THE GENETIC CONTROL OF SPECIES SUSCEPTIBILITY TO INFECTION

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ABSTRACT

The immunological factors controlling the resistance of rats and the suceptibility of mice to Salmonella typhimurium C5 infection have been investigated.

Normal rats of the BN strain were shown to be resistant to doses of Salmonella typhimurium C5 as great as 7.9×10^9 organisms. Normal mice of the Swiss White strain on the other hand, were extremely susceptible to infection with this organism, the LD50 dose being less than 10 organisms.

Normal rats were subsequently shown to possess comparatively high levels of natural antibody in their serum and this antibody was isolated and partially characterized.

The fate of Salmonella typhimurium C5 organisms injected into rats and mice was studied and it was shown that rats were able to control the multiplication of this organism, but could not eliminate it rapidly. Instead, a carrier state was established for a time which was dependent upon the challenge dose. Mice, on the other hand, could not control the infection and the organisms soon multiplied to levels which were fatal.

Rats were shown to produce much greater levels of antibody more rapidly than mice in response to various doses of living vaccine of Salmonella typhimurium C5, as well as to a wide dose range of the killed vaccine prepared from this organism. Furthermore, the presence of antibody in the serum of rats and mice could be correlated with the numbers of organisms that could be recovered.

The numbers of antibody producing cells in the spleens of rats and mice stimulated with the antigens of Salmonella typhimurium C5 were determined and it was shown that rats produce greater numbers of these cells than do mice.

It was also shown that mice could be protected against Salmonella typhimurium C5 infection by the passive transfer of antibody, as well as by the active stimulation of levels of antibody directed against Salmonella typhimurium C5, using an unrelated organism. Conversely, it was shown that both mice that had been made resistant by stimulation of antibody levels, as well as rats, would become susceptible if these antibody levels were depleted.

The immunosupporessive drug Methotrexate was used to prevent synthesis of antibody in rats challenged with Salmonella typhimurium C5 and subsquently it was shown that rats treated with this drug became susceptible to infection with this organism.

Finally, the difference in susceptibility of rats and mice to Salmonella typhimurium C5 infection, was shown to be due to a difference in the ability of rat and mouse lymphoid tissues to respond to the antigens of this organism. This was done by establishing radiation chimaeras.

The significance of these findings is discussed in the light of present knowledge of the genetic control of species susceptibility to infection.

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.

A.IELASI

December, 1970

ERRATA

2nd page of Abstract, line 8, immunosuppressive not immunosupporessive

P. i, line 19, Reduction not Reducation

P. 2, line 13, complements, not compliments

P. 20, line 8, "was not 100 per cent" not "was 100 per cent"

line 9, "was not 100 per cent" not "was 100 per cent"

P. 29, line 15, mouse pox not mous epox

P. 49, line 27, antibody-producing not antibody-producting

line 28, plaquing not plaguing

P. 50, line 8, insert "a" before shaker

P. 51, line 24, should read 1/16 - 1/32 not 1/32 - 1/64

P. 52, line 25, $T_2 - T_1$ not $T_2 T_1$

Table 3. 2 mouse serum haemagglutinating titre should read less than 1/2 not 1/2

in vivo killing end point titre for mouse serum should read less than 1/2 not 1/2

P. 53, line 16, peritoneal not periotoneal

P. 59, line 2, plaquing not plaguing

line 7, plaque not plague

line 22, serological not seriological

Table 3. 8 mercapto-ethanol not mercaptoe-thanol

P. 70, line 10, "persist for at least 7 days" not "persist for 14 days"

line 11, "until 21 days" not "until 28 days"

P. 74, line 17, delete "on" and replace by "and"

line 18, should read "(b) antibody detectable..." not "(b) the antibody is detectable..."

P. 76, line 27, antibody not antigody

p. 98, line 4, injection not injected

P. 101, line 9, should read "... in the overall capacity of rat and mouse lymphoid tissue to respond to S. typhimurium C5 antigens".

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CHAPTER I

INTRODUCTION AND BRIEF ACCOUNT OF SOME FACTORS INFLUENCING THE GENETIC CONTROL OF SPECIES SUSCEPTIBILITY TO INFECTION

In 1883 Metchnikoff carried out the classic experiments which led to his description of the phagocytic cells of both Invertebrates and Vertebrates. The invertebrate cells resembled the mononuclear cells found in vertebrate spleens, livers and lymph nodes and these he termed "macrophages". In the vertebrate circulation he noticed smaller cells with multilobed nuclei which were also phagocytic and these he called "microphages". Metchnikoff observed that the latter cells (leucocytes) were mobilised quickly and were found in association with acute inflammatory conditions. The larger "macrophages" were more sluggish in their response and were found mainly in chronic inflammatory conditions such as malaria, in which these cells could engulf larger particles such as the Plasmodia malariae. These observations led Metchnikoff to present the Cellular Theory of Immunity. He suggested that phagocytic cells of animals played the principal role in defence against infection.

In later studies Clarke and Clarke (1928) and Salt (1956) working with the tadpole (Hyla versicolor) and with the larvae of ichneumon fly (Nemeritus canescens) and the stick insect (Carausius morosus) respectively, showed that the discriminative power of the phagocytic cells described by Metchnikoff was exquisitely fine. So fine, in fact, that these animals could not only distinguish "self" from "non-self", but also they could distinguish their own damaged or autochthonous cells and eliminate them from their circulatory system.

However, in 1888 Nuttal had demonstrated that in the absence of cells, in vitro, B. anthracis and B. subtilis were killed by a heat-labile factor

present in the defibrinated blood of rabbits or dogs. These findings were confirmed by Buchner in 1889. In 1890 von Behring and Kitisato proposed the Humoral Theory of Immunity having shown, in addition to the above-mentioned findings, that the sera of animals receiving repeated injections of sublethal doses of tetanus toxin had acquired the property of specifically neutralizing the toxin produced by the living bacillus. They also demonstrated that resistance could be transferred to susceptable animals with serum from resistant animals. They therefore suggested that these humoral factors - antibodies - were the major factors involved in resistance of animals to bacterial invasion.

These concepts led to the development of two schools of thought concerned with the defence mechanisms of animals. Subsequently, however, it became clear that each of these mechanisms compliments the other in host defence. It has been demonstrated, in studies with bacteria, that phagocytosis is dependent upon humoral factors which act as discriminating factors for phagocytic cells. In fact, soon after these two theories were proposed, Wright and Douglas (1903) established that in vitro phagocytosis of Staphylococcus pyogenes by human leucocytes was much greater in the presence, than in the absence, of human serum. This enhancement of phagocytosis with subsequent killing was not due to serum factors alone, nor to any factor which stimulated or affected the phagocytic cells. Wright and Douglas concluded that the serum contained substances which modified the bacteria and thus rendered them more susceptible to phagocytosis and killing. More recently Robertson and Sia (1927), Rowley and Whitby (1959), Cohn and Morse (1959), Jenkin and Rowley (1959), Rowley (1960) and Jenkin and Benacerraf (1960), have shown that without antibody, little or no phagocytosis of various bacteria can take place.

Weigle (1961), concluded that the presence of specific antibody greatly enhances phagocytosis; whilst the findings of Fenn (1921), Nelson and Lebrun (1956), Potter and Stollerman (1961) and Jenkin and Rowley (1961), all suggest that even inert particles such as bentonite and carbon require combination with serum protein(s), which can even be shown to have some specificity, in order to be recognised as foreign and removed efficiently (Murray, 1963).

Although some schools favour the hypothesis that humoral factors play the major role in immunity, others argue that cellular factors are more important than humoral factors. However, it is clear that animals possess the capacity to recognise foreignness and react either by a cellular reaction and/or a humoral reaction. Furthermore, a case can be made for the participation of both humoral and cellular (phagocytic) factors as essential requirements for host resistance. Therefore, when considering the ability of a particular parasite to multiply in the environment of the various hosts it may invade, or the ability of different parasites to multiply within the same host, we can conclude that the susceptibility or resistance of an animal is dependent upon its ability to mount an immune response and particularly on the rate and magnitude of this response.

The inter-relationships of the humoral and cellular aspects of acquired immunity are clear. However, the role of phagocytic cells has, as yet not been clearly defined. This is due, partly at least, to the fact that there are a large number of different micro-organisms capable of causing infection, which vary in their ability to parasitize their hosts. Suter (1956), points out the fact that many bacteria such as the pathogenic cocci, Pasteurella pestis, Haemophilus influenzae, Bacillus anthracis and Escherichia coli, may be regarded as obligate extracellular parasites capable of multiplying only in an extracellular environment; others, like Salmonella typhi, the brucellae

and the tubercle bacillus as facultative intracellular parasites, capable of intracellular as well as extracellular multiplication. The leprosy bacillus, viruses and rickettsiae are obligate intracellular parasites requiring an intracellular environment for multiplication. These divisions are by no means absolute, since bacteria of the extracellular group may parasitize and multiply in the phagocytic cells of highly susceptible animals. Intracellular parasites are harboured mainly within the phagocytic cells of the reticulo-endothelial system. In both cases, the parasites have an extracellular phase and humoral mechanisms may determine successively, phagocytosis and intracellular destruction. There is, for example, some evidence in brucella-monocyte and salmonella-macrophage systems that specific antibody is necessary for intracellular killing, as well as for ingestion. (Elberg, 1960, Jenkin, 1963). Although the enhancing value of both antibody and serum factors found in the sera of normal animals is not reported by all groups working with monocytes in tissue culture, this may be a reflection only of the degree to which the necessary co-factors were already present in the in vitro system employed.

It has been stated that acquired resistance is the result of a specific antibody response to an invading organism (Raffel, 1949). However, if this correlation of the level of antibodies with resistance is used as a measure of immunity, the degree of correlation appears to be determined by the type of invading organism involved, i.e. whether it is an intracellular or extracellular parasite. In the case of extracellular parasites antibody is clearly required for phagocytosis and subsequent killing. It has been shown that for phagocytosis of staphylococci, pneumococci and haemolytic streptococci to occur, specific serum factors are required and that acquired resistance to infections caused by these organisms is dependent upon a specific antibody response. (Raffel, 1949; Jeter, McKee and Mason, 1961;

Wu and Marcus, 1964). Therefore in this instance a correlation between antibody and resistance is observed.

In the case of immunity to intracellular parasites however, early studies suggested that the situation was much more complex, since there appeared to be little or no relationship between antibody titres and resistance. For example in 1938 Gorer and Schutze found that mice injected with various strains of Salmonellae showed a variable degree of resistance to these organisms. They examined the sera of these mice for the presence of antibodies specific for the "H" and "O" antigens of the various Salmonellae used and found no correlation between the levels of antibodies they could detect and the resistance of these animals to the parasite injected. Furthermore, in 1933 Lurie had observed a clear cut difference in the fate of tubercle bacilli injected into normal as opposed to immune animals. Although he observed that in vivo phagocytosis of these bacilli occurred in both normal and immune animals, the micro-organisms failed to multiply only in the phagocytic cells of the immune hosts. In 1942 Lurie reported a series of experiments which appeared to exclude the participation of antibody in the killing of tubercle bacilli by monocytes of immune animals. "Immune" and "normal" phagocytic cells containing ingested tubercle bacilli from tubercle bacillus immunized and normal rabbits respectively, were injected into the anterior chamber of the eye of normal rabbits - "immune" cells into one eye and "normal" cells into the other. After 10-14 days proliferation had occurred in the eye into which "normal" cells had been injected, the chamber was seen to contain large numbers of intracellular and extracellular tubercle bacilli. The other chamber into which "immune" cells had been injected showed no proliferation and only a few degenerate bacilli were seen, mostly inside phagocytic cells. Viable counts of the contents of the anterior chambers

confirmed the inference that the "immune" cells had inhibited the growth of tubercle bacilli. This experiment suggested that immunity was of a non-humoral type, mediated by phagocytic cells. It was not established however, whether the increased resistance was specific for the tubercle bacilli, although Lurie (1939) had shown that monocytes from tubercle bacilli-injected animals showed a "heightened physiological activity" which expressed itself by an "increased phagocytic capacity for a variety of particulate substances such as carbon particles, staphylococci and colloidin particles". Further evidence for acquired cellular immunity has also been presented in cases of infection with Brucellae, Salmonellae and Listeria, as well as with tuberculosis (Suter, 1953; Elberg, 1960; Saito, Akiyama, Nakano and Ushiba, 1960; Mackaness, 1962 and 1964). It has become apparent however, that the increased bactericidal activity of phagocytes is not specific for the inducing organism (Elberg, Schneider and Fong, 1957; Howard, 1961; Mackaness, 1964). That this cellular immunity is non-specific is also shown by the studies of Boehme and Dubos (1958) who showed that injection of bacterial lipopolysaccharide resulted in increased bactericidal activity of the reticulo-endothelial cells of mice to infections by heterologous organisms. Jenkin and Benacerraf (1960) also demonstrated that macrophages from B.C.G. - immunized mice had enhanced resistance to Salmonellae. Rowley, Auzins and Jenkin (1968) have reported that the increase in "cellular" immunity stimulated by B.C.G., Brucella or Listeria monocytogenes was directed against a wide range of organisms with little or no specificity. In addition, Auzins and Rowley (1962) demonstrated increased levels of the digestive enzymes in phagocytic cells of animals possessing this immunity.

Holland and Pickett (1956, 1958) enhanced the capacity of cultured monocytes from rat, mouse and guinea pig to restrict the intracellular

growth of brucellae by immunizing the animals with low doses of living, virulent strains of organisms, though not with killed or living avirulent strains. They also found evidence of enhanced in vivo destruction of intracellular brucellae in the spleens of immune animals. In 1959 Ushiba, Saito, Akiyama and Sugiyama showed that they could induce immunity to Salmonella enteritidis infection in mice by prior immunization with a living vaccine. This immunity could be passively transferred with peritoneal macrophages, but not with serum. They found that killed vaccines were not effective in inducing this type of immunity to this infection. Mitsuhashi, Hashimoto and Kawakami (1960), and Mitsuhashi, Sato and Tanaka (1961) demonstrated that the immunity induced in mice by living but not killed avirulent Salmonella enteritidis is also associated with more effective destruction of the Salmonella in liver and spleen. In 1963 Jenkin and Rowley clearly showed that living attenuated strains of intracellular parasites proved to be effective vaccines whereas killed suspensions of virulent strains did not, even though the latter induced the formation of high titres of specific antibody.

It is apparent from the above-mentioned reports that increased resistance to infection against facultative intracellular parasites is most certainly associated with more active phagocytic cells which are induced as a result of injection of living vaccines. In general, killed vaccines fail to induce this stimulation and fail to elicit immunity against intracellular parasites. In other words the findings discussed above support the view that the mechanism of immunity to intracellular parasites is due to an altered physiological state of phagocytic cells. This view is further supported by the observation that there is an apparent lack of specificity of this cellular immunity, suggesting that specific antibody plays no role in increased resistance.

However, more recently it has been shown that immunity to some intracellular parasites can be achieved using non-living vaccines (Jenkin and Rowley, 1963; Ribi, Anacker, Brehmer, Goode, Larson, List, Milner and Wicht, 1966). The successful use of such vaccines depended upon the use of adequate doses of antigen as well as the use of a suitable method of preparation of the vaccine. The resistance conferred by killed vaccines is due in the main to the production of specific antibody, since there is little or no stimulation of phagocytic cells. When killed vaccines are used, they can only be protective if specific antibody production is elicited. In other words, the killed vaccines must possess all the antigenic determinants that are present on the surface of the living organisms. Any treatment used to prepare killed vaccines which leads to the destruction or alteration of one or more of these antigenic determinants, may lead to the production of a non-effective vaccine. Unfortunately, however, in many instances the complexity of the antigenic structure of bacteria is such that it is difficult to determine whether antibody against one or other antigenic determinant, or a combination of antigenic determinants is an essential requirement for immunity. It follows, therefore, that it may be impossible to determine whether any of these antigens are destroyed during the preparation of killed vaccine. Attempts to immunize mice against virulent Salmonella typhimurium provide a good illustration of this problem. Before 1963, attempts to immunize mice against this pathogen, using heat killed vaccines had been unsuccessful. However, in 1963, Auzins and Rowley provided a possible explanation for this lack of success. They showed that the virulent strain of Salmonella typhimurium possesses a heat labile surface antigen which must react with antibody before efficient phagocytosis by mouse macrophages can occur in vitro. It seemed possible, therefore,

that failure to immunize mice against this Salmonella strain using heat-killed vaccines was due to destruction of this heat-labile antigen. This hypothesis was confirmed by the experiments of Jenkin and Rowley (1965) who were able to demonstrate that mice could be afforded some protection to Salmonella typhimurium by prior immunization with an alcohol-killed vaccine of this organism. They suggested that the heat labile antigen may be involved in the immunization of mice against virulent strains of Salmonella typhimurium. If this is so, then damage or destruction of such a labile antigen during preparation of killed vaccine would, as already mentioned, undoubtedly account for their inefficiency in immunizing animals against that infection.

That serum factors do participate in the reactions of intracellular parasites with monocytes in in vitro cultures has been demonstrated by Fong, Chin, Akiyama and Elberg (1959). Also, Jenkin and Benacerraf (1960) have shown that naturally occurring opsonins are important in these cellular reactions. Rowley et al., (1968) studied this problem further and concluded that treatment of mice with living vaccines of both Salmonella typhimurium and Salmonella enteritidis 11RX led to immunity to Salmonella typhimurium infection. This immunity they showed, was dependent upon an increased bactericidal activity of the macrophage population but the presence of specific antibody was essential for its expression.

Most of the early experiments, whilst demonstrating that humoral antibody was not required, did not attempt to demonstrate the absence of cell-bound antibody. A previously mentioned example is the work of Lurie (1942). In his and other studies of bacterial - phagocytic cell interaction using intra cellular parasites whether in vivo, (passive transfer of immunity with cells from immune animals), or in vitro (tissue culture phagocytic experiments), the possible presence of cell-bound antibody was not investigated. Furness and Ferreira (1959), Saito, Nakano, Akiyama

and Ushiba (1962), claimed that immunity to Salmonellae is manifested in the phagocytic cells of the animal and is divorced from antibody. In 1959 Ushiba, Saito, Akiyama and Sugiyama had been able to confer antibacterial immunity against Salmonella enteritidis to mice using peritoneal macrophages from immune mice. Although these workers claimed that their experiments demonstrated that humoral antibody was not involved, they did not demonstrate the absence of cell-bound antibody. Rowley, Turner and Jenkin (1964) have ably shown that transfer of peritoneal macrophages from mice rendered immune to Salmonella typhimurium infection, resulted in the transfer of immunity to the normal recipients of these cells. However, they also showed that if these same "immune" macrophages were killed by repeated freezing and thawing, they were still capable of transferring immunity. Furthermore, by treating the cell debris after freezing and thawing with 2M urea, very small quantities of specific 19S macroglobulin type antibody could be eluted. This eluted antibody was used to successfully transfer immunity passively to mice. Thus, this so-called cellular immunity may be attributable, in part at least, to cell-bound antibody. These findings have been confirmed by Mitsuhashi, Saito, Osawa and Kurashige (1967), using Salmonella enteritidis.

Whilst the role of antibody in protection against intracellular parasites has now been established, the reasons for the clear cut correlation between stimulated phagocytic cells and immunity are not so apparent. While the rate and amount of phagocytosis is determined by quantities of specific antibody present, the ultimate control of multiplication and/or elimination of an infecting organism is the function of phagocytic cells. Thus any changes in the numbers of phagocytic cells or in their activity will affect the degree of immunity.

Phagocytic cells are a heterogeneous population. It has been demonstrated in experiments involving excess numbers of macrophages with respect to the numbers of bacteria, under conditions where antibody levels were not limiting, that some bacteria will survive and, in time, multiply within the macrophages (Jenkin and Benacerraf, 1960; Mackaness, 1960; and Jenkin, Rowley and Auzins, 1964). The heterogeneity observed in a normal macrophage population appears to be reduced in populations of stimulated macrophages obtained from mice that have previously been injected with lipopolysaccharide or certain living attenuated bacterial vaccines such as B.C.G. and various Salmonellae (Rowley, 1959; Jenkin and Palmer, 1960; Jenkin and Benacerraf, 1960; Cohn and Morse, 1960). Whether this reduction in heterogeneity is due to an increase to higher levels of activity of individual macrophages or due to the production of a more homogeneous, active population of phagocytes is not clear from these experiments, since they all involve the study of populations and not individual phagocytic cells. Therefore, in an attempt to answer this question, McIntyre, Rowley and Jenkin (1967) studied single cell - bacterial interactions in microdroplets using a micro-manipulator. These workers examined the interactions of mouse peritoneal macrophages and Salmonella typhimurium (virulent for mice) or Listeria monocytogenes (avirulent for mice). They concluded that macrophage populations from normal animals are heterogeneous with respect to their ability to phagocytose and kill at least the two strains of bacteria used in their study. Another conclusion reached from these studies was that little or no phagocytosis of either strain occurred in the absence of specific antibody. Normal unstimulated macrophages in the absence of antibody were incapable of ingesting the virulent Salmonella typhimurium organisms and with the avirulent Listeria monocytogenes only 5.5 per cent phagocytosis occurred. In those few instances where phagocytosis took

place in the apparent absence of antibody, the survival of intracellular bacteria was high - approximately 70 per cent. In the presence of specific antibody, 57 per cent and 32 per cent of normal macrophages were capable of ingesting the virulent and avirulent bacteria respectively, and the intracellular survival was approximately 18 per cent in both cases. That is, in the presence of specific antiserum, the percentage phagocytosis was greatly increased and the intracellular survival of ingested bacteria, greatly decreased. If mice were injected with bacterial lipopolysaccharides or living attenuated vaccines, the resultant stimulated macrophage populations were more homogeneous in that a greater percentage of cells were able to phagocytose and efficiently kill the ingested bacteria. O.75 per cent of stimulated macrophages, in the absence of added specific antibody were capable of ingesting the virulent strain and 18 per cent of the cells took up the avirulent strain. Again the percentage survival of ingested bacteria was high - 60 per cent and 42 per cent respectively. However, in the presence of specific antibody the stimulated macrophages were capable of 51 to 62 per cent and 57.8 per cent phagocytosis of the virulent and avirulent strains respectively, and of these ingested organisms only approximately 10 per cent survived in both cases. The results presented in this study indicate strongly that functional heterogeneity of macrophage populations does exist in vivo. It follows that the factors which alter the phagocytic and bactericidal properties of macrophages will affect the ultimate resistance or susceptibility of the host. In other words, it has now become apparent that immunity to both intracellular and extracellular parasites is determined by the levels of specific antibody present and the ability of phagocytic cells to engulf and kill the invading organism. However, in the presence of specific antibody, normal unstimulated phagocytic cells are capable of controlling and eliminating infections of

extracellular parasites, whilst in the case of intracellular parasites, infection is controlled and the infecting organism eliminated more efficiently by a stimulated or more homogeneous active population of phagocytes.

The immune state, dependent upon the factors discussed, will determine ultimately host resistance or susceptibility. These factors which determine resistance are in turn dependent upon the genetic constitution of the host, their expression being dependent upon other factors which may broadly be termed "environmental". "Environmental" factors include any factors which have an indirect influence on the immune response, that is factors which may modify the potential immune response or the expression of immunity. These include dietary changes or deficiencies, fatigue, temperature and humidity variations, hormones, chemical and chemo-therapeutic agents, and seasonal changes.

Resistance to infection varies with changes in diet. Starvation leads to a decrease in both non-specific resistance and a decrease in the capacity to synthesise antibodies. (Schaedler and Dubos, 1956). Depletion of protein (Guggenheim, Boechler-Czaczkes and Halevy, 1951) or vitamin 'A' (Greene, 1933) have been clearly associated with lowered resistance to infection. Depletion of several of the water-soluble vitamins, especially those of the B-complex, decreases both resistance (Kligler, Guggenheim and Herrnheiser, 1946) and antibody formation (Axelrod, Carter, McCoy and Geisinger, 1947). Conversely, there appears to be no consistent evidence which suggests that an excess of any food factor (over and above the normal requirement) enhances resistance to infection. Fatigue states, whilst they do not actually pre-dispose to infection, are quite likely to activate a latent infection (Boycott and Price-Jones, 1926). Prolonged exposure to heat (Bowen, Gowen and Tauber, 1957) or cold (Levinson, Milzer and Lewin, 1945) appear to depress resistance. It is therefore

not surprising that seasonal changes may affect the general resistance of certain animal populations.

Certain chemical and physical agents may cause a lowered resistance to infection. Such is the case in the natural pathogenesis of tetanus (Bullock and Cramer, 1919). In wounds contaminated with soil, pathogenesis is aided by the presence of ionizable calcium salts which favour the germination of tetanus spores in these wounds. Also silica favours the proliferation of tubercle bacilli in tissues, and is an important cause of predispostion to tuberculosis in certain industrial populations (Gardner, 1920). Hormonal imbalance of the body in general may alter both cellular and humoral aspects of the immune response. Steroid hormones in particular may have a varying effect; there may be an enhancement of resistance or a decrease in resistance depending upon whether they are present in low or high levels respectively (Foley and Aycock, 1944, 1945).

There are factors or agents which are known to influence resistance or susceptibility to infection which are better described as experimental tools than as environmental factors. Benzene (Schnitzer, 1943) or X-rays in large doses (Taliaferro and Taliaferro, 1951), causes tissue damage, particularly to cells of the lymphoid-macrophage system which are involved in the immune response. This results in a subsequent decrease in resistance. Pharmacological agents do not affect resistance unless they are administered in concentrations large enough to decrease the numbers or activity of the cells involved in the immune response (Topley and Wilson, 1964a). Antibiotics and chemotherapeutic agents are well-known for their ability to check the course of infection caused by a particular organism. These agents do not affect host resistance directly, but rather enhance resistance by virtue of their effect on the causative organism. However, for completely effective elimination of the invading organism, it may be

essential that the host has a fully functional immune mechanism, i.e., antibiotics are an aid rather than the sole factor required for resistance and protection against subsequent infection with the same organism. This is clearly indicated by the observations that antibiotics have been shown to suppress the antibody response. Ambrose and Coons (1963) showed conclusively with their in vitro experiments that in the presence of chloramphenicol, the antibody response may be almost completely suppressed.

That genetically determined factors play the major role in determining differences in susceptibility and resistance is illustrated by the variation which exists between the individuals, firstly of different species of animals, and secondly of different strains within the same species, to the same infection under the same conditions. Most of the mammalian pox viruses have a restricted host range under natural conditions, whilst under experimental conditions the range may be wider, particularly when very young animals are used and given large doses of virus (Downie and Dumbell, 1956; Hahon and Wilson, 1960). Smallpox is an infection to which only humans are normally susceptible. However, monkeys have occasionally been found infected with variola, with manifestations of disease quite similar to those in man. Man is also the sole host naturally susceptible to poliomyelitis, although Landsteiner and Popper (1909) did transmit the virus to monkeys and produced lesions which resembled those in man. Other animals are not susceptible to the virus. Cholera too is an excellent example of a disease process due to an infection which is entirely confined to one animal species, man. Natural cholera in animals is unknown, although under experimental conditions a disease simulating cholera can be produced in new born rabbits (Topley and Wilson, 1964b; Jenkin and Rowley 1959a; Dutta and Habbu, 1955).

Salmonella typhi and S. paratyphi are natural pathogens for man and oral administration of typhoid bacilli to laboratory animals such as the rabbit, guinea pig, rat or mouse does not give rise to an infection of this type (Topley and Wilson, 1964c). However a disease resembling human typhoid fever was induced by feeding large numbers of living organisms to young chimpanzees (Edsall, Gaines, Landy, Tigertt, Sprinz, Trapani, Mandel and Benenson, 1960). A classic example of unique susceptibility to infection of a particular species of animal, is that of Myxomatosis in rabbits. In the field, rabbits are 100 per cent susceptible, whilst introduction of the myxoma virus under experimental conditions into sheep, goats, horses, pigs, cattle, dogs, cats, fowls, pigeons, ducks, man, monkeys, guinea pigs, rats, mice, ferrets, hamsters and many other animals, results in no evidence to suggest they are all anything but totally resistant (Fenner, 1959; Fenner and Ratcliffe, 1965).

The interspecies variation in susceptibility to a particular infection may be further illustrated by the Mycobacterium infections. The human strains never produce progressive disease in cattle, regardless of the inoculum size used. However, both humans and cattle are susceptible to the bovine tubercle bacillus. Guinea pigs are susceptible to both the human and the bovine strains. Monkeys and Anthropoid apes are easily infected by both human and bovine types but are completely resistant to the avian tubercle bacillus. Fowls and other domestic birds are completely resistant to mammalian-type bacilli, but susceptible to the avian strain (Topley and Wilson, 1964d). This variation is also quite apparent when pneumococcal infections are studied. Tchistowich (1890) used the eye chambers of rabbits and pigeons in his studies and showed that pigeons are totally resistant to pneumococcal infection, whilst rabbits are very susceptible. In fact, Bull and Bartual (1920) have been able to list, in order, a number of

animal species ranging from those most susceptible to those most resistant to pneumococcal infections. This order is as follows : rabbit, mouse, cat, guinea pig, sheep, man, dog, fowl and pigeon; rabbits being extremely susceptible, and pigeons totally resistant. Turkeys have been shown to be normally resistant to infection with Rous sarcoma virus, whilst chickens are quite susceptible (Harris, 1956). A final example is the difference in susceptibility of the closely related rat and mouse species to a common parasite. Mice have been shown to be more susceptible to Pasteurella pestis infection than are rats (Quan, Wheeler, Goldenberg and Kartman, 1965)

Intra species differences in susceptibility to infection of laboratory animals provide an even better illustration of the genetic control which is operative in determining resistance or susceptibility. This is especially so since the many inbred strains of animals, particularly mice, have become readily available. A large number of studies involve a variety of species including different strains of chickens which have been shown to differ in their susceptibility to Pullorum disease. White Leghorn chickens are more resistant to Salmonella pullorum infection than are Rhode Island Reds and other heavy breeds (Hutt and Crawford, 1960). Ben-Harel (1951) showed that 68 per cent of White Pekin ducks were resistant to Plasmodium lophurae infection, whilst the remaining 32 per cent had little or no resistance. Irwin (1929, 1933) using inbred, selected lines of rats, established strains which were 94 per cent and 42 per cent susceptible respectively when challenged with Salmonella enteritidis. Quan, et al., (1965), working with three strains of wild rats bred in captivity, demonstrated that the Rattus rattus strain was far more susceptible to Pasteurella pestis S-113 infection than the Rattus exulans and Rattus norvegicus strains.

By far the greatest amount of experimental effort on this subject has been directed to the mouse species, and the best evidence available is from the studies of Gowen (1960). His data shows that different strains of mice exhibit great variation in resistance to the same organism. Using a virulent strain of Salmonella typhimurium Gowen selected 10 strains of mice which varied in resistance from 84 per cent (almost complete resistance) to 0.2 per cent (almost complete susceptibility). Many other workers have demonstrated that different strains of mice differ in their susceptibility to particular infectious agents. Schell (1960) showed that Hall institute and C57 BL. mice differed in susceptibility to ectromelia. Boehme, Schneider and Lee (1959) have shown that the bacteria-susceptible virus-susceptible (BSVS) strain of mice is 100 per cent susceptible to Salmonella typhimurium infection, whilst the bacteria-resistant virus-resistant (BRVR) strain is 100 per cent resistant. Mathot, Rothen and Scher (1966) also reported a variation in inbred mouse strain susceptibility to infection with Friend virus.

There is some evidence to suggest that there are sex differences in susceptibility to infection. It appears that there are genetic factors which differ in males and females of a particular species which render females more resistant, or males more susceptible (Washburn, Medearis and Childs, 1965; Topley and Wilson, 1964e).

Whilst the evidence that immunity to infection is genetically determined is quite conclusive, the question regarding the level at which this genetic control is exerted remains unanswered. There appear to be two possible levels at which genetic control could exert its influence - viz., at the level of the phagocytic cells, or at the level of the immune response. If the immune response is the focus at which genetic control is directed then either the inductive phase or the synthesising phase may be affected.

Firstly, the genetic control of resistance or susceptibility may exert its effect at the level of the phagocytic cells, and examples of inherited defects of phagocytic function do exist. The Chediak-Higashi syndrome is inherited as an autosomal recessive trait and is reported to occur in man, mink and cattle. These animals have been shown to have defects in many cell types which results in a very low level of resistance to many infectious agents (Padgett, Reiquam, Hensom and Gorham 1968). Whilst defective phagocytic cells have not been implicated as the sole causative factor in Chediak-Higashi syndrome, they appear to be a major contributing factor. However, there is a syndrome which occurs in children and which is due to a functional deficiency in phagocytic cells. Fatal Granulomatous Disease is inherited as an autosomal recessive trait and children suffering from this disease possess monocytes and polymorphonuclear leucocytes which are unable to efficiently kill certain kinds of ingested bacteria (Rodey, Park, Windhorst and Good, 1969). These deficiencies in genetic control result in the generalized susceptiblity of an animal to infection by a wide range of organisms. However, as we have seen from the examples cited, the variations in resistance or susceptibility between species and between different strains within the same species of animals, are highly specific and restricted to one organism or one group of cross-reacting organisms. Therefore, it is unlikely that these specific variations in resistance could be due to a deficiency in a general mechanism of defense such as the phagocytic cells.

It appears probable therefore that the genetic control of resistance or susceptibility is exerted at the level of the immune response, that is, that whether an animal is resistant or susceptible to a particular infection is dependent on the ability of that animal to synthesise specific antibody. Furthermore, it follows that natural resistance to a particular organism is

due to a difference in the amount of pre-existing "natural" antibodies, or to a more rapid immune response after infection, or both.

Schell (1960b) has shown that resistance to ectromelia virus infection is inherited as a single, autosomal, dominant factor. In comparing the resistance of two strains of mice to infection with ectromelia virus, Schell (1960a) was able to show that the resistant mouse strain was able to synthesise more antibody more rapidly than the susceptible strain. Since the resistant strain was 100 per cent resistant and the susceptible strain 100 per cent susceptible, these experiments indicate that where differences in the immune response exhibited by different strains, or different species of animals occur, these differences are not absolute. The implication is therefore, that even susceptible animals possess some immuno-competent cells capable of producing insufficient quantities of antibody to a particular parasite, compared with resistant animals which produce sufficiently large amounts of antibody to resist infection. Thus the genetic control does not appear to determine the presence or absence of certain immuno-competent cells. However, the actual number of immuno-competent cells present may be genetically determined.

Finally, genetic control of resistance or susceptibility may influence the inductive phase of the immune response. Since macrophages have been strongly implicated as playing an important role in the induction of the immune response by virtue of uptake and/or processing of antigen (Fishman and Adler, 1963; Askonas and Rhodes, 1965), then it is possible that genetic control may be exerted at the level of the macrophage.

An animal may well be capable of mounting an immune response, but this will depend on the form in which the antigen is presented. There is evidence to suggest that an antigen must first be metabolised or reduced to a form which is immunogenic. Uhr and Weissman (1965) suggest that

with some antigens such as bacteriophage, degradation within phagosomes is a necessary prerequisite if antibody formation is to occur. This being the case, an animal may be susceptible to a particular infection by virtue of the inability of its macrophages to handle the particular antigen. Alternately, resistant animals possess macrophages which can process antigen very efficiently. However, as has already been mentioned, specific antibody is necessary for phagocytosis to occur efficiently. Therefore, the inability to handle antigen may be only an apparent one due to a relative lack of immuno-competent cells which are suggested to be the source of "natural" opsonins (Jerne, 1955).

Induction of the immune response not only means the stimulation of lymphoid cells to synthesise antibody in various amounts, but also includes the possibility of inducing tolerance, that is, a state of specific unresponsiveness in which no antibody formation occurs. Since Mitchison (1964) and Shellam and Nossal (1968) have shown that active antibody production and tolerance can be induced simultaneously, and that antibody production or tolerance depends on the effective antigen dose, then it is possible that the ability to produce antibodies may be genetically controlled by factors which are dose-dependent.

Repeated attempts in the past to demonstrate immune responses to synthetic polypeptides composed exclusively of D-amino acids in several animal species have met with failure (Maurer, 1963, 1965; Ben-Efraim, Fuchs and Sela, 1967). That these observations were a manifestation of a dose-dependent effect was proven by Janeway and Sela (1967). They showed that whilst the primary response of mice to the D-isomers was highly dose-dependent, the antibody response to the L-isomers was largely independent of dose. Janeway and Humphrey (1968) were able to explain this phenomenon by showing that following footpad injection the D-polymer

was retained in draining lymph nodes and spleen 200-1000 times more efficiently than was the L-polymer. Furthermore, they showed that the D-polymer was catabolised 22 times more slowly than the L-polymer, thus giving the former a much greater capacity to induce tolerance.

Sobey, Magrath and Reisner (1966), described a strain of mice (Sobey mice) which were defective in their ability to form antibody to bovine serum albumin (BSA). They concluded that the unresponsiveness of these mice was genetically controlled by two or three genes. However, Hardy and Rowley (1968) examined these Sobey mice for their ability to respond to BSA over a wide range of doses and found that these so-called unresponsive mice could produce anti-BSA antibodies but that this ability to respond was dose-dependent. They showed that with low doses of BSA both Sobey mice and Swiss White mice produced similar amounts of antibody. Using a standard (higher) dose of BSA however, the Swiss White mice produced antibody but the Sobey mice were paralysed and produced no antibody. Furthermore, if heat aggregated BSA was used instead of soluble BSA, then both strains of mice responded equally well regardless of the dose used.

It has also been demonstrated that protection against infection can be dependent on dose. Auzins and Rowley (1969) demonstrated a difference in the immune response of Swiss White and Balb/c strains of mice when injected with a standard dose of acetone-killed Salmonella typhimurium C5 to organisms which possess the O-somatic antigens 1, 4, 5 and 12. Only the Balb/c strain failed to respond to antigen 5, and their response to the O-somatic antigens 1, 4 and 12 whilst of the same magnitude was less prolonged than in the Swiss White mice. On the other hand, Jackson and Jenkin (1969) showed that Balb/c and Swiss White mice could be protected against Salmonella typhimurium C5 infection by prior immunization with

O-acetylated galactan. This protection was due to the stimulation of antibody production against O-acetylated galactan which has been shown to cross-react with antigen 5 of Salmonella typhimurium C5 (Jenkin, Karnovsky and Rowley 1967). However, 1 mg. per mouse was required to protect Balb/c mice compared with 200 μg per mouse for Swiss White mice. Therefore, it is probable that antibody against antigen 5 could be produced by Balb/c mice if the correct dose of Salmonella typhimurium C5 vaccine was administered.

These studies suggest that if one could determine the correct dose, most animals - even so-called unresponsive animals - could be made to respond to most antigens. This evidence is again suggestive of the fact that species differences in resistance or susceptibility are not absolute and that even so-called non-responder animals possess some immunocompetent cells capable of producing some antibody.

Since the failure or apparent failure of some animals to respond is limited to one antigen or one group of cross-reacting antigens, it is tempting to suggest that the reason is due to the inability of macrophages to handle that antigen properly. Furthermore, it may be suggested that an important function of macrophages is to handle -"process" or "degrade" an antigen and in so doing alter the antigenic dose in such a manner that it is effective in inducing antibody synthesis rather than tolerance or no response at all. With the implication that macrophages play the major role in induction of antibody formation, the possibility exists that genetic control of the immune response is exerted at the level of the macrophage. Again, however, the actual mode of this control is not clear.

It is apparent from the examples cited and it has been suggested by Sokhey and Chitre (1937) that genetically controlled resistance is important even under natural conditions, that is, in normal animals. Therefore an

important factor which bears careful consideration in a discussion on the development of the immune state is the presence or absence of antibody in the sera of normal animals and the role this antibody plays in immunity.

Burgi (1907) is credited with first reporting the presence of naturally occurring agglutinins, bactericidins and other sensitising antibodies, and many reports of reactions between a variety of bacteria and natural antibodies are reviewed by Skarnes and Watson (1957) and Shilo (1959). It appears that most if not all sera from "normal" animals examined, have been shown to contain natural antibodies (Bailey, 1923, and Springer, Horton and Forbes, 1959).

The origin of natural antibodies is far from certain and in fact there are two distinct schools of thought concerning the origin of this type of antibody. One school proposes that all natural antibodies are the result of a prior antigenic stimulus. This antigenic stimulus may be in the form of a particular antigen which possess specific antigenic determinants or the stimulus may come from some other cross-reacting molecule which shares a determinant group or groups with it. In fact there is a great deal of evidence that many antigenic determinant groups are shared among many bacteria, animal and plant tissues (Jenkin, 1963). The opposing school of thought argues, that since natural antibodies may be invariably present and persist with little or no change throughout the life of an animal, both the level and the specificity of these antibodies, particularly the isohaemagglutinins, are independent of a prior antigenic stimulus and are under direct genetic control (Bernstein, 1930; Hirszfeld, Kernshaw, cited by Boyden 1966).

With regard to the specificity of natural antibodies, Mackie and Finkelstein (1930, 1931, 1932) and Finkelstein (1933) tested the sera of a large number of normal animals such as the ox, sheep, horse, pig,

rabbit, rat, cat, guinea pig, pigeon and man for bactericidal activity against a variety of bacteria including Vibrio cholera, Salmonella species, dysentry bacilli, proteus bacilli, influenza bacilli, Brucella abortus and Brucella melitensis and meningococci. In a large number of cases they demonstrated bactericidal activity which was dependent upon the joint action of complement and a heat stable factor in serum which could be removed by absorption. This absorption specifically removed the bactericidal activity against the homologous organism but had no effect on the bactericidal activity of the serum against other organisms - this remained intact at the preabsorption level. There is further evidence which suggests that the antibody found in the serum of normal animals is no less specific than acquired antibody. Svehag (1964) has shown that the physico-chemical characteristics of natural 19S anti-polio antibody were the same as those of early acquired 19S antibody.

