S A L M O N E L L A   I N F E C T I O N   I N   M I C E

Ronald Bruce Johnson, B.Sc. (Hons.) (Adelaide)

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Department of Microbiology and Immunology,
The University of Adelaide,
Adelaide, South Australia.
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LIST OF ABBREVIATIONS USED IN TEXT

F1097 - *Escherichia coli* F1097

11RX - *Salmonella enteritidis* 11RX

C5 - *Salmonella typhimurium* C5

LPS - lipopolysaccharide

P/S - polysaccharide

DTH - delayed-type hypersensitivity

HU - haemagglutinating unit

EAT - Ehrlich Ascites Tumour

PEC - peritoneal exudate cells

SRBC - sheep red blood cells

F1 - (C57BL/6J♂ X BALB/cJ♀)F1 hybrid mice

i.p. - intraperitoneally

i.v. - intravenously

ABSTRACT

These studies evaluated the ability of several vaccines to protect mice against infection with *Salmonella typhimurium* C5. They not only revealed information on the efficacy of the vaccines examined, but also on fundamental features of host immunity to this infection. The main findings were as follows:

(a) *Salmonella enteritidis* 11RX established a carrier state in (C57BL/6J♂ X BALB/cJ♀)F1 mice, stimulating their reticuloendothelial system and elevating levels of serum antibody specific for *Salmonella typhimurium* C5.

(b) Mice infected with *Salmonella enteritidis* 11RX were moderately resistant to *Salmonella typhimurium* C5.

(c) The injection of *Salmonella typhimurium* C5 polysaccharide into *Salmonella enteritidis* 11RX-infected mice reduced the level of specific antibody, and abrogated their ability to control and eliminate a *Salmonella typhimurium* C5 challenge.

(d) In contrast, the acquisition of specific antibody, by either active immunization or passive transfer, enhanced the resistance of *Salmonella enteritidis* 11RX-infected mice to typhoid.

(e) *In vitro*, *Salmonella typhimurium* C5 organisms could adhere to and were destroyed by activated macrophages harvested from *Salmonella enteritidis* 11RX-infected mice. This required the presence of specific antibody.

(f) Although mice immunized with *Listeria monocytogenes* acquired an activated reticuloendothelial system and

resistance to a homologous challenge, they remained effectively unable to control a Salmonella infection.

(g) An extract of *Coxiella burnetii* stimulated the reticuloendothelial system and induced immunity to *Listeria monocytogenes* and transplanted tumours. However, it conferred resistance to challenge with *Salmonella typhimurium* C5 only in mice which had acquired elevated levels of specific antibody.

(h) Dextran sulphate, which stimulated both humoral and cellular immune responses, protected mice against infection with *Salmonella typhimurium* C5

It is apparent from these observations that the generation of both humoral and cellular immune responses is necessary for resistance to *Salmonella typhimurium*. In fact, the expression of cell-mediated antibacterial immunity appeared to be limited by the availability of specific antibody. The implication is that activated macrophages, like normal macrophages, require opsonins to phagocytose and kill typhoid bacilli. These results are discussed in relation to the current knowledge of immunity to intracellular bacterial parasites.

This thesis contains no material previously submitted by me for a degree in any University and to the best of my knowledge and belief it contains no material previously published or written by another person except where due reference is made in the text.

Ronald Bruce Johnson

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ERRATA

P. 12, line 28	(Plant and Glynn, 1979) not (Rosenstreich, 1980)
P. 14, line 1	(Plant and Glynn, 1979) not (Rosenstreich, 1980)
P. 26, line 13	(Mackaness, 1969; Kaufmann et al., 1979), not (Mackaness, 1969)
P. 35, line 4	(BALB/cJ♀ X C57BL/6J♂), not (C57BL/6J♂ X BALB/cJ♀)
P. 47, line 18	.....Sephadex G100 (Pharmacia) and ....., not .....Sephadex G100 and .....
P. 56, line 4	.....medium using the manufacturer's instructions (Grand ....., not medium (Grand .....
P. 57, line 26	Insert (Preliminary experiments indicated that leaving cultures at 4°C for more than an hour did not result in any increase, or decrease, in the number of rosettes observed.) after ....., did so.
P. 59, line 15	..... in SPF LACA strain or ..... not ..... in SPF or .....
P. 67, line 13	Add ....., at least in the mouse strains studied.
P. 71, line 28	delete .....phagocytose and .....
P. 95, line 18	..... treated ..... not ..... opsonised .....
P. 95, line 28	..... treated ..... not ..... opsonised .....
P. 96, line 3	..... opsonins ..... not ..... antibody .....
P. 96, line 5	..... antibodies to C5 LPS were not detectable in serum from normal mice above ..... not ..... these antibodies were not detectable above .....
P.104, line 6	..... decreased ..... not ..... completely abrogated
P.104, line 14	..... decreased ..... not ..... abrogated .....
P.105, line 22	..... number of bacteria recovered ..... not ..... level of bacteraemia .....
P.106, line 12	..... decreased ..... not ..... abolished .....
P.107, line 13	..... decreased ..... not ..... abrogated .....
P.108, line 14	(.....; North,1973; Kaufmann et al., 1979) not (.....; North, 1973)

CHAPTER 1

INTRODUCTION

Typhoid fever

One of the great achievements of the 20th century has been the prevention and control of communicable diseases in the economically developed world. In the case of typhoid, the disease caused by infection with *Salmonella typhi*, the success has been due largely to improvements in water supply, sanitation and personal hygiene. In some developed countries, incidence of the disease has fallen to such an extent that its occurrence is unusual. International travel is now an important factor in the epidemiology of the disease. In 1975 the incidence of typhoid and paratyphoid fevers in the United States was reported at only 1 case per million of population (W.H.O. World Health Statistics Report, 1976), and a survey by the Center for Disease Control revealed that 33% of these patients acquired the disease outside the United States (Ryder and Blake, 1979).

However, in the developing countries with their lower standards of sanitation, typhoid fever is still a major medical problem. During 1975, Chile, for example, reported 6,011 cases of typhoid which represents an incidence rate of 601 cases per million population (W.H.O. World Health Statistics Report, 1976). In addition to poverty and ignorance, contamination of food and water supplies occasionally occurs as the result of various disasters such as flood and famine and these have also caused quite serious epidemics. An effective public

immunization program is therefore necessary to protect specifically vulnerable populations unless good water supplies and sanitation become available.

Therapy with chloramphenicol was first used in 1948 and since then has changed the course of the disease and improved the prognosis for patients. Although effective in reducing the mortality rate, antibiotic therapy fails to break the chain of infection and unfortunately the morbidity rate remains high. In addition, the recent appearance of episomal resistance to chloramphenicol in strains of *Salmonella typhi*, has raised some apprehension about the future of this form of therapy. In 1972, a typhoid epidemic in Mexico was caused by a strain which was resistant to a number of antimicrobial agents, including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclin (Weissman *et al*., 1973; Rodriguez-Leiva, 1979; Davies, 1979). It would appear that if typhoid fever is to be controlled and eventually eliminated, then some form of effective immunization will be necessary.

Typhoid vaccines have been available since 1896 when Wright injected killed typhoid bacilli into 2 Indian medical officers. The vaccine was assumed to be protective as illness did not occur following inoculation with viable *Salmonella typhi* (Hornick *et al*., 1970a). Despite these early beginnings the currently available alcohol-killed and heat-killed, phenol modified typhoid vaccines have achieved only a modest amount of success. World Health Organisation field trials have shown that parenteral immunization with these conventional vaccines has significantly reduced the incidence of the disease in endemic areas, particularly in children. Not all vaccinated individuals, however, are protected (Hejfec *et al*., 1966).

When the conventional vaccines were evaluated on volunteers, only low levels of protection could be demonstrated and even these were ablated when the challenge was merely increased to a dose which would induce clinical disease in half of the unvaccinated subjects (Hornick _et al._, 1970b; Collins, 1979). Studies on volunteers have also shown that while these killed vaccines induce good humoral immune responses, they fail to induce a cell-mediated immune response (Nath _et al._, 1977). However, even if enteric fever itself induces a cell-mediated immune response in humans, as it certainly does in mice (Blanden, Mackaness and Collins, 1966), the protection conferred appears to be only moderate and quite transient. Relapses, although usually mild, are common and occur in about 10 - 20% of typhoid patients (Hornick _et al._, 1970b). Re-infection with organisms of a different phage type was reported in 1953 when a Royal Air Force Unit in Egypt was exposed to 2 outbreaks of typhoid fever in a period of 5 months. Prior infection with _Salmonella typhi_ appeared to confer only a moderate degree of specific resistance as approximately 20% of the men exposed to both outbreaks contracted the disease twice (Marmion, Naylor and Stuart, 1953). It is therefore important to produce an effective live vaccine. However, attempts to develop living oral typhoid and cholera vaccines have not been particularly successful (Collins, 1979).

Although much progress has been made in reducing the incidence of typhoid, it does appear that, before the disease can be controlled and eliminated, we must be able to protect susceptible populations with an effective immunization program. The situation regarding prophylactic vaccination against typhoid is still unsatisfactory, particularly when compared

to the near absolute success which has been obtained with smallpox vaccination. Before vaccines can be improved, we need to know more about the actual infection and host defence mechanisms.

Pathogenesis

Typhoid fever is a disease which is unique to man. The Salmonella typhi and paratyphi are not known natural pathogens of any other animal species, nor do laboratory animals experimentally infected with these organisms acquire an illness simulating typhoid fever. Chimpanzees, when infected with Salmonella typhi develop symptoms typical of the disease seen in man. Nevertheless, in comparison to man, they are relatively resistant and the infection is less severe (Edsall et al., 1960). Unfortunately these organisms are readily inactivated in mice, and fail to produce a progressive disease. Investigation of this infection has been of little value. The reasons for such striking differences in host susceptibility have yet to be determined (Collins and Carter, 1978).

The most productive experimental model of typhoid has been based on a few strains of Salmonella which cause a typhoid-like disease in mice. As early as 1928 it was realized that a natural pathogen of mice, Salmonella typhimurium, induced such a disease in these animals (Orskov, Jensen and Kobayashi, 1928). Subsequently it was shown that the experimental infection of mice with Salmonella enteritidis could also be used as a satisfactory model for natural systemic Salmonellosis (Kligler and Olitzki, 1931).

The pathogenesis of enteric fever, as it occurs in humans, chimpanzees and mice, is generally characterised by both a

primary intestinal infection and a secondary systemic bacteraemia. Infection is usually the result of ingesting contaminated food and water. The majority of these ingested bacteria move quickly through the gastrointestinal tract due to peristalsis, cause only transient contamination and are of little pathogenic significance. Bacteriological studies have revealed that typhoid bacilli are excreted in stools within 24 hours of ingestion and then frequently continue to be shed for up to several weeks (Hornick *et al*., 1970a). While most regions of the gut are relatively free of Salmonella, the largest number can be found in the caecum and large intestine. However, when intestinal peristalsis is interrupted by morphine treatment, significant infection of the small bowel occurs as well (Miller and Bohnhoff, 1962).

Direct intralumenal inoculation into the caecum, colon and rectum has shown that systemic infection is possible without the involvement of the small intestine (Carter and Collins, 1974). Some workers have suggested that these bacteria may be able to pass through, or between, the epithelial cells of the caecum (Ozawa *et al*., 1973). Although Salmonella in the lower bowel are infective, the primary site of bacterial penetration of the intestinal epithelial lining is thought to occur as a result of an infection of the distal ileal mucosa and its Peyer's patches. Continued bacterial multiplication results in the infection spreading quickly to the distal mesenteric lymph nodes which drain the ileum and caecum. Lymph nodes draining the stomach, duodenum and colon remain free of Salmonella at this stage (Carter and Collins, 1974). However this distribution is certainly not absolute and infection of the tonsilar lymphatics (Gaines *et al*., 1968a; Edsall *et al*.,

1960), upper respiratory tract (Carter, Woolcock and Collins, 1975) and other areas of the gastrointestinal tract have been reported following oral challenge, particularly when larger challenge doses were used (Carter, 1975).

From the regional lymph nodes, the organisms disseminate *via* the thoracic duct to the bloodstream and on to various organs, predominantly the liver and spleen. Further bacterial multiplication results in fever, headache and abdominal pain, followed by toxaemia which causes the patient to pass into a coma and die (Hornick *et al*., 1970a; Davies, 1975). Morbidity and mortality are largely the result of the systemic phase of the disease.

Generally, systemic typhoid is the result of an oral infection, although some workers have shown that other routes of infection may occur experimentally or naturally. Tannock and Smith (1972) routinely infected mice intranasally with *Salmonella typhimurium* while conjunctival inoculation has also been used (Duguid *et al*., 1976). The conjunctival route may be important in some circumstances, as Moore (1957) demonstrated that the transmission of Salmonella infections was prevented when guinea pigs were fitted with goggles. Because bacteria enter the bloodstream after an oral challenge, the parenteral challenge route is often used experimentally, for convenience, as fewer organisms are required to establish a systemic infection (Collins, 1972).

An interesting aspect of typhoid fever is that up to 5% of infected humans become asymptomatic carriers (Huckstep, 1962), with women outnumbering men by a ratio of 3:1 (Hornick *et al*., 1970a). These people act as a reservoir of typhoid fever, and may excrete as many as 10^{11} virulent organisms per

gram of faeces. Frequently these bacilli are carried in foci of infection in the biliary tree. It is assumed that these people are protected by local humoral or cellular immune mechanisms, although this has not been clearly established (Hornick *et al.*, 1970a). Experimentally, *Salmonella typhimurium* has been found to persist in mice, with organisms residing in the liver and spleen (Hobson, 1957a) and the upper respiratory tract (Tannock and Smith, 1971). However, to date, the host-parasite relationship in carriers is poorly understood.

Natural resistance to Salmonella infection

The outcome of a particular Salmonella infection such as typhoid, like any host-parasite interaction, depends on a multiplicity of factors. Host, parasite and environment all participate in determining both the nature of the infection, and finally resistance or susceptibility. Although many of these factors are known, some have yet to be identified. For example, the unique adaptation of *Salmonella typhi* to man is not well understood.

The gastrointestinal tract presents a formidable barrier to enteric pathogens. Collins and Carter (1974) found that conventional mice are surprisingly resistant to an oral challenge of viable *Salmonella enteritidis*. They noted that these organisms were eliminated at an impressive rate within hours of inoculation. The relatively few organisms which remained were able to colonize the intestine and eventually bring about a fatal infection. In another study they reported that within 6 hours of infection, only 0.25% of the inoculum could be recovered from the entire gut (Carter and Collins,

1974). A number of environmental factors have already been identified which can interfere with this capacity of the gastrointestinal tract to eliminate pathogenic Salmonella.

The stomach, with its highly acidic gastric secretions, acts as an important non-specific host defence mechanism by reducing the number of viable bacteria ingested. When this barrier has been compromised, otherwise healthy individuals are vulnerable to enteric infection. Experimentally, bicarbonate is frequently included in an oral challenge to neutralize this acidity. Collins, when investigating the fate of Salmonella enteritidis given orally to CD-1 mice, reported that overnight starvation and pretreatment with bicarbonate increased the relative survival of the challenge inoculum 10-fold (Collins, 1972). Likewise Hornick, Greisman, Woodward, DuPont, Dawkins and Snyder, (1970a), when establishing enteric infections in human volunteers, found that small doses of sodium bicarbonate prior to oral challenge, increased both the rate of illness and the number of organisms which could be recovered in stools. Normally, when bacteria are ingested with solid food, the intragastric retention time is considerable and in normo-acidic subjects this results in a drastic reduction in the number of viable bacterial cells. However, when the stomach is empty, small quantities of contaminated water are able to pass quickly through this organ, thus minimizing its bactericidal potential (Mossel and Oei, 1975; Mossel, 1976). This last observation would help to explain why very few bacteria can occasionally trigger disease, particularly during periods of starvation when animals have been shown to have a predisposition to such enteric infections.

Variations in diet and environmental conditions are known

to affect an animal's natural resistance. Tannock found that depriving mice of food, water and bedding for 48 hours, markedly alters their gastrointestinal microbiota. When mice which had already carried an asymptomatic *Salmonella typhimurium* infection, were subjected to this stress, they were found to harbour more of these bacteria in their bowel (Tannock and Smith, 1972; Tannock and Savage, 1974). Even certain nutritional factors have been shown to modify the composition of the faecal flora and to affect resistance to various experimental infections (Dubos and Schaedler, 1962). Studies such as these have shown that both the normal bacterial flora within the gut, as well as foreign pathogenic bacteria, have a close relationship to various nutritional and related forms of stress.

In fact, these indigenous bacteria do themselves affect the overall ecology of the gastrointestinal tract, and play an important role in contributing to a host's natural resistance. Some of them are so successful at colonizing the gut, particularly the large bowel, that they can proliferate freely and yet at the same time create an environment which is antagonistic to recognised enteric pathogens such as Salmonella or Shigella. Certain obligate anaerobes belonging to the genus Bacteroides, one of the most common groups of inhabitants of the murine colon, have been found to produce acetic and butyric acids. These volatile fatty acids, which can be isolated from both faecal material and anaerobic cultures, have been found to inhibit the growth of *Salmonella typhimurium* (Meynell, 1963) and *Salmonella enteritidis in vitro* (Bohnhoff, Miller and Martin, 1964). Predictably, the antibiotic treatment of conventional mice greatly increased

their susceptibility to *Salmonella typhimurium* (Meynell and Subbaiah, 1963) and to *Salmonella enteritidis* (Bohnhoff and Miller, 1962; Collins, 1970). Bohnhoff and Miller (1962) found that, following the administration of a single dose of streptomycin to CF-1 mice, as few as 10 organisms were sufficient to infect half the mice, whereas 10^6 organisms were normally required to infect half of the conventional CF-1 mice. Repopulation of the intestinal tract of these antibiotic-treated mice with Bacteroides species was found to restore normal resistance (Miller and Bohnhoff, 1963).

Similarly, germ free mice have been found to have a marked increase in susceptibility to an oral challenge with either *Salmonella typhimurium* or *Salmonella enteritidis*. Collins and Carter (1978) reported that conventional mice, when challenged orally with *Salmonella enteritidis*, had an LD_{50} of 5×10^6 organisms, while for germ free mice the LD_{50} was only 3-5 organisms. Abrams and Bishop (1966) obtained similar results using *Salmonella typhimurium*. These studies on germ free mice also revealed that the normal gut flora has a direct impact on the anatomy of the gut, particularly the small intestine. The structure of the lamina propria, the half-life of the mucosal cells and even the surface area are all influenced by exposure to these indigenous bacteria. The reduced intestinal motility found in germ free mice may contribute to their susceptibility to enteric infections by reducing the rate of intestinal emptying (Abrams and Bishop, 1966).

The importance of the normal intestinal flora in resistance to typhoid can be inferred from the many experiments which have demonstrated that both antibiotic-

treated and germ free mice are extremely susceptible to enteric infection. In such mice, Salmonella multiply as well in the gut as in nutrient broth (Meynell and Subbaiah, 1963; Ruitenberg *et al*., 1971).

Finally, there have been some indications that a prior enteric infection with an avirulent organism such as *Escherichia coli*, enhances the natural resistance of the host to more virulent pathogens. Competition for nutritional elements and adherence sites would be the more likely mechanisms for such phenomena (Savage, 1970; Savage and McAllister, 1970; Ozawa *et al*., 1973).

In summary, these studies have revealed that following ingestion, pathogenic bacteria find themselves in a hostile environment, competing against the host's impressive natural defence mechanisms. The level of resistance is influenced by many factors including gastric acidity, intestinal motility, nutritional status and the indigenous microbial flora in the gut itself. Usually, infection only occurs when one or more aspects of the host's natural defences have been compromised or bypassed, or in the event of an overwhelming inoculum such as occurs during an epidemic. It matters not whether one considers typhoid in humans or in various animal models, such as in the mouse, the fact that disease is the exception and not the rule, emphasises the importance of natural resistance mechanisms in the overall Salmonella-host relationship.

Genetics of resistance to infection

To survive, an invading pathogenic bacterium must not only be able to penetrate the external protective barriers of the host, it must also cope with a very powerful host defence - the

immune system. Briefly, resistance to extracellular bacteria such as Diplococcus pneumoniae, Klebsiella pneumoniae and Streptococcus pyogenes correlates well with antibody levels and the 'humoral' immune response. However, with intracellular bacteria such as Salmonella typhi, Listeria monocytogenes and Mycobacterium tuberculosis, resistance is dependent on macrophage activation and the 'cellular' response. During a Salmonella infection of mice this cell-mediated response appears towards the end of the first week. It is manifested by the ability of the infected animals to control bacterial growth and is accompanied by the appearance of delayed-type hypersensitivity reactions to extracts of Salmonellae (Collins and Mackaness, 1968; Collins, 1974). Immunity to typhoid will be discussed more thoroughly in later sections.

Animal and human populations vary markedly in their susceptibility to infectious agents and the involvement of genetic factors is of obvious clinical and veterinary importance. The genetic basis of the immune response has been studied in mice for nearly 50 years, and several studies have indicated that a large number of genes control resistance to various micro-organisms. For example, the gene locus Lsh, which controls the intrahepatic and intrasplenic growth of Leishmania donovani, has been mapped to the proximal end of chromosome 1; a second locus, Ric, controlling resistance to infection with Rickettsia tsutsugamushi, has been mapped to the middle of chromosome 5; and a third locus Ity, influences resistance to Salmonella typhimurium and is located near Lsh on chromosome 1 (Rosenstreich, 1980). Research in this field has accelerated greatly during the past few years, particularly since the development of recombinant inbred mouse strains.

Inbred mouse strains, differing in susceptibility to *Salmonella typhimurium*, were first demonstrated by Webster in 1933 and since then a number of papers have indicated that resistance to this organism is under polygenic control. Gowen (1960) found a gradation in the susceptibility of 10 strains of mice to *Salmonella typhimurium* 11C, which suggested that resistance to this organism is probably due to several factors rather than a single attribute. Similarly Hormaeche (1979a) recently reported inbred mouse strains of low, intermediate and high natural resistance to *Salmonella typhimurium* C5. Survival was found to be dependent on 2 separate events, namely a slow net growth rate during the first 4 days and secondly, the appearance of a cell-mediated response at the end of the first week. A slow net growth rate of bacteria during the early phase of the infection is of critical importance , as it allows the host time to develop the cell-mediated response required to control and eliminate the infection. However, very little is known about the mechanism by which this is achieved. (Hormaeche, 1979a). This parameter of resistance, the early net growth rate, is under the control of a single autosomal gene or gene cluster (Plant and Glynn, 1976; Hormaeche, 1979b). The ability to slow down the early net growth rate can be transferred with bone marrow cells (Hormaeche, 1979c). Inheritance of this trait was not linked to either the ability to mount delayed-type hypersensitivity reactions (Hormaeche, 1979a), coat colour, H-2 genes or antibody responses to ovalbumin or ovomucoid (Plant and Glynn, 1976). The gene was designated Ity, with r and s the respective resistant and susceptible alleles (Plant and Glynn, 1977), and as previously stated, was mapped close to the Lsh

locus on chromosome 1 (Rosenstreich, 1980).

While a slow bacterial net growth rate is important for the host's survival, it is nevertheless insufficient for complete resistance to Salmonella typhimurium C5. Following intravenous inoculation, the infection in DBA/2 mice has a long incubation period, with bacterial net growth being characteristically slow but unchecked and therefore eventually overwhelming the hosts. These mice have a defective cell-mediated response and, as expected, they manifest poor delayed-type hypersensitivity reactions to bacterial antigens (Hormaeche, 1979a). Genetic studies suggest that the cell-mediated response is under complex, polygenic control (Hormaeche, 1979b).

Endotoxins, which are complex lipopolysaccharide molecules, produced by Salmonellae and other Gram-negative bacteria, may play an important role in the pathogenesis of the disease caused by these organisms. Release of these endotoxins from the bacterial cell wall usually occurs after the bacteria are killed. Endotoxins are known to elicit a variety of physiological responses, including fever, hyperglycaemia, abortion and circulatory disturbances, while large doses cause irreversible shock and severe diarrhoea (Davis et al., 1973). It has been reported that male S strain mice are considerably less resistant to infection with Salmonella typhimurium 11C than are the females, and that they will succumb to a large dose of killed organisms of this strain. Death occurs comparatively early and has been attributed to the rapid release of endotoxin from digested bacteria (Gowen, 1960). It is not clear whether this difference in the susceptibility of the sexes is due to a direct linkage of resistance genes or

to some hormonal influences.

Lipopolysaccharides are extremely immunogenic substances, capable of stimulating large specific humoral responses. However, their ability to activate macrophages (Alexander and Evans, 1971) and induce division in and polyclonal immunoglobulin synthesis by B lymphocyte populations has aroused more interest (Andersson, Sjöberg and Möller, 1972). The C3H/HeJ mouse strain is poorly responsive to all of these biological effects of LPS, although its mitogenic response to other polyclonal B-cell activators, such as dextran sulphate and purified protein derivative of tuberculin, is unimpaired (Watson and Riblet, 1974; Coutinho, Gronowicz and Sultzer, 1975; O'Brien *et al*., 1980).

Genetic studies suggested that LPS responsiveness was under the control of a single gene, designated Lps, with d and n the defective and normal alleles respectively. The Lps gene locus has been mapped to the middle of chromosome 4, closely linked to the major urinary protein locus Mup-1 (Watson *et al*., 1978). The C3H/HeJ mice are also highly susceptible to infection with *Salmonella typhimurium* despite their resistance to the toxic effects of endotoxin (Von Jeney, Gunther and Jann, 1977) and the evidence available suggests that this susceptibility is conferred by an autosomal gene, either identical to or closely linked to, the Lps locus (O'Brien *et al*., 1980).

Genetic control of the humoral immune response has also been shown to influence the susceptibility of mice to *Salmonella typhimurium*. An X-linked gene has been described which controls the response of mice to murine typhoid, and it is probably the same as the xid gene which controls B cell

differentiation, (O'Brien *et al.*, 1979). Rowley and Jenkin (1962) suggested that the susceptibility of mice to infection with *Salmonella typhimurium* may be due to their inability to produce certain opsonins and that this partial tolerance to the pathogen was caused by cross-reactivity of the Salmonella antigen(s) to a self component(s). More recently Wooley and Ebringer (1980) demonstrated that CBA mice produced higher titres of agglutinating antibodies to *Salmonella typhimurium* antigens than did BALB/c mice and that this appeared to correlate with resistance to this organism. Similarly, they proposed that the low antibody response of BALB/c mice was probably due to partial tolerance caused by the cross-reactivity between their cell surface antigens and those of the bacteria. Finally, systemic salmonellosis is a recognised complication of sickle cell anaemia and a clinical study has indicated that this correlates with a decrease in the bactericidal capacity of serum obtained from people with this genetic disorder (Hand and King, 1977). These studies on the genetic control of the humoral immune response, highlight the important role played by antibody in resistance to Salmonella infection. It is tempting to speculate that there may be a correlation between the level of the humoral immune response, as indicated by the level of background antibody in serum, and the phenotype of early net growth rate (Hormaeche, 1979a) which is under the control of the Ity gene (Plant and Glynn, 1977) and is evident before the onset of the cell-mediated immune response (Hormaeche, 1979a).

It is interesting to note that the strain distribution of resistance and susceptibility to *Salmonella typhimurium* is totally different from that obtained with *Listeria*

TABLE 1.1

The resistance of various strains of mice to infection with *Salmonella typhimurium* and *Listeria monocytogenes*

Mouse srain[a]	$L.D._{50}$ of Salmonella	$L.D._{50}$ of Listeria
C3H/HeJ	<2	N.D[c]
B10.D2	<10	$2.2x10^5$
BALB/c	$1.8x10^1$	$3.9x10^3$
B10	$1.8x10^1$	$5.0x10^5$
B10.A	$3.1x10^1$	$2.2x10^5$
DBA/2[b]	$3.9x10^1$	$5.0x10^3$
C3H[b]	$1.2x10^2$	N.D[c]
CBA[b]	$1.0x10^3$	$5.0x10^3$
C3H/HeN	$1.0x10^4$	N.D[c]
A/J[b]	$1.8x10^4$	$5.0x10^3$

a. All mice were challenged intravenously

b. These strains were highly resistant to *Salmonella typhimurium* given subcutaneoulsy with $L.D._{50} \geq 2x10^5$

c. Not determined

This table was composed using data presented in papers by Cheers and McKenzie (1978), Hormaeche (1979), O'Brien, Rosenstreich, Scher, Campbell, MacDermott and Formal (1980), Plant and Glynn (1976) and Skamene, Kongshavn and Sachs (1979).

monocytogenes, although both organisms are facultative intracellular bacterial parasites. These striking differences in resistance between different mouse strains are illustrated in Table 1.1, a composite of several workers' observations (Plant and Glynn, 1976; Cheers and McKenzie, 1978; Hormaeche, 1979; Skamene, Kongshavn and Sachs, 1979; O'Brien et al., 1980). For example, the CBA and A/J strains are resistant to Salmonella typhimurium, but susceptible to Listeria monocytogenes, whereas the opposite applies to the B10 and related sublines. BALB/c mice are extremely susceptible to both parasites.

Resistance to Listeria monocytogenes is characterised by both an initial ability to control the growth of the parasite and the early onset of a cell-mediated immune response (Cheers et al., 1978). Genetic studies have revealed that this resistance is under the control of a single autosomal gene, or group of linked genes, which is not linked to the H-1, H-2, H-3, H-4, H-7 or H-8 loci, to the immunoglobulin allotype, to the Thy-1 gene, to the Hc gene specifying C5, nor to coat colour genes (Cheers and McKenzie, 1978). However, the level of specific immunity to Listeria antigens, as indicated by delayed-type hypersensitivity reactions which can be elicited in the footpad of mice previously injected with live Listeria monocytogenes, may be the H-2 linked (Skamene, Kongshavn and Sachs, 1979). Similarly, the adoptive transfer of resistance to naive animals with anti-Listeria immune T cells is restricted by the H-2 haplotype (Zinkernagel et al., 1977).

To summarise, the resistance of mice to Listeria monocytogenes is influenced by genes controlling the cell-mediated immune response. In contrast, resistance to

Salmonella typhimurium, which follows a different strain distribution pattern in mice, appears to be influenced by genes controlling both the humoral and cell-mediated immune responses.

Acquired resistance to Salmonella infection

Resistance to some enteric diseases, such as cholera, can be acquired through the use of oral vaccines. Coproantibody, predominantly of the IgA class, protects immunised individuals by preventing the adherence of the cholera vibrios to the epithelial cells of the intestine (Rowley, 1974; Rowley, 1978). However, there are several reasons why the stimulation of local intestinal immune responses alone, may be inadequate for effective protection against typhoid:

(a) Although typhoid and cholera are usually the result of an oral infection, potentially, typhoid can be contracted by various other non-oral routes (Moore, 1957; Tannock and Smith, 1972; Duguid *et al*., 1976).

(b) Secondly, *Vibrio cholera* is confined to the intestine and causes diarrhoea by producing a toxin which alters the epithelial permeability (Spring, 1969), whereas *Salmonella typhi* is able to penetrate the intestinal lining and the ensuing systemic phase of the disease is largely responsible for the morbidity and mortality (Hornick *et al*., 1970a; Davies, 1975).

(c) Hohmann (1979) fed 2×10^7 *Salmonella typhimurium* C5 organisms to orally immunized LAC strain mice and found that the organisms could be recovered from both the Peyer's Patches and the spleen. It is important to note that irrespective of the level of local

intestinal immunity, some degree of systemic infection appeared inevitable. Naturally the survival of the animals was dependent on their being able to control this systemic infection. Therefore, while the local intestinal immune responses may greatly assist in combating an oral typhoid challenge, the systemic immune responses are crucial for eventual survival and are the sole concern of this thesis.

At present, studies in mice have shown that the acquisition of resistance to systemic Salmonella infection is best achieved through the use of live rather than killed vaccines (Ushiba _et al._, 1959; Rowley, Auzins and Jenkin, 1968; Collins, 1970; Germanier, 1972). The live vaccines invariably conferred excellent protection against subsequent challenge with virulent organisms. For instance, vaccination with a sublethal dose of living _Salmonella enteritidis_ prevented the growth of a virulent strain of _Salmonella enteritidis_ in the liver and spleen (Collins, 1970).

In general, the non-living vaccines are much less effective, although the extent of protection varies, depending on the particular preparation used and on the criterion for assessing resistance. Rowley, Auzins and Jenkin (1968) found that an acetone-killed _Salmonella enteritidis_ vaccine would confer resistance on the basis of increased survival, but only to challenge with the homologous strain. However, Badakhsh and Herzberg (1969), using a "whole-cell residue" of _Salmonella typhimurium_ produced protection against both homologous and heterologous strains of Salmonellae. More recently a number of subcellular fractions of _Salmonella typhimurium_, particularly ribosomal fractions have also been

reported to increase resistance to this organism (Venneman and Berry, 1971; Venneman, 1972; Smith and Bigley, 1972; Misfeldt and Johnson, 1976; Angerman and Eisenstein, 1978; Angerman and Eisenstein, 1980; Kita and Kashiba, 1980). In contrast, Collins (1969a) was unable to demonstrate protection after immunization with alcohol-killed Salmonella vaccines. Although mice vaccinated with alcohol-killed *Salmonella enteritidis* produced high titres of antibody and were able to reduce the size of the initial inoculum, they were unable to control the multiplication rate of the surviving organisms (Collins, 1969b). In a subsequent paper, Collins (1970) also reported that immunization with an alcohol-killed vaccine, given by various routes, delayed the spread of orally introduced *Salmonella enteritidis* to the liver and spleen by 1 to 2 days. While the growth of the pathogen was not prevented, an initial reduction in the viability of the inoculum allowed the mice time to produce an active immune response and so survive the infection. This result emphasises the fact that when resistance is being assessed, it may be necessary to consider both the eventual survival of the infected hosts and the fate of the challenge organisms in the hosts. In addition, it may also account for the occasional reports of protection using killed vaccines (Jenkin and Rowley, 1963; Jenkin and Rowley, 1965; Kenny and Herzberg, 1968; Badakhsh and Herzberg, 1969).

The reasons behind the relative ineffectiveness of the killed Salmonella vaccines have, over the years, been the subject of a few studies and a great deal of conjecture. It is essential that the antigenic determinants present on the surface of the living organisms are not altered or destroyed during the preparation of the killed vaccine. Attempts to immunize mice

against virulent *Salmonella typhimurium* with heat-killed vaccines have failed (Hobson, 1957b), partly because a heat-labile surface antigen is destroyed by this method of killing the bacteria (Auzins and Rowley, 1963). In a later study, a number of *Salmonella typhimurium* vaccines, killed by various methods, were compared for their protective effects. While the heat-killed vaccine was not protective, alcohol and acetone-killed vaccines were found to confer significant protection (Jenkin and Rowley, 1965). The destruction of a heat-labile antigen during the preparation of heat-killed vaccines undoubtedly contributes to their ineffectiveness as prophylactic agents. Unfortunately however, with many vaccines the complex antigenic structure of the bacteria is such that it is difficult, if not impossible, to determine whether any of the antigens are destroyed or modified by the techniques used to prepare the vaccines. Also, the implication of some studies is that the antigens necessary for the production of immunity may only be produced *in vivo* (Germanier, 1970; Germanier and Furer, 1971; Germanier, 1972). While not all rough *Salmonella typhimurium* mutants are suitable as live vaccines, the gal E mutants which are able to synthesise smooth-like LPS *in vivo*, produced a degree of immunity against virulent *Salmonella typhimurium* comparable to that conferred by a sublethal infection with virulent smooth bacteria.

The effect of dose could be another possible reason for the superiority of live vaccines over killed ones. Following immunization with a living vaccine, mice receive constant antigenic stimulation as the organisms continue to multiply and are subjected to the bactericidal mechanisms of the host

defence systems (Pike and Mackenzie, 1940; Hobson, 1957a). On the other hand, killed vaccines generally provide antigenic stimulation over a comparatively short period of time. Therefore it is quite likely that a different antigenic dose would be administered with a live vaccine than with a killed vaccine and that this may also contribute to the different immune responses to the two vaccines.

Finally, it has been suggested that the ineffectiveness of the killed vaccines, in comparison with the live vaccines, is due to their inability to elicit delayed-type hypersensitivity reactions (Mackaness, Blanden and Collins, 1966; Collins, Mackaness and Blanden, 1966; Blanden, Mackaness and Collins, 1966; Collins, 1968a) and therefore their inability to elicit a cell-mediated immune response. This may be due to the fact that the presentation of the antigen and/or its localization in the tissues may be different when live bacteria rather than killed bacteria are used for immunization even if they are given by the same route (Davies, 1975). However, the cell-mediated immune responses can be manipulated by the use of adjuvants. Collins (1973) found that heat-killed suspensions of *Salmonella enteritidis* and *Salmonella pullorum* when suspended in Freund's Complete Adjuvant, were able to induce effective antimicrobial resistance, as determined by the fate of the organisms, against a virulent Salmonella challenge. Similarly, Van Der Meer, Hofhuis and Willers (1977) reported that the injection of killed *Listeria monocytogenes* in association with dextran sulphate, induced resistance against a lethal homologous challenge.

In conclusion, acquired resistance to typhoid is best achieved through the use of live, rather than killed vaccines.

While the reasons behind the superiority of living vaccines are unclear, they may include the possession of important antigenic determinants which are destroyed and/or not expressed on killed bacteria. Use of live vaccines may also ensure that a more suitable dose of antigen is injected and/or that the bacterial antigens are presented to the host's immune system in a manner which results in a cell-mediated response being induced. Nevertheless, killed vaccines, which are generally unable to generate cell-mediated immune responses, are able to confer substantial levels of protection and this demonstrates the important role played by humoral factors in resistance to typhoid.

The cell-mediated immune response to intracellular bacterial infections

Historically, the work of Metchnikoff (1893) first led to the suggestion that the phagocytic cells are a central component of the host defence, and that their functional modification is necessary for the expression of resistance to certain bacteria. However, Lurie (1942) supplied the first direct evidence that resistance to intracellular bacterial parasites is dependent on an alteration in the microbicidal properties of the phagocytic cells. He found that macrophages, harvested from *Mycobacterium bovis* strain BCG-vaccinated rabbits and cultured in the anterior chamber of the eye of normal rabbits, were able to inhibit the growth of ingested tubercle bacilli. In contrast, the macrophages from normal rabbits were unable to control the proliferation of the ingested bacilli. Suter (1953) and then Mackaness, Smith and Wells (1954), independently confirmed this report, using

in vitro culture techniques.

Subsequent experimental evidence revealed that immunity to other facultative intracellular bacterial parasites is also dependent on the development of a similar cellular response. The report that immunity to *Salmonella enteritidis* could be transferred to normal recipients by "immune" macrophages, highlights the importance of these phagocytic cells (Ushiba *et al*., 1959). Similarly, macrophages harvested from animals infected with *Brucella abortus* (Pomales-Lebrón and Stinebring, 1957), *Listeria monocytogenes* (Mackaness, 1962; Armstrong and Sword, 1964) and *Salmonella typhimurium* (Hobson, 1957b;Howard, 1961) have all been shown to possess enhanced *in vitro* microbicidal activity. Interestingly, the addition of specific immune serum to the cultures was frequently found to have little, if any, effect on the microbicidal function of the macrophages. Also, the observation that neither alcohol-killed vaccines, nor serum from immunized mice, would protect mice against an intravenous challenge with either *Salmonella enteritidis* or *Salmonella typhimurium* further implied that antibody plays only a marginal role (Collins, 1969 a & b). However, this conclusion is contraversial and will be discussed in more detail in the next section. Nevertheless, these experiments together suggested that immunity to intracellular bacterial parasites was of a nonhumoral type, mediated by activated or "angry" macrophages with enhanced bactericidal activity.

Similarities between the kinetics of appearance and duration of immunity to intracellular bacterial parasites and delayed-type hypersensitivity and the requirement of live vaccine for their induction eventually led to an explanation

of the mechanism responsible for macrophage activation. Mackaness (1969) demonstrated that mice infected with *Listeria monocytogenes* not only developed resistance to a secondary challenge, but also developed delayed inflammatory reactions when injected with Listeria antigens. Moreover, both immunity and DTH were transferred to normal mice with spleen cells from immune mice. On the basis of this and other evidence, he suggested that in response to such an infection, the host generates a large population of T-lymphocytes which are specifically committed to the bacterial antigens that evoked their production. Phagocytic cells with enhanced bactericidal properties then arise from bloodborne monocytes, as a consequence of their interaction with these antigenically stimulated lymphocytes. The participation of both T-lymphocytes and macrophages in DTH and antimicrobial immunity has since been confirmed by manipulation of the original cell transfer experiments. For instance, the pretreatment of spleen cells of immunized mice with anti-θ serum and complement to specifically remove T-lymphocytes, abolished their ability to transfer both the protective effect (North, 1973a) and DTH (Youdim, Stutman and Good, 1973). Likewise, the destruction of monocyte precursors by irradiation of the recipients also ablates the transfer of cell-mediated immunity (Tripathy and Mackaness, 1969; Volkman and Collins, 1971).

Some investigators were able to separate experimentally DTH and antimicrobial immunity and, as a result, have questioned whether these two phenomena are causally related. Youmans and Youmans (1969) were able to demonstrate resistance to tuberculosis infection in the absence of detectable DTH. Also Raffel (1948, 1950), working with guinea pigs, induced

DTH to tuberculin without the concomittant development of immunity to tuberculosis. In contrast, Dodd (1970) found that guinea pigs undergoing systemic DTH reactions to bovine gamma globulin were resistant to infection with *Listeria monocytogenes*. Although this apparent contradiction is not yet understood, it is possible that these two cellular responses are mediated by different subpopulations of T-lymphocytes. Alternatively, an explanation may be associated with the different sensitivities of the two assays, with the amount and the distribution of the relevant antigens in these animals, or with the contribution of antibody to survival. However, the coincident development of immunity and hypersensitivity during infection with *Listeria monocytogenes* (Mackaness, 1969), *Mycobacterium tuberculosis* (Mackaness, 1971) or *Salmonella enteritidis* (Collins and Mackaness, 1968), suggests at the very least, that these are two closely related phenomena.

While the generation of resistance to intracellular bacterial parasites is dependent on a highly specific immune response, the expression of this resistance is, however, frequently observed to be non-specific. In one such study, macrophages harvested from mice infected with *Mycobacterium bovis* BCG were found to have increased bactericidal activity against apparently unrelated Salmonellae (Jenkin and Benacerraf, 1960). In addition, mice immunized with *Mycobacterium bovis* BCG are resistant to challenge with *Listeria monocytogenes* (Mackaness, 1969), *Mycobacterium fortuitum* (Boehme and Dubos, 1958) and *Salmonella enteritidis* (Howard *et al*., 1959). Similarly, the injection of lipopolysaccharide into mice was found to increase the bactericidal activity of their phagocytic cells against

heterologous organisms (Landy, 1956; McIntyre, Rowley and Jenkin, 1967) and to increase their resistance to challenge with virulent Gram-negative bacteria (Rowley, 1955, 1956). These, and other demonstrations of the apparent non-specific expression of immunity to intracellular bacteria were generally interpreted as providing further evidence that antibody is not involved. However, although the immunity appears non-specific, the participation of antibody cannot be easily excluded. For instance, lipopolysaccharide, which is common to all Gram-negative bacteria, is a potent polyclonal B-lymphocyte activator (Andersson, Sjöberg and Möller, 1972; Ness _et al._, 1976; Dufer _et al._, 1980). In fact, during some infections, the presence of antibody specific for apparently unrelated, heterololous organisms has already been established (Rowley, Auzins and Jenkin, 1968; Ielasi, 1970; Davies, 1975). This question of non-specific immunity will be discussed in more detail in Chapter 4.

Clearly, the acquisition of resistance to infection with facultative intracellular bacterial parasites is associated with the induction and functional expression of macrophage activation. Should this immunity be solely dependent on these more active phagocytic cells and if they are non-specific, as has been suggested, then at the height of resistance one would be able to make two predictions:

(a) Firstly, the non-specifically active macrophages would be able to inactivate _any_ intracellular parasite much more effectively than normal macrophages;

(b) Secondly, these non-specifically active cells, consuming host as well as parasite, would gradually

cause a massive auto-immune disease, unless
macrophage activation is of a very short duration.
Coppel and Youmans (1969) disproved the first prediction with the demonstration that mice immunized and challenged with either Listeria or tubercle bacilli, eliminate the homologous challenge organism more effectively than the heterologous one. The second prediction is obviously incorrect because autoimmune diseases are generally not associated with antimicrobial immunity. Activated macrophages are therefore able to discriminate between "self" and "non-self", and between one invading pathogen and another. Hence, the view that immunity to intracellular bacterial parasites is solely due to an alteration in physiological state of the phagocytic cells does not provide a complete explanation for the specificity of this response. It is well established that antibody enables normal phagocytic cells to recognise a foreign invading pathogen. Therefore antibody would be a logical candidate as the discriminatory factor for activated macrophages and its role in bacterial infections will now be considered.

The humoral response to bacterial infections

The involvement of antibody in combating bacterial infections has become evident since late in the nineteenth century. Notably, von Behring and Kitasato (1890), while investigating immunity to tetanus, found that the serum from immune animals was not only able to neutralize the tetanus toxin, but also could be used to transfer resistance to susceptible animals. They suggested that humoral factors, called antibodies, were important in resistance to bacterial

infections. This hypothesis was supported by the demonstration of a heat-labile factor in defibrinated blood which was capable of killing Bacillus anthracis and Bacillus subtilis in vitro (Nuttal, 1888 and other contemporary studies). Later Wright and Douglas (1903) observed that the presence of serum enhanced the phagocytosis of Staphylococcus pyogenes by leucocytes. They concluded that serum contained substances which rendered the bacteria more susceptible to phagocytosis and killing. These early experiments established that humoral factors were able to kill some bacteria directly, as well as act as discriminating factors for phagocytic cells.

The role of antibody in promoting phagocytosis and killing of microorganisms is particularly noticeable with extracellular parasites, such as staphylococci, pneumococci and haemolytic streptococci. Here the acquisition of resistance to infection with these organisms is dependent on a specific humoral response, and the level of resistance correlates with the level of antibody (Raffel, 1949; Jeter, McKee and Mason, 1961; Wu and Marcus, 1964).

However, the importance of antibody in immunity to Salmonellae and other intracellular parasites is not always readily apparent and has been the subject of much vigorous debate. On the one hand, specific opsonic antibody enhances the phagocytosis and intracellular killing of these organisms. For instance, the presence of serum from rabbits immune to Brucella melitensis greatly reduced the survival of this organism in normal monocyte cultures (Elberg, 1960). Similar results have been reported for various intracellular bacteria (Robertson and Sia, 1927; Rowley and Whitby, 1959; Cohn and Morse, 1959; Jenkin and Rowley, 1959; Rowley, 1960; Jenkin and

Benacerraf, 1960; Weigle, 1961). Further evidence for the importance of antibody was provided by Jenkin (1963) with the demonstration that specific opsonins not only enhanced phagocytosis but also influenced the fate of ingested bacteria. He found that phage P22-treated and normal *Salmonella typhimurium* were both phagocytosed by normal mouse peritoneal macrophages in the presence of antibody specific for the phage or bacteria respectively. Surprisingly, killing of the ingested bacteria only occurred when opsonins specific for the bacteria were used. In a later study, the interaction of mouse peritoneal macrophages with *Salmonella typhimurium* and *Listeria monocytogenes* was examined *in vitro* at the single cell level (McIntyre, Rowley and Jenkin, 1967). Their observations revealed that, in the presence of specific antibody, 57% and 32% of normal mouse peritoneal macrophages were capable of ingesting *Salmonella typhimurium* and *Listeria monocytogenes* respectively, and that the intracellular survival of these organisms was low, approximately 18% in both cases. However, in the apparent absence of this antibody little or no phagocytosis of the bacteria occurred and, when the phagocytosis had occurred, the intracellular survival rate was high, approximately 70%. They also demonstrated that the macrophage populations from immunized mice, in comparison with normal mice, contained a higher proportion of phagocytic cells with an effective bactericidal mechanism. To summarize, specific antibody is clearly required by normal unstimulated macrophages for the expression of their antimicrobial function.

In contrast to these results, Gorer and Schutze (1938) found that the resistance of mice to various strains of Salmonellae bore no relationship to the levels of antibodies

specific for either their flagella (H) or polysaccharide (O) antigens. Over the intervening years, many other workers investigating resistance to intracellular bacterial parasites have reported specific antibody to be of limited value and have questioned its role in immunity to these organisms (Lurie, 1942; Hobson, 1957b, Furness and Ferreira, 1959; Ushiba et al., 1959; Howard, 1961; Blanden, Mackaness and Collins, 1966; North, 1978). The experimental evidence upon which this conclusion is based, falls essentially into 4 categories:

(a) Killed vaccines, although capable of inducing humoral immune responses, are less effective at conferring protection than the living vaccines (Holland and Pickett, 1956, 1958; Ushiba et al., 1959; Collins, 1969 a & b);

(b) Specific antibody from immune animals does not generally confer resistance on passive transfer (Miki and Mackaness, 1964; Mackaness, Blanden and Collins, 1966);

(c) Resistance to intracellular bacterial parasites may be transferred with phagocytic cells (Ushiba et al., 1959; Furness and Ferreira, 1959);

(d) Antimicrobial resistance may be induced in the absence of "detectable" antibody and is apparently non-specific in its expression (Blanden, Mackaness and Collins, 1966; North and Deissler, 1975).

Although these experiments clearly emphasised the importance of macrophage activation in antimicrobial resistance, they do not, as is now apparent from more recent studies, constitute proof for the non-involvement of antibody. For instance, the necessity of a cellular response explains

previous difficulties in conferring resistance with either killed vaccines or passive antibody transfer. Nevertheless, antibody induced as the result of immunization with an alcohol-killed vaccine, can play a vital role in host survival by significantly reducing the size of an orally introduced challenge inoculum and also by delaying the dissemination of the organisms to the liver and spleen (Collins, 1970). Similarly, the participation of antibody cannot be excluded merely because resistance to intracellular parasites can be transferred with phagocytic cells from immune animals. Rowley, Turner and Jenkin (1964) found evidence of antibody on the surface of macrophages which were capable of transferring immunity to Salmonella typhimurium. When eluted, this cytophilic antibody could also transfer resistance to normal animals. Likewise, the experiments demonstrating "non-specific" resistance are far from conclusive. While Blanden, Mackaness and Collins (1966) claimed that Listeria-infected mice are non-specifically resistant to challenge with Salmonella typhimurium, it was difficult to assess the extent of this resistance because they challenged mice of the Swiss-Webster strain with only 2.6×10^3 Salmonella typhimurium, a comparatively low dose (c.f. $L.D._{50}=4 \times 10^3$ organisms). The participation of low levels of cross-reacting antibody in these Listeria-infected mice cannot be excluded on the grounds that the level of opsonic activity in their serum was similar to that in uninfected mice, as these were animals of relatively high natural resistance. Also, while the presence of specific antibody for the somatic antigens of Salmonella typhimurium was not demonstrable in this serum, it is likely that their assay was not sufficiently sensitive to detect

background levels of antibody, particularly as they were using a 1:10 dilution of serum. The presence of such antibody has already been established in *Salmonella enteritidis* 11RX-infected Swiss-Webster mice (Davies, 1975). Ten years later, Zinkernagel (1976) reported that *Listeria monocytogenes*-infected CBA/H mice are not resistant to challenge with *Salmonella typhimurium*. He speculated that in addition to macrophage activation, protective immunity to this organism requires the generation of specific factors, most probably antibodies. Lastly, Meléndez, Gonzalez, Reid, Fuentes and Castillo (1978), demonstrated that the *in vitro* growth of *Salmonella enteritidis* was inhibited by peritoneal exudate cells of immunized mice only in the presence of specific immune serum. However, because a killed *Salmonella typhi* Ty2 vaccine was used to generate these peritoneal exudate cells, some doubts exist about the level of macrophage activation obtained. Nevertheless, this finding also supported the proposal that the mechanism of immunity in salmonellosis is "humoral plus cellular" (Rowley, Auzins and Jenkin, 1968; Kenny and Herzberg, 1968).

In conclusion, the role of antibody in immunity to typhoid, and other intracellular bacterial infections, is a question of fundamental importance which still remains unanswered.

Summary and conclusions

Typhoid fever is a disease caused by infection with *Salmonella typhi* and is unique to man. It is acquired as a result of ingesting contaminated food or water and usually occurs as a medical problem only in the less affluent

communities. Although the infection is characterised by a preliminary intestinal phase, it is the ensuing secondary systemic bacteraemia which is mainly responsible for the morbidity and mortality. At present, public immunization programmes are ineffective and will only be improved when more is understood about the actual infection and host defence mechanisms.

Infection of mice with *Salmonella typhimurium* or *Salmonella enteritidis* has resulted in the production of experimental models of typhoid fever. The mechanism of immunity to these infections is controversial, but is commonly thought to be cellular in nature. However, a variety of experimental evidence suggests that humoral factors are also important. For instance, recent genetic studies have shown that the resistance of mice to infection with *Salmonella typhimurium* is influenced by genes controlling both humoral and cell-mediated immune responses.

The aim of this study was to re-examine the role of the humoral immune response in the resistance of mice to infection with *Salmonella typhimurium*. In particular, it was hoped that such a study would more clearly determine whether activated macrophages require the presence of antibody for the expression of their cell-mediated immune function.

