig. 4.8 shows that the cells responsible for the majority of the cytotoxic activity induced by either NPCs or D 3 IPCs, were of the Thy1.2^{+}, Lyt2.2^{+} phenotype. Hence, considerable amounts of proliferation and cytotoxicity were induced in the C57BL/6 MLNC population after culture with F1 NPCs or D 3 IPCs. The magnitude of the responses induced by either APC suspension were comparable and cells of the same phenotype were induced. The involvement of the T cell subsets in the activities induced was also comparable. Because the target cells used so far expressed only Class I MHC molecules, target cells expressing Class II MHC molecules were used in an attempt to detect L3T4^{+} CTLs.

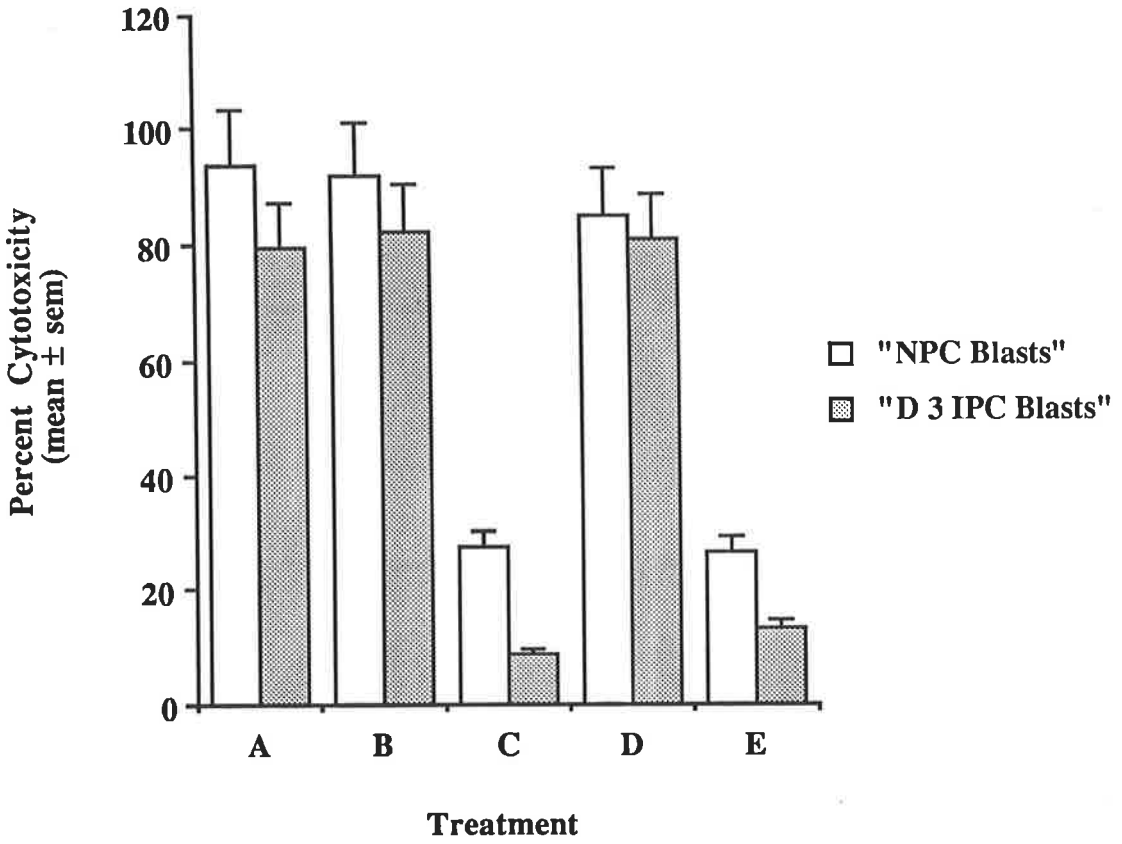


FIGURE 4.8 Phenotype of the allo-Ag-specific cytotoxic cells

Day 5 MLR blasts generated by culturing C57BL/6 NW MLNCs with F1 NPCs or D 3 IPCs in "bulk" cultures, were adjusted to 10^7 cells/ml and incubated with the T cell-specific MoAbs and C, C alone, or culture medium alone for 1 hour at 37°C. After incubation, each population of cells was mixed with ^{51}Cr-labelled P815 at a ratio of 25:1 (in quadruplicate) and standard aliquots incubated for a further 4 hours in a round-bottomed wells. The ^{51}Cr released into the supernatants was measured and the percent cytotoxicity (mean ± sem) was calculated. Effector cells received treatment with either: culture medium (A), C alone (B), α-Thy1.2 + C (C), α-L3T4 + C (D) or α-Lyt2.2 + C (E).

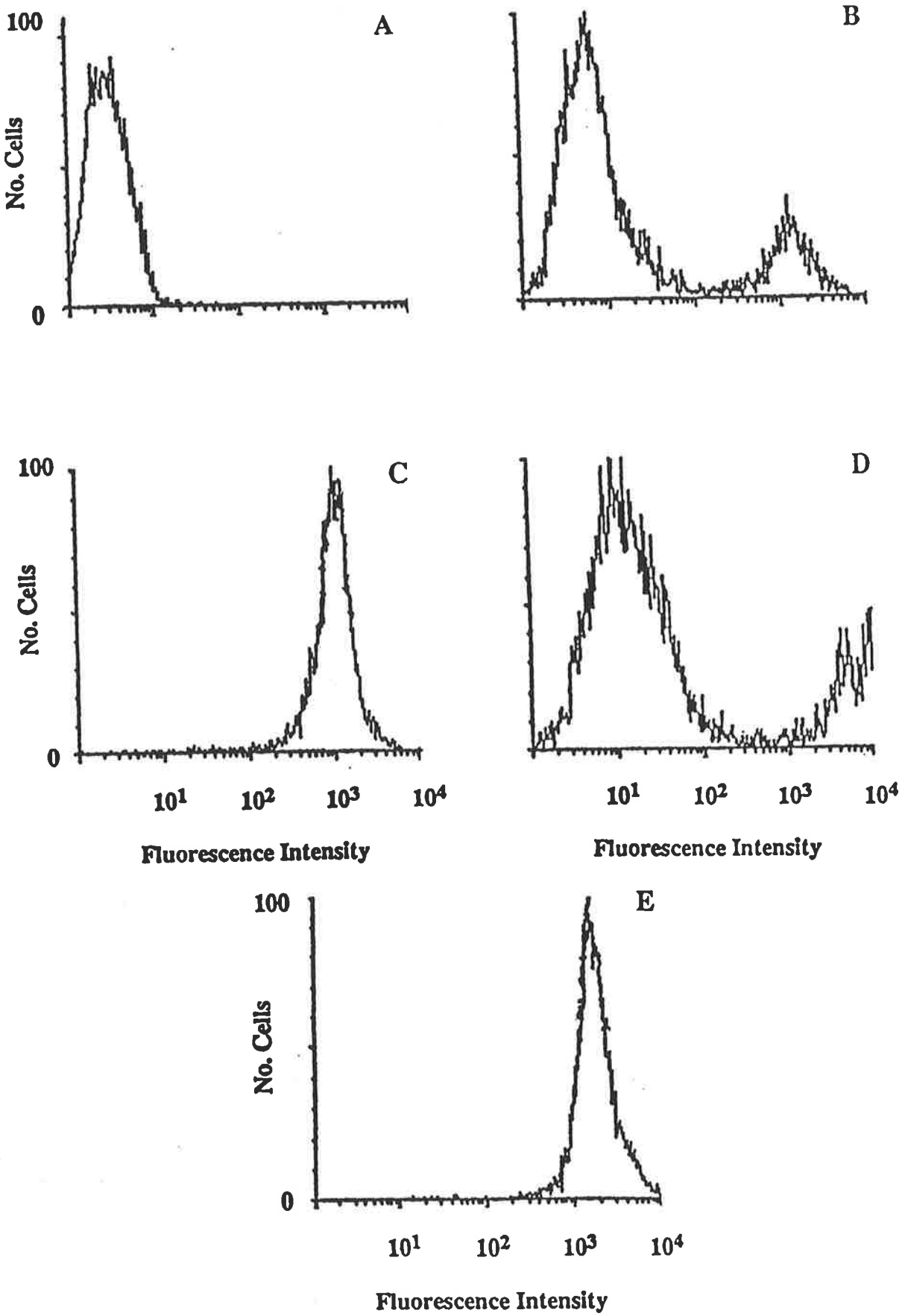
(ii) ***In vitro lysis of ^{51}Cr-labelled ConA blasts***

One possible candidate for the Class II$^+$ target cells was the cell line P388D1, however, because these cells often exhibited relatively high spontaneous release values and also were susceptible to considerable lysis by nonspecific cytotoxic cells (see Chapter 5), another target cell population was used. Target cells expressing both class I and II MHC molecules were obtained by culturing normal spleen cells (obtained from F1 mice) with ConA for 3 days at 37°C. Confirmation of the expression of both Class I and Class II MHC molecules was obtained from the FACScan analysis carried out on ConA blasts which had been labelled indirectly with immunofluorescence (Fig. 4.9 [A-E]). It was clearly evident that this population contained both Class I$^+$ and Class II$^+$ cells and therefore, detection of any L3T4$^+$ CTLs should be possible when using these cells as the targets. After 3 days culture, the ConA blasts were counted and labelled with ^{51}Cr as previously described. Five day C57BL/6 MLR blasts treated with the various MoAbs and C were mixed with the ^{51}Cr-labelled ConA blasts (at a ratio of 25:1) and each combination was assayed in quadruplicate by incubating for 4 hours at 37°C prior to assaying for ^{51}Cr release in the usual manner.

The ^{51}Cr-labelled ConA blasts were shown to be suitable targets because the levels of cytotoxicity detected were comparable to those found against ^{51}Cr-labelled P815 (Fig. 4.10). Clearly, C57BL/6 MLR blasts stimulated by either F1 NPCs or D 3 IPCs contained no L3T4$^+$ CTLs because only Lyt2.2$^+$ CTLs were detected. Therefore, there appeared to be no detectable differences in the ability of NPCs or D 3 IPCs to induce allogeneic responses in the C57BL/6 MLNCs. Finally it was decided to determine whether this applied to other allogeneic combinations. Accordingly, the ability of F1 NPCs and D 3 IPCs to induce a MLR in MLNCs obtained from CBA mice was examined.

FIGURE 4.9 FACScan analysis of ConA blasts

NSCs (10^6 cells/ml) were cultured for 3 days at 37°C in the presence of 1 μg/ml ConA before being labelled with immunofluoresecence using the indirect method. 50 μl aliquots containing $5x10^5$ ConA blasts were each incubated with a particular MoAb, or P/B/A for 1 hour at 4°C (with shaking at 20 minute intervals) before being washed in P/B/A and incubated with a 1 in 10 dilution of SHAM-FITC for 45 minutes at 4°C in the dark. The suspensions were washed thoroughly, resuspended in 1 ml volumes of 1% PFA and analysed on the FACScan. The fluorescence intensities are presented for cells incubated with: P/B/A (A); P/B/A + SHAM-FITC (B); α-Thy1.2 + SHAM-FITC (C); α-Ia^d + SHAM-FITC (D) and α-H-$2D^d$ + SHAM-FITC (E).


A
B
C
D
E
100
0
No. Cells
10^1
10^2
10^3
10^4
Fluorescence Intensity

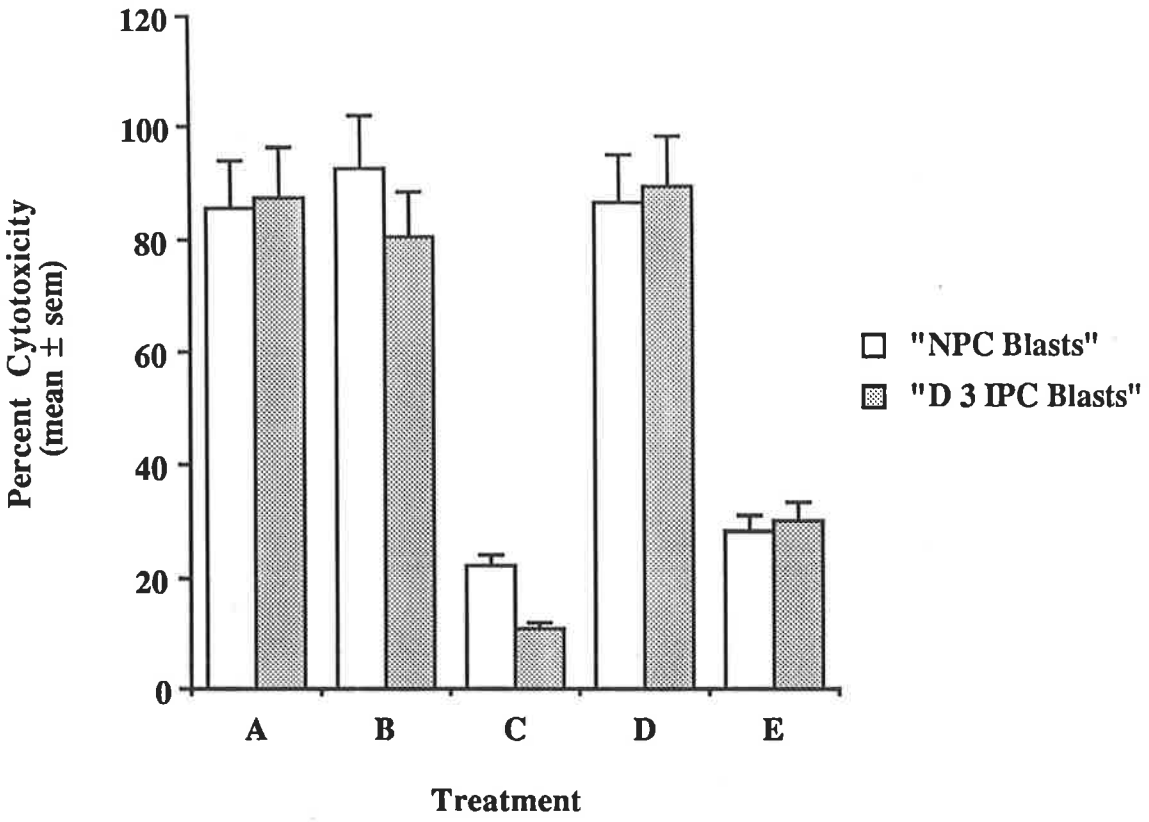


FIGURE 4.10 Detection of cytotoxic activity towards ConA blasts

MLR blasts harvested from 5 day cultures of C57BL/6 NW MLNCs with F1 NPCs or D 3 IPCs were treated with CM, various MoAbs and C or C only at 37°C for 1 hour. Four aliquots of $5x10^5$ cells of each treated population were mixed in 200 µl volumes with $2x10^4$ ^{51}Cr-labelled ConA blasts and incubated for 4 hours at 37°C. The percent cytotoxicity (mean ± sem) was calculated for each replicate set and the results from a typical experiment are presented, illustrating the cytotoxicity of both MLR blast populations after treatment with CM (A), C (B), α-Thy1.2 + C (C), α-L3T4 + C (D) or α-Lyt2.2 + C (E).

4.2.5 Induction of MLR responses by CBA MLNCs in response to (BALB/c x C57BL/6) F1 PCs

4.2.5.1 Proliferation of NW CBA MLNCs induced by F1 PCs

CBA mouse MLNCs were fractionated on NW columns to provide the responder population. NPCs and D 3 IPCs from (BALB/c x C57BL/6) F1 mice were adjusted to 10^7 cells/ml and treated with 10 μg/ml Mitomycin C for 1 hour at 37°C before being washed thoroughly, counted and adjusted to the required concentration. Twenty aliquots of $2x10^5$ NW MLNCs were mixed with $4x10^4$ PCs and cultured for 5 days at 37°C. On the fifth day appropriate dilutions of α-Thy1.2, α-L3T4, α-Lyt2.2 (TIB 150) and α-Lyt2 (YTS169.4) MoAbs and C were added to quadruplicate cultures, which were shaken and incubated for 1 hour at 37°C before measuring proliferation in the usual way. The two types of α-Lyt2 MoAb were used because CBA cells express the Lyt2.1 surface marker, not Lyt2.2 and therefore treatment with α-Lyt2.2 (TIB 150) and C would not deplete the $Lyt2^+$ T cell population. Consequently, a different MoAb specific for Lyt2 (YTS 169.4), which is not allotype restricted, was used to deplete any $Lyt2^+$ T cells proliferating in the CBA MLR blast population (α-Lyt2.2, TIB 150 was included as a control). In addition, the C57BL/6 MLR blasts (generated as described previously) were examined in parallel.

The effects of the various MoAb and C treatments on the proliferation of either CBA or C57BL/6 MLR blasts are summarized in Tables 4.26 [A and B]. As previously, mentioned the majority of the response by C57BL/6 MLNCs induced by either F1 PC population express the $Lyt2.2^+$, $Thy1.2^+$ phenotype, with some involvement of $L3T4^+$, $Thy1.2^+$ cells and both MoAbs specific for the Lyt2 marker were effective in removing most of the proliferative activity. In contrast, only the YTS169.4 α-Lyt2 MoAb was effective in removing a large proportion of the proliferative activity of the CBA MLR blasts, illustrating that the majority of the CBA cells induced to proliferate by either F1 PC population also expressed the $Lyt2^+$, $Thy1.2^+$ phenotype and confirms that TIB 150 is specific for the allelic form of Lyt2 not expressed on CBA cells. Again, there

* $2x10^5$ NW MLNCs obtained from CBA mice (Table A) or C57BL/6 mice (Table B) were cultured with $4x10^4$ (BALB/c x C57BL/6) F1 PCs (20 replicate wells for each mixture) for 5 days at 37°C before determining the proliferation induced by measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 4 hours of culture.

Prior to addition of the [^{3}H]-TdR, mixtures of appropriately diluted MoAbs (1/10 final) and C (1/20 final), C alone (1/20 final) or culture medium were added to 4 replicate wells for each MLNC-APC combination and the trays incubated for 1 hour at 37°C. Presented are the results from a typical experiment expressed as the cpm (mean ± sem) of [^{3}H]-TdR taken up by each quadruplicate group.

TABLE 4.26 Phenotype of the T cells induced to proliferate in different allogeneic systems

A:

	[^{3}H]-TdR uptake (cpm ± sem) by CBA NW MLNCs* after 5 days of culture with F1 NPCs of D 3 IPCs:	
Treatment#	NPCs	D 3 IPCs
C alone	255 099 ± 25 142	205 654 ± 7094
α-Thy1.2 + C	12 133 ± 3624	6114 ± 398
α-L3T4 + C	162 074 ± 19 186	136 980 ± 31 142
α-Lyt2.2 (TIB 150) + C	235 121 ± 29 301	249 426 ± 45 810
α-Lyt2 (YTS 169.4) + C	96 189 ± 8968	128 054 ± 26 123

B:

	[^{3}H]-TdR uptake (cpm ± sem) by C57BL/6 NW MLNCs* after 5 days of culture with F1 NPCs of D 3 IPCs:	
Treatment#	NPCs	D 3 IPCs
C alone	132 009 ± 1399	98 703 ± 2548
α-Thy1.2 + C	8411 ± 520	4661 ± 349
α-L3T4 + C	113 730 ± 5036	74303 ± 2723
α-Lyt2.2 (TIB 150) + C	31 190 ± 2162	20 543 ± 2074
α-Lyt2 (YTS 169.4) + C	30 047 ± 1328	12 183 ± 467

was little or no difference in the response of CBA MLNCs to the F1 NPCs or D 3 IPCs.

4.2.5.2 Cytotoxic activity of CBA MLNCs cultured with F1 PCs

(i) In vitro lysis of ^{51}Cr-labelled P815

MLNCs harvested from CBA mice were cultured with Mitomycin C treated F1 NPCs or D 3 IPCs and on the fifth day of culture the blasts induced were treated with the MoAbs and C, adjusted to the required concentrations and incubated with the ^{51}Cr P815 for 4 hours at 37°C before measuring the amount of radioactivity released into the supernatant in the usual way.

Fig. 4.11 shows that significant cytotoxic activity was induced by both APC populations. It is clear that both NPCs and D 3 IPCs induced Lyt2$^+$, Thy1.2$^+$ cells with considerable cytotoxic activity. To investigate the possibility of cytotoxic L3T4$^+$ T cells in these populations their cytotoxic activity against ^{51}Cr-labelled ConA blasts was also examined.

(ii) In vitro lysis of ^{51}Cr-labelled ConA stimulated blasts

ConA blasts of F1 mice were labelled with ^{51}Cr following the normal procedure and then used in the standard ^{51}Cr release assay. CBA MLR blasts generated by culture with Mitomycin C treated F1 PCs and pretreated with MoAbs and C or untreated, were mixed with the ^{51}Cr-labelled ConA blasts and incubated for 4 hours at 37°C when 100 µl of each supernatant was harvested into separate tubes and the amount of radioactivity measured.

The ^{51}Cr-labelled ConA blasts made good targets as levels of cytotoxicity comparable to those observed with ^{51}Cr-labelled P815 were detected (Fig. 4.12). The only cytotoxicity detected was due to Lyt2$^+$, Thy1.2$^+$ cells. It seemed therefore, that stimulation of MLR by F1 NPCs or D 3 IPCs in CBA MLNCs induced only Lyt2$^+$ CTLs, a conclusion already reached with C57BL/6 MLR blasts.

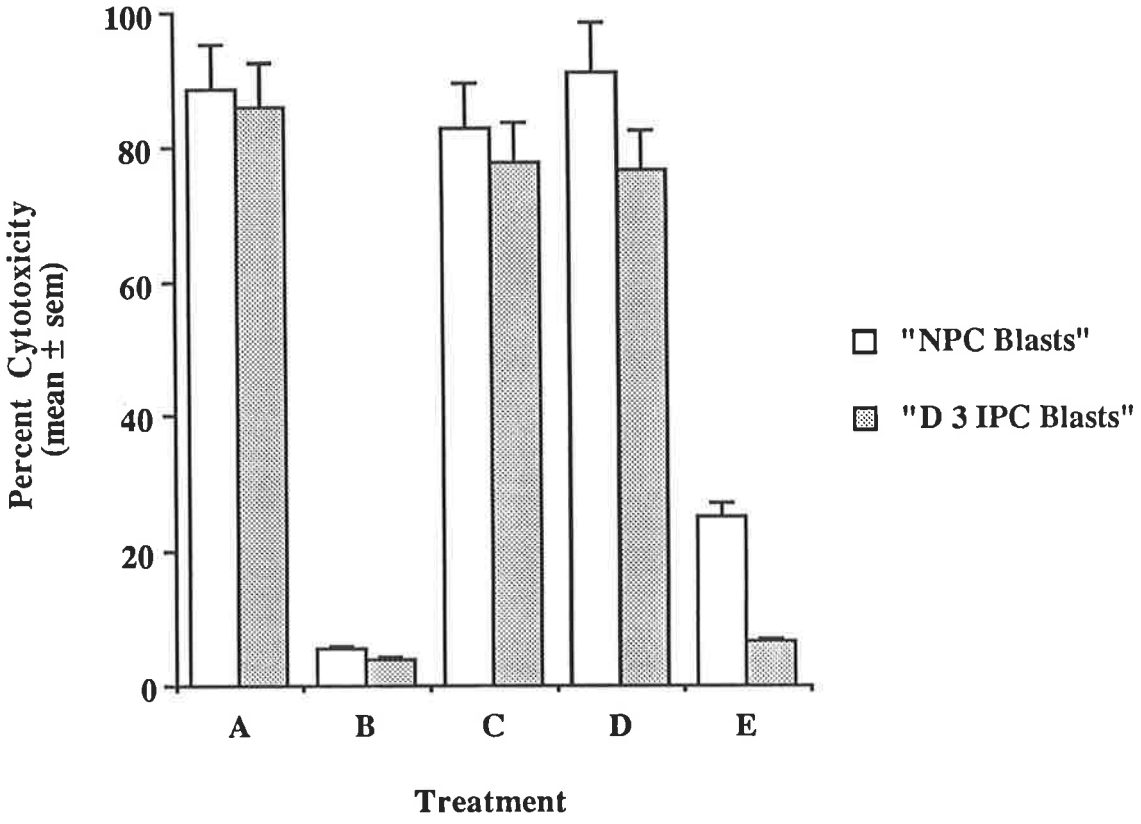


FIGURE 4.11 Cytotoxic activity of CBA MLR blasts

Mixtures of 10^6 CBA NW MLNCs with $2x10^5$ F1 NPCs or D 3 IPCs were cultured in 1 ml volumes in 24 well flat-bottomed trays for 5 days at 37°C. The MLR blasts generated were adjusted to 10^7 cells/ml and incubated with the T cell-specific MoAbs and C, or C alone, for 1 hour at 37°C. Four aliquots of these populations were mixed with ^{51}Cr-labelled P815 at ratios of 25:1 and incubated at 37°C for a further 4 hours in a 96-well round-bottomed tray. The amounts of ^{51}Cr released into the supernatants were measured and the percent cytotoxicity (mean ± sem) was calculated for each replicate set. The cytotoxicity of a typical population of CBA MLR blasts pretreated with C (A), α-Thy1.2 + C (B), α-L3T4 + C (C), α-Lyt2.2/TIB 150 + C (D) or α-Lyt2/YTS 169.4 + C (E) are provided.

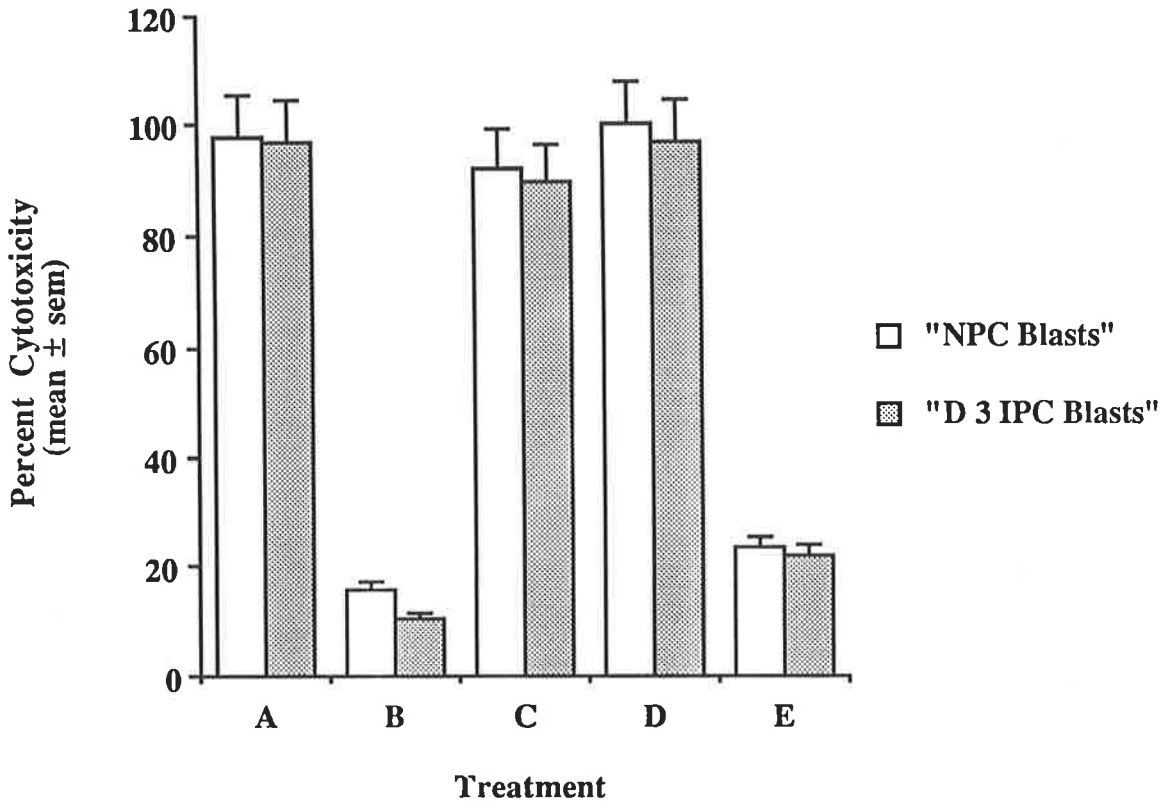


FIGURE 4.12 Detection of L3T4$^+$ CTLs in the CBA MLR blast populations

Five day CBA MLR blasts obtained by culturing $2x10^5$ F1 NPCs or D 3 IPCs with 10^6 CBA NW MLNCs, were treated with either C (A), α-Thy1.2 + C (B), α-L3T4 + C (C), α-Lyt2.2/TIB 150 + C (D) or α-Lyt2/YTS 169.4 + C (E) at 37°C for 1 hour. The treated blast populations were each mixed with $2x10^4$ ^{51}Cr-labelled ConA blasts at ratios of 25:1 (in quadruplicate) in 200 µl volumes in a 96 well round-bottomed tray and incubated for 4 hours. The levels of ^{51}Cr released were measured and the percent cytotoxicity (mean ± sem) calculated for each replicate set.

4.3 Summary and conclusions

The effect of ip immunization of mice with L11RX was investigated. Preliminary experiments revealed that infection with L11RX induced considerable changes in the PC population. Cell yields were increased and there was also a large influx of neutrophils into the peritoneal cavity. The ability of PCs from mice injected ip with L11RX 3 days earlier to stimulate various T cell activities *in vitro* was examined and compared to that of PCs from unimmunized mice.

It was shown that large numbers D 3 IPCs exhibited a reduced capacity to induce both *Salmonella*- and allo-Ag-specific T cell responses *in vitro*. The inability to induce responses was mediated by adherent cell population(s) and attempts were made to identify the cause(s) of this effect.

Pulsing with *Salmonella* Ags *in vivo* removed the inhibitory effect of D 3 IPCs and it was concluded that *in vivo* pulsing probably removed inhibitory cells and/or provided sufficient Ag to induce proliferation. The latter possibilty was supported by the *in vitro* experiments using PFA. Fixation with PFA after pulsing cells with Ag *in vitro* reduced the ability of these cells to stimulate T cells although Ag-pulsed D 3 IPCs showed greater ability to stimulate, suggesting the cells were processing Ag faster than NPCs. This implied that the D 3 IPCs had increased degradative capacity which might partially explain the lack of responses induced in the presence of large numbers of these cells and relatively small amounts of Ag. However, this did not account for their inability to induce allogeneic responses. *In vivo* pulsing with Ag not only provided sufficient Ag for T cell stimulation, but also removed a population(s) of inhibitory cells allowing the cells to induce a MLR (although not at the highest cell number), as well as the *Salmonella*-specific response.

: Addendum
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These "inhibitory" cells were not neutrophils, because removal of neutrophils had no effect on the ability of PCs to stimulate either *Salmonella*- or allo-Ag-specific responses. The cells responsible for this inhibition were adherent, with a demonstrable increase in Ag degradative ability. The likely candidates for these cells

are macrophages, which have been reported to show enhanced metabolic activity and inhibitory activity, following infection with an IBP (see Chapter 1). The slight increase in the responses induced by larger amounts of F11RX in the absence of neutrophils suggests that neutrophils may also contribute to this effect.

The other responses induced by NPCs or D 3 IPCs which were studied were very similar. No difference in the characteristics of the secondary responses induced in the *Salmonella*-purified, primed T cells nor the responses induced in allo-Ag-specific unprimed T cells were detected. Both APC populations induced $L3T4^+$ T cells to proliferate in response to *Salmonella* Ags *in vitro*. The majority of the T cells induced to proliferate in response to allo-Ags of NPCs or D 3 IPCs expressed the $Lyt2^+$, $Thy1.2^+$ phenotype and both populations also induced allo-Ag-specific $Lyt2^+$ CTLs. In other words, no modulation of APC activity as a result of immunization with L11RX was detected by the approaches used. Consequently, to further examine the involvement of the T cell subsets in the immune response to *Salmonella*, the characteristics of the T cells induced following *Salmonella* infection were analysed in some detail, and the results are presented in the following Chapters.

CHAPTER 5

ANALYSIS OF THE FUNCTIONAL CAPACITIES OF T CELLS FROM MICE CHALLENGED WITH *SALMONELLA*

5.1 Introduction

Studies on the immunity to IBPs have demonstrated roles for both cellular and humoral responses and the importance of CMI to infection of mice with *Listeria* is particularly well documented. In 1973, North demonstrated that T cells are required for immunity and DTH reactions to listerial Ags. Earlier work on the MHC restriction of the immune response to *Listeria* provided conflicting data from several groups. However, it is now clear that both Class II restricted $CD4^+$ T cells and Class I restricted $CD8^+$ T cells are involved. Studies using T cell clones have indicated that both $CD4^+$ T cells and $CD8^+$ T cells are required for effective immunity to *Listeria* (Kaufmann *et al.*, 1982 and 1986; De Libero and Kaufmann, 1986). Cooperation between the T cell subsets is necessary for clearance of *Listeria*, with the $CD8^+$ T cells being ultimately responsible for clearance of secondary infections (Kaufmann *et al.*, 1985; Bishop and Hinrichs, 1987; Mielke *et al.*, 1988). The identification of the production of an exotoxin, listeriolysin O, by virulent strains of *Listeria* (Njoku-Obi *et al.*, 1963; Groves and Welshimer, 1977) and its role in enabling organisms to escape from the phagolysosome into the cytosol (Berche *et al.*, 1987a; Tilney and Portnoy, 1989) provided a perfect explanation for the induction of both Class I and Class II MHC restricted T cell responses, since the Class I Ag processing pathway involves the cytosolic degradation of "endogenously" derived Ags whilst "exogenously" derived Ags are generally processed in endososmal compartments and presented in association with Class II MHC products (Allen and Unanue, 1984; Grey

and Chestnut, 1985; Morrison *et al.*, 1986; Nuchtern *et al.*, 1989; Yewdell and Bennink,1989; Guagliardi *et al.*, 1990).

The importance of T cells for protection against *Salmonella* infection was demonstrated by Davies and Kotlarski in 1976, when they found that depletion of T cells decreased the capacity of mice to clear an infection of L11RX and abolished protection against LC5 normally provided by immunization with L11RX. It could be assumed that the type of response induced by *Salmonella* infection is the same as that induced by *Listeria*, but this may not be the case because there is no evidence for production of a "listeriolysin-equivalent" by *Salmonella* and it is generally accepted that *Salmonella* normally remain localized inside phagolysosomes and are not found in the cytosol. Nonadherent, Lyt1^{+}2^{-} T cells obtained from L11RX immunized mice, had been shown to release the LKs, IL 2 and MAF in response to specific Ags and accessory cells *in vitro* in an H-2I (Class II) restricted manner (Attridge and Kotlarski, 1985a). The same cells were also able to transfer DTH reactivity to *Salmonella* Ags to normal, unimmunized mice (Attridge and Kotlarski, 1985b).

The only evidence that infection with live *Salmonella* induced Class I MHC restricted Lyt2^{+} T cells was provided by some preliminary experiments carried out in our laboratory. T cells with cytotoxic activity were detected in IPC suspensions of L11RX immunized mice using a lectin-mediated cytotoxicity assay and it was found that such Lyt2^{+} CTLs were not present after immunization with killed 11RX nor in normal, unimmunized mice (unpublished data). These observations were extended in the present study to establish whether *Salmonella*-specific Lyt2^{+} T cells were induced after infection with L11RX. This included the analysis of the T cell subsets induced by secondary *Salmonella* infections because it has been reported that the activity of Ag-specific Lyt2^{+} T cells was increased during secondary infection with *Listeria* (Mielke *et al.*, 1988).

Whenever the mouse strain used is not specified, F1 mice were used in the experiments described in this Chapter.

5.2 Results

5.2.1 Transfer of DTH reactivity to *Salmonella* antigens

It is well established that development of the ability to mediate DTH reactivity to specific Ags is associated with the induction of CMI (eg. Mitsuyama *et al.*, 1982). Consequently, measurement of the capacity to mediate DTH can provide useful information regarding the form of immune response induced. Infection of mice with the avirulent organism 11RX induces both humoral and CMI responses and provides protection against infection with virulent C5 organisms (Davies and Kotlarski, 1974). The induction of immunity following infection with L11RX has been shown to correlate with the induction of T cells capable of mediating DTH reactivity to C5 Ags (Davies and Kotlarski, 1974) and to 11RX Ags (Attridge and Kotlarski, 1985b).