The role of natural antibodies in promoting phagocytosis of bacteria has been thoroughly investigated. It has been shown that antibodies in the sera of some normal animals are opsonic and enhance the phagocytosis of virulent bacteria not normally readily phagocytosed. Furthermore, it has been shown that naturally occurring antibodies are important in determining the fate of ingested bacteria (Jenkin and Rowley, 1959; Jenkin and Benacerraf, 1960; and Rowley and Jenkin, 1962).

Jerne (1955) and Boyden (1960) have both proposed theories of antibody formation which implicate preformed naturally occurring antibody as the mediator of the primary antigenic stimulus. The antigen-antibody ratio must be such that there is an antigen excess in order that antibody synthesis be stimulated. In antibody excess there will be a depression of antibody synthesis (Rowley and Fitch, 1961; Uhr and Baumann, 1961; Finkelstein and Uhr, 1964; Moller and Wigzell, 1965; Dixon, Jacot-

Guillarmod and McConakey, 1967). Whilst it has been shown that the presence of specific antibodies may have a depressive effect on the immune response, this inhibition is dependent on the quantities of antibody present. Henry and Jerne (1968) have ably demonstrated that the effect of antibodies on the primary immune response was not always negative. Although they confirmed that specific 7S antibody suppresses the primary immune response, they demonstrated that specific 19S antibody enhanced the primary response of mice to sheep red blood cells. The 19S antibody was given in equal molar amounts to the dose of the 7S antibody which was suppressive. These results confirm the reports of Pearlman (1967) who states that whereas 19S and 7S antibodies were effective inhibitors of antibody formation, smaller amounts of 19S antibody were capable of enhancement. Also Segre and Kaeberle (1962) have found that specific antibody and "normal" gamma-globulin both enhance the antibody response of newborn piglets deprived of colostrum.

Finally, Jenkin and Rowley (1959) showed that the serum of many normal animals was protective. Pretreatment of virulent Salmonella typhimurium organisms with these sera was effective in preventing the otherwise lethal effects of injecting these organisms (unopsonised) into susceptible animals.

Whilst antibody and cells have both been strongly implicated as determinants of resistance or susceptibility, it appears that specificity as determined by the antibody molecules is of particular importance in deciding the progression of infection. Upon injection with a particular parasite, antibodies will be produced against all available and different antigenic determinants. The possibility exists that there could be several antigenic determinants present and assay of antibody against one or other of these, independently of the rest, is difficult. Also it is often not clear

whether antibody directed against one particular antigenic determinant is more capable of conferring protection than antibody directed against the other determinants on the same bacterium. Hence the reason that attempts to correlate antibody levels with resistance have met with only limited success. Another difficulty is that the level of antibody production may vary in different strains or species of animal. It has in fact, been shown that a difference in character of the antibody response may be produced to the same chemically defined antigen (Kantor, Ojeda and Benacerraf, 1963; Maurer, 1963; McDevitt and Sela, 1965).

In order to overcome such difficulties, many studies carried out to show that the immune response is under genetic control and the mode of its inheritance have utilized simple antigens. The most commonly used antigens are either chemically defined synthetic polypeptides, or simple haptens such as Dinitrophenol (DNP) conjugated to proteins or synthetic polypeptides. The studies of McDevitt and Tyan (1968) and of Fink and Quinn (1953), indicate that the ability of mice to respond to such antigens as multichain synthetic polypeptides, egg albumin and pneumococcal polysaccharides are definitely under genetic control. McDevitt and Tyan (1968) have also shown that this genetic control is directly related to the process of antibody formation and can be localized to spleen cells, since they can transfer the ability to respond, from high responder to irradiated low responder recipient mice. Carlinfanti, (1947) has studied the ability in man to produce iso-antibodies. By comparing mothers' and fathers' titres with those of their children Carlinfanti deduced that the correlation approximates closely to the theoretical O.5 expected under complete genetic control. This was the case for both anti-'A' and anti-'B' iso-antibodies. Sobey, et al., (1966) have presented evidence which suggests that in rabbits and mice at least,

a relatively simple genetic mechanism controls their ability to produce antibodies to bovine serum albumin. Backcross experiments with non-responding rabbits suggest that unresponsiveness is genetically controlled by two or three genes. Similar results were obtained with mice.

Whilst the variation in susceptibility of animal species to infection is undoubtedly due to differences in their genetic constitution, the actual mode of the genetic control is at the present time still in doubt. Irwin (1929, 1933) working with inbred lines of rats varying in susceptibility to Salmonella enteritidis, concluded that multiple genes were operative and that differences in resistance and susceptibility were controlled by at least partially dominant genetic factors. Schott (1932), working with mice, reached similar conclusions. However, it has since been demonstrated that susceptibility may be inherited either as a dominant or a recessive trait. Susceptibility to pseudo-rabies virus in one strain of mice showed a tendency to be dominant in F1 cross with resistant mice, comparison of a pseudo-rabies-resistant strain of mice with a strain susceptible to mouse typhoid revealed susceptibility to the latter disease to be a recessive trait in the appropriate F1 cross (Bang and Warwick, 1960; Kantock, Warwick and Bang, 1963). These workers concur with the belief that in some cases resistance is dominant, whilst in cases such as mouse hepatitis, susceptibility is the dominant heritable trait. The conclusion is that while genetic constitution is related to resistance to infection, it is best regarded as a composite of several genes, some favouring resistance, and others susceptibility, depending upon the infectious agent (Gowen and Schott 1933a, 1933b).

Webster (1937) by cross-breeding two strains of mice, namely bacteria resistant virus-susceptible (BRVS) and bacteria-susceptible virus-resistant (BSVR), was able to produce a strain which was highly

resistant to infection with various Salmonellae and with St. Louis encephalitis (BRVR). These experiments revealed the pattern of inheritance of resistance to both the bacteria and the virus. Each factor was found to be inherited independently of the other, and as a single Mendelian dominant. The relative quantity of antibody produced to the 'H' and 'O' antigens of Salmonellae by different strains of mice is also thought to be inherited in such a manner (Gorer and Schutze, 1938). Resistance to Yellow Fever in mice is also inherited as a dominant genetic trait. Sabin (1952) observed that the character for susceptibility segregated in the F2 generation and in backcrosses from the susceptible F1 hybrids to the resistant mice.

Schell (1960b) showed by direct measurement of neutralizing antibody, hypersensitivity reactions, and of actively acquired immunity in mousepox-resistant C57 BL. mice, that they responded more effectively than did a mousepox-susceptible, random bred strain of mice. The differences in antibody responses and therefore of resistance in general, were inherited as a single, autosomal, dominant factor, and the C57 BL. mice were homozygous in this respect.

Stern and Davidsohn (1954, Davidsohn and Stern (1949a, 1949b, 1954, have also implicated genetic factors as being responsible for the presence and titre of natural agglutinins, as well as for antibody production in mice. The antibody responses of two inbred strains of mice to two synthetic antigens have also been shown to be under determinant specific quantitative genetic control (McDevitt and Tyan, 1968). The different degrees of resistance and susceptibility demonstrated in mice by Gowen (1960), may be attributed to a number of genes affecting the different factors responsible for resistance or susceptibility to the organism involved. Particular combinations of these genes result in either a high

or low level of resistance.

Studies with artificial antigens, synthetic polypeptides prepared using either the D or L optical isomers of amino acids or both, with and without conjugated haptens, have resulted in the widely accepted hypothesis that the factors controlling the immune response are genetically transmitted as a unigenic, autosomal, Mendelian dominant trait. The precise nature of the qualitative control of the immune response is, however, unknown, (Levine, Ojeda and Benacerraf, 1963a, 1963b; McDevitt and Sela, 1965; Ben-Efraim, et al., 1967). However, recent studies, again using synthetic antigens, indicate that in some cases genetic control is transmitted as a complex mechanism which is both dominant and polygenic (McDevitt and Sela, 1967; Simonian, Gill and Gershoff, 1968).

Genetically controlled factors - antibody and cells - are clearly responsible for determining resistance or susceptibility to infection. As a general rule host resistance or susceptibility is limited to one organism or one group of cross-reacting organisms. Thus in a study of any specific example of species or strain variation in susceptibility to infection, the question which most often remains unanswered is - at what level of the immune response is the resistant animal able to respond more effectively than the susceptible animal?

CHAPTER II

MATERIALS AND METHODS

Bacterial strains:

(1) Salmonella typhimurium C5:

This smooth strain is virulent for mice (LD50 = 100 organisms). (Furness and Rowley (1956). However, it was found to be avirulent for rats.

(2) Salmonella typhimurium M206:

This smooth strain is avirulent for mice (LD50 = approximately 10^6 organisms). This organism was described by Jensen (1929) and Furness and Rowley (1956), and was found to be avirulent for rats.

(3) Salmonella enteritidis Se 795:

This is a smooth strain isolated from fur seals and described by Milner, Jellison and Smith (1957). This organism is virulent for mice but avirulent for rats, and was kindly supplied by Dr. Nancy Atkinson, Department of Oral Biology, Dental School, University of Adelaide.

(4) Salmonella enteritidis 11RX:

This rough strain is avirulent for both mice and rats. This organism was described by Ushiba, Saito, Akiyama, Nakano, Sugiyama and Shirono (1959).

(5) Streptococcus pneumoniae type II:

This is a smooth strain, highly virulent for mice, (LD50 = approximately 50 organisms), and was kindly supplied by Mr. R. G. Bateman, Department of Microbiology, University of Adelaide.

(6) Escherichia coli 2380:

A smooth strain described by Rowley (1954).

Species and strains of animals used:

All animals were kept in air-conditioned rooms, maintained at 22°C, in metal cages. Food and water were supplied ad libitum. The two species of animals used in this study were:-

(1) Rats:

These were of the inbred BN. strain which originated from a brown mutation first described by Silvers and Billingham (1958). These animals were originally obtained from Dr. W. K. Silvers of the Wistar Institute, Philadelphia, Pennsylvania, U.S.A. and were maintained in this department by strict brother sister mating. The body weight of rats used in most experiments, was in the range of 180-280 gm. In any one experiment the variation in body weight of animals used did not vary by more than approximately 20 gm. (Fig. 2.1)

(2) Mice:

These were of the Swiss White strain bred as a closed colony at the Waite Agricultural Research Institute of South Australia. The body weight of mice used in most experiments was in the range of 18-24 gm. In any one experiment the variation in body weight of animals used did not vary by more than approximately 2 gm. (Fig. 2.1).

Maintenance of bacterial strains

Each bacterial culture was checked for uniformity prior to use and prior to storage for further use. Each culture was subcultured into nutrient broth and incubated at 37°C overnight. To verify its purity, a loopful of this culture was streaked onto a dried nutrient agar plate and this was again incubated overnight. If the colony form was uniform a single colony was picked

FIG. 2.1

Photograph showing a rat of the inbred BN strain and a mouse of the Swiss White strain used in this study

off and used to inoculate a nutrient broth. This was grown on a shaker at 37°C for 18 hours. The resultant culture was centrifuged for 30 minutes at 3020 x g and the supernatant decanted. To the deposit a small volume of sterile milk was added and a thick suspension made by agitating with a sterile pasteur pipette. (Prior to use the milk had been centrifuged to separate the cream which was subsequently removed. The milk was then sterilized by autoclaving at 15 lbs/sq.in. pressure for 20 minutes). Approximately 0.5 ml. of the bacterial suspension was placed into the required number of sterile 4 x $\frac{1}{4}$" freeze drying ampoules, (Johnsen and Jorgensen, Ltd., London) and the end of each ampoule plugged with cotton wool. The cultures were then lyophilized in a Speedivac Centrifugal Freeze Drier Model 5 PS (Edwards High Vacuum, Ltd., Sussex, England). Briefly, the method was as follows - the ampoules were centrifuged whilst the pressure was reduced to approximately 200 microns. The ampoules were then held at that reduced pressure over phosphorous pentoxide for 6 hours. When most of the moisture had been removed the vacuum was released and the cotton wool plugs were pushed about three-quarters of the way down the ampoules. After a constriction had been made about half-way along each ampoule, the ampoules were evacuated to a partial pressure of 30 microns and held at that pressure over dry phosphorous pentoxide for 16 hours. Each ampoule was then sealed at the constriction without releasing the vacuum. The freeze dried cultures were labelled and stored at room temperature.

When a particular bacterial strain was required, the appropriate ampoule was opened and the contents suspended in several drops of nutrient broth using a sterile pasteur pipette. The contents were transferred to a bottle of nutrient broth and grown on a shaker at 37°C

for 18 hours. This culture was again checked for uniformity of colony form prior to use. A single colony was used to inoculate another nutrient broth which was grown and used to streak onto nutrient agar slopes in one ounce screw capped bottles. These were incubated overnight at 37° C and stored in the dark at room temperature. Each slope was used for subculture only 10 times. Fresh slopes were prepared from freeze-dried cultures every 3 months.

P^{32} - labelling of bacteria

The technique used to label Salmonella typhimurium C5 with P^{32} was described by Benacerraf, Sebestyen and Schlossman, (1959). The organisms were labelled by allowing them to multiply in a supplemented casamino acid medium to which P^{32} was added as orthophosphate. The medium used consisted of:-

Sodium citrate	0.1 gm.
$Mg\ SO_4 .\ 7\ H_2O$	0.02 gm.
Glucose	0.4 gm.
Casein hydrolysate	2.0 gm.
Distilled water	200 ml.

After the pH of this medium had been adjusted to pH 7.0, it was autoclaved at 15 lbs./sq. inch pressure for 20 minutes.

To 50 ml. of this medium, 1 millicurie of P^{32} as orthophosphate was added and the medium was inoculated with 0.1 ml. of a log phase shaker culture of the organisms in nutrient broth. This was placed on a shaker at 37°C for 18 hours and the resultant culture of bacteria was deposited by centrifugation at 3020 x g for 15 minutes. The deposit was resuspended and washed three times with 50 ml. of physiological saline. Finally the labelled bacteria were resuspended in a small, known volume of casamino acid. The number of organisms/ml. in this

suspension was calculated from optical density measurements at 650 milli-microns wavelength, after calibration with a suspension on which viable bacterial counts had been performed. The suspension was then diluted with casamino acid to give a suspension containing 1×10^9 organisms/ml. This was stored at 4°C and was used for no longer than 5 days after preparation.

If the radioactively labelled bacteria were not used on the day of preparation, the required volume of the suspension was centrifuged at 3020 x g for 15 minutes and the bacteria resuspended in the original volume of casamino acid medium. This was done to ensure that any radioactive label not associated with bacteria was removed.

Opsonisation of bacteria

Equal volumes of bacteria (approximately 10^9 organisms/ml.using P^{32}labelled bacteria) and the serum to be investigated were mixed and incubated at 4°C for 20 minutes. The bacteria were then deposited by centrifugation at 3020 x g for 10 minutes and the deposit washed three times with 20 ml.of physiological saline. The washed, opsonised bacteria were resuspended and suitably diluted for immediate use. During each opsonisation procedure a check was made for any agglutination of bacteria and any reduction in viability of the organisms. For most experiments using unlabelled bacteria, a nutrient broth culture grown at 37°C for 18 hours on a shaker was used.

Bacterial clearance studies

All bacterial clearance studies using isotopically labelled bacteria were carried out in normal Swiss White mice. After opsonisation of radioactively labelled bacteria, a 0.1 ml. volume of bacteria containing approximately 2.5×10^8 organisms was injected intravenously into mice. These mice were bled from the retro-orbital venous plexus with a

calibrated glass pipette, according to the method of Biozzi, Benacerraf and Halpern (1953). 0.02 ml. blood samples were taken from each mouse 1, 2, 3, 5 and 10 minutes after injection of the bacteria and immediately placed on plastic-backed absorbent paper discs. These discs were cut 3 cm. in diameter to fit metal planchette holders. The amount of radioactivity in each sample was then assayed using a thin end window Geiger counter installed in a Nuclear Chicago automatic sample changer C 110 A, (Nuclear Chicago, Chicago U.S.A.), and with an automatic timer C 111 coupled to a model 183 scaling unit to record the results. This apparatus was modified by the insertion of a General Electric helium-filled thin-window Geiger counter tube, (General Electric Co., Schenectady, New York), which increased the sensitivity of the machine threefold.

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. Three or five mice were used to assay each serum dilution.

In vitro bactericidal assay for quantitation of antibody

The technique used was essentially that of Landy, Michael and Whitby, (1962), with minor modifications. Fresh guinea pig serum was used as a source of complement. Immediately prior to each experiment this serum was absorbed at $0^{o}C$ with approximately 10^{9} living organisms/ml. of the strain to be used in the bactericidal assay. This procedure removed any specific antibodies present in guinea pig serum. In the present study the organism used was the M206 strain of Salmonella typhimurium.

After absorption, the serum was centrifuged to remove the bacteria and sterilized by filtration through an 0.8 millimicrons membrane filter (Millipore Corp., Bedford, Massachusetts), and was diluted 1:5 with

0.15 M phosphate buffer at pH 7.4 containing 0.02 per cent Mg Cl_2. The test organism was also diluted in this buffer to a concentration of approximately 2 x 10^4 organisms/ml. Equal volumes of absorbed guinea pig serum and the bacterial suspension were mixed and kept in bulk at 0^oC. The assay tubes were arranged to contain 1 ml. aliquots of a series of 10-fold dilutions of the serum to be assayed. These dilutions were performed in 0.15 M phosphate buffer at pH 7.4 at 0^oC. An equal volume of the absorbed guinea pig serum - bacterial suspension mixture was then added to each tube. Thus each assay tube contained a final dilution of guinea pig complement of 1:20, a final concentration of approximately 5 x 10^3 test organisms and test antiserum serially diluted to cover a range of 10^{-1} to 10^{-9}. Two control tubes were included with each assay:

(a) A tube containing only the phosphate buffer used as the suspending medium and the same number of bacteria added to each "test" tube. Any adverse effects of this medium on the viability of the test organism over the period of assay, would be made evident by a decrease in numbers of bacteria in this tube.

(b) A tube containing only the absorbed serum which was used as a source of complement, plus bacteria. This control was included to ensure that the serum used as a source of complement had been adequately absorbed and did not contain any antibodies specific for the test organism.

Viable counts were performed at this stage by taking 0.1 ml. samples from each tube and plating these on dried nutrient agar plates. The assay tubes in racks were then transferred to a 37^oC waterbath for two and a half hours. After this time viable counts were again taken by taking 0.1 ml. samples from each tube and plating on dried nutrient agar plates. All the agar plates were then incubated at 37^oC for 18 hours and colony counts made. The end point of each antiserum assayed was taken as that dilution of

antiserum which, in the presence of complement, produced 100 per cent killing of the inoculum of test organisms.

In vivo bactericidal assay for quantitation of antibody

The in vivo assay technique used in this study was essentially similar to that described by Whitby and Rowley (1959) with minor modifications. The organism used in this assay was the C5 strain of Salmonella typhimurium. Using an 18 hour nutrient broth culture containing approximately 2×10^9 organisms/ml the organisms were washed three times in sterile physiological saline and finally resuspended in this medium to contain approximately 2×10^5 organisms/ml.

The antiserum to be assayed was diluted in 2-fold steps, also in physiological saline, at 4°C and used to opsonise the bacteria as previously described in this chapter. The opsonised organisms were washed and resuspended to approximately 10^5 organisms/ml. Groups of mice were injected intraperitoneally with 0.2 ml of this suspension containing approximately 2×10^4 organisms. Three mice were sacrificed by cervical dislocation at each of the following times:- 0, 15, 30, 45, 60 and 90 minutes. The peritoneal cavity of each mouse was washed out with 1.5 ml of sterile saline and viable counts performed on each washout sample. This procedure was carried out using increasing dilutions of an antiserum until little or no killing was observed. The end point was taken as the highest dilution of antiserum which produced 75 per cent or greater killing of the bacterial inoculum in 90 minutes. On each day that this assay was performed a control experiment was carried out using unopsonised Salmonella typhimurium C5 organisms to show that these were not killed in the peritoneal cavity of normal mice.

Recovery and storage of immune and normal sera

(a) Rats

The rats were lightly anaesthetised with ether and their blood taken by cardiac puncture using sterile 22 gauge needles fitted to 10 c.c. syringes. The blood was collected into washed, sterile 20 ml. wide-necked McCartney bottles and stood on the bench at room temperature, for 45 minutes. By this time clotting had occurred and care was taken to see that the clot had separated from the glass. To encourage further clot retraction and ensure maximal recovery of serum, the bottles were then kept at 4°C for a further 45 minutes. After this time the serum was withdrawn with sterile pasteur pipettes and centrifuged in conical plastic tubes at 480 x g for 5 minutes. The cell-free, straw-coloured serum was separated from the red cell deposit and dispensed into labelled screw-capped 5 ml bottles in 1 ml. amounts and stored at - 20°C.

When the same animals were to be bled on consecutive days for long periods of time, heart puncture was not employed. In such situations samples of blood were obtained either by amputation of the tip of the tail or by bleeding from the retro-orbital venous plexus using a pasteur pipette.

(b) Mice

Blood samples from mice were obtained by bleeding from the retro-orbital venous plexus using a pasteur pipette. The animals were not anaesthetised. The blood was collected into 20 ml. wide-necked McCartney bottles and the serum recovered and stored in a manner identical to rat serum.

In most cases the serum from several animals was pooled and in these situations the serum consisted of the pool of no less than 5 mice or 3 rats from each experimental group. In all cases the serum was dispensed into small quantities and frozen until use. Each serum sample was frozen

and thawed only once.

Reduction of serum samples with 2-mercaptoethanol

The serum samples to be reduced were diluted 1:2 with an equal volume of 0.2M mercaptoethanol in phosphate buffer at pH 7.0. The mixture was then incubated for one hour at 37°C. Where the treated serum samples were to be used in biological assays, the mercaptoethanol was removed by overnight dialysis against a 500-fold (v/v) excess of phosphate buffer at pH 7.0.

Preparation of alcohol-killed vaccine

The technique used was essentially that of Auzins (1968) with minor modifications. To a known volume of a washed saline suspension of stationary phase Salmonella typhimuium C5 organisms, a 5-fold excess of 70 per cent alcohol pre-chilled to 4°C was added. After thoroughly mixing, the suspension was stood at 4°C overnight then washed three times with sterile physiological saline, and resuspended in sterile saline to a concentration of approximately 10^{10} organisms/ml. To check that all organisms had been killed, multiple 0.1 ml. samples were spread onto dried nutrient agar plates and incubated at 37°C overnight. If the vaccine proved to be sterile, its dry weight was determined and the concentration of the suspension standardized. The vaccine was then dispensed into small volumes in 5 ml. screw capped bottles and stored at - 20°C. Each sample of vaccine was thawed only once and any remaining vaccine was discarded.

Preparation of lipopolysaccharides

All the lipopolysaccharide used in this study was prepared using the phenol/water extraction technique described by Westphal, Luderitz and Bister (1952) using whole organisms. The lipopolysaccharide was purified by alcohol precipitation and high speed centrifugation.

Preparation of polysaccharides

The technique used was essentially similar to that described by Staub (1965). The only variation from this technique was that instead of using acetone dried and powdered bacteria as the starting material, organisms harvested from Roux bottles, washed three times in distilled water and resuspended in distilled water to the appropriate concentration, were used.

Alkali treatment of lipopolysaccharide

Sufficient sodium hydroxide was added to lipopolysaccharide at a concentration of 1 mg/ml. to give a final concentration of 0.02 N NaOH. The solution was incubated at 37°C for 5 hours and then the pH was readjusted to 7.4 with 1 N hydrochloric acid. The volume of this solution was determined and the concentration of lipopolysaccharide standardized to 500 μg/ml.

Sensitisation of Sheep red blood cells

(a) Using lipopolysaccharide

The technique used was essentially that of Crumpton et al., (1958). Sheep red blood cells were washed three times with physiological saline and resuspended to a concentration of 5 per cent v/v in physiological saline. Equal volumes of these cells and a 100 μg/ml. solution of lipopolysaccharide were mixed thoroughly in a roller culture tube with a screw top. The suspension was then incubated at 37°C for 2 hours on a roller apparatus. The red cells were deposited by centrifugation and again washed three times with physiological saline. Finally, the cells were resuspended in saline to give a 1 per cent suspension.

When alkali-treated lipopolysaccharide was used to sensitise sheep red blood cells, the procedure was identical to that used for native lipopolysaccharide. However, the optimal concentration for sensitisation with alkali-treated lipopolysaccharide was determined to be 50 μg/ml.

(b) Using Bovine Serum Albumin

A modification of the method described by Gold and Fundenberg (1967), was used to sensitise sheep red blood cells with bovine serum albumin (BSA). The sensitisation takes place through the agency of chromium ions (Cr^{+++}) using chromic chloride as a source of these ions. Sheep red blood cells were washed three times with physiological saline and resuspended in saline to a 5 per cent v/v suspension. Twenty volumes of this suspension were deposited by centrifugation in a wide bore glass centrifuge tube and the supernatant fluid removed. One volume of a 1 per cent solution of BSA in saline was added and the deposited pellet of red blood cells was resuspended in this solution. One volume of 1 per cent saline solution of chromic chloride was then added with continuous gentle shaking. After thorough mixing, the cells were allowed to stand at room temperature (circa 22°C) for 1 hour. The sensitized cells were then diluted with 50 volumes of saline and washed three times with cold saline.

Indirect Haemagglutination technique

0.1 ml. volumes of serial two-fold dilutions of antiserum were prepared in haemagglutination trays (Baird and Tatlock, London). To each well, 0.1 ml. of a 1 per cent suspension of appropriately sensitized sheep red blood cells was added and, after thorough mixing, the trays were incubated at 37°C for 1 hour. Controls, using unsensitised sheep red blood cells, were included with each assay. The results were read after standing the trays at 4°C overnight and the end point of each assay was taken as the highest dilution of serum producing complete haemagglutination.

Tissue Culture medium

The medium used in the preparation of single cell suspensions required

for detecting antibody producing cells and for injecting into animals was Hank's balanced salt solution to which 5 per cent foetal calf serum had been added. The feotal calf serum was heat inactivated at 56°C for 20 minutes before use.

The Hank's medium was prepared according to the method described by Weller, Enders, Robbins and Stoddard (1952). Two stock solutions designated A and B were prepared.

Solution A contained:-

160 gm.	NaCl
8 gm.	KCl
4 gm.	$Mg\ SO_4 . 7\ H_2O$
2.8 gm.	$CaCl_2$.
1000 ml.	deionised distilled water

Solution B contained:-

3.04 gm.	$Na_2\ H\ PO_4 .\ 12\ H_2O$
1.2 gm.	$KH_2\ PO_4$
20 gm.	glucose
900 ml.	deionised distilled water
100 ml.	O.4 per cent phenol red solution

To prepare the phenol red solution, 1 gram of phenol red was placed in a flask and $^{N}/_{20}$ HaOH was added with shaking until almost all the powder was dissolved. More NaOH was then slowly added until all the phenol red was dissolved and the solution was a deep red colour. Deionised distilled water was added to bring the volume to 250 ml.

The two stock solutions were sterilised by autoclaving at 15 lbs/sq. in. for 20 minutes. The Hank's balanced salt solution was made up by adding 18 volumes of sterile deionised distilled water to 1 volume of stock solution

A and 1 volume of stock solution B. Before adding the heat inactivated foetal calf serum (5 per cent v/v) which was sterilised by filtration through an 0.8 milli-micron membrane filter, the medium was buffered to pH 7.4 with 0.15 M phosphate buffer which had also been sterilised by autoclaving. Fresh medium was prepared from the stock solutions each day it was required.

The Jerne Agar Plaque technique for the detection of Antibody producing cells

The technique used was identical to that described by Jerne, Nordin and Henry (1963) with one slight modification - Hank's balanced salt solution was used as the culture medium instead of Eagle's medium. Heat-inactivated foetal calf serum was used only when spleen cells of two or more animals were pooled: 10 per cent foetal calf serum was used in these instances to minimise clumping of cells. Both normal sheep red blood cells and sheep red blood cells sensitised with lipopolysaccharide were used in the assay.

The Biozzi Immunocytoadherence technique for the detection of antibody producing cells

The technique employed was identical with that described by Biozzi, Stiffel, Mouton, Liacopoulos-Briot, Decreusefond, Bouthillier, (1966). The culture medium used for preparation of single cell suspensions and suspensions of normal and sensitised sheep red blood cells was Hank's balanced salt solution to which 10 per cent heat-inactivated (56^{o}C for 20 minutes) foetal calf serum was added when cells of two or more spleens were pooled.

Sucrose Density Gradient Ultracentrifugation

In order to separate the heavy and light protein fractions of rat and mouse serum for biological analysis, the technique of sucrose density gradient ultracentrifugation was employed.

A gradient was prepared by layering 1 ml. each of 10, 25, 35 and 45 per cent solutions of sucrose (analytical grade reagent) in 1 M saline buffered with 0.15 M phosphate at pH 7.0, in 1/2" diamater x 2" cellulose nitrate centrifuge tubes (Beckman, U.S.A.) 1 ml. of a 1:3 dilution of rat serum or of a 1:2 dilution of mouse serum was layered on the top of this gradient. These gradients were placed in a Spinco SW39 rotor and centrifuged at 35,000 r.p.m. for 18 hours in a Spinco Model L ultra-centrifuge. The gradients were fractionated by puncturing the bottom of the tubes and collecting the effluent with the aid of a drop counting fraction collector (Paton Industries, Adelaide, S.A.). The protein concentration of each fraction were estimated by measuring the optical density of each fraction on a spectrophotometer, (Shimadzu, Kyoto, Japan) at 280 milli-microns and then extrapolating from a standard curve prepared by using various concentrations of BSA. The fractions were then analysed for biological activity.

Immunoelectrophoresis

(a) Preparation of antisera

To prepare rabbit anti-normal rat serum and rabbit anti-normal mouse serum, rabbits were injected with 1 ml. of serum emulsified in Freund's complete adjuvant. This was administered subcutaneously in multiple sites. One month after the subcutaneous injections, the rabbits received 2 intravenous doses of 0.5 ml. of serum each week for 4 weeks. Ten days after the final injection the rabbits were anaesthetised with Sagatal, (Pentobarbitone Sodium - May and Baker, Victoria), and bled by cardiac puncture. The serum was obtained in the manner previously described in this chapter.

(b) Immunoelectrophoresis procedure

The technique employed for the identification of serum protein fractions was essentially that described by Campbell, Garvey, Cremer and Sussdorf (1964). 0.8 per cent Ion agar (Oxoid, U.K.) in Veronal buffer of ionic strength, 0.05 and pH 8.4 was used as the supporting medium.

Folin - Ciocalteu method of Protein estimation

This method for estimation of protein is described by Kabat and Mayer (1961a). Folin - Ciocalteu phenol reagent is added to an alkaline solution of protein to produce a blue colouration. The optical density of each sample was read at 750 milli-microns wavelength using a spectrophotometer and the protein concentration of each test sample was determined from a standard curve which had been prepared using known concentrations of protein.

CHAPTER III

BACKGROUND STUDIES ON THE DIFFERENCES IN BIOLOGICAL ACTIVITY TO SALMONELLA TYPHIMURIUM C5 OF NORMAL RATS AND NORMAL MICE

INTRODUCTION

Jerne (1955, 1960) has in recent years suggested, that natural antibody may play a part in the immune response. In his Natural Selection Theory of antibody formation, Jerne (1955) suggests that the first step in the induction of the immune response is the formation of an antigen-natural antibody complex; the natural antibody being specific serum protein(s). Skarnes and Watson (1957) have ascribed antibacterial functions to naturally occurring antibody, the range of bacteria involved being quite large, but differing for serum samples from different animal species. Their review of many studies in this field supports the hypothesis that natural antibodies may be quite specific and that immunization leads only to an increase in the levels of these antibodies.

It has been observed that natural antibodies are invariably present and persist with relatively little change throughout life. It has therefore been suggested that both the level and the specificity of natural antibodies are genetically determined and that they are independent of antigenic stimulation (Hirszfeld 1926, Bernstein 1930, Kernshaw 1948, 1949 cited by Boyden 1966). However, Jerne and Nordin (1963) studying the numbers of antibody-producing cells detected in the spleens of piglets raised under normal and under germ-free conditions, conclude that natural antibody arises only after antigenic stimulation.

Whilst there is some doubt as to the origin of natural antibodies and their role in the antibody-forming system, there is no doubt that natural

antibodies are of great importance in relation to the discriminative behaviour of phagocytic cells (Boyden, 1966).

It has been clearly established that the rate of phagocytosis of Salmonella typhimurium and the subsequent intracellular killing of this organism is dependent upon the presence of serum factors which are opsonic (Rowley 1958, Whitby and Rowley, 1959 and Jenkin 1962). These opsonic factors were shown to be present in the sera of normal animals of resistant species and this led Jenkin and Rowley (1959) to conclude that any opsonic activity detected in the serum of normal animals may well be accounted for by their natural antibody content. In fact, Reade, Turner and Jenkin (1965) have shown that the opsonic factors to Salmonella typhimurium which are present in normal rat serum resemble macroglobulin antibody. Also, Jenkin and Rowley, (1959), found that the sera of normal rats and pigs which contained a great deal of opsonic activity, could reduce the lethal effects of normally virulent Salmonella typhimurium if these organisms were incubated with either of these serum samples prior to injection into mice. That is, these workers showed that they could passively transfer protection to mice against virulent Salmonella typhimurium by opsonising these organisms with normal rat or normal pig serum. However, at no time were they able to demonstrate that normal mouse serum was opsonic, nor were they able to passively transfer protection to mice with it. Whilst the mice used in the above mentioned studies were the same as those used in the present study, the rats used were of the outbred Hooded Wistar strain.

In order to determine whether the Salmonella typhimurium - resistant rat species did contain natural antibodies in their serum, studies were undertaken to obtain some background information about normal rat serum and to compare this with similar studies using normal mouse serum.

Experiments were designed to investigate the opsonic and other biological activities of normal rat and mouse serum and to characterise the factor(s) responsible for any biological activities detected. Since Reade (1964) has suggested that the natural antibody present in normal rat serum may already be synthesised during foetal life, it was also decided to examine normal rats and mice for the presence of antibody-producing cells, and to attempt to relate the numbers of these cells to the levels of natural antibody detectable in the serum of these two species of animals.

The techniques used to determine the basic differences existing between normal rats and normal mice were as follows:-

1. determination of LD50 doses of the virulent C5 strain of Salmonella typhimurium in rats and mice;

2. determination of antibody levels in the sera of normal rats and normal mice using various techniques including -

   (a) the indirect haemagglutination assay using normal sheep red blood cells and sheep red blood cells sensitised with lipopoly-saccharide extracted from Salmonella typhimurium C5 (C5 LPS).

   (b) the intravenous clearance rate determination of radio-actively (P^{32}) labelled Salmonella typhimurium C5 in mice.

   (c) the in vivo bactericidal assay.

   (d) the in vitro bactericidal assay;

3. investigation of the cellular aspects of the immunological apparatus of normal rats and normal mice including -

   (a) comparison of the in vitro phagocytic capacities of peritoneal macrophages from normal rats and normal mice in the presence and in the absence of serum.

   (b) determination of numbers of antibody-producting cells in the spleens of normal rats and mice using the Jerne plaguing technique (Jerne et al., 1963) and Biozzi immunocytoadherence technique (Biozzi et al., 1966)

Determination of LD50 doses

It has been established that most laboratory strains of mice are highly susceptible to infection with Salmonella typhimurium, whilst rats are resistant (Rowley and Jenkin, 1962). Therefore, it was essential that the degree of susceptibility and resistance respectively, of the mice and rats used in this study, to the C5 strain of Salmonella typhimurium be determined. To do this a wide range of doses of these organisms from a nutrient broth culture grown on shaker for 18 hours at 37°C, was injected into large groups of mice and rats. The dilutions were performed in sterile saline and the organisms injected intraperitoneally in a volume of 0.2 ml. in mice and 1.0 ml. in rats. The LD50 challenge dose (dose required to kill 50 per cent of animals) was calculated according to the method of Reed and Muench (1938).

(a) Mice:

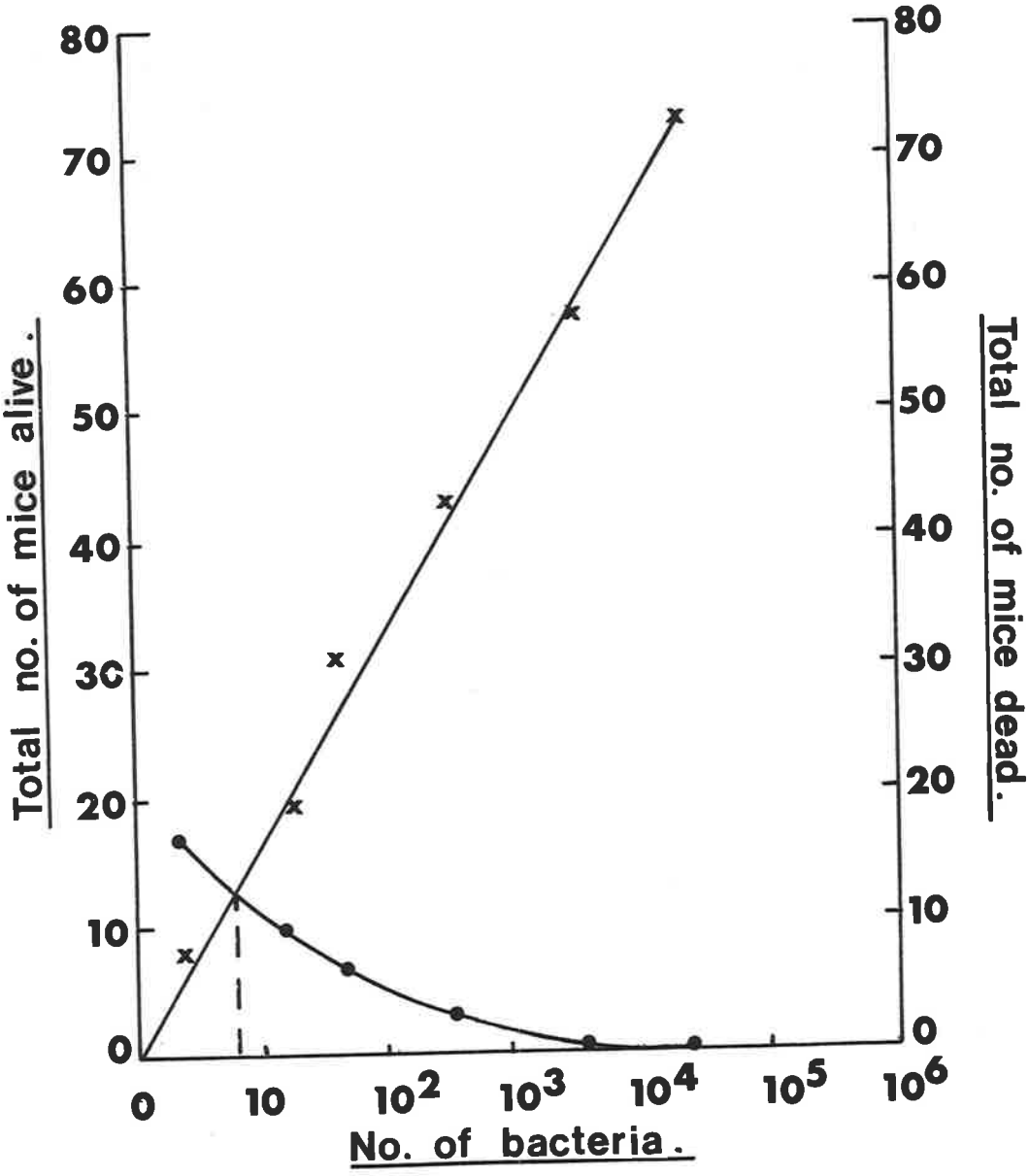
Groups of 30 mice both male and female were used. The mice weighed 18-20 gm. and were 12 weeks old. The results are shown in Fig. 3.1 from which it may be seen that the LD50 dose Salmonella typhimurium C5 for mice is less than 10 organisms.

(b) Rats:

To determine the LD50 dose of Salmonella typhimurium C5 for rats, groups of 20 rats weighing 180-200 gm. were used. Both male and female animals were used and all were 14 weeks old. The rats were challenged with doses of organisms which ranged in number from 7.9×10^3 to 7.9×10^9 in 10 fold steps. Only two rats died in all from all 7 groups challenged and these two animals belonged to the group challenged with 7.9×10^8 organisms. Therefore, three more groups of 20 rats were challenged with the three highest doses that had been previously used. However, no animals died in either of these three goups. Thus an LD50

FIG. 3.1

Determination of LD50 dose of Salmonella typhimurium C5 for normal Swiss White mice using the method of Reed and Muench (1938)


80
70
60
50
40
30
20
10
0
Total no. of mice alive.
Total no. of mice dead.
0
10
10²
10³
10⁴
10⁵
10⁶
No. of bacteria.

dose of Salmonella typhimurium C5 for rats could not be determined, although it is clearly greater than 7.9×10^9 organisms.

It is evident from these results that the two species of animals used in this study exhibit extremes of the susceptibility and resistance range for the organism studied. Because of the great variation in susceptibility exhibited by rats and mice, this model is a particularly suitable one for studying the factors controlling resistance or susceptibility to infection. This variation in susceptibility is probably an indication of some fundamental genetic difference existing between normal rats and normal mice.

Indirect Haemagglutination assays

Normal rat and mouse sera were examined for the presence of antibody able to produce agglutination of sheep red blood cells that had been coated with lipopolysaccharide extracted from Salmonella typhimurium C5 (C5 LPS). Two fold serial dilutions of rat or mouse serum were made in physiological saline in haemagglutination trays. An equal volume of a 2 per cent red blood cell suspension was added and the trays incubated at 37°C for 1 hour. The results were read after standing at 4°C overnight. To determine whether there were any background levels of antibody against normal unsensitised sheep red blood cells in the serum samples assayed, this control was included for each serum sample every time the assay was carried out.