CHAPTER 2

MATERIALS AND METHODS

Animals

Inbred (C57BL/6J♂ X BALB/cJ♀)F1(F1) hybrid mice were used in this study, and they were bred in the University of Adelaide Medical School Animal House from stock which had been obtained from both the Jackson Laboratories, Florida, U.S.A. and the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia. The mice were supplied with food and water *ad libitum*. Generally male mice, 8 - 12 weeks of age were used, although female mice were occasionally used for some experiments.

Bacterial Strains

(a) *Escherichia coli* F1097(F1097) is a hybrid strain which received the O antigenic factors 4, 5, 12 by genetic transfer from *Salmonella typhimurium* (Kiefer, Schmidt, Jann and Jann, 1976). It was originally obtained from Dr.G.Schmidt, Max-Planck-Institut für Immunobiologie, Freiburg, West Germany.

(b) *Listeria monocytogenes* is a small, Gram-positive bacillus which was first described by Murray, Webb and Swan (1926). It was kindly supplied by Dr.R.V.Blanden, John Curtin School of Medical Research, Australian National University, Canberra, and was found to be relatively virulent for mice. The median lethal dose ($L.D._{50}$) for an intravenous challenge of F1 mice was determined to be approximately 2×10^5 organisms

(unpublished data).

(c) Salmonella enteritidis 11RX (11RX) is a rough, Gram-negative bacillus which was first described by Ushiba, et al., (1959). It is relatively avirulent for the F1 mice and the L.D.$_{50}$ dose for an intravenous challenge was determined to be approximately 2 x 10^6 organisms (Davies, 1975).

(d) Salmonella newington is a smooth Gram-negative bacillus which has been described by Kauffman (1969) and was originally obtained from the Salmonella Typing Laboratory, I.M.V.S., Adelaide, South Australia.

(e) Salmonella typhimurium C5 (C5) is a smooth, Gram-negative bacillus which has been previously described by Furness and Rowley, (1956) and Rowley and Whitby (1959). This strain is particularly virulent for F1 mice, as less than 5 organisms will kill more than 50 per cent of the mice (Davies, 1975).

Bacterial culture media

Listeria monocytogenes was routinely grown in either brain-heart infusion broth (Difco) or tryptic-soy broth (Difco), while all other strains were grown in nutrient broth (double strength Bacto Nutrient Broth, Difco, with NaCl 5g/l added). All bacteria were grown on nutrient agar (Blood Agar Base, Difco) as either plate or slope cultures.

Maintenance of bacterial strains

All strains were maintained as lyophilized cultures, stored in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth, using a pasteur pipette. The

contents were then transferred to a bottle of broth and grown on a shaker at 37°C overnight. Purity was checked by streaking a loopful of this culture onto a dried nutrient agar plate and this was also incubated at 37°C overnight. If the colony form was uniform, single colonies were selected and picked off for subsequent storage or use.

When preparing bacterial strains for storage, these colonies were suspended in a small volume of sterile skimmed milk. Approximately 0.2ml aliquots of this thick bacterial suspension were dispensed into sterile 1/4in. x 4in. freeze drying ampoules (Johnson and Jorgensen Ltd.,London), and then the end of each ampoule was plugged with cotton wool. The samples were then lyophilized in a Speedivac Centrifuge Freeze Drier, Model 5PS (Edwards High Vacuum Ltd., Sussex, England).

This technique involved centrifugation of the ampoules whilst the pressure was reduced to 200 microns. Then the ampoules were held at that reduced pressure over phosphorus pentoxide for 6 hours to remove most of the moisture. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and held at that pressure over phosphorus pentoxide for a further 16 hours. They were then sealed at the constriction without releasing the vacuum. Finally the ampoules were labelled and stored at 4°C.

After an ampoule was opened and cultures prepared on nutrient agar plates, single colonies were routinely used to inoculate bottles of broth, which were incubated with shaking at 37°C for 18 hours. Such overnight cultures were then suitable for subculture or experimental use.

Cultures of Salmonella were also set up on nutrient agar slopes in capped bottles and they were kept at 4°C for up to 3 months. These slope cultures were more convenient for regular use than the lyophilized cultures in ampoules; however each slope was only used twice. Similarly, for the sake of convenience, *Listeria monocytogenes* was kept in frozen cultures. These were prepared by placing small samples of broth culture into sterile ampoules which were then sealed and snap-frozen in an alcohol-dry ice mixture. They were stored at -80°C until required. Whenever a culture of *Listeria monocytogenes* was required a fresh ampoule of either lyophilized culture or frozen culture was opened. This was necessary to ensure that the characteristic virulence of the strain was retained.

Preparation of bacteria for injection

Living preparations of the various bacteria were required for both immunizing and challenging the mice. They were prepared by inoculating a 10ml broth with 1.0ml of an overnight broth culture of the strain required. Each inoculated broth was incubated with shaking at 37°C for 3 hours. In this time the subcultures grew to approximately 2×10^9 viable organisms per ml. Each subculture was diluted to the appropriate concentration in cold physiological saline (0.9% w/v). Immediately after dilution, mice were injected with 0.2ml of this suspension, by either the intraperitoneal or intravenous route. The actual number of viable bacteria injected was checked by spreading 0.1ml aliquots of a suitably diluted sample on to nutrient agar plates. After overnight incubation at 37°C, the number of colonies was counted.

Bacterial enumeration of infected animals

The fate of a bacterial inoculum, used to experimentally infect mice, was studied by determining the number of viable organisms in various organs and tissues of the infected animals. Results were expressed as geometric means of the number of viable counts obtained. Briefly, the methods involved were:

(a) Blood: duplicate 0.1ml blood samples were taken from the retro-orbital venous plexus of each animal, using a sterile graduated pasteur pipette. These samples were plated on dry nutrient agar plates which were then incubated at 37°C overnight. The total blood volume of mice was assumed to be 2.0ml.

(b) Peritoneal Cavity: mice were killed by cervical dislocation and swabbed with ethanol (70% v/v). The abdominal skin was reflected and the peritoneal cavity rinsed with 2.0ml of sterile saline. The abdomen was massaged vigorously during the washout to ensure maximum recovery of bacteria. The washout fluid was appropriately diluted and plated in duplicate on dried nutrient agar plates and incubated at 37°C overnight.

(c) Spleens: spleens were removed aseptically and placed in separate sterile bottles containing 5.0ml of sterile saline. After homogenisation the suspensions were appropriately diluted in sterile saline. Duplicate 0.1ml samples were plated on dried nutrient agar plates and incubated at 37°C overnight.

(d) Livers: the technique was essentially the same as for the spleen, with the exception that 10ml of sterile saline was used for homogenisation.

^{32}P-labelling of bacteria

Salmonella typhimurium C5 were labelled with ^{32}P as they multiplied in a supplemented casamino acid medium to which ^{32}P was added as orthophosphate. The technique was originally described by Benaceraff, Sebestyen and Schlossman (1959). The medium consisted of:-

Sodium citrate	0.1gm
$MgSO_4.7H_2O$	0.02gm
Glucose	0.4gm
Casein hydrolysate	2.0gm
Distilled water	200ml

The pH of the medium was adjusted to pH7.0, before it was autoclaved at 15lbs/sq.inch pressure for 20 minutes.

One millicurie of ^{32}P as orthophosphate was added to 50ml of this medium prior to inoculation with 0.1ml of a suspension of log phase bacteria in nutrient broth. The culture was incubated on a shaker at 37°C for 18 hours and the ^{32}P labelled bacteria were deposited by centrifugation at 3020 x g for 15 minutes. The pellet was subsequently washed 3 times with 50ml of physiological saline and finally resuspended in a small, known volume of the casamino acid medium. The number of organisms / ml in this suspension was estimated from optical density measurements at 675 milli-microns wavelength and adjusted to a final concentration of 10^9 organisms / ml. A culture of known viability was used as a reference when estimating viability from optical density measurements. The radioactively labelled bacteria were stored at 4°C and were used for a maximum of 5 days after preparation.

Opsonisation of bacteria

For most experiments, a volume of bacterial suspension (approximately 10^9 organisms / ml) was mixed with an equal volume of serum and incubated at 4^oC for 30 minutes. The bacteria were deposited by centrifugation at 3020 x g for 15 minutes and the resultant pellet was washed 3 times with 20ml of physiological saline. The washed opsonised bacteria were finally resuspended and suitably diluted for immediate use.

Clearance of bacteria from the peritoneal cavity

The technique used for this study was essentially that described by Whitby and Rowley (1959). Normal F1 mice were injected intraperitoneally with approximately 10^4 log phase *Salmonella typhimurium* C5 in 0.2ml saline. At various times after injection, mice were killed and the peritoneal cavity rinsed with 2.0ml of sterile saline using 2.0ml syringes. The abdominal wall was massaged vigorously to ensure the maximum recovery of viable bacteria. Syringes were sterilised in boiling water between washouts. Duplicate 0.1ml samples of each washout were plated onto dried nutrient agar plates, which were then incubated at 37^oC overnight and all colonies were counted. Finally, the rate of killing of injected bacteria was calculated.

Clearance of intravenous bacteria

This technique was originally described by Benacerraf, Sebestyen and Schlossman (1959). F1 mice were injected intravenously with approximately 10^8 isotopically labelled bacteria in a volume of 0.2ml. Each mouse was bled from the retro-orbital venous plexus at various times after injection.

Blood samples (approximately 0.04ml) were taken with disposable heparinized haematocrit tubes and pipetted into 3ml of a sodium carbonate solution (0.1% w/v). The amount of radioactivity in each sample was measured in a well-type scintillation counter (Auto-Gamma Spectrometer, Packard Instrument Company, Downers Grove, Illinois, U.S.A.). The number of bacteria present in each sample was proportional to the number of counts / minute. The results obtained were plotted as $\log_{10}$ concentration of bacteria against time and this exponential expression was used to calculate the phagocytic index (K); where

$$K = \frac{\log C1 - \log C2}{T2 - T1}$$

and where C1 and C2 are the concentrations of bacteria at times T1 and T2.

Clearance of intravenous carbon

This technique was previously described by Jenkin and Rowley (1961). The F1 mice were injected intravenously with 0.2ml of colloidal carbon suspended in physiological saline containing gelatin (1% w/v). Indian ink (Penguin) was used as the source of carbon, and a dose of 16mg / 100gm body weight was used. Each mouse was bled from the retro-orbital venous plexus at various times after injection. Blood samples (approximately 0.04ml) were taken with disposable, heparinized haematocrit tubes and pipetted into 3ml of a sodium carbonate solution (0.1% w/v). The concentration of carbon in each sample was determined from optical density measurements at a wavelength of 675 milli-microns. The results obtained were plotted as $\log_{10}$ concentration of carbon against time and this exponential expression was used to calculate the phagocytic

index (K); where

$$K = \frac{\log C1 - \log C2}{T2 - T1}$$

and where C1 and C2 are the concentrations of carbon at times T1 and T2.

Preparation of alcohol-killed vaccine

An alcohol-killed *Salmonella typhimurium* C5 vaccine was prepared using the technique described by Auzins (1968). A 5-fold excess of 70% alcohol, pre-chilled to $4^{o}C$, was added to a known volume of washed stationary phase bacteria suspended in saline. After standing overnight at $4^{o}C$, the bacteria were washed 3 times and resuspended in sterile physiological saline. The dry weight of the bacteria was determined and the concentration adjusted to 50µg / ml. Viability was checked by spreading multiple 0.1ml samples on dry nutrient agar plates, which were then incubated at $37^{o}C$ overnight. If the vaccine proved sterile, it was dispensed into screw capped bottles and stored at $-20^{o}C$. Aliquots of vaccine were only thawed when required and any surplus was discarded.

Preparation of crude antigen

A method for preparing crude bacterial antigens was described by McCabe (1972). Initially an overnight culture of *Salmonella enteritidis* 11RX was centrifuged. The pellet was washed 3 times and resuspended in physiological saline to a concentration of 2×10^{9} organisms / ml. After boiling for 2 hours the suspension was centrifuged at 12,000 x g at $4^{o}C$ for 30 minutes in a Sorvall centrifuge (Ivan Sorvall Inc., U.S.A.). The supernatant fraction was kept and its dry weight determined.

Finally the concentration of crude antigen was adjusted to 200µg / ml and ethanol was added to a concentration of 0.95% v/v. The antigen was stored at -20°C until it was required for sensitizing sheep red blood cells (SRBC).

Preparation of protein antigen

Protein antigens were prepared from *Salmonella enteritidis* 11RX and *Salmonella typhimurium* C5 by a technique previously described by Ashley, Kotlarski and Hardy (1974). The studies of Ashley (1976) revealed that the antigen preparation contained more than 95% protein, less than 1.3% carbohydrate and no detectable nucleic acid.

An overnight culture of 11RX was washed with distilled water and resuspended to approximately 30mg / ml. The bacteria were disrupted by ultrasonic vibration at 4°C in the presence of B-mercaptoethanol (5mM) and nucleases (DNase 10µg / ml and RNase 10µg / ml; Calbiochem) in buffered magnesium chloride (5mM $MgCl_2$ in 10mM tris-HCl, pH 7.8). The sonicate was centrifuged at 12,000 x g for 15 minutes at 4°C to remove large debris and then at 100,000 x g for 2 hours at 4°C to remove the lipopolysaccharide. Nucleic acid was digested when the supernatant material was dialysed at 37°C for 24 hours against tris buffered magnesium chloride containing 0.01% sodium azide and additional DNase and RNase (10µg / ml of each) to ensure complete digestion. The 11RX antigen was then dialysed extensively against distilled water at 4°C, lyophilised and stored at 4°C.

Preparation of lipopolysaccharides

(a) the technique was described by Westphal, Lüderitz

and Bister (1952) and was used to prepare LPS from the smooth strain, Salmonella typhimurium C5. An overnight culture was washed and resuspended in saline to a concentration of approximately 20mg / ml. The bacterial suspension and a 90% phenol solution were heated to approximately 65°C. Equal volumes of each solution were then mixed and held at a temperature of 68° - 70°C, with stirring, for 20 minutes. After cooling to room temperature the mixture was spun at 700 x g for 20 minutes at 4°C in an M.S.E. centrifuge (Measuring and Scientific Equipment Ltd., England). The aqueous layer was removed and kept at 4°C. The phenol layer was then subjected to re-extraction with an equal volume of water. The aqueous layers from both separations were pooled, dialysed overnight against distilled water at 4°C and then mixed with 5 volumes of ethanol and 0.5gm of sodium acetate. This mixture was left for 4 hours and the resultant LPS precipitate was collected by centrifugation at 700 x g for 20 minutes. After solubilization in distilled water and overnight dialysis against distilled water, the LPS was centrifuged at 1,000 x g for 10 minutes. The supernatant fraction was subjected to further centrifugation at 100,000 x g for 1 hour in a Spinco Ultracentrifuge (Beckman Instruments Inc., U.S.A.). Finally the LPS pellet was dissolved in distilled water, dialysed against distilled water and lyophilized.

(b) The technique of Galanos, Lüderitz and Westphal (1969) was used to prepare LPS from the rough strain Salmonella enteritidis 11RX. Acetone-dried bacteria

were mixed with a phenol-chloroform-ether mixture. After stirring for 5 minutes, the mixture was centrifuged at 12,000 x g for 20 minutes and the supernatant was filtered through a 0.45 micron Millipore membrane. The bacterial residue was again subjected to the phenol-chloroform-ether extraction. Both supernatant fractions were pooled. A rotary evaporator at 30° - 40°C was used to evaporate the chloroform and ether. Distilled water was added dropwise to the residue until the LPS started flocculating. After standing for 2 minutes, the LPS was deposited by centrifugation, washed 3 times with 80% phenol and then with ether. The deposit was dried in vacuo and then dissolved in distilled water warmed to 45°C. Finally the LPS was deposited by centrifugation at 100,000 x g for 4 hours, solubilized in distilled water and lyophilized.

Alkali treatment of *Salmonella enteritidis* 11RX lipopolysaccharide

500μg LPS was dissolved in 0.1ml Hanks' medium. This solution was treated with 0.45ml of 2.5N sodium hydroxide for 2 hours at 37°C and then neutralized with 0.45ml of 2.5N hydrochloric acid. The volume was adjusted to 10ml to give a final concentration of 50μg LPS / ml.

Preparation of polysaccharides

The technique used was essentially similar to that described by Staub (1965). Bacteria were cultured overnight at 37°C, in 15 litres of nutrient broth. Having been washed 3

times in distilled water, they were then resuspended in 200ml of 0.1N acetic acid and boiled under reflux for 2 hours. The suspension was cooled and centrifuged at 7,000 x g for 20 minutes at 4°C to remove whole bacteria, char and boiling chips. The straw coloured supernatant was mixed with 6 volumes of alcohol and left overnight at 4°C. The precipitate which had formed was removed by centrifugation at 7,000 x g for 20 minutes at 4°C and redissolved in 20ml of distilled water. The polysaccharide was then precipitated with between 1 - 6 volumes of alcohol and similarly redissolved in 20ml of distilled water. This procedure was repeated 5 times. Finally the polysaccharide was dissolved in distilled water, dialysed against distilled water, and lyophilised.

Chromatographic analysis of the *Salmonella typhimurium* C5 polysaccharide preparation

The nature of the *Salmonella typhimurium* C5 polysaccharide preparation was characterised by 2 chromatographic procedures, using Sephadex G100 and polymixin B coupled to Sepharose 4B. In both cases the manufacturer's recommendations regarding gel hydration, column packing, sample loading and elution flow rates were adopted. The eluted fractions were monitored for their carbohydrate content using the colorimetric method described by Dubois, Giles, Hamilton, Rebers and Smith (1956). Essentially, to each 2ml fraction, 0.05ml phenol (80% w/w in distilled water) and 5ml concentrated sulphuric acid were added. The blanks were prepared by substituting buffer for the eluate. After standing for 10 minutes, the tubes were shaken and placed in a 30°C water bath for a further 20 minutes. The development of a characteristic yellow-orange colour indicated the presence

of carbohydrate in the eluate, and the intensity of the colour was measured by absorbance at 480nm. The concentration of carbohydrate in each fraction was then expressed in arbitrary units, where an absorbance at 480nm of 1.0 was taken to represent 1 unit of carbohydrate.

(a) Sephadex G100 chromatography was used to separate molecules according to size up to an exclusion limit of 100,000 molecular weight. In the present study, phosphate buffered saline pH 7.4 was used to elute 2ml polysaccharide (5mg / ml) from a 200ml column of Sephadex G100 (Pharmacia Fine Chemicals, Sydney, Australia). Fractions were collected in 2ml aliquots and assayed for carbohydrate content.

(b) Polymixin B / Sepharose 4B chromatography was used to ascertain the extent of endotoxin contamination in the polysaccharide preparation. The polymixin B was coupled to Sepharose 4B gel by the method previously described by Morrison, Roser, Cochrane and Henson (1975). Initially the Sepharose 4B (Pharmacia Fine Chemicals, Sydney, Australia) was cross-linked and desulphated by treatment with epichlorohydrin, aminated with N,N'-diaminodipropylamine and cyanogen bromide and then succinylated with succinic anhydride. Coupling was achieved by mixing 20ml of the treated, packed beads with 100mg polymixin B sulphate (Sigma Chemicals, St.Louis, U.S.A.) in the presence of 5.0g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma Chemicals, St.Louis, U.S.A.) in a

final volume of 53ml. After standing overnight at room temperature, the gel was washed with 1.0mM HCl, distilled water and finally 50mM Tris HCl buffer pH 7.5, containing 1% Triton X 100. Subsequently a column was prepared with this gel and 2ml of the polysaccharide preparation (5mg / ml) was eluted with the Tris HCl buffer described above. Fractions were collected in 2ml aliquots and assayed for carbohydrate content.

Coxiella burnetii extract

Coxiella burnetii has been described by Weiss and Monlder (1975). A commercially available extract (Phase 1, Nine-Mile Strain) of this rickettsial organism was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. It was prepared from the yolk sacs of infected, embryonated eggs, by the method described by Stoker (1953), for use as the antigen in the complement fixation diagnostic test for Q fever in humans. A control antigen, prepared from uninfected eggs, was also used.

Dextran sulphate

Dextran sulphate of molecular weight 500,000 was obtained from BDH Biochemicals Ltd., Poole, England. Prior to injection, it was dissolved in physiological saline to a concentration of 5mg / ml.

Delayed-type hypersensitivity

The injection of protein antigen, prepared from *Salmonella enteritidis* 11RX, into the hind foot pad of

sensitised mice, induced swelling which was easily measured. The antigen was dissolved in saline to a concentration of 1mg /ml. Groups of 5 mice were injected in the right hind foot pad with 0.01ml of the antigen preparation, using a micrometer operated glass syringe fitted with a 30 gauge needle. At various times after this injection the thickness of both hind feet was measured to the nearest 0.05mm using Micrometer dial gauge calipers (Model 130, Mercer, England). The spring which is designed to apply downward pressure on the measuring anvils was removed before the calipers were used. The difference in the thickness of the test and control feet was then expressed as a percentage increase in thickness of the injected foot, compared to the control foot.

Rabbit anti-11RX antiserum

A log phase culture of *Salmonella enteritidis* 11RX, at a concentration of 2 x 10^9 organisms / ml, was washed in saline and heat-killed at 100°C for 1 hour. The bacteria were then washed again and resuspended in saline to a concentration of 10^{10} organisms / ml. A Dutch SPF rabbit was then given a course of 3 intravenous injections over a period of 9 days (Day 0, 2.5 x 10^9 H/K 11RX; Day 5, 5.0 x 10^9 H/K 11RX; Day 9, 9.0 x 10^9 H/K 11RX). On Day 16, the rabbit was bled from the ear and the serum collected was stored at -20°C until required.

Rabbit anti-C5 antiserum

A culture of *Salmonella typhimurium* C5 was washed in saline and then heated to 55°C for 30 minutes. A Dutch SPF rabbit was injected with 10^8 organisms in 0.5ml Freund's Complete Adjuvant, at six different sites (4 intramuscular and

2 subcutaneous). These injections were then followed by 3 intravenous injections of 10^7 H/K C5 on Day 8, 10^8 H/K C5 on Day 9, and finally 10^8 H/K C5 on Day 10. After 6 weeks, the rabbit was bled and the serum collected was stored at -20°C until required.

Mouse anti-C5 antiserum

A group of 40 F1 female mice were given 3 intravenous injections of 40µg of alcohol-killed *Salmonella typhimurium* C5 vaccine at monthly intervals. After a further week, the mice were bled.

Mouse anti-*Listeria monocytogenes* antiserum

A group of 20 F1 male mice were given 3 intravenous injections of 10^4 viable *Listeria monocytogenes* at monthly intervals. After a further week, the mice were bled.

Recovery of serum from normal and immunized mice

Mice were bled from the retro-orbital venous plexus using a pasteur pipette. The blood, collected into sterile, 20ml wide-necked McCartney bottles, was stood on the bench at room temperature for 1 hour, to allow clot formation. Then the bottles were kept at 4°C for a further 1 hour to encourage maximum clot retraction. After this time the serum was withdrawn with a sterile pasteur pipette and centrifuged in conical plastic tubes at 500 x g for 5 minutes. The cell-free, straw coloured serum was then removed and 1ml aliquots were dispensed into labelled screw capped 5ml bottles. Samples were stored at -20°C until required.

Absorption of serum with bacteria

The purpose of this procedure was to deplete some sera of any specific antibodies which may be directed against the bacterial surface. Log phase bacteria were washed 3 times in cold saline and then resuspended in serum at a concentration of approximately 10^{10} organisms / ml. After standing overnight at $4^{o}C$, the bacteria were deposited by centrifugation and the serum was removed with a pasteur pipette. The absorbed serum was then sterilised by filtration through a 0.45 micron Millipore membrane filter (Sartorius, Membranfilter GmbH, Gotten, West Germany) and used immediately.

Sensitisation of sheep red blood cells

(a) The technique of Crumpton, Davies and Hutchison (1958) was used to sensitise sheep red blood cells with lipopolysaccharide. The LPS was dissolved in saline to the appropriate concentration, 100µg / ml for *Salmonella typhimurium* C5 LPS and 50µg / ml for alkali-treated *Salmonella enteritidis* 11RX LPS. SRBC were washed 3 times with physiological saline and resuspended to a concentration of 5% (v/v) in physiological saline. The SRBC suspension was then mixed with an equal volume of the LPS solution, in a roller culture tube (or tubes) with a screw cap, and then incubated at $37^{o}C$ for 2 hours on a roller apparatus. The SRBC were deposited by centrifugation and again washed 3 times with physiological saline. Finally the cells were resuspended in saline to give a 1% (v/v) suspension.

(b) The *Salmonella enteritidis* 11RX crude antigen was

prepared at a concentration of 200μg / ml and was used to sensitise SRBC by the same technique as that described for LPS.

(c) SRBC were sensitised with protein antigens prepared from Salmonella typhimurium C5, using a modification of the technique described by Gold and Fundenberg (1967). Essentially, the sensitisation takes place through the agency of the chromium ions provided by chromic chloride. Prior to use, the SRBC were washed 3 times with physiological saline and resuspended to a 10% (v/v) suspension. The cells in 1.0ml of this suspension were deposited by centrifugation in a wide bore glass centrifuge tube and the supernatant fluid was removed. Then 0.1ml of the protein antigen solution, prepared at a concentration of 1.0mg / ml in saline, was used to resuspend the pellet of SRBC. Chromic chloride was dissolved in saline to a concentration of 0.1% and then 0.1ml of this solution was added to the suspension with continuous gentle shaking. After standing at room temperature for 1 hour, the cells were washed 3 times with cold saline and resuspended in saline to a 1% (v/v) suspension.

Indirect haemagglutination technique

0.1ml volumes of serial two-fold dilutions of antiserum were prepared in haemagglutination trays (Baird and Tatlock, London, England). To each well 0.1ml of a 1% (v/v) suspension of appropriately sensitised sheep red blood cells was added and after thorough mixing, the trays were incubated for 1 hour at 37°C. The results were read after standing the trays

overnight at 4°C and the endpoint of each assay was taken as the highest dilution of serum producing complete haem-agglutination. Unsensitised SRBC were included as controls in each assay.

Haemagglutination inhibition titrations

Having previously titrated the antisera against the appropriately sensitised SRBC, the lowest concentration of serum able to cause complete agglutination was determined. This was defined as containing 1 haemagglutination unit (HU) of antibody per 0.1ml. A dilution of antiserum containing 4HU of antibody per 0.1ml was made. Two-fold serial dilutions of the antigen to be tested were made in 0.1ml of saline in haemagglutination trays, and to each well was added 0.1ml of antiserum containing 4HU of antibody. Initially the trays were incubated at 37°C for 1 hour and then 0.1ml of a 1% suspension of sensitised SRBC was added to each well. The trays were incubated for a further hour and then left overnight at 4°C before the haemagglutination patterns were read. The endpoint of the assay was that concentration of antigen which would completely inhibit haemagglutination by 4HU of antibody.

Tissue culture media

In general, all tissue culture media were prepared with deionised, distilled water (DDW) and analytical grade chemicals. The media were sterilised before use by filtration through a Millipore membrane filter (0.45μm pore size). The foetal calf serum (FCS) used was heat inactivated at 56°C for 30 minutes before use.

When antibiotics were required in the medium, both

penicillin (100 units / ml) and streptomycin sulphate (100 units / ml) were used. For some procedures, heparin was also added to the medium and was used at 5 I.U./ ml.

(a) Hanks' balanced salt solution was prepared according to the method described by Weller, Enders, Robbins and Stoddard (1952). Initially 0.05N sodium hydroxide was added to 1gm phenol red until the powder had all dissolved and the solution was a deep red colour. The volume was adjusted to 250ml with DDW. Two stock solutions were prepared and 100mls of the phenol red solution were added to solution B.

Solution A contained:-

NaCl	160gm
KCl	8gm
$MgSO_4.7H_2O$	4gm
$CaCl_2$	2.8gm
DDW	1000ml

Solution B contained:-

$Na_2HPO_4.12H_2O$	3.04gm
KH_2PO_4	1.2gm
Glucose	20gm
DDW	900ml
0.4% Phenol Red	100ml

The Hanks' balanced salt solution was prepared by mixing 1 volume of Solution A, 1 volume of Solution B and 18 volumes of DDW. The culture medium used for the SRBC rosette and bacterial adherence assays contained 85ml of Hanks' balanced salt solution with the addition of 10ml of 1.4% sodium bicarbonate and 5ml of sterile heat-inactivated FCS.

(b) Dulbecco's modified Eagle's Minimal Essential Medium was used primarily in the *in vitro* tumour cytotoxicity test. This culture medium was prepared from powdered medium (Grand Island Biological Company, Grand Island, New York, U.S.A.) and penicillin and streptomycin were added. The pH of the medium was adjusted to 7.4 by the addition of 1N sodium hydroxide solution. HEPES (4-[2 hydroxyethyl]-1-piperazine ethane sulphonic acid; Calbiochem Australia Pty. Ltd., Carlingford, N.S.W.) was added to a final concentration of 30mM, using a stock solution which contained 1M HEPES and 0.35N sodium hydroxide. Following the addition of solid sodium bicarbonate ($NaHCO_3$) at a final concentration of 3.7mg/ml, the medium was stirred until the $NaHCO_3$ had dissolved. It was then sterilised and its pH was readjusted to pH 7.4 by flushing with 5% CO_2 in air prior to the addition of FCS (10% v/v).

(c) RPMI - 1640 medium was used primarily for the *in vitro* bactericidal test. This was also prepared from powdered medium (Microbiological Associates Inc., Bethesda, U.S.A.) and was supplemented with 30% FCS and buffered with sodium bicarbonate (1.4mg / ml). Antibiotics were never added to this medium and the FCS was always absorbed with the appropriate bacteria and sterilized prior to use.

Peritoneal exudate cells

The free cells in the peritoneal cavity of both normal and immunized mice are described as 'peritoneal exudate cells'. They were harvested by washing out the peritoneal cavity with

2.0ml of heparinised Hanks' balanced salt solution. Within a group, the cells from a number of mice were pooled and centrifuged at 400 x g for 7 minutes. The pellet was resuspended in the appropriate tissue culture medium and the cell concentration was determined. The volume of the cell suspension was then adjusted to the appropriate concentration for use.

Cell counting

Samples of the cell suspension were diluted in saline containing trypan blue (1mg / ml). The viable (unstained) cells were counted using a haemocytometer.

C5-LPS sensitised SRBC rosette assay

One millilitre aliquots of the peritoneal exudate cell suspension, adjusted to approximately 5.0×10^6 cells / ml of Hanks' medium, were placed into sterile glass Leighton tubes, each containing a coverslip resting on 10 x 40mm flat wells. The cultures were incubated at $37^{\circ}C$ for approximately 30 minutes, by which time the majority of the PEC had adhered to each coverslip. The medium and any non-adherent cells were removed from each tube and replaced with 1.0ml of fresh medium. After a further 3 hour incubation, the medium was again removed and replaced with fresh medium containing 0.5% C5-LPS sensitised SRBC. In some groups, the C5-LPS sensitised SRBC were opsonised with 1 HU of mouse anti-C5 antiserum. These cultures were left at $4^{\circ}C$ for at least an hour to ensure that any rosette formation that could occur, did so. Then the coverslips were removed, washed thoroughly in saline, stained with Wright's stain and examined microscopically. The number

of SRBC adhering to each of 100 randomly chosen peritoneal cells was noted in each culture and scored as 0, 1, 2, 3, 4, 5 or $\geq$6. From this data, the average number of SRBC in a rosette around each adherent cell was calculated.

Bacterial adherence assay

The ability of peritoneal exudate cells to bind live Salmonella typhimurium C5 rather than the C5-LPS sensitised SRBC was examined using this assay. Here 10^8 bacteria were added to 'coverslip' cultures of 5×10^6 PEC. In some groups the bacteria had been opsonised with 1 HU of mouse anti-C5 antiserum. After standing for an hour at 4^oC, the coverslips were removed, rinsed with saline, stained with Gram's stain and examined microscopically. While this assay could have been used quantitatively, for convenience it was used as a qualitative assay, to complement the SRBC rosette data.

In vitro bactericidal assay

Peritoneal exudate cells were suspended to a concentration of 5×10^7 / ml in RPMI 1640 medium with 30% FCS. A log phase culture of either Salmonella typhimurium C5 or Listeria monocytogenes was suspended in the same medium to a final concentration of 5×10^5/ ml. In some groups the bacteria had been opsonised with either an F1 anti-C5 antiserum or an F1 anti-Listeria monocytogenes antiserum. Suspensions of cells and bacteria were mixed and dispensed into Microtitre plastic tissue culture trays (M-29 ART; Cooke Instruments) so that each flat-bottomed well contained 10^4 bacteria and 10^6 PEC in 40µl of culture medium. Each experiment also included wells containing only bacteria. The trays were covered with loose

fitting lids (M-42 AR) and incubated at 37°C in the CO_2 incubator (100% humidity, 5% CO_2 in air). After various periods of incubation, the total number of bacteria remaining in some of the wells set up was determined by lysing the peritoneal exudate cells in each well with an equal volume of 1% Triton X 100 and plating the contents of the wells onto dried nutrient agar plates. These were incubated at 37°C to allow colonies of bacteria to grow up and the number of colonies on each plate were counted. The results were expressed as the percentage recovery of the bacterial inoculum used in each assay.

Tumour cell lines

The following tumours were used in the present study:-

(a) Ehrlich Ascites Tumour (EAT), a mammary tumour which was maintained by *in vivo* passage in SPF or F1 mice. It is not mouse strain specific.

(b) B16, a melanoma which arose spontaneously in C57Bl/6J mice. It was maintained by both *in vivo* passage (intraperitoneal or subcutaneous) or by *in vitro* passage in Dulbecco's modified Eagle's Minimal Essential Medium, 10% FCS, 30mM HEPES and 0.22% sodium bicarbonate.

(c) P815, a mastocytoma which was chemically induced in DBA/2 mice and was maintained by *in vitro* passage in Dulbecco's modified Eagle's Minimal Essential Medium, 10% FCS, 30mM HEPES and 0.22% sodium bicarbonate.

^{51}Cr labelling of tumour cells

For *in vitro* experiments 5 x 10^6 washed tumour cells were

incubated in 1ml of Dulbecco's modified Eagle's Minimal Essential Medium with 100µCi of $^{51}Cr(Na_2CrO_4$,100-300mCi/mg chromium, Radiochemical Centre, Amersham, England) for 60 minutes at 37°C with occasional shaking. The labelled cells were cooled to 4°C, washed 5 times with culture medium and resuspended to 10^5 viable tumour cells / ml.

In vitro tumouricidal assay

The technique used was previously described by Ashley (1976). Peritoneal exudate cells were washed and suspended to a concentration of 10^7 / ml in Dulbecco's modified Eagle's minimal Essential Medium with 10% FCS, sodium bicarbonate, 30mM HEPES and antibiotics. Similarly the ^{51}Cr-labelled tumour cells were suspended in the same medium to a concentration of 10^5 / ml. Equal volumes of these suspensions were then mixed and dispensed into Microtitre plastic tissue culture trays (M-29 ART; Cooke Instruments), so that each well contained 10^4 ^{51}Cr-labelled tumour cells and 10^6 PEC in 0.2ml of culture medium. Each experiment also included wells containing only ^{51}Cr-labelled tumour cells. The trays were covered with loose fitting lids and incubated for 20 hours at 37°C in the CO_2 incubator (100% humidity, 5% CO_2 in air).

After 20 hours, quadruplicate wells were assayed for the release of ^{51}Cr into the medium. An automatic pipette with disposable tips was used to remove 0.1ml of medium from each well without disturbing the cells. The amount of radioactivity in each sample was measured in a Packard Instrument Company well-type γ-ray scintillation counter.

The results were expressed as the percentage of cytolysis, which was defined by the equation:

$$\% \text{ Cytolysis} = \frac{\text{experimental}^{51}\text{Cr release-spontaneous release}}{\text{total releasable}^{51}\text{Cr-spontaneous release}} \times 100$$

where the total releasable amount of ^{51}Cr was determined by lysing duplicate 1ml samples of ^{51}Cr labelled tumour cells with 4 drops of chloroform, and where spontaneous release of ^{51}Cr was obtained from tumour cells cultured alone.

In vivo tumouricidal assay

In these experiments an assessment was made of the ability of mice to control the growth of a tumour challenge. Various groups of mice were injected with either 10^6 EAT or 10^5 B16, and their fate was followed for a period of 90 days. The results were expressed both in terms of the percentage survival and of the mean time to death.

CHAPTER 3

AN INTRODUCTORY STUDY ON THE ROLE OF ANTIBODY IN IMMUNITY TO SALMONELLA INFECTIONS

Introduction

Facultative intracellular bacterial parasites have the ability to survive and multiply within the macrophages of a normal host. Extensive studies on the resistance of animals to infection with Brucella abortus (Pomales-Lebrón and Stinebring, 1957; Holland and Pickett, 1958), Listeria monocytogenes (Mackaness, 1962), Mycobacterium tuberculosis (Lurie, 1942) and Salmonella typhimurium (Blanden, Mackaness and Collins, 1966) have all provided evidence that resistance is dependent on the acquisition of macrophages with increased microbicidal activity. The term "activated macrophage" was originally introduced to describe macrophages with enhanced microbicidal activity (Mackaness, 1962). Currently, enhanced tumouricidal (Hibbs, 1975) and trypanosomicidal (Nogueira et al., 1977 a & b) activities are also used as functional indicators of these cells. However, there is some evidence to suggest that these activities may not always be generated together as a consequence of macrophage activation (Wing et al.,1977; North, 1978) and so caution must be exercised when extrapolating results from one system to another.

In physical appearance "activated macrophages" are characteristically larger, have a more ruffled plasma membrane and have a greater number of phagolysosomes and endocytic

vesicles than their unstimulated counterparts. These cells spread rapidly on glass or plastic surfaces and have an increased capacity for phagocytosis (Cohn, 1978; North, 1978). The behavioural changes associated with macrophage activation are also reflected at the biochemical level. Activated macrophages are metabolically more active and have been reported to contain 7 - 8 times the amount of ATP under steady state conditions (Michl, Ohlbaum and Silverstein, 1976). Glucose oxidation and oxygen consumption are also increased (Nathan, Karnovsky and David, 1970). The ability of activated macrophages to generate increased amounts of certain reactive oxygen metabolites during phagocytosis has now been reported by a number of workers (Nathan and Root, 1977; Johnston, Godzik and Cohn, 1978; Weiss, Lo Buglio and Kessler, 1980; Wilson, Tsai and Remington, 1980). Production of one such intermediate, hydrogen peroxide (H_2O_2) is, to date, the best biochemical correlate of the expression of microbicidal and cytotoxic activities (Cohn, 1978; Nathan _et al_., 1979). The dependence of the intracellular fate of _Toxoplasma gondii_ on the generation of these oxygen metabolites suggests that they may actually be the lethal weapon with which macrophages kill these and other organisms (Wilson, Tsai and Remington, 1980). Similarly Weiss, Lo Buglio and Kessler (1980) concluded that the generation of the superoxide ion and hydrogen peroxide played an integral role in the cytotoxic mechanism of human monocytes stimulated with phorbol myristate acetate. Other changes are also apparent in the make-up of activated macrophages, particularly the level of 2 plasma membrane ectoenzymes. Alkaline phosphodiesterase 1 increases between 2- and 3-fold, while 5' nucleotidase is lost with activation. Finally, the

secretion of a number of neutral proteases including collagenase, elastase and plasminogen activator, is an important feature in the behaviour of activated macrophages (Cohn, 1978).

The process of macrophage activation appears, from a variety of experimental evidence, to be mediated by antigenically stimulated, sensitized T-lymphocytes. The importance of these cells is evident from the _in vivo_ demonstrations that T-cells are necessary for the generation of an effective antibacterial immune response (Davies, 1975), and that they can be used to transfer immunity to normal recipients (Mackaness, 1969; Lane and Unanue, 1972; North, 1973). Similarly, when normal macrophages were cultured with sensitized lymphocytes in the presence of specific antigen, macrophage activation was observed _in vitro_ (Simon and Sheagren, 1972; Krahenbuhl, Rosenberg and Remington, 1973). Subsequently it was further realized that the secretion products of antigen-stimulated lymphocytes would also activate macrophages such that they acquired increased listericidal (Fowles et al., 1973), trypanosomicidal (Nogueira, Gordon and Cohn, 1977 a & b) and tumouricidal (Fidler, Darnell and Budman, 1976; Ruco and Meltzer, 1977) capacities. These soluble factors which modulate macrophage function are collectively referred to as lymphokines and while they have not yet been detected in tissue granulomas, some have been demonstrated in the circulation (Salvin, Youngner and Lederer, 1973; Kostiala, McGregor and Logie, 1975).

The level of macrophage activation generated in response to infection with facultative intracellular bacterial parasites appears to be regulated by the availability of bacterial

antigens. Collins (1968), reported that only mice recently infected with *Salmonella enteritidis* or *Salmonella typhimurium* were able to eliminate a superinfecting inoculum in 3 - 5 days. In convalescent animals, the antimicrobial immunity rapidly decayed in the absence of a persisting bacterial population. However, it was quickly regenerated after re-infection with the homologous organism. Similarly Mackaness (1964, 1968) made some observations which are consistent with these results. For example, he found that the state of acquired resistance was effectively recalled only when mice, which had previously been infected with *Listeria monocytogenes*, *Brucella abortus* or *Mycobacterium tuberculosis*, were re-infected with the homologous organism. He also demonstrated that although *Mycobacterium tuberculosis* BCG-infected mice were highly resistant to challenge with *Listeria monocytogenes*, their lymphoid cells were unable to confer protection against challenge with *Listeria monocytogenes*. However, resistance to this challenge was readily transferred when mice received either *Listeria monocytogenes*-sensitized cells, or *Mycobacterium tuberculosis* BCG-sensitized cells and immunizing bacteria simultaneously. This ability of the convalescent animals to mount an anamnestic response has been shown to be dependent on their ability to regenerate large numbers of non-replicating T-cells within the spleen (North and Deissler, 1975). Likewise, the finding that memory cells associated with helper T-cell function appear to be long-lived T-lymphocytes which require the presence of an intact thymus for their maintenance (Simpson and Cantor, 1975), is relevant to this discussion.

The acquired capacity of macrophages to kill intracellular

bacteria such as Listeria, Mycobacteria and Salmonella is clearly an important factor in immunity to the diseases produced by these organisms (Lurie, 1942; Ushiba *et al.*, 1959; Mackaness, 1969; North, 1973). In comparison, the role of antibody is often less obvious and has been a matter of controversy, aspects of which have already been discussed in Chapter 1. Briefly, some workers, observing the significance of macrophage activation (Lurie, 1942; Ushiba *et al.*, 1959), the apparent non-specific expression of antimicrobial resistance (Boehme and Dubos, 1958; Howard *et al.*, 1959; Mackaness, 1964; Rowley, Auzins and Jenkin, 1968) and the limited ability of either killed vaccines or specific antibody to confer protection (Collins, 1969 a & b) have argued that the mechanism of immunity is purely "cellular" in nature. However, the demonstrations that opsonic antibody not only enhances the phagocytosis and intracellular killing of *Salmonella typhimurium* by normal macrophages (Jenkin, 1963; McIntyre, Rowley and Jenkin, 1967) but also plays a vital role in host survival by reducing the size of the challenge inoculum (Collins, 1970), have suggested that this view does not provide a complete explanation for immunity to intracellular bacteria. In keeping with this conclusion, mice immunized and challenged with either *Listeria monocytogenes* or *Mycobacterium tuberculosis* have been shown to eliminate the homologous challenge organism more effectively than the heterologous one (Coppel and Youmans, 1969). Similarly Zinkernagel (1976) observed that mice immunized with *Salmonella typhimurium* were able to control a secondary typhoid infection whereas mice immunized with *Listeria monocytogenes* could not do so. In addition, Davies (1975) found that the immunization of mice

with an alcohol-killed *Salmonella typhimurium* C5 vaccine either before they were infected with *Salmonella enteritidis* 11RX or before they received spleen cells from *Salmonella enteritidis* 11RX-infected mice, greatly enhanced their resistance to challenge with *Salmonella typhimurium* C5. Lastly, recent genetic studies have revealed that genes controlling both humoral and cell-mediated responses influence the resistance of mice to infection with *Salmonella typhimurium* (O'Brien *et al.*, 1979; Wooley and Ebringer, 1980). In view of these and other results, it is reasonable to conclude that both humoral and cellular responses are important in the production of immunity to intracellular bacteria and, particularly, to Salmonellae.

While antibody appears to be important in immunity to typhoid, its role is both ill-defined and poorly understood. It would be extremely valuable to know whether antibody is actually required for the expression of cell-mediated immunity. With this in mind, it was decided to examine *in vitro* and *in vivo*, various aspects of immunity to *Salmonella typhimurium* C5 in the resistant, *Salmonella enteritidis* 11RX-infected, F1 mice. The aim of these studies,which are presented in this chapter, was to confirm the importance of antibody in resistance to typhoid and to delineate more clearly its role in resistance to *Salmonella typhimurium* C5.

The effect of antibody specific for *Salmonella typhimurium* C5 on the adherence of *Salmonella typhimurium* C5 to peritoneal exudate cells *in vitro*

The interaction of activated macrophages with *Salmonella typhimurium* C5 was investigated *in vitro*, in an attempt to

clarify whether these phagocytic cells, like normal macrophages, require opsonic antibody to phagocytose and kill the typhoid bacilli. Peritoneal macrophages are easily removed from both normal and immunized mice by routine lavage techniques, and so are readily available for study of this interaction. The adherence of whole bacteria and lipopolysaccharide-sensitized sheep red blood cells to peritoneal exudate cells from both normal and *Salmonella enteritidis* 11RX-infected mice, was examined to detect any innate ability of either population of phagocytic cells to recognise and bind the pathogen or its "O" somatic antigens. Secondly, antibody specific for *Salmonella typhimurium* C5 was introduced to determine whether these opsonins promoted adherence.

In the first experiment, peritoneal cells were harvested from 10 male F1 mice which had been injected intraperitoneally with 1.0×10^5 *Salmonella enteritidis* 11RX 6 days previously. The activated state of the peritoneal macrophages from such mice has been demonstrated by their ability to kill tumour cells (Ashley and Hardy, 1973) and release high levels of H_2O_2 (Cooper, personal communication). Likewise, normal peritoneal cells were obtained from a similar number of uninfected, age-matched control mice. Twenty monolayers of both types of peritoneal exudate cells were prepared on glass coverslips by the technique described in Chapter 2. After a short incubation, the tissue culture medium on each monolayer was replaced with fresh medium containing a 0.5% (v/v) suspension of opsonized or unopsonized *Salmonella typhimurium* C5 LPS-sensitized sheep red blood cells. One haemagglutinating unit (1HU) of mouse anti-*Salmonella typhimurium* C5 antiserum had been used to opsonize the erythrocytes. Rosette formation

TABLE 3.1

The effect of antibody specific for *Salmonella typhimurium* C5 on the adherence of C5 LPS sensitized SRBC to monolayers of peritoneal exudate cells harvested from either normal or *Salmonella enteritidis* 11RX-infected F1 mice

Peritoneal exudate cell type[a]	F1 anti-C5 antiserum[b]	No. C5 LPS SRBC Bound/PEC[c]
Normal	-	0.07 ± 0.11
Normal	+	2.39 ± 0.04
Activated	-	0.08 ± 0.06
Activated	+	3.12 ± 0.09

a. Peritoneal exudate cells were obtained from either normal mice or mice infected intraperitoneally with 1.0×10^5 *Salmonella enteritidis* 11RX (day -6)

b. 1HU of F1 anti-C5 antiserum was used to sensitize the C5 LPS SRBC

c. The mean number of C5 LPS SRBC adhering to the PEC ± SE was determined from 10 monolayers

was then allowed to occur by leaving the cultures to stand at 4°C for 1 hour. Subsequently, each of the coverslips was washed, stained, and the number of red blood cells rosetting around 100 randomly chosen peritoneal cells was determined microscopically. The results were expressed as an average of the number of erythrocytes bound per glass-adherent peritoneal cell and are shown in Table 3.1.

The lipopolysaccharide-sensitized erythrocytes were able to adhere to the peritoneal macrophages from both normal and Salmonella enteritidis 11RX-infected mice, but only after prior opsonization. The influence of antibody on this adherence appeared to be even more dramatic than the figures indicated, as a significant proportion of the glass-adherent cells did not bind any red blood cells and so reduced the averages slightly. Both peritoneal macrophage populations appeared to have little, if any, innate ability to bind the bacterial somatic antigens in the absence of antibody.

In a complementary study, the adherence of live Salmonella typhimurium C5 to these macrophage monolayers was assessed qualitatively. The "coverslip" cultures of peritoneal exudates from both normal and Salmonella enteritidis 11RX-infected mice were seeded with approximately 1.0×10^{8} opsonized or unopsonized bacteria. As in the previous experiment, 1HU of mouse anti-Salmonella typhimurium C5 antiserum was used for opsonization and adherence occured while the cultures were left to stand at 4°C for at least 1 hour. The coverslips were then washed, stained and examined as described above, using a Leitz Ortholux II microscope. Representative photographs of the stained preparations were also taken (Figure 3.1 and Figure 3.2).

FIGURE 3.1

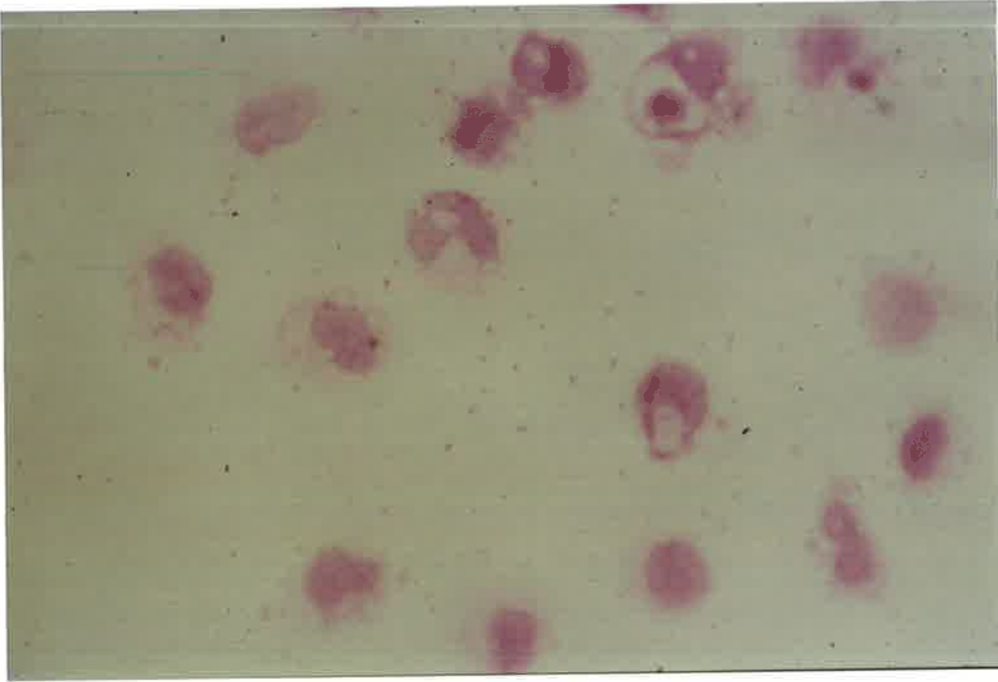
The effect of antibody specific for *Salmonella typhimurium* C5 on the adherence of *Salmonella typhimurium* C5 to monolayers of peritoneal exudate cells harvested from normal F1 mice.

A. Glass-adherent peritoneal cells and *Salmonella typhimurium* C5

B. Glass-adherent peritoneal cells and *Salmonella typhimurium* C5 opsonized with 1HU of mouse anti-*Salmonella typhimurium* C5 antiserum.

The Gram-stained monolayers were photographed at a X500 magnification.