More detailed analysis of the cells responsible for the DTH reactivity was carried out to define the T cell subsets induced by L11RX infection and their functional capacities. In 1985, Attridge and Kotlarski reported that PCs and SCs of mice immunized ip 21 days previously with L11RX (D 21 IPCs and ISCs respectively) were capable of transferring DTH reactivity to soluble 11RX Ags to normal, unimmunized mice. For both the D 21 IPC and D 21 ISC populations, the cells responsible for the reactivity were found to express the $Lyt1^+$ $Thy1.2^+$ phenotype. Since Attridge and Kotlarski (1985b) found that SCs usually transferred less DTH reactivity than the PC populations, the present, more extensive study initially concentrated on the ability of the D 21 IPCs to transfer DTH reactivity to various *Salmonella* Ags.

To partially purify the D 21 IPCs, they were incubated on a plastic dish for 1 hour at 37°C to remove most of the adherent cells. The plastic nonadherent IPCs (P IPCs) were counted, adjusted to the required concentration and mixed with *Salmonella* Ags. The Ags used were F11RX (2.5 μg), L11RX (10^5 organisms) and LC5 ($5x10^3$ organisms). F11RX was used as the "control" Ag and was expected to confirm the results obtained using soluble 11RX Ag (Attridge and Kotlarski, 1985b), as killed or inert Ags are presented in association with Class II MHC molecules and generally react with

L3T4$^+$ T cells. In contrast, live Ags can induce both Class I and Class II restricted T cell responses (Ertl, 1981; Jungi *et al.*, 1982a and 1982b; Leung and Ada, 1982). Therefore, use of live Ags to elicit DTH reactivity may allow the detection of Class I restricted Lyt2$^+$ T cells capable of mediating reactivity to *Salmonella* Ags. [Optimal Ag doses were chosen which induced significant responses in the presence of T cells and minimal swelling when injected alone (data not shown).] Each mixture was transferred into the left hind footpads of 3-4 unimmunized mice in a 50 µl volume per mouse. As controls, 50 µl of P IPCs alone or Ag alone were each injected into the left hind footpads of 3-4 normal mice. The right, uninjected footpads, acted as the negative control for each mouse. The left and right footpads were measured 24 and 48 hours later and the percent swelling calculated with respect to the size of the right footpad.

Representative results from one of a set of three experiments are presented in Fig. 5.1, which show the percent footpad swelling (mean ± sem) elicited by 2.5 µg F11RX, 10^5 L11RX and $5x10^3$ LC5 at 24 hours. Clearly, all three Ags elicited significant responses, with the responses to LC5 and L11RX being consistently smaller than the response induced by F11RX. The swelling observed by 48 hours after the transfer of P IPCs and F11RX had decreased, whilst in the presence of either L11RX or LC5 swelling had increased. Most of this was attributable to growth of bacteria in the footpad because mice injected with live bacteria only showed a similar amount of swelling at 48 hours (compared to 5% at 24 hours, data not shown).

5.2.2 Characterization of the T cell subsets mediating DTH reactivity in response to *Salmonella* antigens

5.2.2.1 Summary of experimental design

Treatment with various MoAbs and C was used to establish the phenotype(s) of the cells mediating DTH activity. The MoAbs employed were α-Thy1.2, α-L3T4 and α-Lyt2.2 and were all used at a final dilution of 1 in 10 and C was used at a final dilution of 1 in 20. Treatment of P IPCs with these MoAbs and C should

FIGURE 5.1 Transfer of DTH reactivity with D 21 IPCs

The T cells in D 21 IPC suspensions were partially purified by removing a large proportion of the adherent cells by allowing them to adhere to plastic during a 1 hour incubation at 37°C. The numbers of plastic nonadherent cells (P IPCs) were adjusted and mixed with the *Salmonellae* Ags to ensure that 10^6 P IPCs were transferred with one of the three different forms of Ag in 50 µl volumes to the left hind footpads of normal F1 mice. The right and left footpads were measured 24 hours later and the relative increase in footpad size was recorded. For each P IPC-Ag combination at least 3 mice were used as recipients and the percent footpad swelling (mean ± sem) was calculated for each group and the results from one representative experiment are presented. The combinations of P IPCs and Ags transferred and the appropriate controls were as follows:

A: P IPCs + 2.5 µg F11RX

B: P IPCs + 10^5 L11RX

C: P IPCs + $5x10^3$ LC5

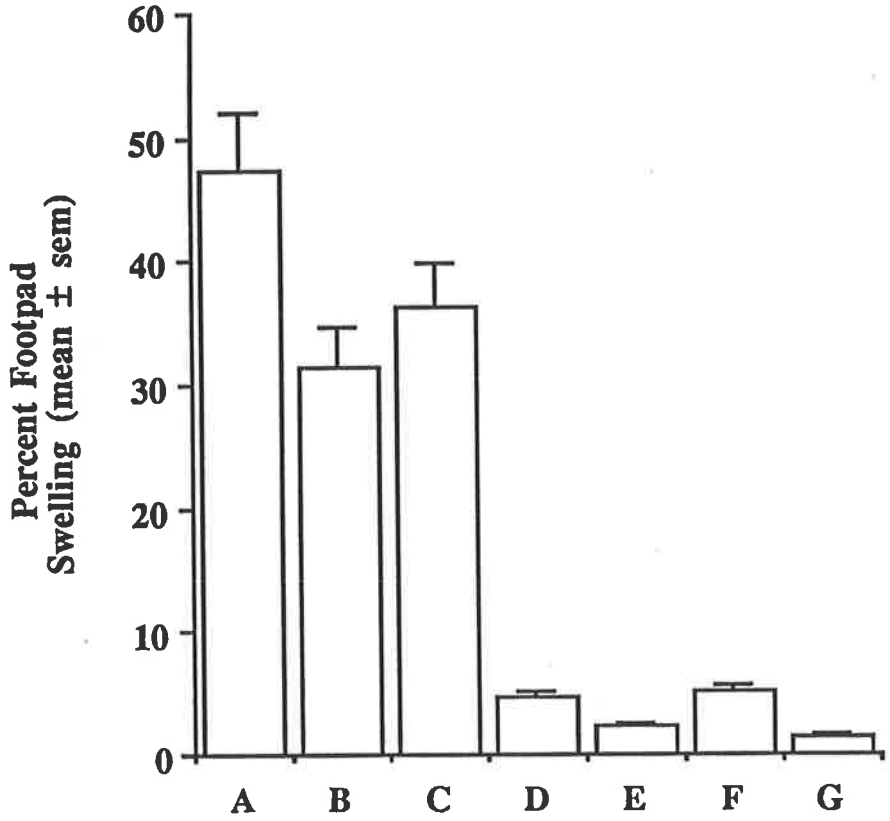
D: 2.5 µg F11RX

E: 10^5 L11RX

F: $5x10^3$ LC5

G: P IPCs

Error bars indicate the sem value for each group of data.


Percent Footpad
Swelling (mean ± sem)
60
50
40
30
20
10
0
A
B
C
D
E
F
G

deplete the P IPCs of either all T cells or only those responding to Ag in association with Class II MHC molecules (L3T4$^+$ T cells) or Class I MHC molecules (Lyt2.2$^+$ T cells), respectively. Therefore, local transfer of these treated cells plus specific Ags to the footpads of normal mice, allows the determination of the phenotpye of the cells responsible for the DTH responses induced. Accordingly, the effect of MoAb and C treatment of P IPCs was assessed by mixing the treated cells with various *Salmonella* Ags, before transferring them to the left hind footpads of groups of 3 or 4 normal mice and taking footpad measurements 24 (and sometimes 48) hours later. The percent footpad swelling obtained for each group was calculated in the usual way.

5.2.2.2 L3T4$^+$ T cells mediate DTH reactivity to F11RX

With the knowledge that killed or inert vaccines are (usually) presented to T cells in association with Class II MHC molecules it was not surprising to find that the cells mediating the DTH reactivity elicited by F11RX expressed the L3T4$^+$, Thy1.2$^+$ phenotype. Fig. 5.2 provides data representative of the observations recorded on 3 separate occasions and illustrates that depletion of either all T cells or only L3T4$^+$ T cells virtually abolished the ability to transfer DTH reactivity to F11RX. These data support the findings reported by Attridge and Kotlarski (1985b) and also the more recent work carried out using IPCs cultured with F11RX *in vitro* for 3 days, which were also capable of transferring specific DTH reactivity (Kotlarski *et al.*, 1989).

5.2.2.3 L3T4$^+$ T cells also mediate DTH reactivity to L11RX

In an attempt to detect Class I restricted Lyt2.2$^+$ DTH effector cells L11RX organisms were also used as the eliciting Ag in this system. Somewhat unexpectedly, the cells mediating DTH activity in response to stimulation with L11RX were found to express the L3T4$^+$, Thy1.2$^+$ phenotype (Fig. 5.3). This experiment was repeated three times and pretreatment with α-Thy1.2 or α-L3T4 and C was consistent in removing virtually all of the ability of the P IPCs to transfer a response to L11RX Ags, while α-Lyt2.2 and C treatment had no effect. Hence, even in the presence of L11RX,

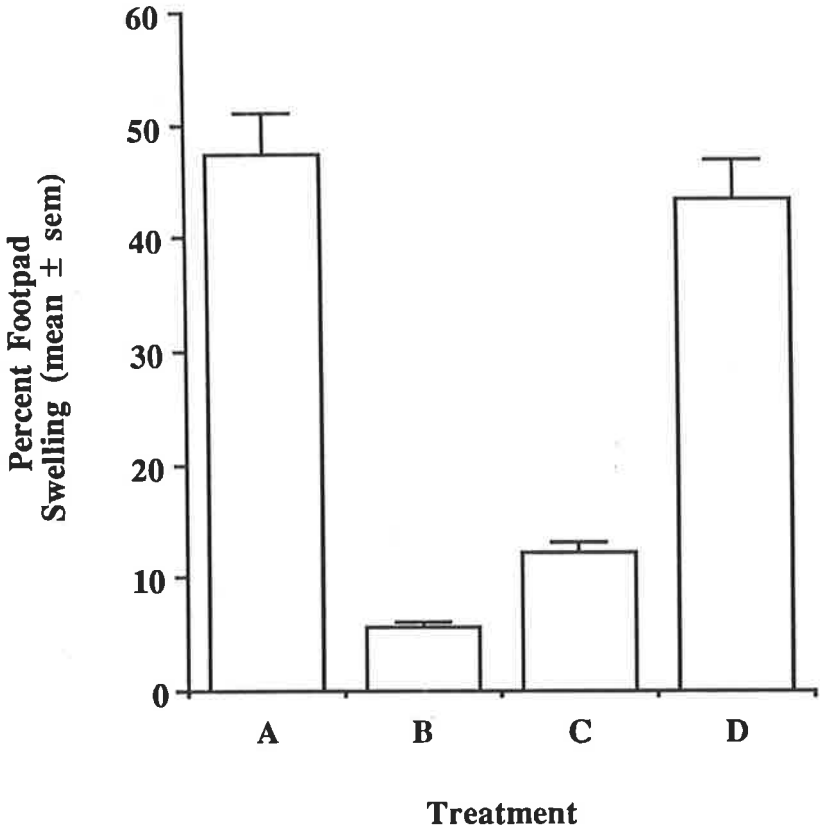


FIGURE 5.2 Phenotype of the T cells mediating DTH to F11RX

P IPCs were pretreated with T cell specific MoAbs and C for 1 hour at 37°C, before mixtures containing 10^6 treated cells and 2.5 μg F11RX in 50 μl were prepared and each injected sc into the left hind footpads of 3 normal mice. 24 hours later the footpads were measured and the percent footpad swellings (mean ± sem) from a representative experiment are provided. P IPCs were treated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D). Error bars indicate the sem value for each group of data.

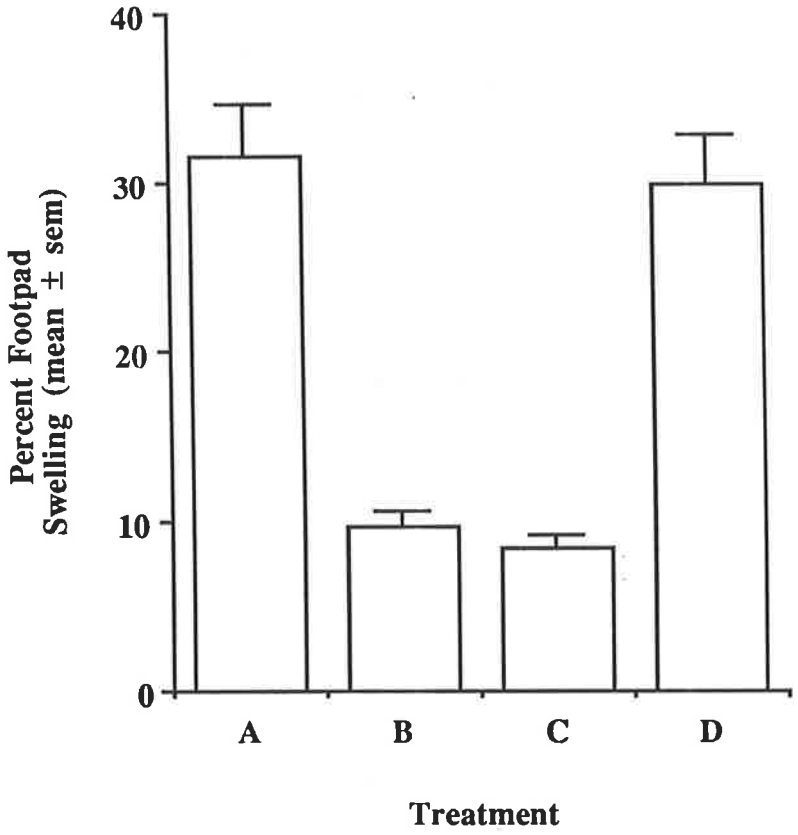


FIGURE 5.3 Phenotype of the DTH effectors induced by L11RX

Results of a typical experiment using P IPCs pretreated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) before mixing with L11RX to provide suspensions that contained 10^6 cells and 10^5 L11RX in 50 µl. These were injected into the footpads of groups of 3 mice and the percent increase in footpad size (mean ± sem) at 24 hours was determined in the usual way. Error bars show the sem values for each group of results.

DTH was mediated by Class II restricted L3T4$^+$ effector T cells. This implied that either there were no specific Class I restricted DTH effector cells induced by *Salmonella* infection or that the Lyt2.2$^+$ T cells represented a minor population which was masked by a larger response by the L3T4$^+$ T cells. Another possibility was that the assay system used was not appropriate for detecting Class I restricted T effector cells because of preferential presentation of L11RX Ags in the context of Class II MHC molecules. Furthermore, a certain amount of "killed" Ag, which may be provided with the live suspension of microorganisms and/or as a result of some killing of L11RX by normal or activated cells in the P IPC population, may have provided sufficient killed organisms for preferential presentation of 11RX Ags with Class II MHC products, resulting in the stimulation of L3T4$^+$ T cells. A way of overcoming this could be to use a virulent organism, like C5, which is more resistant to killing (Davies and Kotlarski, 1976) and should thereby provide a longer lasting source of live Ag.

5.2.2.4 Examination of the T cells mediating DTH in response to LC5

Because immunization of mice with L11RX provides protection against infection with the virulent C5 strain and induces DTH reactivity towards C5 Ags (Davies and Kotlarski, 1974) and since it has been shown that LC5 persist longer than L11RX organisms [in 11RX immunized animals] (Davies and Kotlarski, 1976), LC5 was used to elicit DTH by transferring it locally with P IPCs pretreated with the C and MoAbs. These cells were mixed with LC5 and 50 µl volumes of each Ag-cell mix (containing 10^6 P IPCs and $5x10^3$ LC5) were transferred to the left hind footpads of groups of 3 normal mice and their hind footpads were measured 24 hours later. The same experiment was carried out on three separate occasions and proved to be quite reproducible. Fig. 5.4 presents the data from one of these experiments. Treatment with α-Thy1.2 or α-L3T4 and C removed the ability to transfer DTH reactivity to LC5, whereas removal of the Lyt2.2$^+$ T cells prior to transfer had no influence on the amount of DTH reactivity induced by LC5. Therefore, even when a persisting "live Ag" was used, the DTH response elicited was mediated by Class II restricted L3T4$^+$ T cells.

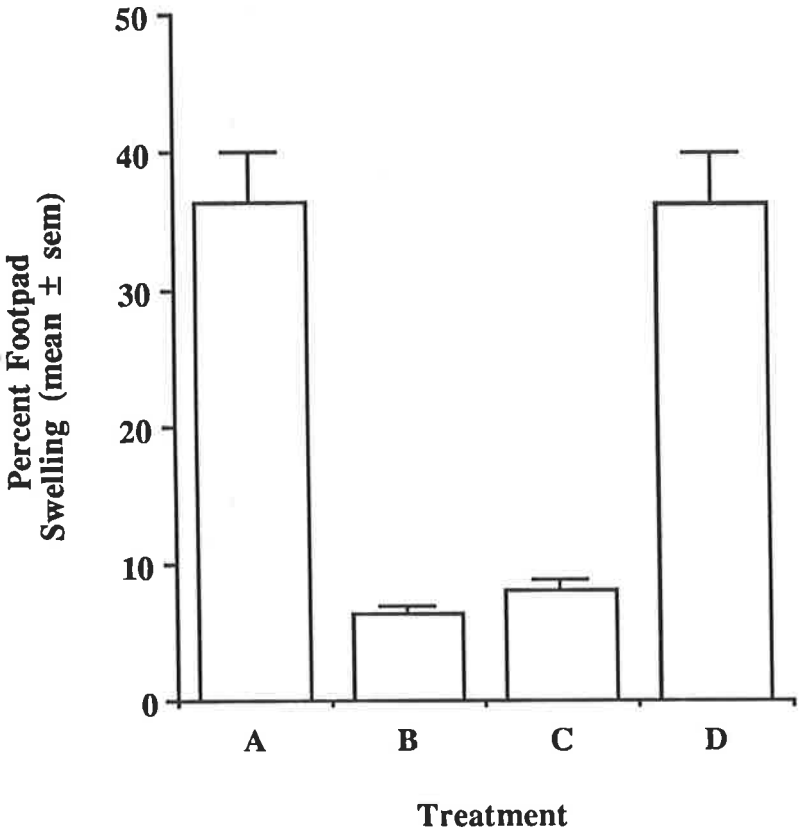


FIGURE 5.4 Phenotype of the DTH effectors induced by LC5

Mixtures of 10^6 MoAb and C treated P IPCs and $5x10^3$ LC5 were injected sc into the left hind footpads of groups of 3 normal F1 mice and the amount of footpad swelling measured 24 hours later and expressed as the percent footpad swelling (mean ± sem) for each group. Treatment of the P IPCs involved an incubation for 1 hour at 37°C with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D).

[Persistence of live Ags was confirmed by bacteria recovery experiments (data not shown).]

5.2.3 Detection of DTH effectors early after immunization with L11RX

It was of interest to determine the level of activity of DTH effector cells induced early after ip immunization with L11RX, because different T cells subsets may be induced at different times during infection. Since a CMI response can be detected by 2-3 days following infection with L11RX (Ashley and Kotlarski, 1982), the ability of PCs and SCs harvested from mice ip immunized with L11RX 4 days earlier (D 4 IPCs and ISCs) to transfer DTH reactivity to normal mice was investigated. Furthermore, preliminary data (reported in Section 5.2.5.1 *(ii)*) demonstrated that as early as two days after infection with live *Salmonella* a population of Lyt2.2$^+$ CTLs were induced (their peak activity occurring around the fifth day and declining thereafter). Therefore, it seemed reasonable to postulate that Lyt2.2$^+$ DTH effector T cells may be present and/or have greater activity earlier than 21 days after immunization.

Before transfer, most of the adherent cells present in these populations were removed by adherence to plastic Petri dishes during a 1 hour incubation at 37°C. The nonadherent cells recovered provided the P IPC and P ISC populations which were counted, adjusted to the required concentration and injected sc into the left hind footpads of groups of 3 normal mice, together with various Ag preparations. Control suspensions of cells or Ag alone were also injected into groups of 3 normal mice. Both hind footpads of all the groups of mice were measured 24 and 48 hours later and the percent swelling induced calculated as normal. The Ag preparations used were F11RX (2.5 μg), S11RX (10 μg), L11RX (10^5 organisms) and LC5 ($5x10^3$ organisms). F11RX and S11RX were included as controls to detect L3T4$^+$ T cells and live organisms were used as the Ags to attempt to detect the presence of Class I restricted Lyt2.2$^+$ T cells able to mediate DTH responses.

Data typical of the results obtained in a set of five individual experiments are outlined in Fig. 5.5 [*(i)* and *(ii)*]. Both P IPCs *(i)* and P ISCs *(ii)* transferred DTH to

FIGURE 5.5 Transfer of DTH reactivity with D 4 IPCs and ISCs

Plastic nonadherent D 4 IPCs and ISCs (P IPCs and P ISCs) were mixed with the various *Salmonellae* Ags, to ensure that 10^6 cells with the appropriate amount of Ag (see below) could be injected sc in a 50 μl volume. For each cell-Ag mixture and the control preparations, groups of 3 mice were used as recipients and the percent footpad swelling (mean ± sem) at 24 hours was calculated for each group. Data illustrating typical responses transferred with either P IPCs or P ISCs are outlined in Fig. 5.5 [*(i)* and *(ii)*], respectively. The test inocula transferred included:

A: Cells + 2.5 μg F11RX

B: Cells + 10 μg S11RX

C: Cells + 10^5 L11RX

D: Cells + $5x10^3$ LC5

E: 2.5 μg F11RX

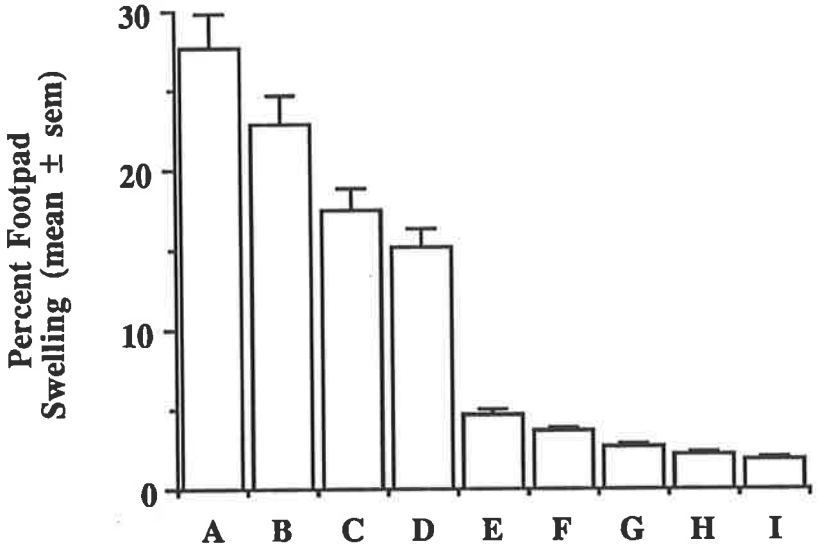
F: 10 μg S11RX

G: 10^5 L11RX

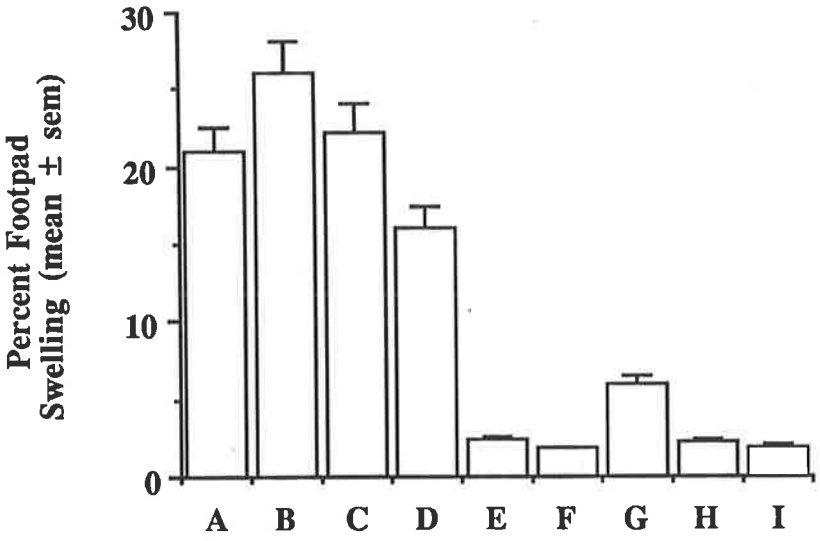
H: $5x10^3$ LC5

I: Cells

(i)


Percent Footpad
Swelling (mean ± sem)
30
20
10
0
A
B
C
D
E
F
G
H
I

(ii)


Percent Footpad
Swelling (mean ± sem)
30
20
10
0
A
B
C
D
E
F
G
H
I

all Ags used, though the responses to F11RX transferred with P ISCs were somewhat lower. Peak responses with both cell types occurred around 24 hours (the swelling elicited by F11RX and S11RX had virtually disappeared by 48 hours, whereas by this time LC5 and L11RX had usually induced a significant amount of swelling even in the absence of cells, indicating that the swelling was caused by bacterial growth within the footpad; data not shown). All responses were lower than those transferred by IPCs or ISCs obtained from mice later after immunization, as reported by Attridge and Kotlarski (1985b) and also shown in Fig. 5.1. However, investigation into the phenotype of the cells mediating the DTH reactivity in these populations yielded inconclusive results. Depletion experiments using MoAb and C, almost completely removed the entire Lyt2.2$^+$ T cell population from these cells, however removal of the L3T4$^+$ T cells was not as complete (as suggested from the data described in Sections 5.2.8.1 and 5.2.8.2). Using these depleted populations to transfer DTH almost identical responses were induced in absence of Lyt2.2$^+$ T cells, even when live organisms were used as the eliciting Ags, suggesting that the L3T4$^+$ T cells were mediating DTH (data not shown). However, depletion of the L3T4$^+$ T cell subset did not consistently reduce the DTH responses transferred, implying that although the numbers of L3T4$^+$ T cells were reduced, sufficient numbers remained to transfer a response (data not shown). Whether the inability to completely abrogate the ability to transfer DTH reactivity simply reflects incomplete depletion of the L3T4$^+$ T cells or the fact that the DTH effector cells were double negative T cells remains to be determined. It is interesting to note that larger numbers of L3T4$^-$, Lyt2$^-$ $\gamma\delta$ T cells appear early after infection with *Listeria* (by approximately day 3) and by the eighth day after infection the proportion of these cells is greatly reduced (Ohga *et al.*, 1990). Similarly, $\gamma\delta$ T cells have been found to appear early during infection with BCG (Inoue *et al.*, 1991). Therefore, these results could be taken to suggest that by the fourth day after infection with *Salmonella* an Ag-specific L3T4$^-$, Lyt2.2$^-$ $\gamma\delta$ T cell population was induced, which was capable of transferring DTH to normal mice in the presence of *Salmonella* Ags. However, more detailed analysis would be required to establish if $\gamma\delta$ T cells were induced by infection with *Salmonella*

and whether they were capable of mediating DTH.

Additional investigations revealed that *in vitro* culture of these cells with ConA, which may have expanded the DTH effector subset and therefore allowed larger responses to be transferred, did not enhance the ability of either of these populations to transfer DTH reactivity (an example representative of several reproducible experiments is provided in Fig. 5.6) Again MoAb and C treatment of these populations yielded inconclusive results (data not shown).

5.2.4 Summary

T cells obtained from the peritoneal cavities of mice immunized with L11RX were able to transfer DTH reactivity to normal, unimmunized mice, with larger responses being transferred when cells harvested late after immunization were used (this also applied for T cells obtained from the spleens of these mice). The cells responsible for mediating this activity were L3T4$^+$ T cells, with no evidence for the involvement of Lyt2$^+$ DTH effector cells even when live bacteria were used to elicit the response. The most obvious interpretation of these data is that primary immunization with L11RX did not induce Class I restricted Lyt2.2$^+$ DTH effector T cells. However, it was possible that a response by a minor Lyt2$^+$ T cell population may have been masked by the Class II restricted L3T4$^+$ T cell response (or a $\gamma\delta$ T cell response). In other words, a more appropriate assay system for detection of effectors of the Lyt2.2$^+$ phenotype may need to be developed - one in which presentation of Ags in association with Class I MHC molecules is favoured.

5.2.5 Detection of CTLs in the PC population from L11RX immune mice

5.2.5.1 Introduction

(i) Summary of assay systems used

Measurement of cytotoxic activity of a T cell population is often used as

FIGURE 5.6 DTH reactivity transferred using ConA cultured P ISCs

The ability of ConA cultured P ISCs prepared from F1 mice 4 days after immunization with L11RX to transfer DTH reactivity was analysed by transferring 10^6 cells with the various Ags to the left hind footpads of normal F1 mice and measuring the footpad sizes 24 hours later. Groups of 3 mice were used for each cell-Ag mixture, as well the control suspensions used and the precent increases in footpad size (mean ± sem) were recorded. The mice received one of the following stimuli:

A: Cells + 2.5 μg F11RX

B: Cells + 10 μg S11RX

C: Cells + 10^5 L11RX

D: Cells + $5x10^3$ LC5

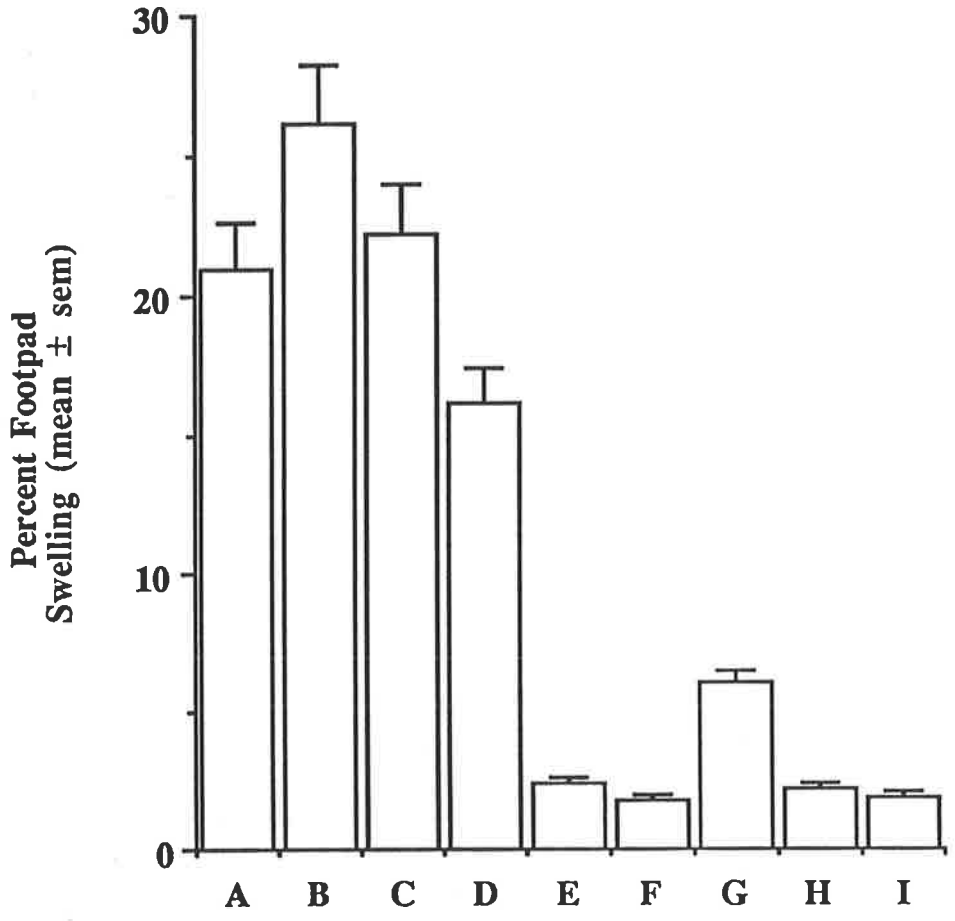
E: 2.5 μg F11RX

F: 10 μg S11RX

G: 10^5 L11RX

H: $5x10^3$ LC5

I: Cells


Percent Footpad
Swelling (mean ± sem)
30
20
10
0
A
B
C
D
E
F
G
H
I

an indication of activation of a CMI response. Measurement of cytotoxic activity usually involves mixtures of effector cell populations and ^{51}Cr-labelled target cells being incubated (in quadruplicate) for 4 hours at 37°C, after which the amount of ^{51}Cr released into the supernatants is measured. Generally, a range of doses of effector cells ($5x10^5$, 10^5 and $2x10^4$) is incubated with $2x10^4$ target cells and the results are expressed as the percent of cytotoxicity (mean ± sem), calculated as described in the Materials and Methods. To detect cytotoxic cells generated following bacterial infection, Ag-pulsed cells are normally used as the target cell population (eg. De Libero and Kaufmann, 1986). However, cytotoxicity can also be detected using a lectin-mediated assay where the target cells are pretreated with the lectin ConA. ConA binds to non-polymorphic regions of MHC molecules on the target cells, enabling them to interact with the TCRs of the T effector cells. Therefore, the lectin-mediated cytotoxicity assay will detect both L3T4$^+$ and Lyt2.2$^+$ CTLs of any specificity, and the phenotype of any cytotoxic cells detected can be determined using MoAbs and C. This procedure was adopted to look for cytotoxic activity of cells from L11RX immunized mice since appropriate *Salmonella* Ag-pulsed target cells were not available at this time. Methods of preparing suitable *Salmonella* Ag-pulsed target cells were being investigated at the same time, with the intention of using these cells to determine whether any Ag-specific CTLs were present in suspensions with demonstrable cytotoxic activity in the lectin-mediated lysis assay. The routine procedure used to define the phenotype of the CTLs involved the pretreatment of NW IPCs with various T cell-specific and subset-specific MoAbs and C. NW IPCs were incubated for 1 hour at 37°C with culture medium, C alone, α-Thy1.2 + C, α-L3T4 + C, or α-Lyt2.2 + C prior to incubating the cells with various target cell populations and determining the cytotoxicity of each effector subpopulation.

(ii) Preliminary observations

CMI can be detected by the second or third day of infection with L11RX, as measured by the clearance of tumour cells *in vivo* and tumour cell killing *in vitro* (Ashley and Kotlarski, 1982), with the majority of this *in vivo* and *in vitro* activity being

attributed to activated macrophages (La Posta *et al.*, 1982). Accordingly, it was concluded that macrophages were activated by LKs released from activated T cells which were present by the second to third day after ip immunization with L11RX. The demonstration that Lyt2$^+$ T cells were able to mediate lectin-mediated cytotoxic activity as early as two or three days after immunization with L11RX (unpublished data, presented in Fig. 5.7 [*(i)* and *(ii)*]), whilst NPCs and IPCs of animals given killed 11RX did not (exhibiting maxima of only 5% cytotoxicity against ConA-treated and untreated ^{51}Cr-labelled P815), was consistent with the conclusion that T cells are activated at this early stage of immunization. These observations also indicated that the peak of activity was likely to occur by the fifth day of infection and showed that Lyt2.2$^+$ CTLs were induced as a result of infection with L11RX. The experiments which follow were designed to further characterize these CTLs.

A standard assay system was developed for the detection of CTLs, which involved incubation of NW fractionated D 4 or 5 IPCs with ^{51}Cr-labelled targets cells at ratios of 25:1, 5:1 and 1:1 (in quadruplicate) in round-bottomed wells for 4 hours at 37° C before measuring the amount of ^{51}Cr released into the supernatant. The bulk of the assays were carried out using ConA-treated target cells, although target cells pulsed with *Salmonella* Ags were also used when they became available.