Normal rat serum consistently agglutinated both C5 LPS coated and untreated red blood cells to a dilution of 1/32 to 1/64. On the other hand, normal mouse serum produced no detectable agglutination (Table 3.2). In order to determine whether the agglutinating antibody detected in normal rat serum was specifically directed against the C5 LPS, or whether there was a background level of antibodies to sheep red blood cells which

agglutinated both sensitised and unsensitised red cells, it was decided to substitute homologous rat erythrocytes for the sheep erythrocytes.

Several normal rats were bled to obtain a pool of rat erythrocytes. The whole blood was immediately mixed with an equal volume of sterile, modified Alsever's solution (Kabat and Mayer, 1961b). The rat red blood cells were stored for one week at 4°C before use. These cells were washed and sensitised with C5 LPS in the same manner as with sheep red blood cells (Ch. II). Using rat red blood cells coated with C5 LPS, normal rat serum again produced agglutination to a dilution of 1/32. There was no detectable agglutination of unsensitised normal rat red blood cells. That is, it appears that using the haemagglutination assay, the antibody in normal rat serum is specifically directed towards the antigens on the lipopolysaccharide extract from Salmonella typhimurium C5.

Clearance of radioactively labelled bacteria

A comparison was made of the ability of serum from normal rats and normal mice to promote the intravenous clearance of radioactively (P^{32}) labelled Salmonella typhimurium C5 in mice, by incubating the radioactively labelled organisms with normal rat or mouse serum. The technique used to radioactively label the bacteria was essentially that of Benacerraf, Sebestyen and Schlossman (1959). Mice were used as the test animal because it had been shown by Jenkin and Rowley (1963) that unopsonised P^{32} labelled Salmonella typhimurium C5 are cleared very poorly, from the circulation of mice. The results are shown in Fig. 3.2 and summarized in Table 3.1. The phagocytic index K, was calculated according to the formula $K = \frac{\log C1 - \log C2}{T2 \quad T1}$ where C1 and C2 are the concentrations of bacteria at times T1 and T2. This method is essentially that described by Biozzi, Benacerraf and Halpern (1953). The results show that there is little or no enhancement of intravenous clearance of P^{32}- labelled

TABLE 3.1

Comparison of intravenous clearance rates of P^{32} labelled Salmonella typhimurium C5 opsonised with normal rat or normal mouse serum

Serum for opsonisation of Salmonella typhimurium C5	PHAGOCYTIC INDEX (K)
Unopsonised control	0.01
Normal mouse serum	0.02
Normal rat serum	0.18

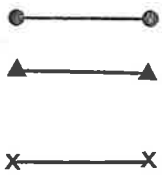
TABLE 3.2

Summary of antibody activities detected in the serum of normal rats and normal mice using different assay techniques

SERUM SAMPLE	Intravenous clearance of P^{32} labelled *Salmonella typhimurium* C5	*In vivo* killing of *Salmonella typhimurium* C5	*In vitro* killing of *Salmonella typhimurium* M206	Haemagglutinating activity against *Salmonella typhimurium* C5 LPS sensitised sheep red blood cells
Normal rat serum	K = 0.18	end point: 1/80	end point:1/1000	1/16 - 1/32
Normal mouse serum	K = 0.02	end point: 1/2	end point: 1/10 - 1/100	1/2

FIG. 3.2

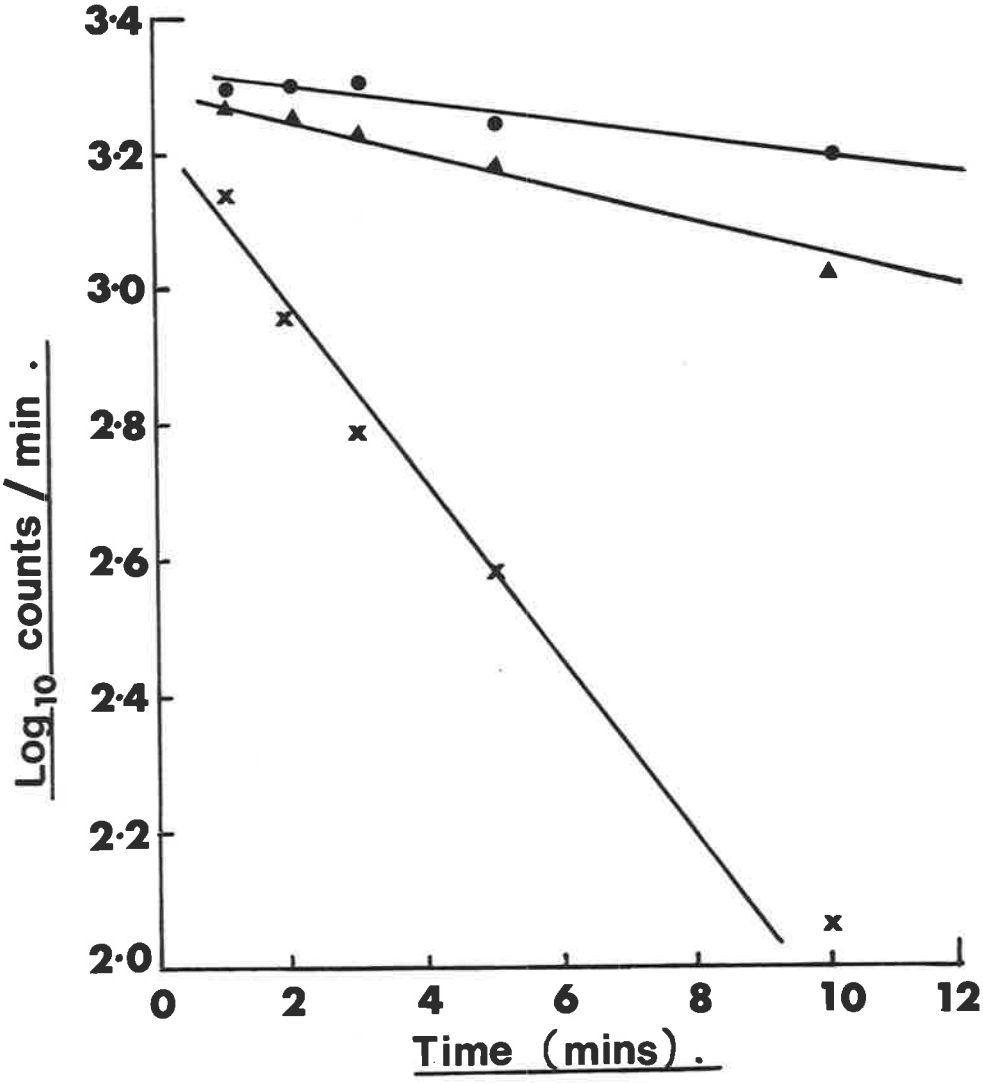
Comparison of the intravenous clearance of P^{32} labelled Salmonella typhimurium opsonised with normal rat and normal mouse serum



unopsonised Salmonella typhimurium C5

normal mouse serum opsonised Salmonella typhimurium C5

normal rat serum opsonised Salmonella typhimurium C5


3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
Log10 counts / min .
0
2
4
6
8
10
12
Time (mins) .

Salmonella typhimurium C5, when normal mouse serum is used. On the other hand, bacteria opsonised with normal rat serum were cleared very rapidly - (Table 3.2). These results confirm the observations of Jenkin and Rowley, (1963).

In vivo bactericidal assays

It has been shown that mice can survive infection with Salmonella typhimurium C5 if these organisms are incubated with normal rat serum prior to challenge (Jenkin and Rowley, 1959). In comparison, normal mouse serum opsonised Salmonella typhimurium C5 were as virulent as unopsonised organisms. Therefore, it was decided to investigate the peritoneal killing in mice of Salmonella typhimurium C5 opsonised with normal rat and mouse serum. The results are expressed as that dilution of normal serum which promotes greater than 75 per cent killing of the inoculum dose of organisms over a 90 minute period. Again it is evident that normal rat serum is more potent than is normal mouse serum. Even neat mouse serum did not enhance peritoneal killing whereas normal rat serum was effective up to a dilution of 1/80. That is, if the organisms were opsonised at a concentration of approximately 1×10^5 organisms/ml. with an equal volume of normal rat serum diluted to a dilution of 1/80, for 20 minutes at $4^{\circ}C$, more than 75 per cent of these organisms were killed after injection into the mouse peritoneum. To determine accurately the degree of enhancement of peritoneal killing by normal rat or normal mouse serum, controls were essential. These consisted of untreated organisms injected into one group of animals and following the fate of these organisms over a 90 minute period. This control was included each time the assay was carried out. The results show that normal rat serum was effective in promoting peritoneal killing of Salmonella typhimurium C5, whereas normal mouse serum was ineffective (Table 3.2).

In vitro bactericidal assays

The next parameter investigated was that of the ability of normal rat and normal mouse serum to exert a bactericidal effect in the presence of complement in vitro against Salmonella typhimurium. Normal rat serum collected in the usual manner (described in Ch.II), if assayed for complement content using haemolysin sensitised sheep red blood cells (Kabat and Mayer, 1961c) consistently produced lysis to a dilution of 1/32. However, normal mouse serum collected in an idential manner possessed no detectable haemolytic activity. Therefore, to ensure that complement was not a limiting factor guinea-pig serum was used as a source of complement.

The technique used was the in vitro bactericidal assay described by Rowley (1968). However, it was found that neither normal rat serum nor normal mouse serum was able to exert a bactericidal effect on the C5 strain of Salmonella typhimurium. If the M206 strain of Salmonella typhimurium was substituted, killing did occur (Rowley - personal communication). Whilst this latter strain is as smooth as and has the same 'O'-somatic antigen as the C5 strain (Furness and Rowley, 1956), it is susceptible to antibody and complement in vitro. Normal rat serum in the presence of its own complement, or in the presence of added guinea pig complement that had been absorbed with Salmonella typhimurium M206, was bactericidal to a dilution of 1/1000. Normal mouse serum was bactericidal only in the presence of added guinea-pig complement to a dilution which varied between 1/10 and 1/100 depending on the batch of serum used. These results show that normal rat serum contains ten to one hundred times more bactericidal antibody, as detected by this method, than does normal mouse serum (Table 3.2).

The results of all serological assays carried out with normal rat and normal mouse serum are compared in Table 3.2.

In vitro phagocytic experiments

It has been suggested (Ch. I) that the genetic control of resistance or susceptibility to a particular infection may be exerted at the level of the macrophage. Therefore, in order to determine whether the difference in susceptibility of rats and mice to Salmonella typhimurium C5 infection, was due to some basic difference in the macrophages from these animals, in vitro phagocytic experiments were carried out.

The aims of the phagocytic experiments were to determine whether peritoneal macrophages from normal rats and normal mice were similar in their ability to phagocytose and kill Salmonella typhimurium C5,

(a) in the absence of any serum,

(b) in the presence of normal rat or normal mouse serum.

1. Washing of glassware

The Leighton tubes used were of pyrex glass and measured 1/2" x 5" with 12 x 45 mm. wells moulded into the walls. These wells were 1.0-1.5 mm. deep. All glassware and stoppers used were washed by boiling in dilute nitric acid for 1 hour and this was followed by rinsing three times in distilled water followed by three rinses in deionised, distilled water. After drying, the tubes and stoppers were autoclaved at 15 lbs/sq. inch pressure for 20 minutes.

2. Culture medium

The culture medium used was Hanks Balanced Salt Solution containing 5 per cent heat inactivated foetal calf serum (Ch. II). Hereinafter this medium will be referred to as the Tissue Culture medium. This medium was buffered with 10 per cent (v/v) of 1.4 per cent sodium bicarbonate solution. Antibiotics were never added to the Tissue Culture medium.

3. Preparationof macrophage monolayers

The macrophages used in this study were mononuclear cells obtained from the peritoneal cavities of normal rats and normal mice. The method used to obtain these cells was similar to that described by Rowley (1958). Mice were killed by cervical dislocation and pinned out on a dissecting board. Their abdominal surfaces were flooded with 70 per cent alcohol and the skin reflected to expose the abdominal wall. Using a 2 ml. syringe fitted with a 2 inch 19 gauge needle, 1.5 ml. of Tissue Culture medium to which 5 i.u./ml. heparin had been added was injected into the peritoneal cavity of each mouse. The abdominal wall was firmly massaged and the injected fluid withdrawn. Approximately 1 ml. was recovered and this contained from 3 to 6 x 10^6 cells/ml. of which approximately 95 per cent were mononuclear cells.

The methods for obtaining rat peritoneal macrophages was essentially the same as the one used above with the exception that 5 ml. of Tissue Culture medium was used for each washout. Approximately 4 ml. of fluid were recovered from each rat and this contained approximately 1-2 x 10^6 mononuclear cells/ml. However, only about 80 per cent of these cells were macrophages. Mast cells were quite prevalent in the rat peritoneal washouts.

The washout fluids from rats were pooled as were those from mice. The cells were spun at 500 x g for 10 minutes, the supernatant was removed and the deposited cells were resuspended and washed once in heparinised Tissue Culture medium. After counting, the cell suspensions were adjusted to contain 7 x 10^6 cells/ml. using heparinised Tissue Culture medium.

0.7 ml. of macrophage suspension i.e. approximately 5 x 10^6 cells, was added to each Leighton tube, the tubes were flushed with a 5 per cent

CO_2 – 95 per cent air mixture, firmly stoppered and incubated in a slightly tilted position for 1/2 - 1 hour. After this time the culture medium from each tube was withdrawn and replaced with unheparinised Tissue Culture medium. After flushing the tubes with CO_2 - air mixture, they were incubated for 2-3 hours prior to use in phagocytic experiments.

4. Phagocytic experiments

Only Leighton tubes containing complete monolayers of spreading macrophages were selected (by microscopic examination) for use in these experiments. 10 tubes were used for each experiment. The Tissue Culture medium was removed from each tube and replaced with 0.7 ml. of unheparinised Tissue Culture medium containing approximately 5×10^3 Salmonella typhimurium C5 organisms, i.e. the ratio of bacteria to cells was of the order of 1:1000. At the same time 10 tubes containing no macrophages were also inoculated with the same number of bacteria. These tubes served as controls for bacterial multiplication throughout the course of the experiment.

The phagocytosis of bacteria was followed over a 60 minute period at 0, 20, 40 and 60 minutes. Immediately after the addition of bacteria viable counts were made by withdrawing the supernatants and plating duplicate 0.03 ml. samples on dried nturient agar plates. Duplicate 0.03 ml. samples from control tubes were similarly plated on agar plates. The macrophage monolayers were washed twice with warmed buffered saline, to remove non-phagocytosed bacteria, and resuspended in 0.7 ml. of unheparinized Tissue Culture medium using a rubber scraper. Duplicate 0.03 ml. samples of the cell suspension were plated on dried nutrient agar plates. The tubes to be used for the 20, 40 and 60 minute intervals were treated in a similar manner after having been flushed with the $C0_2$ - air mixture and incubated at 37^oC for the appropriate time.

The results are shown in Table 3.3 from which it is evident that rat and mouse macrophages are capable of phagocytosing and killing Salmonella typhimurium C5 to approximately the same extent. The results of phagocytic experiments in which Salmonella typhimurium C5 organisms that had been opsonised with normal rat or normal mouse serum were used, are shown in Fig. 3.3. It is again evident that rat and mouse peritoneal macrophages do not differ significantly in their ability to phagocytose and kill Salmonella typhimurium C5 organisms under similar conditions of opsonisation. It is also evident from these results that Salmonella typhimurium C5 organisms opsonised with normal rat serum are more readily phagocytosed and fewer survive than if the organisms are opsonised with normal mouse serum or not opsonised at all.

These results confirm the observations of Rowley and Jenkin (1962).

The percentage phagocytosis that occurred at any one time was calculated according to the following formula:-

$$\frac{A - B}{A} \times 100$$

where A = the bacterial count of the supernatant of control tubes at time Tx.

B = the bacterial count of the supernatant of "test" tubes at time Tx.

The percentage survival of phagocytosed bacteria within macrophages at any one time was calculated according to the following formula:-

$$\frac{C}{A - B} \times 100$$

where C = the bacterial count associated with macrophages at time Tx.

TABLE 3.3

Comparison of the abilities of peritoneal macrophages from normal rats and normal mice to phagocytose and kill Salmonella typhimurium C5

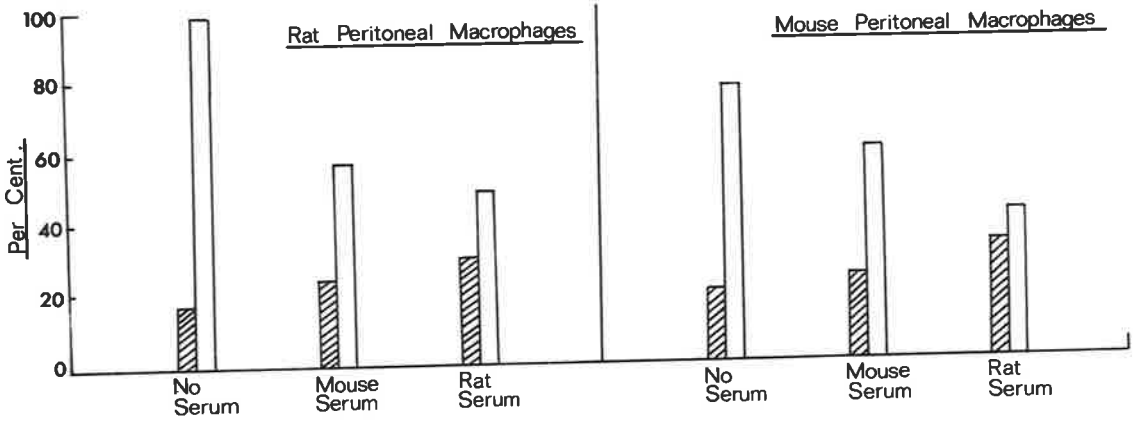
Time (minutes)	Rat peritoneal macrophages		Mouse peritoneal macrophages	
	Percentage Phagocytosis of Salmonella typhimurium C5	Percentage Survival of Salmonella typhimurium C5	Percentage Phagocytosis of Salmonella typhimurium C5	Percentage Survival of Salmonella typhimurium C5
0	7	61	7	71
20	10	65	18	69
40	12	72	11	81
60	11	68	15	85

FIG 3.3

Comparison of the abilities of peritoneal macrophages from normal rats and normal mice to phagocytose and kill Salmonella typhimurium C5.

Percentage phagocytosis

Percentage survival


Rat Peritoneal Macrophages
Mouse Peritoneal Macrophages
Per Cent.
100
80
60
40
20
0
No Serum
Mouse Serum
Rat Serum
No Serum
Mouse Serum
Rat Serum

Detection of antibody producing cells

a) The Jerne plaguing technique

The technique described by Jerne et al., (1963), was used to enumerate the numbers of antibody producing cells in the spleens of normal rats and normal mice. Both normal sheep red blood cells (N RBC) and sheep red blood cells coated with C5 LPS (S RBC) were used. The results show that normal rat spleens contained an average of 6 plague forming cells (PFC) per 10^8 spleen cells against N RBC and an average of 15 PFC per 10^8 spleen cells against S RBC. The numbers of PFC detected in normal mouse spleens were not significantly different, 3 PFC per 10^8 spleen cells against N RBC and 4 PFC per 10^8 spleen cells against S RBC (Table 3.4).

b) The Biozzi immunocytoadherence technique

Normal sheep red blood cells and red cells sensitised with C5 LPS were also used to detect rosette forming cells (RFC) by the technique of Biozzi et al., (1966). Normal rat spleens were shown to contain an average of 4670 and 5650 RFC per 10^8 spleen cells against N RBC and S RBC respectively. The numbers of RFC detected in normal mouse spleens were of the same order namely, 5550 RFC per 10^8 spleen cells using N RBC and 6400 RFC per 10^8 spleen cells using S RBC (Table 3.4).

It is evident from these results that normal rats and normal mice possess approximately the same numbers of antibody producing cells.

The results of all seriological assays are summarized in Table 3.2 from which it can be concluded that significant differences were detected between the activities of normal rat and normal mouse sera. However, no significant differences in either the activity of normal rat and normal mouse peritoneal macrophages, nor in the numbers of detectable antibody producing cells were detected (Tables 3.3 and 3.4).

TABLE 3.4

Comparison of the numbers of Jerne plaque forming cells and Biozzi rosette forming cells detected in the spleens of normal rats and normal mice

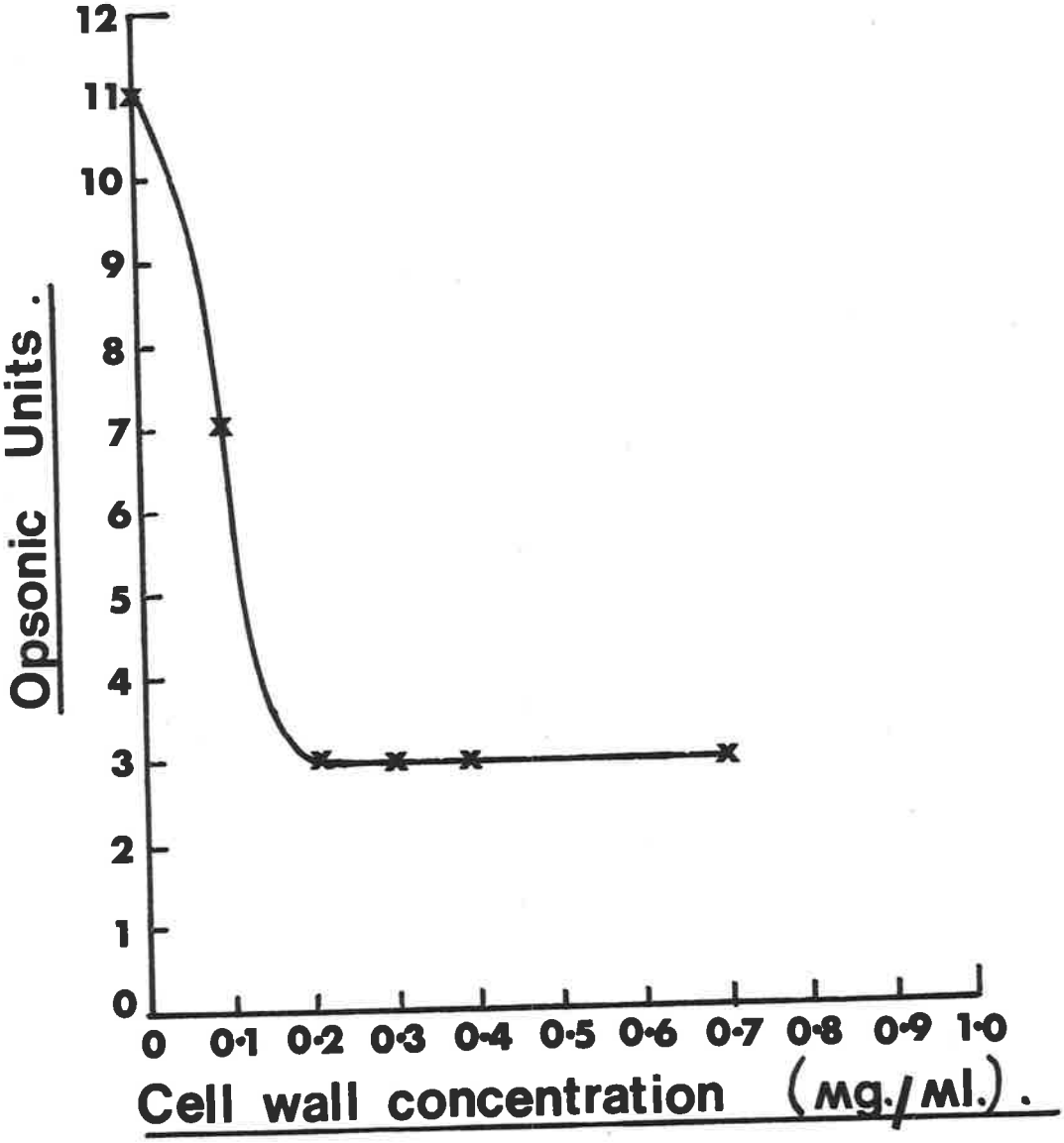
Spleen Cells used	Jerne Plaque forming cells per 10^8 spleen cells		Biozzi Rosette forming cells per 10^8 spleen cells	
Rat	Normal sheep RBC	6	Normal sheep RBC	4670
	Sensitised sheep RBC	15	Sensitised sheep RBC	5650
Mouse	Normal sheep RBC	3	Normal sheep RBC	5550
	Sensitised sheep RBC	4	Sensitised sheep RBC	6400

Isolation, purification and characterization of the opsonic factors in normal rat serum

To investigate more fully the factor(s) in normal rat serum which were opsonic and which possessed bactericidal and haemagglutinating activities, the technique of Turner and Rowley (1963) with minor modifications was employed. Cell walls were obtained by exposing the C5 strain of Salmonella typhimurium to ultrasonic vibrations for 7.5 minutes at a frequency of 20 Kilocycles per second in a Mullard 500 watt apparatus (Mullard equipment Ltd.) Intact cells were removed by centrifugation at 1085 x g for 10 minutes. The cell walls were then deposited by centrifugation for 40 minutes at 27,000 x g. These were resuspended in 0.05 M phosphate buffer at pH 7.0 and then washed three times in the same buffer with alternate centrifugations at 1,085 x g and 27,000 x g to obtain a cell wall preparation free from intact cells. After the final washing the cell walls were resuspended in 20 ml. of 0.01 M phosphate buffer at pH 8.0. Trypsin was added to a final concentration of 100 μg/ml. and the cell walls digested for 2 hours at 37^{o}C. The cell walls were then deposited by centrifugation and resuspended in 0.01 M phosphate buffer at pH 7.0. A dry weight estimation was made on the basis of an optical density reading using a Shimadzu Spectrophotometer at 675 milli-microns. Merthiolate was added to a final concentration of 1/10,000 and the cell walls stored at - 10^{o}C. The normal rat serum required was obtained from a large number of animals by the method described in Chapter II and was stored at - 10^{o}C until required. The rat serum was absorbed with increasing concentrations of trypsinised cell walls in order to determine the optimum concentration required to absorb out the maximum amount of opsonins. The method used to assay the residual levels of opsonins was that of intravenous clearance of P^{32} labelled Salmonella typhimurium C5. The results, shown in Fig 3.4 indicated that 0.2 mg. of trypsinised cell walls per ml. of serum

FIG. 3.4

Determination of the optimal concentration of trypsinised Salmonella typhimurium C5 cell walls required to absorb out the maximum amount of opsonic activity from normal rat serum


Opsonic Units.
12
11
10
9
8
7
6
5
4
3
2
1
0
0 0·1 0·2 0·3 0·4 0·5 0·6 0·7 0·8 0·9 1·0
Cell wall concentration (mg./ml.).

was a suitable concentration for absorbing the opsonins from rat serum. 100 ml. of normal rat serum were then absorbed with 0.1 mg/ml. of trypsinised cell walls in sealed tubes at 4°C for 2 hours on a rotor. The cell walls were removed by centrifugation at 27,000 x g for 20 minutes and the absorbed serum retained for later assay for residual opsonins. The cell walls were washed twice in 0.85 per cent cold saline and both supernatants were stored at - 10°C.

The deposit of washed cell walls was resuspended in 4 ml. of 4M urea in 2M sodium chloride (prepared in borate buffer pH 8.9) and placed on a microid flask shaker (Griffin and George Ltd., London) for 1 hour at room temperature (circa 22°C). The cell walls were removed by centrifugation at 27,000 x g for 20 minutes and the supernatant retained. The deposited cell walls were resuspended in a solution containing 0.1 M formamide and 1.0 M sodium chloride (prepared in acetate buffer pH 4.0) and again placed on a shaker for 1 hour at room temperature. The cell walls were removed as before and the supernatant stored. The urea and formamide elution steps were repeated once more, each elution step being performed in a volume of 4 ml. The four supernatants obtained were dialysed against a large volume of 0.15 M phosphate buffer at pH 7.0 for 48 hours. Protein concentrations of each of the four eluates and of normal rat serum dialysed against the same buffer for the same length of time, were determined by the Folin-Ciocalteu method using dialysed rat serum as a standard (Kabat and Mayer 1961a). The results are shown in table 3.5. The normal rat serum, absorbed with cell walls and each of the four eluates were then used to opsonise P^{32} labelled Salmonella typhimurium C5 and the clearance values were determined. The results are shown in Table 3.6. The supernatants from steps which involved washing of cell walls used to absorb normal rat serum, prior to elution of opsonins, showed no detectable

TABLE 3.5

Protein concentrations for normal rat serum and for elution samples from Salmonella typhimurium C5 cell walls as determined by the Folin-Ciocalteu method

Samples for protein concentration estimation by Folin-Ciocalteu method	Protein concentration mg./ml.
Normal rat serum	47.5
First urea eluate	1.0
First formamide eluate	0.77
Second urea eluate	0.73
Second formamide eluate	0.54

TABLE 3.6

Values for phagocytic indices and opsonic unit values for normal rat serum and for elution samples used to opsonise P^{32} labelled Salmonella typhimurium C5 before intravenous clearance studies

Sample used for opsonisation of Salmonella typhimurium C5 labelled with P^{32} before intravenouse clearance	Phagocytic Index (K)	Opsonic Units per ml.
Unopsonised control	0.01	0
Normal rat serum	0.12	11
First urea eluate	0.08	7
First formamide eluate	0.03	2
Second urea eluate	0.05	4
Second formamide eluate	0.04	3

opsonic activity. The unabsorbed normal rat serum used to opsonise radioactively labelled bacteria was treated in the same way as the eluates. It was placed on a rotor for 2 hours at $4^{o}C$ during cell wall absorption, placed on a shaker at room temperature during the elution steps and finally dialysed against phosphate buffer for 48 hours. This procedure would account for the generalized reduction in opsonic activity which is reflected by the relatively low clearance (K) values obtained for bacteria opsonised with whole, unabsorbed normal rat serum.

Whole normal rat serum contained 11 opsonic units/ml. i.e., 1100 opsonic units in 100 ml. of normal rat serum. The number of opsonic units/ml. is calculated according to the following formula:-

Phagocytic index (K) of test serum - opsonised bacteria. − Phagocytic index (K) of unopsonised bacteria. × 100 × dilution of test serum used to opsonise bacteria.

(Turner, Jenkin and Rowley, 1964)

Normal rat serum absorbed with cell walls was shown to have 5 opsonic units/ml. or 500 opsonic units per 100 ml. remaining after absorption. Therefore approximately 55 per cent of the opsonins originally present in normal rat serum had been absorbed onto the cell walls, or approximately 600 opsonic units in all. The opsonic activity eluted from cell walls was as follows:-

(a) First urea eluate - 7 opsonic units per ml. in 4 ml., that is, a total of 28 opsonic units.

(b) First formamide eluate 2 opsonic units per ml. in 4 ml., that is, a total of 8 opsonic units.

(c) Second urea eluate - 4 opsonic units per ml. in 4 ml., that is, a total of 16 opsonic units.

(d) Second formamide eluate - 3 opsonic units per ml. in 4 ml., that is a total of 12 opsonic units.

Therefore the total activity recovered was 64 opsonic units which represented 10.67 per cent of the 600 opsonic units that had been absorbed onto the cells walls.

The rat serum used for absorption with cell walls, containing 11 units of opsonic activity per ml. had a protein concentration of 47.5 mg. per ml. Thus whole serum contained 0.23 opsonic units per mg. of protein per ml. The eluate contained a total of 64 opsonic units in a protein concentration of 12.16 mg. i.e., 5.3 opsonic units per mg., representing a 23 fold purification of biological activity.

Having shown that the proteins eluated from Salmonella typhimurium C5 cell walls possessed opsonic activity, an attempt was made to characterise the eluted protein. An antiserum, raised in rabbits against whole rat serum (Ch. II), was used in an immunoelectrophoretic analysis of the eluted protein, with whole normal rat serum as a control. The results show that the eluate consisted of β_1 and β_2 macroglobulin (Fig. 3.5).

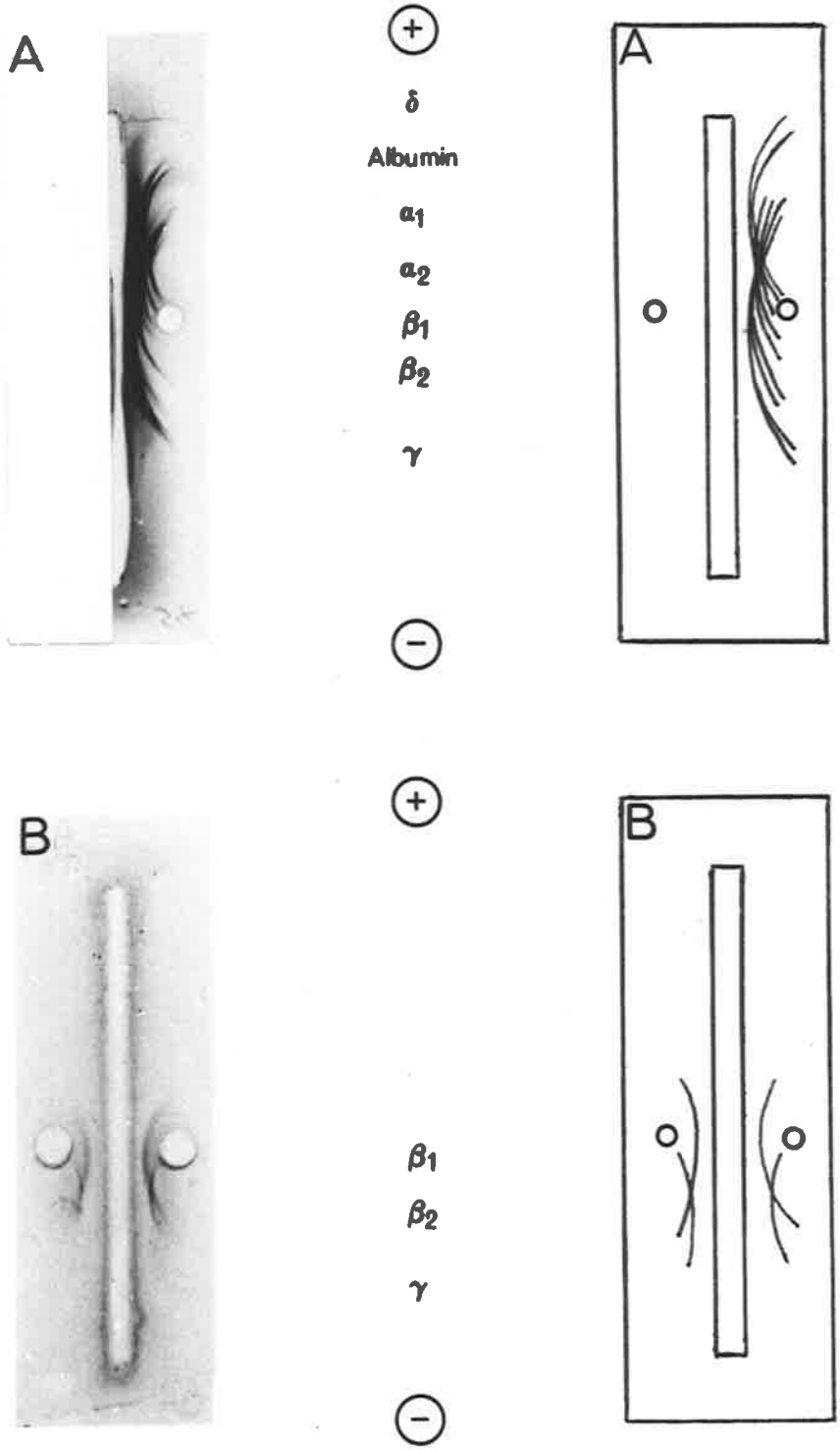
The eluted protein fraction which possessed opsonic activity was also assayed for in vitro and in vivo bactericidal activity, again using normal rat serum as a control. In the presence of fresh guinea pig complement absorbed with the test organism, normal rat serum was bactericidal to Salmonella typhimurium M206 to a dilution of 1/100. The eluted protein assayed at the same time was bactericidal to a dilution of 1/10. Normal rat serum enhanced peritoneal killing of Salmonella typhimurium C5 in mice, to a dilution of 1:40, whilst the eluted protein was active to a dilution of 1:4. When both whole normal rat serum and the eluted protein fraction were treated with 0.2M mercaptoethanol for 1 hour at 37°C and then dialysed against a 500 fold excess (v/v) of phosphate

FIG. 3.5

Immunoelectrophoretic analysis of protein fractions eluted from Salmonella typhimurium C5 cell walls

A Whole normal rat serum in wells, rabbit anti-whole normal rat serum in trough

B Eluted protein samples in wells, rabbit anti-whole normal rat serum in trough


A
+
δ
Albumin
α1
α2
β1
β2
γ
−
A
B
+
β1
β2
γ
−
B

buffer overnight, (Ch. II), all bactericidal and opsonic activity was removed (Table 3.7). It has been shown by Turner, et al., (1964) that although the reduced macroglobulins may undergo partial repolymerization after removal of the mercaptoethanol, loss of biological activity is irreversible.

The precipitin lines obtained with the material eluted from cell walls immunoelectrophoresis are characteristic of β_1 and β_2 macroglobulin. This purified fraction has been shown to possess biological activity which is destroyed upon reduction with 2-mercaptoethanol. It is evident therefore, that normal rats possess in their serum, a biologically active natural antibody which belongs to the macroglobulin class. In support of this, it was also shown that the opsonic activity in normal rat serum resides primarily in that fraction which sediments most rapidly when analysed by sucrose density gradient ultracentrifugation. See Fig 3.6 and Table 3.8. This rapidly sedimenting fraction was also sensitive to treatment with 2-mercaptoethanol. (Table 3.8).

We may conclude therefore, that normal rat serum contains natural antibody which is bactericidal and opsonic for Salmonella typhimurium C5. On the other hand normal mouse serum was found to possess little or no biological activity against Salmonella typhimurium C5. We may also conclude it is likely that the antibody in normal rat serum may play some role in the resistance rats display against infection with the C5 strain of Salmonella typhimurium.

The evidence presented supports the data of Reade et al., (1964), who has shown that the opsonic activity in normal rat serum exists mainly in the macroglobulin fraction of the serum proteins.

TABLE 3.7

Comparison of normal rat serum and eluted protein before and after treatment with 2-mercaptoethanol and used to (i) opsonise P^{32} labelled Salmonella typhimurium C5 before intravenous clearance (ii) assay in vitro bactericidal antibody against Salmonella typhimurium M206 and (iii) assay in vivo bactericidal antibody against Salmonella typhimurium C5

Sample for Assay	Clearance values Opsonic units/ml.		In vitro killing of Salmonella typhimurium M206		In vivo peritoneal killing of Salmonella typhimurium C5	
	untreated	2ME Treated	Untreated	2ME Treated	Untreated	2ME Treated
Normal rat serum	11	Nil	1/100	less than 1/2	1/40	less than 1/2
Eluted protein	7	Nil	1/10	less than 1/2	1/4	less than 1/2

FIG. 3.6

Fractionation of normal rat serum by sucrose density gradient ultracentrifugation, showing pooling of fractions for biological assay

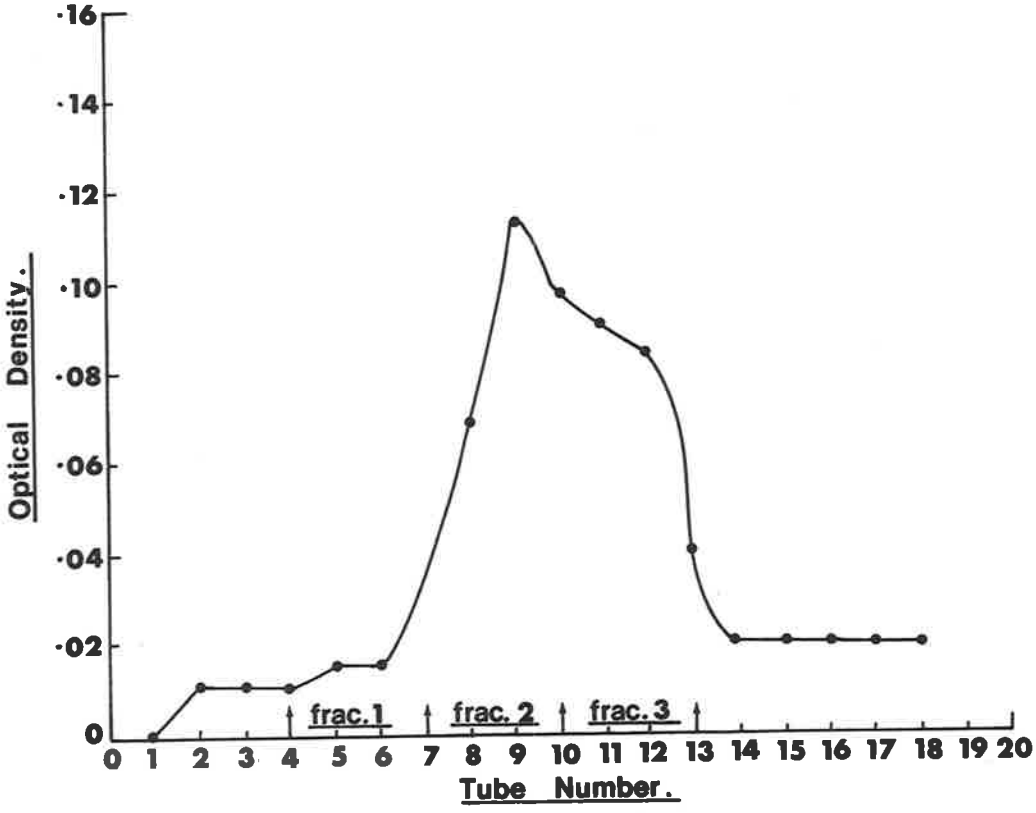

·16
·14
·12
·10
·08
·06
·04
·02
0
Optical Density.
frac. 1
frac. 2
frac. 3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Tube Number.