A



B

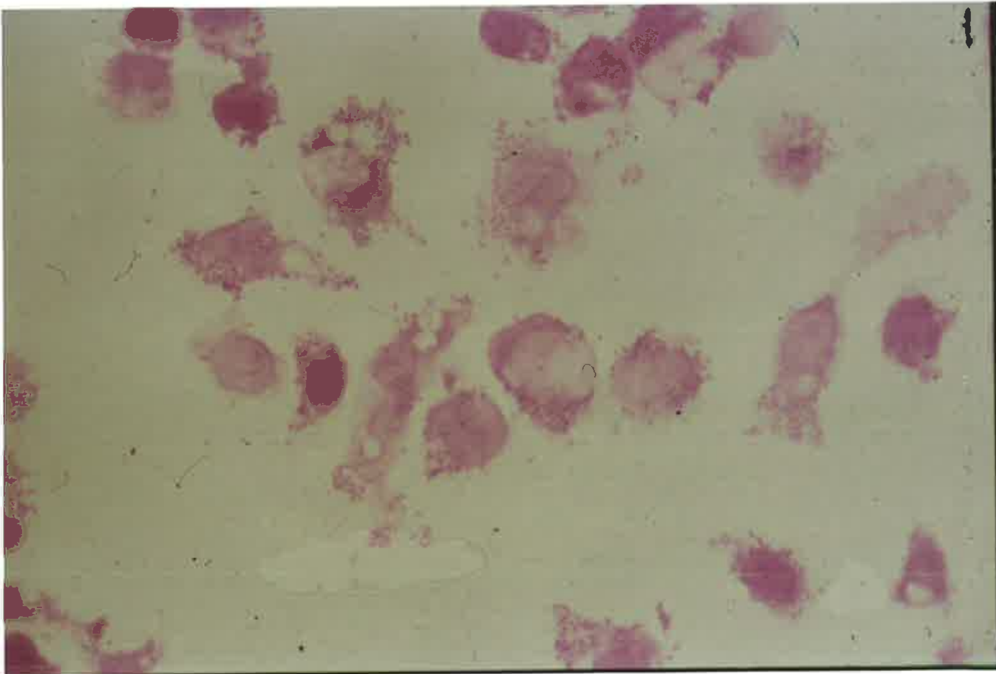


FIGURE 3.2

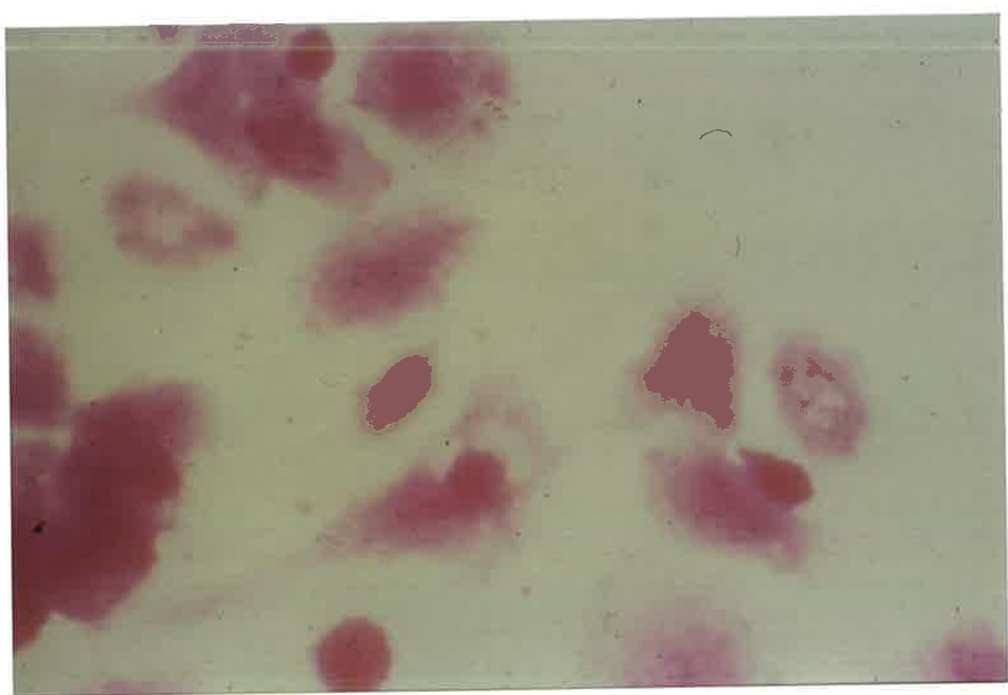
The effect of antibody specific for *Salmonella typhimurium* C5 on the adherence of *Salmonella typhimurium* C5 to monolayers of peritoneal exudate cells harvested from F1 mice infected intraperitoneally with 1.0 x 10^5 *Salmonella enteritidis* 11RX (day -6).

A. Glass-adherent peritoneal cells and *Salmonella typhimurium* C5

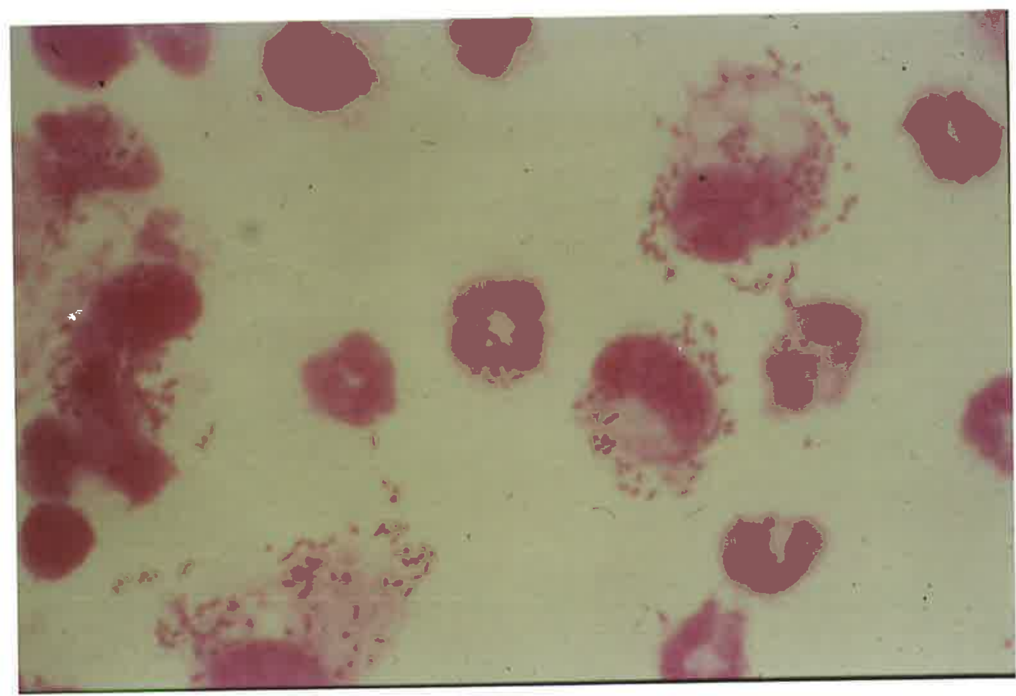
B. Glass-adherent peritoneal cells and *Salmonella typhimurium* C5 opsonized with 1HU of mouse anti-*Salmonella typhimurium* C5 antiserum

The Gram-stained monolayers were photographed at a X625 magnification.

A



B



Both normal and activated peritoneal cells were able to bind only the opsonized bacteria. Although the number of adherent bacteria was difficult to enumerate, it was apparent that both types of phagocytic cells were capable of binding large numbers of bacteria, provided specific antibody was present. In contrast, the unopsonized bacteria were unable to adhere to either macrophage type and most of them were removed when the monolayers were washed.

These studies indicate that both normal and activated glass-adherent peritoneal cells have little, if any, innate ability to recognise and bind *Salmonella typhimurium* or its somatic antigens. Contrary to these observations, Weir and his colleagues have reported that mouse peritoneal macrophages were capable of binding *Corynebacterium parvum* by a mechanism which was not mediated by antibody. Adherence appeared to be due to bridging between carbohydrate residues on macrophage membrane glycoproteins and carbohydrates in the bacterial cell wall, with the involvement of divalent cations (Ögmundsdóttir and Weir, 1976; Weir and Ögmundsdóttir, 1977; Ögmundsdóttir, Weir and Marmion, 1978). Such a "primitive" and, presumably, non-specific recognition mechanism by mononuclear phagocytes is not effective against all foreign invading pathogens, or plays only a relatively minor role in comparison to antibody and so was not detected in these experiments.

The effect of antibody specific for *Salmonella typhimurium* C5 on the ability of peritoneal exudate cells to kill *Salmonella typhimurium* C5 *in vitro*

Although specific antibody appeared to be necessary for the adherence of *Salmonella typhimurium* C5 to activated

macrophages, its effects on the phagocytosis and intracellular killing of this organism are perhaps more crucial to the host-parasite relationship. Hence, the influence of antibody on the ability of both normal and activated peritoneal macrophages to kill typhoid bacilli *in vitro* was investigated.

Five male F1 mice were infected intraperitoneally with 1.3×10^5 *Salmonella enteritidis* 11RX and another 5 were set aside as controls. Six days later all the mice were sacrificed and their peritoneal cavities lavaged. The peritoneal exudate cells thus obtained were mixed with either opsonized or unopsonized live *Salmonella typhimurium* C5 and then dispensed into plastic Microtitre tissue culture trays, so that each flat-bottomed well contained approximately 10^4 bacteria and 10^6 peritoneal macrophages in 40µl of culture medium. The trays were incubated at 37°C and at various times, 3 wells from each group were assayed and the number of viable typhoid bacilli remaining was determined. The results were expressed as the percentage recovery of the bacterial inoculum used and are shown in Figure 3.3. The bacterial inoculum was able to multiply during this experiment and so the usual opsonization procedure was modified slightly, such that any of the 1HU of mouse anti-*Salmonella typhimurium* C5 antiserum used, which had not absorbed to the organisms, was not removed by washing but was left for the opsonization of subsequent progeny.

Peritoneal macrophages harvested from both normal and *Salmonella enteritidis* 11RX-infected mice required the presence of antibody to phagocytose and kill *Salmonella typhimurium* C5. The normal macrophages had a limited bactericidal capacity and, during the first 30 minutes, were able to reduce the size of

FIGURE 3.3

The effect of antibody specific for *Salmonella typhimurium* C5 on the ability of peritoneal exudate cells to kill *Salmonella typhimurium* C5 *in vitro*

A. The fate of 10^4 unopsonized *Salmonella typhimurium* C5

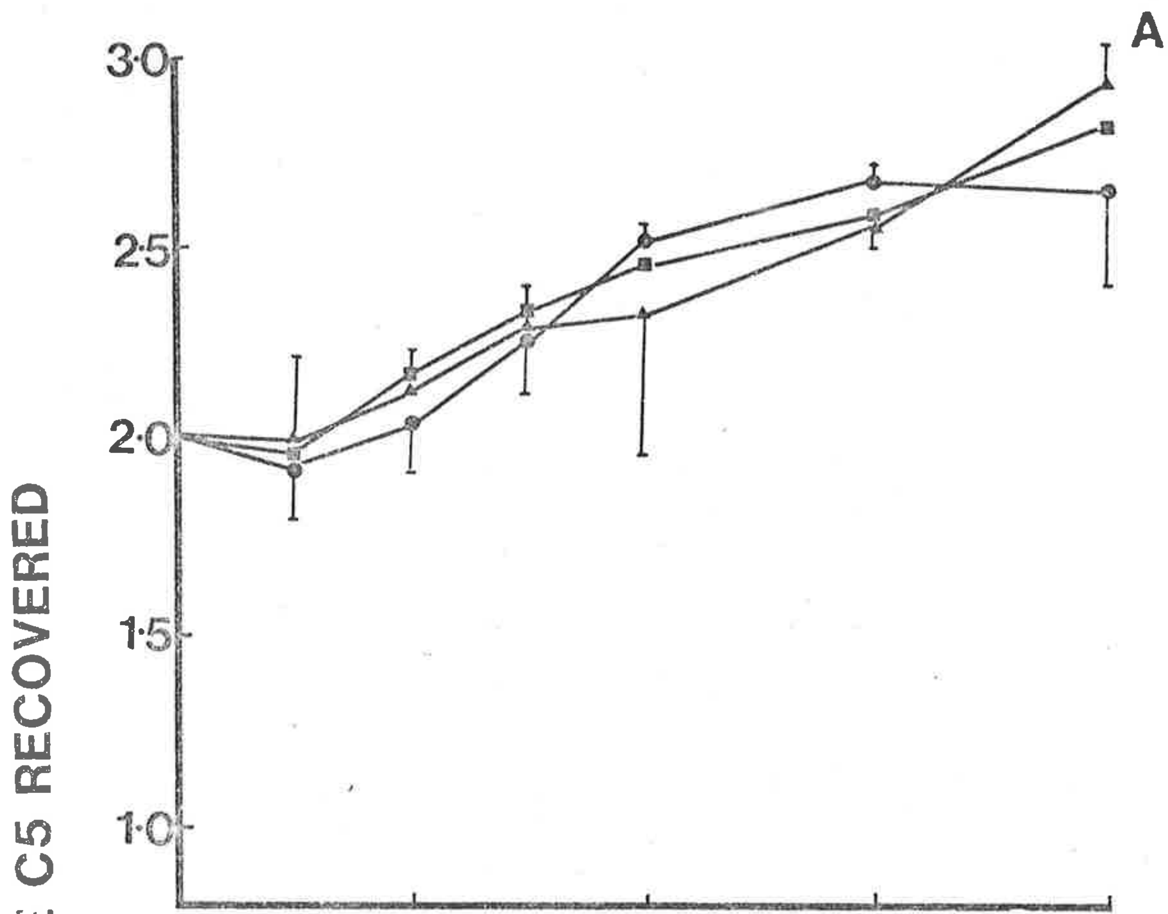
B. The fate of 10^4 *Salmonella typhimurium* C5, opsonized with 1HU of mouse anti-*Salmonella typhimurium* C5 antiserum.

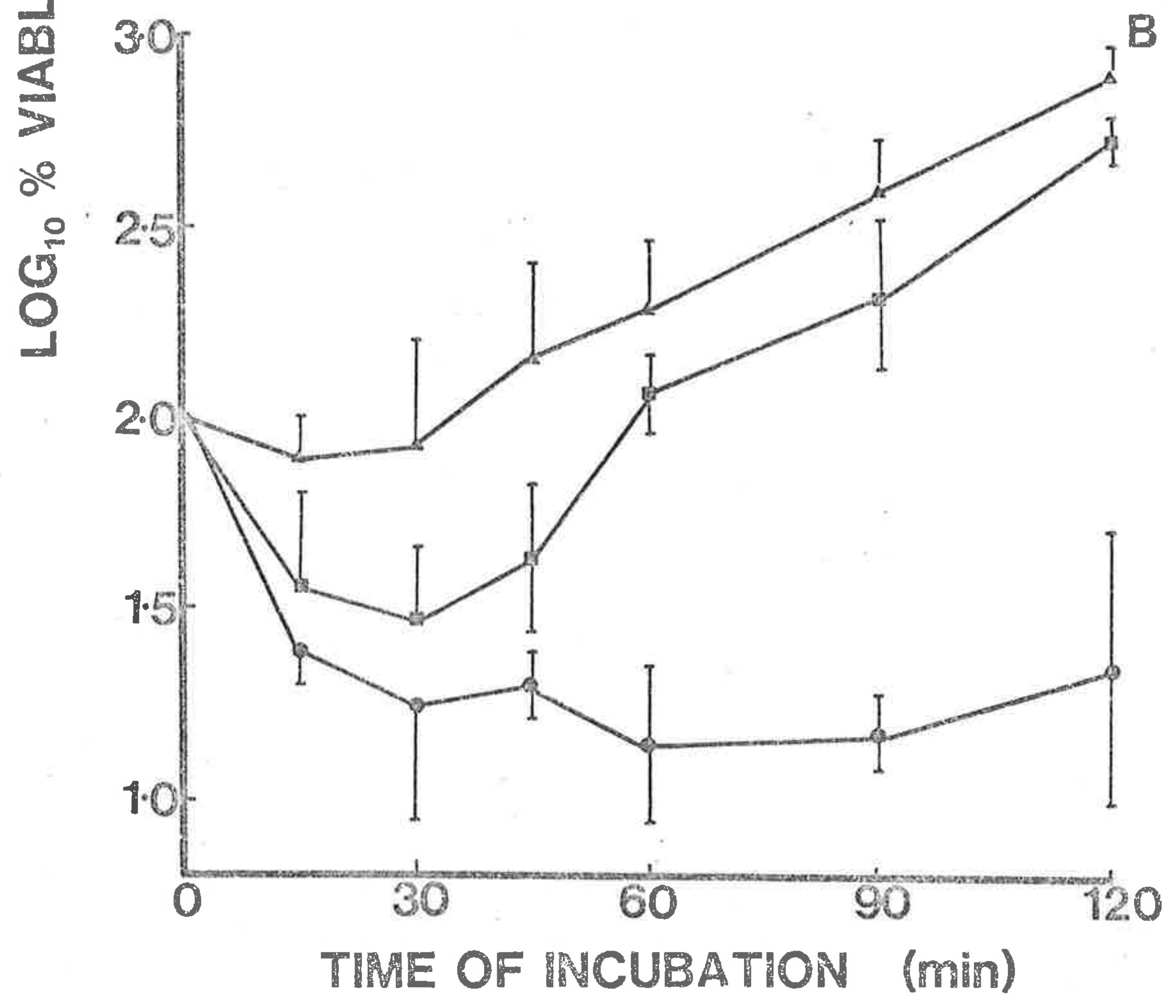
▲ Medium only

■ 10^6 peritoneal exudate cells from normal mice

● 10^6 peritoneal exudate cells from mice infected intraperitoneally with 1.3×10^5 *Salmonella enteritidis* 11RX (day -6).

Each point represents the mean ± S.E. of 3 wells.


A
3·0
2·5
2·0
1·5
1·0
LOG₁₀ % VIABLE C5 RECOVERED


B
3·0
2·5
2·0
1·5
1·0
0
30
60
90
120
TIME OF INCUBATION (min)

the infecting inoculum. However, they were unable to control the subsequent growth of the bacteria which remained. The enhanced bactericidal capacity of the activated macrophages was indicated by their ability to destroy approximately 85% of the initial inoculum, and their ability to contain the growth of the infection for a further 90 minutes. After this time, the bacteria gradually overgrew the cultures (data not shown), perhaps because antibody was limiting and/or the cultured macrophages were undergoing functional changes which reduced their bactericidal potential. The latter possibility was suggested by the finding in preliminary experiments that, after a 3 hour incubation, the activated peritoneal macrophages were less effective in destroying the typhoid bacilli (data not shown). Similar studies carried out with peritoneal exudate cells harvested from *Salmonella enteritidis* 11RX-infected LAC strain mice not only confirmed that these activated cells required the presence of antibody to ingest and kill *Salmonella typhimurium* C5, but also that prior incubation reduced their ability to kill intracellular bacterial parasites (Cooper, personal communication). In general agreement with these observations, phagocytic cells have also been found to be less effective in destroying *Toxoplasma gondii* (Wilson and Remington, 1979), and tumour cells (Kotlarski, personal communication) after brief periods of *in vitro* tissue culture.

Although specific antibody was shown to play a crucial role in the *in vitro* killing of *Salmonella typhimurium* C5 by activated macrophages, its role against other intracellular bacteria is still open to question. Indeed, initial *in vitro* experiments with *Listeria monocytogenes* revealed not only that activated peritoneal macrophages were able to destroy this

parasite in the apparent absence of antibody, but also that a mouse anti-*Listeria monocytogenes* antiserum seemed to have little beneficial effect (data not shown). These observations were in general agreement with previously published reports (Mackaness 1962, 1964 and 1969)and so were not pursued. Nevertheless, these observations highlight the possibility that immunity to these two intracellular bacteria may have fundamentally different requirements. Support for this suggestion has come from recent genetic studies which have demonstrated that the distribution of resistance and susceptibility to these pathogens amongst various inbred mouse strains is very different (Cheers and McKenzie, 1978; Hormaeche, 1979; O'Brien, Rosenstreich, Scher, Campbell, MacDermott and Formal, 1980; Plant and Glynn, 1976 and Skamene, Kongshavn and Sachs, 1979).

While these *in vitro* experiments demonstrated quite clearly that activated macrophages required specific antibody for the adherence, ingestion and eventual destruction of *Salmonella typhimurium* C5, they, like other *in vitro* assays should be interpreted cautiously. The behaviour of activated macrophages adhering to the base of "swimming pool" cultures may have little resemblance to their behaviour *in vivo*. In an attempt to circumvent this problem, the remaining studies in this chapter were concerned with assessing the relative roles of the humoral and cellular immune responses against murine typhoid *in vivo*.

The effect of opsonization with antibody specific for Salmonella typhimurium C5 on the clearance of Salmonella typhimurium C5 from the peritoneal cavity of Salmonella enteritidis 11RX-infected mice

An assessment of the ability of activated peritoneal macrophages to kill *Salmonella typhimurium* C5 *in vivo* can be made from the rate at which these typhoid bacilli are cleared from the peritoneal cavity of *Salmonella enteritidis* 11RX-infected mice. One group of 12 male F1 mice was injected intraperitoneally with 10^4 *Salmonella typhimurium* C5 which had been opsonized with a mouse antiserum raised against this organism, while another group received unopsonized bacteria. Similarly, another 2 groups of mice, which had been infected intraperitoneally with 10^5 *Salmonella enteritidis* 11RX 13 days previously, were also injected intraperitoneally with either the opsonized or the unopsonized *Salmonella typhimurium* C5. Then at 20, 40, 60 and 90 minutes after challenge, 3 mice from each group were sacrificed and the number of viable *Salmonella typhimurium* C5 remaining in the peritoneal cavity was determined. These organisms were distinguished from the immunizing strain by colony morphology. The clearance of the challenge inoculum in these 4 groups is shown in Figure 3.4.

The results demonstrated that the opsonization of the bacteria greatly enhanced their rate of clearance from the peritoneal cavity of both normal and *Salmonella enteritidis* 11RX-infected mice. Nevertheless, the unopsonized bacteria were cleared with reasonable efficiency from the peritoneal cavity of the *Salmonella enteritidis* 11RX-infected mice. This last observation is in sharp contrast to the results of the *in vitro* studies, where the peritoneal macrophages from these

FIGURE 3.4

The effect of opsonization with an F1 anti-*Salmonella typhimurium* C5 antiserum on the clearance of *Salmonella typhimurium* C5 from the peritoneal cavity of either normal mice or mice which had been intraperitoneally injected with 10^5 *Salmonella enteritidis* 11RX 13 days previously.

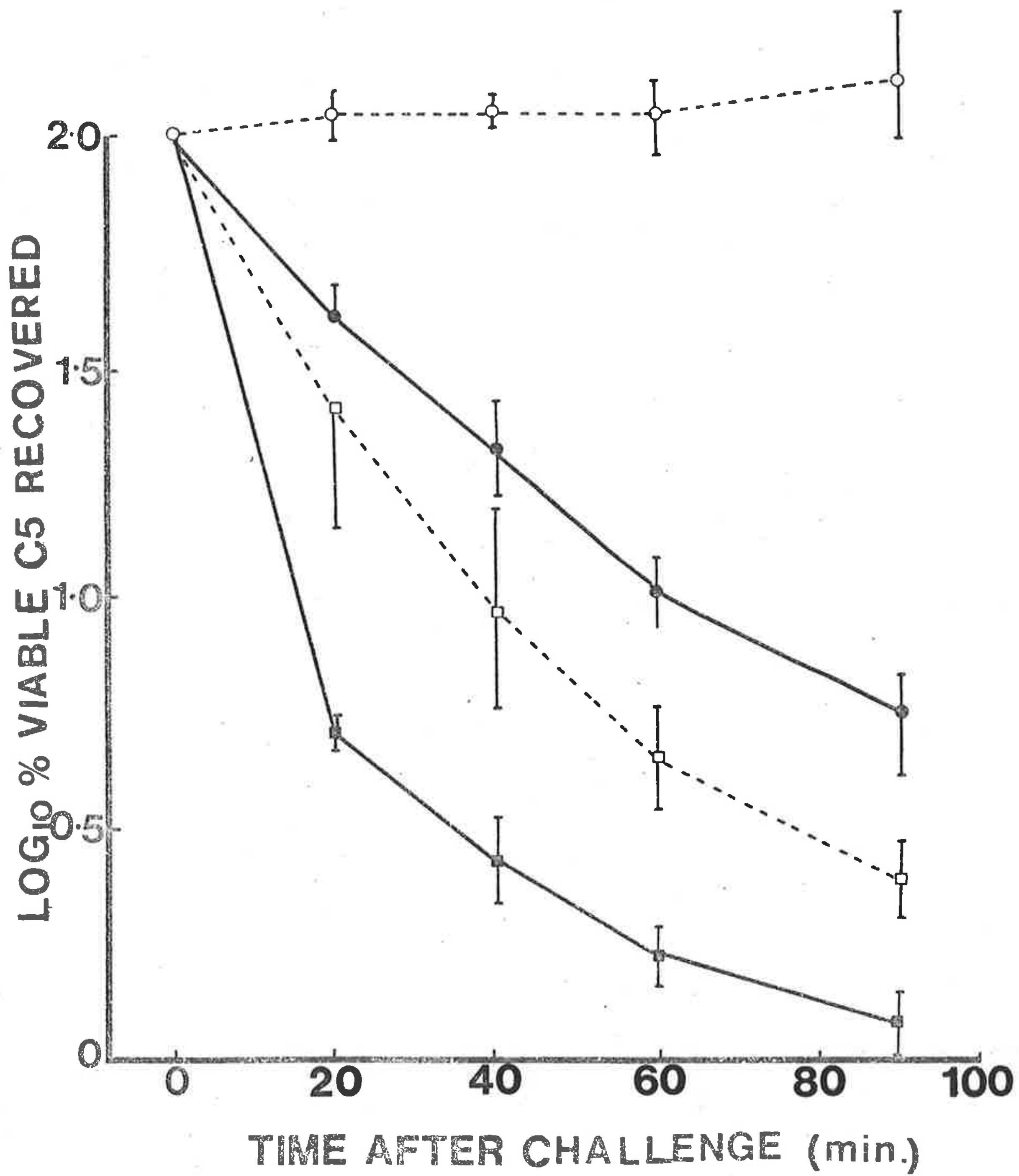
○- - - - -○ Unopsonized C5, normal mice

●———● Opsonized C5, normal mice

□- - - - -□ Unopsonized C5, 11RX-infected mice

■———■ Opsonized C5, 11RX-infected mice

Each point represents the mean ± S.E. of 3 mice.


2·0
1·5
1·0
0·5
0
LOG10 % VIABLE C5 RECOVERED
0
20
40
60
80
100
TIME AFTER CHALLENGE (min.)

immunized mice appeared to require antibody for the expression of their immune function. It is possible that *in vivo*, these activated phagocytic cells may have a limited ability to recognise and phagocytose foreign pathogens in the absence of specific antibody. However, it is equally plausible that these activated macrophages have the same requirement for antibody *in vivo* as they do *in vitro* and that the discrepancy can be attributed to the presence of circulating opsonic antibody in the *Salmonella enteritidis* 11RX-infected mice. Support for this latter hypothesis came from the results of studies outlined in the next chapter. These findings suggested that the serum from *Salmonella enteritidis* 11RX-infected mice contained antibodies specific for *Salmonella typhimurium* C5 (Table 4.1, Figure 4.3) and that these antibodies contributed to the clearance of the typhoid bacilli from the peritoneal cavity of these mice (Figure 4.4). It was also apparent from Figure 3.4 that normal mice were not able to clear the unopsonized *Salmonella typhimurium* C5, a result in keeping with the inability of normal peritoneal macrophages to destroy these organisms *in vitro* (Figure 3.3).

Together, these studies have demonstrated that antibody plays an important role in promoting the destruction of *Salmonella typhimurium* C5 by activated peritoneal macrophages, both *in vitro* and *in vivo*. Although some workers have conceded that specific antibody may play a role in resistance to an intraperitoneal Salmonella infection, they have argued that antibody has little effect on the progress of the systemic disease (Blanden, Mackaness and Collins, 1966; Collins, 1968). Therefore, the importance of antibody in the resistance of *Salmonella enteritidis* 11RX-infected mice to intravenous

challenge with *Salmonella typhimurium* C5, was also assessed, in an attempt to demonstrate that the results of these initial studies with peritoneal macrophages have general implications for phagocytic cells at other anatomical sites. Before commencing this evaluation of the role of the humoral response in immunity to systemic salmonellosis, it was necessary to confirm that *Salmonella enteritidis* 11RX, when administered intravenously, established a carrier state and activated the reticuloendothelial system of these mice.

The growth of *Salmonella enteritidis* 11RX in normal mice

Fifty male F1 mice were injected intravenously with 0.8×10^5 living *Salmonella enteritidis* 11RX. The fate of this infection was followed by periodically sacrificing 5 mice and determining the number of viable organisms which could be recovered from the liver, spleen and peritoneal cavity of each of the mice. The results, shown in Figure 3.5, indicated that *Salmonella enteritidis* 11RX established a carrier state in this strain of mice and were in agreement with earlier reports (Davies, 1975; Ashley, 1976). These avirulent organisms colonized the liver and spleen and then persisted for approximately 38 days, at which time very few, if any, bacteria could be recovered. The steady decline in bacterial numbers, evident after the third day of infection, was presumably due to the development of an effective anti-bacterial cell-mediated immune response (Mackaness, Blanden and Collins, 1966; Blanden, Mackaness and Collins, 1966; Collins, Mackaness and Blanden, 1966).

FIGURE 3.5

The fate of *Salmonella enteritidis* 11RX in F1 mice.

A. The total number of viable 11RX recovered

□——□ Total/ mouse

B. The number of viable 11RX recovered in various organs

○——○ Liver

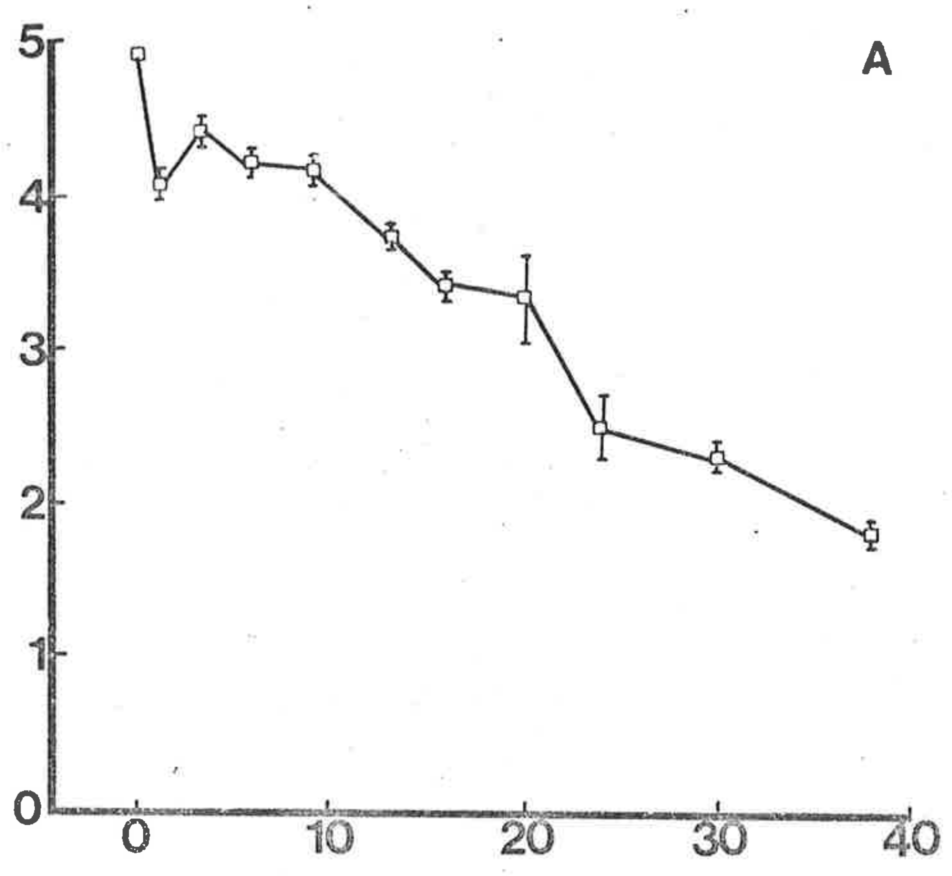
●- - - - -● Spleen

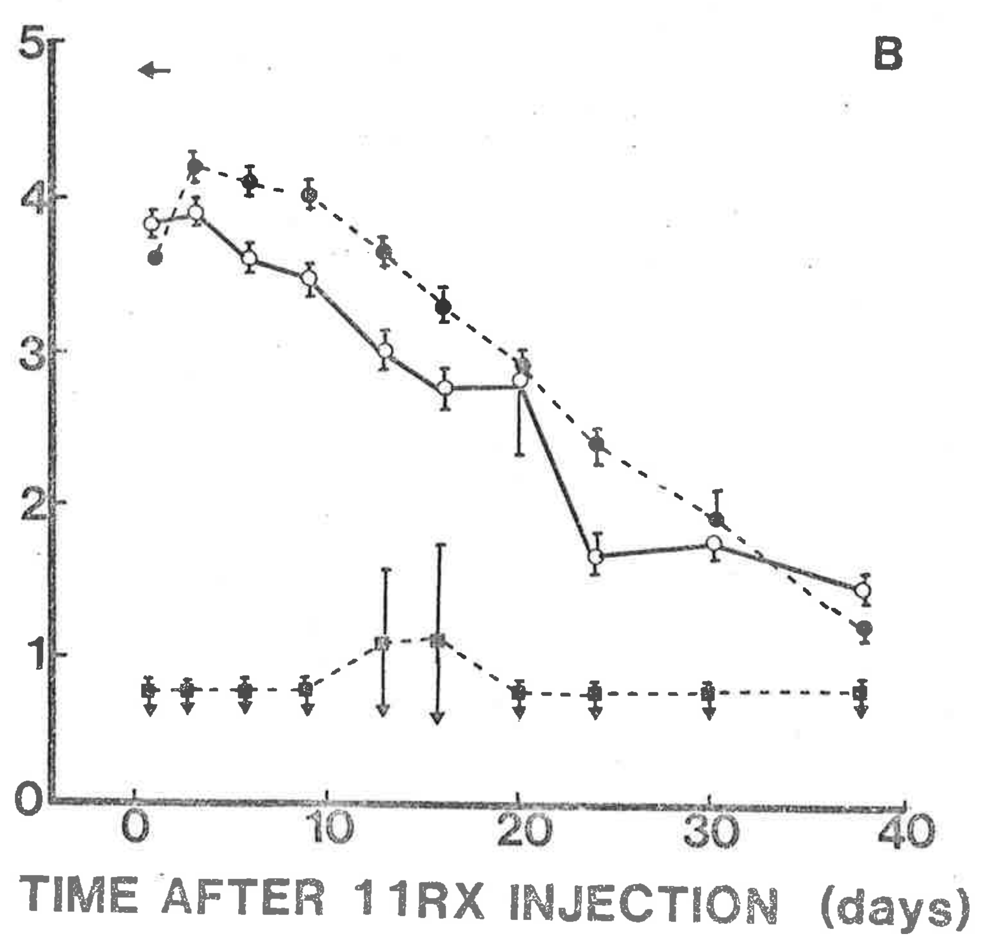
■- - - - -■ Peritoneal cavity

0.8 x 10^5 *Salmonella enteritidis* 11RX were injected intravenously on day 0.

Each point represents the average of the log_{10} values from 5 mice.

The vertical arrows indicate that one or more of the individual values was below the limit of detection. When the individual values were below the limit of detection, these were assumed to be at the limit of detection when calculating the mean.


A
5
4
3
2
1
0
0
10
20
30
40
LOG10 OF VIABLE 11RX RECOVERED


B
5
4
3
2
1
0
0
10
20
30
40
TIME AFTER 11RX INJECTION (days)

The activity of the reticuloendothelial system in mice during an infection with *Salmonella enteritidis* 11RX

The activity of the macrophages in the liver and spleen can be assessed from the speed with which mice are able to clear an intravenous dose of carbon particles from their bloodstream (Jenkin and Rowley, 1961). This study investigated the activation of the reticuloendothelial system which occurred as a result of infection with *Salmonella enteritidis* 11RX. Fifty male F1 mice were injected intravenously with 0.8×10^5 *Salmonella enteritidis* 11RX and periodically the rate of carbon clearance was measured in 5 of these mice by the technique described in Chapter 2. The rate of carbon clearance was also measured in 5 normal age-matched mice, as a control. The results, expressed in terms of a phagocytic index, or K value, are shown in Figure 3.6.

During the course of the Salmonella infection, the carbon clearance rate was greater than that observed in normal mice, particularly from the third to the twenty-fourth day. On day 9, the K value peaked at 0.128 ± 0.012, indicating that the reticuloendothelial system was maximally stimulated at this time. This observed activation of the reticuloendothelial system concurs with an earlier report, where the *Salmonella enteritidis* 11RX organisms were introduced by the intraperitoneal rather than the intravenous route (Hardy and Kotlarski, 1971).

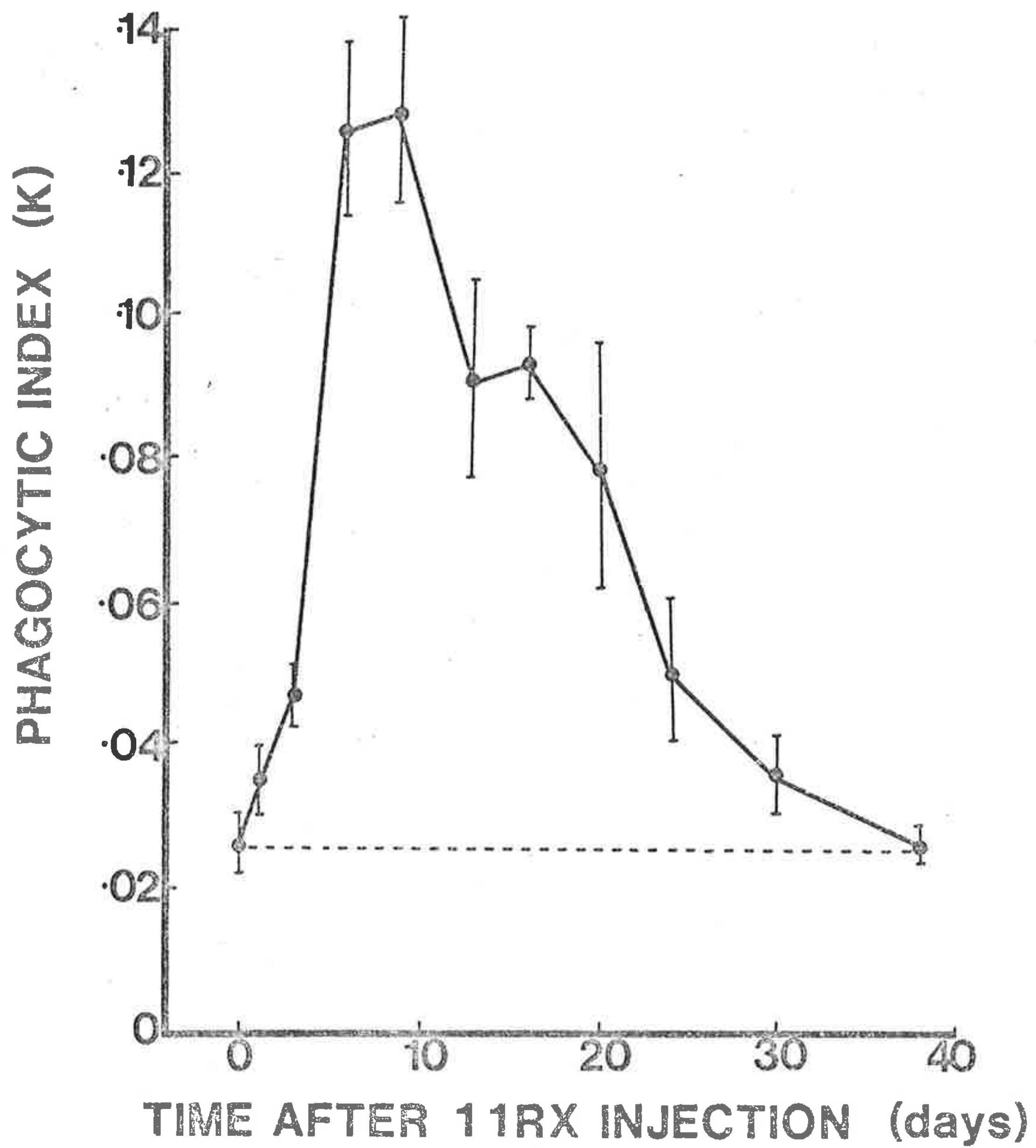
FIGURE 3.6

The clearance of carbon particles from the circulation was measured in F1 mice which had been injected intravenously with 0.8×10^5 *Salmonella enteritidis* 11RX on day 0.

●———● *Salmonella enteritidis* 11RX-infected mice

The dotted line represents the clearance rate found in normal F1 mice.

Each point represents the mean ± S.E. of 5 mice.



PHAGOCYTIC INDEX (K)
·14
·12
·10
·08
·06
·04
·02
0
0
10
20
30
40
TIME AFTER 11RX INJECTION (days)

The effect of antibody specific for Salmonella typhimurium C5 on the resistance of Salmonella enteritidis 11RX-infected mice to challenge with Salmonella typhimurium C5

The enhanced ability of Salmonella enteritidis 11RX-infected mice to clear carbon particles from their bloodstream, together with the declining bacterial load observed during the latter phase of the infection, suggested that these mice had a stimulated reticuloendothelial system, with increased microbicidal activity. This conclusion was supported by the finding that mice immunized with live Salmonella enteritidis 11RX are resistant to challenge with Listeria monocytogenes 2535, Salmonella enteritidis 795, Salmonella paratyphi C and Salmonella typhimurium C5 (Rowley, Auzins and Jenkin, 1968). Subsequently Davies (1975) confirmed the resistance of such mice to infection with Salmonella typhimurium C5. However, he noted that prior immunization with an alcohol-killed Salmonella typhimurium C5 vaccine enhanced the immunity to Salmonella typhimurium C5 produced in response to a Salmonella enteritidis 11RX infection. This effect, thought to be due to an increased level of serum antibody specific for the challenge organism, was particularly marked when a high challenge dose was used (Davies, 1975). However, since this evidence for the role of antibody in protection was rather indirect, an experiment was carried out which was designed to obtain more direct evidence for the role of antibody in protection. The experiment involved the injection of alcohol-killed Salmonella typhimurium C5 vaccine or the passive administration of antibody specific for Salmonella typhimurium C5, to mice which were subsequently challenged intravenously with Salmonella typhimurium C5. Some of these mice were also immunized with Salmonella enteritidis

11RX prior to challenge.

Six groups of 10 male F1 mice were set up as follows:- 3 groups were infected intravenously with 1.0×10^5 Salmonella enteritidis 11RX (day -14), while the other 3 groups were set aside as uninfected controls; 1 of the normal and 1 of the Salmonella enteritidis 11RX-infected groups were injected intraperitoneally with 10µg of an alcohol-killed Salmonella typhimurium C5 vaccine (day -30); another 1 of each type of group received intravenous injections of 0.2ml of antiserum specific for the challenge organisms immediately prior to challenge and twice daily thereafter; the 2 remaining groups were given no additional treatment. All 6 groups were challenged intravenously with 3.7×10^6 Salmonella typhimurium C5 (day 0). The fate of the challenge inoculum in each group was followed for 3 days by sacrificing 3 mice each day and determining the number of viable Salmonella typhimurium C5 organisms that remained in the spleen, liver and peritoneal cavity. The results, expressed in terms of the total number of bacteria recovered, are seen in Figures 3.7 and 3.8.

In Figure 3.7, data for normal mice are shown. These mice were unable to control the Salmonella typhimurium C5 infection because a multiplication of these organisms occurred. Immunization with the alcohol-killed vaccine or the passive transfer of specific antibody had little, if any, effect. The Salmonella enteritidis 11RX-infected mice also appeared to have difficulty in controlling such a high challenge dose of typhoid bacilli (Figure 3.8). However, when these mice received either the alcohol-killed vaccine or the specific antibody, they had little difficulty in reducing the size of the challenge inoculum and controlling the growth of the remaining challenge

FIGURE 3.7

The effect of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine, or the passive transfer of antibody specific for *Salmonella typhimurium* C5, on the ability of normal F1 mice to control the growth of 3.7×10^6 *Salmonella typhimurium* C5 injected intravenously on day 0.

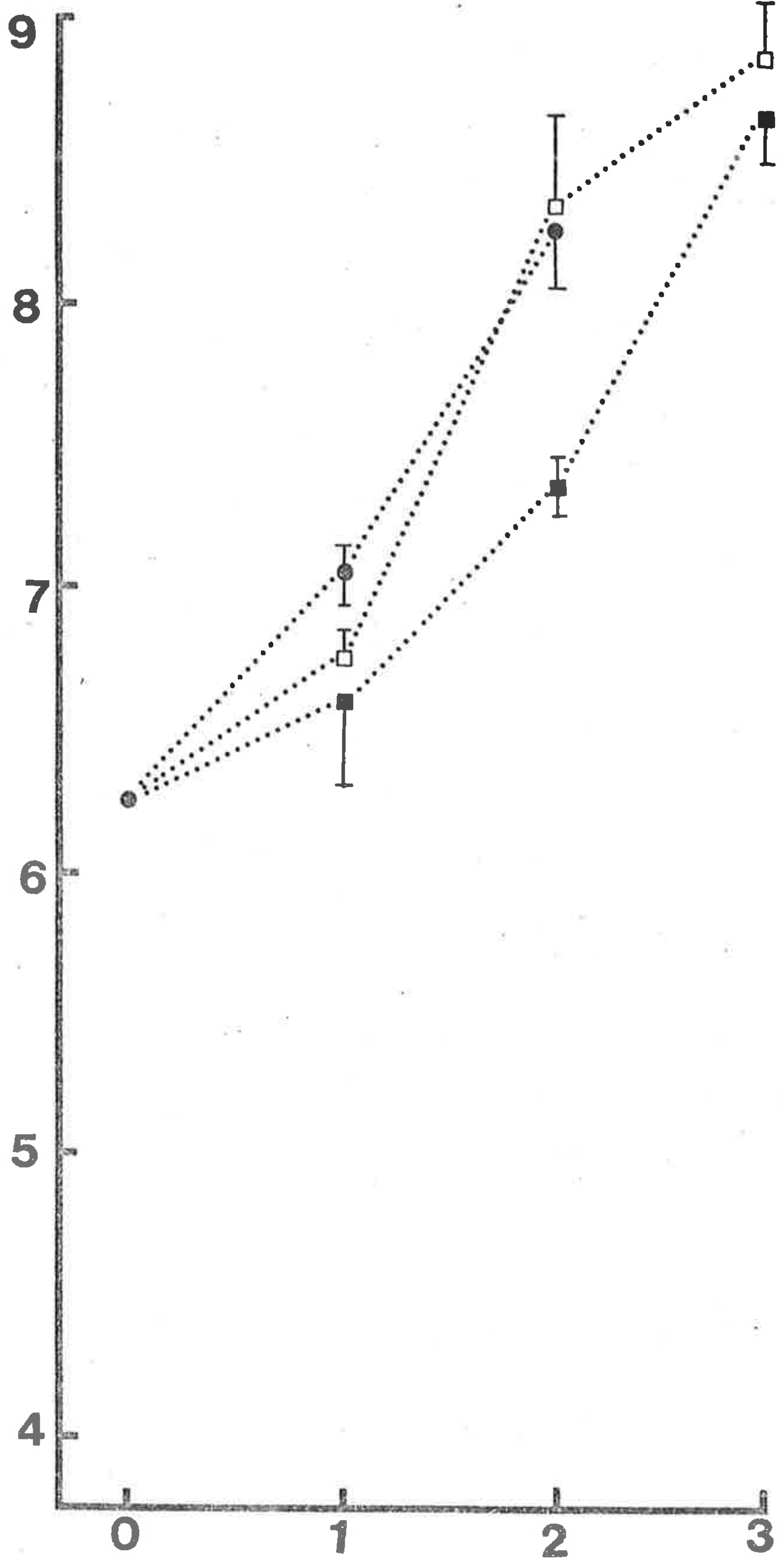
●·········● C5 challenge only

■·········■ 10μg alcohol-killed C5 vaccine i.p. (day -30) and C5 challenge

□·········□ Passive antibody and C5 challenge

The antiserum (0.2ml) was administered intravenously immediately prior to challenge and twice daily thereafter. It was obtained from mice immunized with an alcohol-killed C5 vaccine.

The number of viable bacteria in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen, while very few were recovered from the peritoneal cavity. Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 3 mice.



9
8
7
6
5
4
0
1
2
3
LOG10 OF VIABLE C5 RECOVERED
TIME AFTER CHALLENGE (days)

FIGURE 3.8

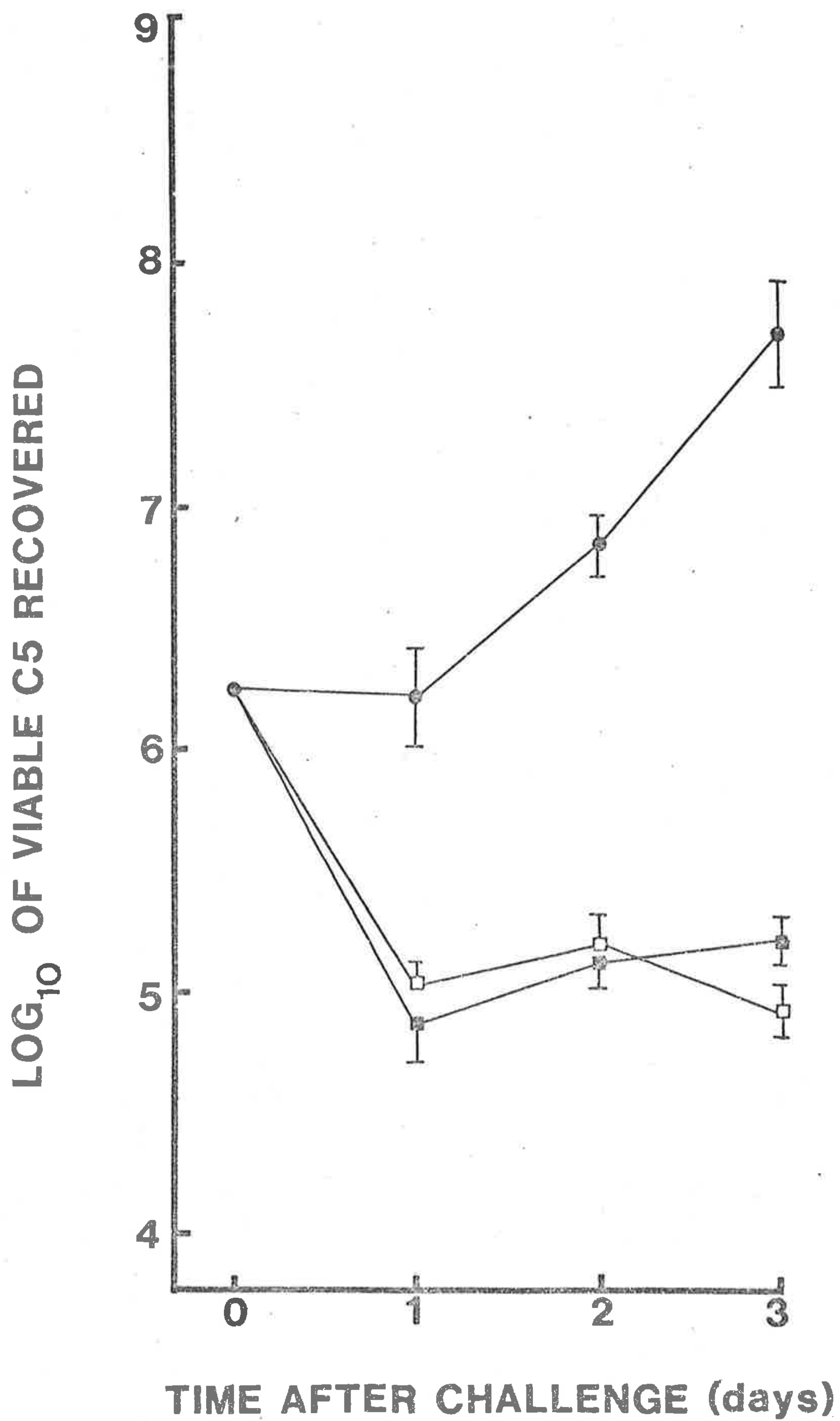
The effect of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine, or the passive transfer of antibody specific for *Salmonella typhimurium* C5, on the ability of *Salmonella enteritidis* 11RX-infected F1 mice to control the growth of 3.7×10^{6} *Salmonella typhimurium* C5 injected intravenously on day 0.

●———● C5 challenge only

■———■ 10μg alcohol-killed C5 vaccine i.p. (day -30) and C5 challenge

□———□ Passive antibody and C5 challenge

All mice were intravenously injected with 1.0×10^{5} *Salmonella enteritidis* 11RX on day -14.
The antiserum (0.2ml) was administered intravenously immediately prior to challenge and twice daily thereafter. It was obtained from mice immunized with an alcohol-killed C5 vaccine.
The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity.
Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 3 mice.



9
8
7
6
5
4
LOG$_{10}$ OF VIABLE C5 RECOVERED
0
1
2
3
TIME AFTER CHALLENGE (days)

organisms. These results demonstrated that specific antibody played an important role in immunity to systemic typhoid and, in so doing, confirmed and extended Davies' (1975) initial observations.

The recall of immunity to *Salmonella typhimurium* C5 in long term *Salmonella enteritidis* 11RX-infected mice

Collins (1968) reported that immediately effective antibacterial immunity was maintained in mice infected with either *Salmonella enteritidis* or *Salmonella typhimurium* only whilst these organisms persisted in the liver and spleen. In addition, reinfection with the homologous organism resulted in a rapid recall of the antibacterial immunity within 3 days. In keeping with these observations, the resistance of *Salmonella enteritidis* 11RX-infected mice to challenge with either *Salmonella typhimurium* C5 or Ehrlich Ascites Tumour has been shown to decline as the immunizing infection is cleared (Davies, 1975; Ashley, 1976). Similarly, the resistance to Ehrlich Ascites Tumour could be recalled by the injection of a *Salmonella enteritidis* 11RX protein antigen preparation (Ashley, 1976). However, it still remained to be shown whether such a protein antigen preparation was capable of recalling immunity to *Salmonella typhimurium* C5 in long-term *Salmonella enteritidis* 11RX-infected mice. It was thought that this recall procedure may be a means of eliciting macrophage activation without the complications of concurrent cross-reacting and polyclonal humoral responses which are associated with any *Salmonella enteritidis* 11RX infection, and so be useful in delineating the role of antibody in immunity to *Salmonella typhimurium* C5. Hence, because of its relevance to

FIGURE 3.9

The effect of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine and a *Salmonella enteritidis* 11RX protein antigen on the ability of normal F1 mice to control the growth of 1.2 x 10^4 *Salmonella typhimurium* C5 injected intraperitoneally on day 0.