5.2.5.2 Lectin-mediated cytotoxicity against various targets

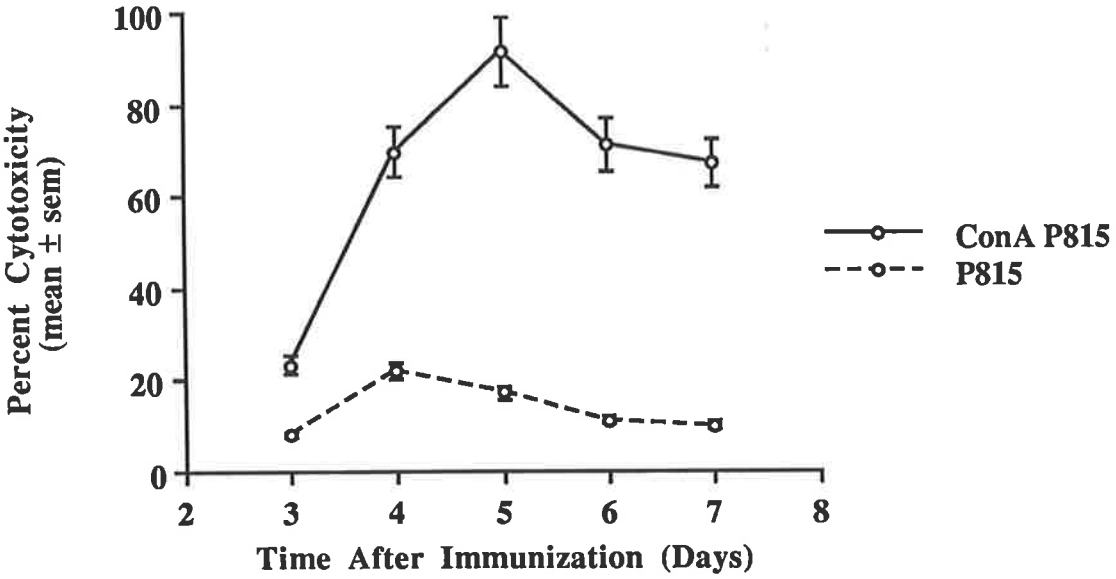
Initially, the ability of the effector cells to lyse ConA-treated P815 was confirmed and compared with the lysis of other target cell populations. Therefore, in the first set of experiments, three different cell lines were used as the target cells. These included the murine mastocytoma P815 which expresses Class I MHC molecules of the H-2^d haplotype, P388D1, a murine macrophage cell line expressing both Class I and II MHC molecules of the H-2^d haplotype and the murine T cell lymphoma EL4, which expresses Class I MHC molecules of the H-2^b haplotype. The ability of the NW IPCs to lyse each of the three target cell populations (pretreated with ConA or untreated) was analysed on at least two occasions and the results from one set of these experiments are

FIGURE 5.7 Cytotoxicity of L11RX IPCs

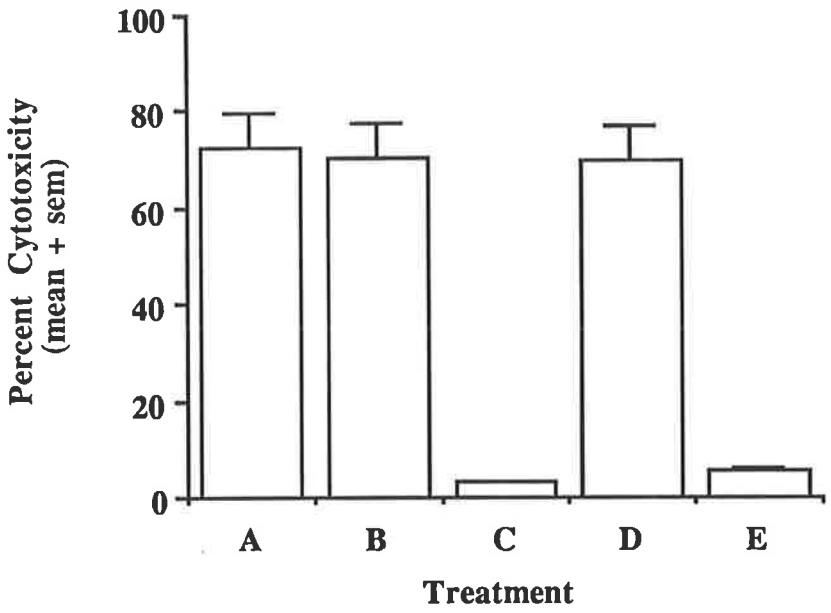
The cytotoxic activity of NW IPCs harvested from mice at various times after ip immunization with L11RX was assessed using the lectin-mediated cytotoxicity assay. The NW IPC suspensions were incubated with ConA-treated ^{51}Cr-labelled P815 in quadruplicate for 4 hours at 37°C and the percent cytotoxicity (mean ± sem) was calculated for each cell suspension. Data typical of several experiments are presented in Fig. 5.7 *(i)*.

The phenotype of the cytotoxic cells was determined by treating the NW D 5 IPCs with T cell-specific MoAbs and C before incubating them with the target cells. Cells were treated with culture medium (A), C (B), α-Thy1.2 + C (C), α-L3T4 + C (D) or α-Lyt2.2 + C (E) and representative data illustrating their cytotoxicity are shown in Fig. 5.7 *(ii)*.

(i)


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
2
3
4
5
6
7
8
Time After Immunization (Days)
ConA P815
P815

(ii)


Percent Cytotoxicity
(mean + sem)
100
80
60
40
20
0
A
B
C
D
E
Treatment

summarized in Fig. 5.8 [A-C]. NW IPCs were able to lyse all three target cell types when they were pretreated with ConA. However, for reasons which were not explored, considerable background lysis of the untreated P388D1 and particularly EL4 cells was also observed and the spontaneous release of ^{51}Cr by EL4 cells was routinely higher (up to 25%) than that of either P815 or P388D1 (averaging approximately 4% or 10% respectively). For this reason, subsequent analysis of the cell types responsible for target cell lysis was carried out using only the P815 and P388D1 cell lines.

5.2.5.3 Phenotype of the cytotoxic cells

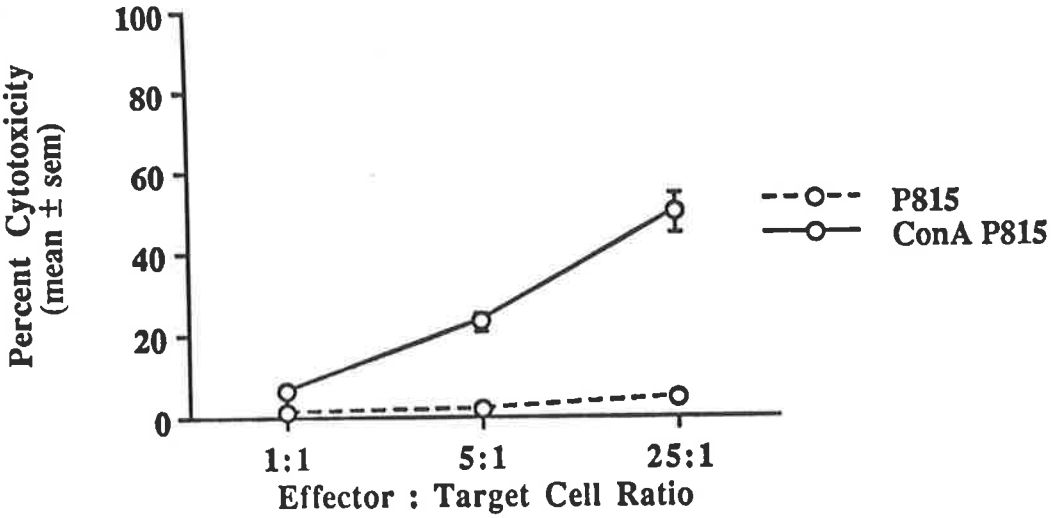
To establish which cells were mediating the cytotoxic activity detected, NW IPCs were pretreated with the MoAbs and C, prior to incubating them with the ConA-treated or untreated ^{51}Cr-labelled P815 or P388D1 cells at ratios of 25:1. These mixtures were incubated for 4 hours at 37°C before measuring the amount of ^{51}Cr released into the supernatants and calculating the percent cytotoxicity detected in the standard way. Typical results obtained are presented in Fig. 5.9 [A and B], which clearly demonstrates that the cells responsible for the lysis of ConA-treated ^{51}Cr-labelled P815 were Lyt2.2$^+$ T cells and support our previous findings (unpublished). Similarly, Lyt2.2$^+$ T cells are responsible for approximately 30% of the lysis of the ConA-treated ^{51}Cr-labelled P388D1. The remaining activity correlates with the background killing observed against untreated P388D1. Data in Fig. 5.9 C also indicate that Thy1.2$^+$ cells were responsible for only small proportion of the cytotoxic activity detected using untreated P388D1.

It is unlikely that the cells responsible for killing of untreated P388D1 were activated macrophages, known to be present after immunization with L11RX, because La Posta *et al.* (1982) demonstrated that both the target cell populations used in this assay were susceptible to lysis by these cells and NW fractionation should have removed them. The kinetics of killing were also not characteristic of activated macrophages. High levels of cytotoxicity were detected in a 4 hour assay which is much less than the 10-20 hours macrophages require to exert such levels of cytotoxic activity

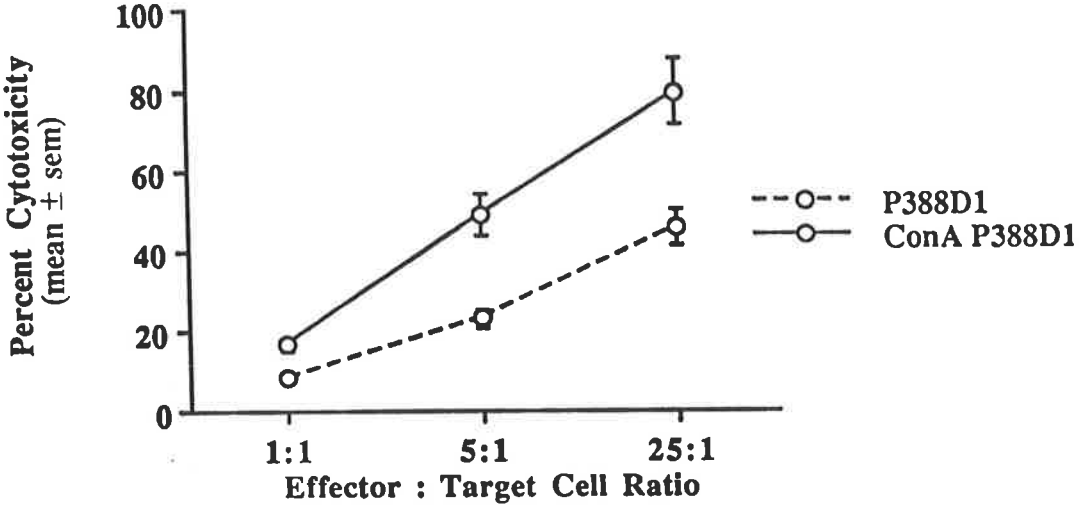
FIGURE 5.8 *In vitro* lectin-mediated cytotoxicity of NW IPCs

NW fractionated D 5 IPCs (NW IPCs) were examined for their capacity to lyse various ^{51}Cr-labelled target cell populations which were pretreated with the lectin ConA or left untreated. NW IPCs were mixed with the ^{51}Cr-labelled target cells at ratios of 1:1, 5:1 and 25:1 (each in quadruplicate) and incubated for 4 hours at 37°C in an atmosphere of 5% CO_2. The amount of ^{51}Cr released into the supernatants of these cultures over this 4 hour period was measured using the gamma counter and the percent cytotoxicity (mean ± sem) was calculated for each effector cell-target cell combination. The data from a typical experiment are provided, illustrating the level of cytotoxic activity against ^{51}Cr-labelled P815 (A), ^{51}Cr-labelled P388D1 (B) and ^{51}Cr-labelled EL4 (C).

A


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1:1
5:1
25:1
Effector : Target Cell Ratio
P815
ConA P815

B


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1:1
5:1
25:1
Effector : Target Cell Ratio
P388D1
ConA P388D1

C

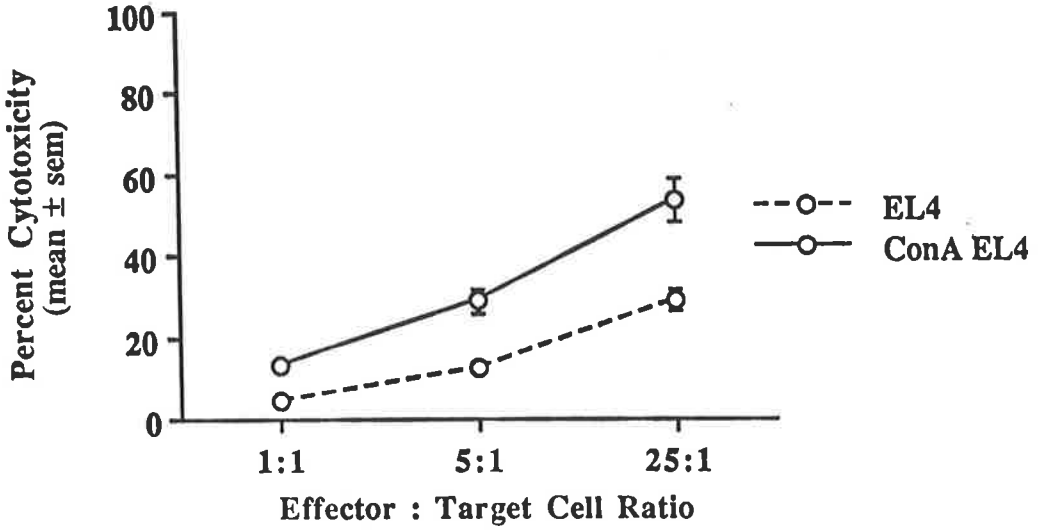
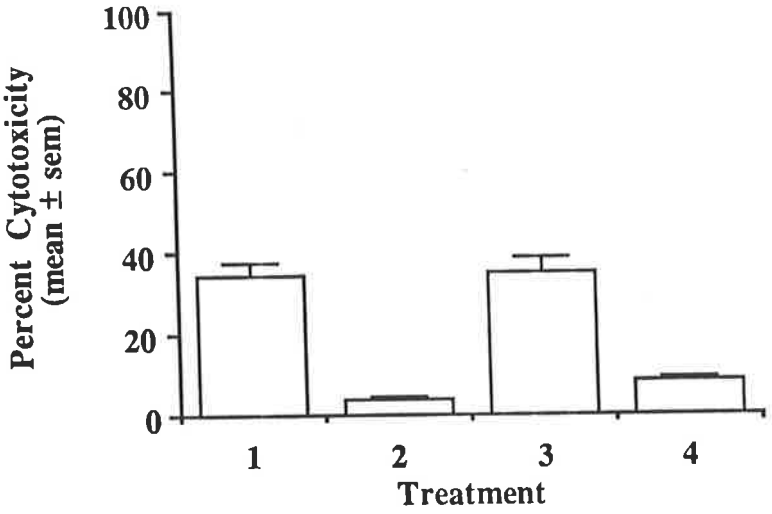

Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1:1
5:1
25:1
Effector : Target Cell Ratio
EL4
ConA EL4

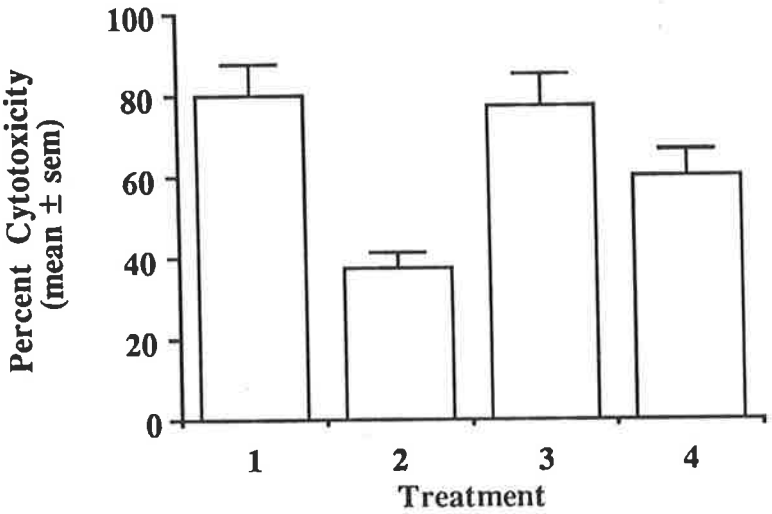
FIGURE 5.9 Phenotype of the cytotoxic cells in NW IPC suspensions

The phenotype of the cytotoxic cells responsible for the lysis of ^{51}Cr-labelled ConA-treated P815 (A), ^{51}Cr-labelled ConA-treated P388D1 (B) and ^{51}Cr-labelled untreated P388D1 (C), was characterized using the standard MoAb and C depletion technique. NW IPCs were treated with either C alone (1), α-Thy1.2 + C (2), α-L3T4 + C (3) or α-Lyt2.2 + C (4) before being added to the ^{51}Cr-labelled target cells and incubated for 4 hours at 37°C. The percent cytotoxicity (mean ± sem) of each treated population was calculated in the usual way.

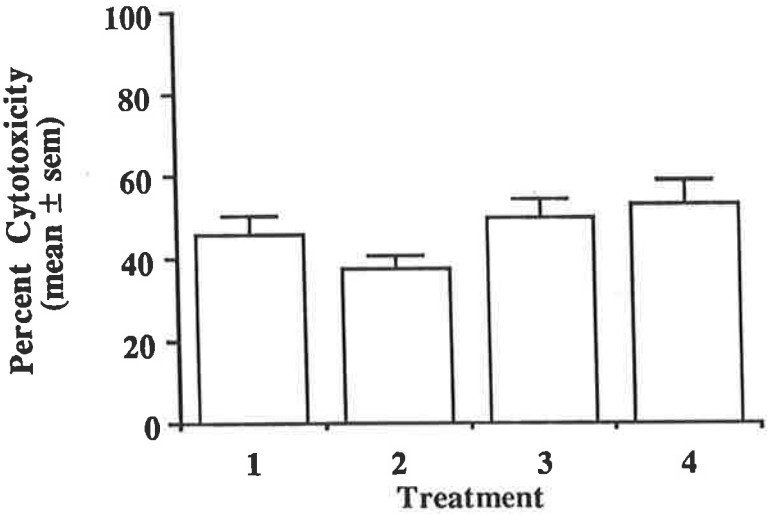
A


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1
2
3
4
Treatment

B


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1
2
3
4
Treatment

C


Percent Cytotoxicity
(mean ± sem)
100
80
60
40
20
0
1
2
3
4
Treatment

(Ashley and Kotlarski, 1982; La Posta *et al.*, 1982).

The most likely explanation is that the cytotoxic activity was due to NK cells, because NW fractionation has been reported to enrich for NK cells if they are present (Roder and Keissling, 1978). This would explain the lysis of untreated P388D1 and not P815 cells, since the latter have been shown to be resistant to killing by NK cells (Roder *et al.*, 1979).

5.2.5.4 F11RX pulsed P815 and P388D1 as the target cells for the detection of specific CTLs

The approach usually used to prepare Ag-pulsed targets suitable for lysis by Lyt2.2$^+$ T cells, is to use well defined Ags and Ag-pulsing methods designed to ensure that target cells were loaded with sufficient Ags presented in association with Class I MHC molecules to be lysed in an Ag-specific manner. Another approach which has been used with *Lm* is to use live organisms as Ags (Kaufmann *et al.*, 1986). Although Ag-pulsing was unlikely to ensure *Salmonella* Ag presentation in association with Class I MHC molecules unless live organisms were used, P388D1 and P815 cells were Ag-pulsed by incubating them with 20 and 100 µg/ml F11RX respectively overnight at 37°C in an atmosphere of 5% CO_2 to confirm that this was the case. The cells were washed and labelled with ^{51}Cr using the normal procedure before being mixed with NW IPCs at the standard ratios and incubated for 4 hours at 37°C. The amount of cytotoxicity was determined as previously described and Fig. 5.10 provides a summary of the data obtained for the 25:1 ratio of effector cells to target cells (representative of three separate experiments). Pulsing the target cells with F11RX did not enhance their susceptibility to lysis by the NW IPCs and did not alter their susceptibility to lysis in a lectin-mediated lysis assay, indicating that this approach was not appropriate for the detection of *Salmonella*-specific CTLs or that few of the CTLs detected in the lectin-mediated assays were specific for *Salmonella* Ags. The latter explanation seemed unlikely, since these CTLs were generated by immunization with L11RX. Since in the DTH system it was possible that Lyt2.2$^+$ T cells were not detected because of the excess

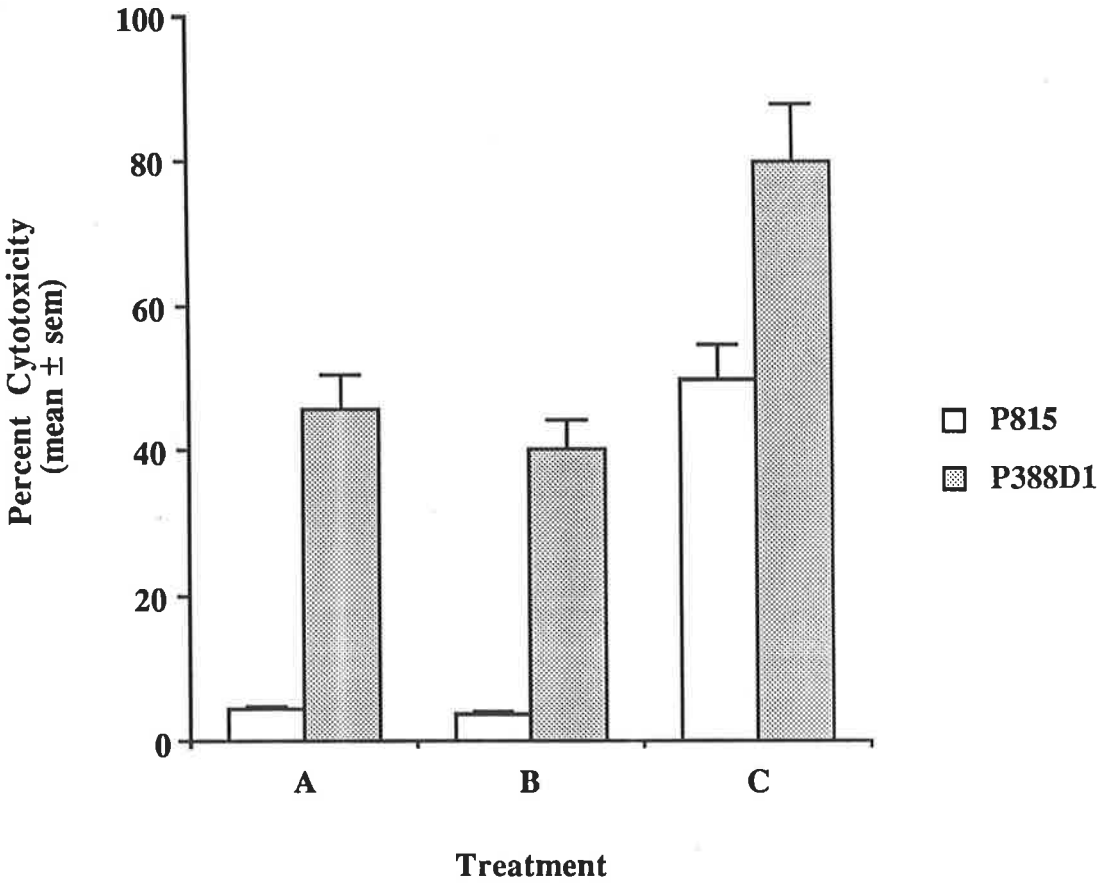


FIGURE 5.10 Comparison of lectin-mediated and Ag-specific cytotoxicity of NW IPCs

P815 and P388D1 were pulsed with F11RX overnight and then labelled with ^{51}Cr in the usual manner (B). An aliquot of both these target cell suspensions were also treated with ConA (C), to ensure that pulsing with the F11RX had not altered their ability to be lysed in the presence of lectin. Untreated P815 and P388D1 cells were also labelled with ^{51}Cr (A), to provide the negative controls. NW IPCs were incubated with these target cells (at a ratio of 25 effector cells : 1 target cell) and the amount of cytotoxicity measured 4 hours later. Data from one of three experiments carried out is provided to illustrate the percent cytotoxicity (mean ± sem) detected against the various target cells in all these experiments.

of L3T4$^+$ T cells, cells were cultured *in vitro* in an attempt to enrich the Lyt2.2$^+$ T cell subset.

5.2.6 *In vitro* expansion of CTLs

5.2.6.1 Purifying the Lyt2.2$^+$ T cells

With the knowledge that Lyt2.2$^+$ CTLs were induced by immunization with L11RX it is possible that if this population was enriched and/or activated *in vitro*, allbeit nonspecifically, it may provide a more sensitive means of detection of Ag-specific cells once the appropriate specific targets became available. Analysis of the NW D 4 IPCs on the FACScan after labelling the cells indirectly with immunofluorescence revealed that NW fractionation increased the homogeneity of the D 4 IPCs (Fig. 5.11 [A, B, E and F]), with the majority of these cells expressing Thy1.2$^+$ and Class I MHC$^+$ with very few Ia$^+$ cells. Fig. 5.11 [C and D] show the relative proportions of L3T4$^+$ and Lyt2.2$^+$ T cells in this population, indicating that there were slightly more L3T4$^+$ T cells than Lyt2.2$^+$ T cells present. Therefore, removal of L3T4$^+$ T cells would increase the relative numbers of Lyt2.2$^+$ T cells present and may enhance the detection of cytotoxic activity.

Incubation of NW IPCs with α-L3T4, α-Ia MoAbs and C was successful in enriching for Lyt2.2$^+$ T cells. FACScan analysis of these cells after labelling indirectly with immunofluorescence (Fig. 5.12 [A-F]) revealed that virtually all cells expressed Class I MHC molecules (F) and that the majority of the cells were Thy1.2$^+$ (B), Lyt2.2$^+$ (D), although a few contaminating Ia$^+$ and L3T4$^+$ cells did remain. The amount of background fluorescence of cells incubated with P/B/A followed by the SHAM-FITC is illustrated in Fig. 5.12 A. This procedure was used to prepare the cells used in the following studies.

5.2.6.2 Proliferation induced by IL 2 or ConA *in vitro*

To expand already active populations or to activate and expand resting

FIGURE 5.11 FACScan analysis of NW D 4 IPCs

NW D 4 IPCs were indirectly labelled with immunofluorescence using a variety of MoAbs. Fig.5.11 [A-F] illustrate the typical fluorescence intensities observed with cells incubated with: P/B/A (A), α-Thy1.2 (B), α-L3T4 (C), α-Lyt2.2 (D), α-Ia (E) or α-H-2K (F).

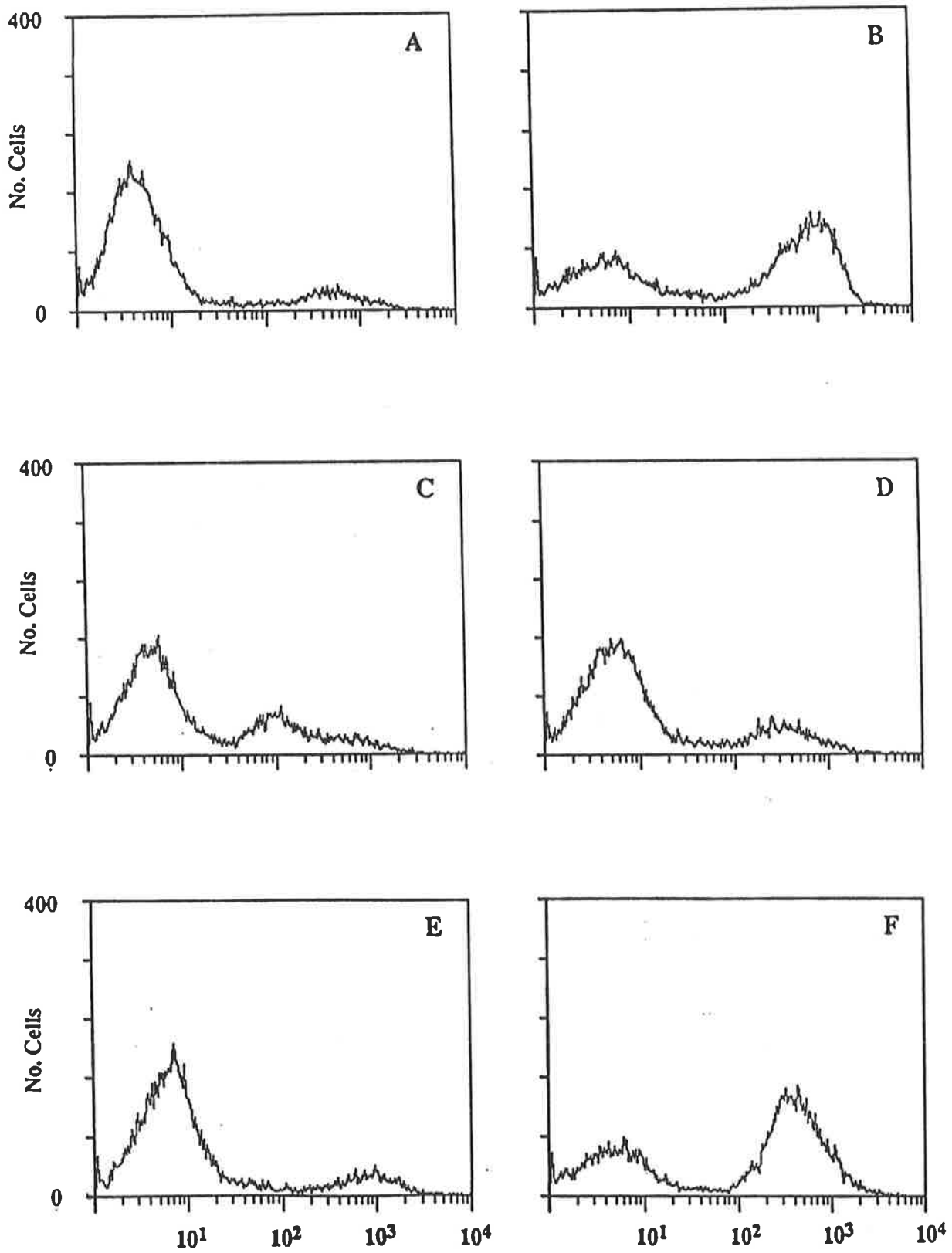
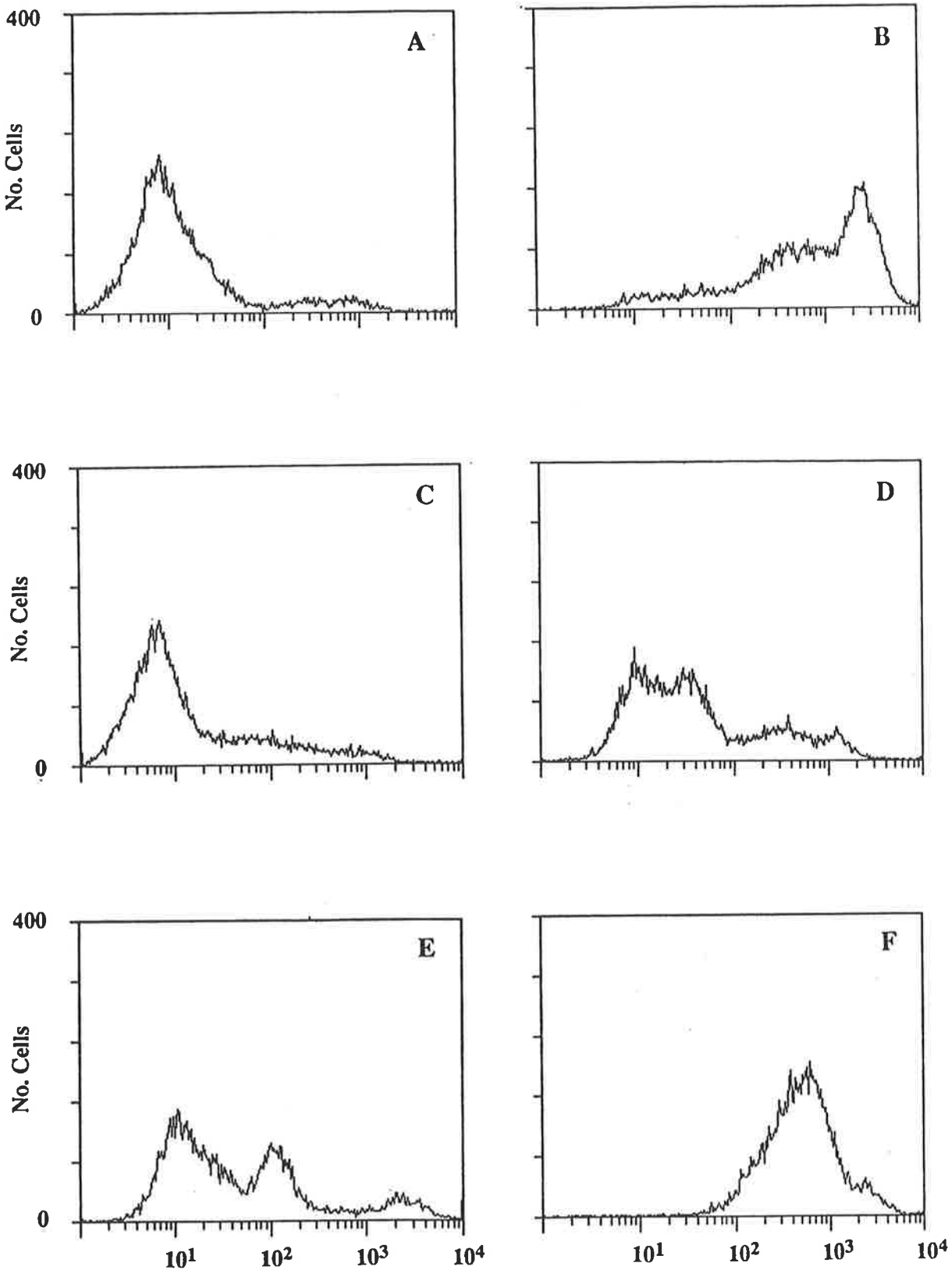

400
No. Cells
0
A
B
C
D
E
F
10^1
10^2
10^3
10^4
Fluorescence Intensity
Fluorescence Intensity

FIGURE 5.12 FACScan analysis of NW D 4 IPCs treated with α-L3T4, α-Ia + C

Lyt2.2$^+$ T cell enriched D 4 IPCs labelled with immunofluorescence using a range of primary MoAbs, were analysed on the FACScan. Using the indirect method for staining, the cells were first incubated with P/B/A (A), α-Thy1.2 (B), α-L3T4 (C), α-Lyt2.2 (D), α-Ia (E) or α-H-2K (F) and then with SHAM-FITC. An example of characteristic results are provided in Fig. 5.12 [A-F].


400
No. Cells
0
A
B
C
D
E
F
10^1
10^2
10^3
10^4
Fluorescence Intensity
Fluorescence Intensity

populations of T cells, D 4 IPCs enriched for Lyt2.2$^+$ T cells were incubated in bulk cultures (10-20 ml volumes) with IL 2 or ConA respectively, for 3 days at 37°C. The T cells proliferating in these cultures were characterized by incubating 4 replicate cultures (10^5 cells/well) of each type with the MoAbs and C for 1 hour at 37°C, prior to determining the amount of proliferation by pulsing with [^{3}H]-TdR for a further 4 hours at 37°C and measuring the [^{3}H]-TdR incorporated by these cells. The results were expressed as the cpm (mean ± sem) of counts obtained for each set of treated cells and very similar results were obtained on three separate occasions. One set of these results are presented in Table 5.1 and they indicate that the 3 day cultures contained proliferating L3T4$^+$ T cells and Lyt2.2$^+$ T cells.

This was confirmed by FACScan analysis. After 3 days of culture with either IL 2 or ConA, the cells were first incubated with P/B/A (A), α-Thy1.2 (B), α-L3T4 (C) or α-Lyt2.2 (D), followed by a 1 hour incubation with SHAM-FITC and analysis on the FACScan. Virtually identical results were obtained for cells cultured with either IL 2 or ConA and Fig. 5.13 [A-D] shows histograms of the fluorescence intensities of cells cultured with IL 2, which illustrate that approximately 80% of the cells present were Thy1.2$^+$ cells and only approximately 15% were L3T4$^+$ cells and 20% Lyt2.2$^+$ cells, suggesting that almost 40% of these cells expressed the Thy1.2$^+$, L3T4$^-$, Lyt2.2$^-$ phenotype (fewer double negative T cells were present in the ConA cultured cells). The identity of these cells was not investigated any further, but as mentioned earlier it was possible that they were γδ T cells which had been expanded as a result of the *in vitro* culture. [The possibility of induction of γδ T cells as a result of *Salmonella* infection will be discussed further in Chapter 7.]