TABLE 3.8

Comparison of phagocytic indices of normal rat serum and fraction of normal rat serum separated on sucrose density gradients

Fraction used to opsonise P^{32} labelled *Salmonella typhimurium* C5 for intravenous clearance	PHAGOCYTIC INDEX (K)	
	Before 2 Mercaptoethanol treatment	After 2 Mercaptoethanol treatment
Unopsonised	0.01	0.01
Normal rat serum	0.13	0.01
Fraction 1	0.11	0.01
Fraction 2	0.05	0.02
Fraction 3	0.01	0.01

CHAPTER IV

THE FATE OF SALMONELLA TYPHIMURIUM C5 INJECTED INTO RATS AND MICE - MEASUREMENT OF BACTERICIDAL ANTIBODY PRODUCED BY THESE ANIMALS AND CORRELATION OF ANTI - BODY LEVELS WITH THE NUMBERS OF ORGANISMS RECOVERED

INTRODUCTION

In 1959 Jenkin and Rowley reported that when mice were challenged with Salmonella typhimurium C5 which had been pretreated with normal rat serum, a lethal infection did not result, indicating that normal rat serum possesses factors capable of limiting multiplication of this organism. These factors were apparently lacking in normal mouse serum since organisms treated with this serum were as virulent for mice as untreated organisms. These observations are supported by the results presented in Chapter III, which show that normal rat serum contains quite high levels of natural antibody when compared with normal mouse serum. These findings suggest that rats and mice may differ in their ability to deal with an infection caused by Salmonella typhimurium C5. To investigate this possibility, studies were undertaken to determine the fate of these organisms injected into rats and mice.

Quantitative data has been published which shows that Salmonella typhimurium multiplies rapidly in mice susceptible to this infection. For example, Hobson (1956, 1957) showed that approximately 10^7 Salmonella typhimurium organisms could be recovered from all moribund mice, irrespective of the number of organisms originally injected. He also showed that challenge of mice with numbers of Salmonella typhimurium varying from 10^3 to 10^5, resulted in a similar mean time to death (MTD). However, outside this range, an increase or decrease in the challenge dose resulted in a shorter or longer MTD respectively. Reduction of the challenge dose to 10

organisms not only prolonged the MTD but also increased the numbers of mice which survived (Hobson, 1957).

Mackaness, Blanden and Collins (1966) pointed out that during the course of an infection with Salmonella typhimurium, those mice which die, represent the innately more susceptible proportion of animals, with the survivors representing the selection of a population of more resistant individuals. Therefore studies involving the enumeration of bacteria recovered from mice over an extended period of time, may result in counts which are somewhat lower than expected since the innately more susceptible animals do not survive for this length of time. Hobson (1956, 1957) and Mackaness et al., (1966) have also shown that whilst some mice will survive infection with Salmonella typhimurium, they are not able to eliminate the infecting organism and continue to harbour the organism in various organs for quite some time. Furthermore, Jenkin and Rowley (1963) showed that mice infected with Salmonella typhimurium and undergoing a terminal bacteraemia, were producing opsonins against this pathogen. This they demonstrated in two ways, namely, by the rapid clearance of bacteria removed from infected mice and reinjected into normal mice and secondly by the very rapid clearance of a superinfecting dose of the same organisms injected into the moribund animals. Therefore, it is clear from the abovementioned findings, that heavily infected mice, even if quite moribund, are able to effectively eliminate a superinfecting dose of the same organism.

Whilst some information is available on the fate of Salmonella typhimurium in mice, there is no information dealing with the fate of this organism in rats. However, since rats are known to be very resistant to infection with this organism, it is likely that they are able to control the rate of multiplication of Salmonella typhimurium, so that the number

of organisms present never attains the level necessary to cause their death. This control of multiplication could result in one of two possible fates of the Salmonella typhimurium injected into rats:

(1) Rats may eliminate the bacteria very rapidly, or

(2) they may react similarly to the more resistant individuals of the mouse population and harbour the organism for some time, at a level insufficient to kill them.

There is also no information available which shows whether any correlation exists between the numbers of Salmonella typhimurium that may be recovered from infected animals and the levels of antibody produced in response to this infection. It would be of some value to know whether a given number of organisms injected into animals of the resistant species and the susceptible species, elicits a better antibody response in the former. If this were so, the possibility exists that resistance or susceptibility to a particular infection is, at least partly, determined by the levels of antibody produced to that infectious agent.

The survival of Salmonella typhimurium C5 after intraperitoneal injection into rats and mice

LD50 studies in mice showed that their MTD when injected with Salmonella typhimurium C5 was similar over a dose range of approximately 10 to 1 x 10^4 viable organisms (Table 4.1). Thus, to ensure accurate enumeration of bacteria recovered from infected animals, the dose chosen as the lower limit for these studies was of the order of 10^4 viable organisms. To investigate and compare the fate of Salmonella typhimurium C5 injected intraperitoneally into rats and mice, large numbers of both species of animals were injected with either approximately 6 x 10^4 ("low" dose) or approximately 1 x 10^7 ("high" dose) viable organisms. Both male and female animals were used in these experiments and they were all 12 weeks

TABLE 4.1

Comparison of Mean Times to death (MTD) of groups of Swiss White mice challenged with different numbers of Salmonella typhimurium C5 organisms

Challenge Dose of Salmonella typhimurium C5 organisms	Percentage Mice that Died	Mean time to death
4	53.4	8.9 days
43	66.7	8.5 days
4.3×10^2	80.0	8.3 days
4.3×10^3	100	8.5 days

old at the beginning of the study. The body weight of the rats used was in the range of 200-230 gm. and the mice weight 18-20 gm. The numbers of viable bacteria that could be recovered from the circulation, peritoneum, spleen and liver of 3 rats and 5 mice were determined at various time intervals after infection, in the following manner:-

(a) In blood: Duplicate 0.1 ml. blood samples were taken from the retro-orbital venous plexus of each animal using a graduated pasteur pipette. These samples were plated on dried nutrient agar plates which were then incubated at 37°C for 18 hours. The total blood volume of rats was assumed to be 20 ml. and that of mice 2 ml.

(b) The Spleen: Spleens were removed aseptically and placed in separate homogeniser tubes containing 3 ml. of sterile saline. After homogenisation using a motorised teflon pestle at 500 r.p.m., the cell suspensions were appropriately diluted in sterile saline. Duplicate 0.1 ml. samples were plated on dried nutrient agar plates and incubated at 37°C for 18 hours.

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

(d) The Peritoneal Cavity: Peritoneal washouts of mice were carried out using 1.5 ml. of sterile saline per animal. This was withdrawn and reinjected several times and the peritoneal cavity firmly massaged to ensure maximum recovery of bacteria. The washout fluid was appropriately diluted and plated in duplicate on dried nutrient agar plates and incubated for 18 hours at 37°C. For rat peritoneal washouts 10 ml. of sterile saline was used, otherwise the technique was the same as for mice.

Using the "low" challenge dose of Salmonella typhimurium C5 in mice - approximately 6×10^4- the organisms recovered were found to be approximately equally distributed between spleen, liver and peritoneum. Blood samples

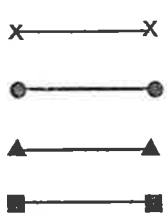
initially contained low numbers of organisms which then increased until day 4 when the level fell slightly and remained constant at approximately 10 per cent of the total organisms recovered from each animal (Fig. 4.1). Fig. 4.2 shows the total numbers of organisms recovered from the blood, peritoneum, liver and spleen of mice. It may be seen that the number of organisms recovered increased rapidly, particularly during the first 4 days after infection, until the seventh day when all the mice had died.

The same challenge dose of Salmonella typhimurium C5 injected into rats resulted in a situation different from that in mice. Whilst the numbers of organisms which could be recovered from spleen, liver and peritoneum were similar, the actual number of organisms recovered was approximately one tenth of those recovered from mice receiving a similar challenge dose. Also, at no time were any organisms recovered from the circulation of rats (Fig. 4.3). The number of organisms that could be recovered remained at a fairly constant level until the seventh day. After this, there was a rapid fall in numbers and by day 21 no organisms could be recovered from any of the sites examined. Fig. 4.2 shows the total number of organisms recovered from rats. It may be seen that there is an initial decrease in the numbers of organisms that could be recovered lasting up to 8 hours after injection. After this time, the bacterial numbers increased to and remained constant at approximately the level originally injected, until day 7. This initial fall in numbers of organisms in rats, as compared with the initial rapid increase in mice, is probably a manifestation of the ability of rats to control this infection.

The higher challenge dose of approximately 1×10^7 Salmonella typhimurium C5 organisms in mice, resulted in a very rapid multiplication of bacteria. The levels of bacteria recovered were similar in the circulation, spleen and liver, whilst, the numbers in the peritoneal cavity were approximately

FIG. 4.1

Recovery of Salmonella typhimurium C5 from the various organs of mice injected intraperitoneally with 6.7×10^4 organisms



Blood

Peritoneum

Spleen

Liver

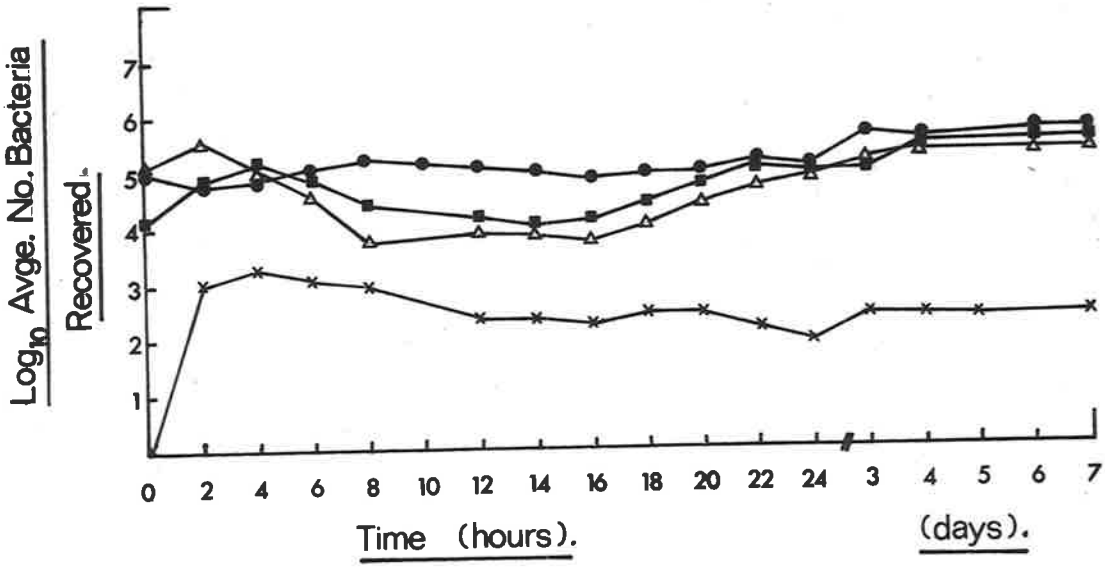

Log10 Avge. No. Bacteria Recovered.
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24 3 4 5 6 7
Time (hours).
(days).

FIG. 4.2

Average total recovery from blood, peritoneum, spleen and liver of Salmonella typhimurium C5 injected intraperitoneally

6.6×10^4 organisms injected into rats

6.7×10^4 organisms injected into mice

The vertical bars represent the range of variation obtained

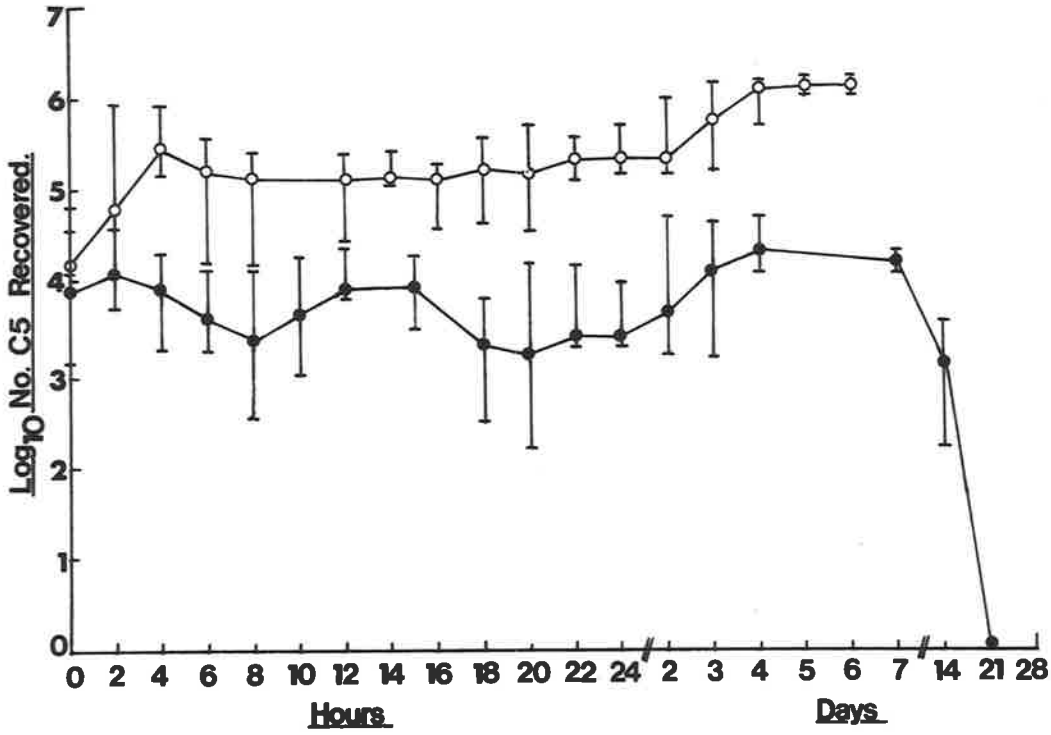
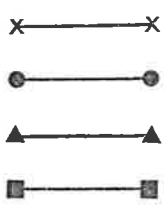

Log10 No. C5 Recovered.
7
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Hours
2 3 4 5 6 7 14 21 28
Days

FIG. 4.3

Recovery of Salmonella typhimurium C5 from the various organs of rats injected intraperitoneally with 6.6×10^4 organisms.

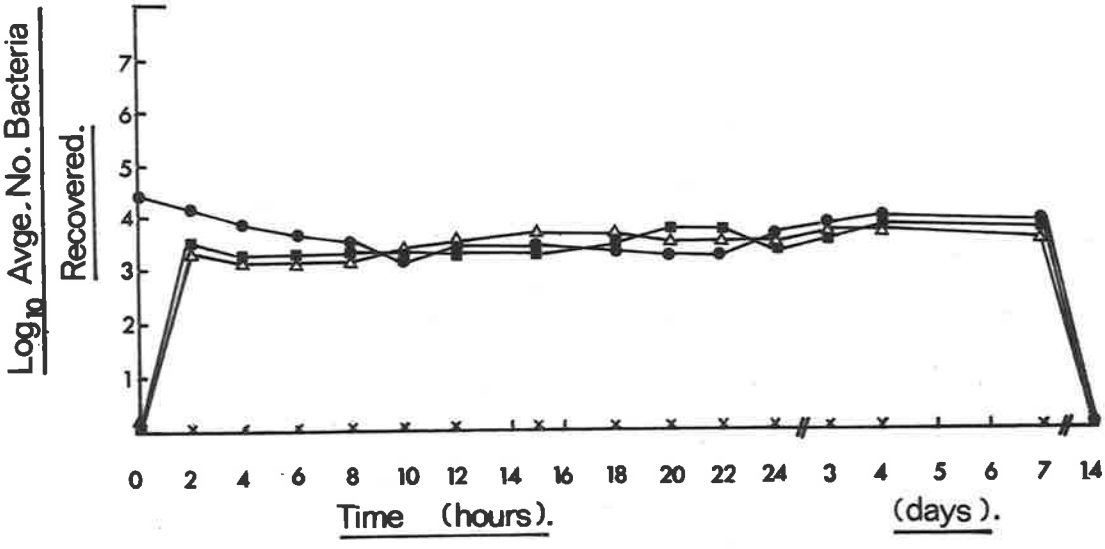


Blood

Peritoneum

Spleen

Liver

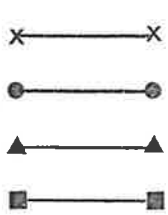

Log10 Avge. No. Bacteria Recovered.
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24 3 4 5 6 7 14
Time (hours).
(days).

10 per cent greater (Fig. 4.4). This rapid increase in numbers of bacteria continued until 24 hours after infection by which time all the mice had died. (Fig. 4.5). Rats receiving the higher challenge dose again presented a situation different from that in mice. Although the numbers of organisms detected in the peritoneal cavity, spleen and liver were again approximately the same, this level was up to 100 fold less than that in mice challenged with the same dose of organisms. In contrast with rats receiving the lower challenge dose, the group receiving the higher challenge dose had a low level of persisting bacteraemia (Fig. 4.6). The bacteraemia persisted for 14 days, whilst organisms in the other sites examined were detected until 28 days after infection. Fig. 4.5 shows that these rats were also able to initially cause a decrease in numbers of detectable bacteria for up to 8 hours, after which the levels increased to and remained constant at, approximately the level at which they were injected. In contrast, mice were at no time able to reduce the number of organisms recoverable, below the level of the original inoculum.

It is clear that in the susceptible species, Salmonella typhimurium C5 organisms multiply and increase steadily in number until all the mice die. The fact that the number of organisms that could be recovered from mice infected with the lower dose of organisms did not appear to increase after day 5 could be explained by the elimination of individual mice innately more susceptible to this strain of Salmonella typhimurium, since a large number of mice died on days 4-5. Thus mice innately more resistant to infection with this organism were the ones being examined. On the other hand, the number of organisms recovered from rats, after an initial decrease, followed by an increase to the original inoculum level, remained at a constant level until either 7 days or about three weeks, depending on the dose used after which time the organisms were rapidly eliminated. In other words, a

FIG. 4.4

Recovery of Salmonella typhimurium C5 from the various organs of mice injected intraperitoneally with 2.0×10^7 organisms



Blood

Peritoneum

Spleen

Liver

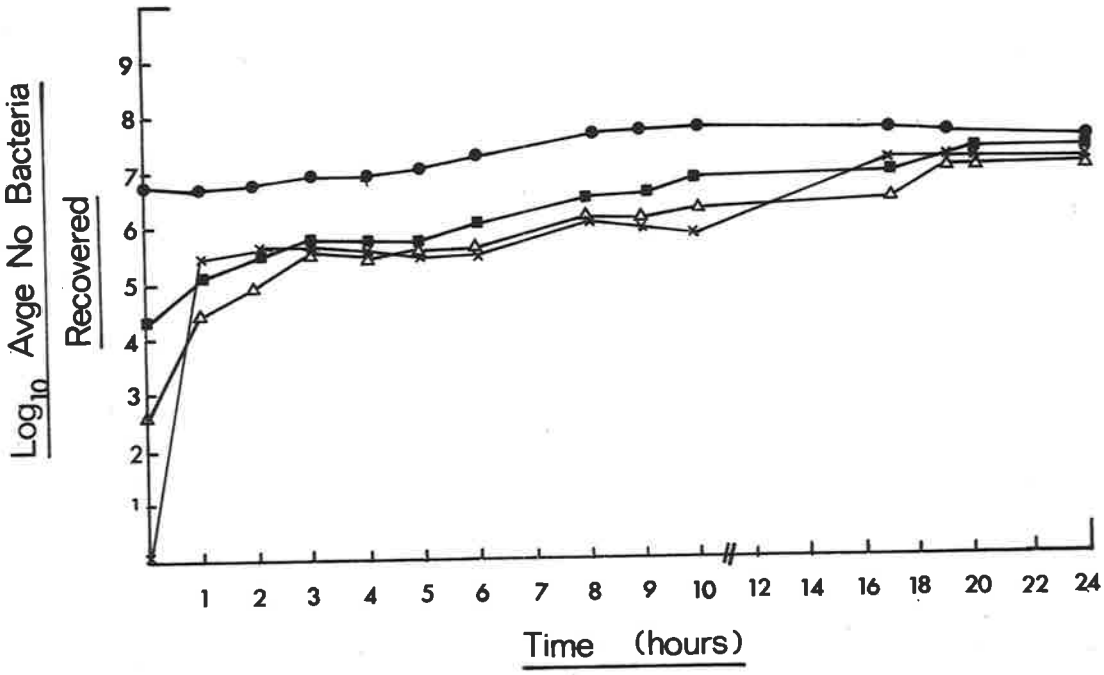

Log10 Avge No Bacteria Recovered
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24
Time (hours)

FIG. 4.5

Average total recovery from blood, peritoneum, spleen and liver of Salmonella typhimurium C5 injected intraperitoneally

1.1×10^7 organisms injected into rats

2.0×10^7 organisms injected into mice.

The vertical bars represent the range of variation obtained

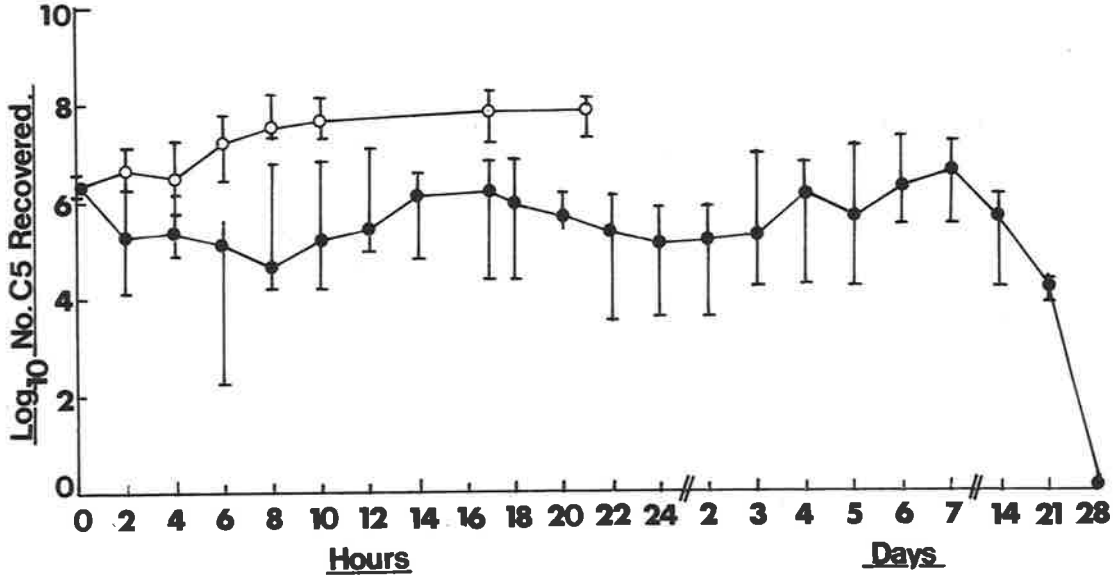
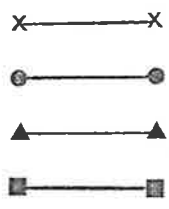

Log10 No. C5 Recovered
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 2 3 4 5 6 7 14 21 28
Hours
Days

FIG. 4.6

Recovery of Salmonella typhimurium C5 from the various organs of rats injected intraperitoneally with 1.1×10^7 organisms

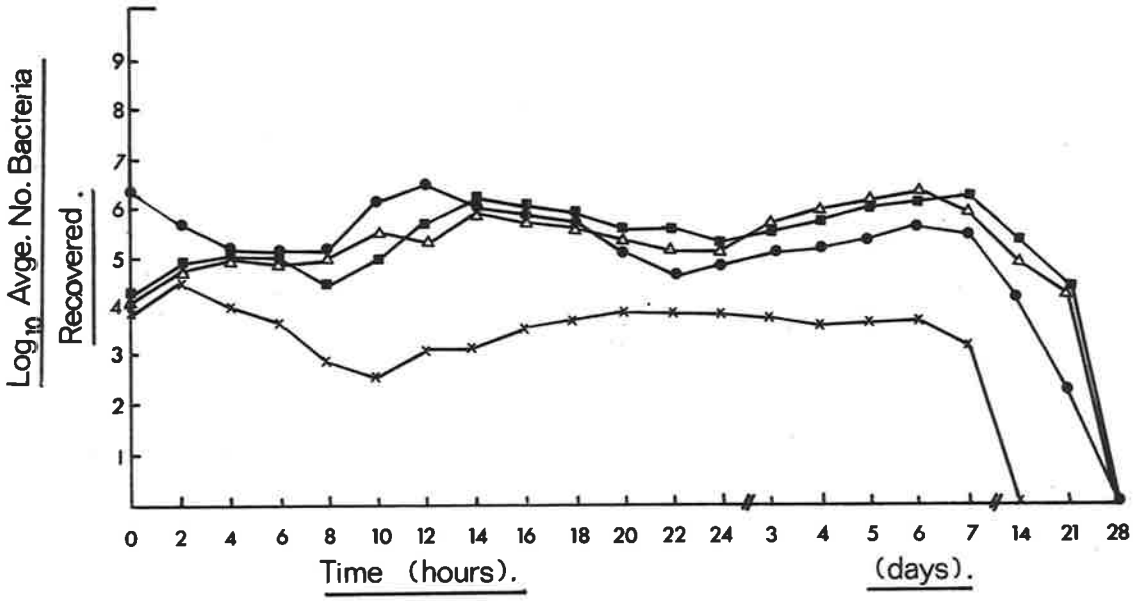


Blood

Peritoneum

Spleen

Liver


Log10 Avge. No. Bacteria Recovered.
9
8
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24 3 4 5 6 7 14 21 28
Time (hours).
(days).

situation similar to the carrier state is established in rats, for a time which appears to be dependent on the dose of organisms injected.

The results of the abovementioned experiments led to the next study which was designed to show whether any correlation exists between the levels of antibody produced by rats and mice and the number of organisms that can be recovered.

The antibody response of rats and mice injected with living Salmonella typhimurium C5

Three main assay techniques are available for the detection and measurement of antibody produced in response to challenge with the antigens of Salmonella typhimurium C5. These include:-

(1) The in vitro bactericidal assay (Landy, Michael and Whitby, 1962)

(2) The in vivo bactericidal assay (Whitby and Rowley, 1959)

(3) The haemagglutination of sheep red blood cells coated with lipopolysaccharide extracted from Salmonella typhimurium C5 (described in Chapter II).

The haemagglutination technique is the least sensitive (Rowley - personal communication) and was therefore not employed. The in vivo bactericidal assay requires large numbers of mice, particularly when a large number of serum samples are to be examined, therefore, initially antibody assays were carried out by the in vitro bactericidal assay.

Preliminary experiments indicated that the peak of antibody production in both rats and mice occurred at about 6 days after injection of Salmonella typhimurium C5. At this time, the numbers of organisms that could be recovered from rats injected with living Salmonella typhimurium C5 were approximately the same as the number of organisms originally injected, whereas in the case of mice, multipliation had occurred (Fig. 4.2). Therefore, the doses of living Salmonella typhimurium C5 organisms used to inject rats

and mice were chosen to ensure that the total number of live organisms recoverable from each species was approximately the same during the 24 hour period when antibody production was at its peak. This was done in an attempt to avoid the possibility that any observed difference in serum antibody titres was a reflection of differences in the amount of antibody adsorbed to live bacteria.

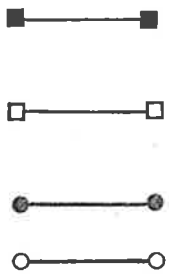
A large number of rats were injected intraperitoneally (i.p.) with 6.4×10^6 living Salmonella typhimurium C5 organisms. Similarly a large number of mice were injected i.p. with 1.3×10^3 living organisms of the same strain. At various intervals following injection, groups of 3 rats and 5 mice were chosen at random for bleeding and for determination of the total number of recoverable living organisms. The serum samples obtained from each species on any one day of bleeding were pooled and the amount of antibody present was determined using the in vitro bactericidal assay system. The results shown in Fig. 4.7 indicate that rats produce much larger amounts of antibody than do mice. At the peak of antibody production rats produce a ten thousand fold greater titre of antibody.

It was not clear from this experiment whether the large difference in the level of antibody production elicited in the two species was due to intrinsic differences in the ability of the two species of animals to respond to Salmonella typhimurium C5 antigens, or was a reflection of the difference in the amount of antigenic stimulus received. In fact, it appeared that the latter could, at least in part, be the case, since rats received an approximately four thousand fold greater number of living organisms than did the mice.

The above experiment was therefore repeated using a weight adjusted dose of viable Salmonella typhimurium C5 organisms. Rats received a dose of 7×10^3 viable organisms per animal, whereas each mouse was given

FIG 4.7

Comparison of in vitro bactericidal antibody responses with recovery of Salmonella typhimurium C5 from rats and mice

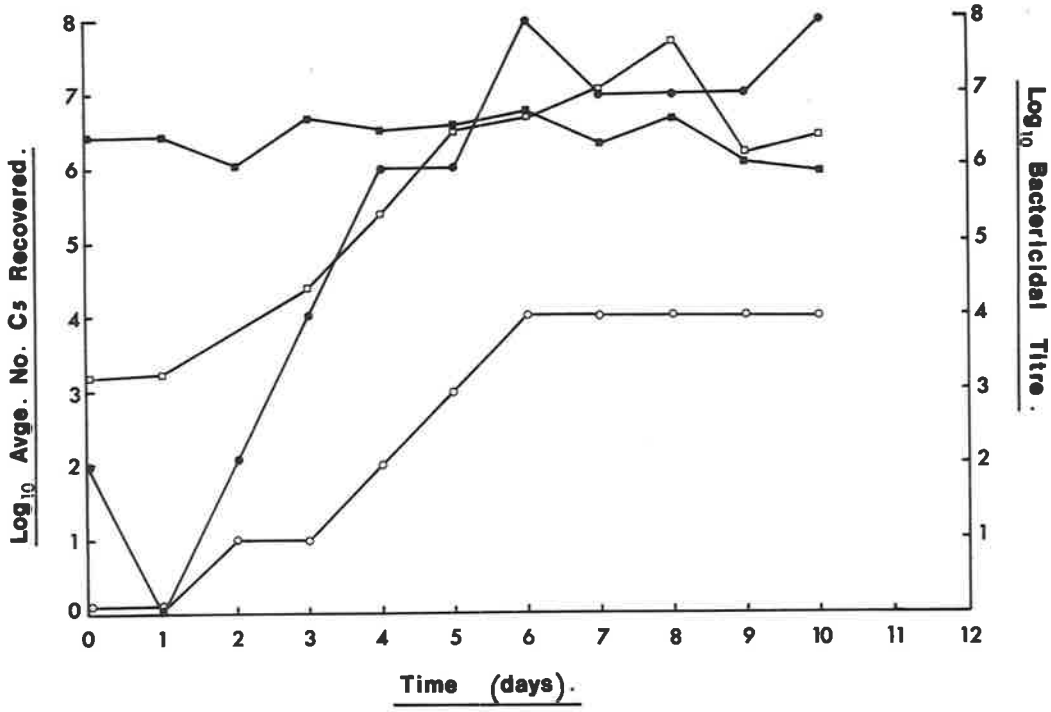


Average total recovery from rats injected intraperitoneally with 6.4×10^6 organisms

Average total recovery from mice injected intraperitoneally with 1.3×10^3 organisms

Bactericidal antibody response of rats

Bactericidal antibody response of mice


Log10 Avge. No. Cs Recovered.
Log10 Bactericidal Titre.
Time (days).
0
1
2
3
4
5
6
7
8
9
10
11
12

a dose of 1×10^3 viable bacteria. Since the challenge dose used represented a higher than LD50 dose for mice, some of the mice died during the time that antibody responses were measured. It is likely, therefore, that a bias was introduced due to the selection of more resistant mice, presumably capable of better antibody production. Nevertheless, from the results obtained (Fig 4.8) it can be seen that even with a weight adjusted dose of antigen, rats respond better than do mice, although the difference in peak serum antibody titre in this experiment was reduced ten fold as compared to the previous experiment. Thus, it appears that there is some intrinsic difference between the ability of rats and mice to respond (produce antibody) to Salmonella typhimurium C5 antigens.

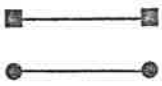
Having detected large differences in the levels of antibody produced by rats and mice, the question of whether the in vitro bactericidal assay system provides an adequate means of measuring levels of protective antibody, had to be considered. This doubt arises because this assay employs the M206 and not the C5 strain of Salmonella typhimurium.

Although both of these strains possess the same O-somatic antigens (Kauffmann, 1966) one cannot be sure that the antibody measured by this method is the same antibody that would be detected if the same sera were assayed by the in vivo bactericidal system which employs the C5 strain of Salmonella typhimurium.

To determine whether this was in fact the case, rats and mice were injected intraperitoneally with 6.4×10^6 and 1.3×10^3 Salmonella typhimurium C5 organisms respectively. These animals were bled at regular intervals and the serum collected as previously described. The sera were then assayed by both the in vitro and the in vivo bactericidal assay techniques. The results of this study are shown in Figs 4.9 and 4.10. It is evident from these results that rats produce much more antibody than do mice and that

FIG. 4.8

Comparison of in vitro bactericidal antibody responses of rats and mice injected intraperitoneally with weight adjusted doses of Salmonella typhimurium C5



7.0×10^3 organisms injected into rats

1.3×10^3 organisms injected into mice

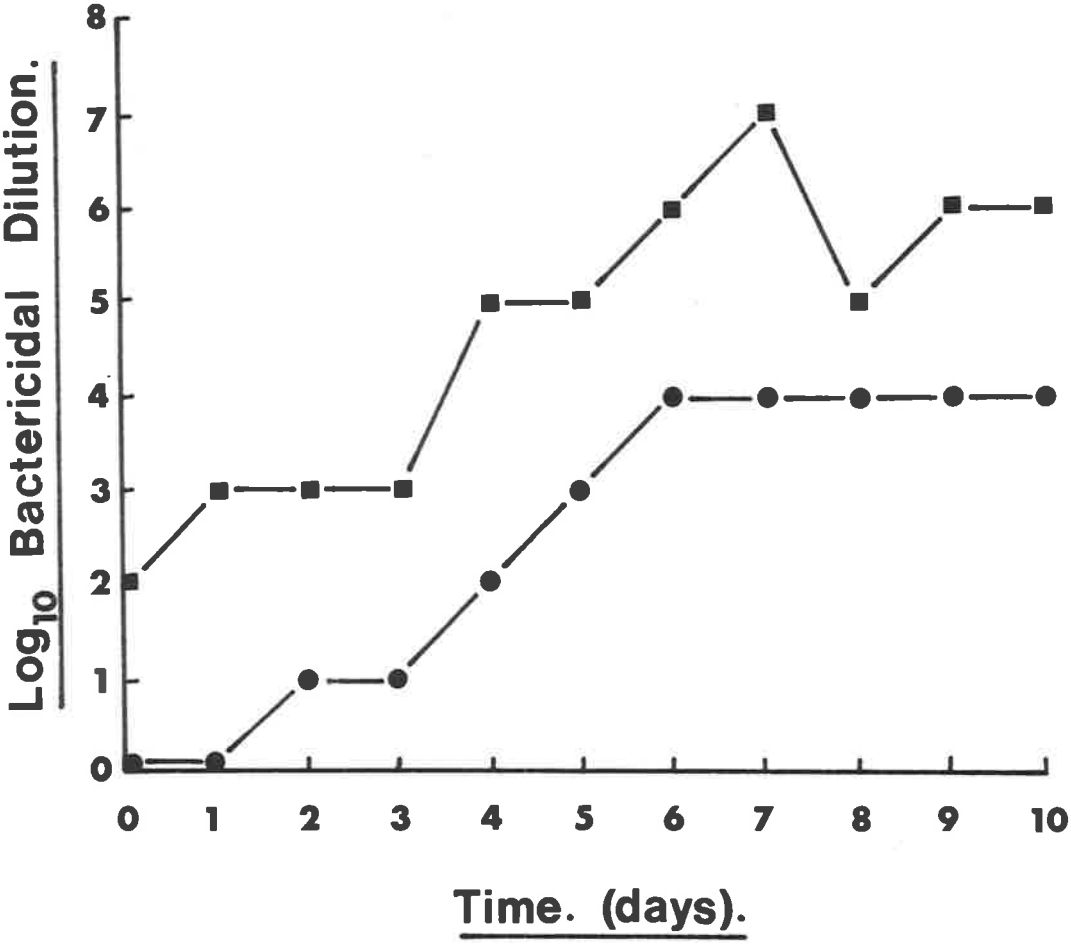
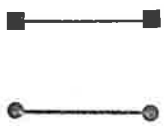

Log10 Bactericidal Dilution.
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10
Time. (days).

FIG. 4.9

Comparison of in vitro bactericidal antibody responses of rats and mice injected with living Salmonella typhimurium C5



6.4×10^6 organisms injected intraperitoneally into rats

1.3×10^3 organisms injected intraperitoneally into mice

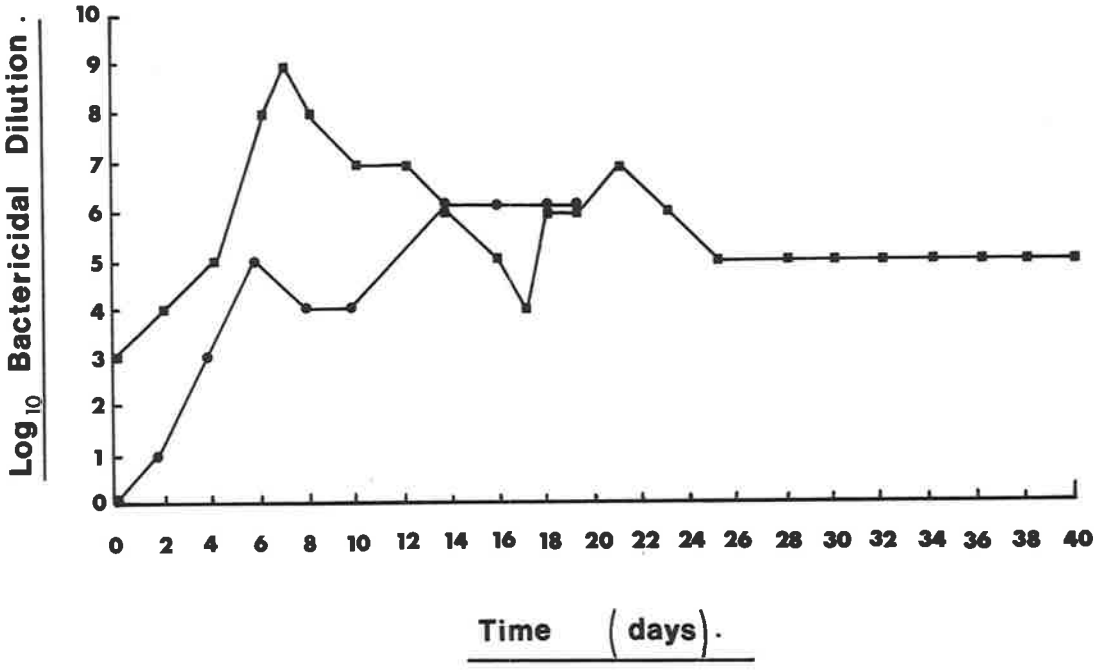
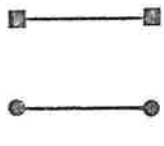

Log10 Bactericidal Dilution.
0
1
2
3
4
5
6
7
8
9
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Time (days).

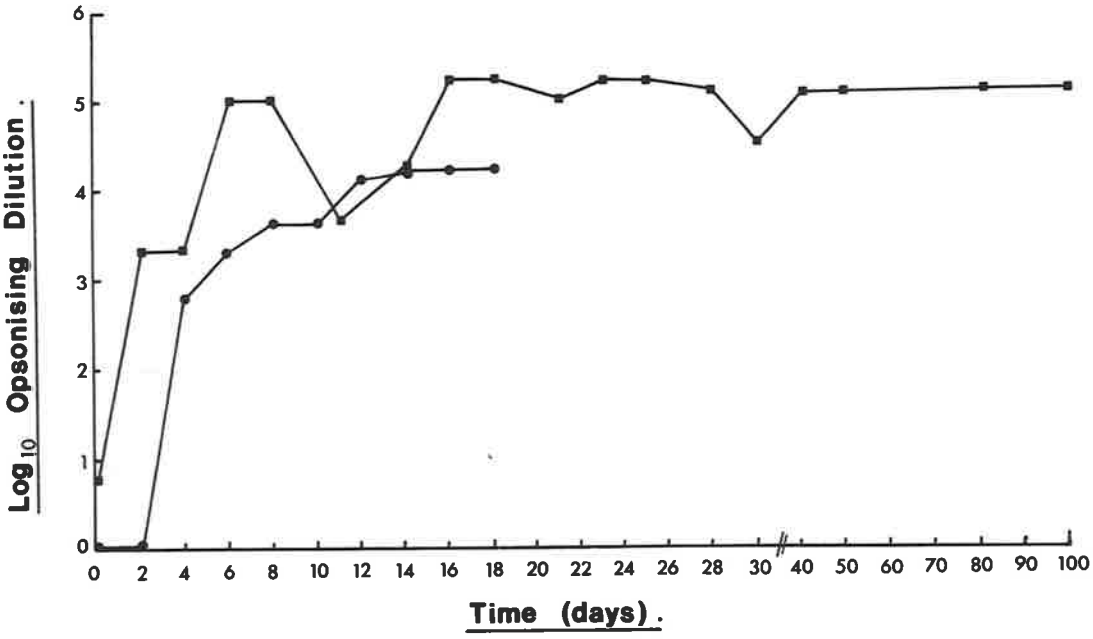
FIG. 4.10

Comparison of in vivo bactericidal antibody responses of rats and mice injected with living Salmonella typhimurium C5



6.4×10^6 organisms injected intraperitoneally into rats

1.3×10^3 organisms injected intraperitoneally into mice


Log10 Opsonising Dilution.
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 40 50 60 70 80 90 100
Time (days).

this difference is independent of the method used to measure antibody levels. However, if the results of the in vitro and in vivo assays are compared, it becomes apparent that the latter method does detect antibody which is different from that detected by the in vitro assay. Fig. 4.10 shows that there are two prominent peaks of antibody detected when the in vivo system is employed. The early peak occurs at days 6-8 and the late peak, which is greater than the early peak, occurs at day 16 and persists until at least day 28. Fig. 4.9 shows that there is an early peak of antibody detectable by the in vitro bactericidal assay, but that there is no prominent late peak of antibody.