□- - - - -□ C5 challenge only

■———■ 10µg alcohol-killed *Salmonella typhimurium* C5 vaccine i.p.(day -24) and C5 challenge

○- - - - -○ 10µg *Salmonella enteritidis* 11RX protein antigen i.p.(day -1) and C5 challenge

●———● 10µg alcohol-killed *Salmonella typhimurium* C5 vaccine i.p.(day -24), 10µg *Salmonella enteritidis* 11RX protein antigen i.p.(day -1) and C5 challenge

Each point represents the geometric mean ± S.E. of the number of viable C5 recovered from the peritoneal cavity of 3 mice.

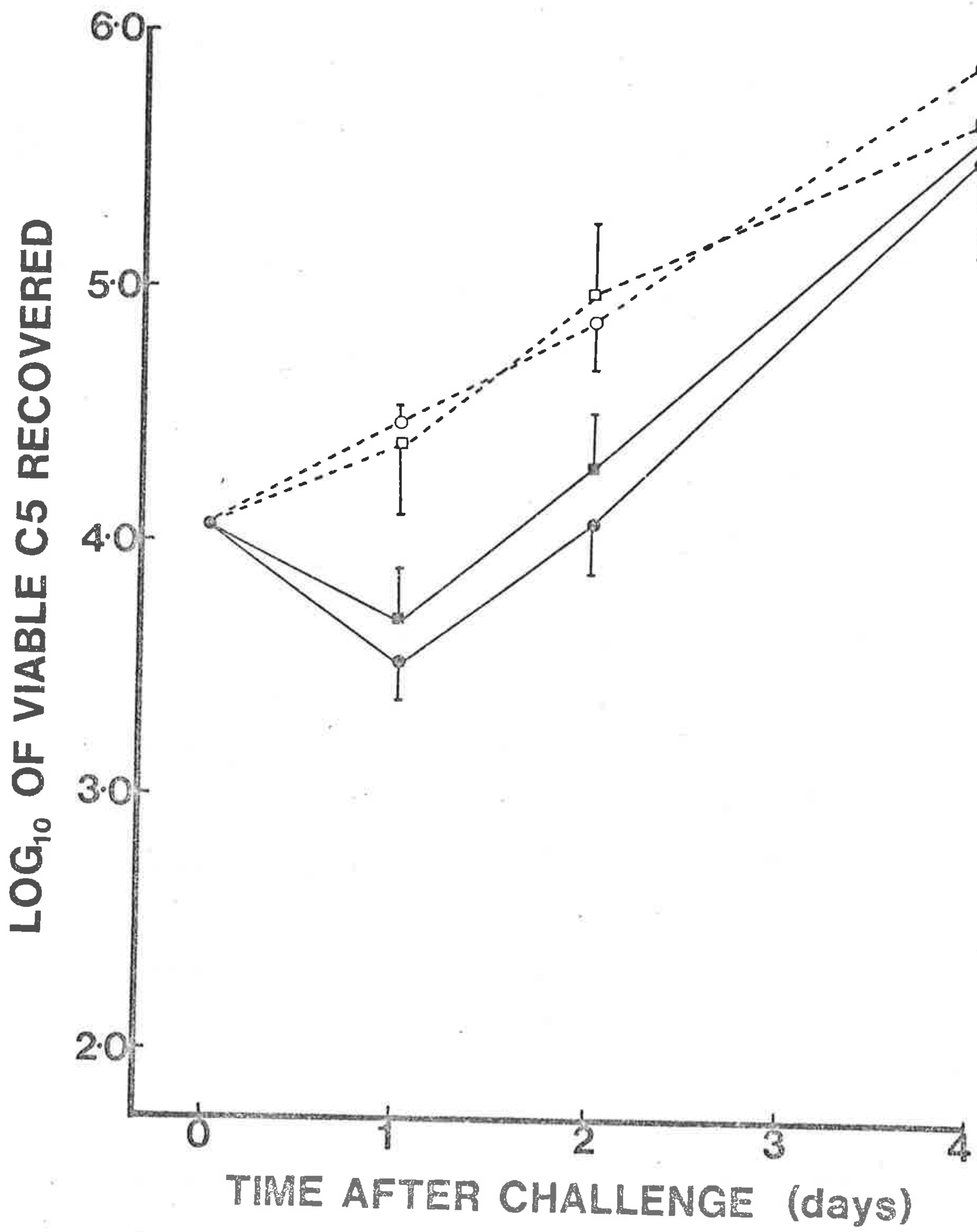

6·0
5·0
4·0
3·0
2·0
LOG$_{10}$ OF VIABLE C5 RECOVERED
0
1
2
3
4
TIME AFTER CHALLENGE (days)

FIGURE 3.10

The effect of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine and a *Salmonella enteritidis* 11RX protein antigen on the ability of F1 mice previously infected intravenously with 3.7 x 10^5 *Salmonella enteritidis* 11RX (day -52), to control the growth of 1.2 x 10^4 *Salmonella typhimurium* C5 injected intraperitoneally on day 0.

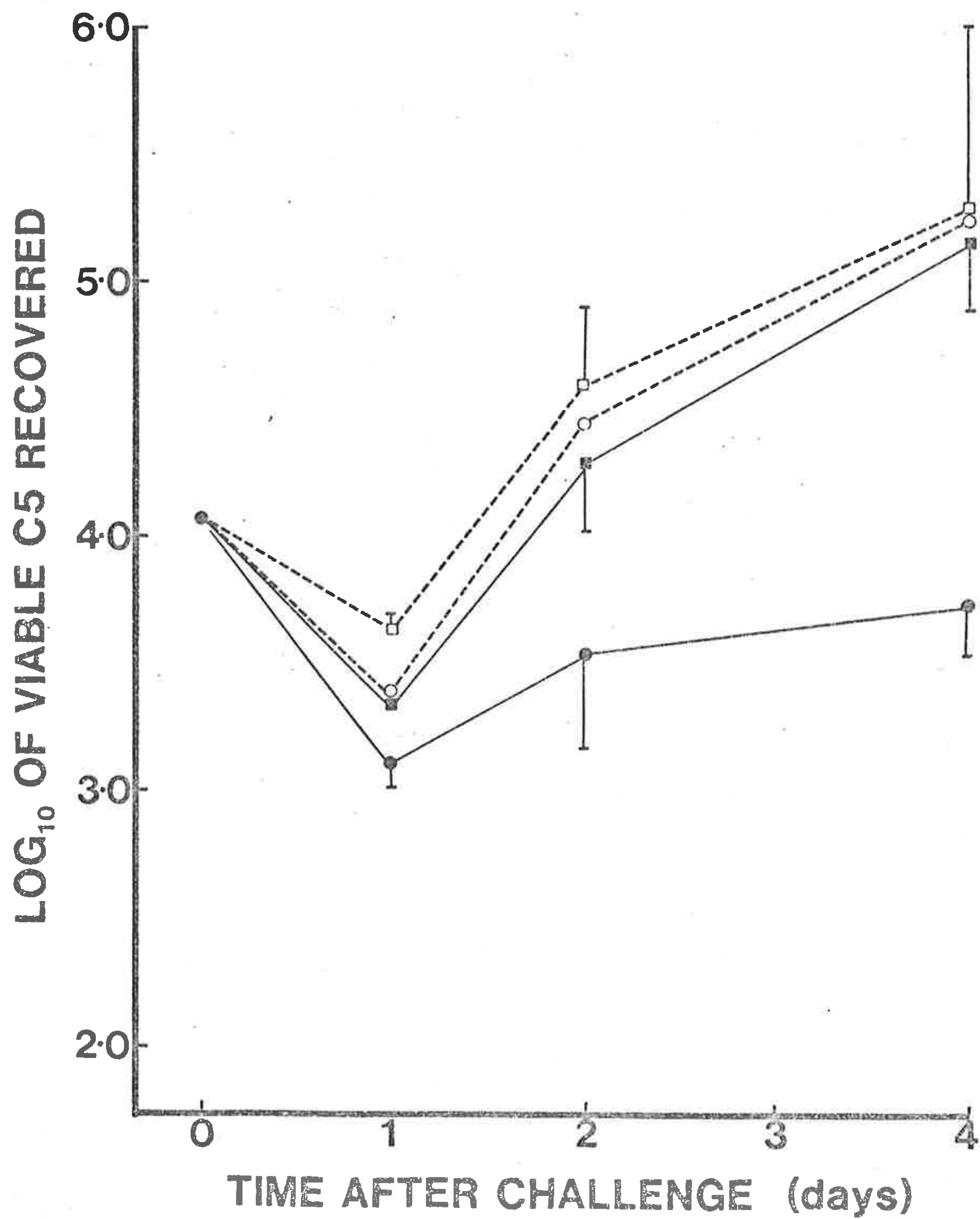
□- - - - -□ C5 challenge only

■———■ 10µg alcohol-killed *Salmonella typhimurium* C5 vaccine i.p.(day -24) and C5 challenge

○- - - - -○ 10µg *Salmonella enteritidis* 11RX protein antigen i.p.(day -1) and C5 challenge

●———● 10µg alcohol-killed *Salmonella typhimurium* C5 vaccine i.p.(day -24), 10µg *Salmonella enteritidis* 11RX protein antigen i.p.(day -1) and C5 challenge.

Each point represents the geometric mean ± S.E. of the number of viable C5 recovered from the peritoneal cavity of 3 mice.


6·0
5·0
4·0
3·0
2·0
LOG10 OF VIABLE C5 RECOVERED
0
1
2
3
4
TIME AFTER CHALLENGE (days)

the objectives of this thesis, an investigation was undertaken of the ability of *Salmonella enteritidis* 11RX protein antigen to recall a cell-mediated antibacterial immune response in *Salmonella enteritidis* 11RX-infected mice which had lost their carrier state (convalescent mice).

For this experiment, 8 groups of 12 male F1 mice were set up as follows:- 4 groups were infected intravenously with 3.7×10^5 *Salmonella enteritidis* 11RX (day -52) while the other 4 groups were set aside as uninfected controls; 1 of the normal and 1 of the *Salmonella enteritidis* 11RX-infected groups were injected intraperitoneally with 10µg of an alcohol-killed *Salmonella typhimurium* C5 vaccine (day -24); another of each type of group received 10µg of *Salmonella enteritidis* 11RX protein antigen intraperitoneally (day -1); yet another of each type of group was injected intraperitoneally with both 10µg of the alcohol-killed *Salmonella typhimurium* C5 (day -28) and 10µg of the *Salmonella enteritidis* protein antigen (day -1), while the 2 remaining groups were administered no additional treatments. All 8 groups were challenged intraperitoneally with 1.2×10^4 *Salmonella typhimurium* C5 (day 0). The progress of this infection in the peritoneal cavity was followed for 4 days by periodically sacrificing 4 mice from each group and determining the number of viable typhoid bacilli which could be recovered from the peritoneal cavity. The fate of the *Salmonella typhimurium* C5 challenge in the uninfected and the convalescent *Salmonella enteritidis* 11RX-infected mice is shown in Figures 3.9 and 3.10 respectively.

Effective antibacterial resistance to *Salmonella typhimurium* C5 appeared to be recalled only when the *Salmonella enteritidis* 11RX-recovered mice received both the

alcohol-killed Salmonella typhimurium C5 vaccine and the Salmonella enteritidis 11RX protein antigen prior to challenge. Such mice were capable of both reducing the size of the challenge inoculum and of controlling its subsequent growth. The administration of either the alcohol-killed Salmonella typhimurium C5 vaccine or the Salmonella enteritidis 11RX protein antigen alone was of little, if any, benefit to the normal or convalescent mice and they remained unable to halt the progress of the challenge infection. These findings implied that the activated macrophages elicited in the longterm Salmonella enteritidis 11RX-infected mice by the homologous protein antigen required the presence of specific antibody to express their microbicidal activity against the typhoid infection. It is also tempting to speculate that an increase in the level of background cross-reacting antibody during an immunizing infection with Salmonella enteritidis 11RX may contribute to the resistance of these mice to challenge with Salmonella typhimurium C5, as the "purely" cell-mediated immune response, recalled in convalescent mice appeared to be insufficient for effective resistance to this pathogen. Further analysis of the resistance of longterm Salmonella enteritidis 11RX-infected mice was not pursued because the state of macrophage activation, specifically recalled in such mice, may not have been comparable, either quantitatively or qualitatively, to that generated during the immunizing infection. In addition, some difficulties were experienced in keeping the convalescent Salmonella enteritidis 11RX-infected mice free of secondary infections for long periods of time. However, the question of background antibody levels contributing to the apparent non-specific resistance of mice recently infected with

Salmonella enteritidis 11RX was not dismissed and studies addressing this issue are presented in the next chapter.

Summary and conclusions

These introductory studies examined various aspects of the resistance of mice immunized with Salmonella enteritidis 11RX, to infection with Salmonella typhimurium C5, with the objective of delineating more clearly the role of antibody in immunity to typhoid.

Activated peritoneal macrophages, harvested from Salmonella enteritidis 11RX-infected mice, were found to have an increased ability to adhere to, ingest and subsequently destroy Salmonella typhimurium C5 in the presence of specific antibody (Table 3.1; Figures 3.1, 3.2 and 3.3). In the absence of specific antibody, the activated phagocytic cells appeared impotent, unable to express their bactericidal activity against the typhoid bacilli. These results suggested that activated macrophages had the same requirement for opsonins as did normal macrophages.

In vivo, the acquisition of specific antibody by either active immunization or passive transfer, enabled the Salmonella enteritidis 11RX-infected mice to control and eliminate a large intravenous challenge of Salmonella typhimurium C5 (Figures 3.7 and 3.8). Similarly, opsonization enhanced the clearance of Salmonella typhimurium C5 from the peritoneal cavities of Salmonella enteritidis 11RX-infected mice (Figure 3.4). However, in the absence of exogenous antibody these mice retained a limited, and yet significant, level of resistance to typhoid. This observation contrasts sharply with the inability of activated macrophages from these mice to kill the unopsonized

bacteria *in vitro*. One explanation for this disparity is that background levels of cross-reacting antibodies *in vivo* permitted limited expression of the antibacterial immunity to occur. This possibility received support from the finding that although a protein antigen preparation from *Salmonella enteritidis* 11RX was capable of recalling macrophage activation in the *Salmonella enteritidis* 11RX-recovered mice, it was not able to restore resistance to *Salmonella typhimurium* C5 (Figures 3.9 and 3.10). Resistance was restored when such mice received additional immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine, which would have elevated specific antibody levels.

Together these results demonstrated that specific antibody plays an important role in immunity to typhoid. Its presence in both *in vitro* and *in vivo* experiments was found to promote the phagocytosis and killing of *Salmonella typhimurium* C5 by activated macrophages. However, care should be exercised when extrapolating from these results to the interactions of other intracellular bacterial parasites with activated macrophages, as immunity to some pathogens such as *Listeria monocytogenes* appears not to involve specific antibody. Perhaps in such situations the activated macrophages may have a "primitive" recognition mechanism other than antibody, or secrete factors capable of effectively killing extracellular bacteria or, on the other hand, the bacteria may have some mechanism of attachment to cells, including phagocytic ones.

CHAPTER 4

THE ROLE OF ANTIBODY IN ACQUIRED APPARENTLY NON-SPECIFIC RESISTANCE TO SALMONELLA INFECTIONS

Introduction

One of the striking features of acquired cellular resistance to intracellular bacterial parasites is that this type of immunity is non-specific in its expression. Numerous reports have established that active immunization with one intracellular bacterial parasite produces significant resistance against challenge with apparently unrelated, heterologous strains. Amongst this evidence is the demonstration that the immunization of mice with *Mycobacterium bovis* BCG confers resistance to challenge with *Mycobacterium fortuitum* (Boehme and Dubos, 1958), *Salmonella enteritidis* (Howard *et al*., 1959) and *Listeria monocytogenes* (Mackaness, 1969). Similarly, mice immunized with *Listeria monocytogenes*, *Brucella abortus* or *Mycobacterium tuberculosis* were resistant to challenge with heterologous bacteria (Mackaness, 1964). The report of Rowley, Auzins and Jenkin (1968) that *Salmonella enteritidis* 11RX produced resistance to *Salmonella enteritidis* 795, *Salmonella typhimurium* C5, *Salmonella paratyphi* C and *Listeria monocytogenes* 2535, is particularly relevant to this thesis. These observations of apparent non-specific cross-resistance were largely responsible for the view, which was generally

accepted, that antibody plays little, if any, role in immunity to intracellular bacterial parasites. However, this conclusion was reached more by implication than by experimental proof. The presence of specific antibodies, and their participation in these systems was always more than a remote possibility and should not have been overlooked. The likelihood that sharing of common enterobacterial antigens and the presence of polyclonal B cell activators on various strains of enteric bacteria are responsible for the induction of specific antibodies in the absence of the more traditional O-somatic cross-reactivity, will be discussed shortly.

One of the pieces of evidence which led to the claim that immunity was mediated by phagocytic cells and was not dependent on antibody was the demonstration that resistance to Salmonella infections could be transferred to normal recipients by "immune" macrophages (Furness and Ferreira, 1959; Ushiba *et al*., 1959; Saito *et al*., 1962). In one such experiment, Ushiba, Saito, Akiyama, Nakano, Sugiyama and Shirono (1959) were able to confer antibacterial resistance against *Salmonella enteritidis* to normal mice, with peritoneal macrophages from immune mice. The degree of immunity transferred was assessed by following the fate of the bacterial challenge in the tissues of the various groups of mice involved in the experiment. Although they claimed that humoral antibody played no role in their experiments, they did not demonstrate the absence of cell-bound antibody. However, Rowley, Turner and Jenkin (1964) investigated the possibility that antibody may be present on the surface of "immune" macrophages and may participate in the expression of resistance. They confirmed the evidence that resistance against Salmonella organisms (*Salmonella typhimurium*

in their instance) could be transferred to normal mice with peritoneal macrophages from immune mice. Immunity was assessed by following the fate of the challenge organisms in the peritoneal cavities of the mice for a period of 90 minutes after challenge. However, they also demonstrated that killed macrophages from immunized mice, or a 19S macroglobulin type antibody eluted from these cells, could also be used to transfer immunity to normal mice. These findings have been confirmed using _Salmonella enteritidis_ (Mitsuhashi _et al._, 1967). The experiments discussed above illustrate not only the importance of antibody in cellular immunity, but also highlight the difficulties in eliminating antibody from these systems.

The existence of shared cross-reactive antigens amongst the bacilli of the _Enterobacteriaceae_ has now been shown by a number of groups. Rabbits immunized with formalin-killed _Escherichia coli_ O14 produced antibodies which not only cross-reacted with the antigens from other _Escherichia coli_ strains, but also with antigens of Salmonella, Shigella, Proteus and other _Enterobacteriaceae_ (Kunin, Beard and Halmagyi, 1962; Kunin, 1963). The similarity of the core region of the lipo-polysaccharides of most Gram-negative bacilli (Lüderitz, Staub and Westphal, 1966) made this structure the most likely candidate for the role of common antigen. Indeed, immuno-chemical studies have demonstrated that preparations of _Escherichia coli_ O14 LPS contained large amounts of an immunogenic "common enterobacterial antigen". This common antigen was composed of only those sugars present in the rough core structure of the _Escherichia coli_ and Salmonella LPS (Hammarstrom, Carlsson, Perlmann and Svensson, 1971). In addition to the common bacterial antigen described by Kunin

(1963), McCabe (1972) reported the presence of another cross-reactive determinant, the Re determinant. He found that both active immunization with, and passive transfer of antibody to the Re rough mutant of *Salmonella minnesota*, protected mice against challenge with the virulent *Escherichia coli* 107 and *Klebsiella pneumoniae*. Hence, during many bacterial infections, either of these common enterobacterial antigens may induce the generation of specific antibodies which could then be an integral part of the mechanism by which animals become resistant to challenge with apparently unrelated bacteria.

Antibody levels to a heterologous bacterial challenge may also be influenced by some bacterial components which have the ability to polyclonally activate B lymphocytes. Amongst the Gram-negative bacteria, a number of cell surface components, including lipopolysaccharide (Andersson, Sjöberg and Möller, 1972), lipoprotein (Melchers, Braun and Galanos, 1975; Bessler and Braun, 1975; Bessler and Ottenbreit, 1977), Protein I and Protein II (Bessler and Henning, 1979), are known to polyclonally activate murine B lymphocytes *in vitro*. Recently, cell wall preparations from a number of Gram-positive organisms have also been shown to induce polyclonal B cell responses (Saito-Taki *et al.*, 1980). Although the properties of most bacterial polyclonal B cell activators were demonstrated *in vitro*, lipopolysaccharide has been shown to have considerable *in vivo* activity (Ness *et al.*, 1976; Peavy, Baughn and Musher, 1978; Dufer *et al.*, 1980). Hence, during a bacterial infection, these polyclonal B cell responses may also contribute some "protective" antibody which could then play a role in resistance against challenge with apparently unrelated bacteria.

Live bacterial vaccines are therefore capable of inducing

resistance to, as well as antibody specific for, apparently unrelated bacteria. While the origins of this antibody are still open to debate, its presence has been established. Rowley, Auzins and Jenkin (1968) demonstrated that not only does *Salmonella enteritidis* 11RX induce resistance to *Salmonella typhimurium* C5, but it also induces humoral antibodies which are bactericidal for *Salmonella typhimurium* M206 *in vitro*. The existence of antibodies specific for *Salmonella typhimurium* C5 in *Salmonella enteritidis* 11RX immunized mice has been reported by Ielasi (1970) and Davies (1975). In the latter, more detailed study, both Swiss-Webster and F1 mice injected with *Salmonella enteritidis* 11RX had increased levels of serum antibody directed against LPS determinants of a wide variety of bacteria. It was interesting to note that there was no obvious correlation between the observed increases in antibody titre and the Kauffmann and White O-somatic antigens present in the LPS preparations used.

While *Salmonella enteritidis* 11RX-infected mice are non-specifically resistant to a range of heterologous bacteria, the results of Davies (1975) demonstrated that presence of high levels of specific antibody facilitated the expression of resistance to *Salmonella typhimurium* C5. The ability of *Salmonella enteritidis* 11RX-infected mice to control and eliminate a *Salmonella typhimurium* C5 challenge dose of 10^4 organisms was found to be enhanced by prior immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine, which induced high levels of specific antibody in the vaccinated mice. When the challenge dose was increased to approximately 10^6 viable organisms, the ability of *Salmonella enteritidis* 11RX -infected mice to control the *Salmonella typhimurium* C5

infection was reduced and the protective effect of the specific antibody was more pronounced. Similarly, the results presented in Chapter 3 have also shown that the presence of specific anti-C5 antiserum is important for both the *in vitro* and *in vivo* interaction of activated macrophages with *Salmonella typhimurium* C5. The implication from these experiments is that the expression of "non-specific" resistance in *Salmonella enteritidis* 11RX-infected mice, to challenge with *Salmonella typhimurium* C5, is dependent on a low level of specific antibody, which becomes the limiting factor when higher challenge doses are used. The purpose of this chapter was to confirm the existence of antibodies to antigens of *Salmonella typhimurium* C5 in *Salmonella enteritidis* 11RX-infected mice and to determine whether they play a role in the "non-specific" resistance of these mice to more modest challenge doses of *Salmonella typhimurium* C5.

The importance of antibody in the "non-specific" resistance of *Salmonella enteritidis* 11RX-infected mice has already been examined using bacterial polysaccharide preparations to deplete these mice of specific antibodies prior to challenge (Ielasi, 1970). He found that the pre-treatment of *Salmonella enteritidis* 11RX-recovered mice with a *Salmonella typhimurium* C5 polysaccharide preparation removed *Salmonella typhimurium* C5 specific antibody activity from their serum and abrogated resistance to challenge with this organism. Similarly O-acetylated galactan, an analogue of the *Salmonella typhimurium* O-somatic antigen 5, was able to abrogate resistance if injected intravenously prior to challenge. In contrast, *Salmonella newington* polysaccharide, which does not cross-react with the O-somatic antigens of *Salmonella*

typhimurium, was ineffective. It appeared that the O-somatic antigens present on the Salmonella typhimurium C5 polysaccharide, and not any contaminating protein components, were responsible for the antibody depletions observed. Although a similar approach was adopted for the present study, it is worth emphasising the differences between the experimental design of this study and that of Ielasi. Firstly, the Swiss-Webster mice used by Ielasi (1970) were naturally more resistant to Salmonella typhimurium C5 challenge than F1 mice. Secondly, and more importantly, Ielasi carried out his experiments with Salmonella enteritidis 11RX injected mice at a time when these mice no longer had detectable activated macrophages and no live organisms of the immunising strain could be recovered from them. In the experimental system used in the present study, the Salmonella enteritidis 11RX injected F1 mice were challenged earlier, to ensure that they had an activated reticulo-endothelial system at the time of challenge. This alteration in experimental design was essential because it allowed an assessment of the ability of activated macrophages to control the growth of a challenge of Salmonella typhimurium C5 under conditions where levels of at least some of the antibodies specific for this organism were depleted by injection of polysaccharide. A possible complication of the system used was that, at the time of challenge, these mice were still infected with Salmonella enteritidis 11RX.

Preparation of polysaccharide

The polysaccharide to be used in this study was obtained from Salmonella typhimurium C5 organisms by mild acid hydrolysis, according to the method of Staub (1965). Two

preliminary experiments were undertaken to characterize the polysaccharide preparation, before it was used in an investigation of the role of antibody in Salmonella infections. The material was analyzed for its homogeneity with regard to size, and its affinity for polymixin B was used to ascertain the extent of lipid A contamination.

The molecular weight profile of *Salmonella typhimurium* C5 polysaccharide

Ten mg of *Salmonella typhimurium* C5 polysaccharide were dissolved in 2ml of phosphate buffered saline (pH 7.4) and applied to a Séphadex G100 column. The material was then eluted with phosphate buffered saline (pH 7.4) in order of decreasing molecular weight. Fractions were collected in 2ml aliquots and assayed for carbohydrate content. The molecular weight profile obtained is shown in Figure 4.1.

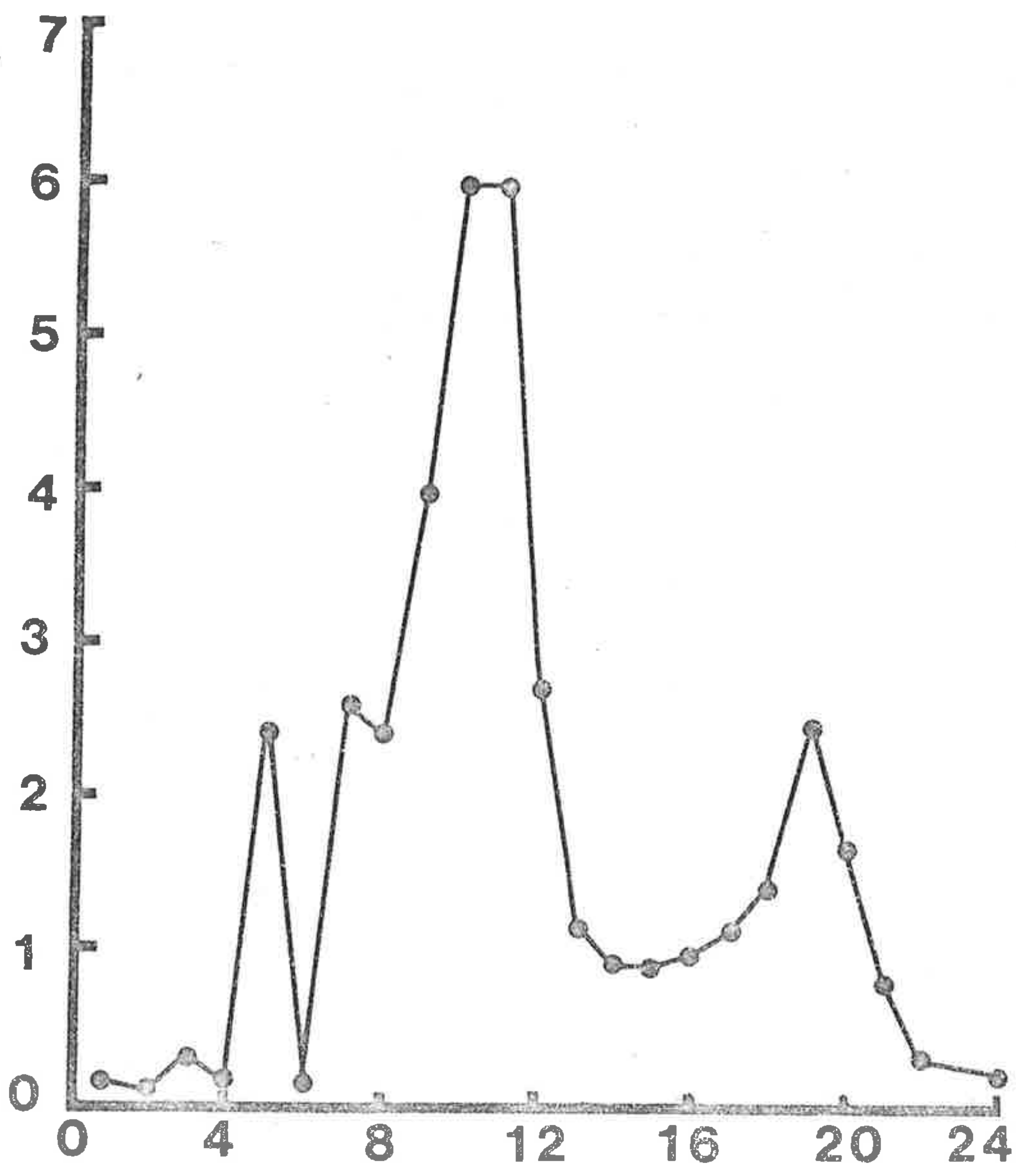
The bulk of the material eluted as a single peak in fractions 7 - 12, while 2 minor peaks were observed in fractions 4 - 6 and 17 - 21. Hence, while the polysaccharide preparation was not completely homogeneous, it was composed of molecules of only 3 different molecular weights.

The affinity of *Salmonella typhimurium* C5 polysaccharide for Polymixin B

Although polysaccharide would undoubtedly absorb antibodies specific for parts of the bacterial surface of the strain used to prepare the polysaccharide, its suitability for use in *in vivo* experiments depended on it being non-toxic. The work of Ielasi (1970) implied that polysaccharides of Salmonella were, indeed, non-toxic. However it was important

FIGURE 4.1

A characteristic pattern for the Sephadex G100 gel filtration of *Salmonella typhimurium* C5 polysaccharide. The material was eluted with phosphate buffered saline, pH 7.4. Fractions were collected and assayed for carbohydrate content as described in Materials and Methods.


CONCENTRATION OF CARBOHYDRATE (ARBITRARY UNITS)
0
1
2
3
4
5
6
7
0
4
8
12
16
20
24
FRACTION NUMBER

to ascertain whether the highly toxic native lipopolysaccharide or lipid A moieties were significant contaminants of the *Salmonella typhimurium* C5 polysaccharide preparation.

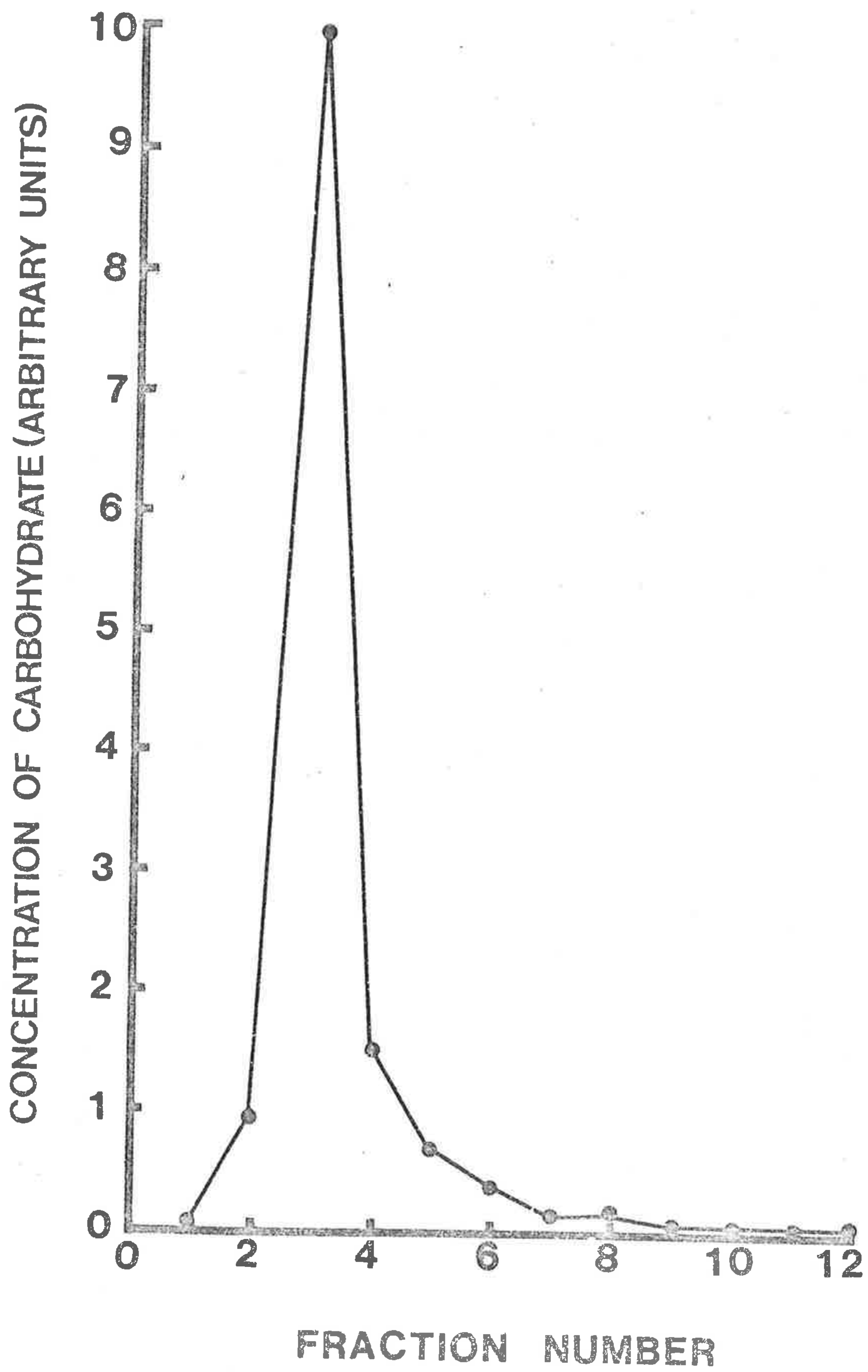
Polymixin B, a cyclic, cationic antibiotic binds to the lipid A portion of lipopolysaccharide (Morrison and Jacobs, 1976). Treatment of a preparation of lipopolysaccharide with polymixin B markedly reduced its mitogenicity both *in vitro* (Jacobs and Morrison, 1976) and *in vivo* (Dufer *et al*., 1980). In this experiment, the ability of lipid A to adhere to Polymixin B was used to determine whether the polysaccharide preparation contained detectable amounts of unhydrolysed lipopolysaccharide.

The *Salmonella typhimurium* C5 polysaccharide (10mg) was dissolved in 2ml of 50mM Tris HCl buffer, pH 7.5, containing 1% Triton X100 and applied to a Polymixin B/Sepharose 4B column. The material was eluted with 50mM Tris HCl buffer, pH 7.5, containing 1% Triton X100 and the 2ml fractions were monitored for carbohydrate content.

As can be seen from the elution profile (Figure 4.2) the polysaccharide was not retarded by the polymixin B column. Similarly, no carbohydrate-containing material was detected when the column was flushed with 3M sodium thiocyanate in 50mM Tris HCl buffer, pH 7.5, containing 1% Triton X100 (data not presented). These results suggested that native lipopolysaccharide was not present in the polysaccharide preparation. This conclusion was supported by the observation that 10mg *Salmonella typhimurium* C5 polysaccharide administered intravenously was not toxic for normal F1 mice, nor did it greatly affect their mean time to death when injected into mice lethally infected with 10^4 *Salmonella typhimurium* C5 (data not

FIGURE 4.2

A characteristic pattern for the Polymixin B / Sepharose 4B gel filtration of *Salmonella typhimurium* C5 polysaccharide. The material was eluted with 50mM Tris HCl buffer, pH 7.5, containing 1% Triton X100. Fractions were collected and assayed for carbohydrate content.


CONCENTRATION OF CARBOHYDRATE (ARBITRARY UNITS)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
FRACTION NUMBER

shown). The question of toxicity was so crucial to the interpretation of the subsequent experiments that 2 further experimental controls were undertaken and are described later in this chapter.

Determination of serum antibody levels to *Salmonella typhimurium* C5 in *Salmonella enteritidis* 11RX-infected mice

Antibodies specific for *Salmonella typhimurium* C5 have been previously detected in *Salmonella enteritidis* 11RX-infected mice (Rowley, Auzins and Jenkin, 1968; Ielasi, 1970; Davies, 1975). For instance Davies (1975), found that the serum of F1 miçe recovering from 2 sequential *Salmonella enteritidis* 11RX infections, contained a high level of antibody specific for *Salmonella typhimurium* C5 (haemagglutination titre of 1/256 with C5 LPS coated SRBC). However, as F1 mice recently infected with *Salmonella enteritidis* 11RX differs as a model system from those described in the earlier reports, it was necessary to confirm that antibody specific for *Salmonella typhimurium* C5 could be detected in these mice.

A group of 20 F1 male mice was infected intravenously with 10^5 *Salmonella enteritidis* 11RX, while a similar group of age-matched controls was set aside. All mice were bled via the retro-orbital venous plexus 13 days later and the sera were stored frozen until just prior to use. The ability of these sera to agglutinate SRBC sensitised with either *Salmonella enteritidis* 11RX LPS or *Salmonella typhimurium* C5 LPS, was subsequently measured. Three other antisera, namely a rabbit anti-*Salmonella enteritidis* 11RX, an F1 anti-*Salmonella typhimurium* C5 and a rabbit anti-*Salmonella typhimurium* C5 (previously described in Chapter 2) were also included in the

TABLE 4.1

Antibody specificities in various mouse and rabbit antisera.

Antisera	Haemagglutination titre		
	11RX LPS SRBC	C5 LPS SRBC	SRBC
Normal F1 serum	1/2	1/2	1/2
F1 anti-11RX serum	1/8	1/8	1/2
Rabbit anti-11RX serum	1/10,000	1/80	1/2
F1 anti-C5 serum	1/32	1/512	1/2
Rabbit anti-C5 serum	1/8	1/4,000	1/2

assay for comparison.

From the results in Table 4.1 the serum from mice infected with *Salmonella enteritidis* 11RX contained antibodies which could specifically agglutinate both C5 LPS and 11RX LPS sensitised SRBC. However, antibody specific for either C5 LPS or 11RX LPS could not be detected in the serum of normal mice. The three other sera were able to agglutinate the C5 LPS and 11RX LPS sensitised SRBC to varying degrees. The high titres observed to 11RX LPS SRBC with the rabbit anti-11RX antiserum, or to C5 LPS SRBC with both the rabbit and F1 anti-C5 antisera were hardly surprising.

Comparison of the opsonic activity of sera from normal and *Salmonella enteritidis* 11RX-infected mice

The opsonic activity of serum is another useful criterion for demonstrating the presence of antibody specific for the bacterial surface. Two groups of 12 normal F1 male mice were injected intraperitoneally with 10^4 *Salmonella typhimurium* C5 which had been opsonized with the sera from either normal or *Salmonella enteritidis* 11RX-infected mice. Another group were similarly injected with unopsonized bacteria. Then at 20, 40, 60 and 90 minutes, 3 mice from each group were sacrificed and the number of viable *Salmonella typhimurium* C5 remaining in the peritoneal cavity was determined. The results are presented in Figure 4.3.

Clearance of the unopsonized *Salmonella typhimurium* C5 was not observed and, in fact, their numbers increased slightly during the period of the assay. In contrast, the rapid clearance of bacteria opsonized with the serum from *Salmonella enteritidis* 11RX-infected mice confirmed the presence of

FIGURE 4.3

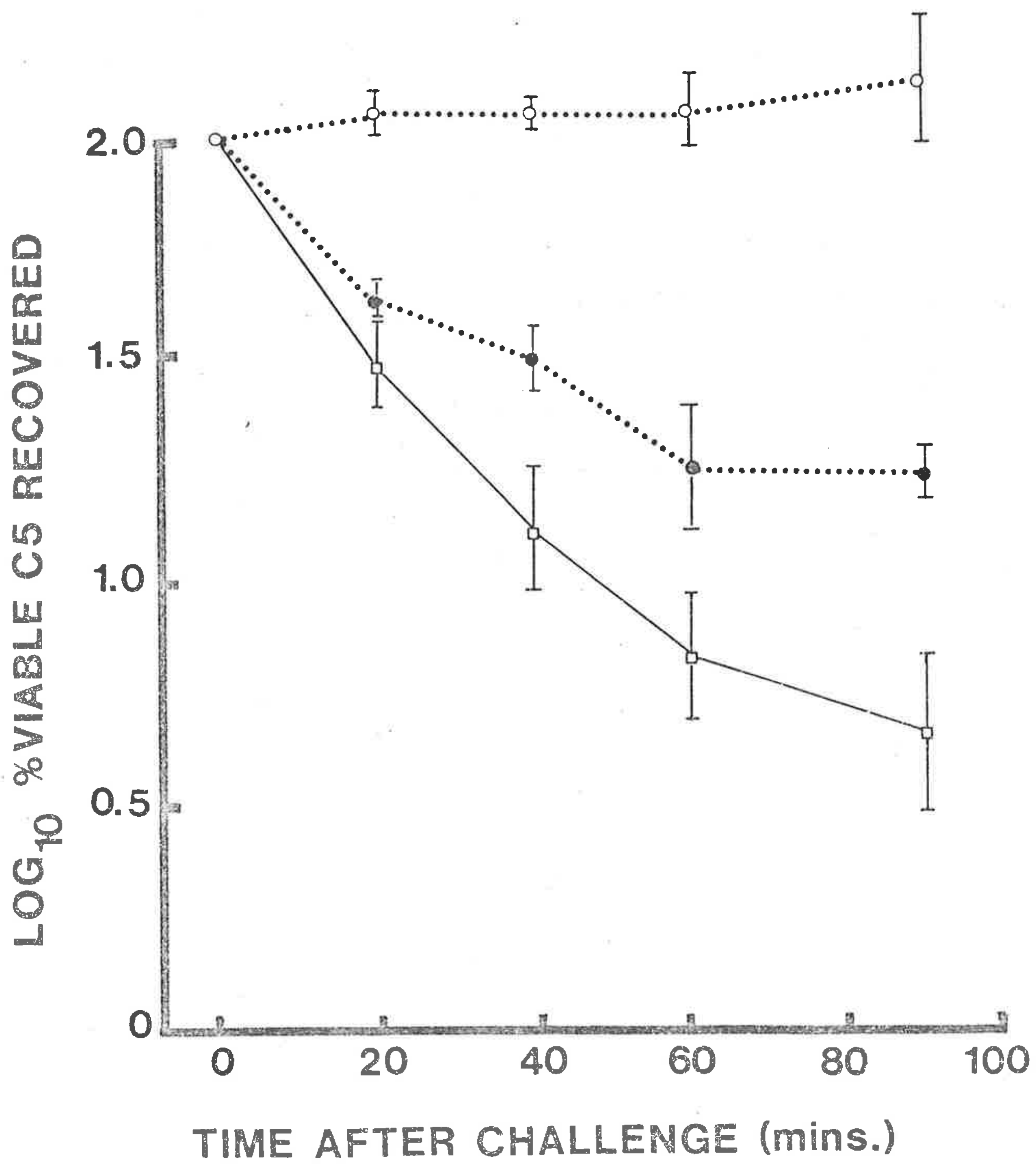
The clearance of opsonized *Salmonella typhimurium* C5 from the peritoneal cavity of normal F1 mice. The bacteria were opsonized with either saline or a variety of mouse sera.

○··········○ Saline controls

●··········● Serum from normal mice

□——————□ Serum from mice injected with *Salmonella enteritidis* 11RX i.v. (day -13)

Each point represents the mean ± S.E. of 3 mice.



2.0
1.5
1.0
0.5
0
LOG10 % VIABLE C5 RECOVERED
0
20
40
60
80
100
TIME AFTER CHALLENGE (mins.)

Salmonella typhimurium C5-specific antibody in these mice. This rather sensitive assay also revealed the presence of low levels of antibody specific for Salmonella typhimurium C5 in the serum from normal mice. In the haemagglutination assay, these antibodies were not detectable above the background agglutination of SRBC.

Absorption of Salmonella typhimurium C5 specific antisera with two polysaccharide preparations

The haemagglutination and clearance assays confirmed that the serum of Salmonella enteritidis 11RX-infected mice contained antibodies specific for Salmonella typhimurium C5. However, to ensure that the Salmonella typhimurium C5 polysaccharide would deplete these Salmonella enteritidis 11RX-infected mice of antibodies specific for Salmonella typhimurium C5, it was necessary to establish that the polysaccharide preparation could specifically inhibit such antibodies in vitro.

In an initial experiment, an antiserum with a high titre to C5 LPS sensitised SRBC (the rabbit anti-C5 antiserum) was absorbed with either Salmonella typhimurium C5 or Escherichia coli 1097, prior to use in a haemagglutination assay. The results, shown in Table 4.2, demonstrate that antibodies directed against the C5 LPS are specifically removed by prior absorption with either strain of bacteria. The titres of antibodies to the other determinants were not greatly affected by this absorption. The only other observation worthy of note was the extremely high titre of antibodies directed against both the lipopolysaccharide and protein antigen determinants.

The demonstration that antibodies specific for C5 LPS could be specifically removed by absorption with bacteria

TABLE 4.2

Antibody specificities in the rabbit anti-C5 serum and the effect of prior absorption with various bacteria.

Haemagglutination titre			
Antigen used to sensitise SRBC	Bacteria used for absorption of Rabbit anti-C5 serum		
	Unabsorbed	C5	F1097
C5 LPS	1/4000	1/256	1/512
C5 Protein Ag	1/8000	1/8000	1/8000
11RX LPS	1/8	1/8	1/8
11RX Crude Ag	1/32	1/8	1/8
Unsensitised SRBC	1/2	1/2	1/2

possessing the appropriate O-somatic antigens, was then extended to show that in fact Salmonella typhimurium C5 polysaccharide preparation could also specifically absorb these antibodies. In the second experiment, various bacterial antigens were investigated for their ability to inhibit the agglutination of both C5 LPS and C5 Protein Antigen sensitised SRBC with either the rabbit or F1 anti-C5 antiserum, and the results are shown in Table 4.3. While the serum from Salmonella enteritidis 11RX-infected mice also contained antibodies specific for C5 LPS, unfortunately the titre was too low for it to be used in this type of assay.

Extremely small quantities (20 - 40ng) of Salmonella typhimurium C5 LPS or polysaccharide specifically inhibited the agglutination of C5 LPS sensitised SRBC with both the rabbit and F1 antisera. In contrast, Salmonella newington polysaccharide did not inhibit the agglutination of the C5 LPS sensitised SRBC. Similarly the agglutination of C5 Ag sensitised SRBC was only inhibited with the protein antigen preparation.

These results demonstrate that antibodies specific for C5 LPS can be removed from the serum by exposure to either the O-somatic antigens on the bacterial surface, or to the polysaccharide preparation. It therefore did not seem unreasonable to expect that the antibodies specific for C5 LPS which were present in Salmonella enteritidis 11RX-infected mice would be removed by the Salmonella typhimurium C5 polysaccharide preparation. This was confirmed by a later experiment, which demonstrated that antibodies specific for C5 LPS were not detectable in Salmonella enteritidis 11RX-infected mice following treatment with Salmonella typhimurium

TABLE 4.3

The ability of various bacterial preparations to inhibit the haemagglutination of sensitised SRBC by two anti-C5 antisera

Preparation used as inhibitor	F1 anti-C5 antiserum		Rabbit anti-C5 antiserum	
	C5LPS SRBC	C5Ag SRBC	C5LPS SRBC	C5Ag SRBC
C5 LPS	0.02	NI[b]	0.02	NI[b]
C5 P/S	0.04	NI[b]	0.02	NI[b]
Control P/S[a]	NI[b]	ND[c]	ND[c]	ND[c]
C5 Ag	0.31	0.04	0.31	0.04

a. *Salmonella newington* polysaccharide

b. No inhibition with 50µg of inhibitor

c. Not done

The results are expressed as the minimum amount of antigen (µg) which inhibited 4 HU of antiserum.

C5 polysaccharide.

The clearance of *Salmonella typhimurium* C5 from the peritoneal cavity of *Salmonella enteritidis* 11RX-infected mice pretreated with *Salmonella typhimurium* C5 polysaccharide

It was shown that the serum of *Salmonella enteritidis* 11RX-infected mice contained antibodies specific for *Salmonella typhimurium* C5 (Table 4.1, Figure 4.3), and that these antibodies greatly enhanced the clearance of *Salmonella typhimurium* C5 from the peritoneal cavity of normal mice. However, whether this antibody also influenced the clearance of *Salmonella typhimurium* C5 from the peritoneal cavity of *Salmonella enteritidis* 11RX-infected mice, remained to be determined. The injection of *Salmonella typhimurium* C5 polysaccharide into *Salmonella enteritidis* 11RX-infected mice prior to the clearance study, was a means of depleting these mice of antibodies specific for the challenge organism, and therefore of analysing whether activated macrophages require antibody for phagocytosis and/or killing as do their unstimulated counterparts.

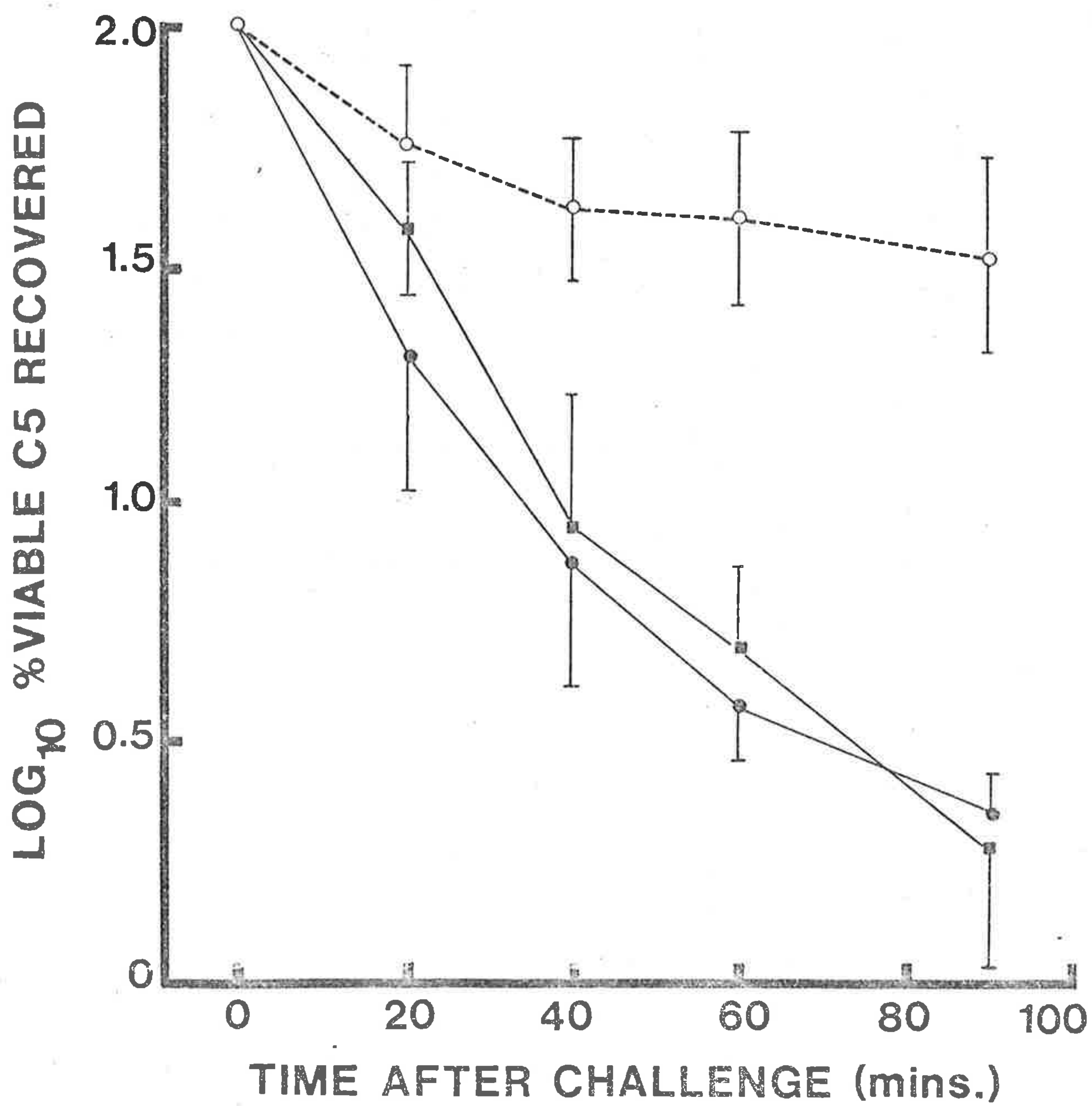
Three groups of 16 F1 male mice were immunized with 10^5 *Salmonella enteritidis* 11RX intraperitoneally 13 days before receiving the *Salmonella typhimurium* C5 challenge. Two hours prior to challenge, 10mg of *Salmonella typhimurium* C5 polysaccharide /mouse were injected intraperitoneally into all the mice of one group, the individuals of a second group received 10mg of *Salmonella newington* polysaccharide, and the third group was set aside as controls. All mice were challenged intraperitoneally with 10^4 *Salmonella typhimurium* C5. At 20, 40, 60 and 90 minutes after challenge, 4 mice from each group

FIGURE 4.4

The effect of polysaccharide pretreatment on the ability of mice to clear 10^4 *Salmonella typhimurium* C5 from their peritoneal cavity. All mice had been intraperitoneally injected with 10^5 *Salmonella enteritidis* 11RX 13 days previously.