5.2.6.3 Cytotoxic activity of *in vitro* cultured IPCs

To assess whether culture with IL 2 or ConA had altered the cytotoxic activity of these cells their lytic potential was assessed using the lectin-mediated assay. Cultured cells were mixed with untreated or ConA-treated ^{51}Cr-labelled P815 (at the standard ratios) and the cytotoxicity measured. Data which are typical of at least three

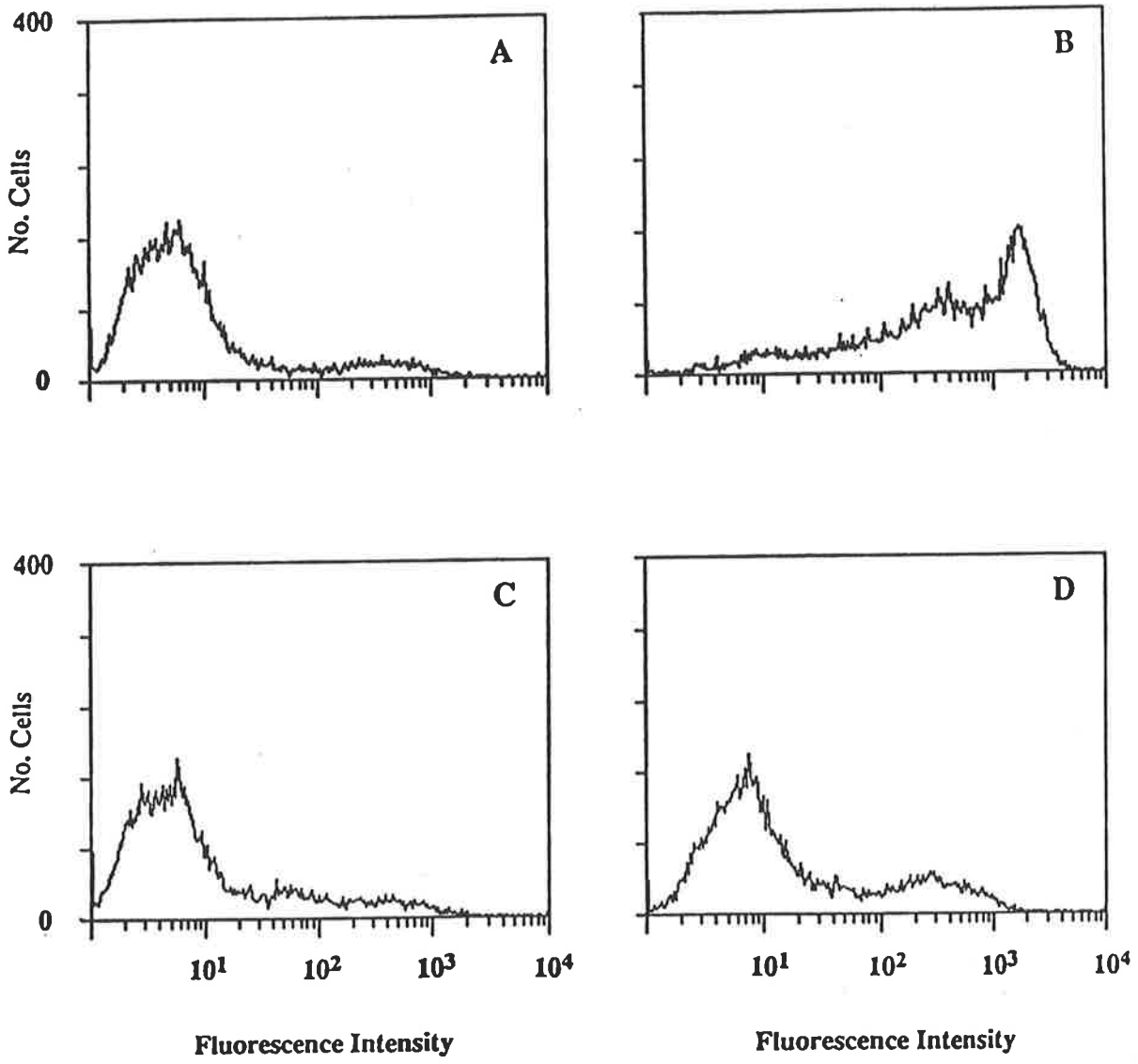
TABLE 5.1 Phenotypes of the proliferating cells in the Lyt2.2$^+$ enriched D 4 IPC suspensions

	[^{3}H]-TdR uptake (cpm ± sem) by Lyt2.2$^+$ enriched D4 IPCs after 3 days of culture with:	
Treatment*	ConA (1 μg/ml)	rIL 2 (10 units/ml)
None	214 790 ± 14 742	198 423 ± 6986
C alone	218 497 ± 19 336	187 454 ± 12 900
α-Thy1.2 + C	41 287 ± 3266	38 236 ± 1290
α-L3T4 + C	165 469 ± 11 514	98 672 ± 3788
α-Lyt2.2 + C	136 555 ± 4656	79 003 ± 9023
α-L3T4, α-Lyt2.2 + C	99289 ± 14 969	50 921 ± 2110

* 10^5 of the *in vitro* cultured cells were added to the wells of a 96-well flat-bottomed tray containing the various MoAbs (1/10 final dilution) and C (1/20 final dilution) [each set up in quadruplicate]. Trays were shaken and incubated for 1 hour at 37°C, before pulsing with [^{3}H]-TdR for a further 4 hours. The thymidine incorporated during this period was measured in the usual way and expressed as the mean cpm ± sem.

FIGURE 5.13 FACScan analysis of Lyt2.2$^+$ T cell enriched IPCs after *in vitro* culture

NW IPCs, treated with α-L3T4, α-Ia + C, were cultured with IL 2 or ConA for 3 days at 37°C, before being examined on the FACScan. Cultured cells were indirectly labelled with α-Thy1.2 (B), α-L3T4 (C) or α-Lyt2.2 (D) as the primary Abs (cells were also incubated with P/B/A as a negative control (A)). The data shown in Fig. 5.13 [A-D] are representative of the results obtained for cells cultured with IL 2.


400
No. Cells
0
A
B
C
D
10^1
10^2
10^3
10^4
Fluorescence Intensity
Fluorescence Intensity

separate experiments are provided in Fig. 5.14 [A and B]. *In vitro* culture of D 4 IPCs enriched for Lyt2.2$^+$ T cells slightly enhanced the cytotoxic activity towards lectin-treated and untreated P815 cells although it was unfortunate that the lysis of untreated P815 was particularly increased following culture with IL 2.

The presence of exogenous IL 2 and presumably some IL 2 endogenously produced by ConA activated T cells, not only increased the activity of CTLs but also activated a population of cells capable of lysing the tumour cells not treated with ConA. It is likely that these cells were lymphokine activated killer cells (LAKs), which can be expanded by *in vitro* culture of cells in the presence of significant amounts of IL 2 (eg. Gunji *et al.*, 1989; Longley *et al.*, 1989; Zychlinsky *et al.*, 1990). Addition of 10 units/ml of IL 2 to the cultures obviously provided sufficient levels of IL 2 for the stimulation of considerable LAK activity, with minimal amounts being provided during culture with ConA. Since γδ T cells have been shown to possess cytotoxic activity (Munk *et al.*, 1990) and such a large proportion of this population appeared to be double negative T cells, the possibility that these cells contributed to the cytotoxicity observed in these studies could not be dismissed.

5.2.7 *In vitro* cytotoxicity of ISCs

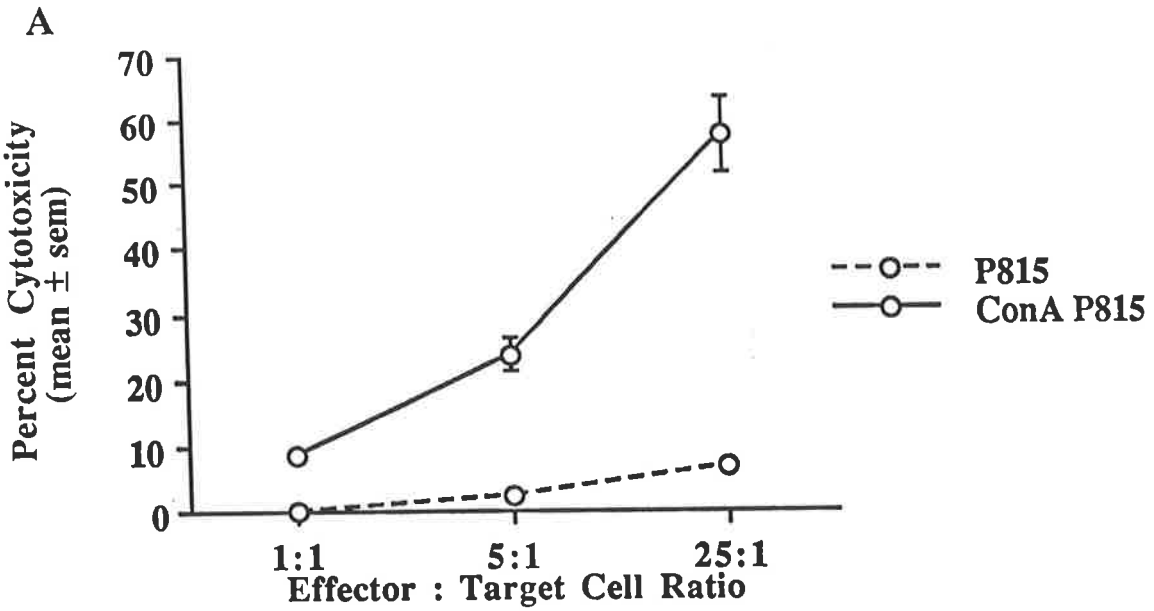
After ip immunization with L11RX both PCs and SCs have been shown to contain *Salmonella*-specific T cells capable of releasing LKs *in vitro* and mediating DTH reactivity in transfer experiments (Attridge and Kotlarski, 1985a and 1985b). Therefore, it was possible that Lyt2.2$^+$ CTLs may also be induced in the spleens of these mice. Initially, a few experiments were carried out to measure the lectin-mediated cytotoxicity of SCs from mice immunized with L11RX ip 4-6 days previously (D 4-6 ISCs), using unfractionated and NW fractionated ISCs (U ISCs and NW ISCs) and ConA-treated and untreated ^{51}Cr-labelled P815. Unlike the D 4 IPCs, U ISCs and NW ISCs exhibited a maximum of only approximately 8% cytotoxicity against both untreated and ConA-treated ^{51}Cr-labelled P815. These data suggested that ip immunization with L11RX either did not induce/generate a CTL population in the spleen, or activated CTLs

FIGURE 5.14 Cytotoxicity of *in vitro* cultured suspensions of D 4 IPCs enriched for Lyt2.2$^+$ T cells

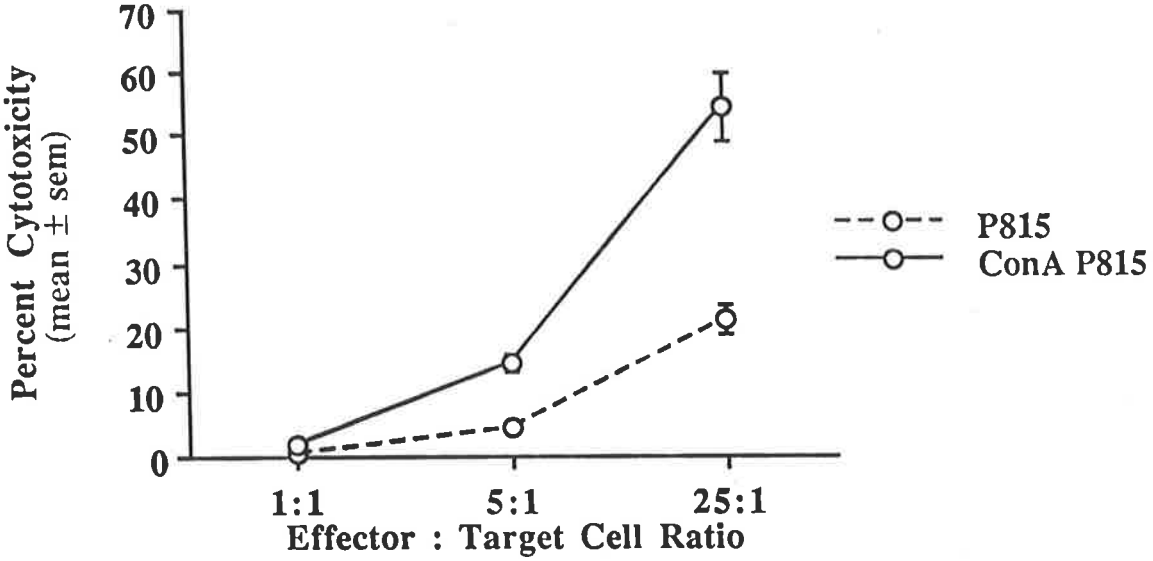
NW α-L3T4, α-Ia + C IPCs cultured with IL 2 or ConA for 3 days at 37°C were assessed for their capacity to kill lectin-treated or untreated ^{51}Cr-labelled P815. The amount of ^{51}Cr released after 4 hours culture of effectors and targets at ratios of 1:1, 5:1 and 25:1, was measured using the gamma counter. Data are presented as the percent cytotoxicity (mean ± sem) detected using ConA-induced (A) and IL 2-induced blasts (B).

The specificity of the cytotoxic responses was confirmed since "LC5 P815" was lysed no more than untreated P815 when ConA stimulated normal spleen cells were used as effector cells. When cells from immunized mice were stimulated *in vitro* with ConA before being used as effectors, significant lysis of the infected target cells was observed only when the effectors were obtained from mice following a secondary *Salmonella* infection.

A


Percent Cytotoxicity
(mean ± sem)
70
60
50
40
30
20
10
0
1:1
5:1
25:1
Effector : Target Cell Ratio
P815
ConA P815

B


Percent Cytotoxicity
(mean ± sem)
70
60
50
40
30
20
10
0
1:1
5:1
25:1
Effector : Target Cell Ratio
P815
ConA P815

that represented only a very minor population which could not be detected, and/or that a minor population of CTLs was induced by infection but required activation to become cytotoxic. Since DTH effector cells can be detected in the spleens of mice infected with L11RX ip, it seemed reasonable to favour the latter possibility and to investigate whether expansion and/or activation of the ISCs *in vitro* would enable the detection of CTLs in these populations, since only activated cells express cytolytic function.

5.2.8 *In vitro* expansion of Lyt2$^+$ T cells from ISCs

5.2.8.1 *In vitro* culture of U ISCs with ConA

Since ISCs did not require removal of adherent cells to proliferate in response to ConA, it was of interest to determine whether NW fractionation and enrichment of the Lyt2.2$^+$ T cells prior to culture with ConA was necessary for the selective expansion and/or activation of the Lyt2.2$^+$ CTLs. [Preliminary experiments indicated that like the IPCs, culturing ISCs with IL 2 resulted in the activation of nonspecific cytotoxic cells (data not shown) and was therefore not used in these studies.] Accordingly, the experiment described above was repeated three times using U ISCs and U ISCs which had been pretreated with either α-L3T4 and C or α-Lyt2.2 and C using the one step procedure. Since past experience had indicated that one treatment with α-L3T4 and C was not sufficient to remove all L3T4$^+$ cells, the MoAb and C treatments were carried out twice in an attempt to improve the efficiency of depletion of the L3T4$^+$ and Lyt2.2$^+$ subsets of T cells. Untreated SCs from a normal, unimmunized mouse (NSCs; although not enriched for the T cell subsets) were also cultured with ConA to compare the effect of culturing on unprimed T cells.

Examination of the proliferative responses of these populations revealed that *in vitro* culture with ConA induced both T cell subsets in the ISC and NSC populations to proliferate (Tables 5.2 [A and B]). Two treatments of U ISCs with α-L3T4 and C prior to culture did not remove all L3T4$^+$ T cells, as proliferating cells of both L3T4$^+$ and Lyt2.2$^+$ phenotype were detected in these cultures, although the number

TABLE 5.2 A Phenotypes of the cells proliferating in suspensions of ISCs cultured with ConA

	[^{3}H]-TdR uptake (cpm ± sem) by ISCs after 3 days of culture with 1 μg/ml ConA:		
	Pretreatment#:		
Treatment*	Culture Medium	α-L3T4 + C	α-Lyt2.2 + C
None	35 996 ± 199	64 561 ± 967	59 038 ± 1548
C	30 853 ± 1217	63 610 ± 5151	58 223 ± 1491
α-Thy1.2 + C	2218 ± 72	2428 ± 55	3865 ± 62
α-L3T4 + C	18 785 ± 317	41 050 ± 889	9230 ± 392
α Lyt2.2 + C	19 836 ± 345	22 383 ± 1171	58 758 ± 2385
α-L3T4, α-Lyt2.2 + C	3560 ± 56	4751 ± 194	9425 ± 380

Prior to culture with ConA, D 4 ISCs were subjected to two treatments with either α-L3T4 + C or α-Lyt2.2 + C (using the one step procedure), or left untreated.

* Each cultured ISC blast population was treated (in quadruplicate) as indicated for 1 hour at 37°C before being pulsed with [^{3}H]-TdR. The cpm (mean ± sem) of [^{3}H]-TdR incorporated was calculated for each population.

TABLE 5.2 B Phenotype of the proliferating cells of a NSC population cultured with ConA for 3 days

Treatment*	[^{3}H]-TdR uptake (cpm ± sem) by NSCs after 3 days of culture with 1 μg/ml ConA
None	85 632 ± 8309
C	90 326 ± 4396
α-Thy1.2 + C	1094 ± 238
α-L3T4 + C	48 633 ± 2761
α-Lyt2.2 + C	31 824 ± 1096
α-L3T4, α-Lyt2.2 + C	3889 ± 288

* ConA cultured NSCs were incubated with the T cell specific MoAbs and C for 1 hour at 37°C in a 96-well flat-bottomed tray prior to being pulsed with [^{3}H]-TdR for a further 4 hours. Each treatment was carried out in quadruplicate and results are expressed as the cpm (mean ± sem) of ^{3}H-thymidine incorporated by each population.

of L3T4$^+$ T cells had been reduced. [It is possible that most had been removed and that selective expansion had occurred during culture.] In this population L3T4$^+$ were responsible for approximately 30% of the proliferation, whereas approximately 50% of the proliferating cells of the untreated ISC population were L3T4$^+$. Two pretreatments with α-Lyt2.2 and C did deplete all Lyt2.2$^+$ T cells, because none of the proliferation detected in this population could be attributed to the Lyt2.2$^+$ T cells. It is interesting to note that the magnitude of responses induced in the two pretreated populations was comparable, indicating that depletion of a particular T cell subset did not affect the overall amount of proliferation induced. The finding that Lyt2.2$^+$ T cells, but not L3T4$^+$ T cells, could be removed from the population may reflect the fact that there are less Lyt2.2$^+$ T cells than L3T4$^+$ T cells in the spleen, making it easier to deplete these cells.

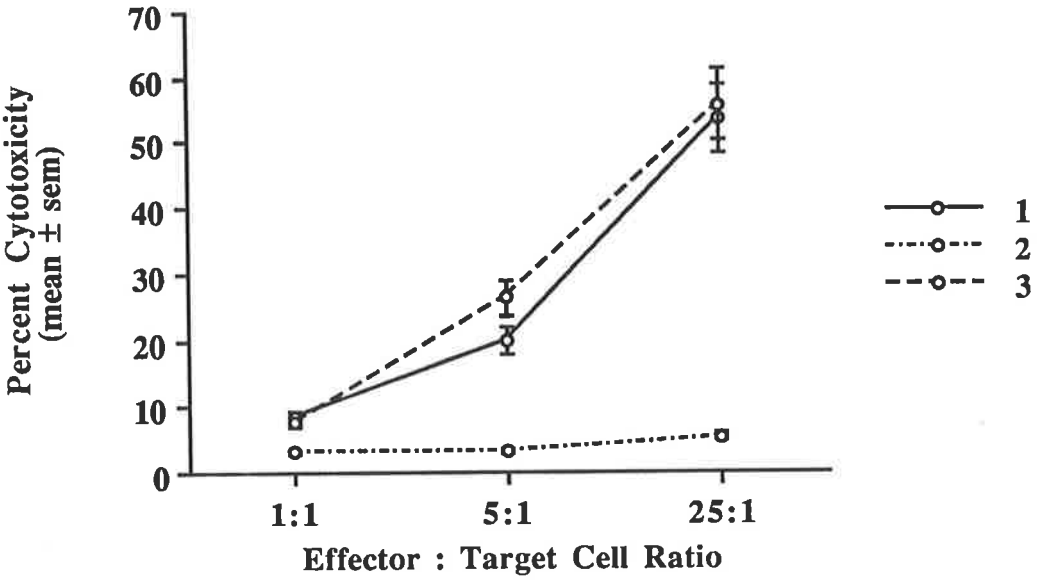
5.2.8.2 Cytotoxicity of U ISCs cultured with ConA *in vitro*

The standard lectin-mediated cytotoxicity assays using untreated and ConA-treated ^{51}Cr-labelled P815 were used to assess the amount of cytotoxicity induced by culturing U ISCs, and U ISCs enriched for L3T4$^+$ or Lyt2.2$^+$ T cells with ConA for 3 days. Since similar proliferative responses were recorded for the NSCs and ISCs after culture with ConA, NSCs cultured with ConA were also examined for their cytotoxic activity and the phenotype of the cytotoxic cells detected in the cultured NSC suspension was established by treating the cells with MoAbs and C prior to adding them to the target cells (using only the 25:1 effector to target cell ratio). In three successive experiments the percent cytotoxicity (mean ± sem) was calculated for each effector cell/target cell combination and Fig. 5.15 [*(i)* and *(ii)*] shows the results from a typical experiment. Clearly, *in vitro* culture of both U ISCs and NSCs with ConA induced populations of cells with considerable cytotoxic activity (approximately 60%). These experiments also confirmed that Lyt2.2$^+$ T cells were responsible for all cytotoxicity induced by *in vitro* culture of both ISCs and NSCs with ConA. Pretreatment of ISCs with α-L3T4 and C did not reduce the amount of cytotoxicity induced by culturing with ConA and the amount of

FIGURE 5.15 Lectin-mediated cytotoxicity of ConA-cultured SCs

D 4 ISCs (1) and populations enriched for either L3T4$^+$ (2) or Lyt2.2$^+$ (3) T cells were cultured with ConA for 3 days at 37°C in an atmosphere of 5% CO_2 and the cytotoxicity of these blasts was assessed using the standard technique. ConA-blasts were incubated with ^{51}Cr-labelled P815 at ratios of 1:1, 5:1 and 25:1 and the amount of cytotoxicity after 4 hours at 37°C was measured and data characteristic of several experiments are provided, showing the percent cytotoxicity (mean ± sem) of these populations (Fig. 5.15 *(i)*). Control NSCs cultured with ConA were also treated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) and their ability to lyse ConA-treated ^{51}Cr-labelled P815 (at a ratio of 25:1) was measured in the usual manner and a typical result is presented in Fig. 5.15 *(ii)*.

(i)


Percent Cytotoxicity
(mean ± sem)
70
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5:1
25:1
Effector : Target Cell Ratio
1
2
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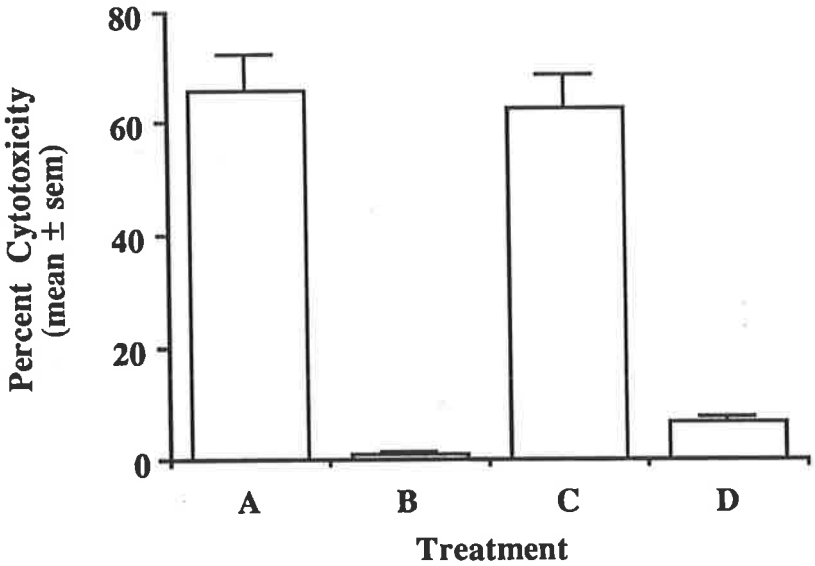
(ii)


Percent Cytotoxicity
(mean ± sem)
80
60
40
20
0
A
B
C
D
Treatment

cytotoxic activity detected in cultures of ISCs pretreated with α-Lyt2.2 and C was negligible (10% maximum). L3T4$^+$ T cells with cytotoxic activity were not detected in any of these experiments. The lysis of untreated P815 by all effector cell populations was minimal (in the range of 3-5%). Because *in vitro* culture of both ISCs and NSCs with ConA induced activation of Lyt2.2$^+$ T cells with very similar levels of cytotoxic activity these studies provided no evidence that *Salmonella* immunization induced Ag-specific Lyt2.2$^+$ CTLs in the spleen.

5.2.8.3 Summary

This series of experiments demonstrated that within a few days of ip immunization of mice with L11RX, Lyt2.2$^+$ CTLs able to mediate lectin-mediated lysis were present in the peritoneal cavity but not in the spleen. Following *in vitro* culture of the IPCs with ConA or IL 2 cytotoxic activity was maintained and even slightly enhanced, but was accompanied by an increase in nonspecific cytotoxic activity, particularly when IL 2 was added to the culture medium. IL 2 did not maintain or expand the activity of the cells in a selective way and also possibly expanded a population of "LAK-type" cells (and possibly some γδ T cells). Lyt2.2$^+$ T cell-mediated cytotoxic activity could be induced in the ISCs by *in vitro* culture with ConA, but the significance of these findings was difficult to interpret because *in vitro* culture of NSCs with ConA for the same length of time also generated Lyt2.2$^+$ CTLs. While it was possible that at least some of the CTLs generated by *in vitro* culture of the ISCs with ConA were *Salmonella*-specific, whereas those detected in cultures of NSCs were not, this could not be confirmed because of the lack of Ag-specific target cells which were not available at this time.

5.2.9 Analysis of the functional capacities of T cells after secondary challenge with *Salmonella*

5.2.9.1 Introduction

Outlined in Chapter 3 is a series of preliminary experiments which were carried out to assess the effect of a second dose of *Salmonella* given to mice primed with 10^5 L11RX on cell yields and the responsiveness of these cells to *Salmonella* Ags. Mice were given either $3x10^4$ LC5 or $8x10^6$ L11RX ip 3 or 6 weeks (respectively) after the primary ip immunization with 10^5 L11RX. As a result of the secondary infection the SC and particularly the PC yields increased, although the ability of these cells to proliferate and release IL 2 *in vitro* were very similar to the responses observed by cells obtained from mice receiving only a single dose of L11RX. The following sections present data on a more detailed analysis of the phenotypes of the cells induced to proliferate following secondary challenge and a characterization of the cells able to mediate DTH reactivity. The induction of CTL activity following secondary challenge was also investigated.

5.2.9.2 Characterization of the T cells induced to proliferate *in vitro*

PCs and SCs were harvested from mice 7, 14 and 21 days after receiving a secondary challenge of either LC5 or L11RX (LC5 or L11RX 2° IPCs and ISCs). 2° NW IPCs, 2° U ISCs or 2° NW ISCs were cultured *in vitro* for 3 days with F11RX to stimulate specific T cells or the nonspecific T cell mitogen, ConA to ensure T cell responsiveness and the phenotype of the proliferating cells was determined in the usual way. Interestingly, the results were the same for cells taken at any timepoint after a secondary challenge with either L11RX or LC5. The amounts [^{3}H]-TdR incorporated by cells harvested 14 days after challenge are provided in Tables 5.3 and 5.4. The proliferative responses of L11RX or LC5 2° NW IPCs to ConA were similar, involving both L3T4$^+$ T cells and Lyt2.2$^+$ T cells (Table 5.3), although there was an indication that L3T4$^+$ T cells contributed less to the proliferation observed for the LC5 2° IPCs. The responses to F11RX, however, were somewhat different. L11RX 2° NW IPCs

TABLE 5.3 Characterization of the proliferating cells present in the L11RX and LC5 2° NW IPCs after culture with F11RX or ConA

		[^{3}H]-TdR uptake (cpm ± sem) by 2° NW IPCs# after 3 days of culture with:	
IPCs#	Treatment*	1 µg/ml F11RX	1 µg/ml ConA
L11RX 2°	None	123 908 ± 1336	175 039 ± 14 816
	C alone	131 435 ± 8050	141 228 ± 3686
	α-Thy1.2 + C	12 677 ± 1240	15 809 ± 1591
	α-L3T4 + C	54 083 ± 5400	52 566 ± 2788
	α-Lyt2.2 + C	129 341 ± 2008	108 573 ± 3937
	α-L3T4, α-Lyt2.2 + C	37 000 ± 478	35 015 ± 2081
LC5 2°	None	265 452 ± 9440	212 612 ± 5301
	C alone	252 973 ± 2920	192 747 ± 5919
	α-Thy1.2 + C	32 173 ± 2410	24 541 ± 2951
	α-L3T4 + C	76 973 ± 2109	139 847 ± 3573
	α-Lyt2.2 + C	173 889 ± 14 784	132 637 ± 3108
	α-L3T4, α-Lyt2.2 + C	42 130 ± 3772	49 293 ± 4315

$2x10^5$ L11RX and LC5 2° NW IPCs were each cultured in 96-well flat-bottomed trays in the presence of ConA or F11RX for 3 days at 37°C. After 3 days, the amount of proliferation was determined by measuring the incorporation of [^{3}H]-TdR during the final 4 hours of culture.

* Before pulsing with [^{3}H]-TdR, four replicate cultures of these cells were treated with the various T cell-specific MoAbs and C for 1 hour at 37°C. Results are expressed as the amount of [^{3}H]-TdR incorporated [cpm (mean ± sem)] by each cell suspension and data representative of a set of five replicate experiments are provided.

TABLE 5.4 Characterization of the proliferating cells present in L11RX and LC5 2° NW ISCs after culture with F11RX or ConA

		[^{3}H]-TdR uptake (cpm ± sem) by 2° NW ISCs# after 3 days of culture with:	
ISCs#	Treatment*	1 µg/ml F11RX	1 µg/ml ConA
L11RX 2°	None	109 638 ± 6976	324 113 ± 14 238
	C alone	90 639 ± 3164	255 760 ± 5443
	α-Thy1.2 + C	37 998 ± 557	27 247 ± 1864
	α-L3T4 + C	58 071 ± 5957	73 977 ± 13 658
	α-Lyt2.2 + C	70 983 ± 2096	159 006 ± 4713
	α-L3T4, α-Lyt2.2 + C	40 127 ± 1959	32 482 ± 2537
LC5 2°	None	42 758 ± 3204	242 298 ± 3978
	C alone	40 193 ± 3735	255 228 ± 6805
	α-Thy1.2 + C	14 974 ± 1176	21 365 ± 1568
	α-L3T4 + C	24 360 ± 5221	186 300 ± 5215
	α-Lyt2.2 + C	28 886 ± 2007	149 605 ± 1236
	α-L3T4, α-Lyt2.2 + C	15 701 ± 1528	31 603 ± 1918

$2x10^5$ L11RX and LC5 2° NW ISCs were cultured with F11RX or ConA in 96-well flat-bottomed trays for 3 days at 37°C, after which the amount of proliferation was determined by measuring the uptake of [^{3}H]-TdR (cpm, mean ± sem) during the last 4 hours of culture.

* Prior to pulsing the cells with [^{3}H]-TdR, appropriate dilutions of the various MoAbs and C were added (in quadruplicate) and the trays incubated for 1 hour at 37°C. Data representative of a set of five replicate experiments are presented.

proliferated well in response to F11RX and, not surprisingly, the cells responsible for the bulk of this proliferation expressed the L3T4$^+$ phenotype. The proliferation induced by F11RX in the LC5 2° NW IPCs was comparable. However, it appeared to be mediated by both L3T4$^+$ T cells and Lyt2.2$^+$ T cells, as treatment with either α-L3T4 or α-Lyt2.2 + C significantly reduced the proliferation and when the two MoAbs were used together a further decrease was observed.

As expected, the proliferative responses of U ISCs and NW ISCs to ConA involved both L3T4$^+$ T cells and Lyt2.2$^+$ T cells and representative data for NW ISCs are provided in Table 5.4. Again, there appeared less involvement of the L3T4$^+$ cells in the proliferative response of T cells obtained from mice given a secondary challenge of LC5. Similar to the responses observed in the SCs from mice receiving only a single dose of L11RX, the 2° ISCs with and without NW fractionation exhibited very inconsistent responses to F11RX. Although no firm conclusions could be drawn from these data, it is interesting to note that when the ISCs did proliferate in response to F11RX, both L3T4$^+$ and Lyt2.2$^+$ T cells were responsible for this proliferation. Depletion with MoAbs and C never completely abrogated the proliferative responses induced by either F11RX or ConA and treatment with both α-L3T4 and α-Lyt2.2 + C did not reduce the responses to the same extent that α-Thy1.2 + C treatment did, particularly in the IPC populations. These data suggested that a population of cells which express the Thy1.2$^+$, L3T4$^-$, Lyt2.2$^-$ phenotype were responsible for this proliferation. It is possible that γδ T cells were responsible for this proliferation and the likelihood of this is discussed in the final Chapter. The fact that treatment with α-Thy1.2 + C could not completely remove the proliferation induced suggested either that a non-T cell population (possibly B cells) was proliferating or simply that the numbers of proliferating cells in these assays were too large to be destroyed by the treatment used.

Thus, in summary, following secondary *Salmonella* infection, particularly with LC5, there was an increased proliferative activity of Lyt2.2$^+$ T cells in the IPCs which was sometimes also seen with the ISCs. It was surprising to find that in the presence of a killed Ag Lyt2.2$^+$ T cells were induced to proliferate (the implications of

this will be discussed later). It was possible that more direct evidence for the existence of Ag-specific Lyt2.2$^+$ T cells in this population could be obtained using live organisms as the Ag. As indicated previously, a system in which live organisms can be used to elicit cellular responses is the transfer of DTH.

5.2.9.3 Transfer of DTH reactivity with L11RX and LC5 2° IPCs and ISCs

PCs and SCs were harvested from mice 7, 14 and 21 days after receiving a second dose of L11RX or LC5 and the T cells partially purified by fractionation on NW columns. Mixtures of 10^6 2° NW IPCs or NW ISCs with 2.5 μg F11RX, 10^5 L11RX or $5x10^3$ LC5 were each injected sc in 50 μl volumes into the left hind footpads of groups of 3-4 normal F1 mice and the size of both hind footpads measured 24 and 48 hours later. The percent increase in footpad size (mean ± sem) was calculated for each group in the usual way, to allow comparison of the responses obtained from each group. This experiment was repeated at least twice at all three timepoints and no significant differences in the abilities of PCs and SCs to transfer DTH over this time period were observed. Furthermore, there appeared to be no difference between the responses transferred with cells from mice receiving either L11RX or LC5 as the secondary challenge. For this reason, only the data showing the responses transferred with LC5 2° NW IPCs and ISCs harvested 14 days after challenge are provided (Fig. 5.16 [*(i)* and *(ii)*]).

Measurement of the footpads at 24 hours revealed that all three Ags were able to elicit significant DTH responses when mixed with the 2° NW IPCs or ISCs. Responses transferred by the ISCs (approximately 20% maximum) were always slightly smaller than those by IPCs (30% maximum). The amount of swelling induced by F11RX was always greater than the responses to either L11RX or LC5 (although there were variations in the maximal responses induced by these Ags between experiments). These data are comparable to those obtained using cells from primary immunized mice, indicating that a secondary infection of *Salmonella* evidently did not enhance the ability of PCs or ISCs to transfer DTH.