This data may be interpreted in two ways:

(1) The in vitro assay detects antibody which is different from that detected by the in vivo assay, or

(2) during the early peak response two types of antibody are produced, namely,

(a) antibody which is detectable by the in vitro bactericidal assay but not by the in vivo assay, or

(b) the antibody is detectable both by the in vitro and the in vivo bactericidal assays,

whereas in the late peak only one type (b) of antibody is produced which is detectable by both the in vivo and the in vitro assay systems.

To determine whether the late antibody peak detected by the in vivo bactericidal assay differed from the antibody comprising the early peak, in vivo bactericidal assays were again carried out on the same sera before and after reduction with 2-mercaptoethanol (2-ME). The serum samples not reduced with 2-ME were dialysed against phosphate buffer under similar conditions to the 2-ME reduced samples in order to provide controls for non-specific reduction in bactericidal activity. The results obtained using

rat serum are shown in Fig 4.11 from which it is evident that whilst the early antibody peak consists of both 2-ME sensitive and resistant antibody, the late peak consists entirely of 2-ME resistant antibody. It is also evident from both Figs 4.10 and 4.11 that this latter peak of antibody is 2 fold greater than the early antibody peak. Reduction by 2-ME of mouse antisera (Fig. 4.12) shows that in contrast to the antibody response in rats, the early response in mice consists almost entirely of 2-ME sensitive antibody. However, later in the mouse response the proportion of 2-ME sensitive antibody to 2-ME resistant antibody decreases.

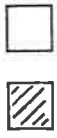
From the results presented above it can be concluded that rats are able to respond more rapidly and produce higher antibody titres than are mice when these animals are challenged with living Salmonella typhimurium C5. Furthermore, if the results of the in vivo bactericidal assays (Fig. 4.10) are compared with the results obtained when examining the fate of Salmonella typhimurium C5 injected into rats (Figs 4.2 and 4.5), it becomes apparent that the late peak of antibody produced by rats occurs immediately prior to and coincident with the time rats begin to eliminate the Salmonella organisms. It appears therefore, that there is a definite correlation between the late peak of antibody and the elimination of Salmonella typhimurium C5. However, there appears to be no apparent correlation of the early antibody peak with a reduction in the numbers of these organisms that can be recovered at that time.

Comparison of the antibody response of rats and mice injected with various doses of alcohol-killed Salmonella typhimurium C5 vaccine

Rats are clearly capable of controlling the Salmonella typhimurium C5 infection, whereas mice are not, and at least initially, rats are much more efficient at killing the C5 strain than are mice. Since it is likely that bacterial antigens provide no antigenic stimulus to the host while they are

FIG. 4.11

Comparison of in vivo bactericidal antibody titres produced by rats in response to challenge with 6.4×10^6 living Salmonella typhimurium C5, before and after reduction with 2-mercaptoethanol.



Dialysed control

2-mercaptoethanol treated

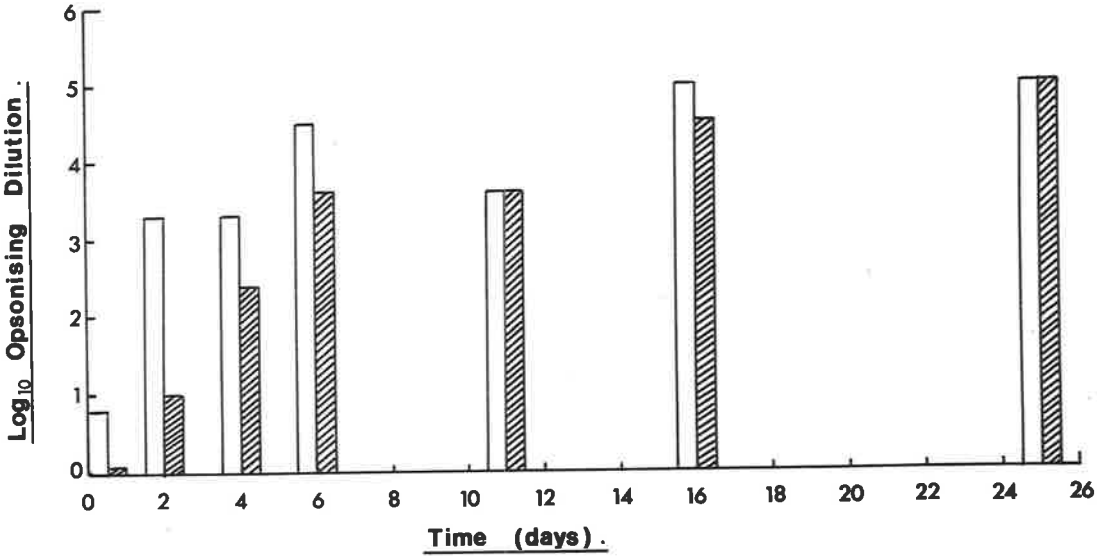
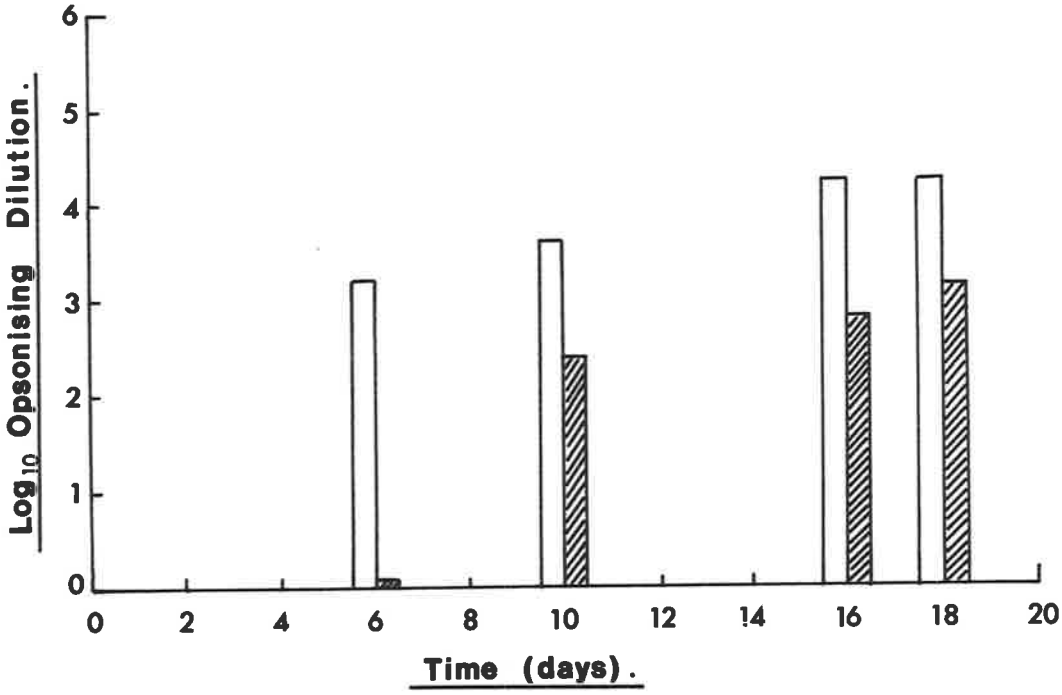

Log10 Opsonising Dilution.
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (days).

FIG. 4.12

Comparison of in vivo bactericidal antibody titres produced by mice in response to challenge with 1.3×10^3 living Salmonella typhimurium C5, before and after reduction with 2-mercaptoethanol.

Dialysed control

2-mercaptoethonol treated


Log10 Opsonising Dilution.
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
Time (days).

part of a living bacterium, it is possible that this species difference in antibody response to Salmonella typhimurium C5 antigens is due to a difference in the effective antigenic stimulus the animals receive.

It seemed necessary, therefore, to compare the antibody response of rats and mice to various doses of killed Salmonella typhimurium C5 vaccine, where the dose of antigen each animal received could be accurately determined.

A large number of both male and female rats and mice, 12 weeks old at the beginning of the study and weighing 190-220 gm. and 18-21 gm. respectively, were injected intravenously with various doses of alcohol-killed Salmonella typhimurium C5 vaccine. The doses used were 0.01 0.1, 1.0, 5.0, 10, 50, 100, 200 and 300 μg. per animal for both rats and mice. The results of these studies are shown in Figs. 4.13, 4.14, 4.15, 4.16 and 4.17. The sera used were obtained by bleeding 3 rats and 5 mice from each group at regular intervals and were pooled for each group. For the purposes of this particular study, the in vitro bactericidal assay was considered an adequate means of detecting antibody. It is evident from the results obtained that whilst antibody levels produced differed most at the upper and lower limits of the dose range used (Figs 4.13, 4.16 and 4.17) rats were able to mount a better immune response than mice to each dose injected.

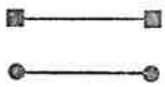
Since the in vitro bactericidal assay system had been used, it was of interest to assay antibody responses to the killed vaccine of Salmonella typhimurium C5 by the in vivo bactericidal system. This was done to confirm the difference in antibody levels produced by rats and mice as detected by the in vitro system and to provide an indication of the levels of protective antigody produced in response to infection with a killed vaccine. Therefore, rats and mice were injected intravenously with a

FIG. 4.13

Comparison of in vitro bactericidal antibody titres produced by rats and mice in response to intravenous injection with alcohol-killed Salmonella typhimurium C5 vaccine

A 0.01 μg. of vaccine injected

B 0.1 μg. of vaccine injected



rat antibody

mouse antibody

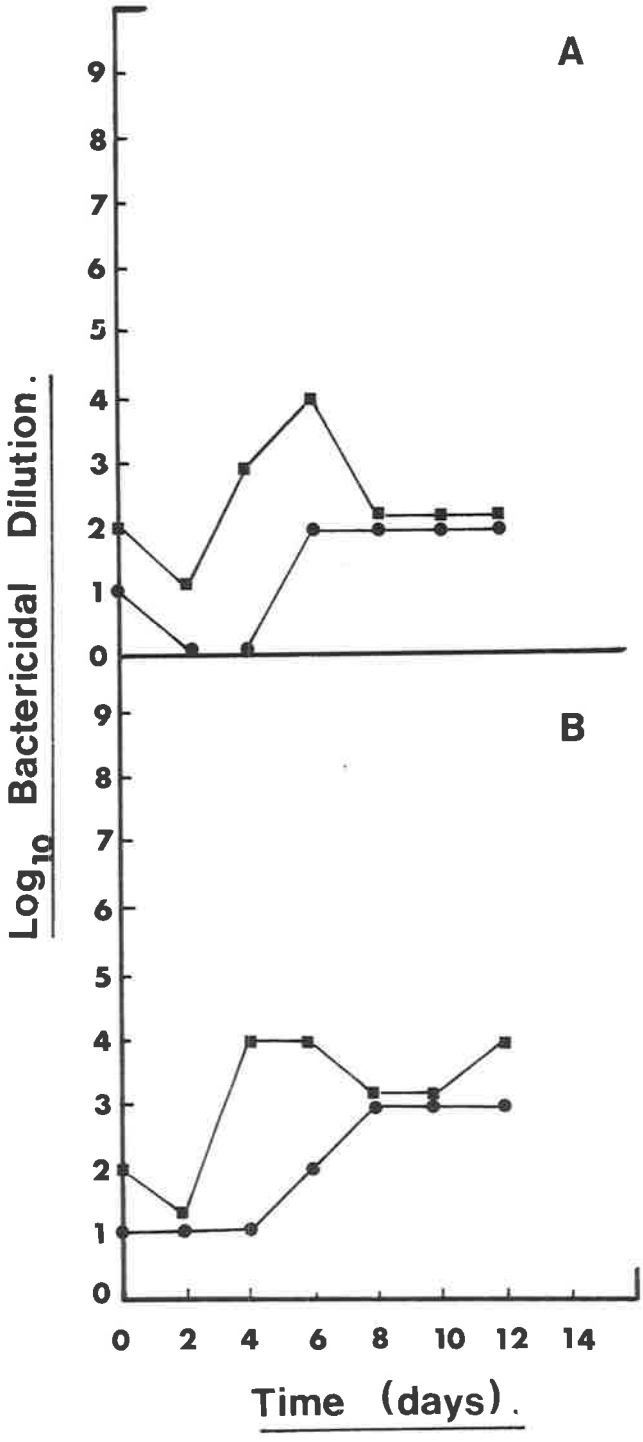
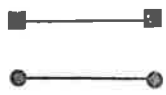

A
B
9
8
7
6
5
4
3
2
1
0
Log10 Bactericidal Dilution.
0 2 4 6 8 10 12 14
Time (days).

FIG. 4.14

Comparison of in vitro bactericidal antibody titres produced by rats and mice in response to intravenous injection with alcohol-killed Salmonella typhimurium C5 vaccine

A 1.0 μg. of vaccine injected

B 5.0 μg. of vaccine injected



rat antibody

mouse antibody

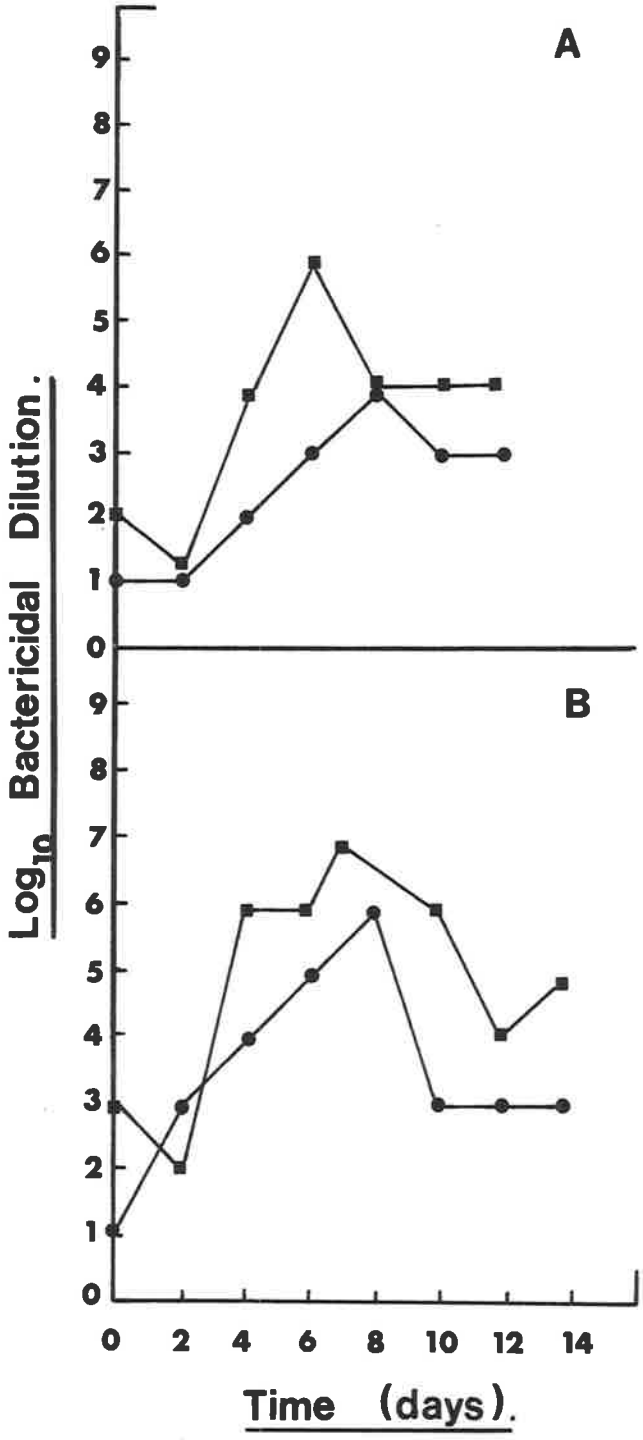
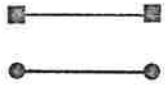

A
B
9
8
7
6
5
4
3
2
1
0
Log10 Bactericidal Dilution.
0
2
4
6
8
10
12
14
Time (days).

FIG. 4.15

Comparison of in vitro bactericidal antibody titres produced by rats and mice in response to intravenous injection with alcohol-killed Salmonella typhimurium C5 vaccine

A 10 μg. of vaccine injected

B 50 μg. of vaccine injected



rat antibody

mouse antibody

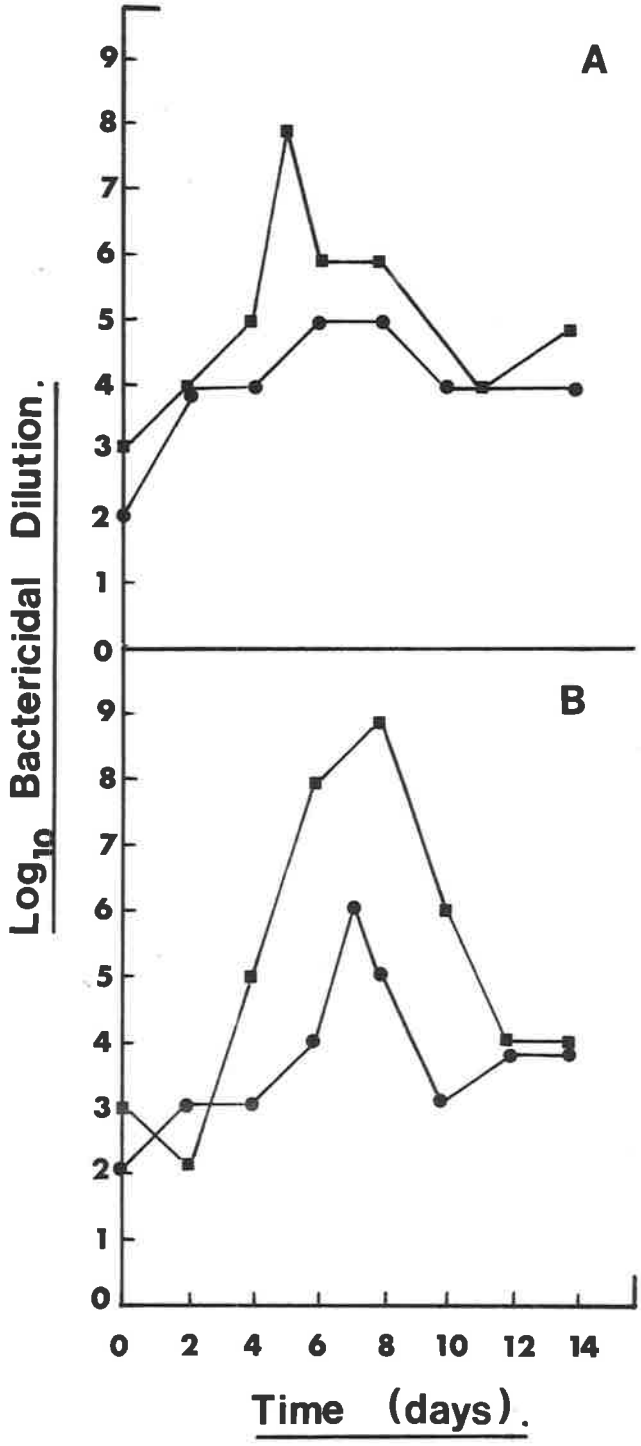

A
B
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Log10 Bactericidal Dilution.
Time (days).

FIG. 4.16

Comparison of in vitro bactericidal antibody titres produced by rats and mice in response to intravenous injection with alcohol-killed Salmonella typhimurium C5 vaccine

A 100 μg. of vaccine injected

B 200 μg. of vaccine injected

rat antibody

mouse antibody

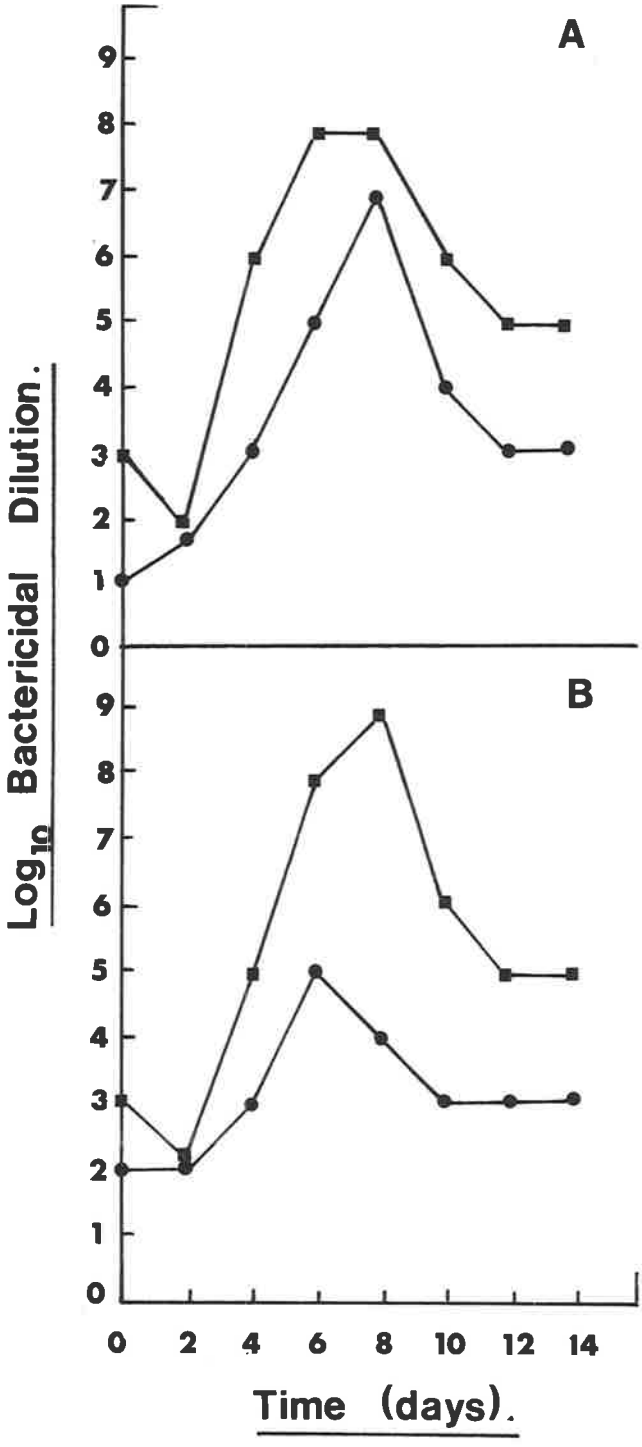
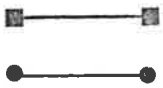

A
B
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Log10 Bactericidal Dilution.
Time (days).

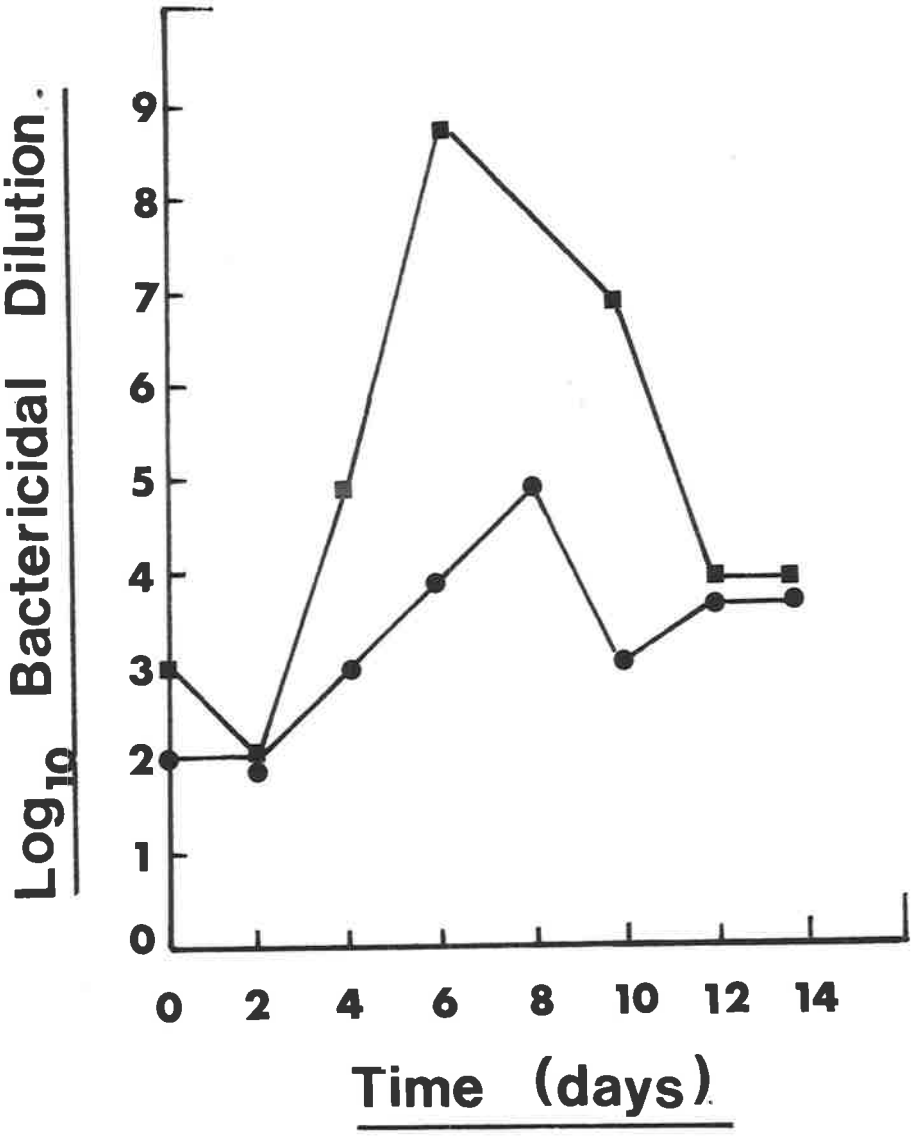
FIG. 4.17

Comparison of in vitro bactericidal antibody titres produced by rats and mice in response to intravenous injection with 300 μg. of alcohol-killed Salmonella typhimurium C5 vaccine



rat antibody

mouse antibody


Log10 Bactericidal Dilution.
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Time (days)

weight adjusted dose of alcohol-killed vaccine - 100 μg. and 10 μg. respectively and the antibody response was followed for a period of 50 days. The results shown in Fig 4.18 again show that rats produce more antibody than do mice. Although a second peak of antibody is not produced by rats in this case, compared with the response of rats to the living vaccine of Salmonella typhimurium C5, it is evident that a relatively high level of antibody is maintained for some time by rats. In comparison mice produce a lower peak of antibody which has a shorter duration.

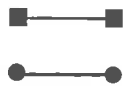
The antibody response of rats and mice immunized with bovine serum albumin

From the results presented it could be said that rats do not die when infected with Salmonella typhimurium C5 organisms because they are able to produce high titres of antibody to this organism. In other words, there appears to be a correlation between the ability of animals to produce high titres of antibody to Salmonella typhimurium C5 and their ability to resist infection with this organism. Mice however, may be susceptible to this infection because they are incapable of producing such good antibody responses. On the other hand, it could be argued that this correlation is only an apparent one which simply reflects the intrinsic difference in the ability of the two species to mount an immune response to any immunogen. Since mice are clearly resistant to a large number of different microorganisms - presumably because they are capable of producing adequate amounts of antibody to these infectious agents - it seemed necessary to determine whether there was any intrinsic difference between rats and mice in their ability to respond to an antigen unrelated to Salmonella typhimurium antigens. Bovine serum albumin was chosen as the antigen to be used.

Three groups of rats and three groups of mice were injected

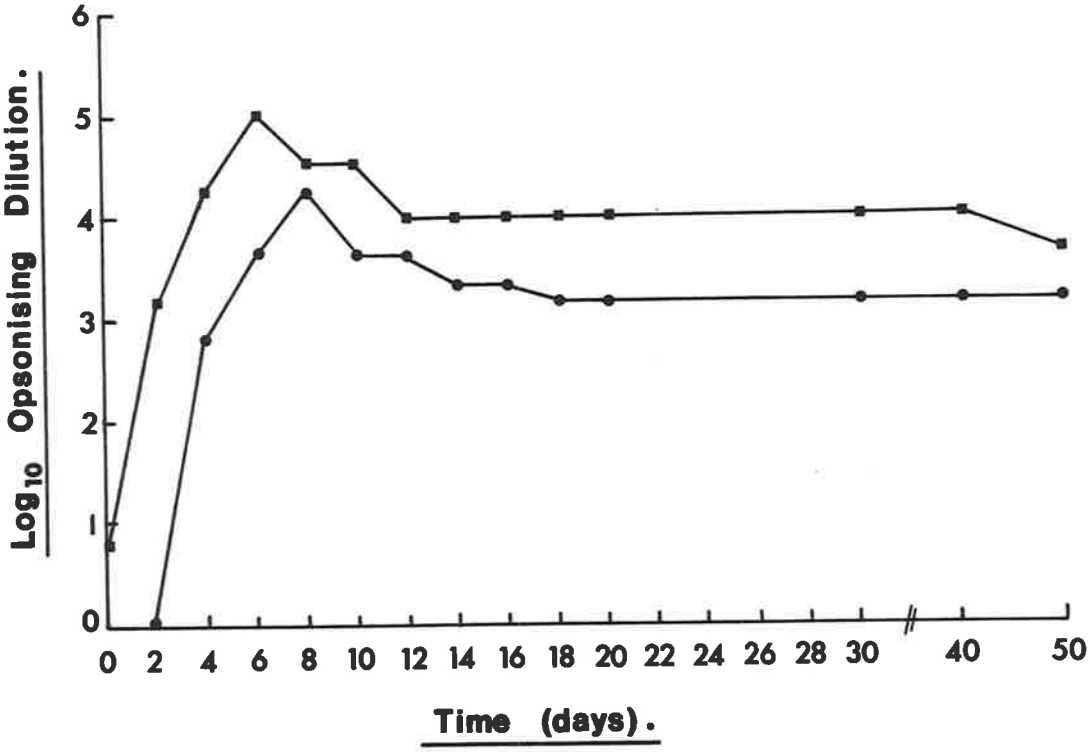
FIG. 4.18

Comparison of in vivo bactericidal antibody response of rats and mice injected intravenously with weight adjusted doses of alcohol-killed Salmonella typhimurium C5 vaccine



rat antibody response to 100 μg. of vaccine

mouse antibody response to 10 μg. of vaccine


Log10 Opsonising Dilution.
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 40 50
Time (days).

subcutaneously at the base of the tail with either 0.1 mg., 1.0 mg., or 10 mg. of bovine serum albumin (BSA) (Commonwealth Serum Laboratories, Parkville, Melbourne) in Freund's incomplete adjuvant. One month later all the animals were injected with 100 μg. of soluble BSA (intraperitoneally). After 10 days the animals were bled and the serum from each group was pooled. The sera were then assayed by the method of haemagglutination of sheep red blood cells coated with BSA as described by Gold and Fundenberg (1967). The results are shown in Table 4.2 from which can be concluded that mice are able to mount an immune response which is equal to that in rats. Thus it is not a general deficiency in the antibody producing system of mice that prevents them from responding effectively to the antigens of Salmonella typhimurium C5, but rather it may be an inherent difficulty to recognise this organism as foreign (Jenkin 1962), or an inability to produce a response as rapidly as do rats.

Conclusions

The results obtained using killed vaccine to immunize rats and mice confirm the findings observed when the living vaccine of Salmonella typhimurium C5 was injected. Rats are able to produce more antibody than mice, irrespective of the dose of antigen used. The lowest dose of antigen induced antibody titres which were one hundred fold higher in rats than in mice, whereas with the highest dose the difference was ten thousand fold.

When mice are injected with living virulent Salmonella typhimurium organisms, they multiply until the mice die from an overwhelming infection (Mackaness et al., 1966). This observation has been confirmed by the present studies which also show that this situation is quite different from that occurring in rats injected with these organisms. The results obtained indicate that Salmonella typhimurium C5 is avirulent for rats because this

TABLE 4.2

Comparison of the ability of rat and mouse serum to haemagglutinate BSA coated sheep red blood cells

Priming dose of BSA (mg.)	Challenge dose of BSA (μg.)	Reciprocal haemagglutination titre of BSA coated sheep red blood cell expressed as Log_2.	
		Mice	Rats
0.1	100	2^{11}	2^{13}
1.0	100	2^{14}	2^{12}
10	100	2^{13}	2^{14}

species is capable of controlling and/or limiting the rate of multiplication of this organism so that an overwhelming infection does not occur. A situation similar to the carrier state described in mice infected with avirulent Salmonellae (Jenkin, Rowley and Auzins 1964; Collins, Mackaness and Blanden 1966) becomes established very shortly after infection, the level of recoverable organisms remaining constant for approximately 7 days or three weeks and being determined by the number of organisms originally injected. However, unlike other previously reported findings dealing with the survival of various intracellular parasites in animals injected with a lower than LD50 dose of organisms (Mackaness, (1962), Mackaness (1964), Mackaness et al., (1966), Gray and Cheers (1967) very little multiplication of Salmonella typhimurium C5 occurs. The total number of bacteria that can be recovered from rats never exceeds the number of viable organisms originally injected. This explains the extremely high degree of resistance to infection with Salmonella typhimurium C5 that rats possess.

Whilst these results do not provide a complete understanding of the actual mechanism involved, which apparently limits multiplication of Salmonella typhimurium C5 in rats, the studies on the comparison of antibody production by rats and mice, injected with either living Salmonella typhimurium C5, or a killed vaccine of the same organism, clearly indicate that rats are capable of producing more antibody than mice. It is tempting to suggest therefore, that this difference in ability to synthesise antibody to Salmonella typhimurium C5 is responsible for the difference in susceptibility of the two species of animals.

Unfortunately however, there appears to be no good correlation between the time of the early peak of antibody and the numbers of bacteria recoverable at or near this time. In fact, antibody production appears to have no detectable effect at all in rats at this time. The carrier state becomes

established within 24 hours of injection of Salmonella typhimurium C5 and is maintained at a constant level for approximately 7 days or three weeks, with no apparent reduction at or near day 6 - the time of the early peak of antibody production.

The results shown in Figs. 4.9 and 4.10 were obtained using the same sera, however, Fig 4.10 presents quite a different picture from that seen in Fig 4.9. In this case a second peak of antibody was detected. This peak was maintained for a comparatively long time - a time which envelops the period of bacterial elimination.

It has been shown (Fig 4.11) that the early antibody peak contains a high proportion of 2-mercaptoethanol sensitive (presumably macroglobulin) antibody and Michael and Rosen (1963) and Robbins (1965) have shown that macroglobulin antibody is more bactericidal molecule for molecule than is 7S antibody. This may suggest therefore, that the correlation between the late peak of antibody, which consists entirely of 7S globulin, and the elimination of bacteria by rats is poor. However, before dismissing the possibility that there is a correlation between antibody levels, elimination of Salmonella typhimurium C5 and resistance to infection caused by this organism in rats, there are several important facts to be considered. Firstly, there is a difference in actual antibody levels existing between early and late peaks. It is evident from Fig 4.10 that the latter peak of antibody is 2-fold greater than the early peak. Secondly, and more importantly, this later peak is maintained for many days. In comparison the early peak of antibody is sustained for only about 2 days, after which time it falls rapidly to a comparatively low titre. It seems quite possible therefore, that the early peak of antibody shows a poor correlation with the number of recoverable organisms, because this level of antibody is maintained for a relatively short time compared with the late antibody peak. Finally, this poor correlation

may well be accentuated by the fact that, as has already been mentioned, the early peak of antibody is composed mainly of 2-mercaptoethanol-sensitive antibody (Fig 4.11) which has a much shorter half-life than has 7S antibody which comprises 100 per cent of the late peak. This would account for the shorter duration of the early antibody peak.

Finally, it is possible that the two peaks of antibody detected by the in vivo bactericidal assay differ in specificity. The antibody comprising the late peak may be specifically directed against those antigenic sites on the bacterium which when complexed with antibody lead to more efficient phagocytosis and killing.

Two different criteria have been used to determine the degree of resistance or susceptibility that an animal possesses to an infection caused by a particular strain of Salmonella. Rowley, Auzins and Jenkin (1968) have taken the ultimate survival of an animal as their criterion for resistance. On the other hand, Collins, et al., (1966) argued that because some animals can survive Salmonella infections without completely eliminating the infecting organism, that complete elimination of bacteria should be the ultimate criterion for resistance.

Since mice are unable to restrict the multiplication of Salmonella typhimurium C5 (Figs 4.2 and 4.5) and invariably die as a result of an overwhelming infection, they are clearly susceptible by either of the two criteria used in the past. In the case of rats, in the early stages of infection with Salmonella typhimurium C5, whilst they are unable to eliminate the infecting organisms, they are clearly able to control their multiplication (Figs 4.2 and 4.5.) and they do not die, therefore using the first criterion (Rowley et al., 1968) one would say that rats are resistant to Salmonella typhimurium C5 infection. However, by the second criterion (Collins et al., 1966) they may only be classed as resistant at the stage when the second peak of antibody is produced

(Fig 4.10) and complete elimination of the infecting organism occurs. Thus the resistance of rats may be said to consist of two phases - an early phase and a late phase.

In summary therefore, the events which occur immediately after challenge of rats and mice with Salmonella typhimurium C5 must play an important part in determining the ultimate resistance of these animals as determined by whether they live or die. This, together with the fact that rats are able to respond more rapidly and produce greater quantities of antibody and the correlation of antibody titres with the elimination of bacteria, suggests that the degree of resistance of animals to Salmonella typhimurium C5 infection, is determined in part by the levels of specific natural antibody that they possess and in part by the antibody response elicited as a result of infection.

CHAPTER V

THE ROLE OF ANTIBODY IN THE PROTECTION OF RATS AND MICE AGAINST INFECTION WITH SALMONELLA TYPHIMURIUM C5

INTRODUCTION

Mackaness (1961) attributed the susceptibility of normal mice infected with Listeria monocytogenes to the capacity of these organisms to survive and multiply within the macrophages of the host. Furthermore he could find no protective factor in the serum of Listeria monocytogenes - convalescent mice which were resistant to further challenge with this organism. These findings, together with the results of phagocytic experiments he carried out led to the conclusion that macrophages were the sole factor responsible for immunity to this infection. Blanden, Mackaness and Collins (1966) studied the mechanism of acquired resistance to Salmonella typhimurium in mice and again concluded that the enhanced bactericidal ability of macrophages was of major importance.

However, McIntyre et al., (1967) in their studies at the single cell level, of the macrophage population resulting from immunization of mice with Listeria monocytogenes, showed that this population of macrophages was different from populations of macrophages of normal mice only because it was more homogeneous. The percentage of cells possessing an effective bactericidal mechanism was increased by immunization with this organism. In other words there appeared to be no alteration in the intrinsic capacity of macrophages to kill. Furthermore, this more homogeneous macrophage population required the presence of specific opsonising antibody for efficient phagocytosis and killing to occur.

Rowley et al., (1968) also showed that immunity to Salmonella typhimurium infection was sometimes associated with increased bactericidal activity of macrophages but that specific antibody was required for the expression of this bactericidal activity.

The results published by Mackaness (1961) may be explained by the presence of cell-bound antibody, which was not investigated. The presence of cell-bound antibody appears likely since Rowley et al., (1964) were able to isolate and purify antibody from the surface of so called immune macrophages from mice infected with Salmonella typhimurium.

The question of correlation of resistance with levels of antibody has been investigated in the case of Salmonella typhimurium infections in mice. Rowley and Whitby (1959) clearly established that the rate at which gram negative organisms, including Salmonella typhimurium, are eliminated from the peritoneal cavities of mice is entirely dependent upon the availability of serum opsonins. Also in 1959, Jenkin and Rowley showed that the sera of some species of normal animals were effective in preventing the otherwise lethal effects of infection with virulent Salmonella typhimurium organisms. They were able to demonstrate that pretreatment of virulent Salmonella typhimurium organisms with horse, pig or rat serum reduced the percentage mortality of mice challenged with these organisms; compared with mice challenged with the same number of untreated (unopsonised) organisms. Further, in 1962 Jenkin concluded that in the absence of serum opsonins, Salmonella typhimurium organisms that are ingested by macrophages, will survive and multiply intracellularly. However, in the presence of pig serum which he had shown to contain opsonins specific for Salmonella typhimurium, the ingested bacteria were killed.

The results presented in Chapter III have shown that in the presence of normal rat serum, normal mouse macrophages are similar to normal rat macrophages in their ability to phagocytose and kill Salmonella typhimurium C5. Furthermore, the results discussed in Chapter IV show that there is a definite correlation between antibody levels and the ability of rats to :-

1. control the multiplication of Salmonella typhimurium C5 and
2. eliminate these organisms completely.

From these results it may be suggested that antibody plays the major role in immunity, both natural and acquired, to infection with Salmonella typhimurium C5.

In 1963 Jenkin and Rowley showed that it was possible to passively transfer immunity to mice with serum. They injected normal mice with serum from mice that had previously been immunized with the avirulent M206 strain of Salmonella typhimurium. Their results show that, upon subsequent challenge of the recipient mice with 1000 lethal doses of the virulent C5 strain of Salmonella typhimurium, some protection of normal mice had been achieved. The protective ability of the passively transferred serum was measured by an increased survival time as well as a decrease in the overall number of deaths. Similar findings were reported by Jenkin et al., (1964).

Therefore, to determine whether antibody which is opsonic and which promotes phagocytosis and killing of Salmonella typhimurium C5 organisms is also protective, passive transfer studies should be carried out.

The data available on the effectiveness of some normal and immune sera in passively transferring protection to animals susceptible to Salmonella typhimurium C5 infections is limited. Therefore, experiments

were carried out to confirm and extend the abovementioned findings. These experiments were however, particularly designed to compare normal and immune serum from both rats and mice, for its ability to passively transfer protection to the susceptible species.

Further studies were carried out to determine the effect of decreasing existing serum antibody levels of both rats and mice, on their ability to kill Salmonella typhimurium C5 organisms and on the overall resistance or susceptibility of these animals to infection with this organism.