●———●	11RX infected mice
■———■	11RX-infected mice injected intraperitoneally with 10mg *Salmonella newington* P/S 2 hours prior to C5 challenge.
○--------○	11RX-infected mice injected intraperitoneally with 10mg *Salmonella typhimurium* C5 P/S 2 hours prior to C5 challenge.

Each point represents the mean ± S.E. of 4 mice.


2.0
1.5
1.0
0.5
0
LOG10 %VIABLE C5 RECOVERED
0
20
40
60
80
100
TIME AFTER CHALLENGE (mins.)

were sacrificed and the number of viable Salmonella typhimurium C5 remaining in the peritoneal cavity was determined. The clearances of the challenge inoculum in these 3 groups are shown in Figure 4.4.

The challenge inoculum was quickly cleared from the peritoneal cavities of Salmonella enteritidis 11RX-infected mice, and this was not affected by treatment with Salmonella newington polysaccharide. In contrast, pretreatment with Salmonella typhimurium C5 polysaccharide drastically reduced the ability of these mice to clear the challenge inoculum. Presumably the capacity of the activated macrophages to phagocytose and kill the invading pathogens was curtailed by the depletion of specific antibody from the peritoneal cavity. However, this interpretation is dependent on the polysaccharide being non-toxic, particularly for the cells of the reticuloendothelial system.

The effect of polysaccharide on the cytotoxic activity of peritoneal exudate cells from Salmonella enteritidis 11RX-infected mice

Although preliminary experiments indicated that Salmonella typhimurium C5 polysaccharide was neither toxic for normal mice nor contained detectable endotoxin contamination, it was necessary to confirm that the preparation had no detrimental effects on the peritoneal cells of Salmonella enteritidis 11RX-infected mice. The fact that these cells are cytotoxic for Ehrlich's Ascites Tumour, in vitro (Ashley and Hardy, 1973), was utilized in an assessment of the effect of polysaccharide on activated macrophages.

Peritoneal exudate cells were obtained from either 1 group

TABLE 4.4

The effect of polysaccharide on the cytotoxic activity of peritoneal exudate cells from F1 mice which had been previously immunised with 0.9×10^5 *Salmonella enteritidis* 11RX i.p. on day -5.

Peritoneal exudate cell type[a]	Polysaccharide treatment		% Cytolysis[b]
	10mg i.p. (day -1)	500µg/well (day 0)	
Normal	-	-	1.7 ± 0.6
Immune	-	-	71.0 ± 1.9
Immune	Control P/S[c]	-	71.3 ± 1.1
Immune	C5 P/S[d]	-	72.2 ± 1.3
Immune	-	Control P/S[c]	72.3 ± 2.9
Immune	-	C5 P/S[d]	81.8 ± 1.2

a. PEC were obtained from either normal or 11RX-infected mice

b. Mean ± S.E. of quadruplicate samples

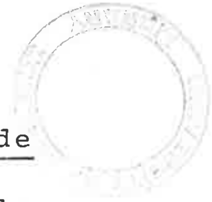
c. *Salmonella newington* polysaccharide

d. *Salmonella typhimurium* C5 polysaccharide

of 4 normal F1 female mice or from 3 similar groups of mice which had been infected intraperitoneally with 0.9×10^5 *Salmonella enteritidis* 11RX 5 days previously. Two of these latter groups were injected intraperitoneally with 10mg polysaccharide prepared from either *Salmonella typhimurium* C5 or *Salmonella newington*. The polysaccharides were injected 2 hours prior to harvesting the peritoneal exudate cells. In addition to exposing activated macrophages to the polysaccharide *in vivo*, the effect of including 500µg polysaccharide / well in the culture medium was also investigated. All cells were assayed for their cytotoxic activity against EAT in a 20 hour ^{51}Cr release assay.

The results, presented in Table 4.4, demonstrate that "immune" cells were able to kill the EAT targets, while normal cells did not have this tumouricidal activity. The pre-treatment of *Salmonella enteritidis* 11RX-infected mice with either polysaccharide preparation did not affect the cytotoxic activity of the peritoneal cells. Similarly, the inclusion of polysaccharide in the culture medium did not reduce the resultant tumouricidal activity.

The observation that the *Salmonella typhimurium* C5 poly-saccharide did not affect the activity of the peritoneal exudate cells is in good agreement with the preliminary experiments which demonstrated that the preparation was neither toxic for normal mice nor contained detectable endotoxin contamination.



The effect of *Salmonella typhimurium* C5 polysaccharide treatment on the humoral immune response of *Salmonella enteritidis* 11RX-infected mice to *Salmonella typhimurium* C5

Before investigating the role of specific antibody in the resistance of *Salmonella enteritidis* 11RX-infected mice to *Salmonella typhimurium* C5, it was important to ascertain whether treatment with *Salmonella typhimurium* C5 polysaccharide had any sustained effects on either the humoral or cellular immune responses to the challenge organism. The duration of antibody depletion following treatment with *Salmonella typhimurium* C5 polysaccharide was determined in this study.

Two groups of 10 F1 male mice were infected with 1.3×10^5 *Salmonella enteritidis* 11RX 13 days before challenge with 2.1×10^4 *Salmonella typhimurium* C5. Two hours prior to challenge, one group was injected with 1mg *Salmonella typhimurium* C5 polysaccharide. The intravenous route of injection was used throughout the experiment. On various days, 3 mice from each group were bled and the sera were stored frozen until required. The sera were then assayed for their level of haemagglutinating antibody to *Salmonella typhimurium* C5 LPS.

Figure 4.5 shows that serum antibodies specific for C5 LPS were not detectable above the background agglutination of unsensitised SRBC for at least 24 hours after polysaccharide treatment. Even after 3 days, the level of specific antibody was significantly less than that in the untreated control group.

The effect of *Salmonella typhimurium* C5 polysaccharide on the activity of the reticuloendothelial system

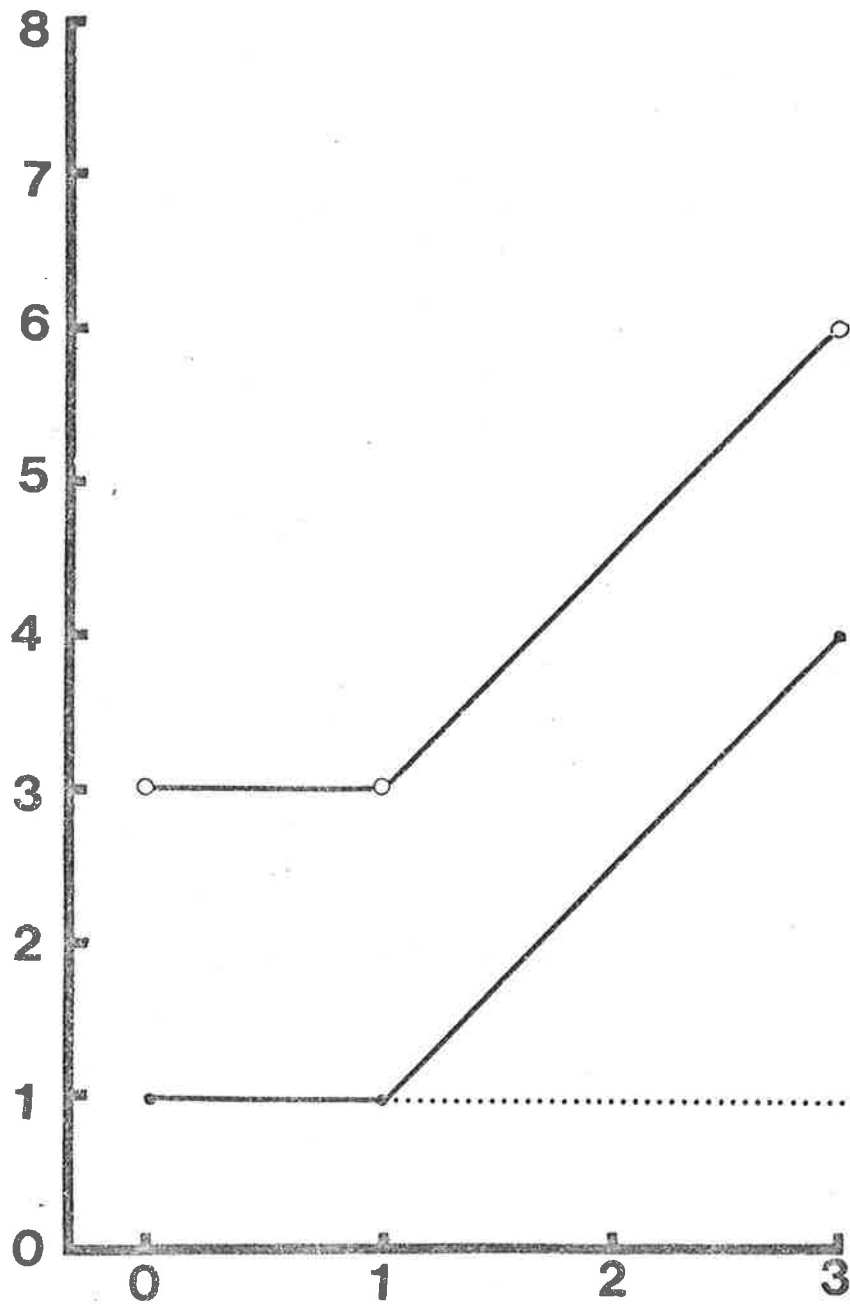
Although polysaccharide treatment did not alter the

FIGURE 4.5

The effect of *Salmonella typhimurium* C5 polysaccharide pretreatment on the haemagglutinating antibody responses to *Salmonella typhimurium* C5 LPS of F1 mice which had been intravenously infected with a dose of 1.3×10^5 *Salmonella enteritidis* 11RX on day -13, and then challenged intravenously with a dose of 2.1×10^4 *Salmonella typhimurium* C5 on day 0.

○——○	11RX-infected mice challenged with C5
●——●	11RX-infected mice injected intravenously with 1mg C5 P/S 2 hours prior to C5 challenge.

The dotted line represents the limit of detection of haemagglutinating antibody.



HAEMAGGLUTINATING ANTIBODY (1/log$_2$)
8
7
6
5
4
3
2
1
0
0
1
2
3
TIME AFTER CHALLENGE (days)

cytotoxic activity of peritoneal exudate cells from *Salmonella enteritidis* 11RX-infected mice, an additional experiment was undertaken to assess what effect it had on the reticulo-endothelial system, and in particular on the phagocytic cells in the liver and spleen. This was achieved by monitoring the ability of mice to clear carbon particles from their blood-stream, following treatment with *Salmonella typhimurium* C5 polysaccharide.

Two groups of 20 F1 male mice were infected with 1.1×10^5 *Salmonella enteritidis* 11RX and then were challenged with 1.5×10^4 *Salmonella typhimurium* C5, 11 days later. Two hours prior to the challenge, 1mg *Salmonella typhimurium* C5 poly-saccharide was administered to 1 group. As in the previous experiment, all injections were given via the intravenous route. At various times after challenge, the rate of carbon clearance was measured in 5 mice from each group. The results were expressed in terms of a phagocytic index, or K value, and are presented in Figure 4.6.

Clearly the *Salmonella typhimurium* C5 polysaccharide had no immediate effect on the activity of the phagocytic cells in the liver and spleen. Neither was there any reduction in the subsequent activation of the reticuloendothelial system, which occurred as a result of the *Salmonella typhimurium* C5 infection.

Taken together, all these experiments established the non-toxic nature of the *Salmonella typhimurium* C5 polysaccharide preparation. Consequently, in the 3 remaining experiments, any effect on the resistance of *Salmonella enteritidis* 11RX-infected mice to challenge with *Salmonella typhimurium* C5, could be ascribed to the ability of the polysaccharide to absorb antibodies specific for the challenge organism.

FIGURE 4.6

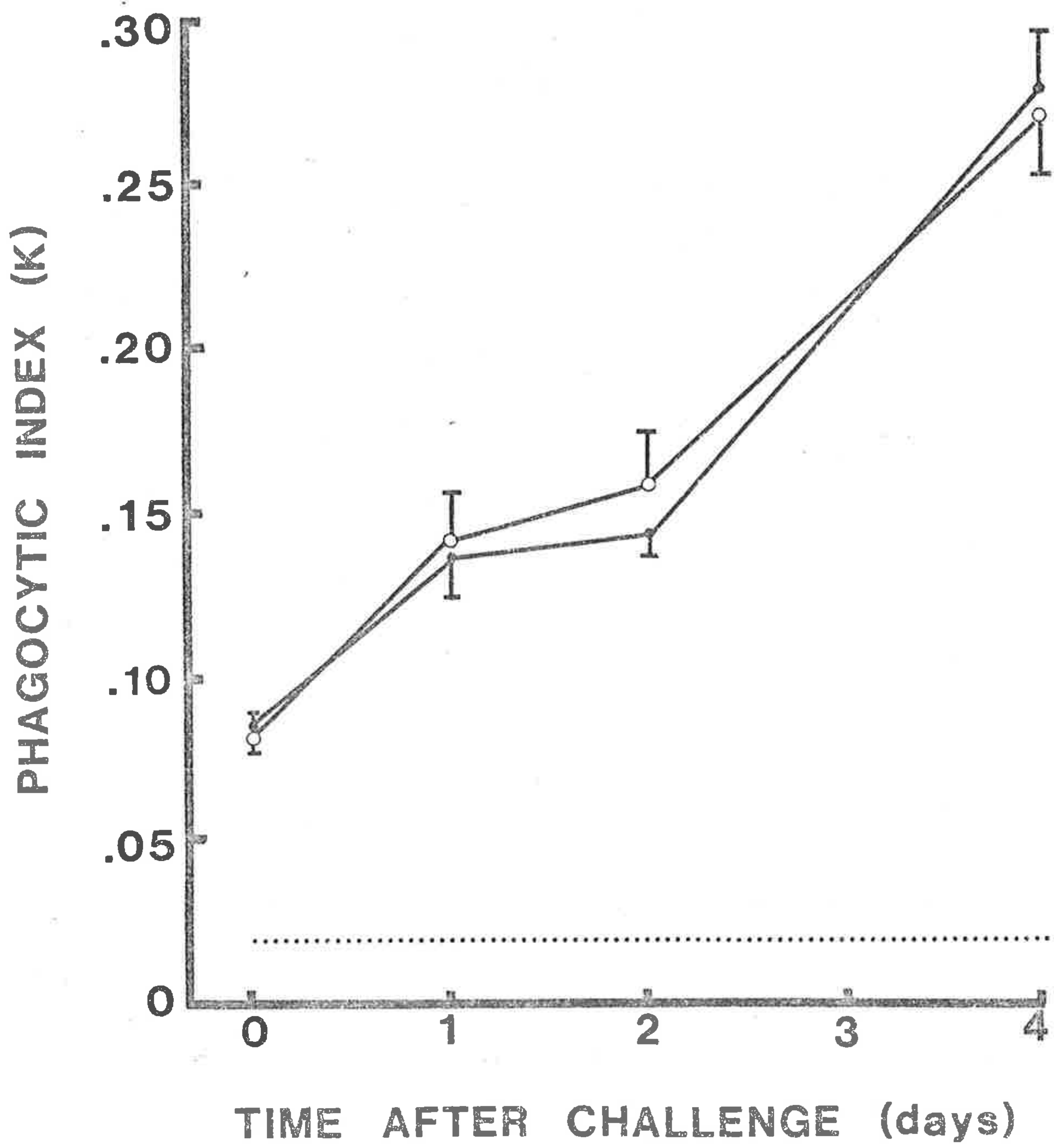
The clearance of carbon particles from the circulation was measured in F1 mice which had been intravenously infected with a dose of 1.1 x 10^5 *Salmonella enteritidis* 11RX on day -11 and then challenged intravenously with a dose of 1.5 x 10^4 *Salmonella typhimurium* C5 on day 0. The effect of *Salmonella typhimurium* C5 polysaccharide pretreatment on the activity of the reticuloendothelial system in these mice was also assessed.

○——○ 11RX-infected mice challenged with C5

●——● 11RX-infected mice injected intravenously with 1mg C5 P/S 2 hours prior to C5 challenge.

The dotted line represents the clearance rate found in normal mice.

Each point represents the mean ± S.E. of 5 mice.


.30
.25
.20
.15
.10
.05
0
PHAGOCYTIC INDEX (K)
0
1
2
3
4
TIME AFTER CHALLENGE (days)

The effect of injecting Salmonella typhimurium C5 polysaccharide on the resistance of Salmonella enteritidis 11RX-infected mice to Salmonella typhimurium C5 infection

The serum of Salmonella enteritidis 11RX-infected mice was shown to contain antibody specific for Salmonella typhimurium C5 (Table 4.1; Figure 4.3). Salmonella typhimurium C5 polysaccharide, known to remove specific antibody activity from serum (Table 4.3), was used to evaluate the role of this antibody in resistance to Salmonella typhimurium C5. In the following 3 experiments, the polysaccharide preparation was administered to Salmonella enteritidis 11RX-infected mice, in progressively greater quantities, in an attempt to abrogate their resistance to Salmonella typhimurium C5. The fate of the challenge inoculum was used as the criterion of resistance in all 3 experiments.

Experiment 1

Three groups of 25 F1 male mice were challenged intravenously with 2.1×10^4 Salmonella typhimurium C5, 13 days after being injected intravenously with 0.7×10^5 Salmonella enteritidis 11RX. Two hours prior to challenge, the mice in one group were injected intravenously with 1mg Salmonella typhimurium C5 polysaccharide/mouse, a second group of mice received 1mg Salmonella newington polysaccharide/mouse while a third group was set aside as a control group.On days 1,3,5,and 7 following challenge, 6 mice from each group were sacrificed and the number of viable Salmonella typhimurium C5 remaining in the liver, spleen and peritoneal cavity was determined. The fate of the challenge inoculum, in each group, is shown in Figure 4.7.

The Salmonella enteritidis 11RX-infected mice were clearly

FIGURE 4.7

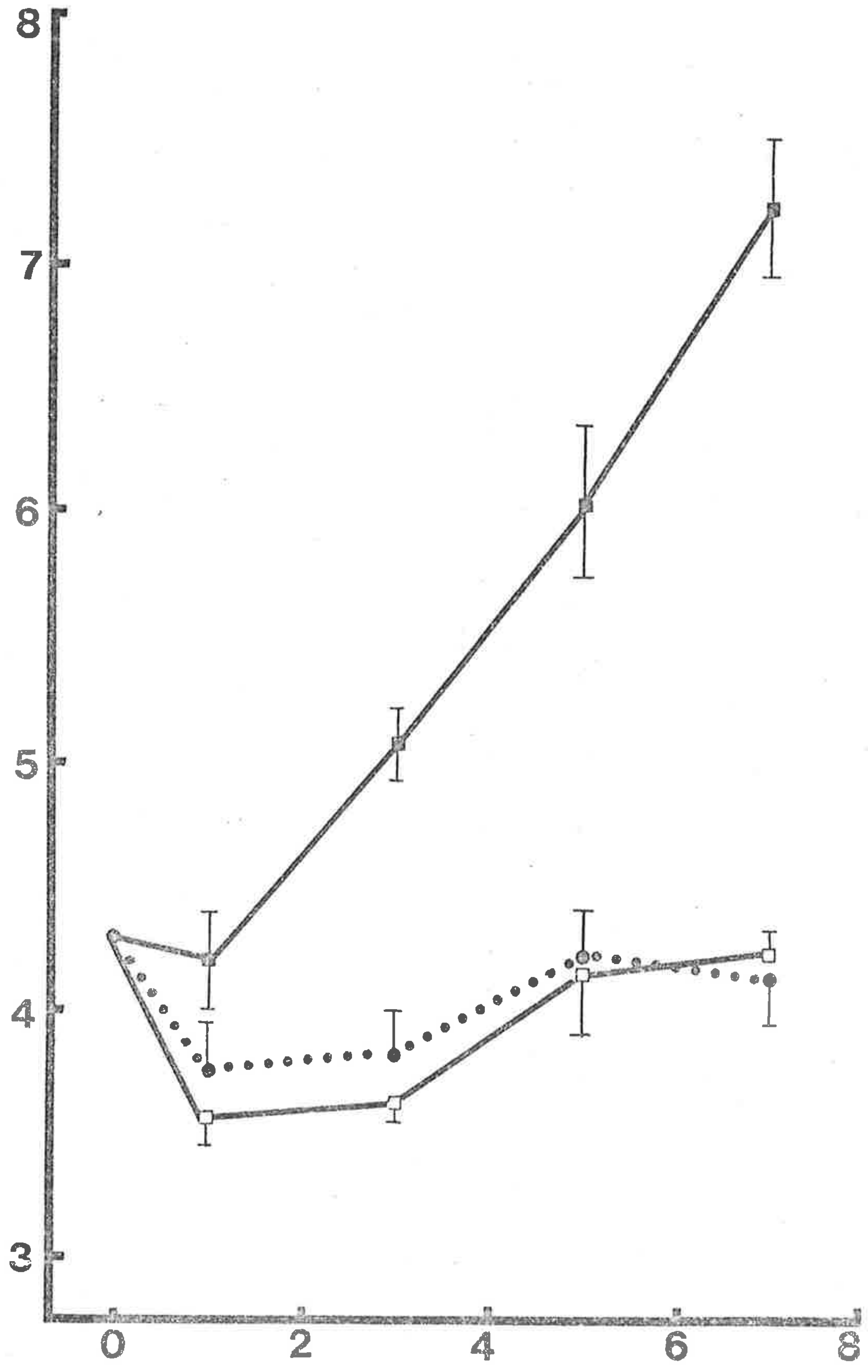
The effect of bacterial polysaccharide pretreatment on the ability of F1 mice to control the growth of 2.1×10^4 *Salmonella typhimurium* C5 injected intravenously on day 0. The mice had previously received 0.7×10^5 *Salmonella enteritidis* 11RX intravenously on day -13.

●••••••● 11RX-infected mice challenged with C5

□——□ 11RX-infected mice injected intravenously with 1mg. *Salmonella newington* P/S 2 hours prior to C5 challenge.

■——■ 11RX-infected mice injected intravenously with 1mg. *Salmonella typhimurium* C5 P/S 2 hours prior to C5 challenge.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity. Each point represents the geometric mean $\pm$ S.E.of the total number of C5 recovered from 6 mice.


8
7
6
5
4
3
LOG$_{10}$ OF VIABLE C5 RECOVERED
0
2
4
6
8
TIME AFTER CHALLENGE (days)

resistant to the Salmonella typhimurium C5 challenge, as they were able to control the growth rate of the challenge organisms during the 7 days of study. Pretreatment with Salmonella newington polysaccharide had no effect on the resistance of the mice. However, pretreatment with Salmonella typhimurium C5 polysaccharide completely abrogated the resistance of the Salmonella enteritidis 11RX-infected mice. In the group of mice injected with the polysaccharide, the Salmonella typhimurium C5 organisms increased in number, and by day 7, these animals contained over 1000-fold more bacteria than the 2 control groups.

These results indicated that Salmonella typhimurium C5 polysaccharide, when injected into Salmonella enteritidis 11RX-infected mice, abrogated resistance to Salmonella typhimurium C5 by depleting these animals of antibodies specific for this organism. Salmonella newington polysaccharide, which does not cross-react with Salmonella typhimurium C5, had no effect and thereby confirmed that the effect of the Salmonella typhimurium C5 polysaccharide was specific and was not due to any non-specific toxic effects.

Experiment 2

Three groups of 20 F1 male mice were injected intravenously with 1.2×10^{5} Salmonella enteritidis 11RX, while a fourth group of age-matched controls was set aside. After 13 days, all mice were challenged intravenously with 1.6×10^{4} Salmonella typhimurium C5. Two hours prior to challenge, 2 of the Salmonella enteritidis 11RX-infected groups were injected intravenously with 1mg polysaccharide prepared from either Salmonella typhimurium C5 or Salmonella newington. This

FIGURE 4.8

The effect of bacterial polysaccharide treatment on the ability of F1 mice to control the growth of 1.6×10^4 *Salmonella typhimurium* C5 injected intravenously on day 0. The mice had previously received 1.2×10^5 *Salmonella enteritidis* 11RX intravenously on day -13.

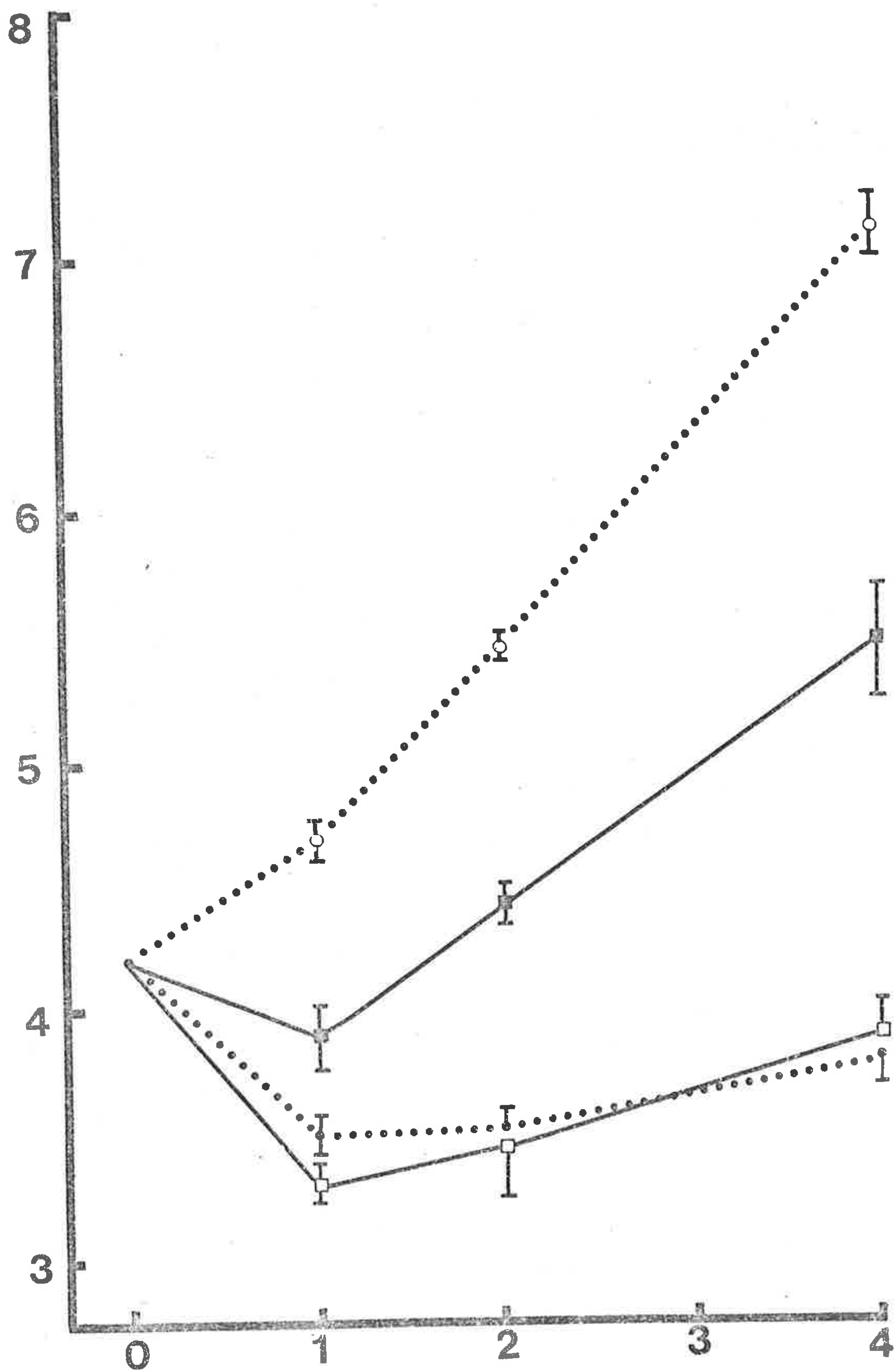
○·········○ Normal mice challenged with C5

●·········● 11RX-infected mice challenged with C5

□———□ 11RX-infected mice injected intravenously with 1mg *Salmonella newington* P/S 2 hours prior to C5 challenge and then once daily thereafter.

■———■ 11RX-infected mice injected intravenously with 1mg *Salmonella typhimurium* C5 P/S 2 hours prior to C5 challenge and then once daily thereafter.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity. Each point represents the geometric mean $\pm$ S.E. of the total number of C5 recovered from 6 mice.


8
7
6
5
4
3
LOG$_{10}$ OF VIABLE C5 RECOVERED
0
1
2
3
4
TIME AFTER CHALLENGE (days)

treatment was continued on a daily basis thereafter. On days 1, 2 and 4 following challenge, 6 mice from each group were sacrificed and the number of viable *Salmonella typhimurium* C5 remaining in the liver, spleen and peritoneal cavity was determined. The fate of the challenge inoculum in each group is shown in Figure 4.8.

Again, *Salmonella enteritidis* 11RX-infected mice were able to control the growth of the *Salmonella typhimurium* C5 challenge. Pretreatment with *Salmonella newington* polysaccharide had little effect on the resistance of these mice. In contrast, the challenge organisms multiplied steadily in both normal and *Salmonella typhimurium* C5 polysaccharide-treated mice. Presumably this latter polysaccharide treatment had not quite depleted the *Salmonella enteritidis* 11RX-infected mice of all antibody specific for *Salmonella typhimurium* C5, as these animals were marginally better off than the normal mice.

Depletion of specific antibody with the injection of 1mg *Salmonella typhimurium* C5 polysaccharide 2 hours before challenge was critical in determining the resistance of *Salmonella enteritidis* 11RX-infected mice. The additional daily treatments given in this experiment appeared to have little effect, as the level of bacteraemia in this experiment was similar to that observed in the first experiment.

Experiment 3

In this, the last experiment of the series, the dose of polysaccharide treatment was increased to 10mg to assess whether antibody depletion had been maximized. Three groups of 20 F1 male mice were challenged intravenously with 2.3×10^4 *Salmonella typhimurium* C5, 14 days after being injected

FIGURE 4.9

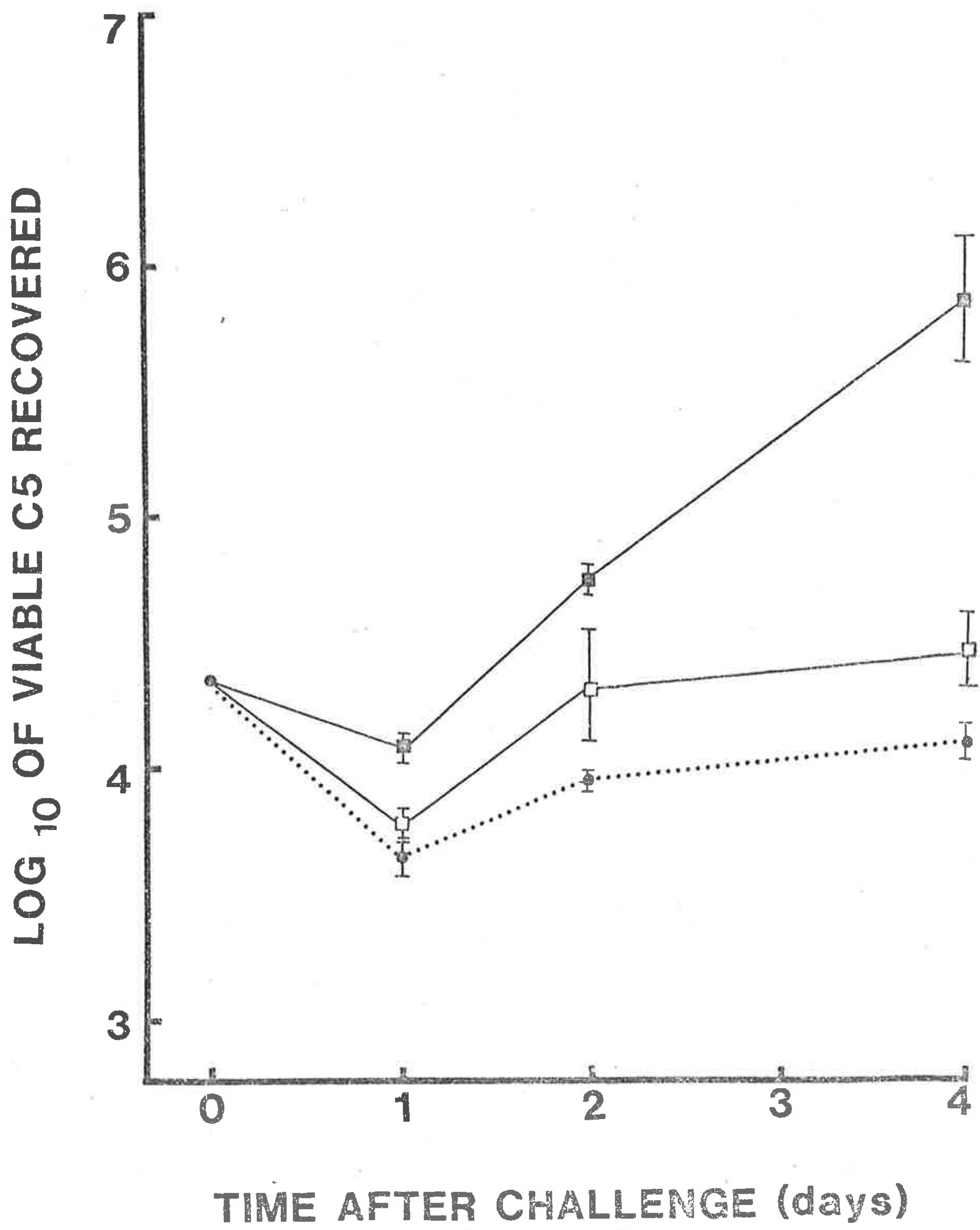
The effect of bacterial polysaccharide pretreatment on the ability of F1 mice to control the growth of 2.3×10^4 *Salmonella typhimurium* C5 injected intravenously on day 0. The mice had previously received 1.1×10^5 *Salmonella enteritidis* 11RX intravenously on day -14.

●⋯⋯⋯● 11RX-infected mice challenged with C5

□——□ 11RX-infected mice injected intravenously with 10mg *Salmonella newington* P/S 2 hours prior to C5 challenge.

■——■ 11RX-infected mice injected intravenously with 10mg *Salmonella typhimurium* C5 P/S 2 hours prior to C5 challenge.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity. Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 5 mice.



7
6
5
4
3
0
1
2
3
4
LOG 10 OF VIABLE C5 RECOVERED
TIME AFTER CHALLENGE (days)

intravenously with 1.1×10^5 Salmonella enteritidis 11RX. Two hours prior to challenge, one group was injected intravenously with 10mg Salmonella typhimurium C5 polysaccharide, a second group received 10mg Salmonella newington polysaccharide, while a third group was set aside as a control group. On days 1, 2 and 4 following challenge, 5 mice from each group were sacrificed and the number of viable Salmonella typhimurium C5 remaining in the liver, spleen and peritoneal cavity was determined. The fate of the challenge inoculum, in each group, is shown in Figure 4.9.

The resistance of Salmonella enteritidis 11RX-infected mice was again abolished by pretreatment with Salmonella typhimurium C5 polysaccharide. In comparison with the 2 earlier studies, increasing the dose of polysaccharide to 10mg had very little effect on the magnitude of the ensuing bacteraemia. Maximal antibody depletion appears to result from the injection of 1mg of Salmonella typhimurium C5 polysaccharide. Although 10mg of Salmonella newington polysaccharide had only a minor effect on the resistance of Salmonella enteritidis 11RX-infected mice, this dose of polysaccharide had a debilitating effect on the mice generally. Immediately after the intravenous injection of either polysaccharide preparation, the mice appeared lethargic for a few hours and the occasional mouse even died in this period. Salmonella typhimurium C5 polysaccharide eluted from the polymixin B column also had this physiological effect, suggesting that it was not due to endotoxin contamination (data not shown). The earlier studies in this chapter, demonstrating that the polysaccharide had no effect on the phagocytic cells of the reticuloendothelial system, also

supported this view. This unpleasant side-effect of treatment with 10mg polysaccharide may have been due to some type of anaphylactic reaction. Whatever the cause, the effect was short-lived and did not seriously interfere with the experiment.

Summary and conclusions

This study not only confirmed that the serum of *Salmonella enteritidis* 11RX-infected mice contains low levels of antibody specific for *Salmonella typhimurium* C5 (Table 4.1; Figure 4.3), but also demonstrated that this antibody plays a central role in the resistance of these mice to challenge with *Salmonella typhimurium* C5. Pretreatment with *Salmonella typhimurium* C5 polysaccharide reduced the level of specific antibody (Table 4.3; Figure 4.5) and abrogated resistance to infection with this organism (Figures 4.4, 4.7, 4.8 and 4.9). In comparison, *Salmonella newington* polysaccharide (O-somatic antigens 3 and 15) had little effect on the resistance of *Salmonella enteritidis* 11RX-infected mice to *Salmonella typhimurium* C5 (O-somatic antigens 1, 4, 5 and 12)

These findings have been interpreted as indicating that although *Salmonella enteritidis* 11RX-infected mice are 'non-specifically' resistant to *Salmonella typhimurium* C5, antibody still plays an important role in the expression of cell-mediated immunity. The implication is that activated macrophages, like normal macrophages, are dependent on the presence of specific antibody to phagocytose and kill the bacterial parasites.

CHAPTER 5

RESISTANCE OF LISTERIA-INFECTED MICE TO SALMONELLA INFECTIONS

Introduction

It is now recognised that a specific cell-mediated immune response is involved in the generation of resistance to infection with facultative intracellular bacteria. Such responses have been demonstrated in studies of brucellosis (Mackaness, 1964), salmonellosis (Blanden, Mackaness and Collins, 1966; Collins, 1971) and tuberculosis (Blanden, Lefford and Mackaness, 1969). However, the extensive investigations of listeriosis revealed more clearly the importance of lymphoid cells in activating macrophages to an increased bactericidal state (Mackaness, 1969; Blanden and Langman, 1972; Lane and Unanue, 1972; North, 1973).

Initially Mackaness (1969) found that during an infection with *Listeria monocytogenes* mice acquired a population of specifically committed lymphocytes which had the capacity to confer protection and delayed-type hypersensitivity upon normal recipients. The transfer of immunity with lymphoid cells from immune mice could be abolished with rabbit anti-mouse lymphocyte globulin (Mackaness and Hill, 1969). Subsequently Lane and Unanue (1972) further characterised the lymphocytes as thymus-derived (T) cells. They found that the *in vitro* pretreatment of spleen cells from immunized mice with anti-θ serum and complement abolished their ability to adoptively transfer resistance to Listeria infections. North

(1973) also reported that θ-bearing lymphocytes were involved in the induction of an effective cell-mediated immune response against *Listeria monocytogenes*. Similarly Krahenbuhl, Rosenberg and Remington (1973), while assessing the ability of phagocytic cells to kill *Listeria monocytogenes*, demonstrated that the *in vitro* activation of macrophages was a T cell dependent process.

The available experimental evidence suggests that in the case of immunity to Salmonella infections, T cells are also essential for macrophage activation. For example, spleen cells from mice infected with *Salmonella gallinarum* have been shown to be capable of transferring delayed-type hypersensitivity to normal recipients (Collins and Mackaness, 1968). More recently Davies (1975) reported that mice required normal numbers of T cells to effectively eliminate *Salmonella enteritidis* 11RX from their liver and spleen, and also to ensure that vaccination with this avirulent organism led to the production of immunity to challenge with the virulent *Salmonella typhimurium* C5.

Immunocompetent T-lymphocytes are important not only for the development of resistance to many intracellular bacterial parasites, but also for the regulation of this response. Asherson and Zembala (1976) found that suppressor T-cells, demonstrable in the spleens of mice 4 days after sensitization with oxazolone or picryl chloride, could suppress the *de novo* development of skin sensitivity in syngeneic normal recipients. Subsequently it was suggested that specific suppressor T-cells may also be responsible for the sharp decline in delayed-type hypersensitivity observed in mice recovering from a bacterial infection (Collins, 1979). The existence of suppressor T-cells

has since been verified in mice infected with Mycobacterium habana (Watson and Collins, 1979), Mycobacterium simiae (Watson and Collins, 1980), and Mycobacterium tuberculosis BCG (Collins and Watson, 1980). In addition, enhancement of the humoral response can also limit cellular responses such as delayed-type hypersensitivity (Mackaness, Lagrange and Ishibashi, 1974; Neta and Salvin, 1974). It is therefore likely that T- and B- lymphocytes, as well as macrophages participate in a complex regulatory network which can either augment or suppress the various immunological components of the defence system (Asherson and Zembala, 1975; 1976; Gershon, 1975).

When elucidating the role of antibody in immunity to typhoid, it is important to know whether the acquisition of activated macrophages alone, is sufficient to confer resistance to this disease. Pertinent to this question is the observation that, although mice infected with either Listeria or Salmonella are resistant to a homologous challenge due to the acquisition of phagocytic cells with increased bactericidal activity, they are however not always resistant to a heterologous challenge. For instance, Zinkernagel (1976) reported that CBA/H mice infected with Listeria monocytogenes were not able to control a challenging infection with Salmonella typhimurium. He suggested that in addition to macrophage activation, specific factors were necessary for immunity to this organism. In contrast, Blanden, Mackaness and Collins (1966) found that Listeria monocytogenes-infected Swiss-Webster mice were resistant to challenge with Salmonella typhimurium. Reconciliation of these conflicting results is difficult as one must consider that many factors, including

the natural resistance of the strains of mice used, the number of immunizing organisms injected, the time of challenge and the size of the challenge inoculum may account for the differences observed. Induction of cross-reacting antibody is not likely to be involved however, because there are marked antigenic differences between these two strains of bacteria. Nevertheless it seemed important to clarify whether *Listeria monocytogenes*-infected mice were resistant to challenge with *Salmonella typhimurium*, because of the implications such a result would have on any analysis of the role of antibody in immunity to intracellular bacterial parasites.

This chapter presents a study of the resistance of Listeria-infected F1 mice to challenge with *Salmonella typhimurium* and an assessment of the role of specific antibody in the level of immunity observed. The aim of this study was to attempt to establish more clearly whether, in addition to macrophage activation, specific factors were required for immunity to *Salmonella typhimurium*. Therefore, before commencing this investigation it was necessary to confirm that *Listeria monocytogenes* was capable of both establishing a carrier state in normal F1 mice and of activating the reticuloendothelial system of these mice.

The growth of *Listeria monocytogenes* in normal mice

Forty male F1 mice were injected intravenously with 1.0×10^4 living *Listeria monocytogenes*. The fate of this infection was followed by periodically sacrificing 5 mice and determining the number of viable organisms which could be recovered from the liver, spleen and peritoneal cavity of each of the mice. The results, recorded in Figure 5.1, indicate

FIGURE 5.1

The fate of *Listeria monocytogenes* in F1 mice

A. The total number of viable Listeria recovered.

□———□ Total / mouse

B. The number of viable Listeria recovered in various organs.

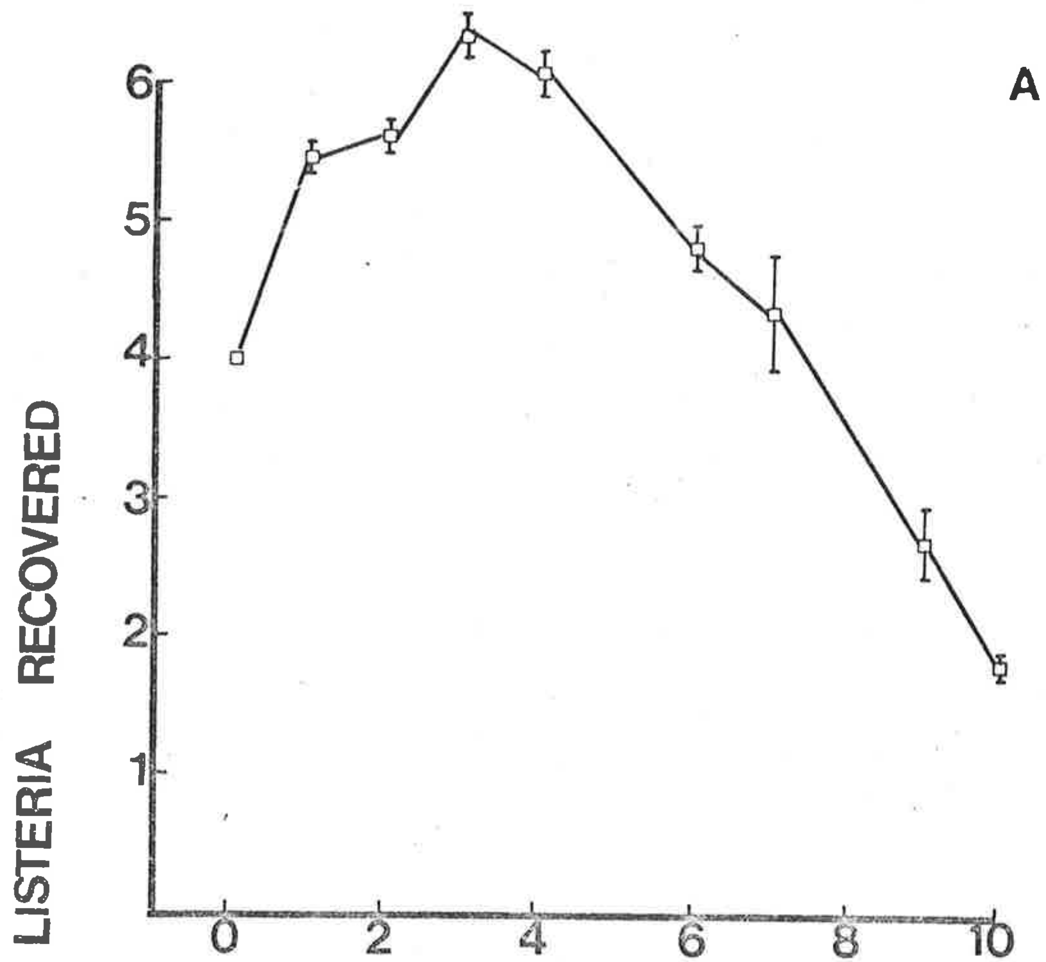
○———○ Liver

●- - - - - - -● Spleen

■- - - - - - -■ Peritoneum

1.0×10^4 *Listeria monocytogenes* were injected intravenously on day 0.

Each point represents the average of the $\log_{10}$ values from 5 mice.


A
6
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3
2
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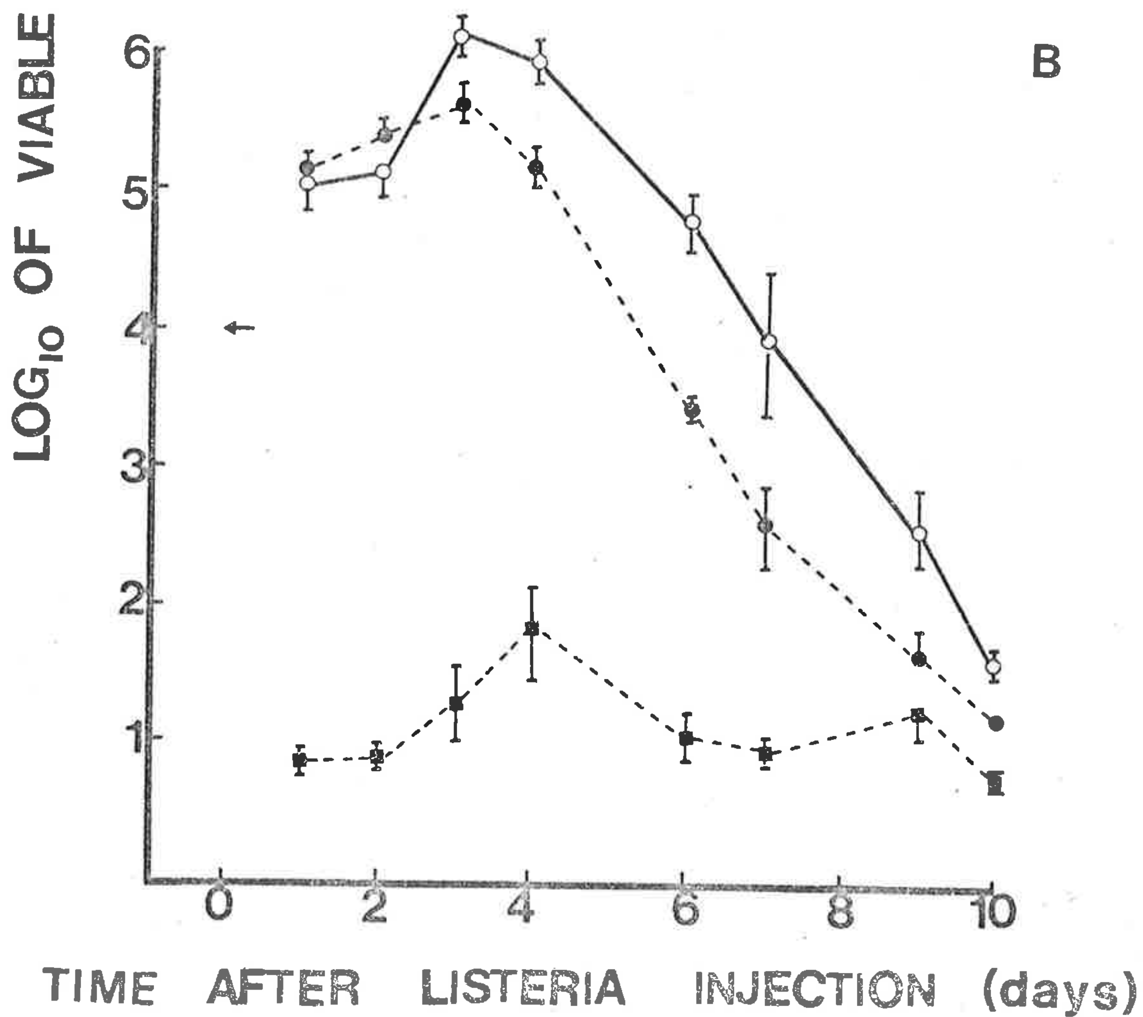
8
10
LISTERIA RECOVERED


B
6
5
4
3
2
1
0
2
4
6
8
10
LOG$_{10}$ OF VIABLE
TIME AFTER LISTERIA INJECTION (days)

that the Listeria established a carrier state, although it was of considerably shorter duration than that induced by Salmonella enteritidis 11RX (Figure 3.4). Essentially, the number of organisms increased during the first 3 days and then gradually declined, with very few organisms being recovered 10 days after infection. Presumably, the development of an effective anti-bacterial cell-mediated immune response by the third day enabled the mice to control and eventually eliminate the infection. Similar results have been previously reported for C57Bl/6 mice (Cheers et al., 1978).

The activity of the reticuloendothelial system in mice during an infection with Listeria monocytogenes

The activity of macrophages in the liver and spleen can be assessed by the ability of the mice to clear an intravenous dose of carbon particles from their bloodstream (Jenkin and Rowley, 1961). In this experiment, 40 male F1 mice were injected intravenously with 1.0×10^4 viable Listeria monocytogenes, while a similar number of normal mice were set aside as uninfected controls. Periodically, the rate of carbon clearance was measured in 5 mice from each group, as described in Chapter 2. The results, expressed in terms of a phagocytic index, or K value, are presented in Figure 5.2.

During the course of the Listeria infection the carbon clearance rate was greater than that observed in normal mice, particularly from the third to ninth day. On day 7, the K value peaked at 0.077 ± 0.005,indicating that the reticulo-endothelial system was maximally stimulated at this time.