FIGURE 5.16 Transfer of DTH reactivity using IPCs and ISCs from mice given a secondary challenge of LC5 14 days earlier

PCs and SCs harvested from mice 14 days after they had received a secondary challenge of LC5 were NW fractionated (LC5 2° NW IPCs and ISCs) and their ability to transfer DTH was investigated. A mixture of 10^6 LC5 2° NW IPCs or ISCs with either 2.5 μg F11RX, 10^5 L11RX or $5x10^3$ LC5 were each injected in a 50 μl volume into the left hind footpads of groups of 3 unimmunized mice. The percent increase in footpad size (mean ± sem) was determined for each group 24 hours later and results of one experiment using LC5 2° NW IPCs and ISCs are shown in Fig. 5.16 [*(i)* and *(ii)*] (respectively). The various groups of mice received the following:

A: Cells + F11RX

B: Cells + L11RX

C: Cells + LC5

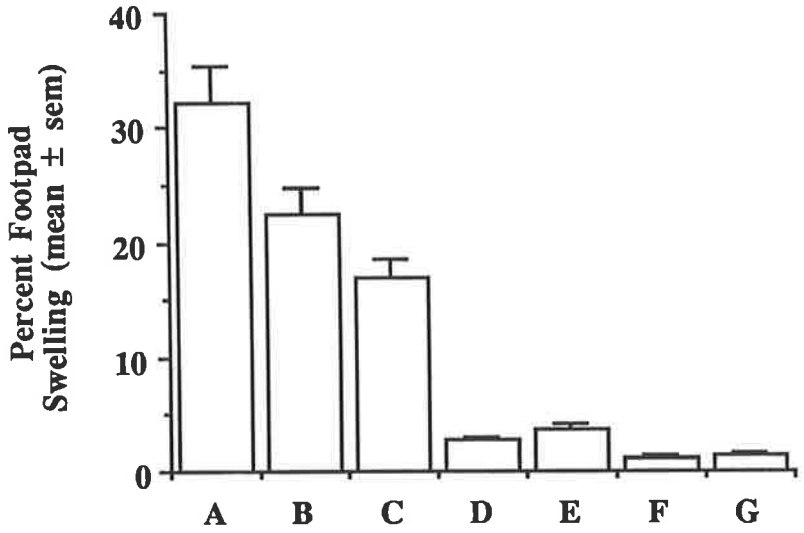
D: F11RX

E: L11RX

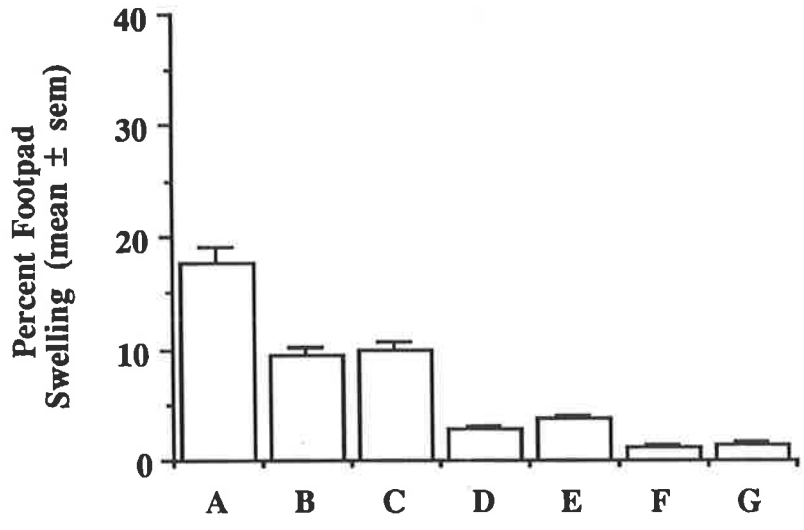
F: LC5

G: Cells

(i)


Percent Footpad
Swelling (mean ± sem)
40
30
20
10
0
A
B
C
D
E
F
G

(ii)


Percent Footpad
Swelling (mean ± sem)
40
30
20
10
0
A
B
C
D
E
F
G

The phenotype of the T cells responsible for the DTH responses obtained in the previous set of experiments was established by using the standard approach involving treatment with MoAbs and C (or C alone). Results using cells harvested from mice 1, 2 or 3 weeks after secondary challenge with either L11RX or LC5 were virtually identical and reproducible. Accordingly, only one set of results using L11RX 2° IPCs and ISCs obtained 14 days after secondary challenge are presented (Fig. 5.17 [*(i)* and *(ii)*], respectively).

It was clear that regardless of whether live or killed Ags were used to elicit DTH, only Class II restricted L3T4$^+$ T cells were involved in the response detected, again implying that either none, or very few, Class I restricted Lyt2$^+$ T cells capable of mediating DTH reactivity were induced by secondary *Salmonella* infections.

5.2.9.4 Summary

Following secondary infection with *Salmonella*, particularly C5, there was enhanced proliferative activity of Ag-specific Lyt2.2$^+$ T cells in both the PC and SC populations. However, no Lyt2.2$^+$ T cells capable of transferring DTH reactivity were detected, even when live organisms were used as the eliciting Ags. This suggested either that no Class I MHC restricted DTH effector cells were induced by infection with *Salmonella* or that, although Ag-specific Lyt2.2$^+$ T cells were induced to proliferate, Ag-specific Lyt2.2$^+$ DTH effector T cells may not have been successfully induced in this system because Ag was presented inappropriately or possibly because the response was masked by a larger Class II restricted response. However, the Lyt2.2$^+$ T cell response, although nonspecific, which was readily detected was cytotoxic activity.

5.2.10 *In vitro* cytotoxicity of L11RX and LC5 2° IPCs and ISCs

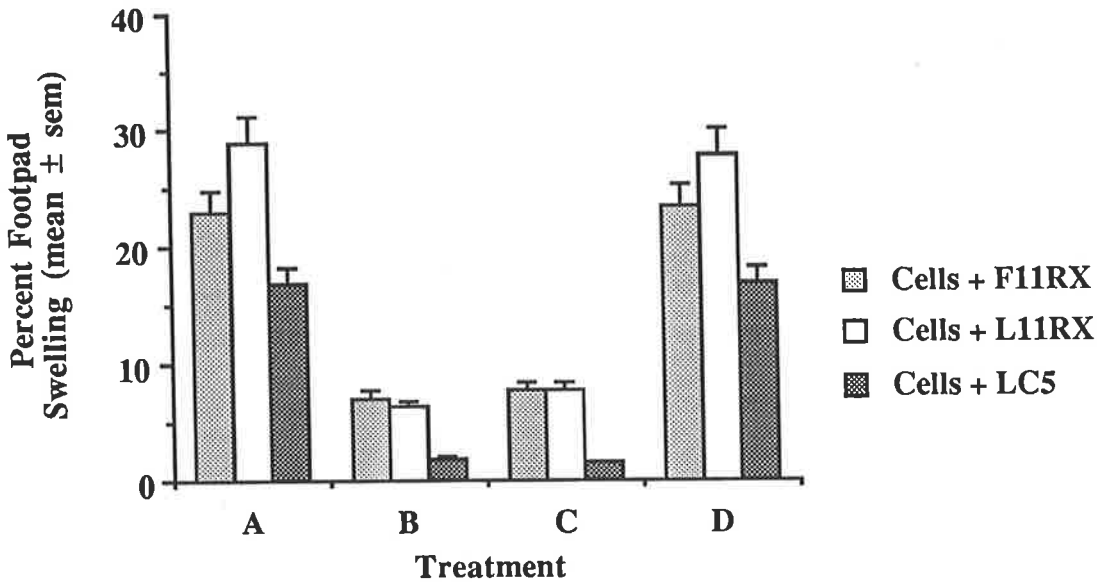
5.2.10.1 Lectin-mediated cytotoxicity of 2° NW IPCs

Because the proliferative activity of Lyt2.2$^+$ T cells was enhanced by secondary challenge with *Salmonella* the effect on the activity of Lyt2.2$^+$ CTLs already

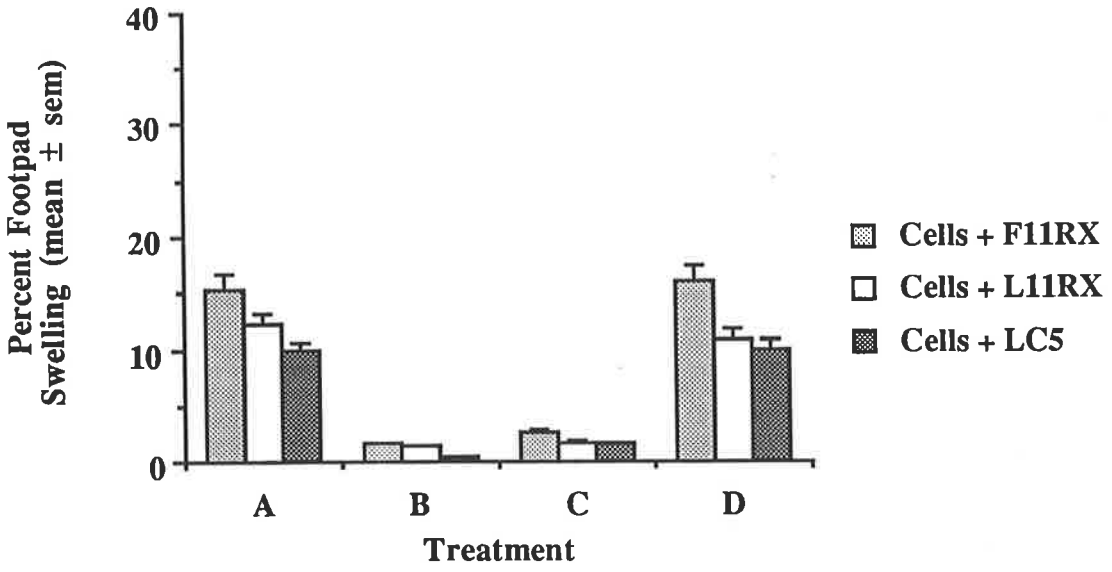
FIGURE 5.17 Phenotype of the DTH effectors in the 2° NW IPC and NW ISC suspensions

2° NW IPCs and ISCs treated with either C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) were each mixed with F11RX, L11RX and LC5, to ensure that in a 50 μl volume, 10^6 cells and the various Ags could be transferred to the footpads of normal, unimmunized mice. Each mixture of cells + Ag was injected into groups of 3 mice and the percent footpad swelling (mean ± sem) at 24 hours was calculated for each group. The results from one experiment using D 14 L11RX 2° NW IPCs and ISCs are shown in Fig. 5.17 [*(i)* and *(ii)*] respectively.

(i)


Percent Footpad Swelling (mean ± sem)
40
30
20
10
0
A
B
C
D
Treatment
Cells + F11RX
Cells + L11RX
Cells + LC5

(ii)


Percent Footpad Swelling (mean ± sem)
40
30
20
10
0
A
B
C
D
Treatment
Cells + F11RX
Cells + L11RX
Cells + LC5

present after primary immunization was investigated. Since peak CTL activity of NW IPCs was detected 5 days after immunization with L11RX ip, the cytotoxic activity of 2° NW IPCs was measured at days 1-5 and days 7, 14 and 21 after a second dose of *Salmonella*, using the standard lectin-mediated assay system.

At all timepoints examined, the CTL activity was measured at least twice and the results shown in Fig. 5.18, expressed as as the percent cytotoxicity (mean ± sem), include representative data from several experiments. For all populations the amount of killing of the untreated ^{51}Cr-labelled P815 was negligible (ranging from 1-5%, with a mean of 3.5 ± 0.9). Even one day after secondary challenge with either LC5 or L11RX, NW IPC populations showed considerable cytotoxic activity towards the ConA-treated targets (45-60%). This activity was significantly elevated above that of NW IPCs from mice immunized with L11RX ip 3 and 6 weeks previously (15% and 8%, respectively) and equivalent to that observed at the peak of response (day 5) after primary immunization (Fig. 5.7 *(i)*). This increased cytotoxic activity was maintained at this level for up to 7 days after secondary challenge and decreased slowly thereafter, as indicated by the decreasing levels of activity by 14 and 21 days after challenge. Undoubtedly, receiving a second dose of *Salmonella* boosted the cytotoxicity of NW IPCs and this increased activity appeared earlier and was maintained for a longer period of time than the peak activity of primary immunized NW IPCs. (Whether LC5 or L11RX was given as the second dose did not appear to make any difference to the response induced.)

5.2.10.2 Analysis of the phenotype of the CTLs in the 2° NW IPCs

The standard procedure of treatment with MoAb and C was used to characterize the cells responsible for the cytotoxicity of LC5 and L11RX 2° NW IPCs. Reproducible results were obtained at least twice for each timepoint and they indicated that the cytotoxic cells in both populations expressed the Lyt2.2$^+$, Thy1.2$^+$ phenotype, as treatment with α-Thy1.2 and C or α-Lyt2.2 and C removed all the lectin-mediated cytotoxic activity. Fig. 5.19 shows the data obtained using NW IPCs harvested from

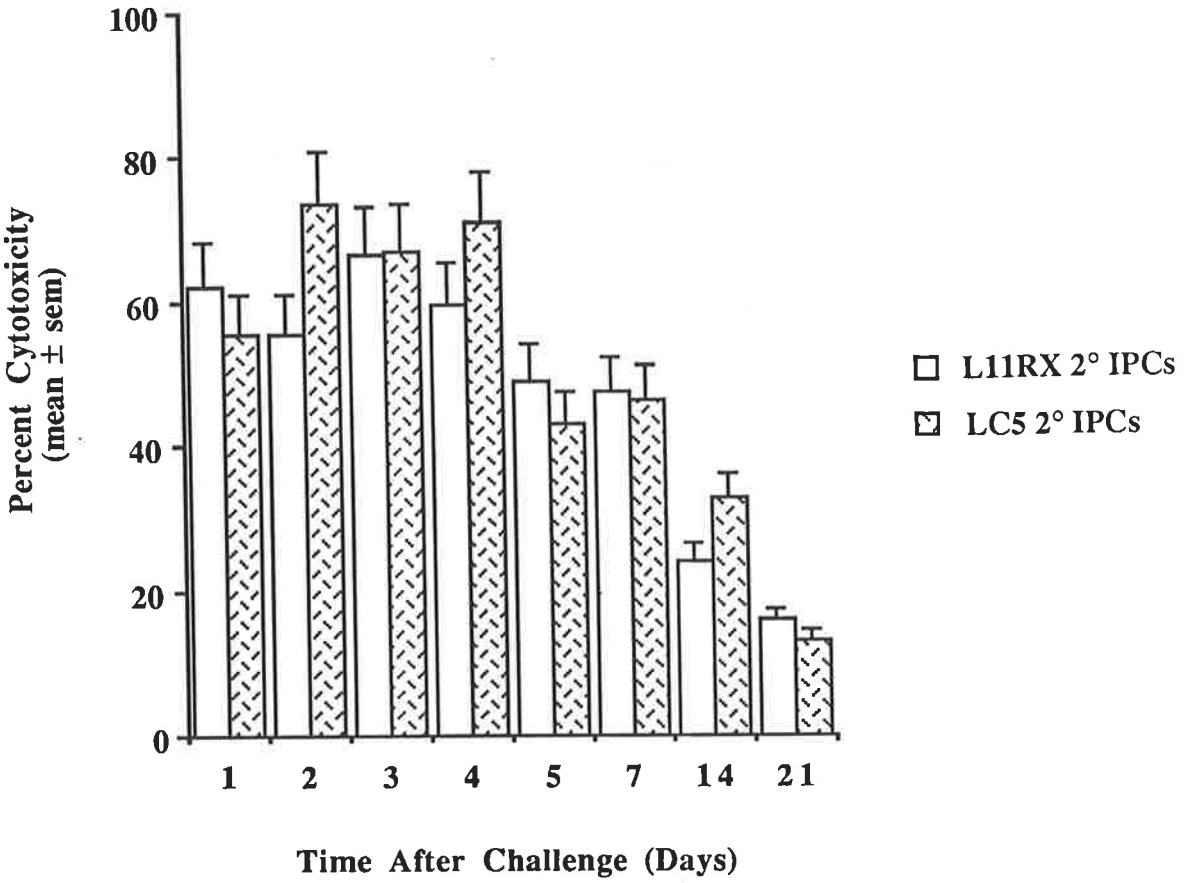


FIGURE 5.18 Lectin-mediated cytotoxicity of LC5 and L11RX 2° NW IPCs

IPCs harvested from mice 1-21 days after receiving a secondary challenge of $3x10^4$ LC5 or $8x10^6$ L11RX were NW fractionated and their cytotoxic activity assessed using the standard lectin-mediated assay. At each timepoint, $5x10^5$ 2° NW IPCs were incubated for 4 hours with $2x10^4$ ConA treated ^{51}Cr-labelled P815 in quadruplicate and the percent cytotoxicity (mean ± sem) was calculated for each suspension. Presented is representative data from several experiments showing the levels of lectin-mediated cytotoxicity of LC5 and L11RX 2° NW IPCs observed at each time point. The amount of killing of untreated ^{51}Cr-labelled P815 was also measured and was always in the range of 0-5% cytotoxicity (data not shown) for each population.

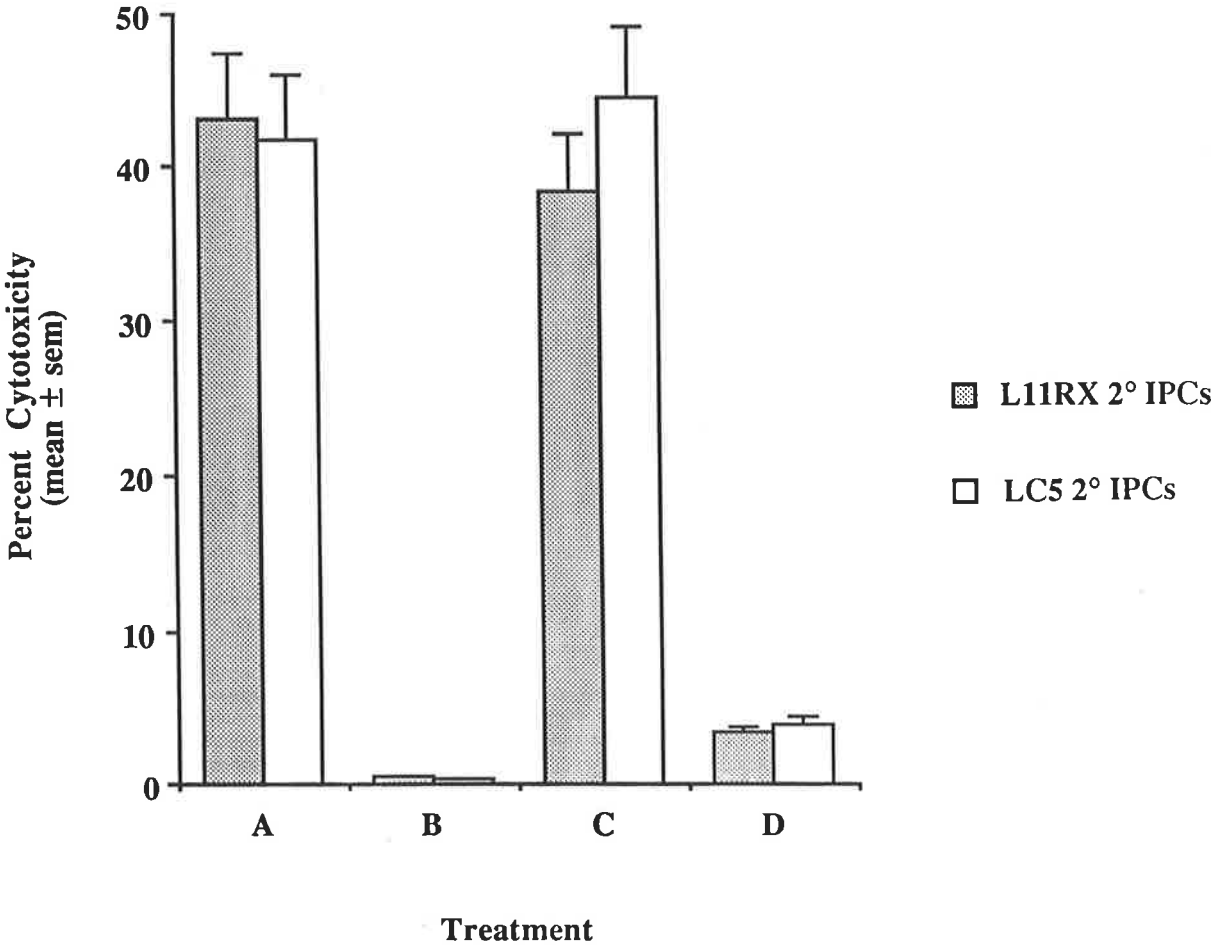


FIGURE 5.19 Phenotype of the cytotoxic cells present in the LC5 and L11RX 2° NW IPC populations

2° NW IPCs (obtained 4 days after challenge) treated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) were mixed with ConA-treated ^{51}Cr-labelled P815 at a ratio of 25 effector cells : 1 target cell (in quadruplicate) and incubated for 4 hours at 37°C, before measuring the amount of ^{51}Cr released by each population and data expressed as the percent cytotoxicity (mean ± sem) for each mixture.

mice 4 days after a secondary challenge with L11RX or LC5 and illustrates the results at all timepoints. Evidently, challenging mice with a second dose of *Salmonella* resulted in the induction of enhanced and longer lasting cytotoxic activity which was mediated by $Lyt2^+$ T cells.

5.2.10.3 *In vitro* cytotoxicity of ISCs from mice given secondary *Salmonella* infection

(i) Lectin-mediated cytotoxicity of 2° ISCs

Since no CTL activity could be detected in spleens of L11RX primed mice unless the cells were cultured *in vitro*, it was of interest to establish whether secondary *Salmonella* infection induced the activation of Ag-specific CTLs in the spleen, correlating with increased activity observed in the PC suspensions. Consequently, the standard lectin-mediated cytotoxicity assay was employed to assess the cytotoxicity of LC5 or L11RX 2° ISCs. Because considerable cytotoxic activity could be detected in the IPCs up to 7 days after a secondary *Salmonella* infection, U ISCs and NW ISCs were harvested from mice 4 days after a secondary infection of L11RX or LC5, incubated with ConA-treated and untreated ^{51}Cr-labelled P815 for 4 hours at 37°C and the levels of ^{51}Cr released into the supernatants measured and the percent cytotoxicity (mean ± sem) was calculated. It became apparent from several experiments that secondary immunization with either L11RX or LC5, had no effect on the cytotoxic activity of ISCs, as minimal lectin-mediated cytotoxicity of approximately 8% (maximum) was observed (data not shown). Hence, secondary immunization did not render the SCs cytotoxic. Since *in vitro* culture of ISCs from L11RX primed mice activated a population of $Lyt2.2^+$ CTLs (the specificity of which is uncertain), it was possible that *in vitro* culture of ISCs from secondary immunized mice would allow the expansion and/or activation of a minor population of Ag-specific CTLs thus enabling their detection. Only LC5 2° ISCs were used for these studies because the effect of secondary C5 infection appeared to enhance the proliferative activity of $Lyt2.2^+$ T cells more than a secondary challenge of L11RX.

(ii) ***Lectin-mediated cytotoxicity of in vitro cultured 2° ISCs***

SCs were obtained from mice 4 days after secondary infection with LC5 and cultured with ConA for 3 days at 37°C, before assessing their cytotoxic activity using the standard lectin-mediated assay. $5x10^5$ ConA cultured LC5 2° ISCs were mixed with $2x10^4$ ConA-treated ^{51}Cr-labelled P815 and incubated for 4 hours at 37°C and the amount of cytotoxicity was determined as previously described. To characterize the phenotype of any CTLs present in the ConA cultured LC5 2° ISCs, aliquots of these cells were treated with the usual MoAbs and C prior to assessing their cytotoxic potential

This experiment was repeated several times and data from one of these experiments is shown in Fig. 5.20, demonstrating the significant level of cytotoxicity (mean ± sem) of the ConA cultured LC5 2° ISCs, observed on each occasion. The level of activity (80%) was comparable to that observed in the primary ISCs and NSCs after *in vitro* culture with ConA (data not shown). ConA cultured ISCs incubated with untreated ^{51}Cr-labelled P815 exhibited minimal cytotoxicity of 5%. As expected, treatment of the ConA cultured LC5 2° ISCs with α-Thy1.2 or α-Lyt2.2 and C abolished virtually all cytotoxic activity. These experiments revealed that *in vitro* culture of LC5 2° ISCs with ConA activated a $Lyt2.2^+$ population able to mediate lectin-mediated cytotoxicity.

5.2.10.4 Summary

Secondary challenge of mice with *Salmonella* increased the cytotoxic activity of $Lyt2.2^+$ T cells in the PC suspensions, which remained elevated for up to seven days after challenge. In contrast, there was no difference in the lectin-mediated cytotoxic activity of the SCs from secondary infected mice, which exhibited no cytotoxic activity unless they were first cultured *in vitro*. However, although these populations were able to mediate lectin-mediated lysis of the target cells, this assay did not establish whether Ag-specific CTLs were present.

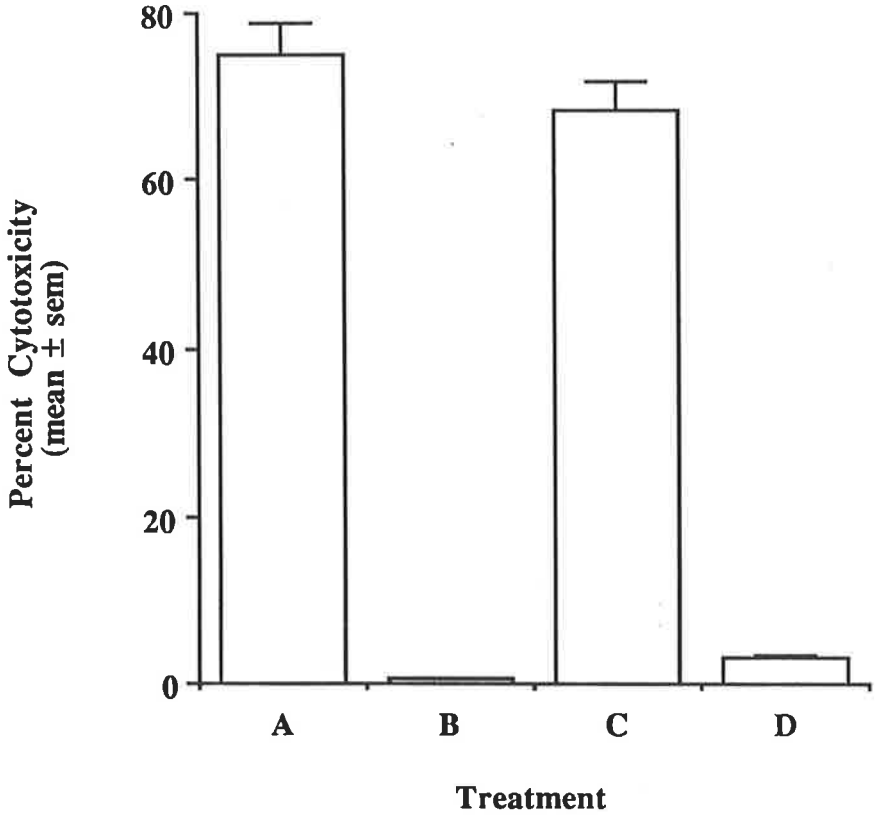


FIGURE 5.20 Cytotoxic activity of ConA cultured LC5 2° ISCs

LC5 2° ISCs (obtained 4 days after challenge) cultured for 3 days with ConA, were mixed with ConA-treated ^{51}Cr-labelled P815 (at the ratio of 25:1, in quadruplicate) and incubated for a further 4 hours at 37°C. The levels of ^{51}Cr released were measured and the percent cytotoxicity (mean ± sem) was calculated for each quadruplicate set. To determine the phenotype of the cells mediating any cytotoxic activity detected, the ConA cultured LC5 2° ISCs had been pretreated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D).

5.3 Summary and conclusions

Secondary infection of mice with *Salmonella*, especially C5, induced Ag-specific L3T4$^+$ and Lyt2.2$^+$ T cells which could be stimulated to proliferate *in vitro*, whilst after a primary infection only L3T4$^+$ T cells could be induced to proliferate. Similarly, secondary infection increased the levels of cytotoxic activity of the PC populations but not in SCs, which was again detected using the lectin-mediated cytotoxicity assay and was mediated by Lyt2.2$^+$ T cells. Cytotoxic activity of SCs was detected only after *in vitro* culture and there appeared to be no difference between the activity induced in the NSC and ISC populations. Comparison of the ability of cells obtained from primary and secondary immunized mice to transfer DTH reactivity to normal mice also revealed little difference between the magnitude of the responses and the cells mediating the responses induced, because even when live organisms were used as the eliciting Ag a significant Class II MHC restricted L3T4$^+$ T cell response was observed. Therefore, either no Lyt2.2$^+$ T cells which can transfer DTH reactivity were induced by infection with *Salmonella* or they were present but a more sensitive system was required for their detection, to prevent them being "masked" by the L3T4$^+$ T cells present.

CHAPTER 6

SALMONELLA-SPECIFIC CLASS I MHC RESTRICTED T CELLS

6.1 Introduction

As in the immune response to *Listeria*, it seemed possible that both the L3T4$^+$ and Lyt2$^+$ T cell subsets were induced following infection with *Salmonella* but the Lyt2$^+$ T cells may represent only a minor population or have more stringent requirements for activation. Evidence for the presence of Class I MHC restricted T cells during *Salmonella* infection was provided by the data presented in Chapter 5 which showed increased involvement of Lyt2.2$^+$ T cells in the proliferation induced by specific Ag in cells obtained from mice with secondary *Salmonella* infections. Similarly, an increase in the Lyt2.2$^+$ T cells able to mediate lectin-mediated cytotoxicity was observed following secondary challenge, although the specificity of these cells was not established. However, on stimulation of these cells with specific Ag in the DTH system, responses by a minor Ag-specific Lyt2.2$^+$ T cell population may have been masked by a larger response by the L3T4$^+$ T cells, because simply supplying Ag to be presented by APCs present in these suspensions may have favoured association with Class II MHC products and subsequently preferentially induced L3T4$^+$ T cells. To activate Lyt2.2$^+$ T cells Ag must be presented in association with Class I MHC molecules. Therefore, development of a stringent transfer system in which Ag is only presented in association with Class I MHC products, may allow Lyt2.2$^+$ DTH effector T cells to be detected. The most obvious approach would be to use Class I MHC$^+$ (Class II$^-$) cells which have been pulsed with *Salmonella* Ags as APCs and to use pure T cell populations. This approach was employed in an attempt to detect Class I restricted Lyt2$^+$ DTH effector T cells and the

Ag-pulsed cells were also used to detect Ag-specific cytotoxic activity.

6.2 Results

6.2.1 Presentation of *Salmonella* antigens in association with Class I MHC molecules

6.2.1.1 Introduction

To preferentially activate *Salmonella*-specific Lyt2.2$^+$ T cells, it was necessary to chose a suitable stimulatory population of Class I$^+$, Class II$^-$ cells pulsed with the appropriate *Salmonella* Ags. Any Ag presented by these cells could only associate with Class I MHC molecules and provided that the relevant antigenic determinants were generated, such Ag-pulsed cells may detect Class I restricted Ag-specific Lyt2$^+$ T cells.

Some cell types have been used by others, including *in vitro* cultured bone marrow derived macrophages which are known to be MHC Class I$^+$ and Class II$^-$, and MHC Class I$^+$ cell lines which are maintained by *in vitro* culture. Several groups have also successfully used cell lines expressing specific Ags for activation or detection of specific Lyt2$^+$ T cells (eg. Townsend *et al.*, 1986; Carbone and Bevan, 1989). One successful approach involved the introduction of small soluble peptides of OVA into the cytoplasm of the cells by the osmotic lysis of pinososmes or Ag-containing endosomes. This ensured access of these Ags to the Class I presentation pathway and their subsequent presentation as complexes of Ag and Class I MHC molecules on the surface of the cell (Carbone and Bevan, 1989). Another approach was to produce a cell line constitutively expressing a particular Ag. This was achieved by transfecting the APC cell line with a gene encoding the expression of the peptide of interest, or by infecting the APCs with a noncytopathic virus (Townsend *et al.*, 1986). Both result in the expression of specific antigenic determinants on the cell surface in the context of Class I MHC molecules.

Similar success using *Listeria*-infected bone marrow derived macrophages

for the detection of *Listeria*-specific Lyt2$^+$ T cells has been reported by De Libero and Kaufmann (1986) and attempts to adopt this approach to detect *Salmonella* Ag-specific Lyt2.2$^+$ T cells were made, but unfortunately were not successful. Introduction of small, soluble *Salmonella* Ags into the cytoplasm of APCs was not an option which could be used in these studies because such a preparation of Ag was not available.

6.2.1.2 Invasion of the cell line P815 with live *Salmonella*

Based upon a modification of the technique described by Isberg *et al.* (1987) for the invasion of HEp-2 cells, a technique of invading the *in vitro* maintained cell line P815 with live *Salmonella* was established. The murine mastocytoma, P815, was chosen because it expresses only Class I MHC molecules of the H-2^d haplotype and is a good stimulator of MLRs, indicating that it can function as an APC (Sprent and Schaefer, 1986). Another reason for this choice was that, being a mastocytoma, it was unlikely to be activated by T cell LKs (eg. IFN-γ) which are known to enhance the activity of macrophages, making them bactericidal for intracellular organisms such as *Salmonella*. In addition, influenza A virus internal Ags were found to be expressed on the surface of infected P815 cells (Yewdell *et al.*, 1981), suggesting that if infection of the P815 cells with *Salmonella* was successful that specific Ags may be expressed on the cell surface and be recognized by specific T cells.

Various techniques of infecting P815 with live *Salmonella* were used before the procedure described in detail in the Materials and Methods section was adopted. P815 cells were mixed with live *Salmonella* at a ratio of 1:100 and centrifuged to ensure contact between the P815 and bacteria. The pelleted cells were incubated at 37°C for 30 minutes, before they were resuspended and washed several times to remove any extracellular bacteria and resuspended in culture medium containing 40 μg/ml Gentamycin to kill any bacteria which had not been completely internalized. After overnight incubation at 37°C, which increased the Ag load within the P815 cells due to bacterial proliferation, the cells were washed, counted and adjusted to the required concentration. P815 invaded with L11RX or LC5 will be referred to as "L11RX P815"

and "LC5 P815" respectively.

To verify that the bacteria had entered the P815 cells, Cytospin smears were made of both "L11RX P815" and "LC5 P815", stained with Giemsa stain and examined using oil immersion microscopy. Photographs of these smears demonstrated that both L11RX and LC5 had successfully entered the cells, and were localized within vacuoles or were free in the cytoplasm (Fig. 6.1 [A and B]). An important fact to note, is that the number of bacteria associated with the P815 cells varied considerably and ranged from none to as many as 20 bacteria per cell. (None of the modifications used to try to achieve a more even form of infection of P815 were successful.) There were virtually no free bacteria outside the cells and none "stuck" around the edges of the cells, supporting the conclusions that the cell-associated bacteria had been internalized.

To determine the viability of the 11RX and C5 organisms within the "L11RX P815" and "LC5 P815", the cells were lysed with 0.2% Triton in saline and aliquots of the lysates were plated onto nutrient agar plates which were incubated overnight at 37°C. The recovery of either L11RX or LC5 organisms was determined in at least 10 separate invasion experiments and was found to be quite variable between experiments and ranged from 1 to 6 bacteria per P815 cell (data not shown). However, it did confirm the presence of live 11RX or C5 organisms within the P815 cells. The ability of the "LC5 P815" or "L11RX P815" to stimulate Lyt2.2$^+$ T cells was investigated in the following set of experiments.