There is a suggestion, from the results presented in Chapter IV, that the antibody produced late in the immune response of rats challenged with Salmonella typhimurium C5, differs from the antibody produced early in the immune response. To determine whether this was so, rats were examined for their ability to deal with a super-infecting dose of Salmonella typhimurium C5 injected at the different times after initial infection, which corresponded with the peaks of antibody formation.

Comparison of the ability of rat and mouse sera to passively transfer protection to mice against infection with Salmonella typhimurium C5

All the mice used were 12 week old males and females and weighed 18-20 gm. at the beginning of the experiment.

(a) Passive transfer of normal rat and normal mouse serum

Normal rat and normal mouse serum was obtained by pooling the sera of a large number of animals. A comparison was then made of the ability of the serum from these two species, to passively transfer protection to normal mice against infection with the C5 strain of Salmonella typhimurium. Protection was assessed in terms of the percentage mortality over a 28 day observation period.

In the first experiment, normal mice were divided into 3 groups of 30 animals as follows:-

1. challenged with 2.16×10^2 unopsonised organisms,
2. challenged with 2.16×10^2 organisms opsonised with normal mouse serum by the method described in Chapter II, or
3. injected intravenously with 0.2 ml. of normal mouse serum on the two days immediately prior to challenge and on the two days immediately following challenge with 2.16×10^2 opsonised organisms.

The results are shown in Fig 5.1

The second experiment using normal rat serum was carried out in a similar manner. Normal mice were again divided into 3 groups of 30 animals and,

1. challenged with 2.86×10^2 unopsonised organisms
2. challenged with 2.86×10^2 organisms opsonised with normal rat serum by the method described in Chapter II, or
3. injected intravenously with 0.2 ml. of normal rat serum on the two days immediately prior to and immediately following challenge with 2.86×10^2 opsonised organisms.

The results are shown in Fig 5.2

These studies show that normal mouse serum provides little or no protection against infection with Salmonella typhimurium C5. In contrast, the passive transfer of normal rat serum resulted in good protection of mice; a 10 per cent mortality of mice resulted when normal rat serum was used whereas 70-80 per cent of mice receiving normal mouse serum died.

(b) Passive transfer of immune rat and immune mouse serum

To obtain the immune sera required for this experiment, 20 rats were injected intraperitoneally with 8.0×10^3 viable Salmonella typhimurium C5 organisms and 50 mice were injected intraperitoneally

FIG. 5.1

The survival of mice challenged with Salmonella typhimurium C5, after passive transfer of normal mouse serum.

mice challenged with $2{\cdot}16 \times 10^2$ unopsonised organisms

mice challenged with $2{\cdot}16 \times 10^2$ organisms opsonised with normal mouse serum

mice receiving two intravenous injections of normal mouse serum, before and after challenge with $2{\cdot}16 \times 10^2$ organisms opsonised with normal mouse serum.

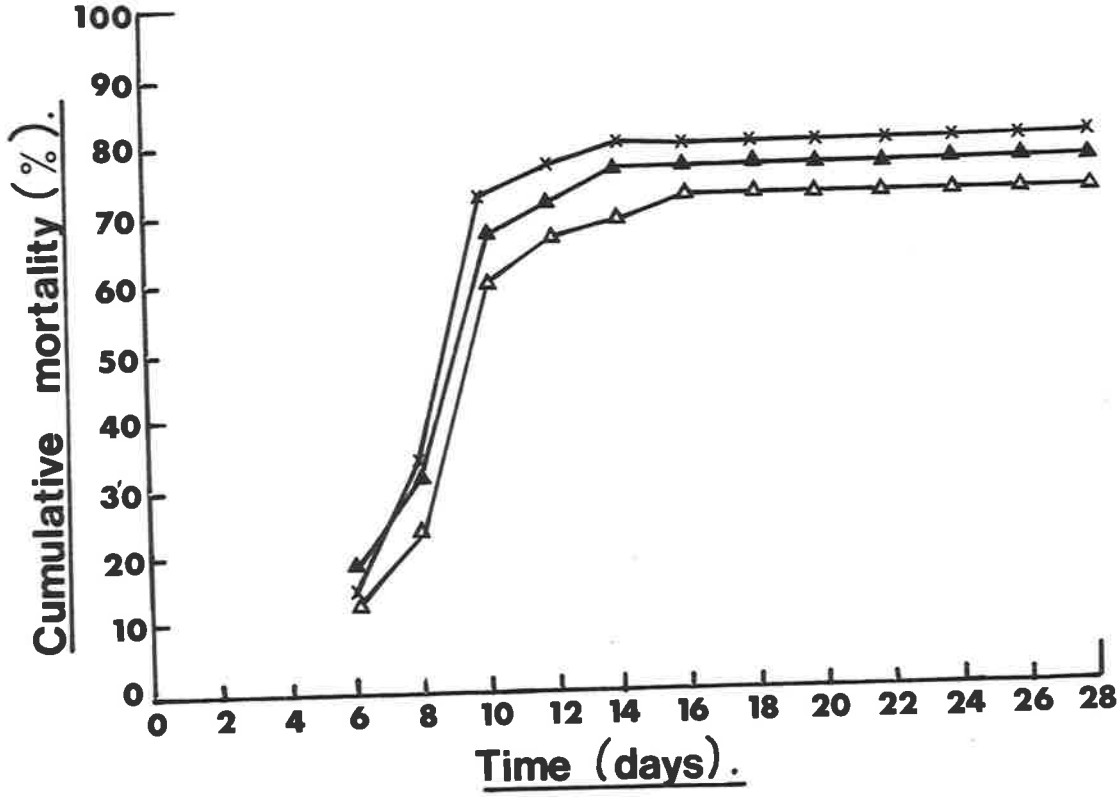

Cumulative mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

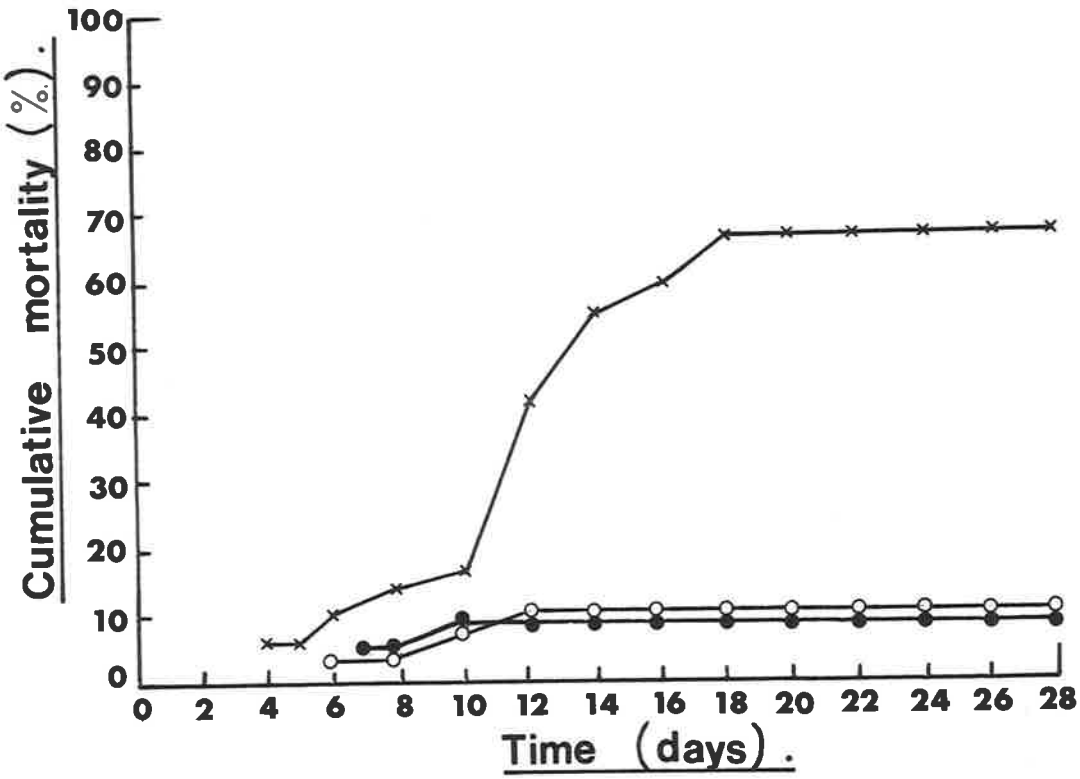
FIG. 5.2

The survival of mice challenged with Salmonella typhimurium C5, after passive transfer of normal rat serum.

mice challenged with $2{\cdot}86 \times 10^2$ unopsonised organisms

mice challenged with $2{\cdot}86 \times 10^2$ organisms opsonised with normal rat serum

mice receiving two intravenous injections of normal rat serum, before and after challenge with $2{\cdot}86 \times 10^2$ organisms opsonised with normal rat serum


Cumulative mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

with 1.0×10^3 viable organisms of the same strain. Six days later all the rats and mice were bled and pools of rat serum and mouse serum prepared in the usual manner. Care was taken to ensure that these and all the sera used in passive transfer experiments were sterile.

The presence of antibody to Salmonella typhimurium C5 in pools of immune rat and immune mouse serum was determined using the in vivo bactericidal assay system. The end points of immune rat and immune mouse serum were found to be 1/25,600 and 1/3200 respectively. Since the antibody titres of the immune sera were greater than those of serum from normal rats and mice, (1/80 and less than 1/2 respectively), the challenge doses of Salmonella typhimurium C5 used were greater than those used in passive transfer experiments with the normal sera.

Using immune rat serum, normal mice were divided into 3 groups of 30 animals and -

1. challenged with 2.0×10^3 unopsonised organisms
2. challenged with 2.0×10^3 organisms opsonised with immune rat serum by the method described in Chapter II, and
3. injected intravenously with 0.2 ml. of immune rat serum on the two days immediately prior to and following challenge with 2.0×10^3 opsonised organisms.

The results are shown in Fig 5.3

Three groups of 30 normal mice were also used to study the passive transfer of protection with immune mouse serum: -

1. a group challenged with 1.0×10^3 unopsonised organisms
2. a group challenged with 1.0×10^3 organisms opsonised with immune mouse serum by the method described in Chapter II, and
3. a group injected intravenously with 0.2 ml. of immune mouse serum on the two days immediately prior to and following challenge with 1.0×10^3 opsonised organisms.

The results are shown in Fig 5.4

FIG. 5.3

The survival of mice challenged with Salmonella typhimurium C5 after passive transfer of immune rat serum

mice challenged with 2×10^3 unopsonised organisms

mice challenged with 2×10^3 organisms opsonised with immune rat serum

mice receiving two intravenous injections of immune rat serum, before and after challenge with 2×10^3

organisms opsonised with immune rat serum

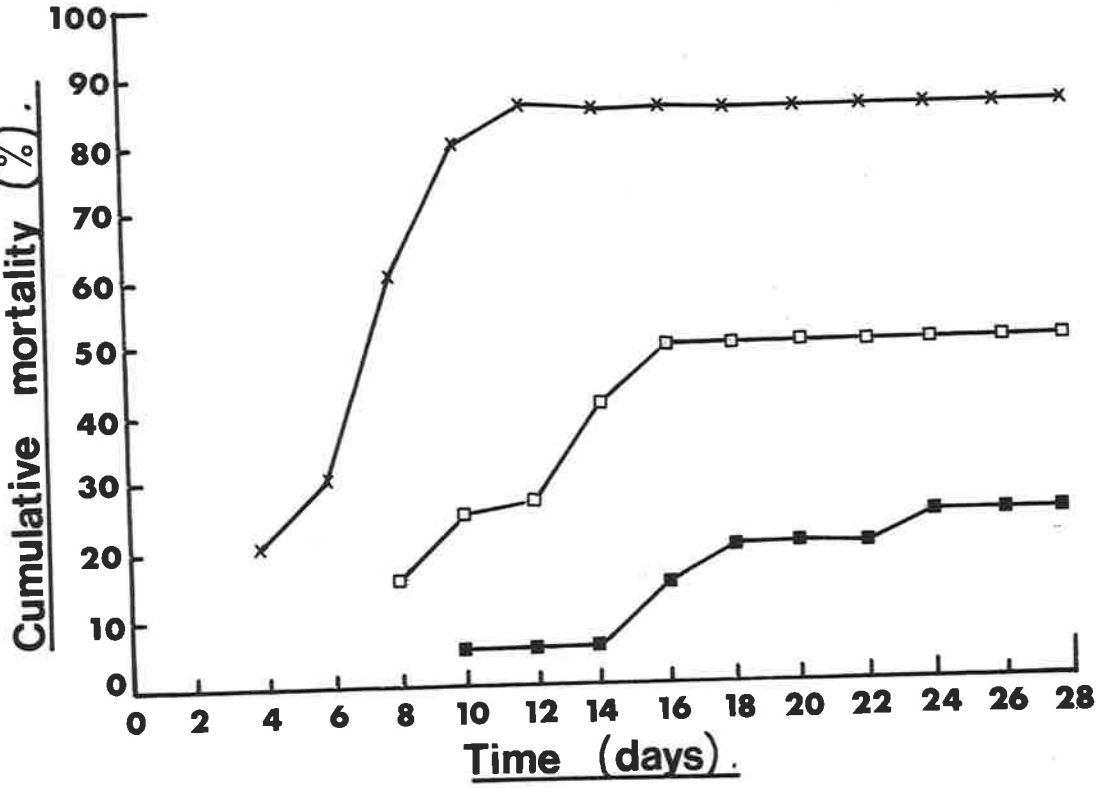
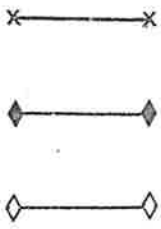

Cumulative mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

FIG. 5.4

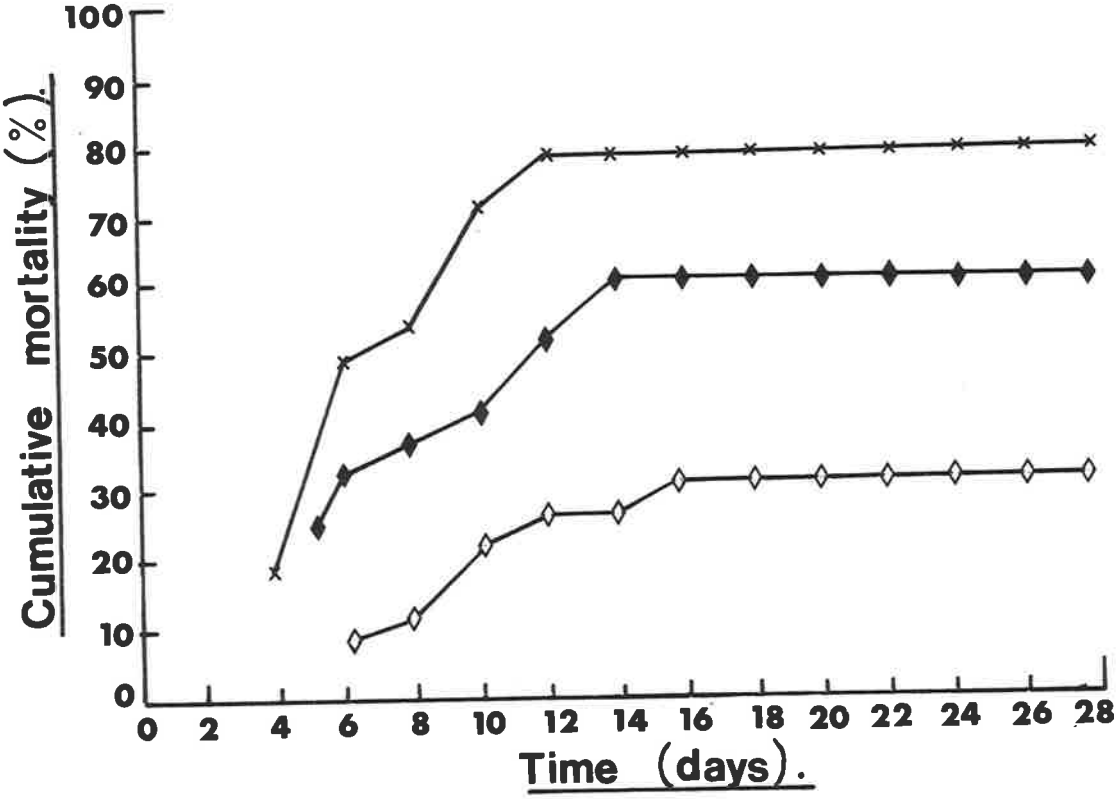
The survival of mice challenged with Salmonella typhimurium C5, after passive transfer of immune mouse serum



mice challenged with 1×10^3 unopsonised organisms

mice challenged with 1×10^3 organisms opsonised with immune mouse serum

mice receiving two intravenous injections of immune mouse serum, before and after challenge with 1×10^3 organisms opsonised with immune mouse serum


Cumulative mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

These experiments show that passive transfer of both immune rat and immune mouse serum, results in the protection of more normal mice challenged with opsonised Salmonella typhimurium C5, than occurs when normal mice are challenged with unopsonised organisms. It is also evident that immune rat serum is more effective in passively transferring protection to normal mice than is immune mouse serum. This result appears to be in keeping with the fact that immune rat serum possesses more bactericidal activity towards Salmonella typhimurium C5 than does immune mouse serum.

The resistance of mice infected with Salmonella enteritidis 11RX to infection with Salmonella typhimurium C5

It has been shown by Rowley et al., (1968) that mice injected with the living vaccine of Salmonella enteritidis 11RX are resistant to infection with Salmonella typhimurium C5. Two weeks after injection of the Salmonella enteritidis 11RX, the mice were challenged with Salmonella typhimurium C5 and were shown to be resistant.

Salmonella enteritidis 11RX is a rough organism which possesses no detectable O-somatic antigens and which does not cross-react with Salmonella typhimurium C5 (Ushiba et al., 1959). It appeared likely therefore, that this method of inducing resistance to Salmonella typhimurium C5 in mice, would provide an excellent model for the investigation of the role of antibody in conferring resistance to these mice.

Rowley et al., (1968) had shown that the resistance of mice injected with Salmonella enteritidis 11RX and challenged with Salmonella typhimurium C5, was due to an increased bactericidal activity of macrophages and specific antibody. It appears then, that 2 weeks after injection of Salmonella enteritidis 11RX, these mice possess large numbers stimulated macrophages. However, approximately 25 days after injection of the organisms into mice, the carrier state is lost, (Neoh 1969 -

personal communication) and therefore, at this time the number of stimulated macrophages present would presumably be minimal. In fact, it has been shown that macrophages taken from the peritoneal cavities of mice 40 days after being injected with Salmonella enteritidis 11RX were identical with peritoneal macrophages from normal mice in their ability to phagocytose and kill Salmonella typhimurium C5 organisms in vitro (Table 5.1).

The resistance of mice injected with Salmonella enteritidis 11RX to challenge with Salmonella typhimurium C5

Large numbers of 10 week old male and female normal mice weighing 18-20 gm. at the beginning of the experiment, were injected intraperitoneally with approximately 1×10^6 Salmonella enteritidis 11RX organisms. After 40 days 20 mice were chosen at random and examined for the presence of these organisms using the technique described in Chapter IV. No organisms were recovered from the circulation, peritoneal cavity, spleen or liver of any of these mice. These mice, treated with Salmonella enteritidis 11RX and that have lost the carrier state, will be referred to as 11RX-recovered mice.

To determine whether these mice were still resistant to infection with Salmonella typhimurium C5, a group of 60 11RX-recovered mice and a similar group of normal mice were challenged with 1×10^5 Salmonella typhimurium C5 intraperitoneally. The 11RX-recovered mice were all resistant, whereas the normal mice were all susceptible.

To determine how specific for Salmonella typhimurium C5 the resistance acquired by 11RX-recovered mice was, a group of 30 of these mice and a group of 30 normal mice were challenged with 5×10^2 pneumococcus type II organisms by the intraperitoneal route. The 11RX-recovered mice and the normal mice were found to be equally susceptible to the infecting organism, since 80 per cent and 90 per cent of the animals

TABLE 5.1

Comparison of the phagocytic abilities of peritoneal macrophages from normal mice and from 11RX-recovered mice of unopsonised Salmonella typhimurium C5 organisms

Normal Mouse Peritoneal Macrophages		Peritoneal Macrophages from 11RX-recovered Mice	
Percentage Phagocytosis	Percentage Survival	Percentage Phagocytosis	Percentage Survival
10	95	11	93

respectively, died within a period of 28 days. This observation suggests that there is some specificity in the resistance conferred to mice by recovery from Salmonella enteritidis 11 RX infection

The fate of Salmonella typhimurium C5 injected into 11RX-recovered mice

The fate of Salmonella typhimurium C5 injected into 11RX-recovered mice was investigated to see whether these mice could deal with this organism as well as rats, that is, control its multiplication and then eliminate it completely. Accordingly, 30 rats and 50 11RX-recovered mice were challenged with 4×10^4 Salmonella typhimurium C5 intraperitoneally.

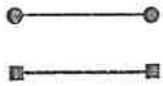
From the results presented in Chapter IV it is reasonable to assume that the fate of organisms in the peritoneal cavity is indicative of the overall ability of rats and mice to cope with this infection. Therefore, at regular intervals, the peritoneal cavities of 3 rats and 5 mice were washed out as described in Chapter IV and 0.1 ml. samples of each washout fluid plated on dried nutrient agar plates to determine the number of bacteria recovered. It is evident from the results presented in Fig 5.5, that mice rendered resistant by virtue of their recovery from Salmonella enteritidis 11RX infection are just as capable as normal rats in controlling the multiplication of Salmonella typhimurium C5 and eliminating these organisms from their peritoneal cavities.

The role of antibody in the resistance of 11RX-recovered mice to infection with Salmonella typhimurium C5

It has not yet been established whether antibody or stimulated macrophages (Rowley et al., 1968) plays the major role in controlling Salmonella typhimurium C5 infection in 11RX-recovered mice. Therefore, an experiment was designed to determine whether any antibody, specific for Salmonella typhimurium C5, could be detected in the serum of 11RX-recovered mice. Pools of serum from normal mice, normal

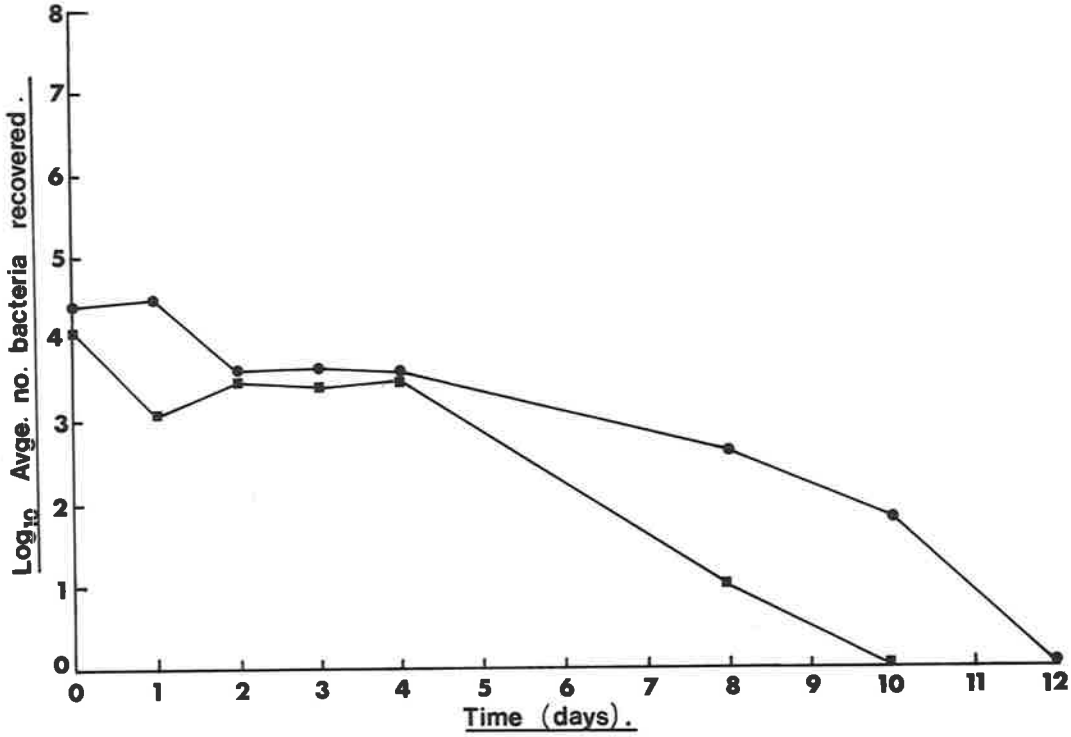
FIG. 5.5

Recovery of Salmonella typhimurium C5 from the peritoneal cavities of rats and 11RX- recovered mice challenged with 4×10^4 organisms intraperitoneally



11RX - recovered mice

normal rats


Log10 Avge. no. bacteria recovered.
0
1
2
3
4
5
6
7
8
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (days).

rats and 11RX-recovered mice were assayed for the presence of antibody to Salmonella typhimurium C5 using the in vivo bactericidal assay technique described in Chapter II. The results are shown in Fig 5.6 from which it is evident that serum from 11RX-recovered mice, is similar to normal rat serum in its ability to promote peritoneal killing of Salmonella typhimurium C5. Both of these sera contained more bactericidal antibody than did normal mouse serum.

To determine whether the antibody detected in the serum of 11RX-recovered mice plays any role in protection, an attempt was made to deplete the serum antibody levels of these mice prior to challenge with Salmonella typhimurium C5. Firstly, a group of 25 11RX-recovered mice were injected intravenously with 10 mg. of the polysaccharide extracted from Salmonella typhimurium C5 (C5 polysaccharide). Two hours later, these mice were bled and the serum collected and pooled. At the same time, similar numbers of normal mice and 11RX-recovered mice not injected with C5 polysaccharide were bled and the serum from each group was pooled. These sera were then assayed for bactericidal antibody content by the in vivo bactericidal assay technique described in Chapter II. The results are shown in Fig. 5.7 and it is evident that injection of C5 polysaccharide into 11RX-recovered mice does reduce the ability of their serum to promote peritoneal killing of Salmonella typhimurium C5. Furthermore, Fig. 5.7 again shows that serum from 11RX- recovered mice is very effective in promoting peritoneal killing of this organism.

Having established that the serum antibody levels of 11RX-recovered mice can be depleted by the injection of C5 polysaccharide, the susceptibility of these animals to challenge with Salmonella typhimurium C5 was investigated. Therefore, 30 11RX-recovered mice were injected intravenously with 10 mg. of C5 polysaccharide and two hours later

FIG. 5.6

Comparison of the ability of normal mouse serum, normal rat serum and serum from 11RX - recovered mice, to promote the peritoneal killing in mice, of Salmonella typhimurium C5.

———— Normal mouse serum

—·—·— 11RX - recovered mouse serum

— — — — normal rat serum

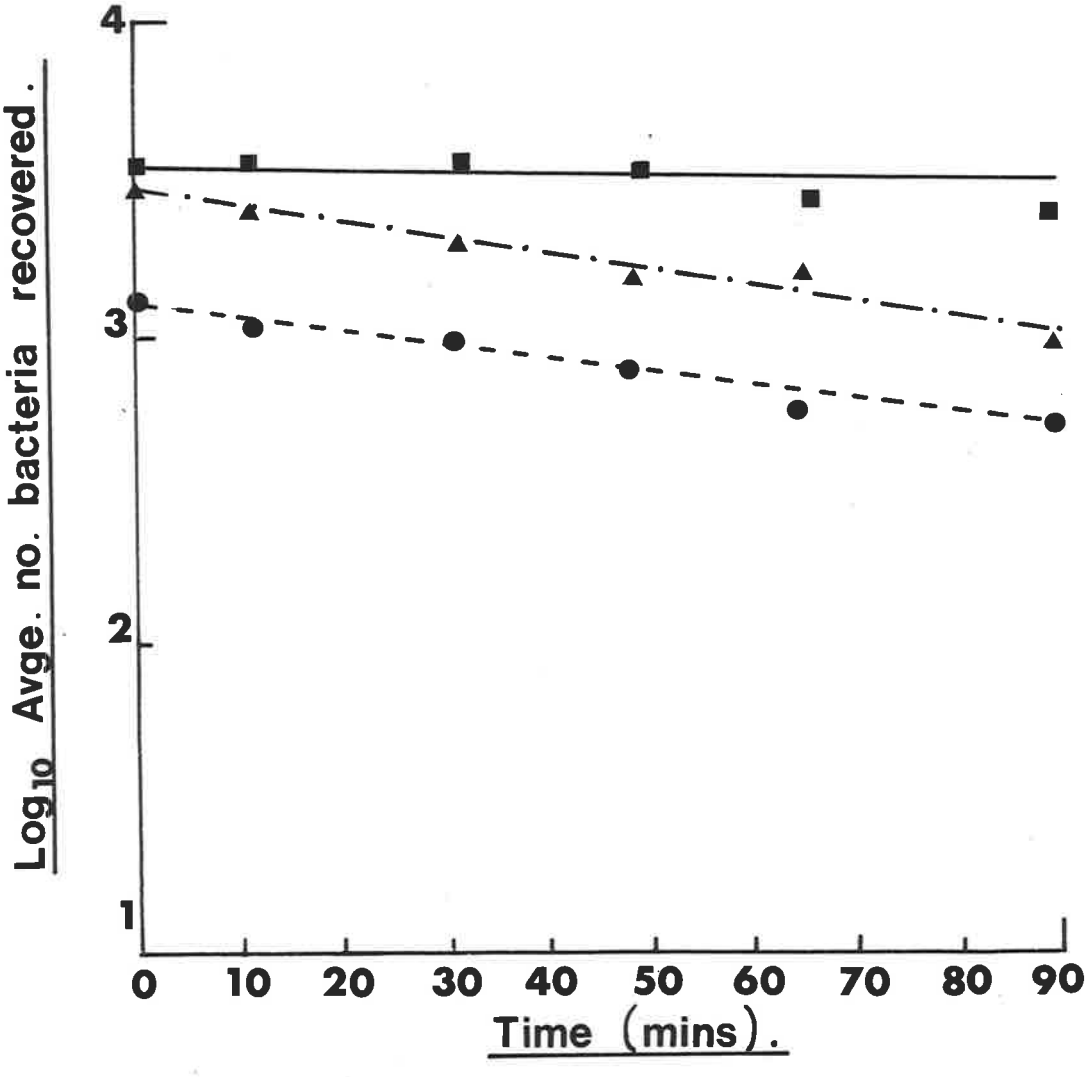
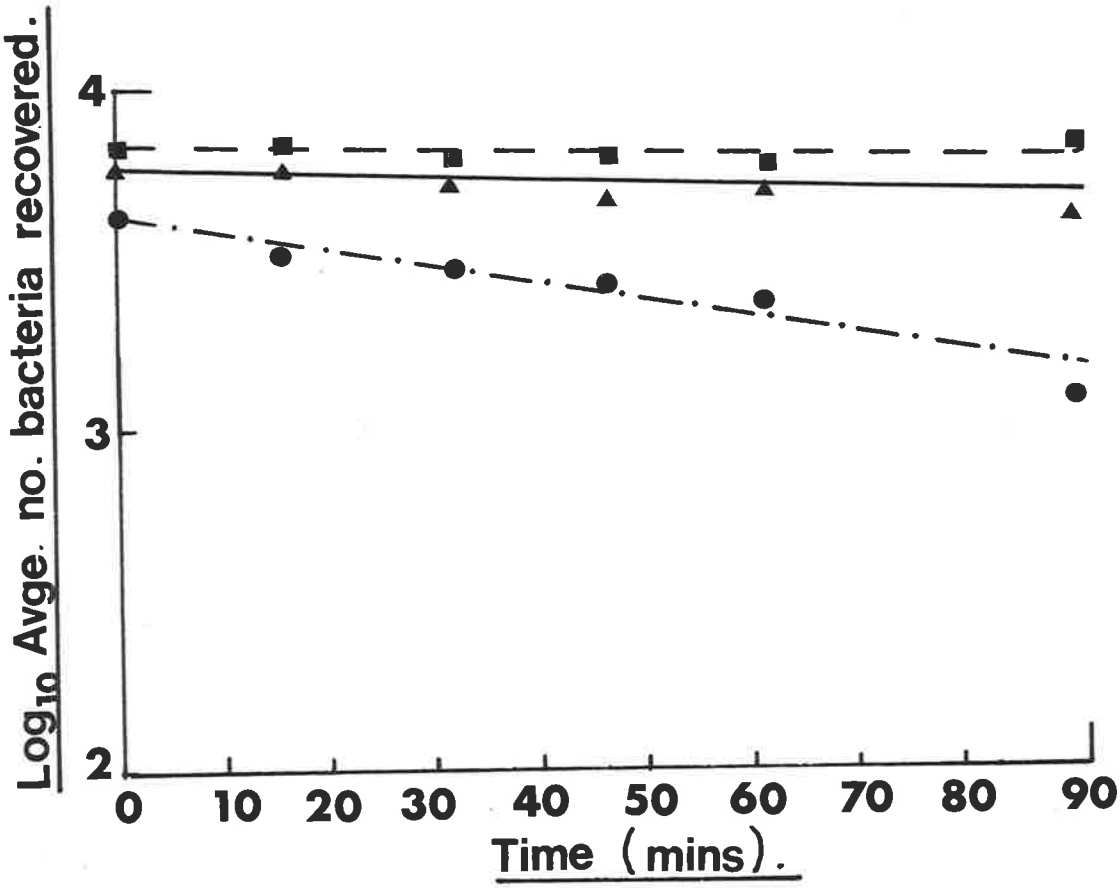

4
3
2
1
Log10 Avge. no. bacteria recovered.
0
10
20
30
40
50
60
70
80
90
Time (mins).

FIG. 5.7

The effect on the in vivo bactericidal activity of serum from 11RX - recovered mice, after injecting C5 polysaccharide intravenously into these mice.

- – – – serum from 11RX - recovered mice injected with C5 polysaccharide
- ——— normal mouse serum
- —·—·— serum from 11RX - recovered mice not injected with C5 polysaccharide


Log10 Avge. no. bacteria recovered.
4
3
2
0
10
20
30
40
50
60
70
80
90
Time (mins).

were challenged with 4.9×10^5 organisms intraperitoneally. Three control groups of mice were included in this experiment, namely: -

1. a group of 30 11RX-recovered mice not injected with C5 polysaccharide and challenged with 4.9×10^5 Salmonella typhimurium C5 organisms,
2. a group of 30 normal mice challenged with 4.9×10^5 Salmonella typhimurium C5 organisms, and
3. another group of 30 normal mice received only an intravenous injection of 10 mg. of C5 polysaccharide. This was done to ensure that the C5 polysaccharide alone had no toxic effects on the mice.

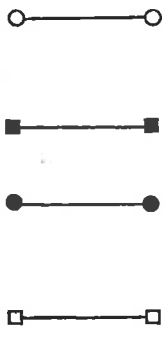
From Fig 5.8 it is apparent that treatment of 11RX-recovered mice with relatively large quantities of C5 polysaccharide, removes their acquired resistance to Salmonella typhimurium C5, since these mice were just as susceptible to Salmonella typhimurium C5 infection as normal mice which had no prior exposure to Salmonella enteritidis 11RX. It is also evident from Fig. 5.8 that the injection of C5 polysaccharide alone has no adverse effects on normal mice and that 11RX-recovered mice receiving no C5 polysaccharide injection are quite resistant.

Chemical analysis of the C5 polysaccharide preparation used in the abovementioned experiments indicated approximately 10 per cent contamination with protein. Therefore, the possibility existed that this protein component was responsible for at least some of the antibody depletion following the injection of C5 polysaccharide into 11RX-recovered mice. To determine whether this was so, two experiments were carried out:-

1. using a polysaccharide with antigens which cross-react with those of Salmonella typhimurium C5 but which contains no protein, and
2. using Salmonella newington polysaccharide which does contain bacterial protein, but no cross-reacting polysaccharide antigens.

FIG 5.8

The effect of injecting C5 polysaccharide on the acquired resistance of 11RX-recovered mice challenged with 4.9×10^5 Salmonella typhimurium C5 organisms.

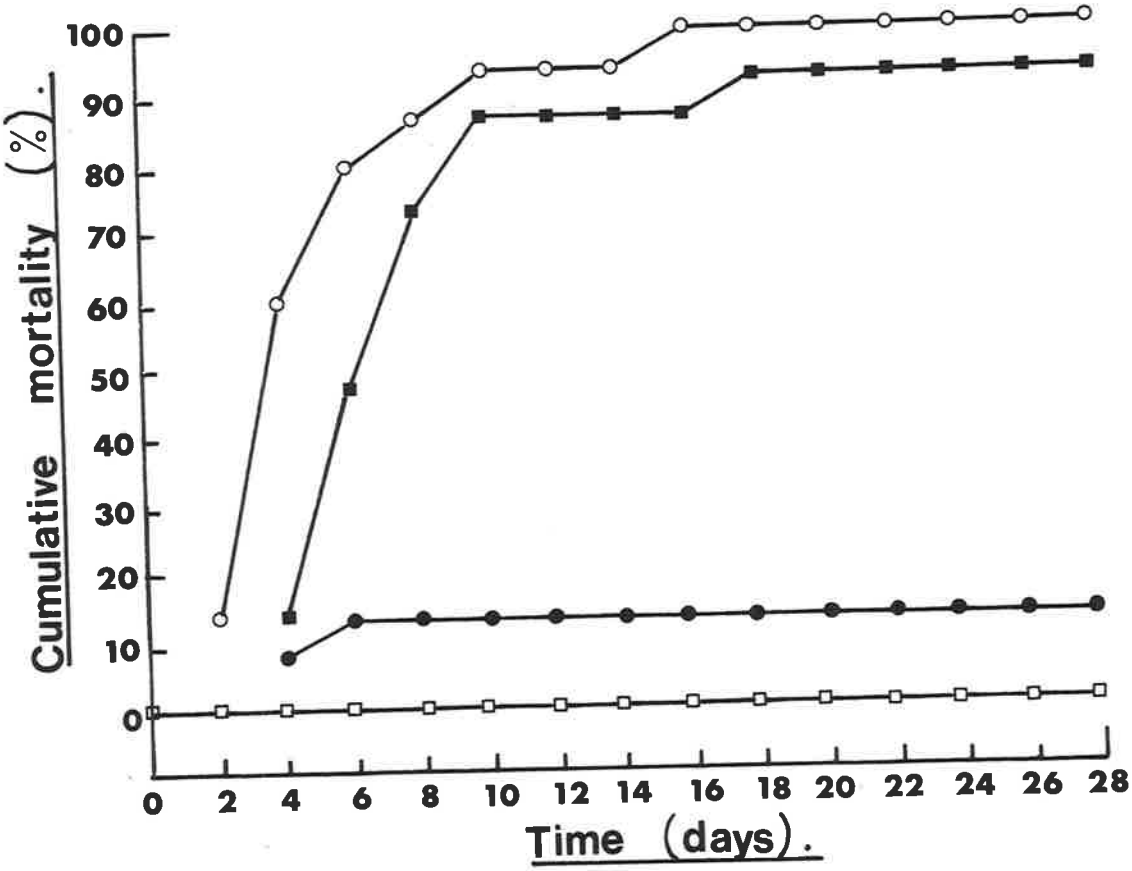


11RX-recovered mice injected with C5 polysaccharide

Normal mice

11RX-recovered mice not injected with C5 polysaccharide

normal mice injected with C5 polysaccharide only


Cumulative mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

Firstly, O-acetylated galactan (kindly provided by Dr. G.D.F. Jackson of this department), which contains no protein and which has been shown to cross-react with O-antigen 5 of Salmonella typhimurium (Jenkin, et al., 1967) was injected intravenously into a group of 30 11RX-recovered mice. 10 mg. of O-acetylated galactan was injected intravenously 2 hours prior to challenge with 8.2×10^4 Salmonella typhimurium C5 organisms intraperitoneally. At the same time 2 other groups of 30 mice were challenged with the same number of organisms - a group of normal mice and a group of 11RX-recovered mice not injected with O-acetylated galactan. The results in Fig. 5.9 show that 11RX-recovered mice injected with O-acetylated galactan are as susceptible to Salmonella typhimurium C5 infection as are normal mice. These results suggest that the O-specificities of C5 polysaccharide and not the protein component are responsible for the antibody depletion that occurs.

Secondly, if the resistance acquired by 11RX-recovered mice to infection with Salmonella typhimurium C5 is due to antibodies specifically directed against the O antigens of C5 polysaccharide, then it is probable that injection of Salmonella newington polysaccharide will not cause the antibody depletion necessary to make 11RX-recovered mice susceptible. To examine this possibility 2 groups of 30 11RX-recovered mice were challenged with 9.1×10^4 Salmonella typhimurium C5 intraperitoneally. Two hours prior to challenge, one of these groups was injected intravenously with 10 mg. of polysaccharide prepared from Salmonella newington. This organism possesses O antigens 3 and 15 (Kauffmann 1966) which do not cross-react with Salmonella typhimurium C5. The results showed that Salmonella newington polysaccharide was completely ineffective in inducing susceptibility to Salmonella typhimurium C5 in 11RX-recovered mice.