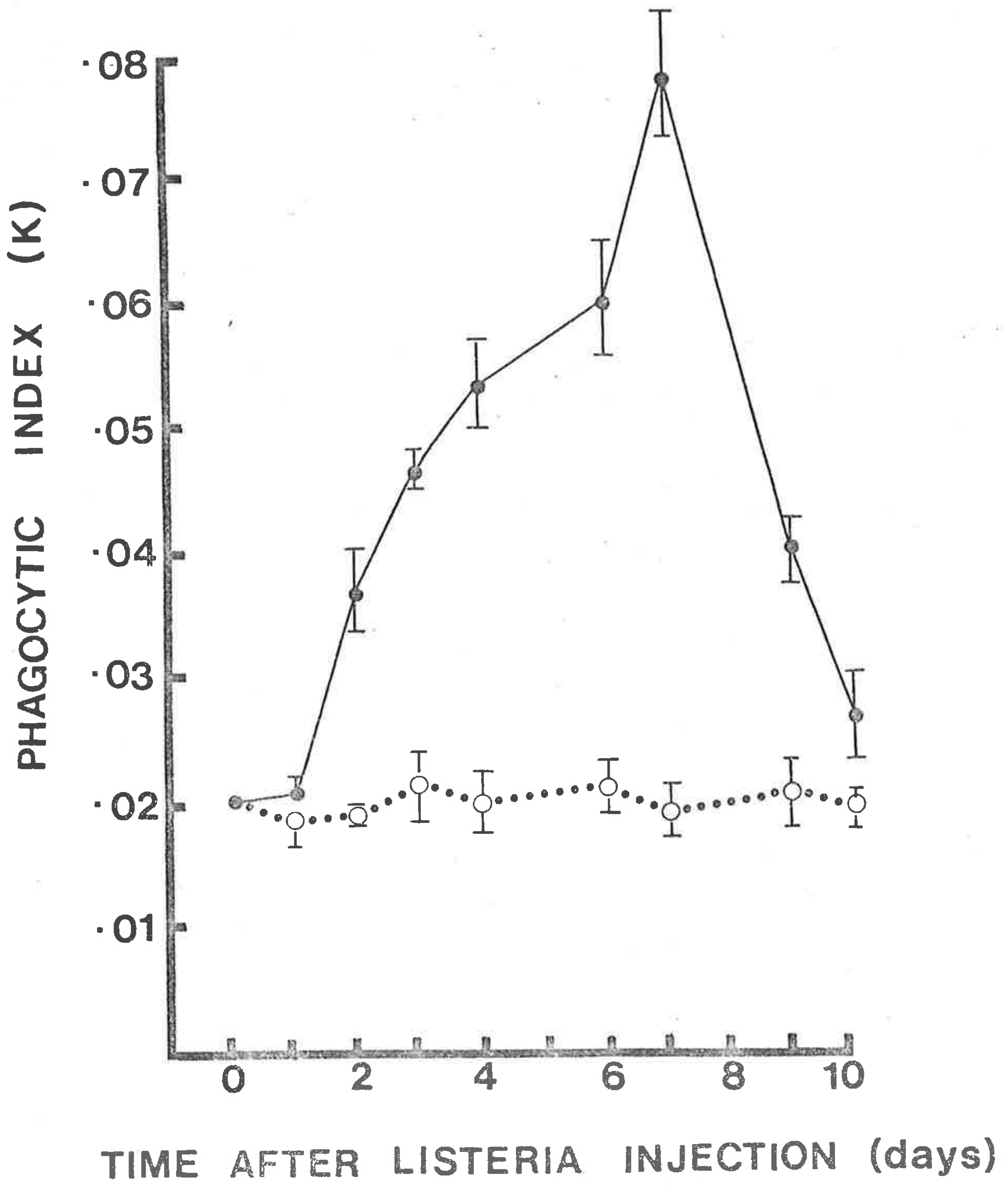
FIGURE 5.2

The clearance of carbon particles from the circulation was measured in F1 mice which had been injected intravenously with 1.0×10^4 Listeria monocytogenes on day 0.

O--------O Normal mice

●————● Listeria monocytogenes-infected mice

Each point represents the mean $\pm$ S.E. of 5 mice.


PHAGOCYTIC INDEX (K)
·08
·07
·06
·05
·04
·03
·02
·01
0
2
4
6
8
10
TIME AFTER LISTERIA INJECTION (days)

The resistance of Listeria monocytogenes-infected mice to a secondary challenge

The fall in bacterial numbers observed in the mice during the latter phase of the Listeria infection implied that the mice had acquired a population of macrophages with enhanced bactericidal activity. This conclusion was supported by the carbon clearance data which demonstrated that Listeria-infected mice had a stimulated reticuloendothelial system.In order to gain more conclusive evidence of macrophage activation, the resistance of the Listeria-infected mice to a secondary challenge was determined.

Twenty-five male F1 mice were immunized with an intravenous injection of 1.1×10^4 Listeria monocytogenes while an equal number of age-matched controls were set aside. After 7 days, all the mice were challenged intravenously with 1.1×10^6 Listeria monocytogenes. In the first part of the experiment, the fate of this challenge dose in each group was investigated, by determining the number of viable Listeria which could be recovered from the liver, spleen and peritoneal cavity of 5 of the mice on days 1 - 3 after challenge. It is apparent from the results, seen in Figure 5.3A, that the Listeria-immunized mice were able to control and eliminate a secondary challenge whereas normal mice were not resistant because the challenge inoculum multiplied during the time period studied.

In the second part of this experiment, the survival of the remaining mice was followed for 15 days and the results are recorded in Figure 5.3B. As expected, the Listeria-immunized mice were resistant while the normal mice quickly succumbed to the infection. Together, these results show that a non-lethal

FIGURE 5.3

The effect of a prior sublethal infection of Listeria monocytogenes on the resistance of F1 mice to a homologous challenge with 1.1×10^6 Listeria monocytogenes i.v. on day 0.

A. The fate of the challenge inoculum

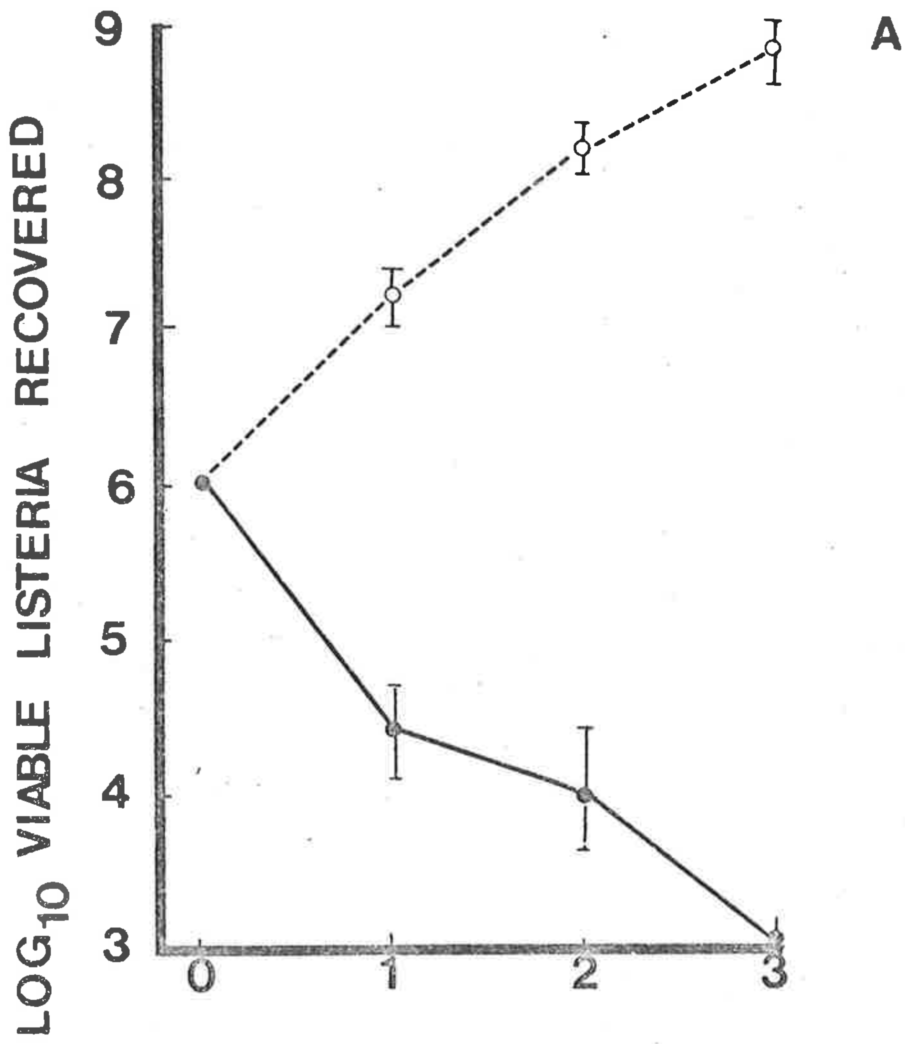
The number of viable Listeria in the liver, spleen and peritoneal cavity of each mouse was determined at various times after challenge. The majority of bacteria were found in the liver and spleen, while very few organisms were recovered from the peritoneal cavity. Each point represents the geometric mean ± S.E. of the total number of Listeria recovered from 5 mice.

B. The survival of the mice after challenge

Survival was followed for 15 days on groups of 10 mice.

O- - - - - - -O Normal mice

●————● Mice immunized by i.v.injection of 1.1×10^4 Listeria monocytogenes (day -7).


A
LOG10 VIABLE LISTERIA RECOVERED
9
8
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6
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2
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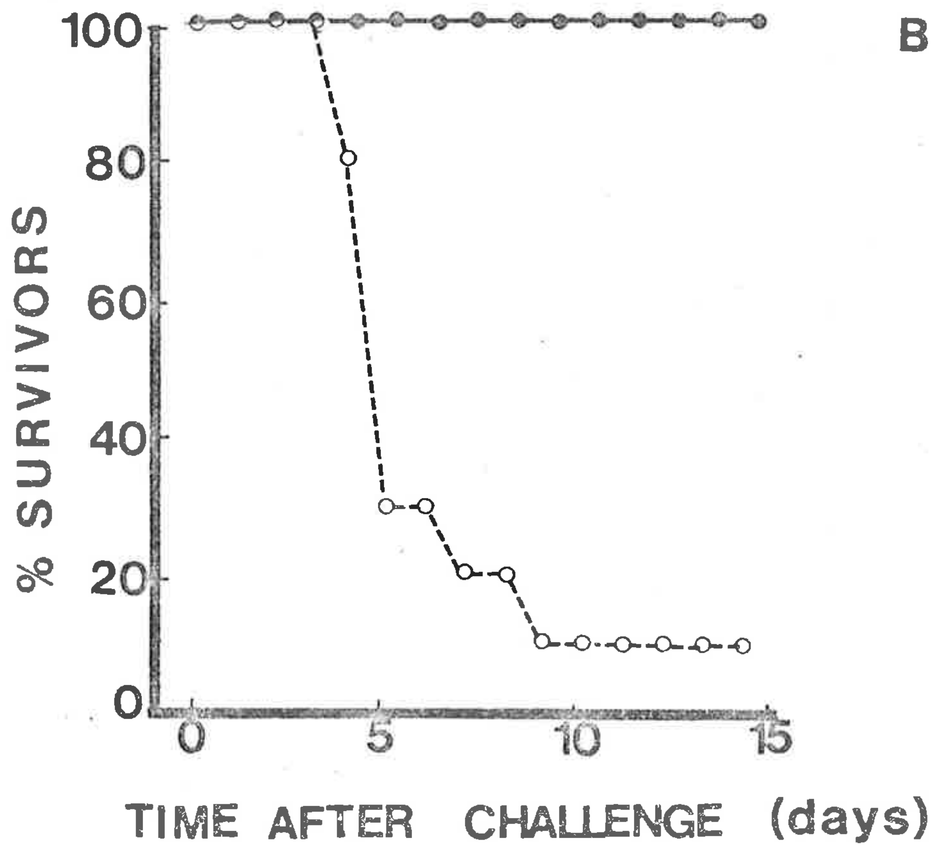

B
% SURVIVORS
100
80
60
40
20
0
0
5
10
15
TIME AFTER CHALLENGE (days)

infection with *Listeria monocytogenes* conferred protection against a normally lethal, homologous challenge. While such a finding was not new, it was important to this study because it gave a positive indication that the phagocytic cells of the infected F1 mice were activated. The prophylactic value of this cell-mediated immune response was then assessed against a heterologous challenge with *Salmonella typhimurium* C5.

The resistance of *Listeria monocytogenes*-infected mice to challenge with *Salmonella typhimurium* C5

Two groups of 45 male F1 mice, one group having been injected intravenously with 1.0×10^4 *Listeria monocytogenes* 7 days earlier, and the other group consisting of age-matched controls, were challenged intravenously with 1.4×10^4 *Salmonella typhimurium* C5. As in the previous study, resistance was assessed in terms of both the fate of the challenge inoculum and of the subsequent survival of the mice. The progress of the typhoid infection in each group was monitored by intermittently determining the number of viable bacteria in the liver, spleen and peritoneal cavity of 5 mice. Similarly, the survival of the mice was followed for 28 days.

The results shown in Figure 5.4 indicate that while Listeria-infected mice had some marginal advantage over the normal mice, they were nevertheless unable to control the ensuing salmonellosis which eventually led to their demise. This observation concurs with the report of Zinkernagel (1976) that in CBA/H mice, infection with *Listeria monocytogenes* did not confer resistance to *Salmonella typhimurium*. He proposed that immunity to this organism was dependent on the presence of specific factors as well as activated macrophages. It seems

FIGURE 5.4

The effect of a prior sublethal infection with *Listeria monocytogenes* on the resistance of F1 mice to a heterologous challenge with 1.4×10^4 *Salmonella typhimurium* C5 i.v. on day 0.

A. The fate of the challenge inoculum

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen, while very few organisms were recovered from the peritoneal cavity.

Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 5 mice.

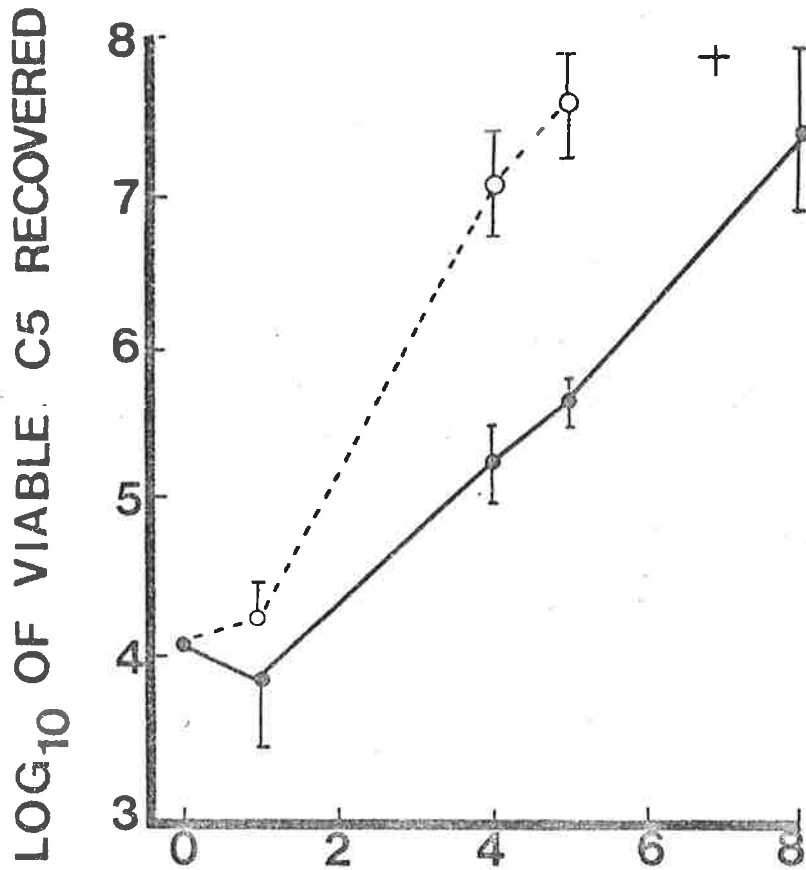
B. The survival of the mice after challenge

Survival was followed for 28 days on groups of 25 mice.

o-------o Normal mice

●———● Mice immunized by i.v. injection of 1.0×10^4 *Listeria monocytogenes* (day -7).

† indicates that all mice died.


LOG10 OF VIABLE C5 RECOVERED
8
7
6
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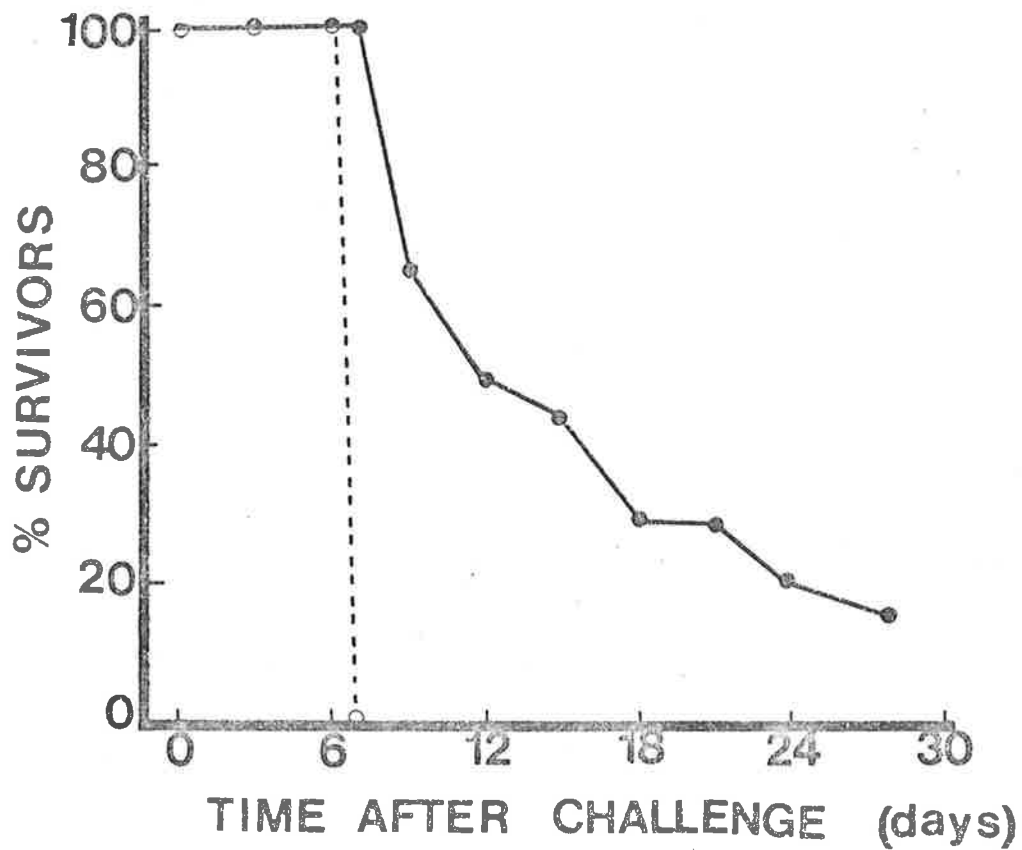
8


% SURVIVORS
100
80
60
40
20
0
0
6
12
18
24
30
TIME AFTER CHALLENGE (days)

most likely that the necessary factors are specific antibodies, either humoral or cytophilic, although they may be inducible enzymes which can deal more effectively with the *Salmonella typhimurium*.

The effect of specific antibody on the ability of *Listeria monocytogenes*-infected mice to clear radiolabelled *Salmonella typhimurium* C5 from their circulation

The effect of specific antibody on the clearance of *Salmonella typhimurium* C5 from the bloodstream of *Listeria monocytogenes*-infected mice was investigated in order to determine whether the availability of opsonic antibody was limiting the expression of resistance to salmonellosis. In this experiment, 1.0×10^8 radiolabelled *Salmonella typhimurium* C5, either opsonized or unopsonized, were injected intravenously into each of 10 mice which had been immunized intravenously with 1.0×10^4 *Listeria monocytogenes* 7 days previously. For comparison, normal age-matched controls were similarly injected with the suspensions of radiolabelled bacteria. Colloidal carbon was also included in the suspensions so that an assessment could be made of any non-specific stimulatory effects that the opsonins may have had on the activity of the phagocytic cells. The clearance rates of the bacteria and the carbon from the circulation were measured and the resultant phagocytic indices are recorded in Table 5.1.

It is apparent that the presence of exogenous antibody, while having little, if any, effect on the fate of the carbon particles, caused a significant increase in the clearance of the radiolabelled bacteria in both groups of mice. This ability of specific antibody to increase the rate of

TABLE 5.1

The effect of specific antibody on the ability of normal and *Listeria monocytogenes*-infected F1 mice to clear carbon particles or radiolabelled *Salmonella typhimurium* C5 from their bloodstream.

Mice	Anti-C5 Antiserum[a]	Phagocytic Index[b]	
		Carbon	^{32}P-C5
Normal	-	0.037 ± 0.020	0.035 ± 0.012
	+	0.036 ± 0.005	0.183 ± 0.037
Listeria-infected[c]	-	0.073 ± 0.017	0.089 ± 0 050
	+	0.091 ± 0.033	0.230 ± 0.052

a. The mouse anti-C5 antiserum was present in the suspensions at a dilution of 1/50

b. Each phagocytic index represents the mean ± S.E. of 10 mice

c. 1.0×10^4 *Listeria monocytogenes* were injected intravenously on day -7

phagocytosis of Salmonella typhimurium C5 in Listeria-infected mice suggested that the availability of these factors may be limiting the expression of resistance to salmonellosis.

The effect of specific antibody on the resistance of Listeria monocytogenes-infected mice to challenge with Salmonella typhimurium C5

The protection afforded by specific antibody or immunization with live Listeria monocytogenes, or a combination of both these treatments was determined by assaying the ability of mice to control the growth of a Salmonella typhimurium C5 infection. In the following 3 separate experiments, the dose of the challenge inoculum was varied in an attempt to optimize any beneficial effects of the antibody, which was provided by either passive transfer of serum from immunized mice or active immunization of the mice used for challenge.

Experiment 1

In this experiment, 4 groups of 26 male F1 mice were challenged intravenously with 2.3×10^6 Salmonella typhimurium C5. By design, 2 of these groups had been infected with 0.8×10^4 Listeria monocytogenes 7 days previously while the other 2 groups were uninfected. Two hundred µl aliquots of an antiserum which had been raised in F1 mice against an alcohol-killed Salmonella typhimurium C5 vaccine, were injected into 1 Listeria-infected group and 1 control group, immediately prior to challenge and twice daily thereafter. The fate of the Salmonella infection in each of the groups is shown in Figure 5.5.

FIGURE 5.5

The effect of immunization with live *Listeria monocytogenes* and the passive transfer of antibody specific for *Salmonella typhimurium* C5 on the ability of F1 mice to control the growth of 2.3×10^6 *Salmonella typhimurium* C5 injected intravenously on day 0.

□-------□ C5 challenge only

○-------○ 0.8×10^4 *Listeria monocytogenes* i.v. (day -7) and C5 challenge

■——■ Passive antibody and C5 challenge

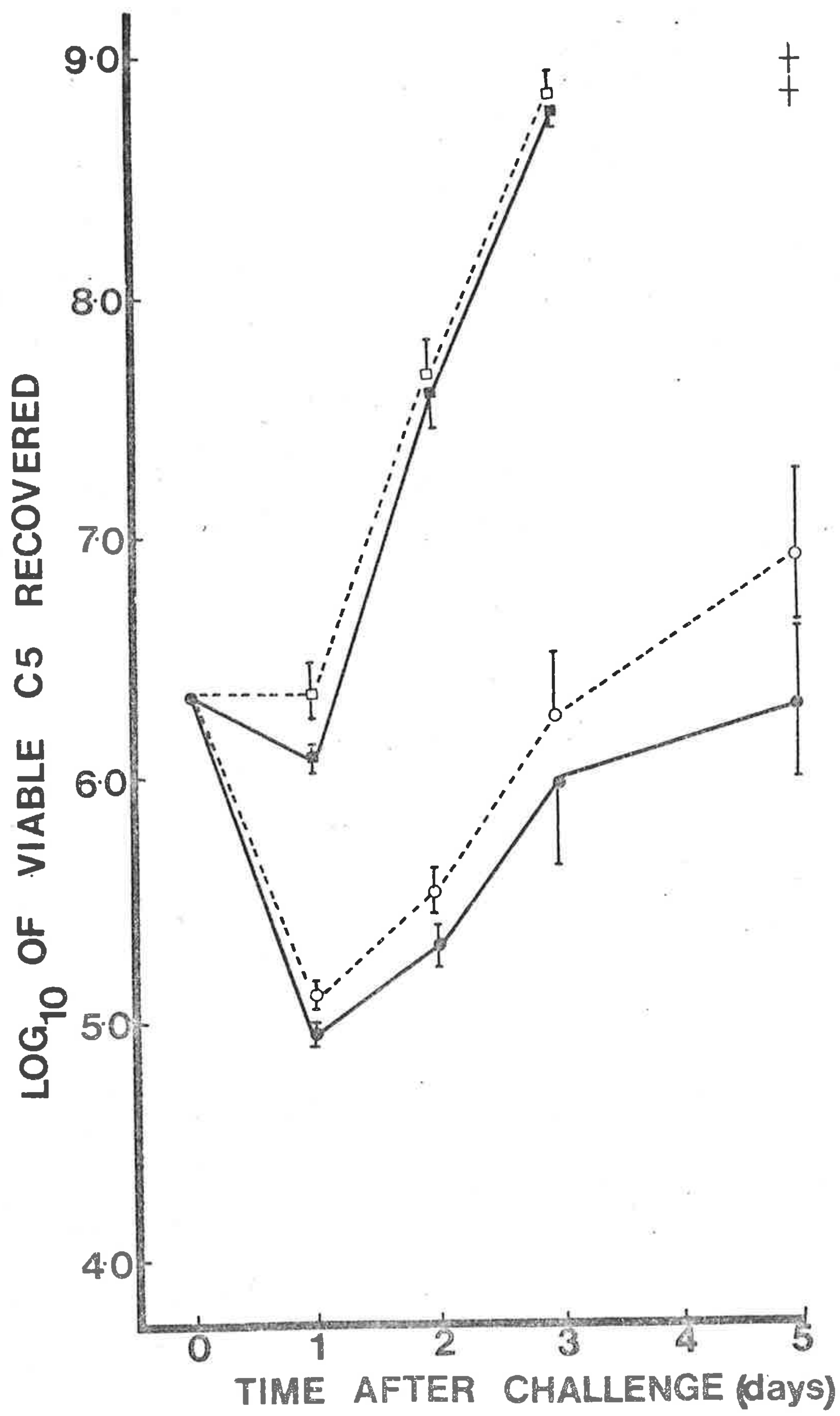
●——● 0.8×10^4 *Listeria monocytogenes* i.v. (day -7), passive antibody and C5 challenge.

The antiserum (0.2ml) was administered intravenously immediately prior to challenge and twice daily thereafter. It was obtained from mice immunized with an alcohol-killed C5 vaccine.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few organisms were recovered from the peritoneal cavity.

Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 5 mice.

† indicates that all mice died.



9·0
8·0
7·0
6·0
5·0
4·0
LOG10 OF VIABLE C5 RECOVERED
0
1
2
3
4
5
TIME AFTER CHALLENGE (days)

The normal mice were unable to control the rapid growth of the Salmonella and quickly succumbed to the infection. In contrast, the Listeria-infected mice were able to significantly reduce the size of the challenge inoculum during the first 24 hours, after which time bacterial numbers increased steadily although at a slower rate than in the normal mice. The passive transfer of specific antibody to Listeria-infected mice had only marginal beneficial effects. This result was somewhat surprising. Previous observations by Zinkernagel (1976) and Blanden, Mackaness and Collins (1966) and the clearance data presented in Table 5.1 had suggested that the Listeria-immunized mice had a stimulated reticuloendothelial system which should have been able to control and eliminate the challenging infection, especially in the presence of specific antibody. In the following 2 experiments, specific antibody was induced in the mice used for subsequent challenge by active immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine. This was done in an attempt to overcome the possibility that the amount of antibody transferred was not sufficient to obtain adequate opsonisation of the challenge organisms.

Experiment 2

Groups of 26 male F1 mice were injected intravenously with 10µg of alcohol-killed *Salmonella typhimurium* C5 vaccine (day -28), or 1.2×10^4 live *Listeria monocytogenes* (day -7) or both or nothing. All mice were challenged intravenously with 1.5×10^6 *Salmonella typhimurium* C5 and the progress of the infection in each group can be seen in Figure 5.6.

Again the Salmonella multiplied rapidly in normal mice.

FIGURE 5.6

The effect of immunization with live *Listeria monocytogenes* and an alcohol-killed *Salmonella typhimurium* C5 vaccine on the ability of F1 mice to control the growth of 1.5×10^6 *Salmonella typhimurium* C5 injected intravenously on day 0.

□- - - - - - - -□ C5 challenge only

○- - - - - - - -○ 1.2×10^4 *Listeria monocytogenes* i.v. (day -7) and C5 challenge

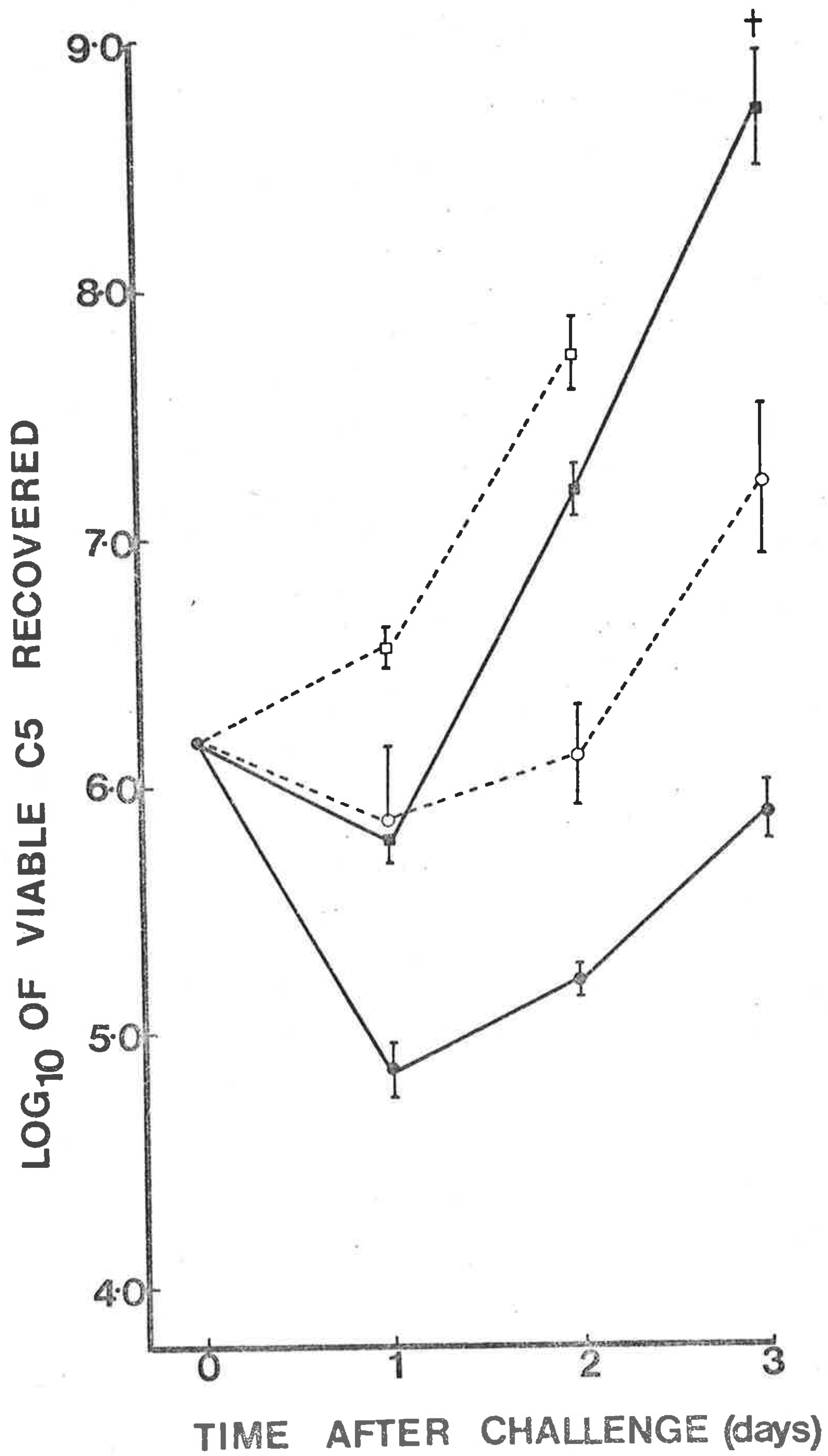
■————■ 10µg alcohol-killed C5 vaccine i.v. (day -28) and C5 challenge

●————● 10µg alcohol-killed C5 vaccine i.v. (day -28), 1.2×10^4 *Listeria monocytogenes* i.v. (day -7) and C5 challenge.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity.

Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 4 mice.

† indicates that all mice died.


9·0
8·0
7·0
6·0
5·0
4·0
LOG10 OF VIABLE C5 RECOVERED
0
1
2
3
TIME AFTER CHALLENGE (days)
†

The 3 groups of vaccinated mice were able to reduce the size of the challenge inoculum, but failed to control its subsequent growth. The doubly immunized mice, primed for both humoral and cell-mediated responses, were able to cope with the Salmonella challenge better than the other groups of mice, but they were certainly not resistant to the challenge, as judged by their ability to control multiplication of the challenge organisms. Because these mice were eventually overwhelmed by this comparatively large challenge, their ability to resist a lower challenge dose was investigated in the third experiment of the series where a 100-fold lower challenge dose was used.

Experiment 3

As in the previous experiment, groups of 26 female F1 mice were injected intravenously with 10µg of alcohol-killed Salmonella typhimurium C5 vaccine (day -28), or 0.8×10^4 live Listeria monocytogenes (day -7) or both or nothing. Subsequently, all the mice received 1.4×10^4 Salmonella typhimurium C5 intravenously. The fate of this lower challenge dose is represented in Figure 5.7and, as in the 2 preceding experiments, none of the experimental groups were able to efficiently eliminate these pathogenic bacteria.

Taken together these experiments demonstrate that mice immunized with live Listeria monocytogenes were not able to control a challenging infection with Salmonella typhimurium C5 and this was supported by the observation that those mice which were not sacrificed during the course of these experiments suffered from a chronic and invariably fatal disease (data not shown). Although Blanden, Mackaness and Collins (1966) found

FIGURE 5.7

The effect of immunization with live Listeria monocytogenes and an alcohol-killed Salmonella typhimurium C5 vaccine on the ability of F1 mice to control the growth of 1.4×10^4 Salmonella typhimurium C5 injected intravenously on day 0

□-------□ C5 challenge only

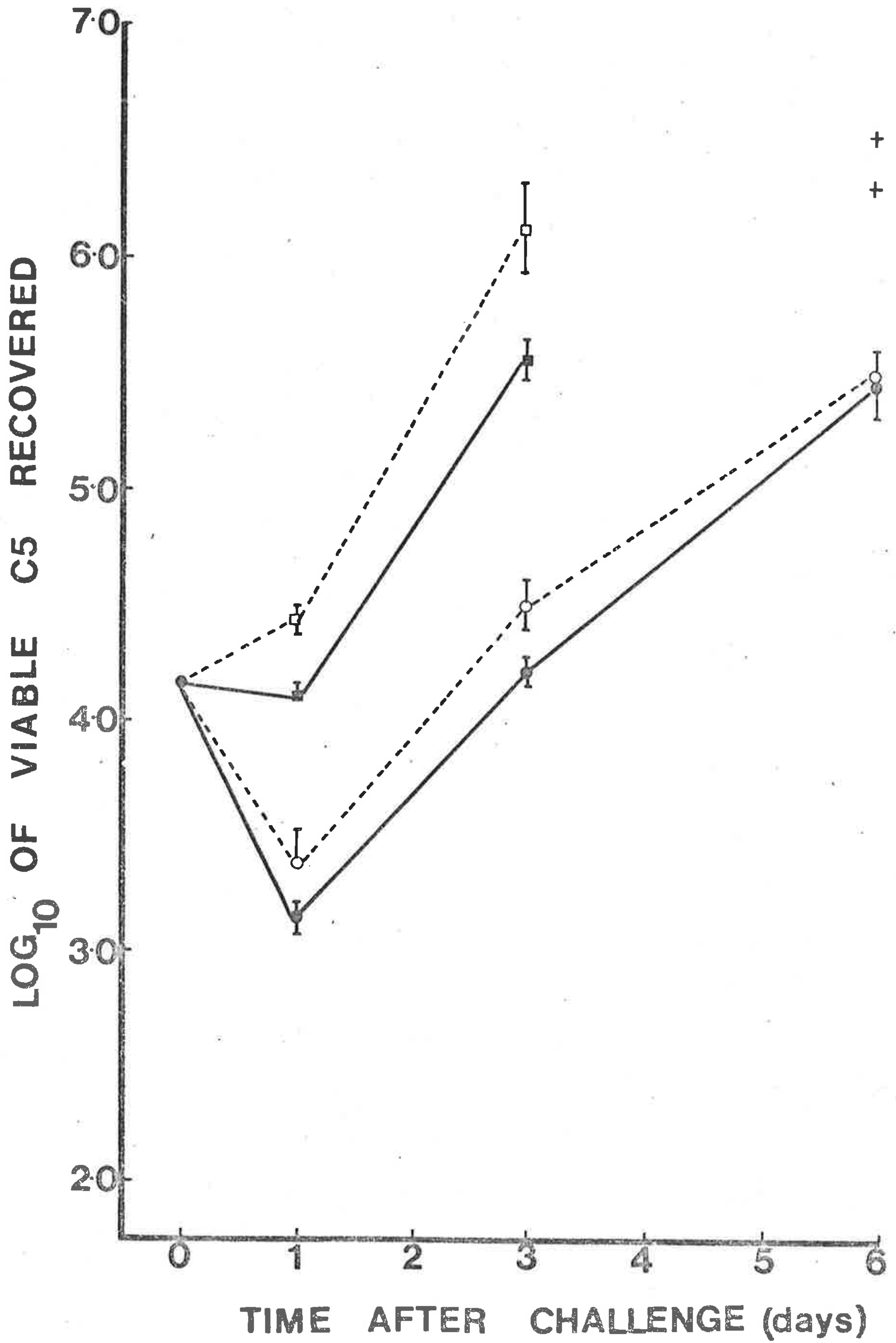
○-------○ 0.8×10^4 Listeria monocytogenes i.v. (day -7) and C5 challenge

■———■ 10μg alcohol-killed C5 vaccine i.v. (day -28) and C5 challenge

●———● 10μg alcohol-killed C5 vaccine i.v. (day -28), 0.8×10^4 Listeria monocytogenes i.v. (day -7) and C5 challenge.

The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen while very few were recovered from the peritoneal cavity.
Each point represents the geometric mean ± S.E. of the total number of C5 recovered from 4 mice.

+ indicates that all mice died.



7·0
6·0
5·0
4·0
3·0
2·0
LOG10 OF VIABLE C5 RECOVERED
0
1
2
3
4
5
6
TIME AFTER CHALLENGE (days)
+
+

that Listeria-infected mice were non-specifically resistant to *Salmonella typhimurium*, the implication from this study is that the level of immunity conferred is probably very low. In keeping with this conclusion, it should be remembered that in these earlier studies Swiss-Webster mice were challenged with less than an L.D.$_{50}$dose, which is unlikely to reveal anything more than marginal "non-specific" resistance.

It was also found that the addition of specific antibody by passive transfer or active immunization was of only marginal benefit, as it did not significantly enhance the resistance of these mice. While this latter observation was disappointing it does not necessarily preclude a role for antibody, but merely illustrates that resistance is a complex immunological phenomenon which cannot always be easily dissected.

There did appear to be a profound difference in the way *Salmonella enteritidis* 11RX-and *Listeria monocytogenes*-infected mice dealt with *Salmonella typhimurium* C5, even when specific antibody was not limiting. It is plausible that such differences may reflect either the state or degree of macrophage activation, or the maintenance of macrophage activation in these mice. The extremely effective cell-mediated response generated in *Salmonella enteritidis* 11RX-infected mice may be due, in part, to persistence of the organism providing continual antigenic stimulation and to the ability of *Salmonella typhimurium* C5, or its digestion products, to maintain the level of macrophage activation. It has already been shown that cytoplasmic protein antigens from *Salmonella typhimurium* C5 are capable of both eliciting delayed-type hypersensitivity reactions (Davies, 1975) and of

recalling anti-tumour immunity (Vingelis, Ashman and Kotlarski, 1980). Recently it has been reported that as a consequence of infection with Listeria monocytogenes, BALB/c mice suffered from thymus atrophy and a loss of T-lymphocytes from the blood and spleen (Mandel and Cheers, 1980). The T-lymphocyte depletion in the spleen was particularly striking as numbers of Thy-1^+ cells dropped to 4% of control values at 2 - 4 days post-infection. This, as yet unexplained observation has important implications for this study. So too do the studies which suggest that suppressor T-cells are generated after some intracellular bacterial infections (Watson and Collins, 1979, 1980; Collins and Watson, 1980). The presence of suppressor T cells or/and the loss of T cells in the Listeria-infected F1 mice may prevent the generation of an effective cell-mediated immune response against the typhoid bacilli and so contribute to the susceptibility of these mice to this infection. The following experiments were designed to examine this possibility.

The immunocompetence of Listeria monocytogenes-infected mice

The ability of Salmonella enteritidis 11RX to induce a cell-mediated immune response in normal and Listeria monocytogenes-infected mice was determined.

Salmonella enteritidis 11RX was chosen as the immunizing strain in preference to the virulent Salmonella typhimurium C5 because of practical considerations, and delayed-type hypersensitivity reactions were used to assess the development of a cell-mediated immune response

Five male F1 mice were infected intravenously with both 10^4 Listeria monocytogenes (day -14) and 10^5 Salmonella

enteritidis 11RX (day -7), while 3 appropriate control groups were also set aside. On day 0, 10μg of Salmonella enteritidis 11RX protein antigen was injected into the hind foot pad of all of the mice. Any ensuing delayed-type hypersensitivity reactions were revealed after 48 hours by a marked increase in the foot pad thickness. The results of 3 such experiments are recorded in Table 5.2.

Prior infection with Listeria monocytogenes appeared to impair the development of immunity to Salmonella enteritidis 11RX, because the delayed-type hypersensitivity reactions in the doubly infected mice were significantly smaller than those in the mice which had only been infected with Salmonella enteritidis 11RX. The protein antigen failed to elicit a response in either the normal or Listeria-infected mice. Although the Listeria-infected mice were resistant to a homologous challenge, it appeared from these findings that their immune system was in a state of reduced responsiveness to heterologous antigenic stimuli. While there are other possible explanations for our results, mostly related to antigenic load and cell trafficking, the idea of decreased immunocompetence in Listeria-infected mice was supported by the observation that these mice occasionally succumbed to challenge with the normally avirulent Salmonella enteritidis 11RX. In one notable instance, not only did mice infected with Listeria monocytogenes die after an "immunizing" dose of Salmonella enteritidis 11RX, but so also did normal mice which had received spleen cells from the Listeria-infected mice (data not shown).

In their study on the histopathology of listeriosis, Mandel and Cheers (1980) found the duration of T-cell

TABLE 5.2

The hypersensitivity responses of normal, *Listeria monocytogenes*-infected and/or *Salmonella enteritidis* 11RX-infected mice to 10µg of *Salmonella enteritidis* 11RX protein antigen.

Infections given to F1 mice[a]	DTH at 48 hours[b]		
	Exp.1	Exp.2	Exp.3
None	1.5 ± 1.0	2.0 ± 2.0	1.6 ± 0.8
L. monocytogenes	0.9 ± 0.7	3.2 ± 1.7	3.5 ± 0.8
S. enteritidis 11RX	25.7 ± 2.4	14.8 ± 2.7	26.3 ± 4.7
L. monocytogenes and *S. enteritidis* 11RX	11.8 ± 2.8	6.1 ± 4.9	5.1 ± 1.1

a. Mice were injected intravenously with either 10^4 *Listeria monocytogenes* (day -14) and/or 10^5 *Salmonella enteritidis* 11RX (day -7)

b. Percentage increase in foot pad thickness at 48 hours Each estimate represents the mean ± S.E. of 5 mice.

depletion to be quite short, beginning 2 days and recovering 6 - 8 days post-infection. If T-cell depletion did contribute to the observations reported above, it appeared possible that the effects may be even more marked if the *Salmonella enteritidis* 11RX were given earlier, at a time corresponding to the period of maximum T-cell depletion. In this last study which followed similar lines to the previous one, 10^5 *Salmonella enteritidis* 11RX were injected intravenously into mice either 1, 3 or 6 days post-infection with *Listeria monocytogenes*. The dose of Listeria was lowered to 10^3 organisms to reduce the possibility that the mice were being "over infected". As before, 7 days after initiating the salmonellosis, 10μg of *Salmonella enteritidis* 11RX protein antigen was injected into the hind foot pad of all the mice.

The results (Table 5.3) demonstrate that the protein antigen elicited good delayed-type hypersensitivity responses in the Salmonella-infected mice, but failed to do so in both normal and Listeria-infected mice. The swelling in the doubly infected mice was again less than that in the Salmonella-infected mice. However, the level of "suppression" was less than in the previous experiments, which may have been due to the lower dose of Listeria used. The time course of the reduced responsiveness did not closely parallel the period of T-cell depletion described by Mandel and Cheers (1980). This may have been because suppressor T cells played a role in the system studied here or because, after the Salmonella were administered, 7 days were allowed to elapse before the mice were tested for delayed-type hypersensitivity.

TABLE 5.3

The hypersensitivity responses of mice given Salmonella enteritidis 11RX 1, 3 or 6 days after infection with Listeria monocytogenes. Delayed-type hypersensitivity responses were measured 48 hours after the injection of 10μg of Salmonella enteritidis 11RX protein antigen.

Infections given to F1 mice[a]	DTH at 48 hours[b]		
	Time of Listeria Infection		
	Day -8	Day -10	Day -13
None	1.7 ± 1.7	2.1 ± 1.5	1.8 ± 1.8
L. monocytogenes	1.8 ± 1.8	3.9 ± 2.5	2.3 ± 2.5
S. enteritidis 11RX	21.3 ± 2.2	25.0 ± 8.6	29.0 ± 5.8
L. monocytogenes and S. enteritidis 11RX	7.9 ± 8.5	18.4 ± 3.2	17.5 ± 4.8

a. Mice were injected intravenously with either 10^3 Listeria monocytogenes (day -8, -10 or -13) and/or 10^5 Salmonella enteritidis 11RX (day -7)

b. Percentage increase in foot pad thickness at 48 hours Each estimate represents the mean ± S.E. of 5 mice.

<u>Summary and conclusions</u>

Listeria monocytogenes is a facultative intracellular bacterial parasite which produces an acute, potentially lethal infection in mice. As a consequence of such an infection F1 mice acquire an activated reticuloendothelial system which is reflected not only by an enhanced ability to clear colloidal carbon, but also increased resistance to a secondary challenge.

This infection was thought to be an ideal system to study the role of antibody in immunity to *Salmonella typhimurium* C5, especially as the Listeria is a Gram-positive organism, the surface of which is unlikely to contain antigens which cross-react with those of the Gram-negative Salmonella. Subsequent studies revealed that the Listeria-infected mice were not non-specifically resistant to *Salmonella typhimurium* C5, supporting the concept that immunity to typhoid is dependent on the presence of specific antibody, as well as activated macrophages. Unfortunately however, while the addition of specific antibody greatly enhanced the initial phagocytosis of the Salmonella, it was of little long-term benefit. This result does not necessarily preclude a role for antibody, but it does imply that several factors may be contributing to the susceptibility of the Listeria-infected mice to salmonellosis. Reduced delayed-type hypersensitivity responses in these mice suggested that a state of reduced immunocompetence, caused by T-cell depletion and/or suppression, may be one of the factors contributing to their susceptibility.

CHAPTER 6

RESISTANCE TO SALMONELLA TYPHIMURIUM IN MICE PRETREATED WITH NON-LIVING VACCINES

Introduction

Over the years investigators have evaluated the efficacy of various vaccines to protect mice against infection with *Salmonella typhimurium*. Such studies have not only revealed information on the potency of these vaccines, but also on the fundamental features of the host-parasite interaction in this infection (Angerman and Eisenstein, 1980).

Briefly, live vaccines and killed bacteria administered in complete Freund's adjuvant have been found to induce both cellular and humoral immunity (Mackaness, Blanden and Collins, 1966; Kenny and Herzberg, 1967; Collins, 1972) whereas killed bacteria and lipopolysaccharide elicit only humoral immunity (Herzberg, Nash and Hino, 1972; Svenson and Lindberg, 1978). Generally, the live bacterial vaccines have been found to confer the greatest degree of protection (Mackaness, Blanden and Collins, 1966; Rowley, Auzins and Jenkin, 1968; Angerman and Eisenstein, 1978), and this has led to the suggestion of developing live-cell vaccines for human use (Collins, 1969; Fahey and Cooper, 1970; Germanier, 1972). However, such an approach has some inherent disadvantages, including the danger of virulent revertants (Angerman and Eisenstein, 1980).

The majority of typhoid vaccines in use in the world today consist of a variety of killed whole cell vaccines. Studies on mice have shown that such non-living vaccines confer substantial levels of protection, although they are inferior to the living vaccines. This demonstrates an important role played by humoral factors (Kenny and Herzberg, 1967; Rowley, Auzins and Jenkin, 1968; Herzberg, Nash and Hino, 1972). However, these vaccines are far from perfect and in fact are considerably toxic, due mainly to their lipopolysaccharide content. The emergence of protective subcellular fractions, particularly ribosomal fractions, has renewed interest in perfecting the non-living vaccines. Ribosomal immunogens derived from Mycobacterium tuberculosis, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus pyogenes, Neisseria meningitidis, Vibrio cholera, Brucella abortus, Listeria monocytogenes, Francisella tularensis and Histoplasma capsulatum have all been shown to be protective against challenge with the homologous organism (Misfeldt and Johnson, 1976).

In one study, the protective and toxic properties of a ribosomal vaccine prepared from Salmonella typhimurium W118-2 were compared with those of an acetone-killed whole cell vaccine, purified lipopolysaccharide and living cells in CD-1 mice. The ribosomal vaccine was found to be more protective and less toxic than the other non-living vaccines. In fact only the ribosomes and live cells afforded significant protection at 6 months after immunization (Angerman and Eisenstein, 1978; Angerman and Eisenstein, 1980). This ability of ribosomal vaccines to confer high levels of protection, comparable to those obtained with living attenuated strains, was initially

described by Venneman (1972). The ribosomal preparations appear superior to the other non-viable salmonella vaccines as they can induce cell-mediated immunity (Venneman and Berry, 1971; Smith and Bigley, 1972; Margolis and Bigley, 1972).

Identification of the active substances responsible for protection in the ribosomal preparations has been the subject of considerable debate. Venneman (1972) reported that a purified ribonucleic acid fraction was highly protective. In addition, ribosomal protein (Smith and Bigley, 1972; Johnson, 1973) and a high molecular weight glycoprotein or mucopolysaccharide (Houchens and Wright, 1973) have each been suggested as the immunogenic moiety. There has also been some evidence that contaminating lipopolysaccharide contributed to the immunogenicity of the ribosomal preparations (Eisenstein, 1975; Hoops et al., 1976). However Misfeldt and Johnson (1976) reported that endotoxin could not totally account for the effectiveness of the ribosomal vaccines. More recently the ribosomal fraction from Salmonella typhimurium LT2 was further purified by gel filtration. One fraction contained a heat stable antigen, rich in ribonucleic acid and free of endotoxin, which was capable of evoking cellular resistance in mice (Kita and Kashiba, 1980). Finally the protective effects of ribosomal vaccines appear to be strain dependent, as C57BL/6J mice are not protected against lethal challenge (Misfeldt and Johnson, 1976).

In summary, all of these non-viable salmonella vaccines are able to increase the resistance of mice to Salmonella typhimurium. While some vaccines, such as the ribosomal preparations, are able to stimulate cell-mediated responses, all are capable of inducing either specific or polyclonal

antibody responses. Definitive analysis of the resultant immune responses to these vaccines is invariably complicated by the effects of lipopolysaccharide contamination. For the purposes of this thesis, it appeared as though these vaccines alone would not be useful tools in clearly delineating the role of antibody in immunity to *Salmonella typhimurium*.

In addition to non-viable salmonella vaccines, a number of other biological materials can confer resistance to a variety of parasites. Non-viable vaccines prepared from *Coxiella burnetii*, the rickettsial agent which causes Q fever, are among some of the more recently reported examples. In guinea pigs, the intralesional injection of formalin-killed *Coxiella burnetii* has been shown to cause tumour regression (Kelly *et al*., 1976). Similarly, guinea pig macrophages activated *in vivo* by these killed organisms, produce extracellular factors capable of killing *Listeria monocytogenes in vitro* (Kelly, 1977). Clark (1979) also provided convincing evidence that a commercially available extract of *Coxiella burnetii* was able to protect mice against challenge with 3 protozoan parasites, namely *Babesia microti*, *Babesia rodhaini* and *Plasmodium vinckei petteri*. From the evidence available it does appear that these protective effects are due to a nonprotein component of *Coxiella burnetii*, which stimulates the cellular rather than humoral immune response. These initial results suggest that non-viable *Coxiella burnetii* vaccines may prove to be useful immunopotentiating agents and so warrant further investigation.

The quest for materials which will enhance the immune response to a given antigen has continued for many years. Compounds such as aluminium salts (April and Wardlaw, 1966), mycobacteria (White, Coons and Connolly, 1955) and polyanions

(Diamantstein _et al_.,1971 a & b) are known to enhance the humoral immune responses, whereas Freund's complete adjuvant (Raffel 1948) and polyanions (McCarthy, Arnold and Babcock, 1977; L'Age-Stehr and Diamantstein, 1977) have been used for increasing cell-mediated immune responses. While the Freund's adjuvant is more widely used, it has a complex chemical nature and so polyanions have some advantage in that they are chemically more defined (McCarthy, Arnold and Babcock, 1977).