6.2.2 Detection of Class I MHC restricted Lyt2.2$^+$ DTH effector cells

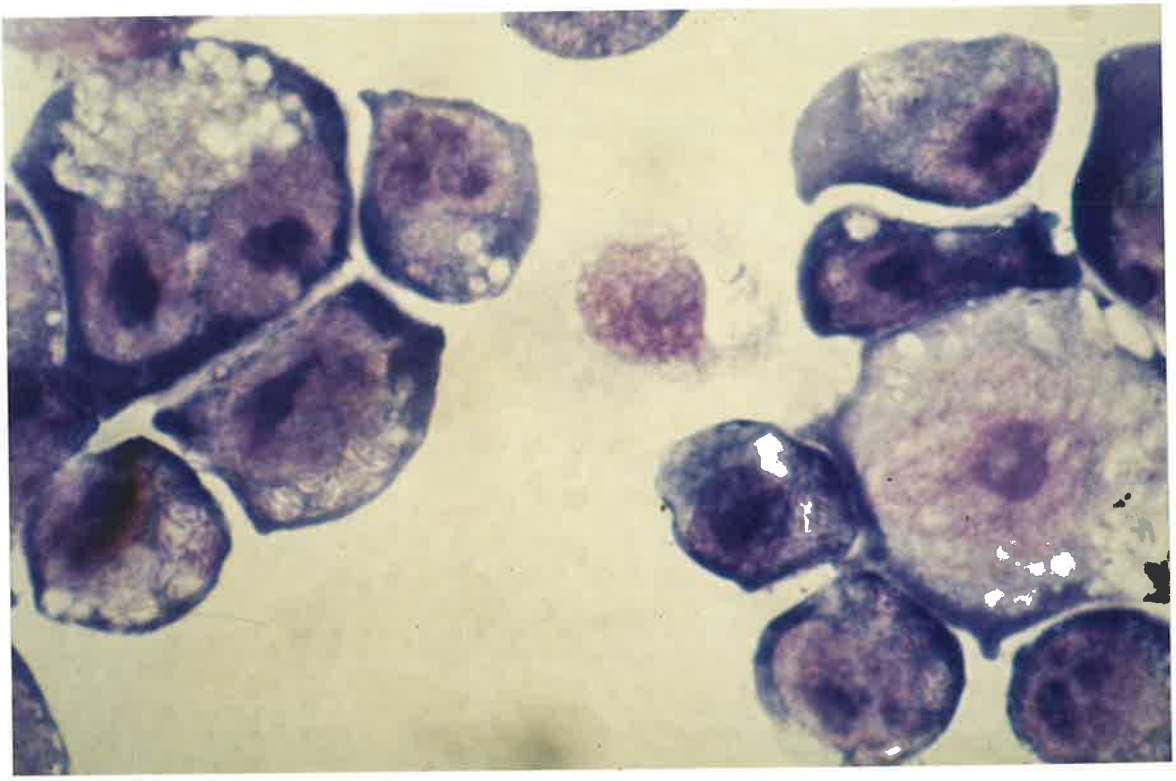
6.2.2.1 Lyt2.2$^+$ T cells induced by secondary C5 infection

Because secondary infection should increase the chance of detecting Class I restricted DTH effector T cells, cells harvested from mice given a secondary challenge of LC5 (which increased the PC yields and the Lyt2.2$^+$ T cell proliferative activity to a greater extent than a secondary challenge with L11RX) were initially used to establish whether "LC5 P815" would present *Salmonella* Ags in association with Class I

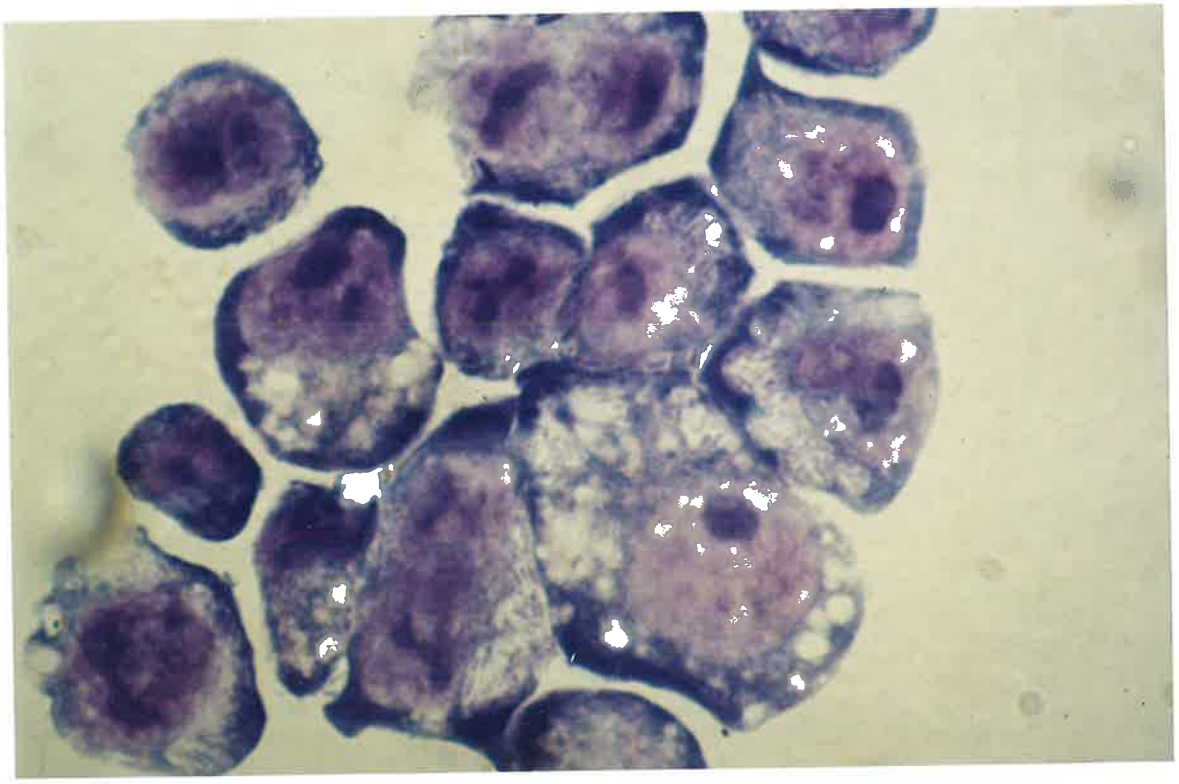
FIGURE 6.1 Photographs of "L11RX P815" and "LC5 P815"

Cytospin smears of P815 cells infected with L11RX ("L11RX P815") or LC5 ("LC5 P815") were prepared, stained with Giemsa stain and viewed under oil immersion microscopy. Photographs of representative populations of "L11RX P815" (A) and "LC5 P815" (B) were taken at 1000 times magnification.

A



B



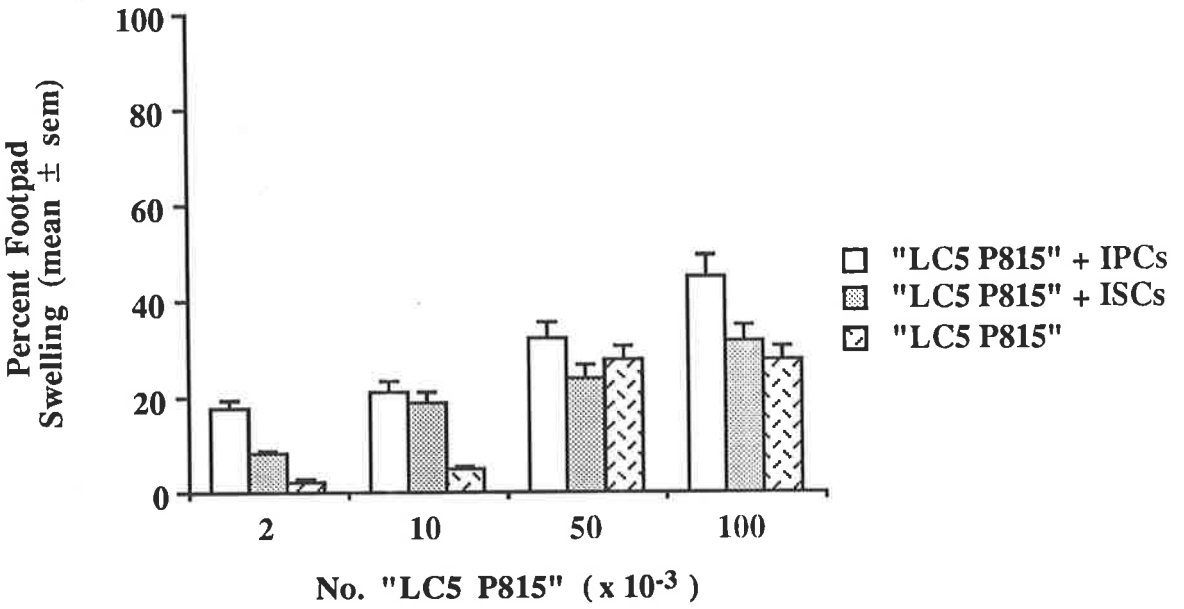
MHC products. To reduce the chance of providing killed Ags to the T cells and therefore inducing a Class II restricted response, "LC5 P815" were used as the Ag-pulsed APCs so that if any live organisms escaped from the P815 and were exposed to macrophages remaining in the T cell population, C5 would be more resistant than 11RX to killing by these cells, thereby increasing the possibility of inducing a Class I MHC restricted Lyt2.2$^+$ T cell response. The NW IPCs and NW ISCs of F1 mice used in these assays were treated with α-Ia and C for 1 hour at 37°C, prior to mixing with the "LC5 P815", to ensure removal of as many of the APCs present in these suspensions as possible. To further limit the involvement of external APCs able to present Ag in association with Class II MHC products, the recipient mice chosen for these experiments were C57BL/6 mice. Theoretically, this should dramatically reduce the participation of recipient APCs in the elicitation of DTH mediated by L3T4$^+$ T cells, because C57BL/6 mice are H-2^b haplotype and it has been shown previously that in the F1 mice used in these studies Class II restricted responses to *Salmonella* Ags are preferentially restricted to the H-2^d haplotype (Attridge and Kotlarski, 1985a). Therefore, any presentation of Ags by recipient H-2^b APCs in association with Class II MHC molecules should not elicit significant L3T4$^+$ T cell-mediated responses.

In the first instance, a range of numbers of "LC5 P815" were examined for their ability to elicit DTH responses in LC5 2° IPCs and ISCs and the footpad swelling was measured 24 and 48 hours after the cells were transferred. This was repeated twice and it became evident that by 24 hours mixtures of "LC5 P815" and either IPCs or ISCs elicited significant responses, which were comparable to the DTH responses induced by LC5 in the F1 system (Fig. 6.2 A). However, the swelling which resulted when larger numbers of "LC5 P815" alone (10^5 and $5x10^4$) were injected accounted for approximately 75% of the footpad swelling induced in the presence of IPCs and virtually 100% of the responses in the presence of ISCs. For all doses of "LC5 P815" tested, by 48 hours the amount of swelling in the footpads injected with only "LC5 P815" was comparable to that observed in the presence of the IPCs or ISCs and presumably indicated bacterial growth (Fig. 6.2 B). The data suggested that 10^4 "LC5 P815" would

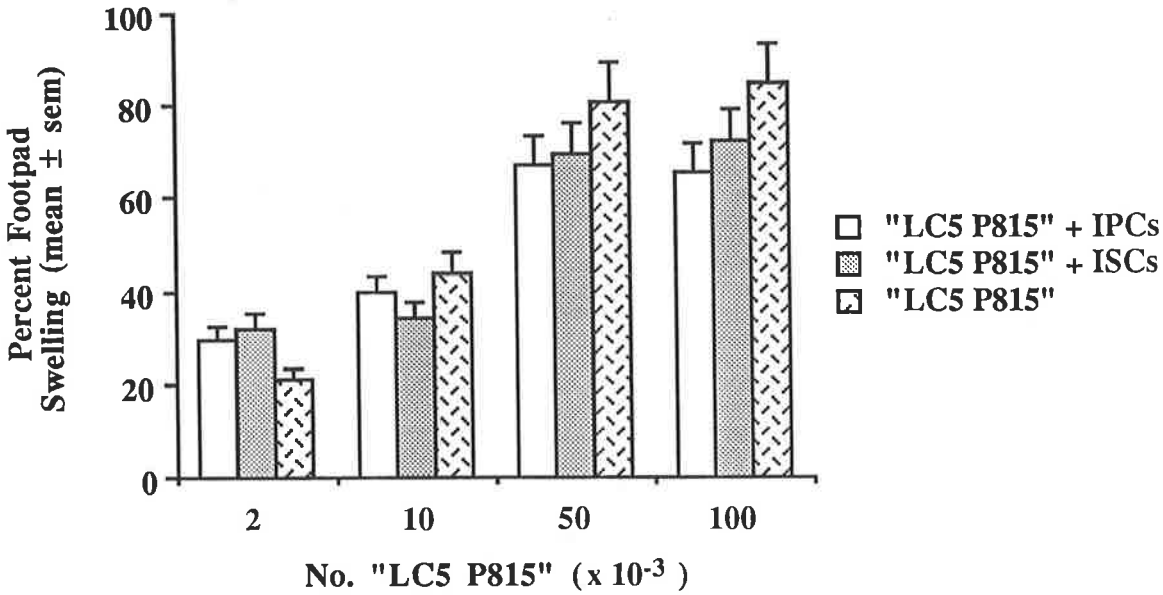
FIGURE 6.2 DTH reactivity induced by "LC5 P815"

A range of doses of "LC5 P815" were mixed with day 14 LC5 2° NW IPCs or ISCs which had been treated with α-Ia + C, to ensure that 10^6 IPCs or ISCs and $2x10^3$, 10^4, $5x10^4$ or 10^5 "LC5 P815" would be transferred in a 50 µl volume. Suspensions of "LC5 P815" alone (all doses) were also transferred to determine the amount of swelling induced by the injected "LC5 P815" cells alone. Each suspension was injected sc into the left hind footpads of groups of 3 normal, unimmunized C57BL/6 mice and left and right hind footpad sizes were recorded 24 and 48 hours later and results are expressed as the percent footpad swelling (mean ± sem). Representative results at 24 hours (A) and 48 hours (B) are presented from one of a set of three experiments.

A


Percent Footpad Swelling (mean ± sem)
100
80
60
40
20
0
2
10
50
100
No. "LC5 P815" (x 10-3)
"LC5 P815" + IPCs
"LC5 P815" + ISCs
"LC5 P815"

B


Percent Footpad Swelling (mean ± sem)
100
80
60
40
20
0
2
10
50
100
No. "LC5 P815" (x 10-3)
"LC5 P815" + IPCs
"LC5 P815" + ISCs
"LC5 P815"

provide the optimum dose to induce significant swelling in the presence of the T cells, with little background swelling in the absence of T cells at 24 hours. It was also encouraging to note that when secondary primed IPCs and ISCs mixed with 10^4 or $5x10^3$ LC5 were transferred to the C57BL/6 recipients, minimal DTH responses were observed (5% maximum). Additional controls of IPCs and ISCs alone and uninfected P815 in the presence and absence of IPCs or ISCs, resulted in only 0-3% swelling. Together these data suggested that the swelling induced in the presence of "LC5 P815" occurred as a result of *Salmonella* Ags presented by the P815.

6.2.2.2 Phenotype of the T cells which mediate DTH to "LC5 P815"

The standard procedure for determining which T cells were responsible for transferring the DTH reactivity was employed. During the α-Ia and C treatment, used to purify secondary primed T cells, IPCs and ISCs were also treated with α-Thy1.2, α-L3T4 or α-Lyt2.2. Mixtures of 10^6 treated cells and 10^4 "LC5 P815" were transferred to groups of 3 normal C57BL/6 mice and their footpads were measured 24 hours later. The percent footpad swelling (mean ± sem) was calculated for each group. This was repeated at least twice using IPCs and ISCs harvested from mice 7, 14 and 21 days after the secondary challenge with LC5 and no significant differences with respect to time after challenge were observed. The data presented in Fig. 6.3 from one of these timepoints and are representative of all the results obtained. Treatment of IPC or ISC populations with α-Thy1.2 or α-Lyt2.2 and C completely removed the ability of these cells to transfer DTH reactivity, indicating that the cells mediating the DTH reactivity induced by "LC5 P815" were Lyt2.2$^+$ T cells. Hence, after secondary immunization with LC5 there are Lyt2.2$^+$ T cells in both PC and SC populations capable of transferring specific DTH reactivity to normal mice. Detection of these cells was made possible by the more stringent assay system employed, which limited Ag presentation to the context of Class I MHC products.

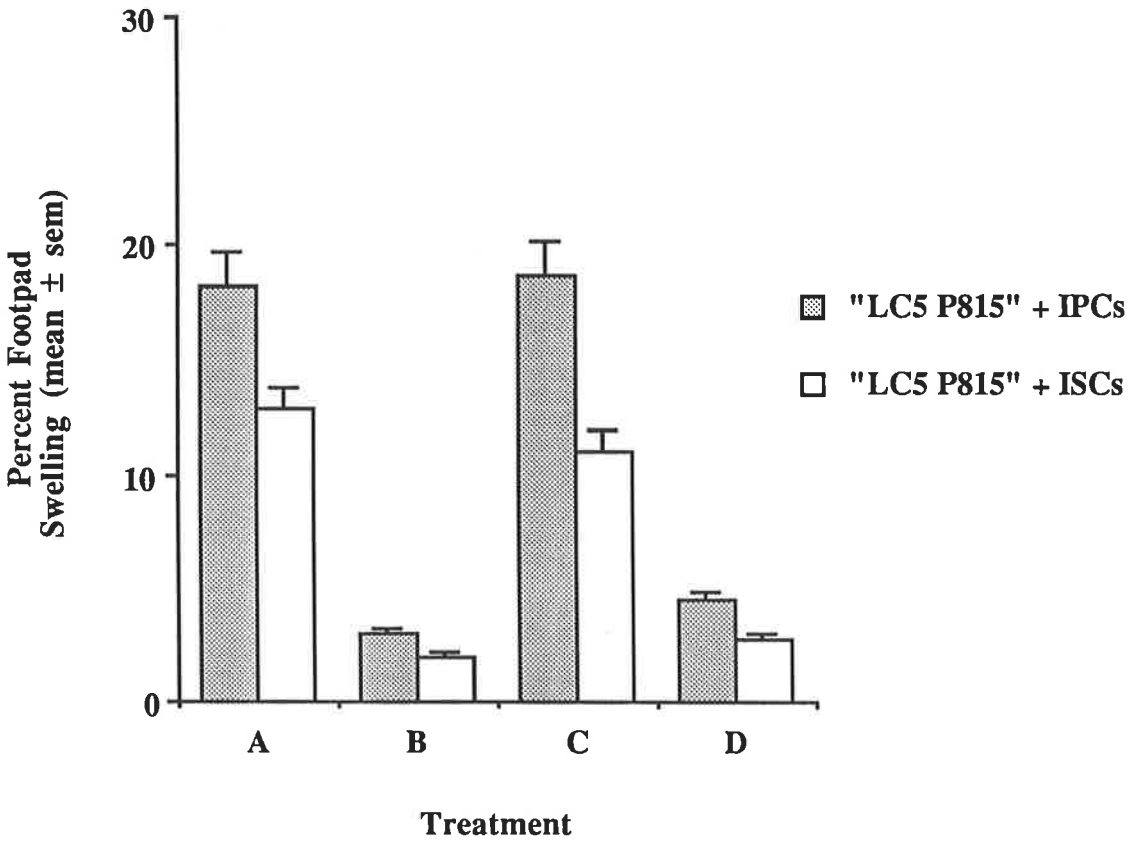


FIGURE 6.3 Phenotype of the DTH effectors induced by "LC5 P815"

LC5 2° NW IPCs and ISCs (harvested 14 days after secondary challenge) treated with α-Ia and C were also treated (or not) with T cell specific MoAbs at the same time and used to determine the phenotype of the cells mediating the DTH responses induced by "LC5 P815". The data presented were obtained using 10^6 LC5 2° IPCs and ISCs treated with α-Ia + C (A), α-Ia, α-Thy1.2 + C (B), α-Ia, α-L3T4 + C (C) or α-Ia, α-Lyt2.2 + C (D), mixed with 10^4 "LC5 P815" and transferred to the left hind footpads of groups of 3 normal C57BL/6 mice. The amount of footpad swelling was measured 24 hours later and data are presented as the percent increase in footpad size (mean ± sem) for each population.

6.2.2.3 Induction of Lyt2.2$^+$ DTH effector T cells by primary immunization

(i) Detection of Class I restricted Lyt2.2$^+$ DTH effector T cells after ip immunization with L11RX

It was also of interest to determine whether specific Lyt2$^+$ DTH effector T cells could be detected after primary immunization with L11RX. Since larger DTH responses could be transferred with PCs and SCs obtained from mice later after infection, D 21 IPCs and ISCs were initially studied to determine whether Lyt2.2$^+$ DTH effector cells were present. NW D 21 IPCs and ISCs were treated with α-Ia and C and 10^6 purified cells were mixed with 10^4 "LC5 P815" and transferred in 50 µl volumes to 3 or 4 normal C57BL/6 mice. To establish the phenotype of the cells mediating the responses induced, the purified, primed T cells were also treated with the T cell-specific MoAbs during the α-Ia and C treatment and were used in the local footpad transfer experiments with the "LC5 P815" as described. Measurement of the footpads at 24 hours revealed some very interesting results.

It was obvious that transfer of ISCs and "LC5 P815" resulted in little or no footpad swelling (approximately 5%, the same as the swelling detected in response to the lymphoid cells or "LC5 P815" alone) and the MoAb and C treatments had no influence on this at all. In contrast, however, considerable DTH reactivity (35%) was detected following transfer of IPCs and "LC5 P815" (Fig. 6.4). The cells mediating this activity expressed the Lyt2.2$^+$, Thy1.2$^+$ phenotype, as treatment with α-Thy1.2 + C (B) or α-Lyt2.2 + C (D) almost completely removed the ability to transfer DTH reactivity (while treatment with α-L3T4 + C (C) or C (A) alone did not). Again the transfer of IPCs with LC5 resulted in minimal amounts of swelling (5% maximum), confirming that the stimulation of the DTH effector cells occurred as a result of presentation of the *Salmonella* Ags by the invaded P815. By 48 hours the swelling observed in all groups injected with "LC5 P815" could be attributed to the growth of LC5 within the footpad (data not shown). It appears, therefore, that by 21 days after a primary ip infection of L11RX, Lyt2.2$^+$ DTH effector T cells were present in the peritoneal cavity but not in the

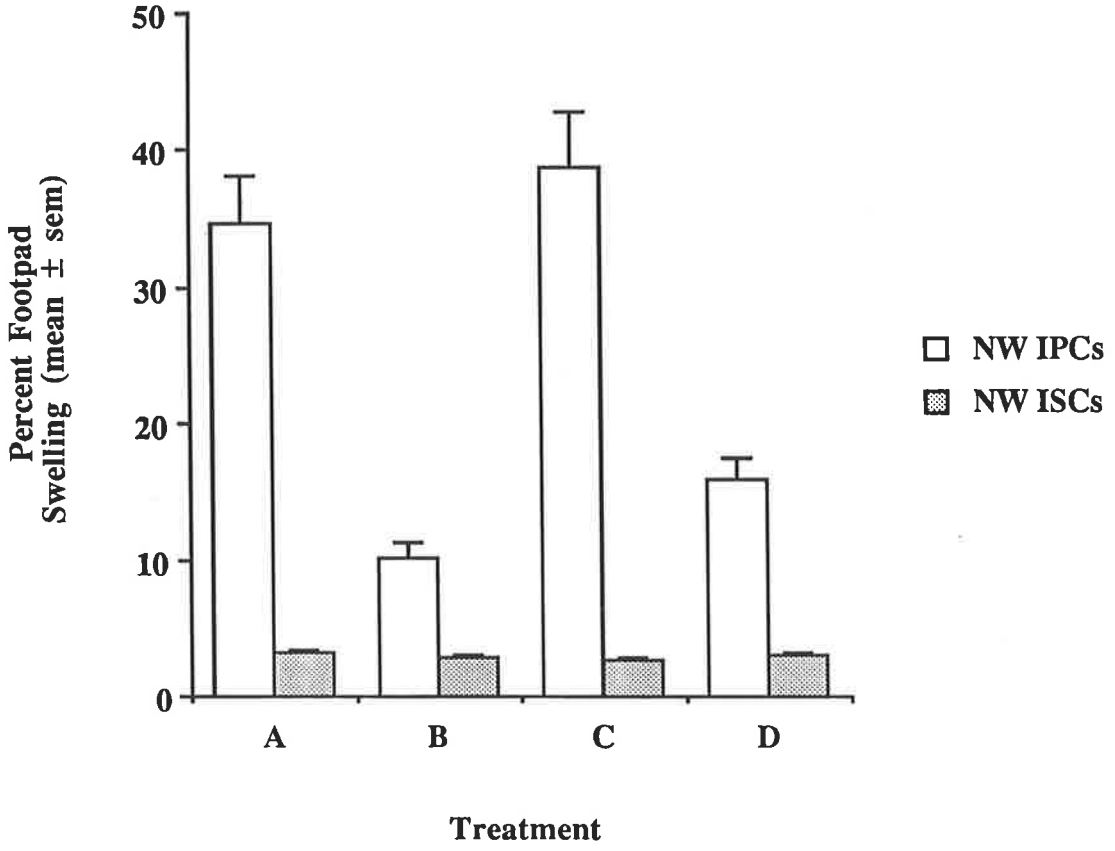


FIGURE 6.4 Examination of the ability of "LC5 P815" to induce DTH responses in IPCs and ISCs from L11RX immunized mice.

D 21 IPCs and ISCs were fractionated on NW and treated with α-Ia + C (A), α-Ia, α-Thy1.2 + C (B), α-Ia, α-L3T4 + C (C) or α-Ia, α-Lyt2.2 + C (D). Each of these populations was mixed with "LC5 P815" such that 10^6 cells and 10^4 "LC5 P815" could be transferred in 50 µl volumes to the left hind footpads of 3 normal C57BL/6 mice. 24 hours later both the left and right footpads were measured and the percent footpad swelling (mean ± sem) was determined for each group.

spleen. From the earlier data presented in Sections 5.2.2 and 5.2.3 it is obvious that the ability of L3T4$^+$ T cells to transfer DTH is greater at 21 days than at 4 days after ip infection with L11RX. It was particularly interesting, therefore, to determine whether this also applied to the Lyt2.2$^+$ DTH effectors.

(ii) ***Are Class I restricted DTH effectors present early after immunization?***

With the knowledge that the peak of activity of Lyt2.2$^+$ CTLs occurred 5 days after immunization with L11RX, the presence of Lyt2.2$^+$ DTH effectors in D 4 IPC and ISC suspensions was investigated by assessing the ability of D4 IPCs and ISCs to transfer DTH reactivity when stimulated with "LC5 P815".

Mixtures of NW D 4 IPCs or ISCs, treated with α-Ia and C to remove APCs, (10^6) and 10^4 "LC5 P815" were each transferred in 50 µl volumes to the left hind footpads of 3 normal, unimmunized C57BL/6 mice. The appropriate controls of cells and LC5, cells, LC5 and "LC5 P815" alone were also included. The thickness of both the right and left footpads were measured 24 and 48 hours later and the percent of footpad swelling (mean ± sem) was calculated as usual. To evaluate the phenotypes of the cells mediating any DTH reactivity observed, the NW, α-Ia + C IPCs and ISCs were also treated with the T cell specific MoAbs. The results from a representative experiment supplied in Fig. 6.5 clearly indicate that no DTH responses were transferred when T cells purified from either the D 4 IPC or ISC suspensions were used (experimental and control suspensions resulted in only minimal footpad swelling [approximately 3%]). Treatment with the T cell-specific MoAbs had no effect on these results. Again, the swelling observed by 48 hours after transfer had increased (reaching almost 60% swelling) and could be entirely attributed to multiplication of C5 organisms (data not shown). Therefore, Lyt2.2$^+$ DTH effector T cells were not present in the PC or SC suspensions obtained from mice 4 days after infection with L11RX.

6.2.2.4 Summary

Lyt2$^+$ T cells capable of transferring DTH reactivity to normal mice were

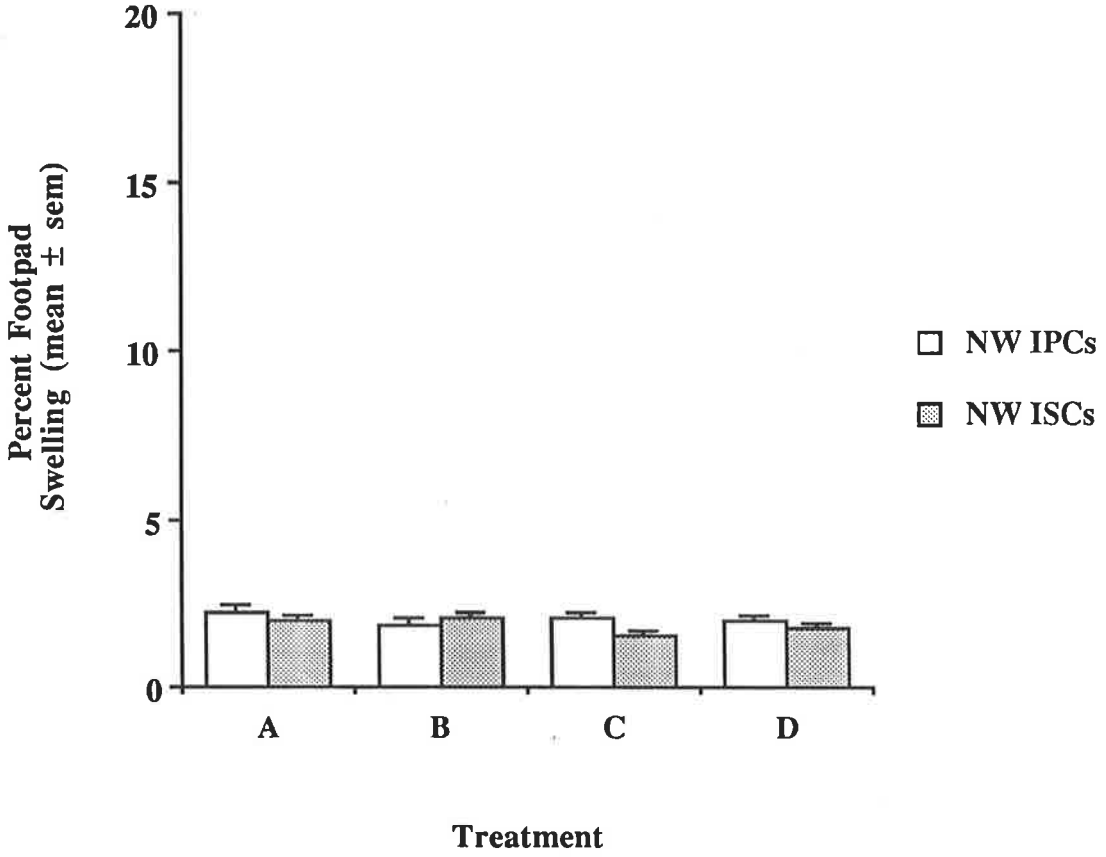


FIGURE 6.5 Investigation into the presence of Class I restricted DTH effector cells early after immunization with L11RX

NW fractionated D 4 IPCs and ISCs were treated with α-Ia + C (A), α-Ia, α-Thy1.2 + C (B), α-Ia, α-L3T4 + C (C) or α-Ia, α-Lyt2.2 + C (D) and each suspension mixed with "LC5 P815" to ensure that 10^6 cells and 10^4 "LC5 P815" could be transferred in 50 µl to the left hind footpads of groups of 3 normal C57BL/6 mice. Both hind footpads were measured 24 hours later and the percent increase in footpad size (mean ± sem) determined for each group in the usual way and a representative set of results is provided.

present in both the spleens and peritoneal cavitites of mice after secondary immunization with LC5 and in the peritoneal cavity after ip immunization with L11RX (but not early). However, the responses by the L3T4$^+$ T cells were much stronger and/or more easily induced than those by Lyt2$^+$ T cells. Lyt2.2$^+$ DTH effector T cells could only be detected using a stringent system which restricted presentation of Ags to an association with Class I MHC molecules. After a secondary challenge with *Salmonella* the involvement of Lyt2.2$^+$ T cells in mediating DTH reactivity was enhanced, whilst the activity of L3T4$^+$ DTH effector T cells remained unchanged.

6.2.3 Ag specificity of Lyt2.2$^+$ CTLs induced by *Salmonella* infection

6.2.3.1 Analysis of the specificity of Lyt2$^+$ CTLs generated in the peritoneal cavity during secondary infection

Although very useful in detecting CTLs, the lectin-mediated cytotoxicity assay provides no information on the specificity of the CTLs detected. Detection of specific Lyt2$^+$ CTLs requires an appropriate target cell expressing the relevant antigenic determinants in the context of Class I MHC molecules. As "LC5 P815" were found to reliably detect Class I restricted DTH effector T cells, it seemed reasonable to use the "invaded P815" to detect specific Class I restricted CTLs.

Since considerable lectin-mediated cytotoxic activity was detected from days 1-7 after secondary infection, IPCs were harvested from mice 4 days after a secondary challenge of LC5 or L11RX, NW fractionated and examined for their ability to kill "L11RX P815". [Preliminary experiments carried out revealed that "LC5 P815" and "L11RX P815" were equally as effective as target cells (data not shown) and that "L11RX P815" exhibited lower spontaneous release values than "LC5 P815" and was therefore used for the majority of this work.] "L11RX P815" were labelled with ^{51}Cr in the normal manner, mixed with a range of doses of the 2° NW IPCs and incubated for 4 hours at 37°C, after which the amount of ^{51}Cr released was measured and the percent cytotoxicity calculated. To assess the reproducibility of these data, this experiment was

repeated at least five times and very similar results were obtained on each occasion.

Data from a typical experiment are shown in Fig. 6.6 [A and B]; significant amounts of cytotoxicity against "L11RX P815" were detected in both NW IPC populations. This activity accounted for approximately 25-50% of the total activity detected in the lectin-mediated assay. The Ag-specific cytotoxicity mediated by LC5 2° NW IPCs was consistently greater than that detected in the L11RX 2° NW IPCs. These data indicated that the invasion of P815 with L11RX generated sufficient antigenic determinants for the detection of Ag-specific CTLs. The finding that only 25-50% of the cytotoxicity detected using the lectin-mediated assay was attributable to *Salmonella*-specific cells could be interpreted as suggesting that the remaining activity was nonspecific. Alternatively, a more likely explanation is suggested from the Cytospin smears of the infected P815 (Fig 6.1) which clearly illustates that a significant proportion of these cells did not contain bacteria and would therefore not present the appropriate antigenic determinants in association with Class I MHC molecules on their surface for recognition by the Ag-specific CTLs. In contrast, virtually all ConA-treated P815 cells would have the potential to be lysed because ConA nonspecifically binds to the invariant portions of MHC molecules and this may account for the reduced amount of CTL activity detected in the Ag-specific system.

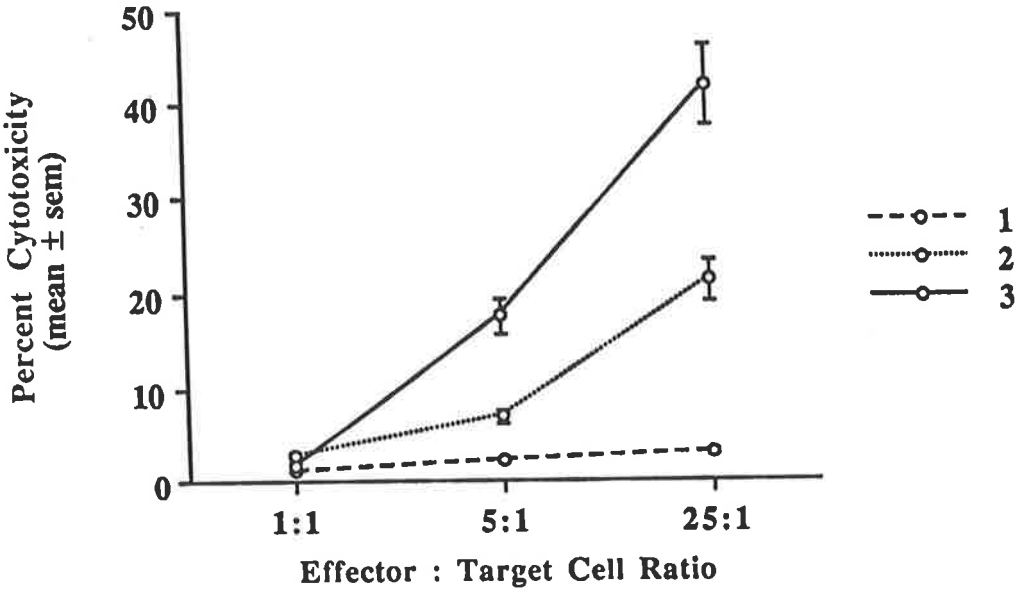
To characterize the CTLs mediating the *Salmonella*-specific cytotoxic activity, the 2° NW IPC populations were pretreated with the various MoAbs and C before being incubated with the "L11RX P815" (at a ratio of 25 effector cells to 1 target cell) and the percent cytotoxicity was calculated for each suspension, as previously described. This experiment was reproduced four times and representative data from one of these experiments are shown in Fig. 6.7. The depletion of either all T cells or Lyt2.2$^+$ T cells completely abrogated the cytotoxic activity of these cells, confirming that the *Salmonella*-specific CTLs were undoubtedly Lyt2.2$^+$ T cells. Therefore, *Salmonella*-specific Lyt2.2$^+$ CTLs were present in the peritoneal cavity after secondary challenge with either L11RX or LC5.