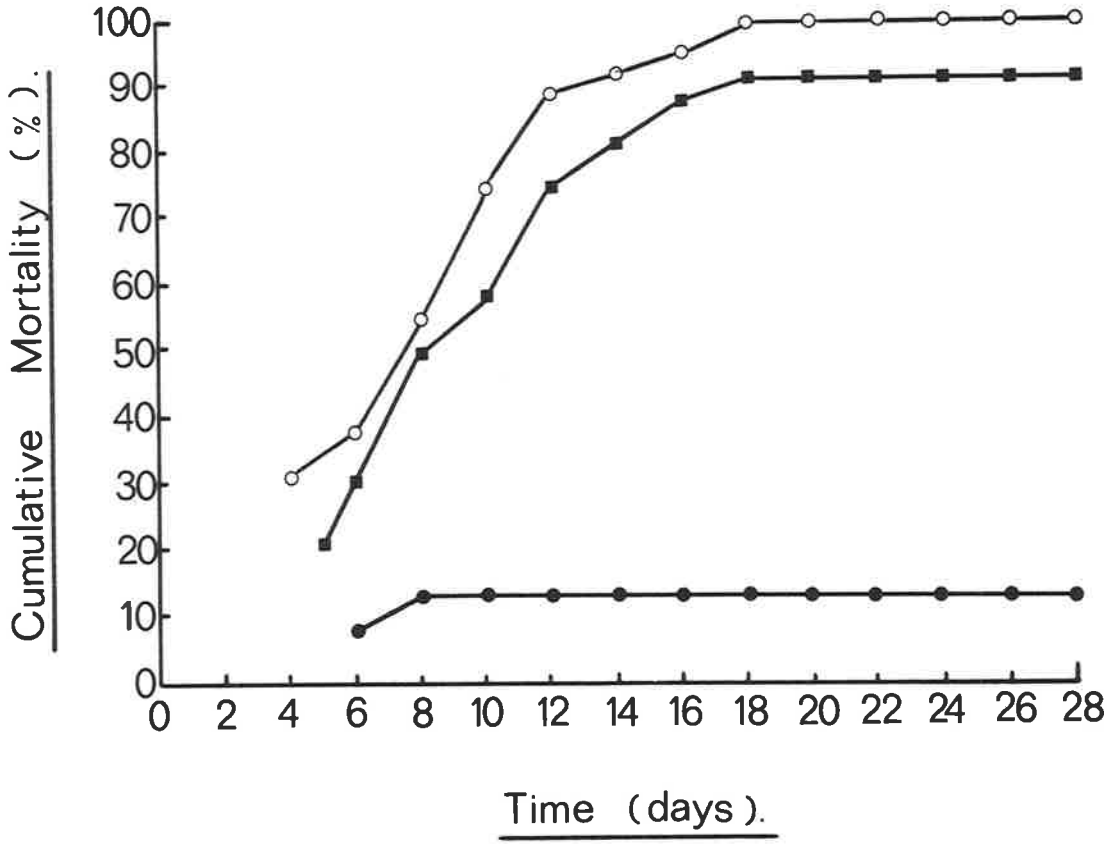
FIG. 5.9

The effect of injecting O-acetylated galactan, on the resistance of 11RX - recovered mice challenged with $8 \cdot 2 \times 10^4$ Salmonella typhimurium C5 organisms intraperitoneally.

11RX - recovered mice injected intravenously with 10 mg. of O-acetylated galactan

Normal mice challenged with Salmonella typhimurium C5

11RX - recovered mice not injected with O-acetylated galactan


Cumulative Mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

The fate of Salmonella typhimurium C5 injected into 11RX-recovered mice treated with C5 polysaccharide

It has been shown that 11RX-recovered mice are resistant and are able to control the multiplication of Salmonella typhimurium C5 and eventually eliminate the infecting organisms completely from the peritoneal cavity (Fig 5.5). However, after the injection of C5 polysaccharide into 11RX-recovered mice, they become as susceptible to challenge with Salmonella typhimurium C5 as are normal mice. Therefore, it remains to be determined, whether the fate of Salmonella typhimurium C5 organisms is the same in 11RX recovered mice injected wtih C5 polysaccharide as it is in normal mice.

In an attempt to answer this question two groups of 60 11RX-recovered mice, one receiving 10 mg. of C5 polysaccharide and the other not,were challenged with 4.9×10^5 Salmonella typhimurium C5 organisms intra-peritoneally. At regular intervals thereafter, 5 mice from each group were sacrificed and the blood, peritoneal cavity, spleen and liver of each mouse was examined for the presence of the organism of challenge. Bacterial enumeration studies were carried out as described in Chapter IV and the total numbers of organisms recovered were plotted as a function of time. The results presented in Figs. 5.10 and 5.11 show that in 11RX-recovered mice injected with C5 polysaccharide, Salmonella typhimurium C5 organisms multiply rapidly until they reach a level which is fatal for the mice. In contrast, the 11RX-recovered mice not injected with C5 polysaccharide are able to control the infection and eventually completely eliminate all the organisms.

It can be concluded from these studies that the resistance of Salmonella enteritidis 11RX-recovered mice to infection with Salmonella typhimurium C5 is due to an increase in serum antibody levels.

FIG. 5.10

The fate of $4{\cdot}9 \times 10^5$ Salmonella typhimurium C5 organisms injected intraperitoneally into 11RX - recovered mice

— — — — organisms recovered from 11RX - recovered mice that had been injected with 10 mg. of C5 polysaccharide intravenously

—·—·— organisms recovered from 11RX - recovered mice not injected with C5 polysaccharide

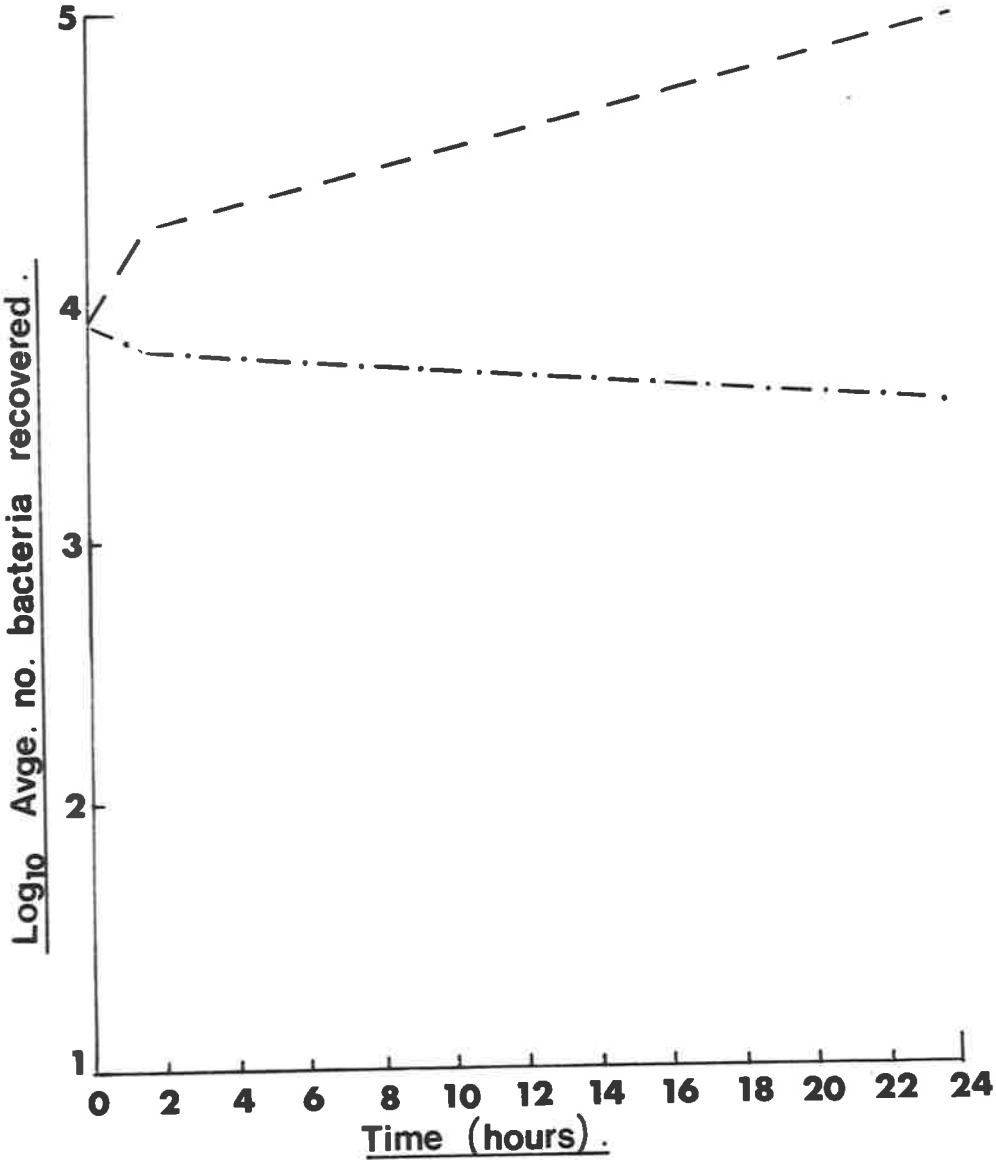
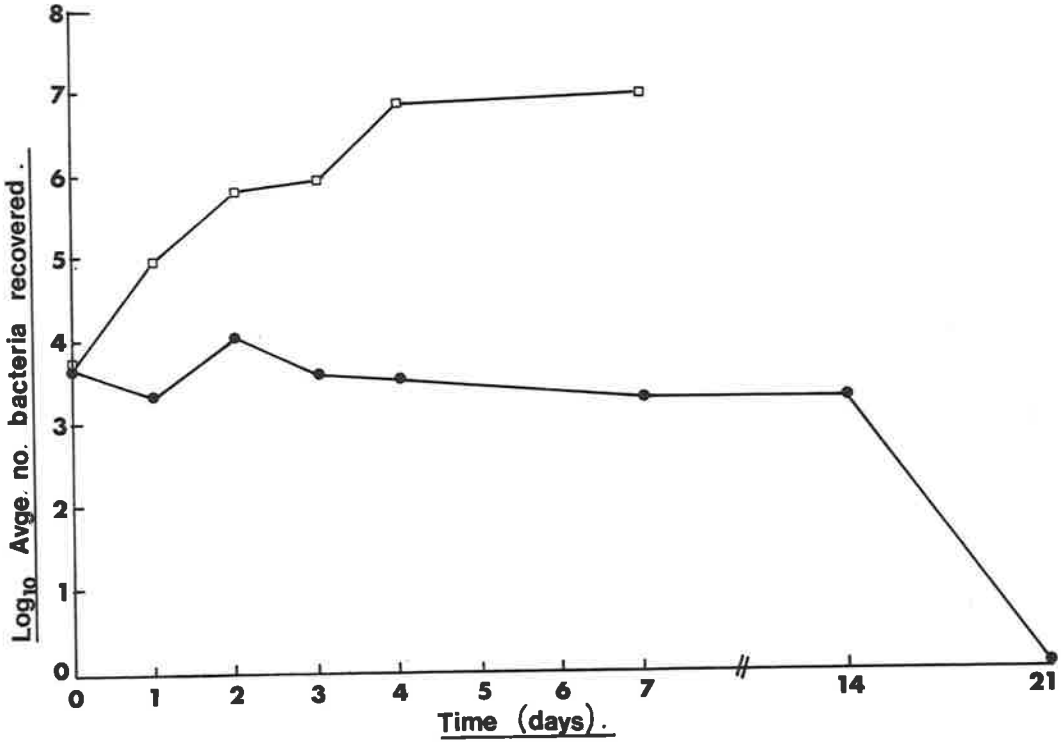

5
4
3
2
1
Log10 Avge. no. bacteria recovered.
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours).

FIG. 5.11

The fate of $4 \cdot 9 \times 10^5$ Salmonella typhimurium C5 organisms injected intraperitoneally into 11RX - recovered mice

organisms recovered from 11RX - recovered mice that had been injected with 10 mg. of C5 polysaccharide intravenously.

organisms recovered from 11RX - recovered mice not injected with C5 polysaccharide


8
7
6
5
4
3
2
1
0
Log10 Avge. no. bacteria recovered.
0
1
2
3
4
5
6
7
14
21
Time (days).

The effect of C5 polysaccharide upon antibody levels and on the susceptibility of rats to Salmonella typhimurium C5 infection

The results presented in Chapter IV suggest that rats injected with even very large doses of Salmonella typhimurium C5 do not succumb to an overwhelming infection of this organism because:-

(a) they possess levels of natural antibody to Salmonella typhimurium C5, which enables them to restrict the multiplication of this organism, and

(b) they are capable of mounting a vigorous immune response to this organism quite rapidly.

Which of these two factors is more important is not evident from the results presented. The following experiments were carried out to try to elucidate this question.

Since the levels of natural antibody to Salmonella typhimurium C5 that are detectable in the serum of normal rats, are no greater than the levels of antibody to this organism that are found in the serum of 11RX-recovered mice, an attempt was made to deplete rats of their natural antibody to Salmonella typhimurium C5 with C5 polysaccharide.

The effect of C5 polysaccharide injection into normal rats on their natural antibody levels

To determine the effect on natural antibody levels of injecting C5 polysaccharide into normal rats, ten 12 week old male rats weighing 260-280 gm. were injected intravenously with 10 mg. of C5 polysaccharide. One hour later 5 rats from this group and 5 rats from a similar group of normal rats not injected with C5 polysaccharide, were lightly anaesthetised with ether and bled by cardiac puncture. Ninety minutes later, 5 rats from each group were again bled. The blood taken at each time from each group of rats was pooled and the serum collected in the manner described in Chapter II. The sera were then assayed for opsonins by intravenous clearance of P^{32} labelled Salmonella typhimurium

C5 in normal mice (method described in Chapter II). Fig. 5.12 shows that the ability of serum from rats injected with C5 polysaccharide to promote the clearance (phagocytosis) of Salmonella typhimurium C5 is markedly reduced when compared with the serum from normal rats.

To determine whether the injection of C5 polysaccharide into rats also decreased their ability to kill Salmonella typhimurium C5, 18 normal rats, similar to those used above, were injected intravenously with 10 mg. of C5 polysaccharide. One hour later, this and another group of 18 rats not injected with C5 polysaccharide, were injected intraperitoneally with 2.02×10^4 Salmonella typhimurium C5 organisms. At 0, 15, 30, 45, 60 and 90 minutes after challenge, 3 rats from each group were sacrificed and their peritoneal cavities washed out with 10 ml. of sterile saline. Viable counts of each of the washout fluids were then made to determine the number of bacteria recovered. The results show that rats injected with C5 polysaccharide were able to kill only 27 per cent of the inoculum of bacteria, whereas normal rats killed 66 per cent of the inoculum (Fig. 5.13).

The effect of injecting C5 polysaccharide on the susceptibility of rats to Salmonella typhimurium C5 infection

To determine whether the reduction in antibody levels resulting from injection of C5 polysaccharide into rats, did affect their resistance to Salmonella typhimurium C5, 25 ten week old male and female rats weighing 180-200 gm. were injected intravenously with 10 mg. of C5 polysaccharide. One hour later these rats were challenged with 2×10^7 Salmonella typhimurium C5 organisms injected intraperitoneally. Six hours after challenge, these rats received an intravenous injection of another 5 mg. of C5 polysaccharide and were then observed for a 28 day period. The controls included in this experiment consisted of:-

FIG. 5.12

The effect of injecting C5 polysaccharide into rats, on the intravenous clearance rate of P^{32} labelled Salmonella typhimurium C5 injected into these animals

—————— rats injected with 10 mg. C5 polysaccharide

– – – – normal rats

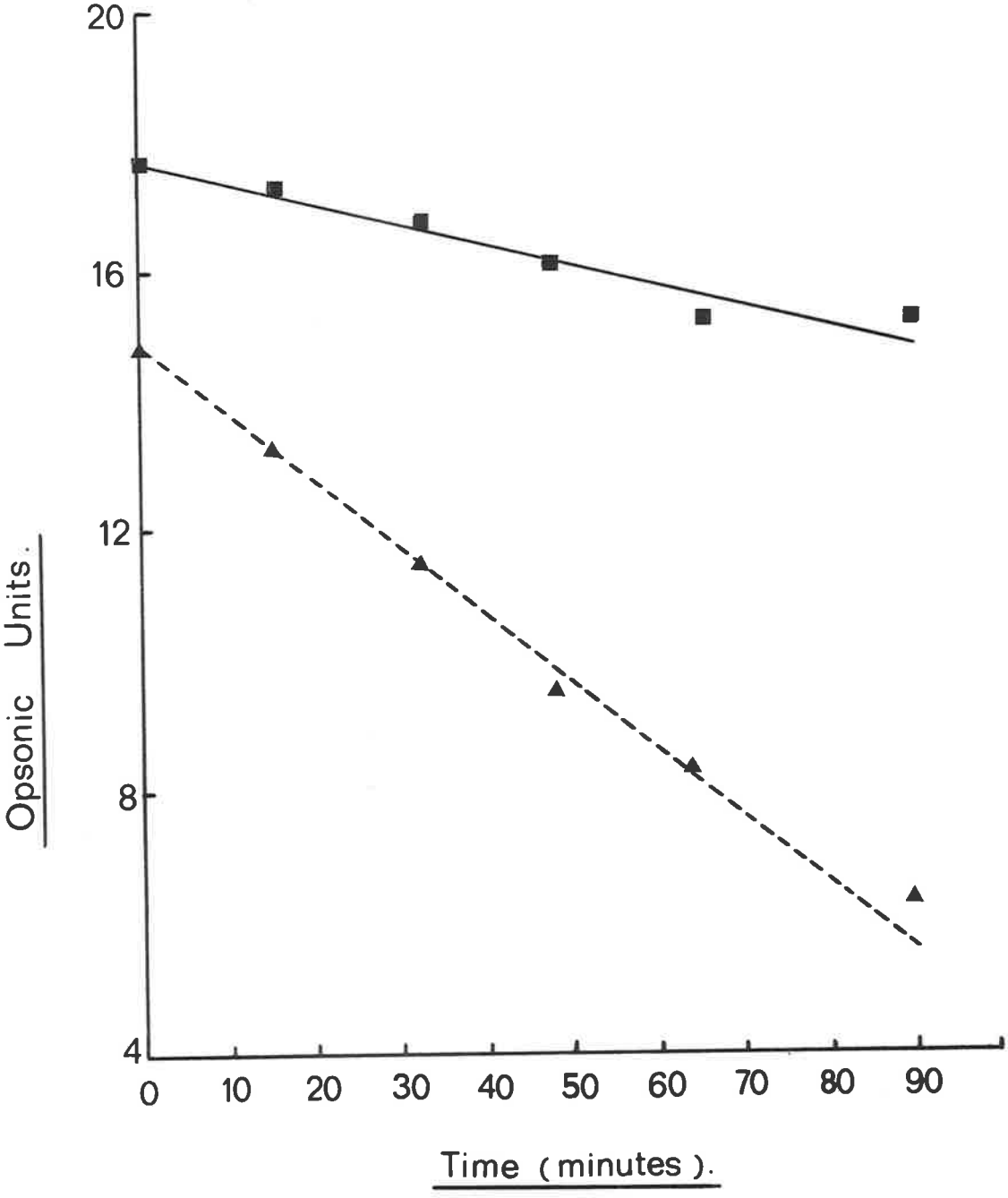
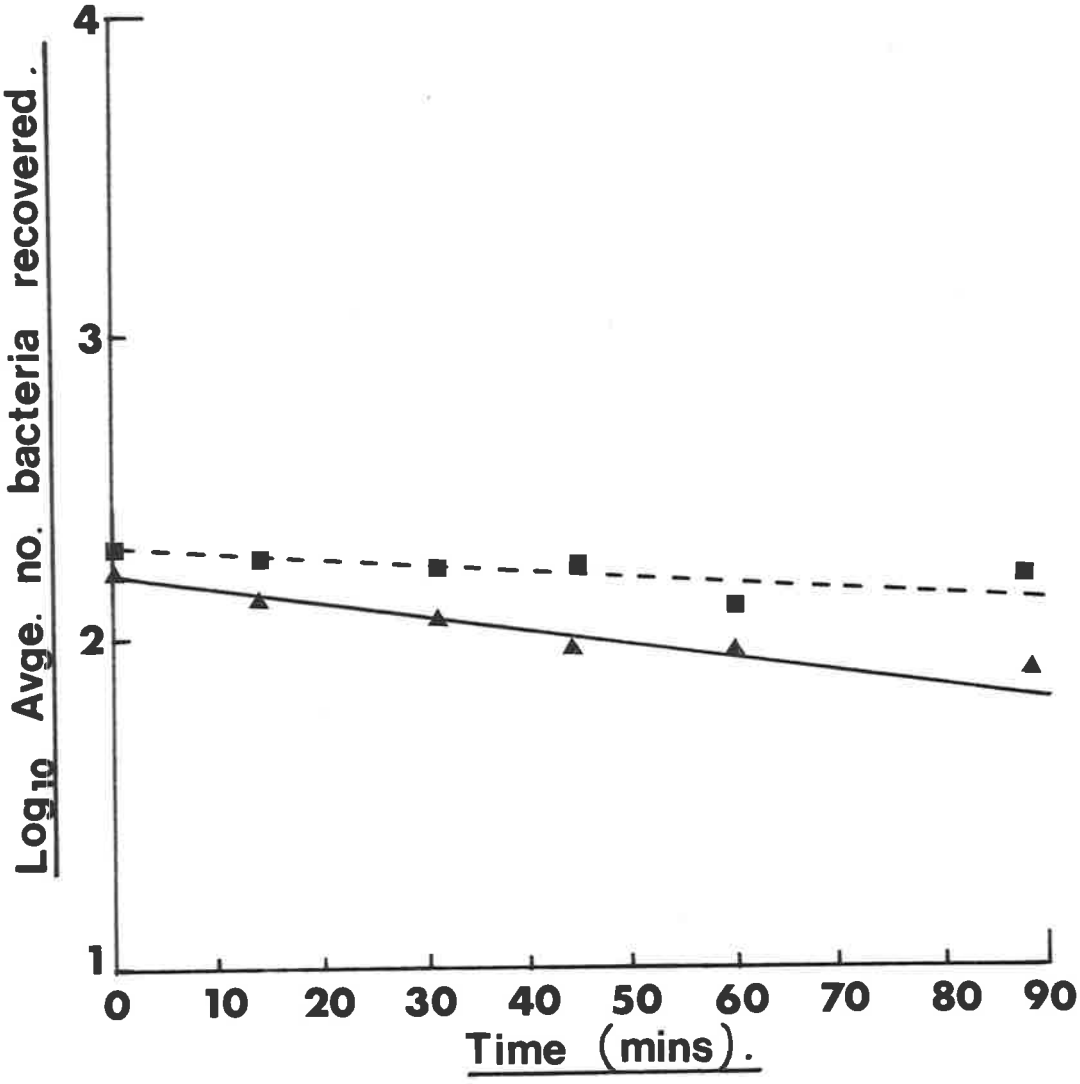

20
16
12
8
4
Opsonic Units.
0
10
20
30
40
50
60
70
80
90
Time (minutes).

FIG. 5.13

The effect of injecting 10 mg. of C5 polysaccharide intravenously into rats, on the ability of these animals to kill an inoculum of 2×10^4 Salmonella typhimurium C5 organisms injected intraperitoneally.

– – – – rats injected with C5 polysaccharide

——— normal rats


4
3
2
1
Log10 Avge. no. bacteria recovered.
0
10
20
30
40
50
60
70
80
90
Time (mins).

1. rats receiving no C5 polysaccharide and challenged with 2×10^7 Salmonella typhimurium C5 organisms, and
2. rats injected with C5 polysaccharide but not challenged.

The results show that injection of C5 polysaccharide into rats does induce susceptibility to Salmonella typhimurium C5 infection in the animals (Fig. 5.14).

Variations in the biological activity of antibody produced at different stages of the immune response of rats to Salmonella typhimurium C5

It is apparent from the results described in Chapter IV that rats and mice differ in their ability to produce antibody to the antigens of Salmonella typhimurium C5. It is also evident that the antibody produced by rats differs in its effect upon these organisms. The antibody comprising the early peak is able to control the multiplication of Salmonella typhimurium C5 organisms, whereas the antibody comprising the late peak appears to effect the complete elimination of these organisms (Figs. 4.10, 4.2 and 4.5). In an attempt to explain this observation, rats were examined for their ability to eliminate a superinfecting dose of Salmonella typhimurium C5 injected at the times corresponding with the occurrence of the two peaks of antibody.

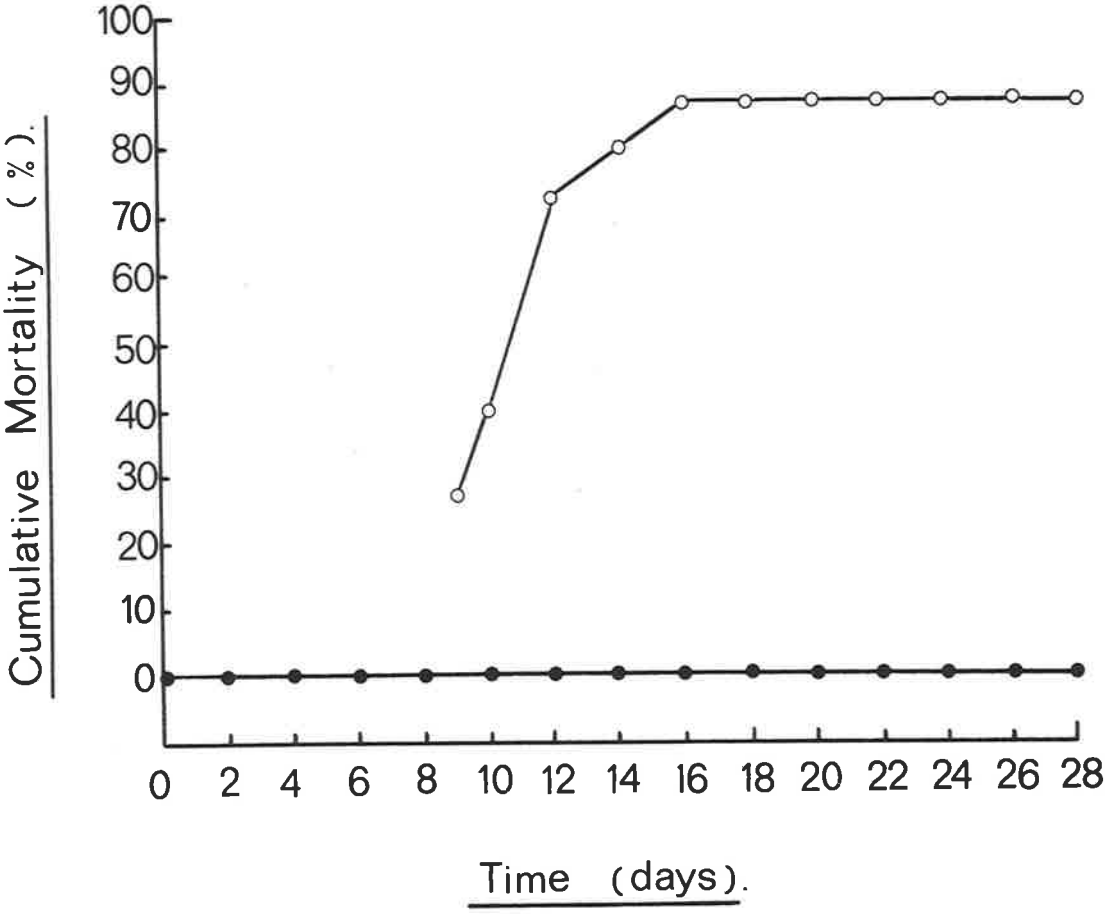
Ten week old male and female rats weighing 180-210 gm. at the beginning of the experiment were challenged with approximately 2×10^7 Salmonella typhimurium C5 organisms intraperitoneally. The days of challenge were arranged such that on the day the clearance studies were to be carried out, there were 4 groups of animals :- one group of normal rats and 3 groups challenged 6, 12 and 21 days previously. The ability of these animals to eliminate a superinfecting dose of P^{32} labelled Salmonella typhimurium C5 injected intravenously, was examined using the technique described for clearance studies in mice (Chapter II). However, in this case, for ease of handling, the rats had to be lightly

FIG. 5.14

The effect of injecting 15 mg. of C5 polysaccharide intravenously into rats, on the resistance of these animals to challenge with 2×10^7 Salmonella typhimurium C5 organisms intraperitoneally.

rats injected with C5 polysaccharide

rats injected with C5 polysaccharide only or rats challenged with 2×10^7 Salmonella typhimurium C5 organisms only.


Cumulative Mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

anaesthetised with ether before commencement of the experiment. The radioactively (P^{32})labelled organisms were prepared by the technique described in Chapter II.

It was not clear from the results presented in Chapter IV, whether antibody or stimulated macrophages were responsible for the elimination of bacteria which occurred in rats. Therefore, on the same day that the Salmonella typhimurium C5 clearances were carried out, 4 groups of rats identical with those described above, were examined for their ability to eliminate a superinfecting dose of an unrelated organism. Radioactively (P^{32}) labelled Escherichia coli 2380 was the organism used.

Table 5.2 shows that the clearance value (K) of a superinfecting dose of Salmonella typhimurium C5 is the same on days 6 and 12 after challenge and that this value is identical with that in normal rats (day O). However, at 21 days the clearance value is significantly higher and this correlates well with the loss of the carrier state which occurs at this time (Fig. 4.5). It is also evident from Table 5.2 that the factors which cause an enhanced clearance of Escherichia coli on days 6 and 12 are no longer active on day 21. The enhanced clearance of Escherichia coli evident on days 6 and 12, is probably due to non-specific stimulation of phagocytic cells. However, since the clearance rate of this organism on day 21 is the same as it is in normal rats (day O), it is clear that the number of stimulated macrophages present at this time is minimal.

Therefore, it can be concluded that the factors responsible for the rapid clearance and elimination of Salmonella typhimurium C5 organisms are specific antibodies.

TABLE 5.2

Clearance values of radioactively (P^{32}) labelled organisms from the circulation of rats at different stages of their antibody response to Salmonella typhimurium C5

	Clearance Value (K)			
TIME / ORGANISM USED	DAY O	DAY 6	DAY 12	DAY 21
Salmonella typhimurium C5	K = 0.18	K = 0.18	K = 0.18	K = 0.25
Escherichia coli 2380	K = 0.06	K = 0.09	K = 0.12	K = 0.06

Conclusions

The results obtained from the experiments described above, confirm the evidence that antibody is the most important factor upon which both rats and mice depend for their resistance to Salmonella typhimurium C5 infection. Furthermore, studies of rats challenged with Salmonella typhimurium C5 have shown that there is protective antibody present in the serum of normal rats and that these rats are able to produce very large quantities of this antibody at a rapid rate. The clearance studies described, show that the antibody produced by rats varies in its activity possibly because of a difference in its specificity at different stages of the immune response.

Whilst normal mouse serum has been shown to contain no detectable protective antibody, it is clear from the passive transfer studies that immune mouse serum does contain some protective antibody. However, it is evident that mice challenged with Salmonella typhimurium C5, cannot produce enough of this antibody or produce it too slowly.

CHAPTER VI

THE EFFECT OF IMMUNOSUPPRESSION AND IRRADIATION ON THE RESISTANCE OF RATS TO INFECTION WITH SALMONELLA TYPHIMURIUM C5

Introduction

The results presented in the preceeding chapters suggest that the resistance of rats of the BN strain to Salmonella typhimurium C5 infection, is due to the levels of natural antibody to Salmonella typhimurium C5 that they possess and their ability to produce much greater quantities of antibody, to this organism, more rapidly than mice. This in turn would suggest that there is a difference in the overall capacity of rats and mice to resist infection in general. However, as yet it has not been possible to determine whether any one particular factor, immunological or environmental, is responsible for this difference.

The aim of the studies to be reported in this chapter, has been to investigate the possibility that the difference in the resistance of rats and mice to Salmonella typhimurium C5 infection, is reflected by a difference in the capacity of rat and mouse lymphoid tissues to respond to the antigens of Salmonella typhimurium C5. In particular an attempt was made to simplify the study of antibody production by examining, in a controlled environment, the cells responsible for antibody production. Two approaches were adopted, namely, by the use of an immunosuppressive drug and by the production of radiation chimaeras.

Immunosuppressive drugs

The effects of metabolic inhibitors on the immune response have been more thoroughly investigated only over the past decade or so. Although the mechanism by which these drugs interfere with the immune response is not completely understood, they are known to interfere with nucleic acid

metabolism and hence the development of antibody producing cells (Friedman and Buckler, 1963).

In 1958 and 1961 Uphoff reported the depressive effect of Methotrexate on apparently non-humoral immune mechanisms. Since that time there have been reports that metabolic inhibitors completely inhibit both "priming" and the primary immune response. In 1959 Schwartz, Eisner and Dameshek studied the effect of the purine analogue 6-mercaptopurine on the immune response to bovine serum albumin injected into rabbits. They demonstrated that at a dose of 6mg/Kgm. no serum antibodies were detected, that is, the primary immune response to bovine serum albumin was completely suppressed.

In 1963 Thomas, Baker and Ferrebee examined the effect of Methotrexate on the antibody response of dogs to attenuated distemper virus vaccine. Dogs were injected subcutaneously with 0.1 mg/Kgm. of Methotrexate three times per week for 6 weeks commencing at the time of vaccination, which consisted of one intramuscular injection of attenuated, egg-adapted virus. Antibody levels were determined at regular intervals and it was shown that some dogs had no detectable antibody in their serum. These dogs eventually died and were shown to have large numbers of the attenuated virus in their circulation.

Methotrexate has also been shown to have a profound effect on the induction time of the antibody response to sheep red blood cells in Sprague-Dawley rats. The peak and average antibody titres were also markedly reduced. These animals received one intraperitoneal injection of 20-50 per cent of the LD50 dose of Methotrexate each day for 5 consecutive days. The antigen was administered at various times during and after commencement of Methotrexate therapy. Some of these treated animals showed no detectable agglutinins to sheep red blood cells in their sera for 42 days

after antigen administration (Santos and Owens, 1964).

Actinomycin D and the chaemotherapeutic agent Chloramphenicol, have been shown by Butler (1961), to be effective inhibitors of the primary immune response. Chloramphenicol has also been shown to inhibit priming of mice injected with purified diphtheria toxoid, (Cruchard and Coons, 1964). Butler and Coons (1964) showed that mice given a primary immunizing dose of diphtheria toxoid and then treated with 6-mercaptopurine, chloramphenicol or other metabolic inhibitor drugs, possessed no detectable serum antibody subsequent to a secondary stimulus with the same antigen. That is, these drugs prevented priming of these animals.

In the light of the abovementioned reports, it was decided to treat rats with a metabolic inhibitor with the aim of perhaps reducing natural antibody levels and hence inducing susceptibility to Salmonella typhimurium C5 infection. The drug chosen was Methotrexate (Lederle, American Cyanamid Co., Pearl river, N.Y.). Methotrexate was chosen for the following reasons:-

1. The work of Thomas et al., (1963), suggested that this drug may induce susceptibility to infection, since some of the dogs used in their experiments died from viraemia caused by an attenuated virus.

2. The doses used in the experiments cited above were comparatively low and multiple doses could be administered for relatively long periods of time without notable toxic side effects.

3. Because the doses used in the abovementioned experiments were effective in preventing primary antibody responses to a number of antigens.

4. The mode of action of Methotrexate is quite well understood. Methotrexate is an antimetabolite which interferes with the participation of folinic acid in nucleic acid synthesis. Thus this drug is used as an antineoplastic agent because it disrupts the mitotic process. It is especially

indicated in the treatment of malignant neoplasms which consist of cells which proliferate rapidly and constantly and which have exceptionally high nucleic acid synthesis and turnover rates. Antigenic stimuli result in a population of rapidly dividing, antibody producing cells and therefore it is quite probable that Methotrexate will interfere with this cell division and subsequent antibody production. At the same time, it is possible that cells with a lower metabolic and turnover rate, such as macrophages, will be less affected by low doses of Methotrexate, thus producing little or no interference with the phagocytic function of these cells.

Radiation chimaeras

If there is a difference in the capacity of rat and mouse lymphoid tissue to respond to the antigens of Salmonella typhimurium C5, radiation chimaeras should provide an excellent model for the study of this problem. Another approach would be to transplant spleen or lymphatic cells from presensitised to normal animals. However, the problem with such a model would be to show conclusively that the injected or transplanted cells were responsible for antibody production and not the recipients own tissues. Another problem is the probability of a homograft reaction occurring, especially if inbred animals are not used.

Sub-lethal irradiation may curtail the host's immune mechanism temporarily, but transplantation of mature lymphoid cells from a foreign donor could still result in a graft versus host reaction. Thus any slight genetic incompatibility between hosts and donor could affect the synthesis of antibody by donor cells. However, if the host animals were lethally irradiated and the haemopoietic and lymphoid organs were restored with immunologically immature cells, then the possibility of a homograft reaction could be avoided even when host and donor are genetically incompatable.

That is, protection of lethally irradiated animals by the proliferation of transplanted donor haemopoietic, (primitive, multipotential), cells. In 1957 van Bekkum and Vos showed that lethally irradiated animals could be injected with bone marrow from syngeneic, allogeneic or xenogeneic hosts shortly after exposure to the irradiation and that in each case they could successfully establish radiation chimaeras.

A variety of techniques have been used to demonstrate that restoration of haemopoietic and lymphoid tissues in radiation chimaeras is due to the transplantation and proliferation of a complete donor haemopoietic system into the irradiated host (Lindsley, Odell and Tausche, 1955, Makinodan, 1956, Nowell, Cole, Habermeyer and Roan, 1956, Makinodan and Anderson 1957, Ford, Ilbery and Loutit, 1957).

The particular aim of this study then, was to establish a mouse into rat chimaera by lethally irradiating rats and transplanting them with mouse haemopoietic tissue. If this could be done, then these animals would be examined to determine their susceptibility to Salmonella typhimurium C5 infection, their ability to produce antibody to this organism and finally to determine whether any correlation exists between the ability to produce antibody and susceptibility to this organism.

The numbers of antibody producing cells produced by rats and mice in response to stimulation with the antigens of Salmonella typhimurium C5

Prior to examining the effects of immunosuppressive drugs and irradiation on the resistance of rats to infection with Salmonella typhimurium C5, the numbers of antibody producing cells resulting from the stimulation of both rats and mice with this organism were determined. A large group of 12 week old male and female rats and mice, weighing 190-210 gm. and 20-22 gm. respectively, were injected intravenously with 100 μg. of an alcohol-killed vaccine of Salmonella typhimurium C5. At regular intervals

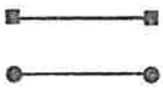
thereafter 3 rats and 3 mice were sacrificed and their spleens examined for the numbers of antibody producing cells present. The technique used was that of Biozzi et al., (1966), using both normal sheep red blood cells and sheep red blood cells sensitised with C5 lipopolysaccharide. The resultant number of antibody producing cells detected in each animal was calculated by subtracting the number of antibody producing cells detected using normal sheep red blood cells, from the number detected using lipopolysaccharide sensitised cells. From the results obtained (Fig. 6.1), it is evident that the capacity of rat lymphoid tissues to respond better to the antigens of Salmonella typhimurium C5 than mouse lymphoid tissues, is reflected in a greater number of antibody producing cells which is sustained for a longer time. This data correlates with the results presented in Chapter IV which show that rats produce greater quantitites of antibody than do mice.

The use of Methotrexate to induce susceptibility to Salmonella typhimurium C5 in rats

To determine whether rats would become susceptible to Salmonella typhimurium C5 infection if they were treated with Methotrexate prior to challenge, groups of twenty 12 week old male and female rats weighing 190-200 gm. were injected with various doses of Methotrexate prior to challenge. It was shown that rats treated with Methotrexate prior to challenge with this organism were susceptible. It was found that the minimum dose of Methotrexate which had no observable toxic effect on the animals and which was effective in inducing susceptibility to 100 per cent of rats challenged with Salmonella typhimurium C5, was 0.25 mg/Kgm. injected intraperitoneally each day for 4 consecutive days prior to challenge. The minimum number of organisms which brought about the death of 100 per cent of the animals was approximately 2×10^3. Rats injected with Methotrexate using the abovementioned schedule and receiving no challenge were observed for up

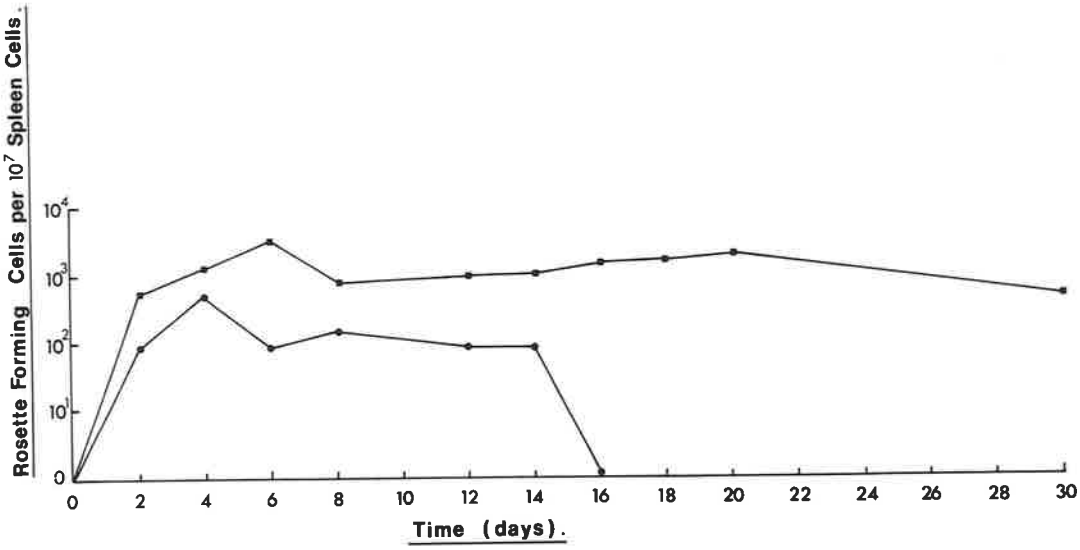
FIG. 6.1

Numbers of antibody-producing cells detected in the spleens of rats and mice injected intravenously with 100 μg. of an alcohol-killed vaccine of Salmonella typhimurium C5



antibody-producing cell response in rats

antibody-producing cell response in mice


Rosette Forming Cells per 10^7 Spleen Cells.
10^4
10^3
10^2
10^1
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time (days).

to 50 days after treatment, with no observable toxic effects. This schedule was the standard treatment used in all subsequent studies with Methotrexate.