Dextran sulphate, a polyanion with a molecular weight of 500,000 is a B cell mitogen (Diamantstein _et al_., 1973; Moreno, Hale and Ivanyi, 1977) and a potent adjuvant for both humoral (Diamantstein _et al_., 1971 a & b) and cell-mediated immunity (McCarthy, Arnold and Babcock, 1977; L'Age-Stehr and Diamantstein, 1977). However, while dextran sulphate is capable of potentiating the immune response to an antigen, it produces a complex spectrum of effects which are influenced by dosage, route and time of injection. Whilst dextran sulphate potentiates delayed-type hypersensitivity to sheep red blood cells when given with the antigen, it delays or even totally suppresses the expression of delayed type hypersensitivity when administered by a different route to that of the eliciting antigen (L'Age-Stehr and Diamantstein, 1977). Also, McCarthy and Babcock (1978) demonstrated that this immunoregulator is capable of simultaneously stimulating and suppressing 2 indicators of the cell-mediated immune response in the same animal.

Although dextran sulphate is known to activate the complement system via the alternate pathway (Hadding _et al_., 1973), morphological evidence suggests that the prime target of dextran sulphate is the mononuclear phagocyte (Hahn and

Bierther, 1973) and this is supported by cell transfer experiments (L'Age-Stehr and Diamantstein, 1977). It has been reported to impair macrophage activity by the inhibition of phagosome-lysosome fusion (Hart and Young, 1975; Goren *et al.*, 1976). Presumably the adjuvant properties of dextran sulphate are related to reduced antigen degradation as a consequence of this effect on macrophages.

Increased susceptibility to infection with *Listeria monocytogenes* is a direct result of the impaired macrophage function (Hahn, 1974; Hahn and Bierther, 1974). However prior activation of the mononuclear phagocyte system with heat-killed *Bordetella pertussis* organisms prevented this loss of resistance due to dextran sulphate (Finger *et al.*, 1978).When mice are injected with killed *Listeria monocytogenes* in association with dextran sulphate, they subsequently become protected against a lethal homologous challenge (Van Der Meer, Hofhuis and Willers, 1977). Extension of these findings to other intracellular bacteria is obviously important, but has yet to be established.

One of the main objectives of the studies presented in this chapter was to assess the prophylactic value of a commercial *Coxiella burnetii* extract and dextran sulphate against infection with *Salmonella typhimurium* C5. Secondly, as the *Coxiella burnetii* extract appears to stimulate the cell-mediated immune response and dextran sulphate affects both the humoral and cell-mediated immune responses, it was hoped that these materials would prove to be useful in delineating the role of antibody in resistance to *Salmonella typhimurium* C5.

Throughout this chapter the experimental regimes were adopted after consideration of the experiences of Clark (1979)

with a Coxiella burnetii extract and of Van Der Meer, Hofhuis and Willers (1977) with dextran sulphate.

Determination of serum antibody levels to Salmonella typhimurium C5 in mice pretreated with either the Coxiella burnetii extract or dextran sulphate.

Initially it was necessary to establish what effect the injection of the Coxiella burnetii extract or dextran sulphate had on the serum antibody levels to Salmonella typhimurium C5. One group of 5 F1 male mice was injected intravenously with 10µl of Coxiella burnetii extract on day -21, similarly another group received 1mg of dextran sulphate intravenously on day -7, while a third group of age-matched controls was set aside. All mice were bled on day 0, the sera were collected and stored frozen until just prior to use. The haemagglutination of C5 LPS SRBC was chosen as a suitable indicator of the antibody levels to Salmonella typhimurium C5.

The data presented in Table 6.1 indicates that the pretreatment of mice with dextran sulphate resulted in an elevation of the serum antibody levels to both C5 LPS SRBC and SRBC. In view of the much publicized B lymphocyte mitogenic activity of dextran sulphate (Diamantstein et al., 1973; Moreno, Hale and Ivanyi, 1977; Wetzel and Kettman, 1981) such a result was not unexpected. In comparison, the Coxiella burnetii extract appeared to have no polyclonal effect whatever. Antibody levels to C5 LPS SRBC were not detectable above the low background levels of anti-SRBC antibodies. This particular result is compatible with the report that the anti-protozoal activity of mice injected with a Coxiella burnetii extract is

TABLE 6.1

The serum antibody levels to *Salmonella typhimurium* C5 in F1 mice after pretreatment with either a *Coxiella burnetii* extract or dextran sulphate.

Treatment given to mice[a]	Haemagglutination titre	
	C5 LPS SRBC	SRBC
Control[b]	1/2	1/2
Cox. ext.[c]	1/2	1/2
DS[d]	1/32	1/16

a. 5 mice per group

b. Normal F1 mice

c. 10µl *Coxiella burnetii* extract was injected i.v. (day -21)

d. 1mg dextran sulphate was injected i.v. (day -7).

radio-resistant and therefore is evidently not caused by enhanced production of specific antibody (Clark, 1979).

Comparison of the opsonic activity of serum from normal and *Coxiella burnetii*-treated mice.

The opsonic activity of serum from mice pretreated with the *Coxiella burnetii* extract was investigated in order to more accurately assess whether this treatment had any effect on the antibody levels to *Salmonella typhimurium* C5.

Two groups of 12 normal F1 male mice were injected intraperitoneally with 10^4 *Salmonella typhimurium* C5 which had been opsonized with the serum from either normal or *Coxiella burnetii*-treated mice. Then at 10, 20, 40 and 80 minutes, 3 mice from each group were sacrificed and the number of viable *Salmonella typhimurium* C5 remaining, was determined. The results are presented in Figure 6.1.

The slow clearance of *Salmonella typhimurium* C5 from the peritoneal cavity indicated that both sera contained only low levels of specific opsonins. The treatment with the *Coxiella burnetii* extract again appeared to have no effect on the level of antibody to *Salmonella typhimurium* C5.

The activity of the reticuloendothelial system in F1 mice after the intravenous injection of a *Coxiella burnetii* extract.

Non-viable *Coxiella burnetii* vaccines have been reported to activate guinea pig macrophages and this has been implicated in both antitumour and antibacterial activities (Kelly, 1977).

The present study was done to evaluate the effect of the

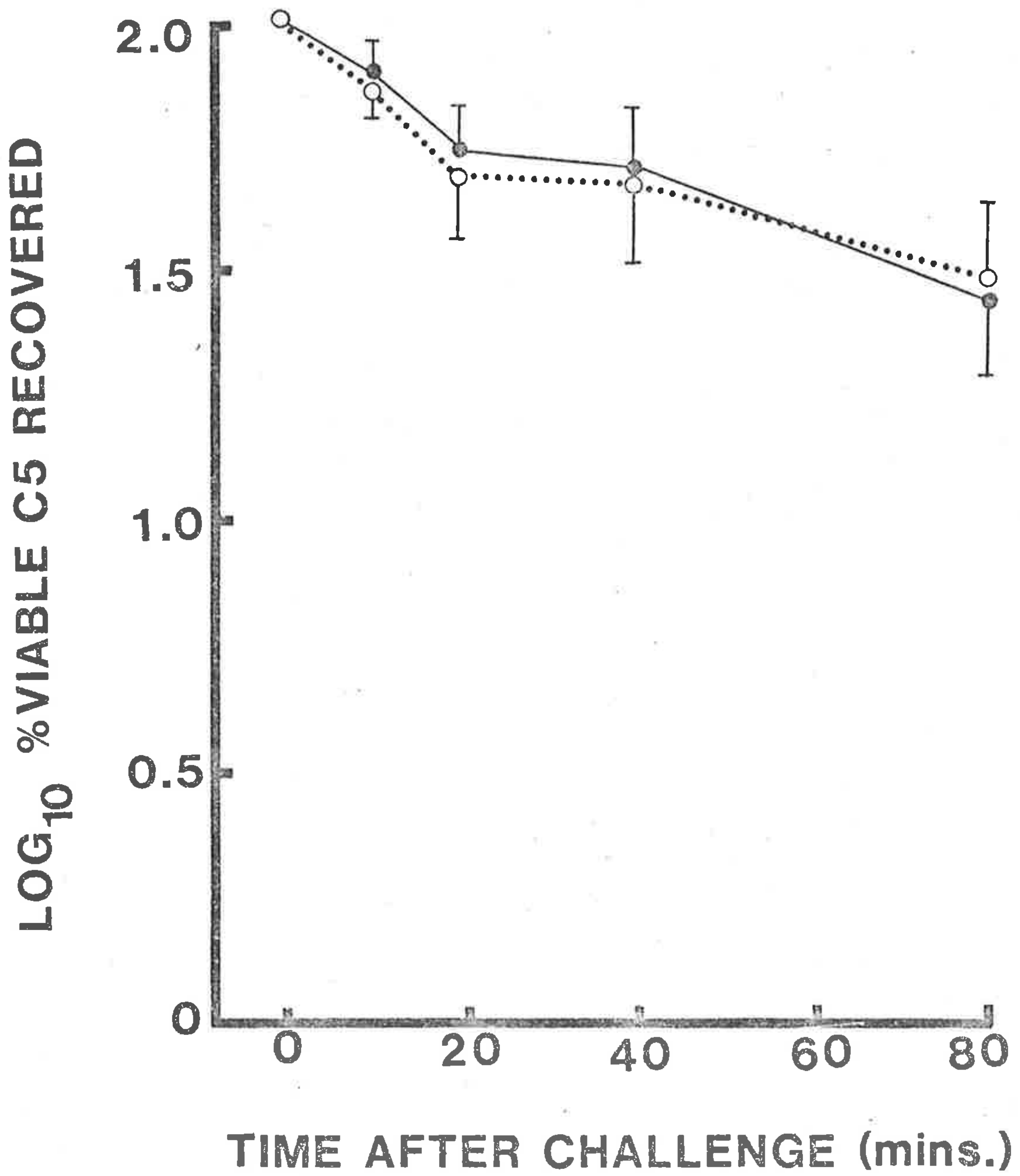
FIGURE 6.1

The clearance of opsonized Salmonella typhimurium C5 from the peritoneal cavity of normal F1 mice. The bacteria were opsonized with the sera from either normal or Coxiella burnetii treated mice.

O••••••••O Serum from normal mice.

●———● Serum from mice pretreated with 10µl Coxiella burnetii extract (day -21).

Each point represents the mean ± S.E. of 3 mice.


2.0
1.5
1.0
0.5
0
LOG10 %VIABLE C5 RECOVERED
0
20
40
60
80
TIME AFTER CHALLENGE (mins.)

Coxiella burnetii extract on the reticuloendothelial system. Monitoring the clearance rate of carbon particles from the circulation was used to assess macrophage activity in the liver and spleen. Forty mice were injected intravenously with 10µl *Coxiella burnetii* extract. At each time point, the rate of carbon clearance was measured in 4 mice, after which they were sacrificed. The results were expressed in terms of a phagocytic index, or K value, and are presented in Figure 6.2.

Considerable activation of the reticuloendothelial system occurred following the administration of the *Coxiella burnetii* extract. Increased clearance rates were observed from day 10 - 30, after which time they appeared to return to normal. Twenty-one days after injecting *Coxiella burnetii* extract the reticuloendothelial system was maximally stimulated and remained so for over a week. In subsequent experiments this time was chosen as being most suitable for investigating the resistance of these mice to both neoplastic and bacterial challenges.

The resistance of mice pretreated with a *Coxiella burnetii* extract to challenge with Ehrlich Ascites Tumour.

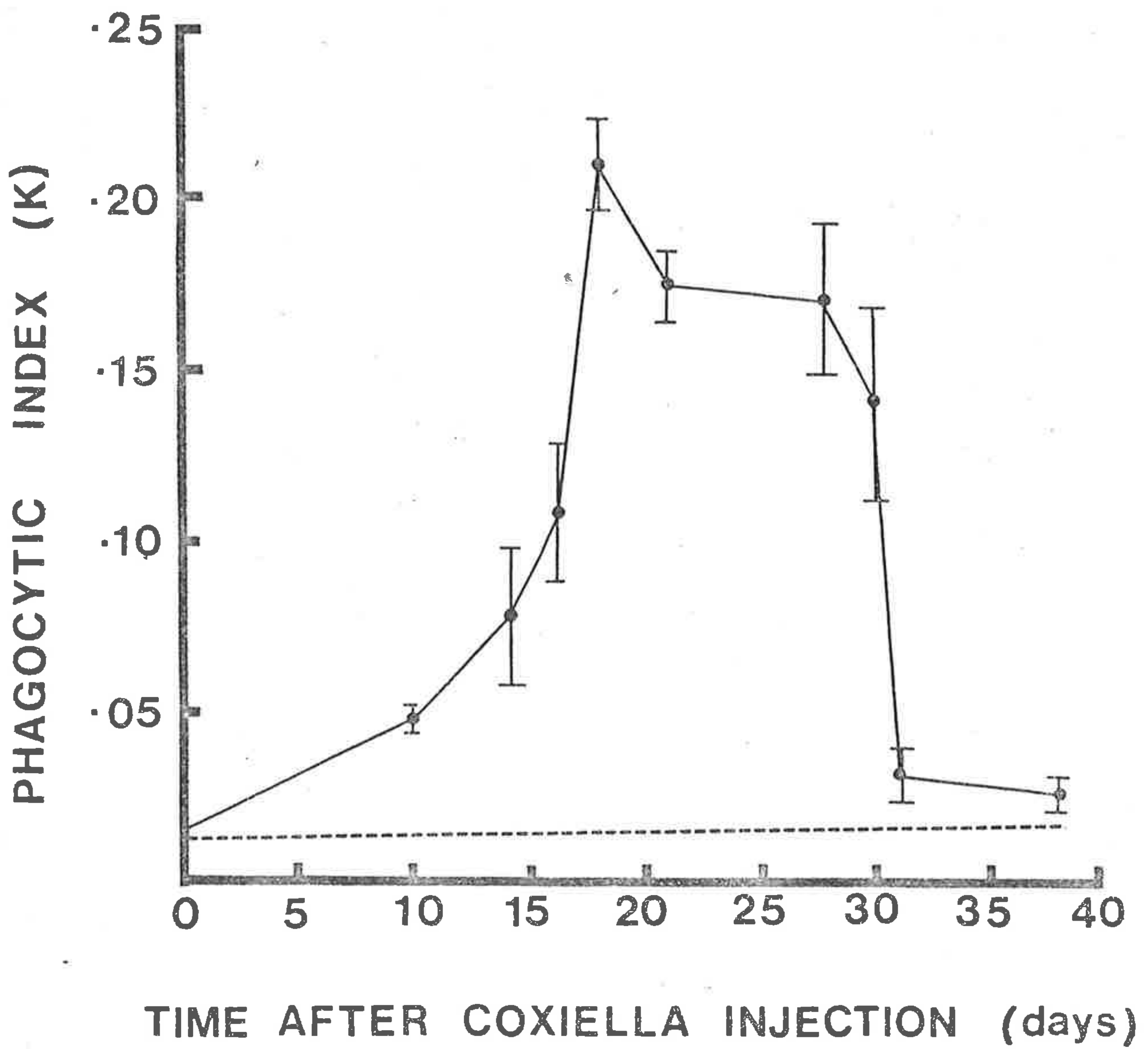
Ehrlich's Ascites Tumour (EAT), is a mammary tumour which can be successfully transplanted into all strains of mice. Normal F1 mice are highly susceptible and when challenged intraperitoneally with 10^6 EAT usually succumb within 20 days. However, *Salmonella enteritidis* 11RX-infected mice are highly resistant to transplanted EAT (Hardy and Kotlarski, 1971; Ashley and Hardy, 1973), and this has been attributed to the acquisition of activated macrophages (La Posta, Ashley and

FIGURE 6.2

The clearance of carbon particles from the circulation was measured in F1 mice which had been injected intravenously with 10μl *Coxiella burnetii* extract on day 0.

●———● *Coxiella burnetii*-treated mice

The dotted line represents the clearance rate found in normal F1 mice. Each point represents the mean ± S.E. of 4 mice.



PHAGOCYTIC INDEX (K)
·25
·20
·15
·10
·05
0
5
10
15
20
25
30
35
40
TIME AFTER COXIELLA INJECTION (days)

Kotlarski,personal communication). If _Coxiella burnetii_ extract could activate the reticuloendothelial system of mice, one would predict that such treatment would confer resistance to transplantable tumours, particularly EAT.

The resistance to Ehrlich Ascites Tumour was evaluated in mice which had been pretreated with _Coxiella burnetii_ extract. The intraperitoneal route was used to both immunize and challenge the mice because EAT only survives in the peritoneum. Groups of F1 male mice received 10µl _Coxiella burnetii_ extract at 1, 2, 3, 4, or 5 weeks prior to challenge with 10^6 EAT. Age-matched controls were also challenged. The survival of the mice was recorded for 90 days and the results are seen in Table 6.2.

While all groups receiving the _Coxiella burnetii_ extract showed some increased resistance, both in terms of survival and mean time to death, maximum resistance was found to occur during the 2 - 4 weeks post-immunization period. This finding that mice pretreated with _Coxiella burnetii_ extract were resistant to EAT was not only interesting, it also supported the carbon clearance data, indicating activation of the reticuloendothelial system.

In a subsequent experiment mice were pretreated with 10µl of either the _Coxiella burnetii_ extract or a control extract 21 days before challenge with EAT. The aim was to determine whether the resistance was specifically induced by a rickettsial component or a substance acquired during the extraction procedure used to prepare the vaccine. The survival was followed for 40 days and the results appear in Figure 6.3.

The manufacturer's control antigen was not at all

TABLE 6.2

The effect of prior treatment with a *Coxiella burnetii* extract on the resistance of mice to challenge with Ehrlich Ascites Tumour.

Treatment[a]	No.Survivors[b]	%Survival[b]	M.T.D.[c]
None (Controls)	0/8	0	20.0
Cox.ext.(day-7)	7/9	57	25.3
Cox.ext.(day-14)	8/11	81	22.0
Cox.ext.(day-21)	10/13	83	28.0
Cox.ext.(day-28)	9/11	75	30.2
Cox.ext.(day-35)	3/8	37	27.8

a. 10μl *Coxiella burnetii* extract was injected intraperitoneally prior to challenge at the times indicated.

b. All mice were injected intraperitoneally with 10^6 EAT on day 0 and their survival was followed for 90 days.

c. Mean time to death (days).

FIGURE 6.3

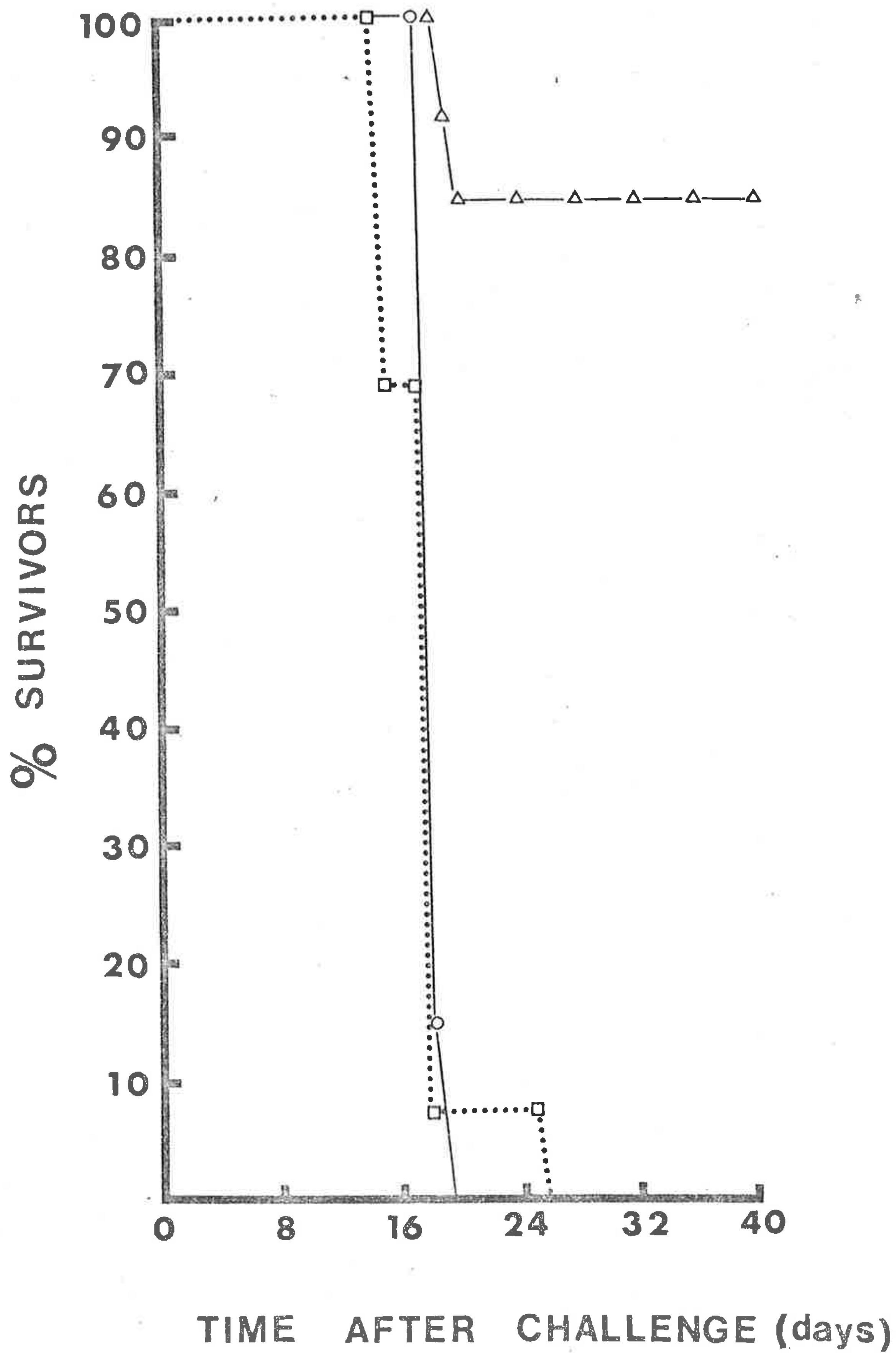
The effect of pretreatment with either a *Coxiella burnetii* extract or a control extract on the survival of F1 mice after an intraperitoneal challenge with 10^6 Ehrlich Ascites Tumour on day 0.

O———O Normal mice

△———△ 10µl *Coxiella burnetii* extract was injected i.p. (day -21)

□··········□ 10µl control extract was injected i.p. (day -21)

Survival was followed for 40 days on groups of 20 mice.



100
90
80
70
60
50
40
30
20
10
% SURVIVORS
0
8
16
24
32
40
TIME AFTER CHALLENGE (days)

protective and so obviously a component of *Coxiella burnetii* is responsible for the protective effects.

The resistance of mice pretreated with a Coxiella burnetii extract to challenge with B16.

The murine melanoma B16, is particularly lethal when transplanted into C57Bl/6J or F1 mice. Complete resistance to the highly metastatic B16 has been found more difficult to achieve than resistance to EAT (Kotlarski and Goh, personal communication). It was decided to investigate what effect intravenous pretreatment with *Coxiella burnetii* extract had on the resistance of mice to intravenous challenge with B16. When injected intravenously via the tail vein, B16 usually grows in the lungs (Kotlarski and Goh, personal communication).

Ten F1 male mice were injected with 10µl *Coxiella burnetii* extract 21 days prior to challenge with 10^5 B16. Age-matched controls were also challenged. The survival of both groups of mice was followed for 40 days.

The results, seen in Figure 6.4, demonstrate that pretreatment with *Coxiella burnetii* extract was beneficial to the mice and prolonged their survival by nearly a week. However, despite some systemic stimulation of the host defences, *Coxiella burnetii* extract was not able to confer complete protection and all mice died within 32 days of challenge. The extent of systemic activation of the reticuloendothelial system is more clearly seen in a subsequent section which examines the resistance of these mice to intravenous challenge with *Listeria monocytogenes*.

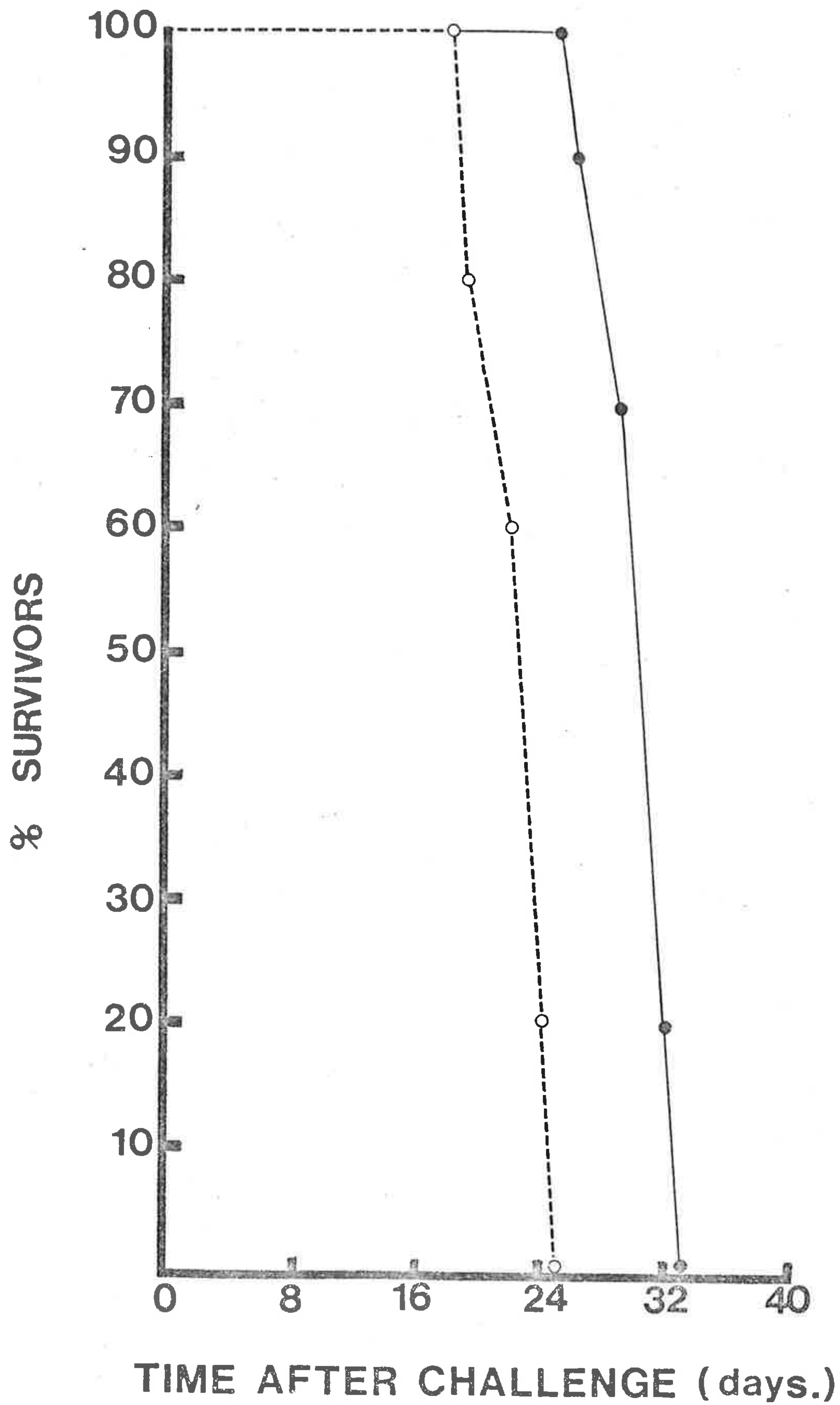
FIGURE 6.4

The effect of pretreatment with a *Coxiella burnetii* extract on the survival of F1 mice after an intravenous challenge with 10^5 B16 on day 0.

○--------○ Normal mice.

●————● 10µl *Coxiella burnetii* extract was injected i.v. (day -21).

Survival was followed for 40 days on groups of 10 mice.



100
90
80
70
60
50
40
30
20
10
% SURVIVORS
0
8
16
24
32
40
TIME AFTER CHALLENGE (days.)

The *in vitro* tumouricidal activity of peritoneal exudate cells from mice pretreated with a *Coxiella burnetii* extract.

Activated macrophages, harvested from animals infected with intracellular bacterial parasites, have the capacity to recognize and destroy neoplastic cells *in vitro* (North, 1978). Previous work from this laboratory established that the peritoneal exudate cells of mice infected with *Salmonella enteritidis* 11RX, were cytotoxic for a variety of tumours, including EAT (Ashley and Hardy, 1973) and P815 (La Posta, personal communication). In this experiment, the *in vitro* tumouricidal activity of peritoneal exudate cells from mice pretreated with a *Coxiella burnetii* extract not only confirmed macrophage activation but also supported the observations made on the *in vivo* resistance of these mice to challenge with EAT.

Groups of 4 F1 female mice received 10µl *Coxiella burnetii* extract intraperitoneally 1, 7, 14 or 21 days prior to the cytotoxicity assay. The peritoneal exudate cells from these and control mice were then assessed for their ability to kill ^{51}Cr labelled EAT and P815 targets.

The peritoneal exudate cells of mice pretreated with a *Coxiella burnetii* extract were cytotoxic for both EAT and P815 (Table 6.3). Maximum tumouricidal activity was observed 7 and 14 days after the pretreatment. Although the per cent cytolysis was low in this assay, these findings have since been independently confirmed and extended in a more detailed study. In that study, the level of cytolysis was found to be greater and comparable with that obtained with peritoneal exudate cells from *Salmonella enteritidis* 11RX-infected mice (La Posta, personal communication).

TABLE 6.3

The *in vitro* cytotoxic activity of peritoneal exudate cells from F1 mice pretreated with a *Coxiella burnetii* extract.

Treatments given to mice[a]	% Cytotoxicity[b]	
	EAT	P815
None (Controls)	-0.5 ±1.0	-2.6±1.4
Cox.ext.(day-1)	3.1 ±2.1	0.6±0.9
Cox.ext.(day-7)	11.6 ±2.8	16.4±0.4
Cox.ext.(day-14)	7.6 ±1.6	14.2±0.6
Cox.ext.(day-21)	0.49±1.6	4.1±2.1

a. 10µl *Coxiella burnetii* extract was injected intraperitoneally into groups of 4 mice at the times indicated

b. Two tumour cell lines, EAT and P815, were labelled with ^{51}Cr and used as targets.

The resistance of mice pretreated with a *Coxiella burnetii* extract to challenge with *Listeria monocytogenes*.

The carbon clearance and tumour resistance data have shown that *Coxiella burnetii* extract stimulates the reticulo-endothelial system. Traditionally the term "activated macrophages" was introduced to describe macrophages with enhanced microbicidal activity from animals with acquired resistance to infection with facultative intracellular bacteria (North, 1978). One such intracellular parasite, *Listeria monocytogenes*, is frequently chosen as a model organism for the study of cell-mediated resistance (Zinkernagel *et al.*, 1977; Cheers and McKenzie, 1978) and macrophage activation (Kelly, 1977; Pederson, Bennedsen, Rhodes and Larsen, 1979). The purpose of this experiment was to evaluate the effect of *Coxiella burnetii* extract on the resistance of mice to *Listeria monocytogenes* and so gain more conclusive evidence of macrophage activation.

One group of 24 F1 male mice was injected intravenously with 10µl *Coxiella burnetii* extract on day -21 while a second group of age-matched controls was set aside. All mice were challenged intravenously with 10^6 *Listeria monocytogenes* on day 0. The survival of the mice was followed for 24 days and the results are seen in Figure 6.5.

Pretreatment with the *Coxiella burnetii* extract conferred resistance to challenge with *Listeria monocytogenes*. As an additional observation, these mice showed no visible signs of disease and it is not beyond possibilities that the 2 mice which died did so from non-listerial causes, particularly as these mice are prone to fighting. Unfortunately post mortems were impractical, and so the exact cause of death is not known.

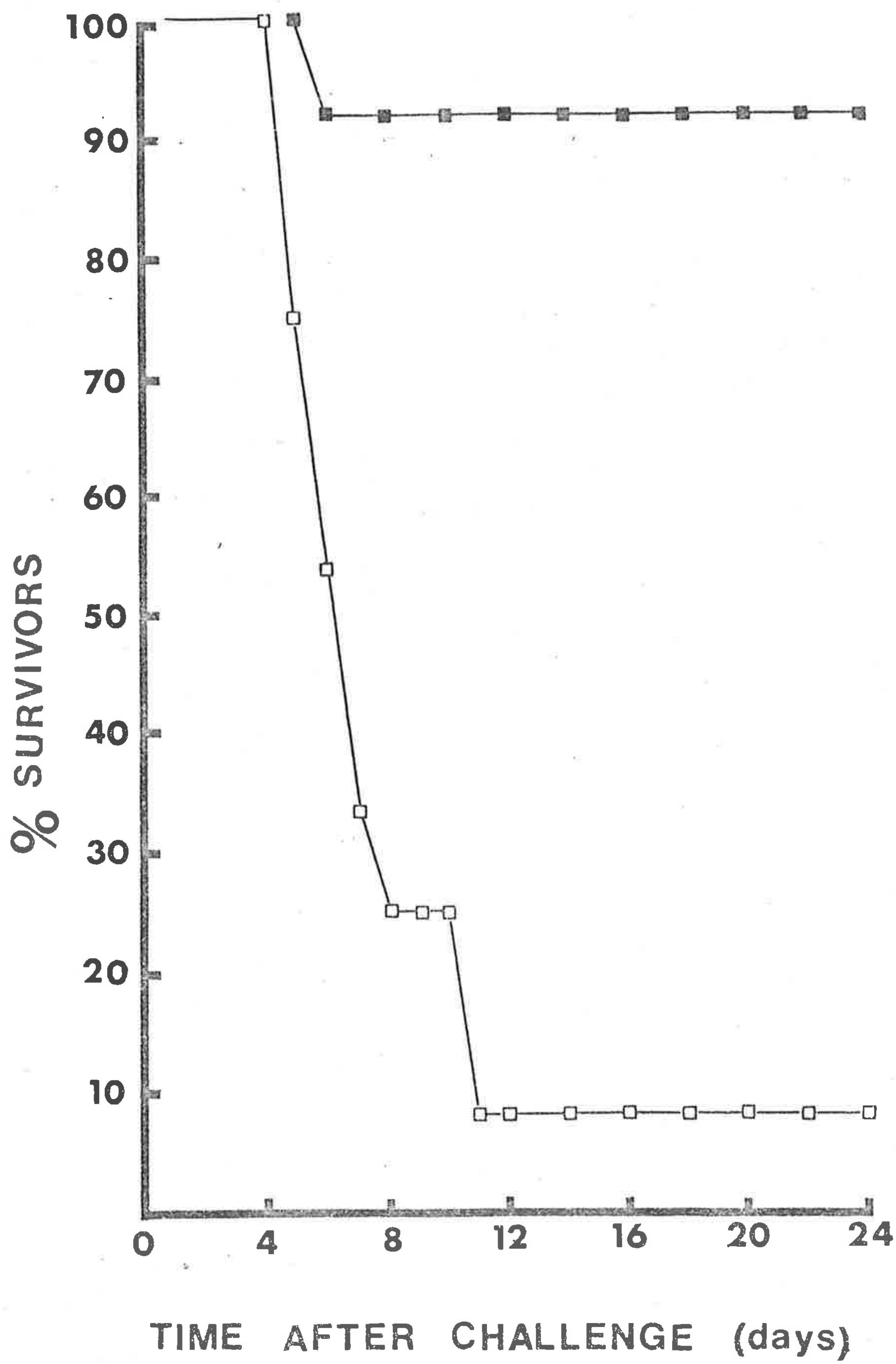
FIGURE 6.5

The effect of pretreatment with a *Coxiella burnetii* extract on the survival of F1 mice after an intravenous challenge with 10^6 *Listeria monocytogenes* on day 0.

□———□ Normal mice

■———■ 10μl *Coxiella burnetii* extract was injected i.v. (day -21).

Survival was followed for 24 days on groups of 24 mice.



100
90
80
70
60
50
40
30
20
10
% SURVIVORS
0
4
8
12
16
20
24
TIME AFTER CHALLENGE (days)

In contrast, all the normal mice were susceptible and quickly succumbed to the listerial challenge. Only 2 mice managed to survive and they may have inadvertently received a slightly lower challenge inoculum. The ability of the *Coxiella burnetii* extract to confer resistance to *Listeria monocytogenes* as well as Ehrlich Ascites Tumour is convincing evidence that it is a potent immunopotentiating agent capable of stimulating the reticuloendothelial system.

The effect of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine and a *Coxiella burnetii* extract on the survival of F1 mice after challenge with *Salmonella typhimurium* C5

Having established that the *Coxiella burnetii* extract was able to activate the reticuloendothelial system, its prophylactic value against infection with *Salmonella typhimurium* C5 was then assessed. Secondly, because the *Coxiella burnetii* extract had no apparent effect on the level of humoral antibody specific for *Salmonella typhimurium* C5, it appeared to be a useful tool in delineating the role of antibody in resistance to this infection. Previous studies have demonstrated, on the basis of survival, that killed vaccines or the passive transfer of antibody, specific for a particular strain of Salmonella, would protect mice against an homologous challenge (Jenkin and Rowley, 1963; 1965; Kenny and Herzberg, 1968; Venneman and Berry, 1971 a & b). However, these procedures were not able to induce effective protection when the assessment was made on the basis of ability to control the multiplication of the challenge organism. These

observations were consistent with the conclusion that anti-Salmonella immunity was cellular in nature (Blanden, Mackaness and Collins, 1966; Mackaness, Blanden and Collins, 1966). Much of the debate regarding the role of antibody in protection to Salmonella infections has arisen because the criteria for the assessment of effective protection have varied. Therefore, when discussing the efficacy of the *Coxiella burnetii* extract, or the role of antibody, it was important to consider any increase (or decrease) in the survival of immunized mice given an appropriate challenge inoculum and in the growth rate of the challenge inoculum in putatively immune and control animals. The latter aspect will be considered following studies dealing with the former aspect.

F1 mice were immunized with the *Coxiella burnetii* extract to stimulate the reticuloendothelial system and with an alcohol-killed *Salmonella typhimurium* C5 vaccine, to generate high levels of specific antibody (Davies, 1975). The degree of protection afforded by either vaccine alone or in combination was assessed in terms of the survival of the mice after challenge with *Salmonella typhimurium* C5. In 4 separate experiments, the route of administration and the dose of challenge were varied. The groups of mice studied, the treatments they received, the challenge doses given and the survival observed are shown in Tables 6.4, 6.5, 6.6 and 6.7.

Experiment 1.

Groups of 20 F1 male mice received 10µl *Coxiella burnetii* extract, or 10µg alcohol-killed *Salmonella typhimurium* C5 vaccine,or both, intraperitoneally 21 days prior to challenge

TABLE 6.4

The effect of immunization with an alcohol-killed Salmonella vaccine and a Coxiella extract on the resistance of F1 mice to challenge with *Salmonella typhimurium* C5.

Treatments given to mice[a]	% Survival[b]	M.T.D.[c]
None (Controls)	0	5.0
Cox. ext.[d]	0	5.6
EtOH-C5[e]	0	6.5
Cox.ext.and EtOH-C5[f]	75	12.6

a. All groups of 20 mice were challenged with 1.3×10^4 C5 i.p. (day 0)

b. Survival at 28 days after challenge

c. Mean time to death (days)

d. 10µl *Coxiella burnetii* extract was injected i.p. (day -21)

e. 10µg alcohol-killed *Salmonella typhimurium* C5 was injected i.p. (day -21)

f. 10µl *Coxiella burnetii* extract and 10µg alcohol-killed *Salmonella typhimurium* C5 were both injected i.p. (day -21).

and age-matched controls were set aside. All mice were challenged intraperitoneally with 1.3×10^4 Salmonella typhimurium C5 and their survival at 28 days was recorded in Table 6.4.

The normal mice were susceptible to the Salmonella typhimurium C5 infection, as were mice pretreated with either the Coxiella burnetii extract or the alcohol-killed Salmonella typhimurium C5 vaccine alone. However, the mice which had received both vaccines were highly resistant, and even the 5 mice which died had a significantly longer mean time to death.

Apparently the generation of both humoral and cellular immune responses were necessary for resistance.

Experiment 2.

The second experiment was similar to the first in that groups of 20 F1 male mice received 10µl Coxiella burnetii extract, or 10µg alcohol-killed Salmonella typhimurium C5 vaccine, or both, intraperitoneally 21 days prior to challenge and age-matched controls were set aside. However, the intraperitoneal challenge dose was increased to 1.7×10^6 Salmonella typhimurium C5. The survival of the mice was followed for 28 days and the results are seen in Table 6.5.

Normal mice quickly succumbed to the higher challenge dose, as did the mice pretreated with either vaccine alone. The group of mice which had received both vaccines, was still highly resistant to the Salmonella typhimurium C5 infection.

Taken together, the results of both these experiments suggested that the Coxiella burnetii extract was protective against intraperitoneal challenge with Salmonella

TABLE 6.5

The effect of immunization with an alcohol-killed Salmonella vaccine and a Coxiella extract on the resistance of F1 mice to challenge with *Salmonella typhimurium* C5.

Treatments given to mice[a]	% Survival[b]	M.T.D.[c]
None (Controls)	0	4.0
Cox. ext.[d]	0	5.7
EtOH-C5[e]	0	5.4
Cox.ext.and EtOH-C5[f]	80	14.2

a. All groups of 20 mice were challenged with 1.7×10^6 C5 i.p. (day 0)

b. Survival at 28 days after challenge

c. Mean time to death (days)

d. 10µl *Coxiella burnetii* extract was injected i.p. (day -21)

e. 10µg alcohol-killed *Salmonella typhimurium* C5 was injected i.p. (day -21)

f. 10µl *Coxiella burnetii* extract and 10µg alcohol-killed *Salmonella typhimurium* C5 were both injected i.p. (day -21).

typhimurium C5, but only in combination with an alcohol-killed Salmonella typhimurium C5 vaccine. What was a little surprising was the fact that the resistance of these mice had not diminished with the higher challenge, and this aspect will be discussed later.

In the two remaining experiments, resistance to intravenous rather than intraperitoneal challenge with Salmonella typhimurium C5 was investigated.

Experiment 3.

Groups of 24 F1 male mice received 10µl Coxiella burnetii extract, or 10µg alcohol-killed Salmonella typhimurium C5 vaccine, or both, intravenously 21 days prior to challenge and age-matched controls were set aside. All mice were challenged intravenously with 2.8×10^4 Salmonella typhimurium C5 and their survival at 28 days is shown in Table 6.6.

As expected, normal mice were susceptible, as were the mice which had received either vaccine alone. However, the mice which had received both vaccines were resistant. The generation of both humoral and cellular immune responses was again necessary for resistance, although the level of protection was far from absolute.

Experiment 4.

The final experiment was similar to the third in that groups of 13 F1 male mice received 10µl Coxiella burnetii extract, or 10µg alcohol-killed Salmonella typhimurium C5 vaccine, or both, 21 days prior to challenge. The intravenous

TABLE 6.6

The effect of immunization with an alcohol-killed Salmonella vaccine and a Coxiella extract on the resistance of F1 mice to challenge with *Salmonella typhimurium* C5.

Treatments given to mice[a]	% Survival[b]	M.T.D.[c]
None (Controls)	0	7.0
Cox.ext.[d]	0	9.3
EtOH-C5[e]	0	7.1
Cox.ext.and EtOH-C5[f]	62.5	10.5

a. All groups of 24 mice were challenged with 2.8×10^4 C5 i.v. (day 0)

b. Survival at 28 days after challenge

c. Mean time to death (days)

d. 10µl *Coxiella burnetii* extract was injected i.v. (day -21)

e. 10µg alcohol-killed *Salmonella typhimurium* C5 was injected i.v. (day -21)

f. 10µl *Coxiella burnetii* extract and 10µg alcohol-killed *Salmonella typhimurium* C5 were both injected i.v. (day -21)

challenge dose was increased to 2.4 x 10^6 *Salmonella typhimurium* C5, the survival of the mice was followed for 28 days and the results are seen in Table 6.7.

Normal mice quickly succumbed to the higher challenge dose, as did mice pretreated with either vaccine alone. The group of mice which had received both vaccines was still relatively resistant to the *Salmonella typhimurium* C5.

Considering the results of this and the other 3 experiments, the *Coxiella burnetii* extract was protective against both intraperitoneal and intravenous challenge with *Salmonella typhimurium* C5, but only in combination with an alcohol-killed *Salmonella typhimurium* C5 vaccine. This dependence of resistance upon prior immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine implied that antibody was important for the full expression of cell-mediated immunity, which had been generated as a consequence of the treatment with the *Coxiella burnetii* extract. Immunization with the alcohol-killed *Salmonella typhimurium* C5 vaccine alone was insufficient for resistance and usually only slightly increased the mean time to death. These results support the proposal that the mechanism of immunity in salmonellosis is 'humoral plus cellular' (Rowley, Auzins and Jenkin, 1968; Kenny and Herzberg, 1968), rather than purely cellular (Mackaness, 1962).

One noticeable feature of all 4 experiments was that the protection generated in mice treated with the *Coxiella burnetii* extract was not absolute. The choice of 10μl of *Coxiella burnetii* extract, used in this study, was based on the observations of Clark (1979) that this was the minimum effective dose necessary to protect mice against challenge with

TABLE 6.7

The effect of immunization with an alcohol-killed Salmonella vaccine and a Coxiella extract on the resistance of F1 mice to challenge with *Salmonella typhimurium* C5.

Treatments given to mice[a]	% Survival[b]	M.T.D.[c]
None (Controls)	0	3.4
Cox. ext.[d]	0	6.3
EtOH-C5[e]	0	5.3
Cox.ext.and EtOH-C5[f]	61.5	11.0

a. All groups of 13 mice were challenged with 2.4 x 10^6 C5 i.v. (day 0)

b. Survival at 28 days after challenge

c. Mean time to death (days)

d. 10µl *Coxiella burnetii* extract was injected i.v. (day -21)

e. 10µg alcohol-killed *Salmonella typhimurium* C5 was injected i.v. (day -21)

f. 10µl *Coxiella burnetii* extract and 10µg alcohol-killed *Salmonella typhimurium* C5 were both injected i.v. (day -21).

Babesia microti. However, when challenging mice with the lethal *Babesia rodhaini* or *Plasmodium vinckei petteri* he routinely used 200µl of extract. Perhaps the level of protection to *Salmonella typhimurium* C5 would be increased if a greater quantity of extract was used, although other factors, such as the time of challenge, may also have an effect.

Although comparisons between separate experiments should be treated cautiously, the level of protection observed in the doubly vaccinated mice, to the larger challenge was not markedly different from that observed to the lower challenge, despite the one-hundred-fold difference in the size of the inoculum. As this occurred when both the intraperitoneal and intravenous routes were used, it is tempting to speculate that in this system, the size of the inoculum does not have a great bearing on the eventual survival of the mice. Efficient humoral and cell-mediated immune responses at the time of challenge may initially be able to control most infecting inocula. Perhaps later, during the carrier state, when the effect of the *Coxiella burnetii* extract is beginning to wane, other factors may determine whether small numbers of bacteria in various infective foci, may begin to multiply and become a serious problem to some mice.

Another implication of these experiments is that greater resistance resulted, in the doubly immunized mice, when the intraperitoneal rather than the intravenous route of administration was used. This finding is in general agreement with earlier studies which have established that the route of challenge influences immunity to intracellular parasites (Blanden, Mackaness and Collins, 1966; Collins, 1968, 1969b). Their studies demonstrated that immunized mice were more

resistant to intraperitoneal rather than intravenous challenge with *Salmonella enteritidis*. The reason for this was that the antibody in the peritoneum reduced not only the viability of the inoculum, but also the rate of dissemination of bacteria to other organs. It is likely that this is also responsible for my observations.

The effect of pretreatment with a *Coxiella burnetii* extract and the passive transfer of antibody specific for *Salmonella typhimurium* C5 on the ability of F1 mice to control the growth of a *Salmonella typhimurium* C5 challenge.

The immunization of mice with both the alcohol-killed *Salmonella typhimurium* C5 vaccine and the *Coxiella burnetii* extract clearly conferred protection against infection with *Salmonella typhimurium* C5. In the following 2 experiments, the efficacy of the *Coxiella burnetii* extract and the role of antibody were assessed by following the fate of *Salmonella typhimurium* C5 during the first few days of this infection. The importance of the growth rate of the challenge organisms as a criterion for resistance, has already been discussed. Antibody was introduced by passively transferring serum specific for *Salmonella typhimurium* C5 to infected mice during the period of study, rather than by direct immunization as was used previously. This serum was obtained from mice which had been immunized with an alcohol-killed *Salmonella typhimurium* C5 vaccine and its titre to C5 LPS SRBC was 1/512 (Chapter 4).

The design of the experiment was as follows. Ten μl of *Coxiella burnetii* extract were injected intravenously into mice 21 days prior to challenge, while 0.2ml of antiserum was

FIGURE 6.6

The effect of pretreatment with a *Coxiella burnetii* extract and the passive transfer of antibody specific for *Salmonella typhimurium* C5 on the ability of F1 mice to control the growth of 1.0×10^4 *Salmonella typhimurium* C5 injected intravenously on day 0.

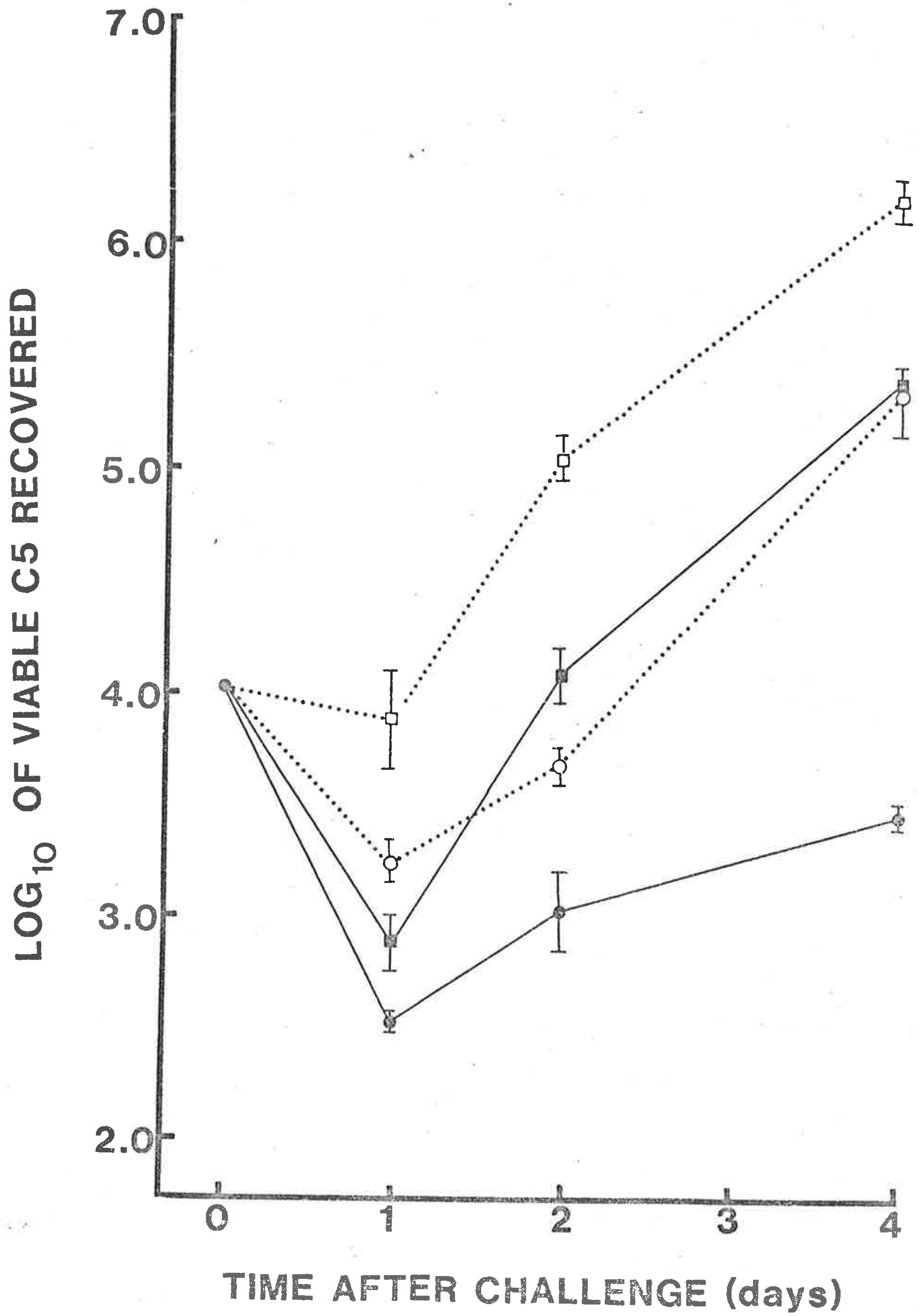
□··········□ C5 challenge only

○··········○ 10μl *Coxiella burnetii* extract i.v. (day -21) and C5 challenge

■——————■ Passive antibody and C5 challenge

●——————● 10μl *Coxiella burnetii* extract i.v. (day -21), passive antibody and C5 challenge.

The antiserum (0.2ml) was administered intravenously immediately prior to challenge and twice daily thereafter. It was obtained from mice immunized with an alcohol-killed C5 vaccine.
The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen, while very few were recovered from the peritoneal cavity.
Each point represents the geometric mean $\pm$ S.E. of the total number of C5 recovered from 4 mice.