FIGURE 6.6 Analysis of the specificity of the CTLs in the LC5 and L11RX 2° NW IPCs

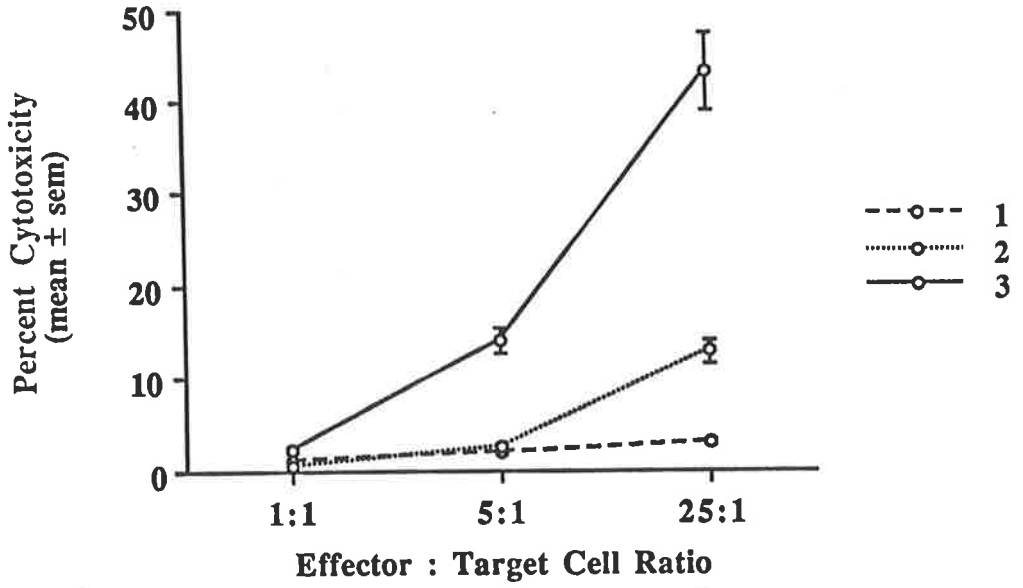
P815 (1) and "L11RX P815" (2) were labelled with ^{51}Cr in the usual manner and an aliquot of the "L11RX P815" was also treated with ConA (3). 2° NW IPCs were mixed with each of these target cell populations at effector:target cell ratios of 25:1, 5:1 and 1:1 and incubated at 37°C for 4 hours. After this incubation, the amounts of ^{51}Cr released into the supernatants of these cultures were determined and the percent cytotoxic activity (mean ± sem) detected using D 4 LC5 2° NW IPCs (A) and D 4 L11RX 2° NW IPCs (B) are shown.

The specificity of the cytotoxic responses was confirmed since "LC5 P815" was lysed no more than untreated P815 when ConA stimulated normal spleen cells were used as effector cells. When cells from immunized mice were stimulated *in vitro* with ConA before being used as effectors, significant lysis of the infected target cells was observed only when the effectors were obtained from mice following a secondary *Salmonella* infection.

A


50
40
30
20
10
0
Percent Cytotoxicity
(mean ± sem)
1:1
5:1
25:1
Effector : Target Cell Ratio
1
2
3

B


50
40
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Percent Cytotoxicity
(mean ± sem)
1:1
5:1
25:1
Effector : Target Cell Ratio
1
2
3

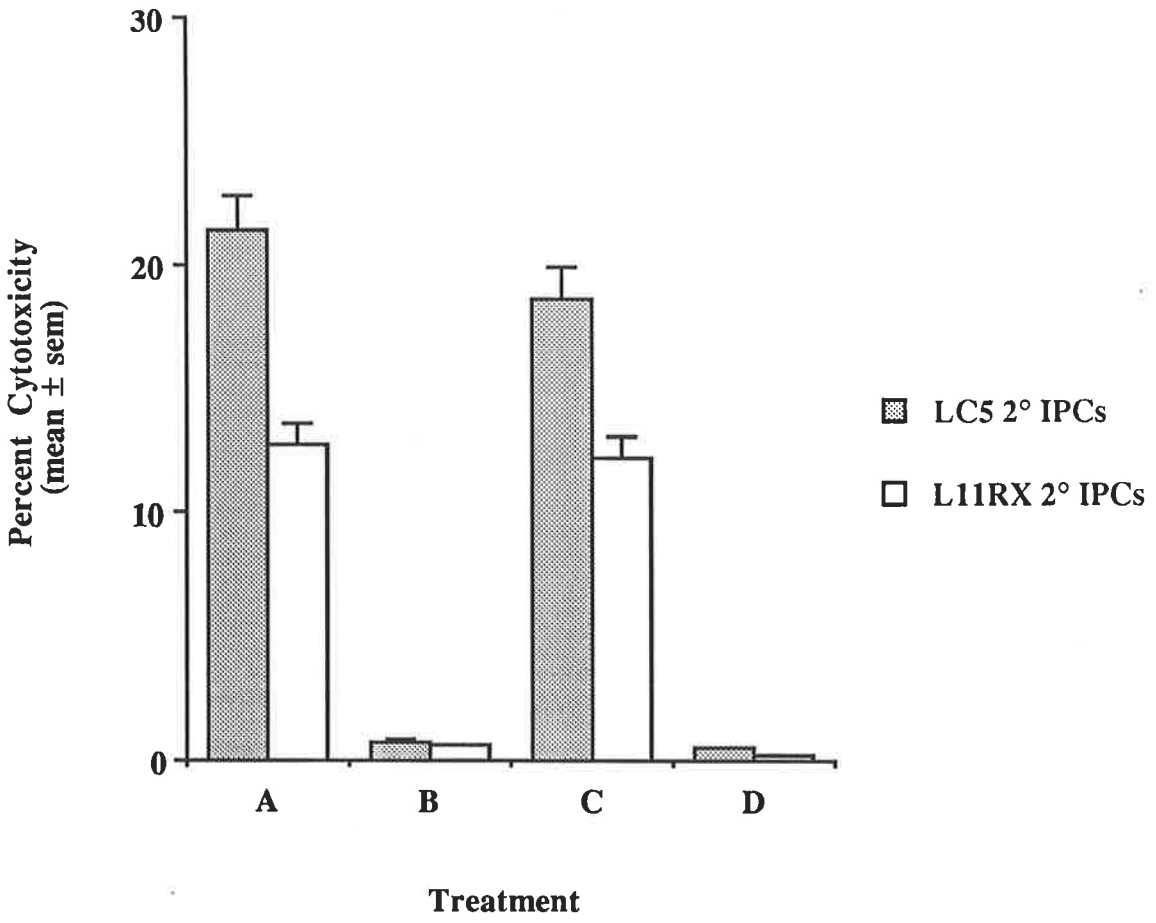

FIGURE 6.7 Phenotype of the *Salmonella*-specific cytotoxic cells of the 2° NW IPCs

Day 4 LC5 and L11RX 2° NW IPCs were treated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) and their ability to lyse "L11RX P815" was investigated. Combinations of $5x10^5$ treated 2° NW IPCs and $2x10^4$ ^{51}Cr-labelled "L11RX P815" (in quadruplicate) were incubated for 4 hours at 37°C, after which the levels of ^{51}Cr in the supernatants of these cultures were measured as usual. Similar results obtained in several experiments are presented, showing the percent cytotoxicity (mean ± sem) for each population.

6.2.3.2 Studies on the specificity of the CTLs induced after primary immunization with L11RX

The same system was used to examine the specificity of the CTLs induced by primary immunization with L11RX. NW IPCs harvested from mice 5 days after immunization, were incubated with ^{51}Cr-labelled "L11RX P815" for 4 hours, the amount of ^{51}Cr released measured and the percent cytotoxicity was calculated as described previously.

The data shown in Fig. 6.8 represent the results from one of four separate experiments, illustrating that IPCs obtained from mice ip immunized with L11RX 5 days earlier contain some *Salmonella*-specific CTLs. The amount of activity detected using the ^{51}Cr-labelled "L11RX P815" represented approximately 20% of the total activity detected using the lectin-mediated assay. All CTL activity detected using the lectin-mediated assay had been found to be mediated by Lyt2.2$^+$ T cells, implying that the CTLs able to kill the "L11RX P815" were also Lyt2.2$^+$ T cells. This was confirmed using the standard MoAb and C depletion treatments prior to incubating the NW IPCs with the ^{51}Cr-labelled P815. A summary of the results observed in three individual experiments are provided in Fig. 6.9. Hence, even after a single dose of *Salmonella* small numbers of specific Lyt2$^+$ CTLs were induced.

6.2.3.3 Detection of specific CTLs in *in vitro* cultured SCs

To determine the specificity of the Lyt2.2$^+$ CTLs previously detected in the ConA cultured SC suspensions using the lectin-mediated cytotoxicity assay, ISCs harvested from mice receiving only a single dose or two doses of *Salmonella* and NSCs were cultured with ConA for 3 days and the cytotoxic activity towards ^{51}Cr-labelled "L11RX P815" (at a ratio of 25 effector cells to every target cell) was measured after a 4 hour period at 37°C. Data typical of four experiments are presented in Fig. 6.10 and illustrate that ConA cultured NSCs and ISCs from mice after a single dose of L11RX, did not kill the ^{51}Cr-labelled "L11RX P815". In contrast, however, cultured LC5 2° ISCs had considerable cytotoxic potential, exhibiting approximately 45% cytotoxicity, which

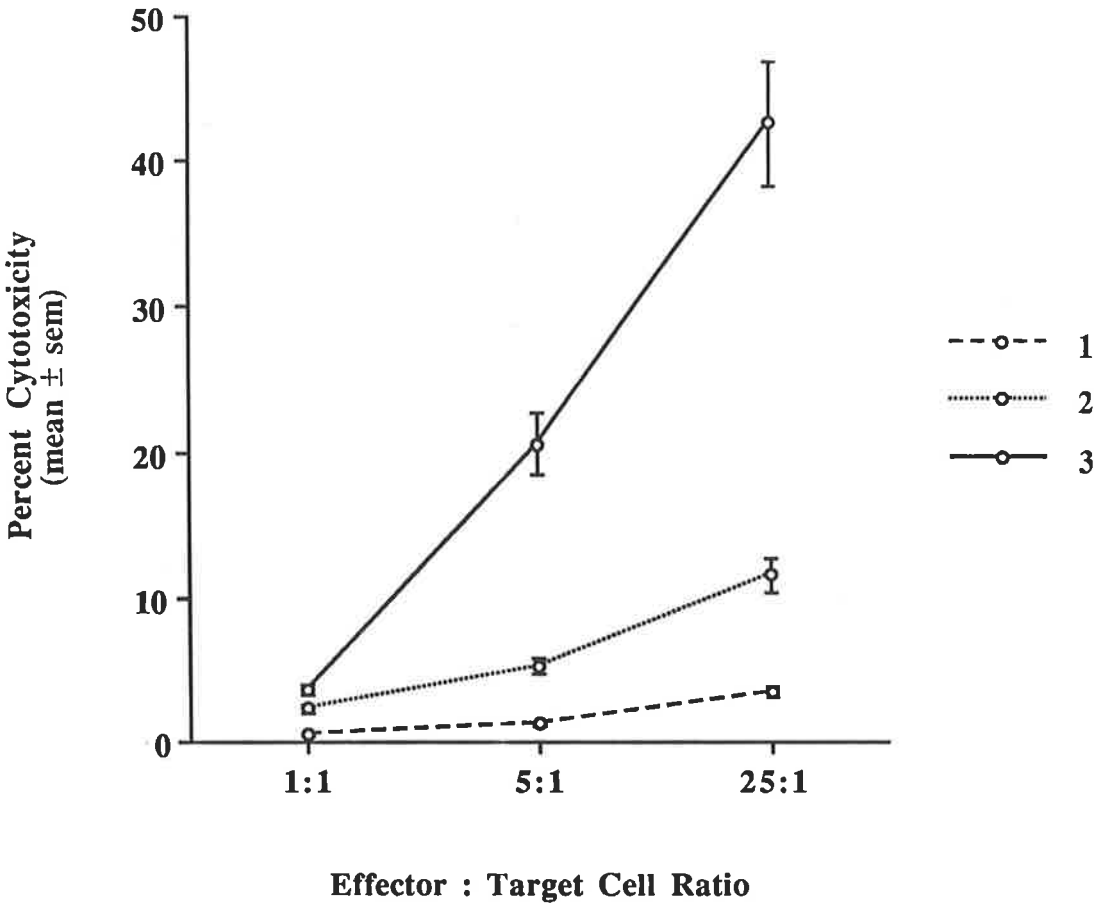

FIGURE 6.8 Analysis of the specificity of CTLs induced after primary immunization with L11RX

NW D 5 IPCs were incubated with ^{51}Cr-labelled "L11RX P815", which had been treated with ConA (3) or left untreated (2), and uninfected ^{51}Cr-labelled P815 (1) at effector:target cell ratios of 25:1, 5:1 and 1:1 (in quadruplicate) and the ^{51}Cr released after 4 hours incubation was measured. Results are presented as the percent cytotoxicity (mean ± sem) for each effector cell-target cell combination from a representative experiment.

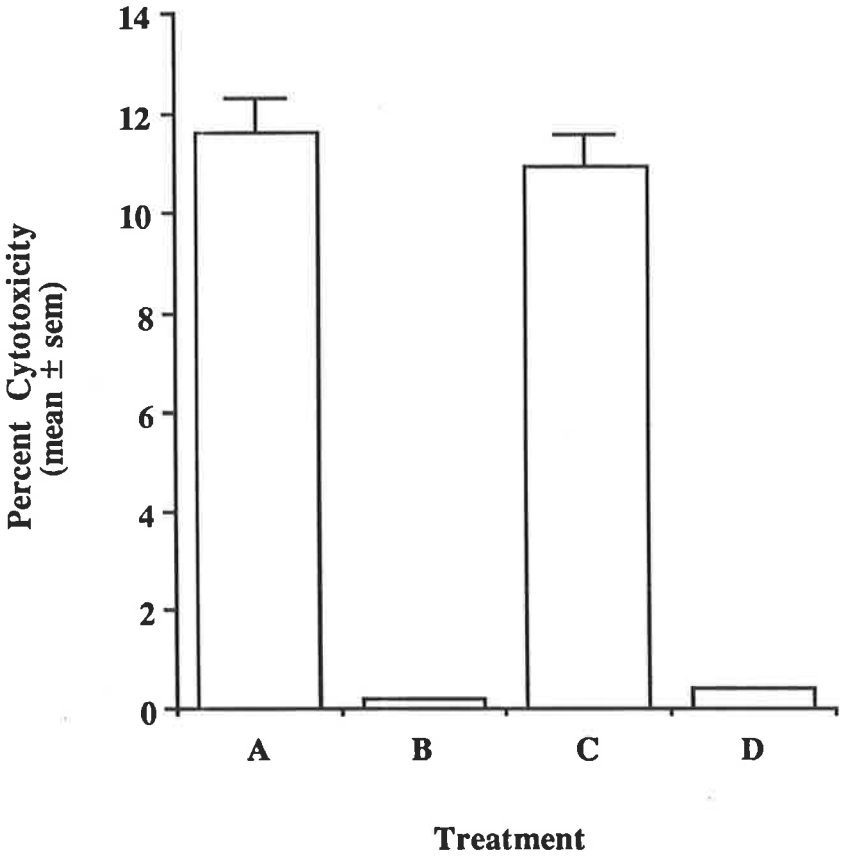

FIGURE 6.9 Phenotype of the *Salmonella*-specific cytotoxic cells present in NW D 5 IPC suspensions

NW D 5 IPCs were treated with either C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) and their ability to lyse "L11RX P815" was measured. Mixtures of the treated NW IPCs and the ^{51}Cr-labelled target cells at a ratio of 25:1 were incubated for 4 hours at 37°C, after which the amount of ^{51}Cr released was measured. Typical data from one of three identical experiments, expressed as the percent cytotoxicity (mean ± sem) for each effector cell population are presented.

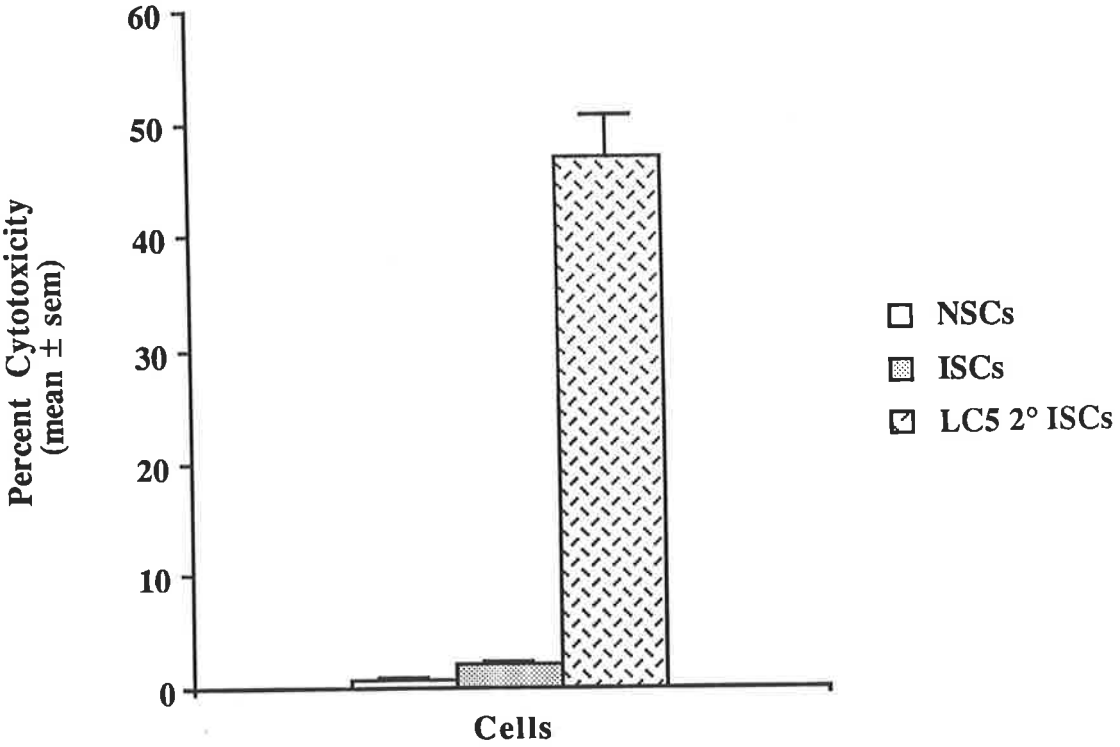

FIGURE 6.10 Examination of the specificity of the cytotoxic activity of ConA cultured SC populations

NSCs, D5 ISCs and LC5 2° ISCs (obtained from mice 5 days after secondary challenge) were cultured with ConA for 3 days, after which the ability of these populations to lyse "L11RX P815" was determined. ConA cultured SCs were incubated in quadruplicate with ^{51}Cr-labelled "L11RX P815" for 4 hours at 37°C (at ratios of 25:1) and the amount of ^{51}Cr in each supernatant was measured. Results from a typical experiment expressed as the percent cytotoxicity (mean ± sem) for each suspension are provided.

accounted for almost 50% of the total cytotoxic activity detected using the lectin-mediated assay. The amount of killing of uninvaded P815 by all three populations was minimal (approximately 3-5% cytotoxicity).

To determine the phenotype of these *Salmonella*-specific cytotoxic cells, ConA cultured LC5 2° ISCs were depleted of all T cells or either T cell subset by treatment with MoAbs and C and the remaining cytotoxic activity towards the "L11RX P815" was measured in the usual manner. Representative data from one of three identical experiments showing the percent cytotoxicity (mean ± sem) for each group of cells are provided in Fig. 6.11. Not surprisingly, depletion of cells expressing either the Thy1.2$^+$ and/or the Lyt2.2$^+$ phenotype removed all cytotoxic activity. Therefore, a population of *Salmonella*-specific Lyt2$^+$ CTLs was induced by secondary immunization with LC5, which required *in vitro* culture with ConA to induce the activation of their cytotoxic activity.

6.2.3.4 Summary

Similar to the observations reported in the *Listeria* system, Ag-specific Lyt2$^+$ T cells with cytotoxic activity were induced by infection with *Salmonella*, the activity and/or numbers of which were enhanced during a secondary infection. Such Ag-specific CTLs were present in the peritoneal cavities of mice as early as five days after primary immunization with L11RX, but did not appear in the spleens until a secondary challenge was given and required *in vitro* activation to be detected.

6.3 Summary and Conclusions

Intraperitoneal immunization of mice with *Salmonella* induced specific L3T4$^+$ DTH effector T cells in the spleens and peritoneal cavities (Chapter 5), whilst Lyt2.2$^+$ CTLs and Lyt2.2$^+$ DTH effectors were induced in the peritoneal cavities only. Following a second dose of *Salmonella*, the L3T4$^+$ DTH effector T cells appeared unchanged, but the involvement of the Lyt2.2$^+$ T cells was enhanced. A secondary

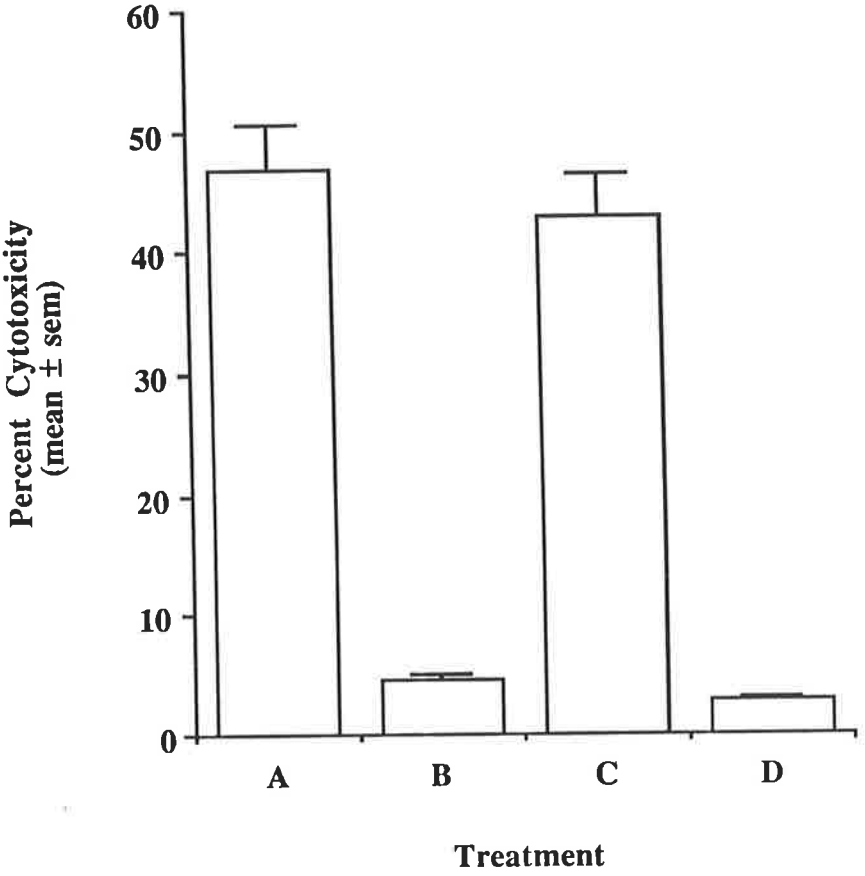

FIGURE 6.11 Phenotype of the *Salmonella*-specific CTLs in the ConA cultured LC5 2° ISC suspension

To characterize the phenotype of the cytotoxic cells, ConA cultured LC5 2° ISC suspensions were treated with C alone (A), α-Thy1.2 + C (B), α-L3T4 + C (C) or α-Lyt2.2 + C (D) before being incubated with the ^{51}Cr-labelled "L11RX P815" (at ratios of 25:1). Presented are the results obtained from one of three similar experiments, showing the percent cytotoxicity (mean ± sem) of each population.

infection with LC5 induced specific Lyt2.2$^+$ DTH effector T cells in both the peritoneal cavities and spleens of such mice. Coinciding with this, following a second dose of *Salmonella*, increased numbers of Ag-specific Lyt2.2$^+$ CTLs and/or Lyt2.2$^+$ CTLs with increased activity were found in the peritoneal cavity and persisted for up to 7 days. Ag-specific Lyt2.2$^+$ CTLs induced in the spleens of mice with a secondary C5 infection were only detected after *in vitro* culture with ConA. Therefore, during a secondary *Salmonella* infection, particularly C5, there was an increase in the activity and/or numbers of *Salmonella*-specific Lyt2.2$^+$ T cells. The implications of these observations will be discussed in the following Chapter.

CHAPTER 7

DISCUSSION

This study was carried out to obtain a detailed understanding of the cells involved in the immune response to *Salmonella* infection. It followed two main avenues of investigation. One approach was to look at the possibility that ip infection with L11RX modulated APC function of PCs, which resulted in the induction of cell-mediated as well as humoral immune responses. The other involved the characterization of the T cells induced by ip infection with *Salmonella* and the effect that secondary *Salmonella* infections had on T cell responses.

7.1 Analysis of the APC activity after *Salmonella* infection

7.1.1 Introduction

Activation of unprimed T cells requires Ag processing and presentation by APCs in association with Class I or Class II MHC products to ensure that appropriate nonspecific secondary signals can be provided to the T cells (Doherty *et al.*, 1976). Depending on the assay system used, these include IL 1 and/or IL 6 and other ill-defined cytokines (Oppenheim *et al.*, 1986; Hurme, 1987; Unanue and Allen, 1987; Kawakami *et al.*, 1989). Several groups have presented evidence indicating that the accessory cell requirements for the induction of T cell proliferation and IL 2 release for cells which mediate CMI are different from those necessary for the activation of T helper cells which provide help for B cells (Ramila and Erb, 1983; Ramila *et al.*, 1983; Erb *et al.*, 1985; Shigeta *et al.*, 1986), and there is also evidence suggesting that the form of immune response(s) induced may be determined by the type of APCs involved in presenting any particular Ag (Ramila *et al.*, 1985; Townsend *et al.*, 1986). Therefore, in the context of

immune responses to *Salmonella* Ags, it seemed reasonable to postulate that the APC(s) normally present in unimmunized animals are "programmed" to induce the "standard" humoral immune responses and that modulation of APC function is required before CMI responses are induced. As an extension, it follows that the live vaccines may be able to modulate the function of the stimulating APC population so that both humoral and cell-mediated responses are induced. This would account for the often reported observations that killed vaccines of *Salmonella* (and other IBPs) usually induce only humoral responses whereas live vaccines induce CMI responses as well.

The most likely candidates for the APCs involved in the immune response to ip infection with *Salmonella* are macrophages, which are present in large numbers in the peritoneal cavities of normal mice and are activated following ip infection with IBPs like *Salmonella*, *Listeria* and *Mycobacteria* (Mackaness, 1969; North, 1973; Cheers *et al.*, 1978) and show increased nonspecific bactericidal activity (Elberg *et al.*, 1957). Increases in the tumouricidal activity of peritoneal macrophages have also been reported following infection with BCG and *Salmonella* (Meltzer and Oppenheim, 1977; Ashley and Kotlarski, 1982; Britz *et al.*, 1982; La Posta *et al.*, 1982).

Macrophage activation has also been associated with an increase in synthesis, expression and/or secretion of enzymes, certain complement proteins, membrane proteins like Class II MHC products and soluble products such as IL 1 (Unanue, 1981; Adams and Hamilton, 1984). In this context, it is interesting to note the recent report that infection of macrophages with *Mycobacteria* induced IL 6 secretion by these cells (Flesch and Kaufmann, 1990), because this cytokine (in conjunction with IL 1) has been implicated in augmenting Ag presentation to T cells (reviewed by Wong and Clark, 1988). A variety of agents, including IBPs, can modulate the expression of Class II MHC products on the surface of macrophages. Intraperitoneal infection of mice with *Listeria* has been reported to result in an increase in expression of Class II MHC molecules on peritoneal macrophages (Beller *et al.*, 1980) in a T cell dependent manner (Lu *et al.*, 1981). IFN-γ released by activated T cells has been identified as the major cytokine involved in macrophage activation (North, 1981; Havell *et al.*, 1982; Kaufmann

and Hahn, 1982) and modulation of Class II MHC expression on macrophages (Beller, 1984). A correlation between an increase in Class II MHC product (I-A and I-E) expression by macrophages and an increase in their ability to stimulate Ag-specific primed T cell proliferation *in vitro* has been found using mice infected with *Mycobacteria* (Kaye and Feldmann, 1986). Interestingly, most of the cells with increased levels of expression of Class II MHC products did not contain intracellular organisms - presumably because live *Mycobacteria* (and to a lesser extent killed organisms) were found to *suppress* IFN-γ induced Class II MHC expression by macrophages (Kaye *et al.*, 1986). It follows that it would be interesting to know whether modulation of the expression of Class I MHC products on the macrophage (APC) populations of IBP-infected mice also occurs because Class I restricted Ag-specific $Lyt2^+$ T cells have been identified during infection of mice with *Listeria* and *Mycobacteria.*

The changes in expression of Class II MHC products and the release of cytokines required for T cell activation by macrophages of IBP-infected animals are consistent with the possibility that these cells play an important role in the induction of effective immune responses to these bacteria. Further support for the role of macrophages as accessory cells involved in the induction of Class I and Class II MHC restricted immune responses to infectious organisms is provided by a recent report from Debrick *et al.* (1991). They showed that macrophages act as accessory cells for $CD8^+$ CTLs during *in vivo* priming with influenza virus and that macrophages have the ability to ingest exogenous Ag in particulate form and present it in association with Class I MHC products. With this is mind, it seemed reasonable to compare the APC function of PCs obtained from mice ip immunized with L11RX (IPCs) with that of PCs from normal mice (NPCs). Since Ashley and Kotlarski (1982) reported that CMI could be detected 2-3 days after infection with *Salmonella*, these studies concentrated on the APC activity of PCs obtained from mice ip immunized with L11RX 1-3 days earlier.

7.1.2 Responding T cell populations

Ideally, analysis of APC activity should involve the induction of

Salmonella Ag-specific primary T cell responses. However, the induction of *in vitro* primary T cell responses to Ags other than allo-Ags has proven quite difficult and most studies of APC function are, therefore, carried out using *in vivo* primed T cells as the responding population. To ensure that Ags are presented to T cells only by the "test" APCs, it is necessary to use purified T cell suspensions, with little, if any, contaminating APC activity. When these studies were commenced, the standard way to assess the APC activity of cell suspensions was to determine their ability to restimulate primed T cells to proliferate and release LKs (IL 2) *in vitro* in response to specific Ags and T cell mitogens. Present studies indicated that a T cell suspension suitable for such *in vitro* experiments could only be obtained from PCs, because IL 2 release was never detected from T cells prepared from spleens (consistent with previous data of Attridge and Kotlarski, 1985a). [Detailed analysis of the cytokines released by SCs may clarify what "types" of T cells were induced in the spleens during *Salmonella* infection.] It was somewhat surprising to find that secondary challenge of L11RX immunized mice with either L11RX or LC5 did not enhance the PC yields above those obtained from mice 14 days after a primary infection with L11RX and that there was no expansion in the numbers of responsive T cells.

Although these PC suspensions contained responsive T cells, it is important to note that they could not be induced to proliferate unless adherent cells were removed prior to *in vitro* culture. There are many reports that cell suspensions obtained from IBP-infected mice exhibit diminished T cell responsiveness to antigenic and mitogenic stimuli (Bona *et al.*, 1976; Florentin *et al.*, 1976; Bennet *et al.*, 1978; Ellner, 1978; Orbach-Arbouys and Poupon, 1978; Allen and Moore, 1979; Navalkar *et al.*, 1980; Turcotte, 1981; Brett, 1984). Several groups have implicated an adherent macrophage-like population of cells in this process. For instance, Klimpel and Henney (1978) showed that SCs harvested from BCG-infected mice inhibited stimulation of normal T cell proliferation by ConA and allo-Ags, that the cells mediating this activity resided in an adherent, phagocytic cell population which lacked membrane associated Thy1 molecules and that they could be removed by passage on NW columns. They

proposed that a "suppressor" macrophage-like population was induced following BCG infection. Similarly, plastic or NW adherent, peritoneal exudate cells with macrophage morphology obtained from *Listeria*-infected animals, inhibit Ag-specific T cell proliferation (Jungi, 1980; Kaufmann *et al.*, 1982). In fact, Jungi (1980) was able to demonstrate that PC suspensions containing more than 10% macrophages always inhibited T cell proliferation. Furthermore, spleen cells from mice infected with live *Salmonella typhimurium* have been reported to exhibit reduced responsiveness to T (and B) cell mitogens, which was at least partially mediated by an adherent population of cells (Deschenes *et al.*, 1986). Consistent with the conclusions of others, and based on similar, circumstantial evidence it seemed likely that the cells involved in the suppressive activity induced by 11RX infection were macrophages. Although NW fractionation removed the inhibitory effect, this procedure removed more than just macrophages and neutrophils - some T cells were lost as well, since the amounts of IL 2 released by NW IPCs were always less than those released by U IPCs. Therefore, selective removal of the macrophages from the IPC suspension is necessary to positively identify macrophages as the inhibitory population.

It is possible that the inability of the U IPCs to proliferate reflects the heterogeneity of this population, because large numbers of macrophages and neutrophils may have sterically interfered with the APC-T cell interactions necessary for activation of T cell proliferation. In other words, NW fractionation may have simply ensured that adequate T cell-accessory cell interactions could occur. However, since removal of most of the neutrophils from the U IPC population had no effect on the proliferative responses of these T cells, it seems unlikely that this is the explanation. It was also established that "classical" Lyt2.2$^+$ suppressor cells were not involved. This possibility was examined because it has been shown quite recently that the spleens of mice infected with BCG contain a suppressor T cell population which belongs to the CD8$^+$ subset (Sussman and Wadee, 1991). Following *in vitro* activation of these CD8$^+$ T cells with factors derived from *M. tuberculosis*, a soluble suppressive factor was released which was able to suppress lymphocyte proliferation. Furthermore, treatment of adherent cells harvested

from the peripheral blood of Mantoux-positive individuals with *Mycobacteria* has been reported to induce the release a suppressor cell activating factor (SCAF) that activated $CD8^+$ suppressor T cells; these in turn could suppress lymphocyte proliferation and LK release by lymphocytes treated with a variety of mitogens or Ags (Wadee *et al.*, 1980 and 1983; Wadee and Rabson, 1981).

The mechanism of suppression was not determined. Although macrophages have been shown to release prostaglandin E (PGE) which is able to inhibit T cell proliferation by blocking IL 2 secretion (Chouaib *et al.*, 1985), this was an unlikely mediator because indomethacin, which inhibits PGE synthesis (Webb and Nowowejski, 1977), was added to all cultures. In addition, F11RX induced the release of considerable amounts of IL 2 from U IPCs, despite its failure to induce T cell proliferation. It is unlikely that other factors, similar to SCAF were released by activated macrophages, because supernatants harvested from these cultures exhibited no suppressive activity (Vordermeier *et al.*, 1990, and additional data, not shown).

7.1.3 Analysis of the APC activity

Although preliminary examination of the PCs harvested 1, 2 or 3 days after ip injection with L11RX revealed that infection induced a cellular exudate, an influx of neutrophils and changes in the morphology of the peritoneal macrophages, no change in the APC activity of these PCs was detected until the third day of infection, when a minor population of inhibitory, adherent cells was detected. A more detailed analysis indicated that the inhibitory effect of D 3 IPCs was at least partially nonspecific and not simply due to increased destruction of Ag by macrophages with enhanced metabolic/degradative ability because inhibition was also observed when D 3 IPCs were used to stimulate primary allogeneic responses. It was also established that this suppressive activity persisted for a reasonably long period of time (up to 6 weeks post infection, data not shown) and was similar to the kinetics of appearance of the suppressor cells induced by mycobacterial infection (Turcotte *et al.*, 1978; Collins and Watson, 1979).

An alternative explanation for the lack of induction of T cell proliferation is that it is mediated by the direct effects of the APCs. Since induction of T cell activation requires Ag processing and presentation of adequate amounts of Ag-MHC product complexes and the production of secondary signals by the APCs, it is possible that alteration of one or more of these activities could affect the ability of these APCs to stimulate T cell responses. There are three obvious ways in which this could occur. Firstly, if the level of expression of MHC products by APCs is modulated by infection, then this could change the amounts of Ag-MHC complexes presented by the APCs to the T cells and therefore, modify their ability to stimulate T cell proliferation. Secondly, the metabolic activation of macrophages (APCs) following infection with *Salmonella* may have increased their capacity to degrade Ags, subsequently limiting the amount of Ag available for presentation to the T cells. Finally, it is also possible that infection with *Salmonella* induced a change in the profile of cytokines released by the APCs and although the signals required for T cell activation may still be being produced, other cytokines could be released which antagonize the activity of the T cell stimulatory signals thereby preventing T cell activation.

The fact that the first signal for T cell activation requires Ag processing and presentation of adequate amounts of correct Ag-MHC complexes is supported by work carried out by Matis *et al.* (1983), who concluded that the magnitude of the proliferative response of specific T cell clones is a function of the product of Ag concentration and the number of Class II MHC (Ia) molecules expressed on APCs. This was based on the findings that *(i)* there was an inverse relationship between the Ag concentration required for maximum proliferation and the number of APCs present in culture, *(ii)* a decrease in the number of relevant Class II MHC molecules per APC resulted in the need for an increase in Ag concentration for a maximal response, and *(iii)* in the presence of α-Ia Abs, higher concentrations of Ag were required for maximal response. Results of adding large amounts of F11RX to *in vitro* cultures were consistent with these findings and also confirm reports that large amounts of Ag prevent the induction of T cell proliferation *in vitro* (Hecht *et al.*, 1983; Matis *et al.*, 1983; Ceredig

and Corradin, 1986; Suzuki *et al.*, 1988). Therefore, this suggests that if L11RX did induce modulation of expression of Class II MHC products on macrophages, different amounts of Ag would be required when D 3 IPCs, instead of NPCs, were used as APCs. However, as mentioned, FACScan analysis revealed no remarkable changes in the cell surface expression of Class II (nor Class I) MHC molecules on PCs following *Salmonella* infection which could explain the inability of large numbers of D 3 IPCs to induce T cell proliferation. [Since this effect appeared to be mediated by a minor population of cells it is possible that the MHC expression on a minor population of cells was modulated by 11RX infection, but no evidence for this was obtained from the preliminary FACScan analysis carried out in these studies.] The possibility that infection with L11RX had modulated the expression of MHC products on the APCs was also addressed by measuring the Ag dose responses induced by the APCs and by their ability to induce allogeneic responses. Although not accounting for the failure to induce allogeneic responses, some evidence was obtained to suggest that the inability of D 3 IPCs to induce proliferation of *Salmonella*-primed T cells was at least partly because insufficient Ag-MHC product complexes were generated due to increased, rapid degradation of Ag by the adherent cell population. Use of the fixative PFA provided more direct evidence that D 3 IPCs had enhanced Ag degradative/processing activity, which may have contributed to the lack of T cell responses observed in the presence of large numbers of these cells and the standard (and reasonably low) dose of F11RX. It follows that the inability to induce allogeneic responses may occur as a result of differences in the processing of "self peptides", resulting in the generation of "different" MHC molecules on the APC surface which could influence the magnitude of T cell proliferation induced. With this in mind, it is important to consider the possibility that the inability of D 3 IPCs to induce *Salmonella*-specific and allo-Ag-specific T cell responses may simply be coincidental and might be mediated by completely unrelated mechanisms.

Interestingly, *in vivo* pulsing of D 3 IPCs with Ag resulted in the selective removal of the inhibitory population observed with both the *Salmonella*- and allo-Ag-specific T cell responses, suggesting that these effects at least were mediated by

the same population of cells. Cytospin smears of these suspensions showed that the NPCs contained macrophages with large numbers of cell-associated bacteria, whilst the macrophages of the D 3 IPCs had fewer bacteria per cell. This suggested that the inhibitory subpopulation present in D 3 IPCs had ingested large numbers of F11RX before becoming attached to the surface of the peritoneal cavity. This conclusion was supported by the finding that macrophages of *in vivo* Ag-pulsed D 14 IPCs contained only small numbers of bacteria, like those of D 3 IPCs, whereas the adherent population of D 14 IPCs pulsed with a similar amount of F11RX for 1 hour at 37°C *in vitro* contained a small portion of macrophages with very large numbers of bacteria per cell as well as many macrophages with numbers comparable to those seen in suspensions of the *in vivo* Ag-pulsed cells (data not shown). The cell yields recovered from mice following *in vivo* pulsing with F11RX were only slightly reduced and examination of the cell profiles of these populations revealed little change, suggesting that only a minor portion of cells had been removed and supporting the earlier conclusion that relatively small numbers of inhibitory cells were present in the D 3 IPC population.

The final possibility that the inhibitory effect of the D 3 IPCs was mediated by soluble suppressive factors, or by the antagonistic activities of "new" cytokines released by a minor adherent cell population was considered and although this seemed the most logical explanation, no evidence was obtained to support this possibility.

See Addendum Comment 1

7.1.4 Characterization of the T cells stimulated by Ag presented by NPCs and D 3 IPCs

In the presence of *Salmonella* Ags, both types of APCs induced mainly *Salmonella*-specific L3T4$^+$ T cells to proliferate. There was no evidence that Lyt2.2$^+$ T cells were induced to proliferate, although all the proliferating T cells (Thy1.2$^+$) were never completely removed by treatment with α-L3T4, α-Lyt2.2 and C, indicating the presence of a double negative T cell subset. The demonstration that most γδ T cells belonged to the double negative T cell population (Meuer *et al.*, 1984), makes it reasonable to postulate that *Salmonella* Ags induced some γδ T cells to proliferate.

These observations are consistent with the large amount of evidence from human and murine systems which demonstrates that a significant proportion of γδ T cells recognize mycobacterial Ags, including mycobacterial heat shock proteins (Augustin *et al.*, 1989; Haregewoin *et al.*, 1989; Holoshitz *et al.*, 1989; Janis *et al.*, 1989; Modlin *et al.*, 1989; O'Brien *et al.*, 1989; Kabelitz *et al.*, 1990; Pfeffer *et al.*, 1990; Havlir *et al.*, 1991; Inoue *et al.*, 1991), and the suggestion that γδ T cells may participate in host resistance against these and other bacterial pathogens. Furthermore, recent work carried out by Munk *et al.* (1990) showed that γδ T cells exist in the peripheral blood of many healthy donors and that these can be readily expanded and activated by *in vitro* stimulation with *Mycobacteria* and several Gram positive bacteria. Such activated γδ T cells expressed IL 2 receptors, secreted IL 2 and lysed *M. tuberculosis*-pulsed target cells. Using a murine model, Griffin *et al.* (1991) were able to demonstrate that substantial numbers of γδ T cells accumulated in mice given primary mycobacterial infections, in parallel to the accumulation of αβ T cells. Further supporting the possibility that γδ T cells play a part in the defence against invasion by various pathogens, was the report from Ohga *et al.* (1990) that during an ip infection of *Lm* γδ T cells appear in the peritoneal cavity before αβ T cells can be detected. The fact that a significant proportion of the γδ T cells recognize phylogenetically conserved stress/heat shock proteins, which have been implicated as immunodominant Ags (Holoshitz *et al.*, 1989, O'Brien *et al.*, 1989; Raulet, 1989), raises the possibility that at least some of the γδ T cells may be involved in the first line of defence by recognizing and eliminating stressed autologous cells, such as infected cells (Koga *et al.*, 1989; Young and Elliot, 1989). Recognition of these highly conserved Ags may also account for the cross protection observed during the early stages of infection. Recent work carried out by Hiromatsu *et al.* (1992) has demonstrated a protective role for γδ T cells in the early stages of infection with *Lm*. Depletion of γδ T cells by *in vivo* administration of MoAbs against γδ-TCRs reduced the ability of these mice to clear the bacteria during the early stages of the infection. *In vitro* analysis of γδ T cells from *Listeria*-infected mice revealed that these cells released MAF and macrophage chemotactic factor, and proliferated *in vitro* in response to PPD from

M. tuberculosis or a heat shock protein of *M. bovis* (but not to heat killed *Listeria*; Hiromatsu *et al.*, 1992). *S. typhimurium* organisms inside macrophages have been shown to respond by increasing their synthesis of a number a bacterial proteins, including the two heat shock proteins GroEL and DnaK, which are immunodominant Ags for many infectious organisms (Buchmeier and Heffron, 1990). Therefore, it is possible that ip infection with *Salmonella* induced γδ T cells specific for various *Salmonella* Ags, including the heat shock proteins, which could respond *in vitro* to F11RX. Clearly, a more detailed analysis of these responses is required to confirm the presence of *Salmonella*-specific γδ T cells, but preliminary experiments carried out in our laboratory using α-γδ TCR MoAbs and C have indicated that such a population of γδ T cells is generated during *Salmonella* infection and can be induced to proliferate *in vitro* when cultured with ConA (Bertram, unpublished).

Because initial analysis of the role of MHC products in MLR reactions *in vitro* indicated that L3T4$^+$ T cells played a major role in the response by providing "help" in the form of LKs like IL 2, which were required for the activation and/or expansion of effector Lyt2.2$^+$ T cells (reviewed by Fitch, 1986), MLR assays were carried out with NPCs and D 3 IPCs to assess whether L11RX induced modulation of the APC activity, as assessed by a change in the phenotypes of the T cells induced to respond. It was somewhat surprising (and disappointing) to find that the allo-Ags present on both F1 NPCs and D 3 IPCs induced a large proliferative response involving Lyt2.2$^+$ T cells, with only a minor contribution from cells expressing the L3T4$^+$, Thy1.2$^+$ phenotype. All the cytotoxic activity generated was allo-Ag-specific and mediated by Lyt2.2$^+$ T cells and depletion of T cell subsets prior to culture revealed that the L3T4$^+$ T cells made only a small contribution to the initiation of the proliferative and the CTL responses. Even when none, or very few L3T4$^+$ T cells were present, Lyt2.2$^+$ T cells were still induced to proliferate and become cytotoxic, indicating that activation of the Lyt2.2$^+$ T cells in this system could occur independently of the L3T4$^+$ T cell subset, although L3T4$^+$ T cells could provide some "help" for the activation and/or expansion of the Lyt2.2$^+$ T cells. These data and conclusions are consistent with

the findings of others who have shown a functional overlap between the T cell subsets - that both T cell subsets can express cytotoxic activity and have the ability to proliferate and release IL 2 *in vitro* (MacDonald *et al.*, 1980; Andrus *et al.*, 1981; Tite and Janeway, 1984; Heeg *et al.*, 1987a and 1987b). Several groups have also observed L3T4$^+$ T cell-independent activities of Lyt2.2$^+$ T cells in *in vitro* culture of allogeneic lymphoid cells (Singer *et al.*, 1984; von Boehmer *et al.*, 1984; Mizuochi *et al.*, 1985 and 1986; Sprent and Schaefer, 1985 and 1986) and also using *in vivo* systems (Sprent *et al.*, 1986; Heeg *et al.*, 1988).

As discussed in Chapter 1, likely candidates for the APCs involved in the activation of unprimed T cells required for induction of CMI to IBPs (and for induction of MLRs) were macrophages, which were present in large numbers in both NPC and D 3 IPC populations. Each PC suspension expressed both Class I and Class II MHC products and therefore had the potential to stimulate both L3T4$^+$ and Lyt2.2$^+$ T cell subsets. The identity of the APCs responsible for induction of allo-Ag-specific responses in unprimed T cells remains controversial (Sprent and Schaefer, 1990), because various T cell subsets appear to require different allo-APCs (Sprent and Schaefer, 1989) producing different APC-derived costimulator molecules for the induction of maximal responses (Weaver and Unanue, 1990). Although Inaba *et al.* (1987) were able to show that *in vitro* cultured spleen or peritoneal macrophages possessed no APC function for unprimed CD8$^+$ T cells, Sprent and Schaefer (1989) found that fresh, thioglycollate induced Class II$^-$ macrophage populations were able to elicit very strong MLRs by CD8$^+$ T cells, suggesting that activation of macrophages was required to generate a Class I$^+$, Class II$^-$ subset which could elicit allogeneic CD8$^+$ T cell responses. Therefore, if macrophages require activation to be able to induce Class I restricted allogeneic responses then this would explain why the Lyt2.2$^+$ T cell subset predominated in the proliferation observed in response to the D 3 IPCs. However, no direct evidence demonstrating that infection with L11RX induced a Class II$^-$ macrophage population similar to that characterized by Sprent and Schaefer (1989) was obtained. [Use of double colour immunofluorescent labelling techniques would allow the detection of such a

population (if present) on the FACScan.] However, since the NPCs used in these studies were not activated and also induced mainly Lyt2.2$^+$ T cell responses, activation of the macrophages was not crucial for the induction of Lyt2.2$^+$ T cells in this system. Interestingly, a recent report from McCormack *et al.* (1991) demonstrated that a subset of normal mouse splenic macrophages could elicit allo-Ag-specific CD8$^+$ T cell responses by unprimed CD8$^+$ T cells. Hence, evidence is available which illustrates that in some systems, macrophages are capable of inducing significant allo-Ag-specific Class I restricted T cell responses, without the involvement of L3T4$^+$ T cells. It was unfortunate that the NPCs of F1 mice induced mainly Lyt2.2$^+$ T cells, because this did not permit the analysis proposed - the possible modulation of APC function following L11RX infection to favour the induction of Lyt2.2$^+$ T cells.

7.2 L3T4$^+$ T cells and Lyt2.2$^+$ T cells are induced during *Salmonella* infections

Induction of both L3T4$^+$ and Lyt2$^+$ T cells in mice infected with *Listeria* is explained by the suggestion that production of listeriolysin O allows these organisms to escape phagolysosomes and to enter the cytosol, thus providing ready access to the Class I Ag processing pathway required for the induction of Lyt2$^+$ T cells (discussed extensively in Section 1.2.5.2). Because no such factor has been described for either *Mycobacteria* or *Salmonella* it might be expected that Lyt2$^+$ T cells are not induced following infection with these organisms. However, this is not true because Lyt2$^+$ T cells have been reported to play a role in immunity to infection with *Mycobacteria* (Orme and Collins, 1984; Müller *et al.*, 1987; Roch and Bach, 1990). In this context, the demonstration that macrophages infected with *M. microti* had reduced levels of expression of MHC Class II MHC products (Kaye *et al.*, 1986), despite the fact that the level of expression of Class II MHC molecules on uninfected cells was increased during infection (Kaye and Feldmann, 1986), may be highly significant and possibly, could also apply to *Salmonella*. It could be argued that Class II$^-$ macrophages infected

with IBPs present specific Ags of these organisms only in association with Class I MHC molecules.

Two obvious mechanisms can be proposed to explain how antigenic determinants of IBPs like *Salmonella*, which do not readily exit phagolysosomes as immunogenic *Listeria* organisms do, may associate with Class I MHC products and activate $Lyt2^+$ T cells. One possibility is that bacteria multiplying within the infected macrophages "break out" of the phagolysosomes into the cytosol of the cell and/or that some bacterial Ags "leak" into the cytosol, thereby gaining access to the Class I Ag processing pathway. Evidence obtained in the present studies support the possibility that *Salmonella* organisms multiplying within vacuoles of infected cells can "break out" and enter the cytosol, as the Cytospin smears of P815 cells infected with 11RX or C5 clearly show bacteria within vacuoles and also "free" in the cell cytoplasm. A second mechanism may be provided by the direct binding of exogenous peptides to Class I MHC products. Some evidence is available supporting both these possibilities. Recent work carried out by Harding *et al.* (1991) has shown that small amounts of liposome-encapsulated Ag were released from the endocytic compartments into the cytosolic compartment and were subsequently presented in association with Class I MHC products. Indirect evidence that small, exogenous antigenic peptides may be able to bind to Class I MHC products is available. For example, Carbone *et al.* (1988), demonstrated that small peptides of OVA appeared to associate directly with Class I MHC molecules of Class II^- cell lines (without being processed via the endogenous pathway), because such Ag-pulsed cells were able to induce $CD8^+$ Ag-specific CTLs. It follows that any *Salmonella*-derived peptides released by macrophages during degradation of the bacteria may associate with Class I MHC products directly and be presented to the appropriate T cells. Another alternative is that at least some exogenously derived Ags degraded within the endosomal compartment may be able to associate with Class I MHC products. Because Class I MHC products on activated T cells can recycle through endosomes (Tse and Pernis, 1984), it is possible that, whilst in endosomes, they may be able to bind to exogenously derived antigenic peptides. The very recent work carried out by Hochman

et al. (1991) using fluorescent β_2-microglobulin to label membrane Class I MHC molecules, has demonstrated the constitutive endocytosis of Class I MHC molecules and dissociation of β_2-microglobulin within the endosomes of ConA activated T cells and lymphoma cells of mice. They have proposed that although this process is reversible, it is likely that in the recycling endosomes of T cells Class I MHC molecules undergo conformational changes, with dissociation and reassociation of β_2-microglobulin resulting in changes in the peptide binding site. It follows that if this process occurs in APCs, it might play an important role in Ag presentation. Therefore, although there is evidence for such recycling only in T cells, it is possible that during infection with IBPs Class I MHC products of APCs may also recycle through endosomes, associate with processed antigenic peptides present in these compartments, be re-expressed on the cell surface and subsequently induce specific Lyt2$^+$ T cells.

However it happens, results presented from these studies show that *Salmonella* Ags do access the Class I Ag processing pathway. Intraperitoneal infection of mice with *Salmonella* induced Ag-specific L3T4$^+$ and Lyt2.2$^+$ T cells and, like secondary infection with *Listeria*, the activity of Lyt2.2$^+$ T cells was enhanced by secondary immunization with *Salmonella*, particularly C5 which was shown to persist longer than L11RX. Although secondary challenge with *Salmonella* increased the involvement of Ag-specific Lyt2.2$^+$ T cells, the detection of these cells proved to be more difficult than the detection of Ag-specific Lyt2$^+$ T cells in other systems, in which the use of live Ags was sufficient to demonstrate their presence. The use of live organisms to elicit Ag-specific DTH reactivity has successfully detected Ag-specific Class I restricted DTH effector T cells in several other systems. For example, Jungi *et al.* (1982b) reported that when live *Listeria* organisms were used as the eliciting Ag, transfer of DTH to normal rats was restricted to the A region of the rat MHC (a region which is homologous to the murine H-2K locus), whilst a listerial extract induced a B region restricted response (which is equivalent to the mouse I or Class II region of the MHC). Similarly, the cells responsible for the DTH to Sendai virus were found to be Class II restricted T cells when killed virus was used as the eliciting Ag, while both Class I and

Class II MHC restricted T cells were induced by live virus (Ertl, 1981). However, using live *Salmonella* as the antigenic stimulus to elicit DTH induced similar responses in all primed T cell populations, with L3T4$^+$ T cells mediating all the activity observed. These data were interpreted as indicating that fewer Lyt2$^+$ T cells were induced by *Salmonella* infection than *Listeria* infection, (and were masked by the preferential induction of Class II restricted responses) and therefore implied that a more stringent system was needed to detect these cells. This was confirmed using a system where the presentation of *Salmonella* Ags was restricted to an association with Class I MHC molecules, which induced a response by Lyt2.2$^+$ DTH effector T cells. Since approximately 35% footpad swelling was the maximum response induced by the "LC5 P815", it is possible that all the Ag-specific Lyt2.2$^+$ DTH effector T cells present were not activated, because of insufficient Ag and/or inadequate processing and presentation of Ag by the invaded P815. It was evident from the Cytospin smears of the "LC5 P815" that only some of the cells contained bacteria. In addition, although "LC5 P815" were able to induce Lyt2.2$^+$ T cells, P815 is not regarded as a "classical" APC line and therefore, may not possess all the attributes necessary for efficient processing and/or presentation of Ag to T cells, despite the fact that such cell lines (including P815 and EL4) have been successfully used by others to present specific Ags to Class I MHC restricted T cells (eg. Carbone *et al.*, 1988) and allo-Ag to unprimed T cells (Sprent and Schaefer, 1986). The presence of Lyt2.2$^+$ T cells capable of mediating DTH reactivity was enhanced by secondary *Salmonella* infection and although not much information is available with other IBPs, these data are consistent with the observations made with *Listeria*.

The *in vitro* cytotoxicity assay also detected Lyt2.2$^+$ T cells in cell suspensions harvested from *Salmonella*-infected mice. Induction of CTLs following infection with other IBPs such as *Listeria* and *Mycobacteria* has been reported by several groups. Recent studies have demonstrated that T cell populations from *M. leprae*- and *M. tuberculosis*-infected individuals contained CD4$^+$ CTLs which lysed monocytes/macrophages pulsed with mycobacterial Ags (Mustafa and Godal, 1987; Ottenhoff *et al.*, 1988; Hancock *et al.*, 1989; Kumararatne *et al.*, 1990; Boom *et al.*,

1991). In addition, Ag-specific Lyt2$^+$ CTLs have been detected in the draining lymph nodes of mice following intradermal infection with irradiated *M. leprae* (Chiplunkar *et al.*, 1986). Similarly, *Listeria*-specific Class I and Class II restricted T cell clones, established from T cell suspensions harvested 6-8 days after infection with *Listeria* and capable of lysing *Listeria*-infected target cells, were detected by Kaufmann *et al.* (1986 and 1987, respectively).

The initial work to detect the presence of specific L3T4$^+$ and/or Lyt2.2$^+$ CTLs following infection with *Salmonella* was carried out using the lectin-mediated cytotoxicity assay. This revealed that a peak Lyt2.2$^+$ CTL (but no L3T4$^+$ CTL) response was induced in the PC population five days after infection with L11RX and secondary infection with either C5 or 11RX enhanced their cytotoxic activity. Although the kinetics of induction of CTLs by other IBPs has not been well characterized, it is interesting to note that to establish *Listeria*-specific cytotoxic T cell clones, T cells were also harvested from mice reasonably early (6-8 days) after infection with *Listeria* (Kaufmann *et al.*, 1986 and 1987). Once an Ag-specific cytotoxicity assay was developed, using ^{51}Cr-labelled "L11RX P815" as the target cells, it was found that up to 50% of the activity detected by the lectin-mediated assay was mediated by *Salmonella*-specific Lyt2.2$^+$ CTLs (with greater Ag-specific activity being detected during secondary infection, particularly with C5). In contrast, no cytotoxic activity was detected in the SC suspensions obtained from mice following primary or secondary *Salmonella* infections unless the cells were activated by *in vitro* culture with ConA. Lyt2.2$^+$ CTLs were detected in all ConA cultured SC suspensions using the lectin-mediated lysis assay, but *Salmonella*-specific Lyt2.2$^+$ CTLs were present only in the SCs obtained from mice with a secondary infection of C5, suggesting that secondary C5 infection had induced, but not activated, a population of Ag-specific Lyt2$^+$ CTLs in the spleen, since only activated cells express lytic activity. Similarly, Lyt2$^+$ CTLs have been detected in the spleens of *Listeria*-infected mice following *in vitro* culture of these cells with irradiated, infected accessory cells and IL 2 (Kaufmann *et al.*, 1986). As in the DTH system, it was likely that not all the Ag-specific CTLs were detected using the

"L11RX P815" as targets, because a significant proportion of the P815 cells did not contain any bacteria and therefore, were unlikely to be presenting specific Ags in the context of Class I MHC molecules. Obviously, a more effective method of Ag-pulsing needs to be developed to allow a more accurate study of the specific CTLs (and possibly DTH effectors) induced. An obvious choice would be to "load up" an appropriate cell population with well defined peptides of *Salmonella* Ags, via the osmotic lysis of pinosomes (as described by Moore *et al.*, 1988) to ensure that virtually all cells present Ag in association with Class I MHC products on their surface. A pure preparation of the low molecular weight 11RX Ag described by Vordermeier and Kotlarski (1990) would be useful for such an approach.

In addition to these functional assays which indicated an increase in the activity of Ag-specific Lyt2.2$^+$ T cells after secondary infection with *Salmonella*, it was interesting to find that there was also an increase in the proliferative activity of Lyt2.2$^+$ T cells. As discussed earlier (Section 7.1.2), initial examination of the proliferative responses of the *Salmonella* primed T cells prepared from PC or SC suspensions revealed no differences in their responsiveness to F11RX or ConA. However, more detailed analysis of the responding cells demonstrated that secondary infection with *Salmonella*, especially C5, induced an increase in the proliferative activity of the Lyt2.2$^+$ T cell subset. It was surprising to find that even in the presence of killed Ag (a form of Ag which is usually presented in the context of Class II MHC products), Class I restricted T cells were induced to proliferate, suggesting that, following secondary infection APCs able to present Ags in the context of both Class I and Class II MHC products were present. As in T cell suspensions obtained after primary immunization with L11RX, the results obtained suggested that a population of $\gamma\delta$ T cells may also have been induced to proliferate in these cultures.

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Although both L3T4$^+$ and Lyt2.2$^+$ T cell subsets have been detected following infection with *Salmonella*, the roles played by these populations in the effective clearance of bacteria remain to be established. It is possible that both may play a role in controlling/eliminating *Salmonella* organisms and there are obviously two mechanisms

by which this could be mediated. Firstly, Ag-specific L3T4$^+$ and/or Lyt2$^+$ CTLs could induce the direct lysis of infected cells exposing the bacteria released to uptake and killing by activated macrophages. [Only Ag-specific Lyt2.2$^+$ CTLs, but no L3T4$^+$ CTLs, were induced following primary and secondary infections with *Salmonella*.] A second mechanism involved in controlling bacterial infection may result from the release of cytokines by T cells responsible for the activation of macrophages and activation and/or expansion of effector T cell subsets. Preliminary data from Nauciel (1990) have indicated that L3T4$^+$ T cells play a role in the clearance of an infection of a temperature sensitive mutant of *S. typhimurium* and Boom *et al.* (1991) have established the importance of the CD4$^+$ T cells in controlling the growth of *M. tuberculosis*. These T cells activate macrophages by secreting cytokines and are also able to lyse Ag-pulsed monocytes/macrophages. A likely role for the Lyt2.2$^+$ CTLs in the control of the infection, would be to lyse infected macrophages which are unable to kill the bacteria they contain, thus releasing the bacteria for uptake and killing by activated macrophages. The presence of Class I restricted CTLs would be particularly advantageous if the expression of Class II MHC products was down-regulated on macrophages infected with live organisms, as has been observed with *Mycobacteria* (Kaye *et al.*, 1986), because the Lyt2.2$^+$ CTLs could still lyse these cells. It is possible that since all cells express Class I MHC products, whilst only a proportion express Class II MHC molecules, Class II restricted L3T4$^+$ CTLs may not even be required for such a role in immunity to IBPs. The fact that a large number of microorganisms, including *Salmonella*, have been shown to enter mammalian cells other than macrophages (Finlay and Falkow, 1989), and that the genetic determinants in *Salmonella* which are essential for expression of the invasive phenotype have been identified (Elsinghorst *et al.*, 1989), suggest that *Salmonella* organisms may have the potential to invade a wide range of (non-phagocytic) cell types *in vivo* which do not express Class II MHC products. If this did occur, there would be an even greater need for Class I MHC restricted CTLs and other T effector cells to be induced.

As already suggested, cytokines released by Ag-specific T cells may play

an important part in the clearance of bacterial infection. L3T4$^+$ T cells may also play an essential role by providing help for the activation and/or expansion of specific Lyt2.2$^+$ T cells (as in the case of *Listeria*). L3T4$^+$ T cells (and possibly the Lyt2.2$^+$ T cells) may release IFN-γ and other ill-defined cytokines which activate macrophages which are required to kill IBPs more effectively (Nathan *et al.*, 1983; Khor *et al.*, 1986; Weyser *et al.*, 1987) and/or modify the Ag presenting function of APCs by regulating Class II (and possibly Class I) MHC expression (eg. Sztein *et al.*, 1984). Support for this possibility has been provided by numerous groups who have evidence for the participation of the LKs IFN-γ and TNF in the clearance of IBP infections (Bancroft *et al.*, 1981 and 1987; Buchmeier and Schreiber, 1985; Havell, 1988; Kindler *et al.*, 1989; Nakano *et al.*, 1990; Tite *et al.*, 1991).

7.3 Concluding remarks

This study provides a basis for more detailed analysis of the functional roles of T cell subsets in immunity to *Salmonella*. With hindsight, it may be useful to analyse the APC function of IPCs using purified, primed T cells obtained from mice with a secondary C5 infection, as such suspensions have been shown to contain larger numbers of Lyt2.2$^+$ T cells capable of proliferating *in vitro*. Furthermore, more detailed examination of the APC activity of cells present following secondary infection may also provide information related to the modulation of APC activity during infection with live bacteria, because APCs present in the T cell suspensions prepared from mice with secondary *Salmonella* infections were capable of inducing Lyt2.2$^+$ T cells to proliferate even when F11RX was used as the Ag. It has become apparent that analysis of the cytokines released by the various APC populations, using recently developed assays (eg. Dallman *et al.*, 1991; Feldmann *et al.*, 1991; Sander *et al.*, 1991), may provide a more useful method for characterizing their ability to induce various T cell subsets.

Furthermore, it would be interesting to establish whether the L3T4$^+$ and Lyt2.2$^+$ T cells detected in these studies comprise distinct functional subpopulations

within each subset, *ie.* whether the *Salmonella*-specific L3T4$^+$ T cells can be subdivided into the Th1 and Th2 groups proposed by Mosmann *et al.* (1986) and whether this also applies for the Lyt2.2$^+$ T cells. Recent work carried out by Tsukada *et al.* (1991) demonstrated the dissociated development of T cells mediating DTH and T cells responsible for immunity to *Lm* and that, although both populations were initially categorized as Th1 cells, T cells mediating protection were further characterized by their ability to release IFN-γ. Detailed characterization of the cytokines released by T cells prepared from the spleens (and peritoneal cavities) of *Salmonella*-infected mice is necessary since the existence of Class II MHC restricted T cells which are able to mediate DTH reactivity and release MAF/IFN-γ, but which cannot release detectable amounts of IL 2, have been detected, and do not "fit" the proposed division of L3T4$^+$ T cells into the Th1 and Th2 subsets (described by Mosmann *et al.*, 1986). Therefore, identification of other cytokines released by 11RX primed splenic T cells would clarify the "types" of T cells induced in the spleens following *Salmonella* infection. It is possible that the T cells mediating *Salmonella*-specific DTH reactivity or cytotoxicity (or protection) represent distinct subpopulations of cells, which could be categorized by cell surface markers, such as the CD45 or Ly6C molecules (see Chapter 1), and by the cytokines they release.

Thus in summary, the new approaches for the analysis of APC and T cell function which have become available since these studies were undertaken provide the most obvious way to extend the studies reported in this thesis. Definition of the profiles of cytokines released by both the APCs and T cells of *Salmonella* immunized mice, obtained after both primary and secondary challenge with *Salmonella*, should indicate whether APCs are modified by *Salmonella* infection and enable further characterization of the T cell subsets induced and their involvement in the clearance of infection.

APPENDIX

Certain material presented in this thesis has been published or manuscripts are in preparation for publishing.

Published material:

Kotlarski, I., Pope, M., Doherty, K. and Attridge, S. R. (1989) The *in vitro* proliferative response of lymphoid cells of mice infected with *Salmonella enteritidis* 11RX. *Immunol. Cell Biol.* **67:** 19-29.

Vordermeier, H-M., Pope, M., and Kotlarski, I. (1990) Presentation of *Salmonella* antigens by peritoneal cells of normal and *Salmonella*-infected mice. *Immunol. Cell Biol.* **68:** 161-172.

Manuscripts in preparation:

Pope, M. and Kotlarski, I. The effect of *Salmonella* infection on murine T cell responses. I. Detection of *Salmonella*-specific L3T4$^+$ and Lyt2.2$^+$ T cells which can proliferate *in vitro* and mediate DTH reactivity.

Pope, M., Kotlarski, I., and Doherty, K. The effect of *Salmonella* infection on murine T cell responses. II. Induction of Lyt2.2$^+$ CTLs following primary and secondary *Salmonella* infection.

Pope, M. and Kotlarski, I. Antigen presenting characteristics of peritoneal cells of *Salmonella enteritidis* 11RX-infected mice.

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ADDENDUM

Comment 1 - Re: Data presented in Table 3.2; 3.17; 4.3; 4.4; 4.10; 4.14 and 4.20 and in Fig 4.4.

Although most of these data have been discussed extensively in the text, a consistent finding which deserves some emphasis is that addition of high concentrations of Ag (100 μg/ml) to *in vitro* cultures containing NW IPCs and NPCs resulted in smaller proliferative responses than those obtained using lower doses of Ag. These data are consistent with the earlier reports of Matis *et al.* (1983) and Lamb *et al.* (1983; *J. Exp. Med.*, **157:**1434-1447) and the recent observations of Kulkarni *et al.* (1991; *Immunol. Cell Biol.*, **69:**27-), using other Ags. These workers concluded that the magnitude of Ag-specific T cell proliferation induced was dependent on the concentration of the Class II MHC coded products expressed on APCs and the amount of Ag available, with excessive amounts of Ag being inhibitory. The finding that T cell proliferative responses to high doses of F11RX and NPCs were altered in the presence of anti-Ia monoclonal antibodies supports this view (Table 3.17).

In contrast, the reverse effect was observed when 10^5 D3 IPCs were used to present Ag, where low doses of Ag did not induce proliferation, whilst increasing Ag concentration to 100 μg/ml resulted in some proliferation. Explanations based on increased Ag degradation have been considered in the text and found to be inadequate, since such doses of D3 IPC were also unable to stimulate allogeneic T cells. With hindsight, these observatons may also be related to the observations highlighted above. This is because reexamination of the data presented in Fig. 4.4 indicates that D3 IPC do contain a subset (or subsets) of cells expressing high levels of both Class I and Class II MHC products which are absent or at much lower levels in suspensions of NPC. Therefore, it is possible that the inhibitory cells detected in D3 IPC suspensions were these highly activated cells which express large amounts of MHC products on their surface and remain in the peritoneum after F11RX injection.

Finally, it is worth emphasising that different cells may be induced to proliferate in response to large amounts of F11RX, and/or their specificity may be different from the specificity of the T cells induced to proliferate with standard amounts of Ag. Vordermeier

and Kotlarski (1990) have established that the T cells induced to proliferate *in vitro* are specific for *Salmonella* Ags and that some of these are shared by other members of the *Enterobacteriaceae*. As indicated in the text, unpublished observations indicate that proliferating populations may also contain γδ T cells. The proportion of these may increase when large doses of F11RX are used and some B cells may have also been induced to proliferate, especially when U IPC were used. Further detailed examination of the cells induced to proliferate and characterisation of D3 IPC subsets is obviously required to distinguish between these possibilities.

Comment 2 - Re: Difference in numbers of bacteria associated with NPC and IPC (p.106).

It is unlikely that this differeence reflects the larger numbers of cells in the peritoneum following immunization since less that 1/10 IPC contained any bacteria and those that did usually contained only 3 or 4, with an absolute maximum of 20 organisms, which was seen very rarely.

Comment 3 - Re: Data in Table 4.12.

It is important to note that the interpretation of these results is made difficult by the possibility that some adherent cells were lost during the 2 hour incubation. If the cells lost during the 2 hr incubation were actively phagocytic and were also responsible for mediating the inhibitory activity, then the idea of increased degradation of Ag by these suspensions is weakened.

Comment 4 - Re: Data in Table 4.25.

An alternative explanation for these data is that the $L3T4^+$ and $Lyt2^+$ subsets were activated quite independently, with no synergism being required. To distinguish between these alternatives would require further experiments using highly purified suspensions of the two T cell subsets.