7.0
6.0
5.0
4.0
3.0
2.0
LOG10 OF VIABLE C5 RECOVERED
0
1
2
3
4
TIME AFTER CHALLENGE (days)

injected immediately prior to challenge and twice daily thereafter. Groups of 12 F1 male mice received either treatment alone, or a combination of both, or none. The fate of the 2 challenge doses of *Salmonella typhimurium* C5 in these mice is shown in Figures 6.6 and 6.7.

Experiment 1, using 1.0×10^4 C5 as the intravenous challenge dose.

The growth rate of *Salmonella typhimurium* C5 in normal mice was rapid. However, despite an initial reduction of the challenge inoculum, the growth rate of the challenge organisms in mice receiving only the *Coxiella burnetii* extract or the antiserum, paralleled that observed in normal mice. Not only was the greatest initial reduction in the challenge inoculum observed in mice receiving both treatments, but they were also the only group able to control the growth rate of the challenge. After 4 days there were approximately 100 - 1,000-fold fewer bacteria in these mice than in any of the other groups (Figure 6.6).

Hence, in mice, the *Coxiella burnetii* extract was able to confer the ability to control the growth of a *Salmonella typhimurium* C5 challenge, but only in the presence of antibody. In the following experiment, the degree of this resistance was assessed by increasing the size of the challenge inoculum.

Experiment 2, using 1.6×10^6 C5 as the intravenous challenge dose.

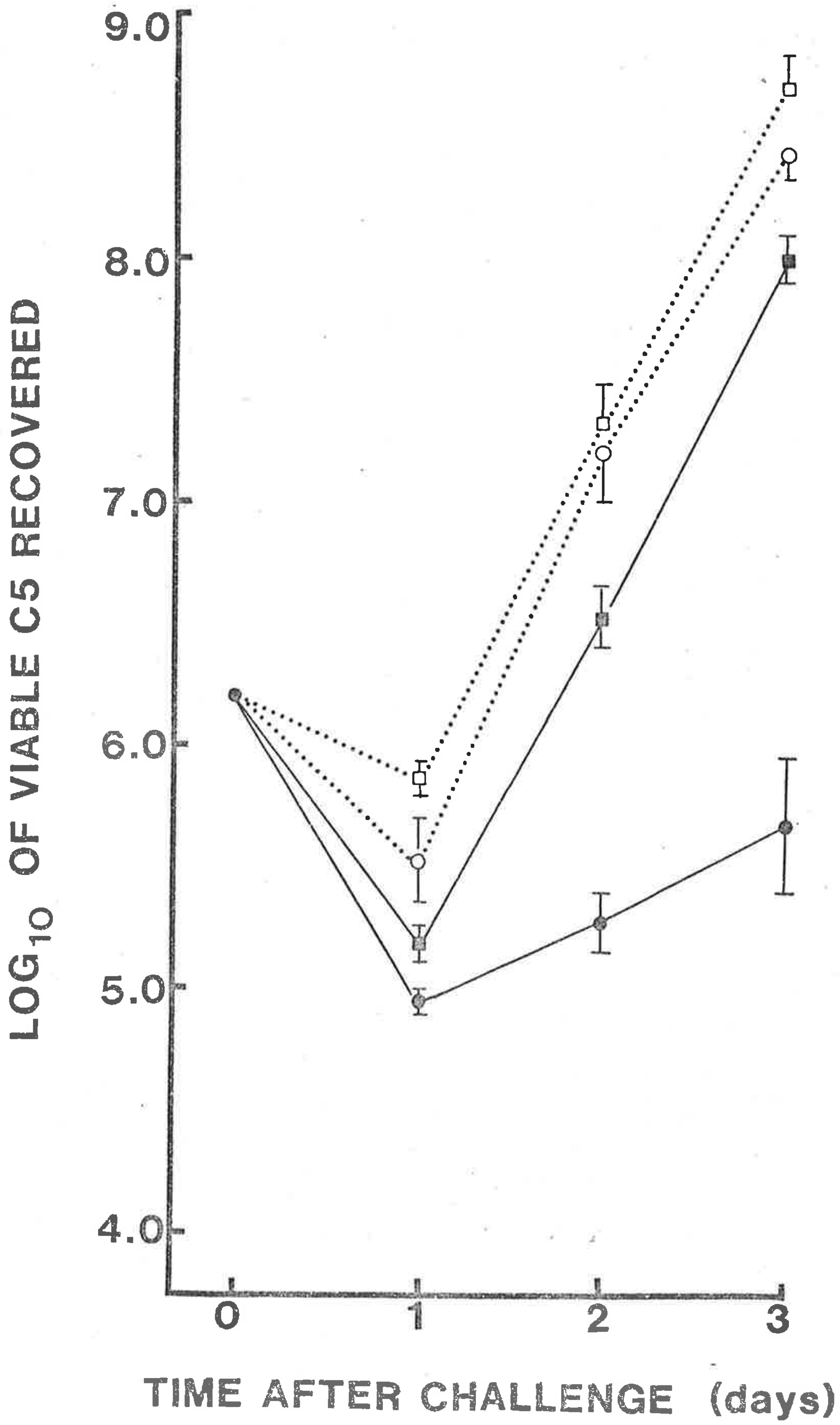
The group of mice which had received both the *Coxiella*

FIGURE 6.7

The effect of pretreatment with a *Coxiella burnetii* extract and the passive transfer of antibody specific for *Salmonella typhimurium* C5 on the ability of F1 mice to control the growth of 1.6×10^6 *Salmonella typhimurium* C5 injected intravenously on day 0.

□·············□	C5 challenge only
○·············○	10µl *Coxiella burnetii* extract i.v. (day -21) and C5 challenge
■————■	Passive antibody and C5 challenge
●————●	10µl *Coxiella burnetii* extract i.v. (day -21), passive antibody and C5 challenge.

The antiserum (0.2ml) was administered i.v. immediately prior to challenge and twice daily thereafter. It was obtained from mice immunized with an alcohol-killed C5 vaccine.
The number of viable C5 in the liver, spleen and peritoneal cavity of each mouse was determined. The majority of bacteria were found in the liver and spleen, while very few were recovered from the peritoneal cavity.
Each point represents the geometric mean $\pm$ S.E. of the total number of C5 recovered from 4 mice.



9.0
8.0
7.0
6.0
5.0
4.0
LOG$_{10}$ OF VIABLE C5 RECOVERED
0
1
2
3
TIME AFTER CHALLENGE (days)

burnetii extract and the passive antibody, was able to control even this large challenge dose of Salmonella typhimurium C5. Again, this was the only group able to do so. The growth rate of organisms in mice receiving either treatment alone, was very rapid and was almost identical to that observed in normal mice. The rapid growth rate seen with the higher challenge dose (Figure 6.7) would account for the early deaths found when the survival was investigated (Table 6.7).

The results of these 2 experiments demonstrated that a combination of Coxiella burnetii extract and passive antibody was able to confer on mice the ability to control the growth of a Salmonella typhimurium C5 challenge. Either treatment alone was relatively ineffective. While the doubly treated mice were able to limit the growth of the challenge organisms, they were not able to eliminate them during the period of study. This may be due to insufficient Coxiella burnetii extract and the reasons for this have already been discussed. The small quantity of antibody transferred may also have limited the expression of resistance.

The studies with the Coxiella burnetii extract implied that the generation of both humoral and cellular immune responses was necessary for resistance to Salmonella typhimurium C5. This being the case, a substance, such as dextran sulphate, which supposedly affects both of these immune responses, may confer resistance to this infection. In the remaining experiments the efficacy of this polyanion was investigated.

<u>The activity of the reticuloendothelial system in F1 mice after the intravenous injection of dextran sulphate</u>

A number of studies have demonstrated that dextran sulphate is capable of potentiating both humoral (Diamantstein *et al*., 1971 a & b) and cell-mediated (McCarthy, Arnold and Babcock, 1977) immune responses. However, as has already been discussed, this polyanion was able to produce a complex spectrum of effects depending on the individual experimental conditions (L'Age-Stehr and Diamantstein, 1977; McCarthy and Babcock, 1978) and this is probably due to its effects on the activity of macrophages (Hart and Young, 1975; Goren *et al*., 1976). For this reason the effect of dextran sulphate on the activity of the reticuloendothelial system was assessed by monitoring the rate of clearance of carbon particles from the circulation.

Forty five F1 female mice were injected intravenously with lmg dextran sulphate and then the rate of carbon clearance was measured daily in 5 of these mice, after which they were sacrificed. Five age-matched controls were also assessed for their ability to clear an intravenous injection of carbon particles. The results, expressed in terms of a phagocytic index or K value, are shown in Figure 6.8.

Following the injection of dextran sulphate, the phagocytic index of mice fluctuated during the 9 day study. The clearance rate in mice 1 and 2 days after treatment was less than that in control mice, and from 3 to 9 days after treatment, the clearance rate exceeded that in control mice, the peak occurring on day 7. These observations imply that the dextran sulphate initially suppresses and then stimulates the reticuloendothelial system. However, as can be seen from a

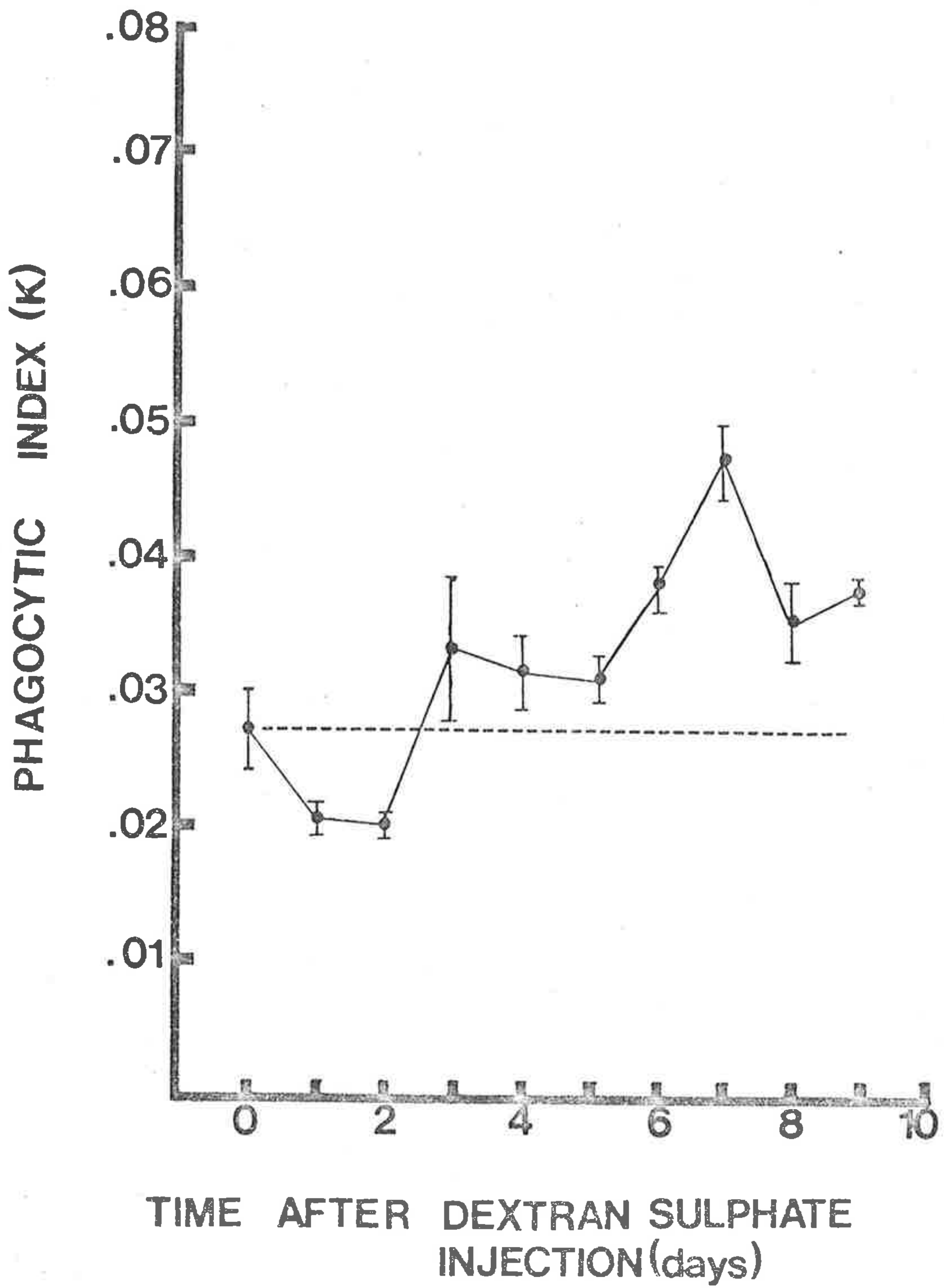
FIGURE 6.8

The clearance of carbon particles from the circulation was measured in F1 mice which had been injected intravenously with 1mg dextran sulphate on day 0.

●————● dextran sulphate-treated mice

The dotted line represents the clearance rate found in normal F1 mice.

Each point represents the mean $\pm$ S.E. of 5 mice.



PHAGOCYTIC INDEX (K)
.08
.07
.06
.05
.04
.03
.02
.01
0
2
4
6
8
10
TIME AFTER DEXTRAN SULPHATE INJECTION (days)

TABLE 6.8

The effect of pretreatment with dextran sulphate on the ability of F1 mice to clear carbon particles from their bloodstream. The phagocytic indices obtained with other agents have been included for comparison.

Treatments	Phagocytic Index[a]
None (Controls)	0.027 ± 0.007
D.S. (day -1)[b]	0.020 ± 0.001
D.S. (day -7)[b]	0.047 ± 0.003
11RX[c]	0.085 ± 0.004
Listeria[d]	0.077 ± 0.005
Cox. ext.[e]	0.173 ± 0.010

a. Each phagocytic index represents the mean ± S.E.of 5 mice

b. 1mg dextran sulphate was injected i.v. (day -1 or -7)

c. 10^5 *Salmonella enteritidis* 11RX were injected i.v. (day -11)

d. 10^4 *Listeria monocytogenes* were injected i.v. (day -7)

e. 10µl *Coxiella burnetii* extract was injected i.v. (day -21).

summary of the data (Table 6.8), these effects of dextran sulphate are minimal, especially when compared to those of other agents studied, such as *Salmonella enteritidis* 11RX, *Listeria monocytogenes* and *Coxiella burnetii* extract. Confirming the relatively low level of reticuloendothelial stimulation in mice treated 7 days previously with 1mg dextran sulphate, is the observation that such mice are susceptible to an intravenous challenge of 10^6 *Listeria monocytogenes* (data not shown). In comparison, mice immunized with *Salmonella enteritidis* 11RX, *Listeria monocytogenes* or *Coxiella burnetii* extract are highly resistant to this organism.

Seven days after the intravenous administration of 1mg dextran sulphate, mice had both an increased level of serum antibodies to *Salmonella typhimurium* C5 LPS (Table 6.1) and a slightly stimulated reticuloendothelial system (Figure 6.8, Table 6.8). The resistance of these mice to *Salmonella typhimurium* C5 was therefore assessed.

The effect of treatment with dextran sulphate on the survival of F1 mice after challenge with *Salmonella typhimurium* C5

In this, the final experiment, groups of 33 F1 male mice were injected intravenously with 1mg dextran sulphate 1 or 7 days prior to challenge, while age-matched controls were set aside. All mice were challenged intravenously with 1.4 x 10^4 *Salmonella typhimurium* C5 and their survival was followed for 28 days.

The results obtained (Table 6.9) demonstrated that treatment with dextran sulphate 7 days before challenge conferred resistance to challenge with *Salmonella*

TABLE 6.9

The effect of pretreatment with dextran sulphate on the resistance of F1 mice to challenge with *Salmonella typhimurium* C5.

Treatments given to mice[a]	% Survival[b]	M.T.D.[c]
None (Controls)	0	7.9
D.S. (day -1)[d]	0	5.0
D.S. (day -7)[d]	81.8	11.8

a. All groups of 33 mice were challenged with 1.4×10^4 C5 i.v. (day 0)

b. Survival at 28 days after challenge

c. Mean time to death (days)

d. 1mg dextran sulphate was injected i.v.(day -1 or -7).

typhimurium C5. However, mice treated with dextran sulphate 1 day before challenge were definitely not resistant and in fact died significantly earlier than the control mice.

This pattern of resistance paralleled the carbon clearance rates. Presumably the dextran sulphate initially inhibited macrophage activity, reducing the ability of the mice to clear carbon particles from their circulation and rendering them acutely susceptible to infection with *Salmonella typhimurium* C5. With the passage of time, the reticuloendothelial system became stimulated and the mice were resistant to infection with *Salmonella typhimurium* C5. The level of resistance to infection with *Salmonella typhimurium* C5 was surprising, considering the minimal nature of reticuloendothelial stimulation and the susceptibility of these mice to *Listeria monocytogenes*.

It is, therefore, tempting to suggest that the resistance observed was mainly due to the ability of dextran sulphate to enhance the rate of induction and/or degree of immunity which is normally induced by the challenge inoculum itself i.e. that the dextran sulphate was acting as an adjuvant. An analysis of the mechanism by which dextran sulphate induced resistance to *Salmonella typhimurium* C5 was outside the scope of this thesis, and so remains to be elucidated. The important point was that resistance to *Salmonella typhimurium* C5 was observed in mice pretreated with dextran sulphate and this complemented the study with the *Coxiella burnetii* extract.

Summary and Conclusions

The main objective of this study was to assess the

prophylactic value of a commercial *Coxiella burnetii* extract and dextran sulphate against infection with *Salmonella typhimurium* C5. The results not only demonstrated that both materials could induce resistance, they also highlighted the importance of antibody in resistance to this infection.

The *Coxiella burnetii* extract was shown to stimulate the reticuloendothelial system (Figure 6.2), to induce resistance to tumours (Table 6.2, Figures 6.3, 6.4) and to induce resistance to *Listeria monocytogenes* (Figure 6.5). Presumably all of these effects are related and can be attributed to the acquisition of activated macrophages in these animals. The demonstration of tumouricidal peritoneal exudate cells *in vitro* confirmed the existence of activated macrophages in these animals (Table 6.3).

In addition to these findings, the *Coxiella burnetii* extract was found to have no detectable effect on the level of humoral antibodies specific for *Salmonella typhimurium* C5 (Table 6.1, Figure 6.1). This latter property made it an ideal tool for evaluating the role of antibody in resistance to *Salmonella typhimurium* C5. The presence of specific antibody in mice pretreated with a *Coxiella burnetii* extract was critical for their resistance to this infection. Specific antibody was either administered passively (Figures 6.6, 6.7), or generated as a consequence of immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine (Tables 6.4, 6.5, 6.6 and 6.7).

In comparison, dextran sulphate was found to enhance the level of serum antibody specific for *Salmonella typhimurium* C5 LPS (Table 6.1). The dextran sulphate was also capable of stimulating the reticuloendothelial system, although not to

the same degree as the Coxiella burnetii extract (Table 6.8). However such mice are highly resistant to challenge with Salmonella typhimurium C5 (Table 6.9).

In conclusion, the generation of both humoral and cellular immune responses was necessary for resistance to Salmonella typhimurium C5. Specific antibody was found to be of critical importance in the expression of cell-mediated immunity against this intracellular parasite.

CHAPTER 7

DISCUSSION

Introduction

Although the conventional killed typhoid vaccines are able to induce specific humoral immune responses, they have achieved only limited success in protecting humans against infection with *Salmonella typhi*. The inability of these vaccines to generate cell-mediated immune responses has been suggested as one of the reasons for their failure (Nath *et al*., 1977). No doubt the efficacy of prophylactic vaccination will be improved only with a greater understanding of the pathogenesis of the disease and of the host defence mechanisms.

The infection of mice with either *Salmonella enteritidis* or *Salmonella typhimurium* has, as discussed in detail in Chapter 1, proved to be the most useful experimental model of enteric fever. While the mechanism of immunity to these intracellular bacterial parasites has been the subject of much vigorous debate, it unfortunately remains poorly understood. Briefly, several workers, finding that resistance to infection with Salmonellae was dependent on the acquisition of macrophages with increased bactericidal activity, suggested that the mechanism of immunity was cellular rather than humoral in nature (Ushiba *et al*, 1959; Howard, 1961; Blanden, Mackaness and Collins, 1966). However, this conclusion is controversial and, as previously discussed, the involvement of antibody in these studies is difficult to dismiss. For instance, antibody

generated in response to mitogenic or cross-reacting antigenic determinants in the living bacterial vaccine, may well participate in the expression of what often appears to be non-specific antibacterial resistance. In comparison, others have observed that specific antibody promoted the phagocytosis and killing of *Salmonella typhimurium in vitro* (Jenkin, 1963; McIntyre, Jenkin and Rowley, 1967) and also conferred a significant level of protection against salmonellosis *in vivo* (Rowley, Auzins and Jenkin, 1968; Kenny and Herzberg, 1968; Venneman and Berry, 1971b). They concluded that both humoral and cellular responses were involved in resistance to these pathogens.

Therefore, in order to evaluate the relative importance of both humoral and cellular factors in immunity to *Salmonella typhimurium* C5, it seemed worthwhile to examine the level of resistance conferred by stimulating the reticuloendothelial system of mice with various immunopotentiating agents, and also to assess the effect of antibody in each case. It was thought that such an investigation would delineate more clearly the role of antibody in the expression of immunity to this intracellular bacterial parasite. The results of these studies will be discussed in the following sections:

(a) The ability of various immunopotentiating agents to stimulate the reticuloendothelial system.

(b) The efficacy of live *Salmonella enteritidis* 11RX as a vaccine against infection with *Salmonella typhimurium* C5.

(c) The role of antibody in acquired apparently non-specific resistance to challenge with *Salmonella typhimurium* C5.

(d) The efficacy of live *Listeria monocytogenes* as a vaccine against infection with *Salmonella typhimurium* C5.

(e) The efficacy of a *Coxiella burnetii* extract as a vaccine against infection with *Salmonella typhimurium* C5.

(f) The efficacy of dextran sulphate as a vaccine against *Salmonella typhimurium* C5.

(g) Immunity to Salmonella infection.

The ability of various immunopotentiating agents to stimulate the reticuloendothelial system.

In these studies, mice were treated with four immunopotentiating agents, namely living *Salmonella enteritidis* 11RX (a Gram-negative bacterium), living *Listeria monocytogenes* (a Gram-positive bacterium), an extract from *Coxiella burnetii* (a rickettsial substance), or dextran sulphate (a high molecular weight polyanion) and then subsequently examined for their resistance to challenge with *Salmonella typhimurium* C5. However, before discussing either their prophylactic value or the implications of these results for the mechanism of immunity to murine typhoid, it was necessary to consider what effects these agents had on the immune system. It was particularly important to determine whether macrophage activation had occurred, since resistance to intracellular bacterial parasites is usually associated with the development of a cell-mediated immune response.

Activated macrophages have been shown to have increased phagocytic, bactericidal and tumouricidal capabilities (North,

1978). The phagocytic activity of macrophages in the liver and spleen can be assessed from the ability of mice to clear an intravenous dose of carbon particles from their bloodstream (Jenkin and Rowley, 1961). The 4 immunopotentiating agents tested using this technique were each found to stimulate the reticuloendothelial system to varying degrees (Figures 3.6, 5.2, 6.2, 6.8; Table 6.8). *Salmonella enteritidis* 11RX established a carrier state in the F1 mice and persisted for at least 38 days (Figure 3.5). During the course of this infection the carbon clearance rate was greater than that observed in normal mice, indicating enhanced phagocytic activity (Figure 3.6). It was apparent that as the infection cleared, the activity of the reticuloendothelial system gradually returned to normal pre-infection levels. These results are in general agreement with earlier reports (Hardy and Kotlarski, 1971; Davies, 1975; Ashley, 1976). *Listeria monocytogenes* also established a carrier state but only of approximately 10 days duration (Figure 5.1). Similarly, these Listeria-infected mice were able to clear carbon particles from their bloodstream quite rapidly. However, the period for which the reticuloendothelial system remained activated was correspondingly shorter (Figure 5.2). Surprisingly, mice injected with the *Coxiella burnetii* extract were capable of rapid clearance of an intravenous dose of carbon particles for nearly 30 days after injection of the extract (Figure 6.2). Presumably this non-viable substance was able to persist for a considerable period of time. Lastly, the bi-phasic fluctuation of the phagocytic indices seen in Figure 6.8, suggested that dextran sulphate initially suppressed and then stimulated the reticuloendothelial system. When compared on

the basis of their ability to elevate the carbon clearance rates, the *Coxiella burnetii* extract appeared to be the most potent, followed by the live *Salmonella enteritidis* 11RX and *Listeria monocytogenes*, with dextran sulphate being the least effective. However, it is difficult to determine whether these differences reflect inequalities in either the number of macrophages stimulated, the level of macrophage activation reached, or both.

Classically, activated macrophages were identified by the expression of enhanced antimicrobial resistance (North, 1978). Hence, resistance to infection with *Listeria monocytogenes* is another means of evaluating the state of macrophage activation. Rowley, Auzins and Jenkin (1968) have established that mice infected with *Salmonella enteritidis* 11RX are resistant to challenge with *Listeria monocytogenes* as well as a number of other organisms including *Salmonella typhimurium* C5. Previous reports have also shown that in mice, a sublethal infection with *Listeria monocytogenes* confers protection against a Listeria challenge (Mackaness, 1969; Cheers *et al*., 1978) and this was verified in Figure 5.3. However, although *Listeria monocytogenes*-immunized mice are resistant to a homologous challenge, their resistance to a heterologous challenge with *Salmonella typhimurium* C5 is questionable and will be discussed later. Thus, it appeared that mice infected with either *Salmonella enteritidis* 11RX or *Listeria monocytogenes* acquired a population of phagocytic cells with enhanced bactericidal activity. This conclusion was supported by the observation that bacterial numbers declined during the latter phase of their respective carrier states (Figures 3.5, 5.1).

The induction of protective immunity to *Listeria*

monocytogenes can be achieved only by vaccination with sublethal numbers of live Listeria or with killed organisms in combination with certain adjuvants (Van Der Meer, Hofhuis and Willers, 1977). It was therefore a little surprising that mice pretreated with the *Coxiella burnetii* extract survived a normally lethal infection with *Listeria monocytogenes* (Figure 6.5). Nevertheless, this finding is consistent with the carbon clearance data which demonstrated clearly that this non-viable vaccine stimulated the reticuloendothelial system (Figure 6.2). Another functional property of activated macrophages, their ability to recognize and destroy neoplastic cells, has been well documented (Ashley and Hardy, 1973; Hibbs, 1975; Fidler, Darnell and Budman, 1976; Ruco and Meltzer, 1977; North, 1978). Hence the demonstration that peritoneal exudate cells, harvested from the *Coxiella burnetii* extract-treated mice, were cytotoxic towards both EAT and P815 tumour cells *in vitro* (Table 6.3) provided additional evidence of macrophage activation. Furthermore, mice pretreated with the *Coxiella burnetii* extract were found to be more resistant than normal mice to transplanted tumours *in vivo* (Table 6.2; Figures 6.3, 6.4). Maximum resistance to EAT was shown to occur 2 - 4 weeks post-immunization (Table 6.2), corresponding with the period when the carbon clearance data indicated that the reticuloendothelial system was stimulated (Figure 6.2).

Van Der Meer, Hofhuis and Willers (1977) reported that pretreatment with dextran sulphate alone conferred little protection against *Listeria monocytogenes*. Preliminary experiments confirmed that mice injected with dextran sulphate remained highly susceptible to infection with these organisms (data not shown), despite their slightly enhanced ability to

clear carbon particles from their bloodstream (Figure 6.8; Table 6.8). Together, these results suggested that this polyanion was capable of stimulating the reticuloendothelial system to only a limited extent, such that the macrophages were insufficiently activated to kill Listeria monocytogenes, or there were insufficient in number to do so.

Lastly, while discussing the effects of Salmonella enteritidis 11RX, Listeria monocytogenes, Coxiella burnetii extract and dextran sulphate on the immune system, it is important to consider that these agents may also potentiate the immune response to other antigens. In fact the adjuvant activity of Salmonella enteritidis 11RX (Davies, 1975) and dextran sulphate (Diamantstein et al, 1971 a & b; McCarthy, Arnold and Babcock, 1977; L'Age-Stehr and Diamantstein, 1977; Van Der Meer, Hofhuis and Willers, 1977) has already been documented. Similarly, Bordetella pertussis (Finger, Emmerling and Brüss, 1970; Finger et al., 1978) and Corynebacterium parvum (Halpern et al, 1964; Howard, Scott and Christie, 1973) have been shown to activate macrophages and act as adjuvants. Hence, in addition to their effects on macrophage activity, these immunopotentiating agents may also influence host resistance to some virulent bacterial parasites by ensuring that the infected animal mounts a rapid immune response to the foreign pathogen.

The efficacy of live Salmonella enteritidis 11RX as a vaccine against infection with Salmonella typhimurium C5

Infection with the rough strain, Salmonella enteritidis 11RX, has been shown to stimulate the reticuloendothelial system of mice (Hardy and Kotlarski, 1971; Figure 3.5) and to

confer long-lasting protection against challenge with the virulent Salmonella typhimurium C5 (Rowley, Auzins and Jenkin, 1968; Ielasi, 1970; Davies, 1975). In addition, the Salmonella enteritidis 11RX-infected mice were found to require the presence of normal numbers of T-cells for the generation of effective resistance, and they also developed delayed-type hypersensitivity reactions to antigens of Salmonella typhimurium C5 (Davies, 1975). These findings, together with those of other workers (Mackaness, Blanden and Collins, 1966; Collins, Mackaness and Blanden, 1966; Collins and Mackaness, 1968; Collins, 1974) indicated that a cell-mediated immune response was involved in resistance to typhoid.

However, the role of antibody in immunity to these Salmonella infections is a matter of controversy. Mackaness, Blanden and Collins (1966) demonstrated that the course of a Salmonella typhimurium C5 infection in normal mice was not significantly influenced by either the passive transfer of serum from actively infected or vaccinated animals, nor by immunization with heat-killed organisms. They suggested that circulating antibody was of little importance in the expression of acquired resistance to this disease. In contrast, Davies (1975) found that prior immunization with an alcohol-killed Salmonella typhimurium C5 vaccine greatly enhanced the ability of Salmonella enteritidis 11RX-infected mice to control and eliminate a Salmonella typhimurium C5 challenge, particularly when high challenge doses were used. On the basis of these results he concluded that the protective effect of specific antibody could only be expressed in the presence of "cellular activation" produced by live Salmonella enteritidis 11RX.

The present study examined this hypothesis further. In

keeping with Davies' (1975) observations, *Salmonella enteritidis* 11RX-infected mice which had been immunized with an alcohol-killed *Salmonella typhimurium* C5 vaccine were found to have little difficulty in coping with a large, intravenous challenge of *Salmonella typhimurium* C5 (Figure 3.7). Both antibody and activated macrophages were clearly required, as mice which received only the live *Salmonella enteritidis* 11RX, or the alcohol-killed *Salmonella typhimurium* C5 vaccines, were unable to limit the progress of the disease (Figures 3.7 and 3.8). Finally, the observation that the passive transfer of specific antibody had effects similar to active immunization with the alcohol-killed *Salmonella typhimurium* C5 vaccine, provided more direct evidence for the importance of specific antibody in immunity to systemic typhoid.

It is particularly relevant to this discussion that Collins (1968b) also noticed an element of specificity in the expression of immunity to Salmonellae. He found, using the smooth strains of *Salmonella enteritidis* and *Salmonella typhimurium*, that vaccination with either one protected mice against an intravenous infection with the other. However, the immune response to the homologous organism was more rapid and effective than the response to the heterologous organism. Coppel and Youmans (1969) made similar observations while working with Listeria and tubercle bacilli. In view of the above studies, it is possible that elevated levels of specific antibody may be one of the reasons behind the more effective inactivation of the homologous rather than the heterologous organisms.

Similarly, when *Salmonella typhimurium* C5 were injected intraperitoneally, specific antibody was found to contribute

significantly to the expression of resistance. Specific antibody appeared to increase the rate at which typhoid bacilli were inactivated in the peritoneal cavity of normal mice (Figure 3.4). While mice which had been recently infected with Salmonella enteritidis 11RX were able to clear Salmonella typhimurium C5 rapidly from their peritoneal cavity, this clearance rate was also enhanced by prior opsonization of the bacteria (Figure 3.4). In another closely related experiment, immunization with an alcohol-killed Salmonella typhimurium C5 vaccine was shown to facilitate the recall of resistance to Salmonella typhimurium C5 in long-term Salmonella enteritidis 11RX-recovered mice (Figures 3.9 and 3.10). Although a protein antigen preparation from Salmonella enteritidis 11RX is known to activate macrophages and recall immunity to EAT (Ashley, 1976), it is not by itself able to restore effective antibacterial immunity.

Some of the above results are consistent with those described by Collins (1969a). He found that while the opsonization of Salmonella enteritidis 5694 with hyperimmune serum failed to protect normal mice, it did promote the phagocytosis and killing of this organism during the initial phase of the infection. However, in contrast to the findings presented here, he demonstrated that serum treatment had no effect on the fate of Salmonella enteritidis 5694 in Salmonella gallinarum-vaccinated mice (Collins, Mackaness and Blanden, 1966; Collins, 1969b). This discrepancy may be attributable to the fact that Salmonella enteritidis 5694 and Salmonella gallinarum are antigenically related and are known to share the O-somatic determinants 1, 9 and 12. It is quite possible that opsonization of Salmonella enteritidis 5694

prior to injection had little effect, as opsonins for this organism were already available after vaccination with *Salmonella gallinarum*. His conclusion that antibody plays only a marginal role is therefore questionable.

Lastly, in an attempt to gain more definitive evidence for the role of antibody in immunity to Salmonella infections, some aspects of the macrophage-bacterium interaction were investigated *in vitro*. Peritoneal macrophages harvested from both normal and *Salmonella enteritidis* 11RX-infected mice appeared to have little, if any, innate ability to recognise and bind *Salmonella typhimurium* or its somatic antigens. The adherence of either whole bacteria or lipopolysaccharide sensitised sheep erythrocytes to these normal and activated phagocytic cells was found to be dependent on the presence of specific antibody (Figures 3.1 and 3.2; Table 3.1). Interestingly, Weir and his colleagues have shown that mouse peritoneal macrophages were capable of binding *Corynebacterium parvum* by a mechanism which was not mediated by antibody (Ögmundsdóttir and Weir, 1976; Weir and Ögmundsdóttir, 1977; Ögmundsdóttir, Weir and Marmion, 1978). Such a "primitive" recognition mechanism by mononuclear phagocytes is presumably not effective against *Salmonella typhimurium* C5, or plays only a relatively minor role in comparison to antibody and was not detected in these experiments.

Specific antibody also appeared to influence the ability of phagocytic cells to express their bactericidal potential against *Salmonella typhimurium* C5 (Figure 3.3). Activated macrophages from *Salmonella enteritidis* 11RX-infected mice were able to phagocytose and kill the typhoid bacilli *in vitro*, but only in the presence of serum specific for these organisms.

Peritoneal exudate cells from normal mice displayed limited bactericidal activity. Similar findings have also been reported by Meléndez, González, Reid, Fuentes and Castillo (1978), who have shown that anti-*Salmonella typhi* Ty2 antiserum specifically enhanced the ingestion and intracellular killing of *Salmonella enteritidis* by macrophages from immunized mice.

There is little doubt from these results that humoral as well as cellular factors, play an important role in immunity to *Salmonella typhimurium* C5.

The role of antibody in acquired apparently non-specific resistance to challenge with *Salmonella typhimurium* C5.

Infection with *Salmonella enteritidis* 11RX has been shown to protect mice against subsequent challenge with *Listeria monocytogenes* 2535, *Salmonella enteritidis* 795, *Salmonella paratyphi* C or *Salmonella typhimurium* C5 (Rowley, Auzins and Jenkin, 1968). This and other reports of the apparent non-specific expression of antimicrobial resistance have been interpreted by some, as providing evidence that the mechanism of immunity is purely cellular in nature and that specific antibody plays little, if any, role. However, the sharing of common antigens amongst various members of the *Enterobacteriaceae* (Kunin, Beard and Halmagyi, 1962; Kunin, 1963; Hammarstrom *et al.*, 1971; McCabe, 1972) and the ability of some bacterial cell wall components to polyclonally activate B lymphocytes (Andersson, Sjöberg and Möller, 1972; Melchers, Braun and Galanos, 1975; Ness *et al.*, 1976; Bessler and Ottenbreit, 1977; Peavy, Baughn and Musher, 1978; Bessler and Henning, 1979; Dufer *et al.*, 1980) makes it difficult, if not impossible to preclude the participation of antibody from these

systems.

Experiments described in Chapter 3 demonstrated that, *in vivo*, the acquisition of specific antibody by either active immunization or passive transfer, enabled *Salmonella enteritidis* 11RX-infected mice to eliminate a large intravenous challenge dose of *Salmonella typhimurium* C5. *In vitro*, the presence of immune serum was essential, if activated peritoneal macrophages from these mice were to bind and eventually destroy the typhoid bacilli. These findings suggested that in *Salmonella enteritidis* 11RX-infected mice, the expression of resistance to challenge with *Salmonella typhimurium* C5 is dependent on low levels of specific antibody which becomes the limiting factor when high challenge doses are used. The studies discussed here were designed to determine, firstly, whether the serum of mice infected with *Salmonella enteritidis* 11RX contained antibodies specific for *Salmonella typhimurium* C5 and, secondly, whether these antibodies were involved in the resistance to this pathogen.

Antibodies specific for *Salmonella typhimurium* C5 were detected in the serum of mice which had been infected 13 days previously with 10^5 *Salmonella enteritidis* 11RX. Their presence was evident from the ability of the serum to both agglutinate C5 LPS sensitized sheep erythrocytes (Table 4.1) and to enhance the clearance of typhoid bacilli from the peritoneal cavity of normal mice (Figure 4.3). The existance of these antibodies in *Salmonella enteritidis* 11RX-immunized mice supported several earlier observations (Rowley, Auzins and Jenkin, 1968; Ielasi, 1970; Davies, 1975).

Polysaccharide, prepared from *Salmonella typhimurium* C5 by mild acid hydrolysis, was found to be non-toxic for normal F1

mice (data not shown) and had no detrimental effects on the activity of the reticuloendothelial system (Table 4.4; Figure 4.6). Although the preparation was not homogeneous (Figure 4.1), it appeared to be free of contaminating unhydrolysed lipopolysaccharide (Figure 4.2). Extremely small quantities (20 - 40ng) of Salmonella typhimurium C5 polysaccharide specifically inhibited the agglutination of C5 LPS sensitized SRBC with hyperimmune antisera (Table 4.3).

When injected intravenously into Salmonella enteritidis 11RX-immunized mice, Salmonella typhimurium C5 polysaccharide reduced the level of specific antibody (Figure 4.5) and abrogated resistance to infection with Salmonella typhimurium C5 (Figures 4.7, 4.8 and 4.9). In contrast, the administration of Salmonella newington polysaccharide, which does not cross-react with the O-somatic antigens of Salmonella typhimurium C5, had little effect, as these mice remained able to control the growth of the challenge organisms. Similarly, pretreatment with Salmonella typhimurium C5 polysaccharide specifically reduced the ability of the Salmonella enteritidis 11RX-infected mice to clear typhoid bacilli from their peritoneal cavity (Figure 4.4). Presumably, in each of these 4 experiments, the capacity of the activated macrophages to phagocytose and kill the invading pathogens was curtailed by the depletion of specific antibody.

These findings are consistent with those of Ielasi (1970) and suggest that although Salmonella enteritidis 11RX-infected mice are 'non-specifically' resistant to challenge with Salmonella typhimurium C5, antibody still plays an important role in the expression of cell-mediated, antibacterial immunity.

<u>The efficacy of live *Listeria monocytogenes* as a vaccine against infection with *Salmonella typhimurium* C5</u>

At the commencement of this study, the efficacy of live *Listeria monocytogenes* as a vaccine against infection with *Salmonella typhimurium* was not clearly defined. In 1966, Blanden, Mackaness and Collins reported that Swiss-Webster mice infected with *Listeria monocytogenes* were resistant to challenge with *Salmonella typhimurium* C5 in the apparent absence of antibody capable of opsonizing or agglutinating these organisms. Although they claimed that an enhancement of the phagocytic cells' bactericidal ability was largely responsible for this acquired immunity, their studies failed to preclude the involvement of low levels of cross-reacting antibody. In contrast, Zinkernagel (1976) found that *Listeria monocytogenes*-immunized CBA/H mice were not able to control a challenge infection with *Salmonella typhimurium* C5, and he concluded that in addition to macrophage activation, specific factors were necessary for protective immunity. Reconciliation of these conflicting results is difficult, especially with regard to the role of antibody in immunity to typhoid.

The studies presented in Chapter 5 demonstrated that F1 mice infected with *Listeria monocytogenes* were not resistant to challenge with *Salmonella typhimurium* C5. These results were compatible with those of Zinkernagel (1976) and suggested that the acquisition of activated macrophages alone is insufficient for effective immunity to Salmonella infections. Surprisingly, neither the passive transfer of specific anti-serum (Figure 5.5) nor active immunization with an alcohol-killed *Salmonella typhimurium* C5 vaccine (Figures 5.6 and 5.7)

enabled the *Listeria monocytogenes*-infected mice to control and eliminate a *Salmonella typhimurium* C5 challenge. However, prior opsonization of the typhoid bacilli with specific antibody was found to greatly enhance their clearance from the bloodstream of these mice (Table 5.1). Therefore, while a shortage of specific humoral factors may have limited the expression of resistance, it was not the sole reason for the susceptibility of *Listeria monocytogenes*-infected mice to a heterologous challenge.

The *Salmonella enteritidis* 11RX-immunized mice appeared better able to cope with a *Salmonella typhimurium* C5 infection than the *Listeria monocytogenes*-immunized mice, even when specific antibody was not limiting. Such a marked difference in resistance may reflect either the state or degree of macrophage activation, or the maintenance of macrophage activation in these mice. The more effective cell-mediated response in the *Salmonella enteritidis* 11RX-infected mice may be partly attributable to the persistence of these organisms (Figure 3.5) providing continual antigenic stimulation and to the ability of *Salmonella typhimurium* C5 or its digestion products to maintain the level of macrophage activation. Cytoplasmic protein antigens from *Salmonella typhimurium* C5 have been shown to elicit delayed-type hypersensitivity reactions (Davies, 1975) and recall anti-tumour immunity (Vingelis, Ashman and Kotlarski, 1980) in *Salmonella enteritidis* 11RX-immunized mice.

In contrast, the carrier state for *Listeria monocytogenes* was shown to be relatively short (Figure 5.1) and mice infected with these organisms were unable to mount delayed-type hypersensitivity reactions to cytoplasmic proteins

prepared from Salmonella (Tables 5.2 and 5.3). Also, the development of immunity to Salmonella enteritidis 11RX appeared to be impaired in the Listeria-infected mice (Tables 5.2 and 5.3). This state of reduced responsiveness to heterologous antigenic stimuli may be another factor contributing to their susceptibility to Salmonella typhimurium C5. Recently Mandel and Cheers (1980) found that BALB/c mice suffered from thymic atrophy and T-lymphocyte depletion following infection with Listeria monocytogenes. Similarly, others have shown that suppressor T-cells are generated after some intracellular bacterial infections (Watson and Collins, 1979, 1980; Collins and Watson, 1980). In view of these studies it is possible that the presence of suppressor T-cells or/and the loss of T-cells, may be responsible for the decreased immunocompetence seen in Listeria-infected F1 mice.

While mice immunized with Listeria monocytogenes acquired an activated reticuloendothelial system and resistance to a homologous challenge, they remained unable to control a challenge with Salmonella typhimurium C5. Evidence was provided which suggested that reduced immunocompetence and a lack of specific antibody were 2 factors contributing to this susceptibility.

The efficacy of a Coxiella burnetii extract as a vaccine against infection with Salmonella typhimurium C5

Over the years, a variety of vaccines have been used to immunize mice against infection with Salmonella typhimurium. While live bacterial vaccines were generally found to bestow the greatest degree of protection (Mackaness, Blanden and Collins, 1966; Rowley, Auzins and Jenkin, 1968; Angerman and

Eisenstein, 1980), killed whole cell vaccines and some sub-cellular fractions nevertheless conferred substantial levels of resistance (Kenny and Herzberg, 1967; Herzberg, Nash and Hino, 1972; Misfeldt and Johnson, 1976; Angerman and Eisenstein, 1978). As all Salmonella vaccines are capable of inducing specific and/or polyclonal humoral responses, the superiority of the live vaccines is thought to be due to their ability to elicit a cell-mediated response. Unfortunately, definitive analysis of the resultant immune responses is difficult and invariably complicated by the effects of lipopolysaccharide contamination.

Recently, non-viable vaccines prepared from Coxiella burnetii, the rickettsial agent which causes Q fever, have been shown to be useful prophylactic agents against a range of parasites. Kelly and his colleagues have demonstrated that the injection of formalin-killed Coxiella burnetii into guinea pigs resulted in enhanced macrophage listericidal activity (Kelly, 1977) and in regression of a transplantable hepato-cellular carcinoma (Kelly et al., 1976). Subsequently, Clark (1979) observed that a commercially available extract of Coxiella burnetii protected mice against the protozoan parasites Babesia microti, Babesia rodhaini and Plasmodium vinckei petteri. The available evidence suggests that a non-protein rickettsial component with an ability to stimulate macrophages, may be the active principle responsible for these findings.

Preliminary studies have revealed that in F1 mice the Coxiella burnetii extract was able to stimulate the reticulo-endothelial system (Figure 6.2), enhance resistance to trans-planted tumours (Tables 6.2 and 6.3; Figures 6.3 and 6.4) and

confer protection to *Listeria monocytogenes* (Figure 6.5). These results are consistent with the previous reports and provide further evidence for macrophage activation. Interestingly, this immunopotentiating agent appeared to have no effect on serum antibody levels to *Salmonella typhimurium* C5 (Table 6.1, Figure 6.1). This last observation suggested that the *Coxiella burnetii* extract would be useful in determining whether the acquisition of activated macrophages alone confers protection against challenge with *Salmonella typhimurium* C5.

Pretreatment with the *Coxiella burnetii* extract was found to confer resistance to infection with *Salmonella typhimurium* C5, but only in mice vaccinated with alcohol-killed typhoid bacilli (Tables 6.4, 6.5, 6.6 and 6.7) or receiving specific antibody by passive transfer (Figures 6.6 and 6.7). Mice immunized with only the rickettsial extract were unable to control the growth of the *Salmonella typhimurium* C5 and eventually succumbed to the infection. The critical importance of specific antibody in these experiments implied that the generation of both humoral and cellular responses were necessary for immunity to *Salmonella typhimurium* C5.

Although immunization with the *Coxiella burnetii* extract conferred considerable resistance to *Salmonella typhimurium* C5, it did appear to be slightly less effective as a vaccine than the live *Salmonella enteritidis* 11RX. In these experiments the protection was not absolute, as some of the mice failed to survive. It is possible that the level of immunity generated may have been limited by the dose of extract used, or the time of challenge.

The efficacy of dextran sulphate as a vaccine against infection with *Salmonella typhimurium* C5

The prophylactic value of dextran sulphate as a vaccine against infection with *Salmonella typhimurium* C5 was investigated because it is known to potentiate both humoral (Diamantstein *et al*., 1971 a & b) and cell-mediated immune responses (McCarthy, Arnold and Babcock, 1977; L'Age-Stehr and Diamantstein, 1977). This high molecular weight polyanion has been shown to produce a complex spectrum of effects which are influenced by dosage, route and time of injection (L'Age-Stehr and Diamantstein, 1977; McCarthy and Babcock, 1978). It affects mainly phagocytic cells (Hahn and Bierther, 1973; L'Age-Stehr and Diamantstein, 1977), inhibiting phagosome-lysosome fusion (Hart and Young,1975; Goren *et al*., 1976). In keeping with these reports, the carbon clearance data (Figure 6.8; Table 6.8), indicated that dextran sulphate initially suppressed and then stimulated the reticuloendothelial system. Likewise, the observation that dextran sulphate elevated serum antibody levels to both C5 LPS-sensitized and un-sensitized sheep erythrocytes (Table 6.1), supported earlier demonstrations that it was mitogenic towards B lymphocytes (Diamantstein *et al*., 1973; Moreno, Hale and Ivanyi, 1977).

The injection of dextran sulphate into F1 mice also resulted in the acquisition of resistance to challenge with *Salmonella typhimurium* C5 (Table 6.9), presumably due to increases in both specific antibody levels and macrophage activity. However, it is also possible that its adjuvant properties contributed to this resistance, particularly as the level of macrophage activation appeared to be minimal. Prior to the onset of resistance, the immunized mice were acutely

susceptible, dying even before the control mice. Together these findings suggested that, following the administration of dextran sulphate , the activity of the reticuloendothelial system was initially impaired and then, after several days, became stimulated.

In conclusion, the demonstration that mice pretreated with dextran sulphate survived infection with *Salmonella typhimurium* C5, supported the hypothesis that both humoral and cellular factors are involved in immunity to this intracellular bacterial parasite.

Immunity to Salmonella infection

The experiments presented in this study have shown that the generation of both humoral and cellular immune responses was necessary to protect mice against Salmonella infection. Stimulation of the reticuloendothelial system, *per se*, conferred little resistance to challenge with *Salmonella typhimurium* C5. However, the expression of antibacterial immunity was greatly enhanced by the presence of specific antibody. From this it was concluded that activated phagocytic cells require specific antibody in order to bind and subsequently destroy these pathogenic organisms. These findings have confirmed and extended several earlier demonstrations that antibody plays an important role in immunity to typhoid (Jenkin, 1963; McIntyre, Rowley and Jenkin, 1967; Rowley, Auzins and Jenkin, 1968; Kenny and Herzberg, 1968; Collins, 1970; Meléndez *et al*., 1978; Angerman and Eisenstein, 1980).

Similarly, the demonstration that serum from mice immunized with *Salmonella enteritidis* 11RX, contained low

levels of antibody specific for _Salmonella typhimurium_ C5 was also consistent with previous reports (Rowley, Auzins and Jenkin, 1968; Ielasi, 1970; Davies, 1975). These opsonins were generated in the absence of O-somatic cross-reactivity and appeared to contribute significantly to host resistance. Pretreatment with _Salmonella typhimurium_ C5 polysaccharide depleted _Salmonella enteritidis_ 11RX-infected mice of specific antibody and abrogated their resistance to typhoid. These results have shown that at least one live bacterial vaccine is capable of inducing antibody specific for an apparently unrelated bacterium. It is therefore likely that low levels of background antibody may participate in other cases of apparent non-specific cross-resistance (Boehme and Dubos, 1958; Howard _et al_., 1959; Mackaness, 1964; Blanden, Mackaness and Collins, 1966).

Another interesting observation made during the course of this study was that resistance to _Salmonella typhimurium_ C5 did not always correspond with resistance to _Listeria monocytogenes_ and _vice versa_. For instance, while mice immunized with either live _Listeria monocytogenes_ or a _Coxiella burnetii_ extract were resistant to challenge with _Listeria monocytogenes_, they remained susceptible to _Salmonella typhimurium_ C5. On the other hand, dextran sulphate conferred protection against infection with _Salmonella typhimurium_ C5, but not to _Listeria monocytogenes_. It is not unreasonable to conclude that at least some of the critical factors in host resistance may differ from one intracellular bacterial parasite to another. Therefore, one should be extremely careful when generalizing from immunity to Listeria to other organisms, such as Salmonella. It is also

apparent from such anomalies that immunity to intracellular bacterial parasites is not as "non-specific" as some previous studies have implied (Howard et al., 1959; Ushiba et al., 1959; Blanden, Mackaness and Collins, 1966; North, 1978; Collins, 1979).

Several workers have now been able to transfer resistance to Listeria with T-lymphocytes from immunized mice (Mackaness, 1969; Mackaness and Hill, 1969; Lane and Unanue, 1972; North, 1973; Zinkernagel et al., 1977). In marked contrast, the scarcity of reports dealing with the adoptive transfer of immunity to Salmonella is probably significant. In one reported study, Davies (1975) demonstrated that it was difficult to transfer protection against Salmonella typhimurium C5 with spleen cells from immunized mice. He found it necessary to immunize the recipient F1 mice with an alcohol-killed Salmonella typhimurium C5 vaccine prior to the transfer of spleen cells from the immune donors. The inference from these observations is that the transfer of cell-mediated immunity to normal mice is insufficient to confer protection against typhoid bacilli and that the availability of specific opsonins in the recipient mice is one of the factors limiting their resistance. The data presented here is not only consistent with this conclusion, but also provides an explanation as to why the availability of specific antibody is important - activated macrophages require specific antibody to phagocytose and kill the Salmonella typhimurium C5 organisms. One would therefore predict that the transfer of immunity to Salmonella would be more successful when specific antibody was not limiting in the recipient mice.

Lastly, as a direct extension of this work, it is tempting to suggest that vaccines against typhoid fever in humans would be more successful if they contained antigens capable of inducing both humoral and cell-mediated responses. While the inability of current killed vaccines to induce a cell-mediated response is an obvious limitation, it is important not to underestimate the value of specific antibody when developing new vaccines.

Summary

This study has provided further evidence that, in addition to macrophage activation, specific antibody also plays an important role in immunity to Salmonella infections. Indeed, the very expression of cell-mediated immunity to these intracellular bacterial parasites appeared to be dependent on, and limited by, the availability of these opsonic factors.

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