Having determined that rats treated with this immunosuppressive drug were susceptible to challenge with Salmonella typhimurium C5, experiments were designed to investigate:-

1. The fate of this organism injected into rats treated with Methotrexate,

2. The effect of Methotrexate treatment on natural and acquired antibody levels and

3. The effect of Methotrexate treatment on the phagocytic function of macrophages from these animals.

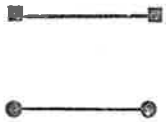
The fate of Salmonella typhimurium C5 organisms injected into rats treated with Methotrexate

A large group of Methotrexate-treated and normal rats were challenged intraperitoneally with 2.8×10^7 Salmonella typhimurium C5 organisms. At regular intervals for 24 hours, 3 rats from each group were sacrificed and the numbers of organisms that could be recovered were determined. Fig. 6.2 shows that the ability of Methotrexate-treated rats to control the multiplication of Salmonella typhimurium C5, is markedly reduced when compared with that of normal rats. The number of organisms that can be recovered from Methotrexate-treated rats increases rapidly over 24 hours, whereas normal rats are able to reduce the number of organisms initially and then control the multiplication of these organisms.

Having shown that treatment of rats with Methotrexate induces susceptibility to infection with Salmonella typhimurium C5 and that these animals were no longer able to control the multiplication of these organisms, the ability of these animals to produce antibody after challenge and the levels of natural antibody after Methotrexate treatment and before challenge were investigated.

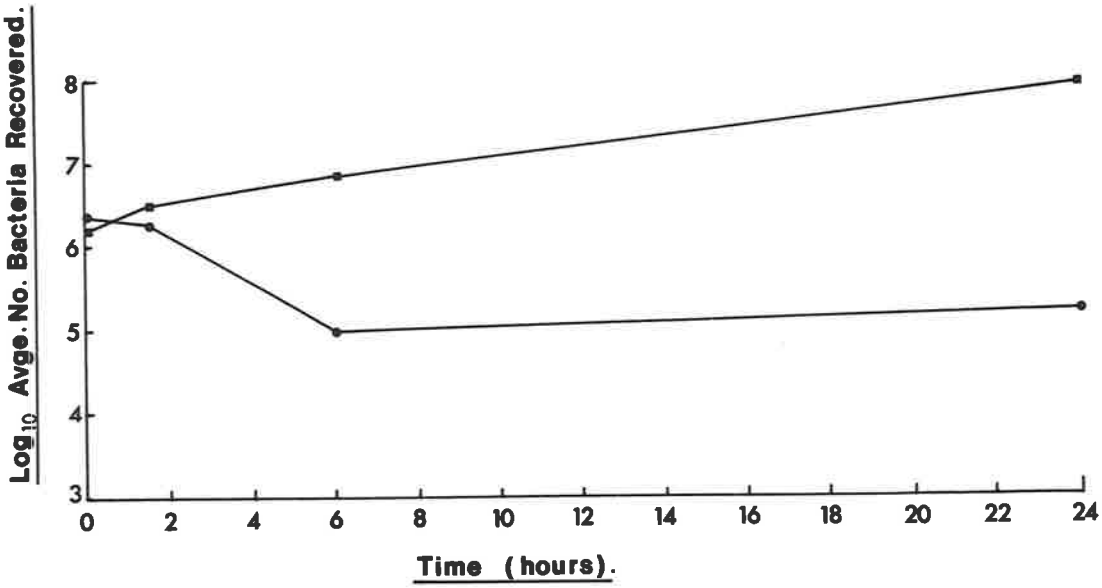
FIG. 6.2

The recovery of Salmonella typhimurium C5 organisms from the peritoneal cavities of rats injected with methotrexate



rats treated with methotrexate and challenged with $2 \cdot 8 \times 10^7$ organisms intraperitoneally

normal rats challenged with $2 \cdot 8 \times 10^7$ organisms intraperitoneally


Log10 Avge. No. Bacteria Recovered.
8
7
6
5
4
3
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours).

Antibody production by rats treated with Methotrexate

To determine the effect of Methotrexate treatment on natural antibody levels and to compare the ability of Methotrexate treated rats and normal rats to produce antibody, rats treated with Methotrexate were bled at 24 hours after the last injection of Methotrexate and the serum collected and pooled. These rats and a group of normal rats were then injected intravenously with 100 μg. of alcohol-killed Salmonella typhimurium C5 vaccine. At regular intervals, at least 3 rats from each group were bled and the serum from each group collected and pooled. The sera were then assayed for their bactericidal antibody content using the in vivo bactericidal assay technique described in chapter II. Table 6.1 shows that treatment of rats with Methotrexate reduces the natural antibody level. It is also evident that Methotrexate treatment markedly reduces the ability of rats to produce antibody to the vaccine. Table 6.1 also shows that by day 20 after injection with vaccine, the Methotrexate-treated rats are beginning to recover their antibody-producing ability. This in turn shows that Methotrexate injected at the levels used in this study produces no permanent damage to lymphoid tissues and that if one is to maintain a depressed capacity to produce antibody, the Methotrexate treatment must also be maintained.

The effect of Methotrexate on the phagocytic function of rat macrophages

To ascertain whether Methotrexate therapy affected the phagocytic function of rats, liver perfusion studies were carried out. Two groups of 12 week old male rats weighing 250-280 gm. were used. One group received one intraperitoneal injection of 0.25 mg/Kgm. of Methotrexate daily for 4 days whilst the other group received no Methotrexate. The commencement of Methotrexate therapy was staggered and the animals

TABLE 6.1

Comparison of the bactericidal antibody levels in the sera of Methotrexate-treated and normal rats before and after challenge with alcohol-killed Salmonella typhimurium C5 vaccine

	End point of bactericidal antibody as detected by the in vivo bactericidal assay	
Time	Normal Rats	Methotrexate-treated Rats
Day O	1/80	less than 1/2
Day 3	1/6400	less than 1/2
Day 6	1/102,400	less than 1/2
Day 12	1/25,600	less than 1/2
Day 20	1/25,600	1/1600

separated into sub-groups such that on the day the liver perfusions were carried out, several rats had received the last injection of Methotrexate 1, 6 and 12 days previously. This was done to determine whether the effects of Methotrexate treatment were evident soon after the last injection and whether these effects were sustained.

The technique of perfusing livers was similar to that described by Howard and Wardlaw (1958) with the following modifications:-

1. The rats were injected intravenously (via tail vein) with heparin (100 i.u.) immediately after anaesthetising with Sagatal (60 mg/Kgm.)

2. The oesophagus was not cut for access to the portal vein.

3. The cannulation was carried out using T2 PVC surgical tubing, 0.039 inch bore and 0.079 inch external diameter (Camelec, Camden, South Australia).

4. The heart was not excised after cannulating the inferior vena cava.

5. The whole abdomen was prevented from drying out by covering with a cotton wool pad moistened with physiological saline.

6. The perfusion apparatus used in these studies was constructed from two liebig condensers. The perfusion fluids were heated by circulating water through the water jackets of the condensers using a Techne TE4 Triac control unit (Techne (Cambridge) Ltd., Duxford, Cambridge, U.K.)

7. Ringer-Locke solution (Parker 1961) was used as the perfusing fluid. This medium was used to dilute an 18 hour nutrient broth culture of Salmonella typhimurium C5 to approximately 1×10^4 organisms per ml. as required.

8. After perfusing the liver free from blood with Ringer-locke solution, the Ringer-Locke solution containing the bacteria was perfused through at the constant rate of 5 ml. per minute for 20 minutes. Viable counts of each 5 ml. fraction collected were made and to determine whether any

bacterial multiplication had occured during the experiment, viable counts of the Ringer-Locke solution containing bacteria were made before beginning the perfusion and after its completion.

The results of the perfusion experiments (Table 6.2) show that there was no significant difference in the ability of normal and Methotrexate-treated rats to phagocytose Salmonella typhimurium C5 organisms. Normal rats displayed an average of 29 per cent uptake of organisms by the liver, whereas Methotrexate-treated rats showed an average of 32 per cent uptake by their livers.

It can be concluded from these experiments therefore, that the susceptibility to Salmonella typhimurium C5 infection induced in rats by treatment with Methotrexate, is due to a depression in the levels of natural and acquired antibody with no apparent depression of phagocytic function.

The susceptibility of mouse into rat radiation chimaeras to infection with Salmonella typhimurium C5

Jenkin and Rowley (1963a) have shown that Hooded Wistar rats, made tolerant to mouse tissues by injecting newborn rats intravenously with mouse lymphoid cells, were susceptible to Salmonella typhimurium C5 infection. The basis for these studies was the hypothesis proposed by Jenkin (1962), that mice are susceptible to Salmonella typhimurium C5 infection because they share antigenic determinants with this organism and hence cannot mount an adequate immune response. Thus, if rats were made tolerant to mouse tissues, they should also be made susceptible to infection with this organism.

In an attempt to repeat this experiment with the BN strain of rats, approximately two hundred newborn rats were injected intravenously via the orbital branch of the facial vein with a single cell suspension of between 1×10^7 and 8×10^7 spleen, liver and thymus cells from foetal mice at term. The newborn rats were injected at various times after birth between 6 and

TABLE 6.2

Comparison of liver uptake of Salmonalla typhimurium C5 by normal and Methotrexate-treated rats

	Number of Bacteria Perfused	Average number of Bacteria Recovered	Percentage Recovery	Percentage Uptake by Liver
Normal Rats	9.1×10^3/ml.	6.45×10^3/ml.	71	29
Methotrexate Treated Rats	9.1×10^3/ml.	6.2×10^3/ml.	68	32

48 hours and were challenged with up to 1 x 10^7 Salmonella typhimurium C5 organisms when they had reached the age of 6 - 8 weeks. However, it was not possible to induce susceptibility to Salmonella typhimurium C5 organisms in any of the rats injected with mouse cells.

Therefore an alternate method was used in an attempt to induce susceptibility to Salmonella typhimurium C5 in rats. Rats were lethally irradiated prior to injection with mouse cells and were challenged some time later.

Method used to establish mouse into rat radiation chimaeras

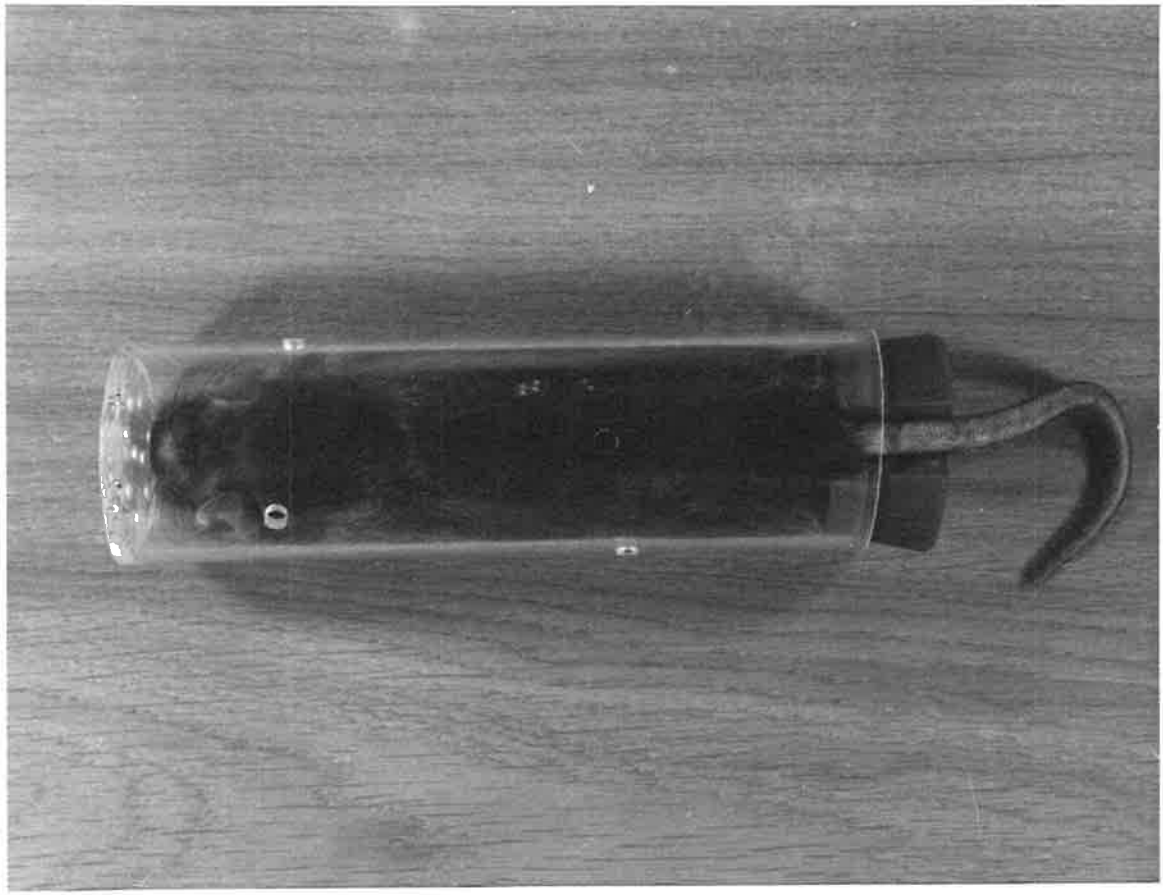
Before attempting to establish radiation chimaeras, it was necessary to determine the LD100 dose of x-irradiation for the strain of rats used in this study. The rats were placed in adequately ventilated perspex cylinders of 2 1/4 inch internal diameter and 8 inches in length (Fig. 6.3). The animals were then irradiated using a Philips Deep X-ray unit (250 KV. and 12 ma.) using a 1 mm. copper and a 1 mm. aluminium filter. The Focal Skin Distance was 50 cm. The LD100 dose of x-irradiation under these conditions was 900 rads instant dose of total body x-irradiation. This dose of x-irradiation produced death of all the animals within 21 days.

The irradiated animals were then divided into 3 groups -

1. irradiated rats given no cells
2. irradiated rats injected with xenogeneic mouse haemophoietic tissue, and
3. as control, irradiated rats injected with isogeneic rat haemopoietic cells.

FIG. 6.3

Photograph showing rats in perspex containers during x-irradiation



The mouse haemopoietic tissue used for transplanting into irradiated hosts was obtained from the femoral bone marrow of adult mice and from the livers of foetal mice. Rat haemotpoietic tissue was obtained from the femora and tibiae of adult rats. These tissues were suspended in Hanks balanced salt solution (Chapter II) to which 10 per cent heat inactivated (56^{o} for 20 minutes), foetal calf serum had been added. They were then gently pressed through a fine nylon mesh (400 holes per cm.^{2}) and then passed through needles of decreasing size until a single cell suspension was obtained. Viable cell counts of the suspension were made using the method of Trypan blue exclusion. The cell concentrations were then adjusted so that at least 5×10^{7} viable cells were injected into each irradiated animal.

The irradiated rats that were to receive haemopoietic cell transplants were 9 week old males and females weighting 150-170 gm. at the time of irradiation and were injected with these cells within 4 hours of irradiation. The route of administration of cell transplants was always intravenous. The irradiated rats injected with either rat or mouse haemopoietic cells, a group of irradiated rats not injected with cells and a group of normal rats that were neither irradiated nor injected with cells, were then observed for a period of at least 30 days. The progress of these 4 groups of animals was followed by observing any changes in weight that occurred. Fig. 6.4 shows that irradiated rats transplanted with both rat and mouse haemopoietic cells, initially suffered a slight loss in weight, but then recovered so that 30 days after irradiation they were similar in weight to normal untreated rats of the same age. In contrast, the irradiated rats receiving no haemopoietic cell transplant showed a dramatic loss in weight from which they do not recover.

FIG. 6.4

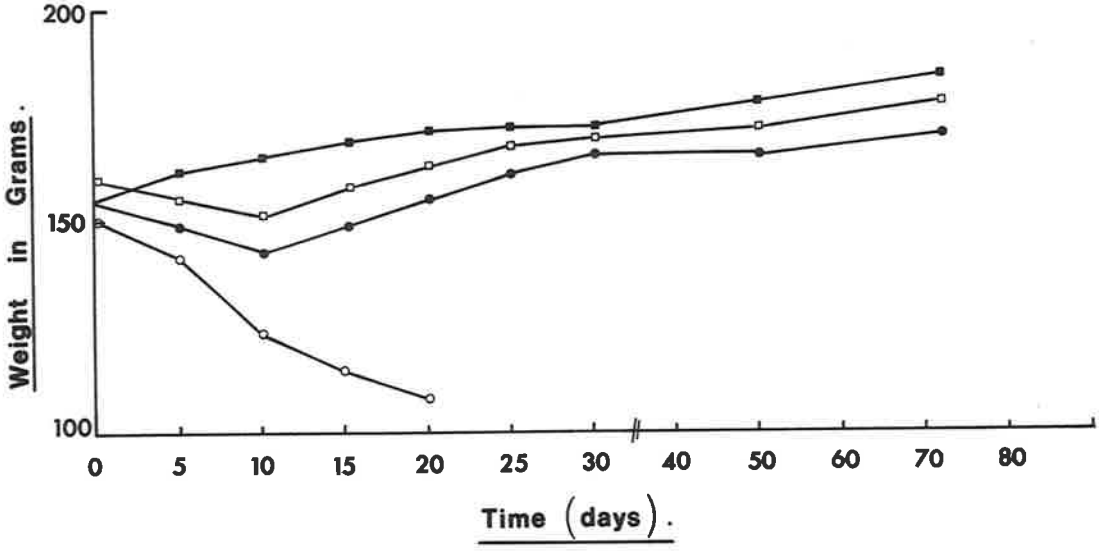
Weight changes in rats after x-irradiation and haemopoietic tissue replacement therapy.

normal, non-irradiated rats

irradiated rats receiving rat haemopoietic tissue intravenously after irradiation

irradiated rats receiving mouse haempoietic tissue intravenously after irradiation

irradiated rats receiving no haemopoietic tissue replacement therapy


200
150
100
Weight in Grams.
0
5
10
15
20
25
30
40
50
60
70
80
Time (days).

The survival of mouse into rat radiation chimaeras

The irradiated rats injected with mouse haemopoietic cells have been observed for long periods of time (greater than 100 days), with no signs of "secondary disease" which has been shown to occur in chimaeras 30-40 days after treatment (Congdon and Urso, 1957 and Trentin 1957). None of the characteristics of "secondary disease" which include loss of weight, epilation, the occurrence of skin lesions as well as primary histophathologic lesions of lungs, kidney, gut or liver were observed up to 100 days after irradiation and haemopoietic cell transplantation. However at this time the "normal" post-irradiation signs of greying of the fur and cataracts were evident.

Some deaths were observed in the mouse cell injected groups of irradiated rats, but these occurred at an earlier time than the secondary disease syndrome is normally observed (Trentin 1956, Uphoff, 1957, van Bekkum and Vos 1957). These deaths occurred at about two weeks after irradiation, at the time when the rats receiving no haemopoietic cell transplant were dying. The dead and dying animals were autopsied and found to contain massive numbers of bacteria. However, these deaths were comparatively few in number and the overall survival rate of irradiated rats injected with mouse cells was 90-100 per cent. In comparison, the survival rate of irradiated rats injected with rat cells was invariably 100 per cent.

The survival of mouse into rat radiation chimaeras challenged with Salmonella typhimurium C5

Thirty, 40 and 50 days after irradiation and transplantation, the surviving animals were challenged intraperitoneally with Salmonella

typhimurium C5. Normal rats are known to be resistant to 5×10^5 organisms and therefore this inoculum was chosen for no other reason than convenience. Using this challenge dose, the non-irradiated normal rats and the irradiated rats transplanted with rat haemopoietic tissue were shown to be 100 per cent resistant. However, the irradiated rats injected with mouse haemopoietic cells showed a 100 per cent mortality when challenged with this dose of organisms. Normal mice were equally as susceptible to this same number of organisms (Fig. 6.5). It is probable that the irradiated rats injected with mouse cells would be equally susceptible to a much smaller challenge dose of Salmonella typhimurium C5 organisms but because of a shortage of irradiated animals LD50 studies were not carried out.

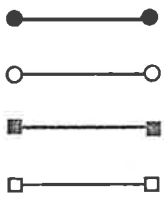
It is evident therefore, that post-irradiation protection of rats using xenogeneic mouse haemopoietic tissues can be achieved satisfactorily. However, these animals exhibit a susceptibility to Salmonella typhimurium C5 infection which is somewhat characteristic of normal mice. It appears most probable therefore, that the mouse cells which presumably proliferate and repopulate the lymphoid organs of lethally irradiated rats, do not have the capacity to mount an immune response of sufficient magnitude to provide adequate protection against this infection. On the other hand, rat cells appear to be able to proliferate and reconstitute the damaged lymphoid organs and thence respond effectively and resist infection with Salmonella typhimurium C5

Origin of the antibody synthesised by mouse into rat radiation chimaeras

Before any definite conclusions could be drawn from these studies, the existence of a true chimaeric state had to be confirmed. That is, it

FIG. 6.5

The survival of mouse into rat radiation chimaeras challenged with $5 \cdot 0 \times 10^5$ Salmonella typhimurium C5 organisms intraperitoneally

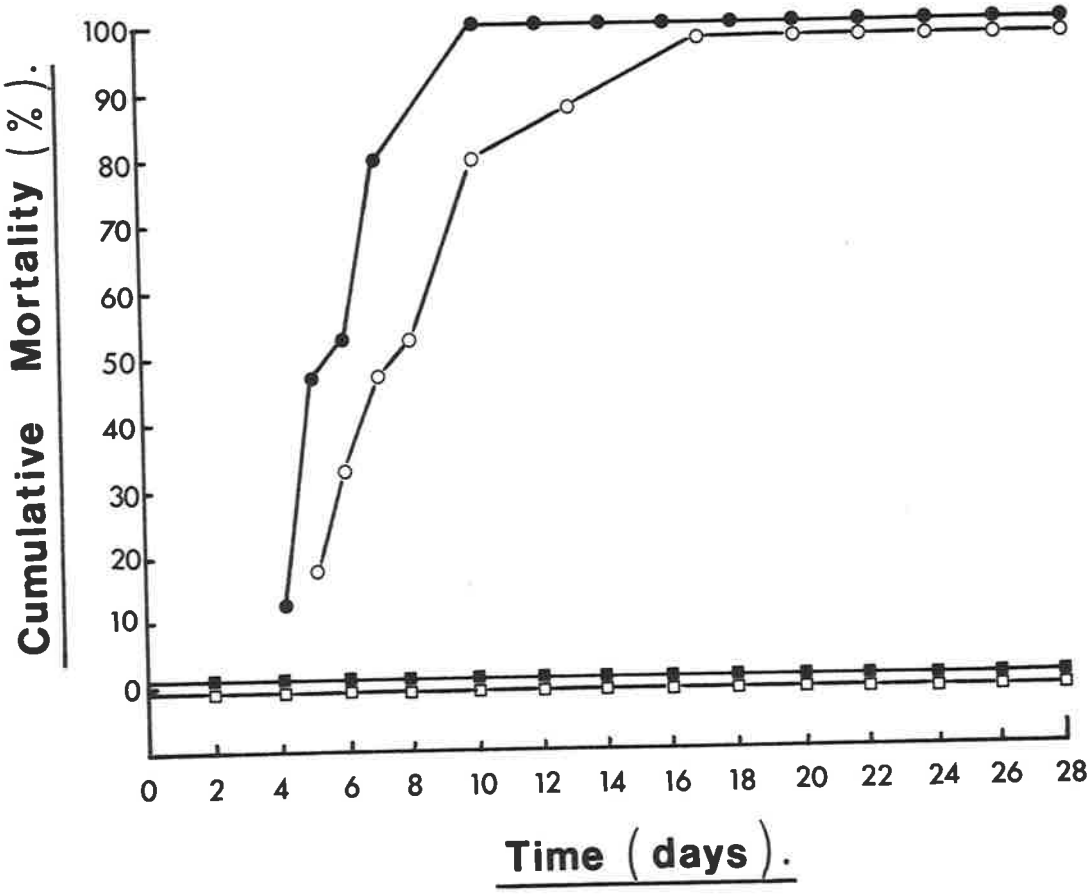


mouse into rat radiation chimaeras

normal mice

rat into rat radiation chimaeras

normal rats


Cumulative Mortality (%).
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days).

had to be determined whether the transplanted mouse haemopoietic tissues had proliferated and repopulated the lymphoid organs of the irradiated rats. The approach used was to examine the serum of irradiated rats that had received mouse cells for the presence of mouse immunoglobulins.

The studies of Makinodan, Gengozian and Congdon (1956) showed that if irradiated animals treated with bone marrow were injected with an antigen within 6 hours of this treatment, they were able to mount a normal antibody response. These irradiated animals had partially recovered the ability to produce antibody by 15 days after treatment and by one month this recovery was complete. The results of these experiments were used as a basis for the procedure to be followed in the present study. Irradiated rats were injected intravenously with 6.8×10^7 mouse haemopoietic cells together with 200 μg. of alcohol-killed Salmonella typhimurium C5 vaccine, within 4 hours of irradiation. Fifteen days later several animals were bled and the serum collected, pooled and stored.

The method used to look for the presence of mouse antibodies in this serum was by a slight modification of the immunodiffusion technique described by Ouchterlony (1958). The antiserum used was a rat anti-mouse serum raised in the following manner. A 20 ml. pool of normal mouse serum was precipitated with ammonium sulphate according to a slight modification of the method of Kendall (1938). Briefly, saturated ammonium sulphate was added to the serum in a ratio of 1:2 v/v and the precipitate was removed by centrifugation and dissolved in water. Saturated ammonium sulphate was again added in the same ratio and the precipitate removed by centrifugation and dissolved in a small volume of water.

The residual ammonium sulphate was removed by dialysis against an excess of distilled water. This method gives a preparation which contains almost all of the immunoglobulins with a relatively low albumin content. The protein concentration was determined by the method of Folin-Ciocalteu as described in Chapter II. A group of rats was then injected with 10 mg. of the mouse immunoglobulin preparation in complete Freund's adjuvant. Each animal received 1 ml. of the preparation and this was injected into the 4 footpads and subcutaneously at the base of the tail. Three weeks later each rat received another injection of 10 mg. of mouse immunoglobulin in complete Freund's adjuvant intraperitoneally. Ten days later all the animals were bled and the serum collected and pooled. The antiserum was raised in rats to prevent the formation of antibodies against any antigenic determinants of mouse immunoglobulins which may cross-react with those of rat immunoglobulins.

From the results shown in Fig. 6.6 it is clear that mouse into rat radiation chimaeras do possess mouse cells which are capable of synthesising immunoglobulins. Therefore, it is not unreasonable to assume that the lymphoid organs of these irradiated rats have, at least partially, been repopulated with mouse cells.

Duration of the chimaeric state

Finally, to determine whether this chimaeric state is of long duration, irradiated rats that had been transplanted with mouse haemopoietic tissue 12 weeks earlier, were bled and then injected intravenously with 200 μg. of alcohol-killed Salmonella typhimurium C5 vaccine. The rats were bled again 6 days after the injection and then both pools of sera were examined for the presence of mouse immunoglobulins using the Ouchterlony (1958) immunodiffusion technique. Fig. 6.6 shows that even 12 weeks after

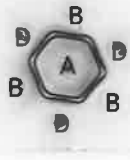
FIG. 6.6.

Ouchterlony immunodiffusion pattern showing mouse protein production by mouse into rat radiation chimaeras.

A	rat anti-mouse serum
B	serum from irradiated rats injected intravenously with mouse haemopoietic tissue and 200 μg. of alcohol-killed Salmonella typhimurium C5 vaccine within 4 hours of irradiation and bled 15 days later
C	serum from irradiated rats transplanted with $6{\cdot}8 \times 10^7$ mouse haemopoietic cells 12 weeks prior and bled before injection with alcohol-killed vaccine of Salmonella typhimurium C5
D	serum from irradiated rats transplanted with $6{\cdot}8 \times 10^7$ mouse haemopoietic cells 12 weeks prior, injected intravenously with 200 μg. of alcohol-killed Salmonella typhimurium C5 vaccine and bled 6 days later


B
A
B
B


D
C
A
C
D
D
C


B
D
D
A
B
B
D

irradiation and mouse cell transplantation the chimaeras are capable of producing mouse immunoglobulins after stimulation with Salmonella typhimurium C5 vaccine.

Conclusions

The difference in the capacity of rat and mouse lymphoid tissues to respond to the antigens of Salmonella typhimurium C5 has been established.

The depression of natural and acquired antibody levels due to the injection of low doses of the antimetabolite Methotrexate, results in the susceptibility of rats to Salmonella typhimurium C5 infection. However, these low levels of Methotrexate do not interfere with the phagocytic function of macrophages from those animals, since it has been shown that liver uptake of Salmonella typhimurium C5 organisms is similar in both normal and Methotrexate-treated rats. Presumably therefore, the depressed levels of antibody detected in the serum of rats treated with Methotrexate, are a reflection of the reduction in numbers of antibody producing cells - Friedman and Buckler (1963) have shown that Methotrexate selectively inhibits the proliferation of immunologically active cells and Rivarola, Friedman and Lawrence (1967) have shown that this drug reduces the number of antibody producing cells as detected by the Jerne plaquing technique.

The depressed activity of rat lymphoid tissue induced by Methotrexate can be compared with the lymphoid activity of mouse haemopoietic tissues transplanted into lethally irradiated rats. The mouse tissues in mouse into rat chimaeras do produce antibody, but in obviously inadequate levels (as is the case with normal mice), since these radiation chimaeras are susceptible to Salmonella typhimurium C5 infection. In contrast, the irradiated rats injected with rat bone marrow cells must produce adequate amounts of antibody since they are quite resistant to infection with this organism.

These results confirm the data presented in the previous chapters - antibody both natural and acquired is essential for protection against infection with Salmonella typhimurium C5.

CHAPTER VII

DISCUSSION

The aim of the studies reported in this thesis is to attempt to determine some of the immunological factors which influence the susceptibility of two species of animals to infection with the C5 strain of Salmonella typhimurium. The two species of animals used were chosen because it has been shown by previous workers that they represent the two extremes of susceptibility to infection with Salmonella typhimurium C5. An LD50 could not be determined for the resistant species - the rat - but it was shown to be greater than 7.9×10^9 organisms when the intraperitoneal challenge route was used. The susceptible species is a strain of mice which is highly susceptible to Salmonella typhimurium C5 infection. Using the intraperitoneal challenge route, the LD50 was shown to be less than 10 organisms.

As pointed out in the introduction to this thesis, there is a great deal of controversy regarding the factors which play a role in determining immunity to Salmonella infections. There is no clear cut evidence to indicate whether humoral or cellular factors play the predominant role in immunity that would satisfy all the workers in this field. However, it is certain that most of these workers accept the finding that specific antibody probably plays some role in protection. The present study has been restricted mainly to a study of humoral factors involved in immune defense mechanisms to Salmonella typhimurium C5 infection in rats and mice, in an attempt to determine whether the difference in susceptibility of these two species of animals is under genetic control, expressed at the level of antibody synthesis.

An hypothesis, with some evidence to support it, has been proposed to explain why mice are susceptible to infection with Salmonella typhimurium C5. Jenkin (1962) presented evidence that there is an antigenic relationship between this organism and certain antigens of the susceptible host. He suggested that mice fail to recognise the organism as foreign and thus do not produce an adequate immune response. Furthermore, Jenkin and Rowley (1959) showed that there are factors in the serum of normal rats which are, "extremely effective in preventing the lethal effects of Salmonella typhimurium", as demonstrated by serum transfer experiments into mice. These factors account for the opsonic activity detected in the serum of normal rats and were assumed to be natural antibodies.

There is little doubt that the serum from many species of normal animals, including rats, contains natural antibodies which have been shown to be specific as tested by the in vitro bactericidal assay using a wide variety of bacteria (Gibson, 1930 and 1932, Mackie and Finkelstein, 1930, 1931 and 1932). Furthermore, it has been shown quite conclusively that natural antibodies are important in determining protection against infection by gram negative and gram positive bacteria (Lovell, 1951 and Wellman, Liebke and Engel, 1962).

The data presented in chapter III show the results of examining normal rat and normal mouse serum for the presence of natural antibodies to Salmonella typhimurium C5. Several techniques were used in an attempt to detect and measure this antibody and it was found that normal rat serum contains quite high levels of natural antibody. In contrast, normal mouse serum contains little or no detectable natural antibody (Table 3.2).

The one disadvantage of the model chosen to study species susceptibility to infection is that it is difficult to choose any one assay system which will give a measure of protective antibody. Therefore, to overcome this problem several assay techniques were employed, namely,

a) the indirect haemagglutination assay,

b) the intravenous clearance of radioactively labelled organisms,

c) the *in vitro* bactericidal assay, and

d) the *in vivo* bactericidal assay.

To determine the nature of the natural antibody in the serum of normal rats, which specifically promotes opsonisation of *Salmonella typhimurium* C5, the technique described by Turner and Rowley (1963) was used. The antibody was eluted from *Salmonella typhimurium* C5 cell walls and was shown to be macroglobulin. This finding is in agreement with the results published by Reade *et al.*, (1965), who showed that the natural opsonins specific for *Salmonella typhimurium* isolated from both foetal and neonatal hooded wistar rats were predominantly β macroglobulins.

Since rats possess natural antibodies to *Salmonella typhimurium* whilst mice do not, the question which arises is whether this difference is under genetic control or is merely a reflection of a difference in antigenic stimulation received by these animals.

All rats and mice used in the present study were housed in the same animal rooms and fed on the same diet. Thus the likelihood of exposure to Salmonella antigens was similar for both species of animals. Furthermore, the results described in chapter VI show that lethally irradiated rats given normal mouse haemopoietic cells were susceptible to *Salmonella typhimurium* C5 infection, whereas similar rats transplanted with normal rat haemopoietic tissues were not susceptible. These observations are compatible with the view that the difference in natural antibody levels to *Salmonella typhimurium* in rats and mice is in fact a genetically determined species difference. This conclusion is supported by the studies of Reade

and Jenkin (1964) and Reade et al., (1965) which showed that both foetal and neonatal rat serum possessed low levels of opsonic activity for Salmonella typhimurium C5. Furthermore, if new born mice were suckled on rats, these animals were no more resistant to infection with Salmonella typhimurium C5 than were mice reared by their own parents (Reade and Rowley - personal communication).

The present study has not produced any clear cut evidence to indicate the level at which this genetic control is exerted. It does not seem possible to explain the difference in natural antibody levels to Salmonella typhimurium, which exist between the serum of normal rats and normal mice, in terms of a difference in the number of antibody producing cells because normal rats and normal mice have similar numbers of antibody producing cells per 10^7 spleen cells (Table 3.4). It is, however, possible that the techniques employed to detect antibody producing cells did not detect all the cells producing antibody specific for Salmonella typhimurium, because the antigen used in the assays was lipopolysaccharide extracted from Salmonella typhimurium C5 and not the whole organisms. Alternately, it is possible that the antibody producing cells detected in rat spleens are producing larger amounts of antibody per cell than similar cells detected in mouse spleens.

Another aspect which deserves mention is the fact that using the normal technique of haemolysis of sensitized sheep red blood cells, fresh normal mouse serum appears to lack complement whereas fresh normal rat serum consistently lyses the sheep red blood cells to a dilution of 1/16 - 1/32. Beernink and Steward (1967 and 1968) have shown that when E. coli or Salmonella strains are exposed to guinea pig serum, serum proteins other than immunoglobulins adhere to their surface. Some of these serum proteins were shown to be related to the complement system and the authors suggest that, since they adhere very strongly to the

bacterial surface, they may play a role in phagocytosis and intracellular killing. It is possible, therefore, that some components of complement may contribute to the greater opsonic activity of normal rat serum as compared to normal mouse serum and thus, indirectly contribute to the difference in susceptibility to infection of these two species. This does not, of course, account for the finding that normal rat serum possesses higher levels of natural antibody than normal mouse serum as measured by the in vitro bactericidal or indirect haemagglutination assays.

Whilst there is a great deal of controversy about whether humoral antibody or cellular factors play the major role in determining immunity to Salmonella infection, there is no doubt that phagocytic cells have an important role in protection. Therefore, to determine whether there was any difference in the phagocytic ability of macrophages from normal rats and normal mice, in vitro phagocytic experiments were carried out. The results (Table 3.3) confirm the data Rowley and Jenkin (1962) obtained with a different strain of rats and show that rat and mouse macrophages have similar abilities to phagocytose and kill Salmonella typhimurium C5 under identical conditions of opsonisation. It follows, that the innate resistance of rats to Salmonella typhimurium C5 infection cannot be accounted for solely in terms of highly efficient phagocytic cells.

Two different approaches are used to assess resistance of animals, or the effectiveness of vaccinating procedures to Salmonella infections. The first technique relies on determination of the death or survival rate of animals challenged with a particular strain of Salmonella. By this criterion rats are obviously resistant, since they invariably survive infection even with 7.9×10^9 Salmonella typhimurium C5 organisms. However, Collins et al., (1966), Collins (1968) and Collins and Mackaness (1968) pointed out that this technique gives an assessment of immunity only in terms of an "all-or-nothing" basis, since no account is taken of

the fact that the animals which do survive may have suffered a subclinical or even a severe clinical infection, followed by recovery. They suggested that a more accurate technique for determining resistance to Salmonella infections, is a combination of overall survival figures together with bacterial enumeration studies, which give an indication of whether the challenged animals possess the ability to control and progressively eliminate the challenge organism without the development of a clinical infection.

Studies of the fate of Salmonella typhimurium C5 injected intraperitoneally into normal rats and normal mice indicated that the rats used in this study could be classed as resistant to Salmonella typhimurium C5 even using the latter criterion for resistance. Furthermore, the results obtained lead to the conclusion that natural antibody plays a part in the control of this infection, although no direct correlation can be observed between the presence of natural antibody to Salmonella typhimurium C5 and complete elimination of this organism because a carrier state becomes established within 24 hours of challenge. Rats injected with Salmonella typhimurium C5 are initially apparently able to kill some of these organisms because there is a fall in the total number of organisms that can be recovered. However, about 8 hours after challenge, the total number of organisms that can be recovered begins to increase and the organisms soon reach approximately the level of the original inoculum and remain constant at this level until the organisms are eliminated at a time which is determined by the size of the original inoculum (Figs. 4.2 and 4.5). The results do not, of course, indicate whether during the carrier state :-

a) the rate of multiplication of bacteria is equivalent to the rate at which these organisms are being killed, or
b) the organisms enter a "stationary phase" in which there is no killing and no multiplication.

In contrast, when Salmonella typhimurium C5 organisms are injected into mice, an immediate increase in the total number of organisms that can be recovered occurs, with no indication of any killing. The organisms continue to multiply rapidly until they reach a level which is fatal for the mice (Figs. 4.2 and 4.5).

While on the basis of the results of the present study the conclusion that normal rats can control the multiplication of Salmonella typhimurium C5 because they possess low levels of natural antibody to this organism is an inevitable one, nevertheless the findings are somewhat surprising because they differ from what might be predicted from the observations that other workers have made on Salmonella infections in mice. For example, Collins (1969b), found that mice, vaccinated with alcohol-killed Salmonella enteritidis to elicit quite high titres of antibody, as measured by bacterial agglutination, in direct haemagglutination using red blood cells sensitised with Salmonella enteritidis lipopolysaccharide and the in vitro bactericidal test, were not able to control the multiplication of a challenge dose of the same organism. At best, the immunized mice were able to reduce the size of the initial viable population, but the subsequent rate of multiplication of surviving organisms was virtually the same as in control, non-immunized animals. Collins therefore concluded that the ability of mice to control and eliminate Salmonella infections depends on a modification of the functional activity of phagocytic cells.

It is difficult to reconcile the present findings, using rats, with those reported by Collins, because the question which remains unanswered is - why should this natural antibody be so much more effective in bringing about the control of bacterial multiplication ? One possibility is that there is a difference in the biological activity of natural antibody present in normal rat serum and antibody induced by the injection of a killed vaccine into mice.

This can only be regarded as a tentative explanation because no direct evidence exists for the possibility that different antibodies to Salmonella strains may have different biological activities. The suggestion is, nevertheless, made because of the results obtained when serum from rats injected with live or killed Salmonella typhimurium C5 was examined for antibody content using various antibody assay techniques.

A study of the antibody response of both rats and mice injected intraperitoneally with live Salmonella typhimurium C5 indicated that rats produced much more antibody than mice and that the antibody produced by rats reached high levels very rapidly (Figs. 4.9 and 4.10). The results also show that as detected by the in vitro bactericidal assay, there is one prominent peak of antibody produced by both rats and mice occurring at about 6 - 8 days after challenge. However, using the in vivo bactericidal assay, rats are seen to produce two prominent peaks of antibody - the first occurs at 6 - 8 days after challenge and corresponds to the peak detected by the in vitro assay. The second peak occurs at about day 16 after challenge and this peak persists for several weeks. It seems possible therefore, that the antibody detected by the in vivo bactericidal assay is different from that detected by the in vitro bactericidal assay. In other words, antibodies to Salmonella typhimurium C5 which have different biological properties may exist. As already stated, however, these results cannot be regarded as providing indisputable evidence that this is so, because alternate explanations exist.

Antibody produced early in the response to this organism may be less avid than that produced later, or the antibody produced late in the immune response may differ in specificity from that produced early. Alternately, the early and late responses may consist of antibodies with the same specificities but differ in that the late peak contains more

antibody of a particular specificity which is conducive to phagocytosis and elimination. Because of the limitations of the antibody assay techniques these questions could not be resolved.

It is interesting to note that if the results obtained using the in vivo bactericidal assay for antibody are compared with the results of the bacterial recovery studies, it becomes apparent that the late peak of antibody produced by rats coincides with the time during which the Salmonella typhimurium C5 organisms are being eliminated. Therefore, it appears that there is a correlation between acquired antibody levels and the total elimination of these organisms. It is realized however, that these results do not provide conclusive evidence that the second peak of antibody is responsible for the elimination, because the correlation may just be a chance occurrence. This correlation is not apparent when comparing the early peak of antibody with the total number of organisms that can be recovered. However, there is a correlation between the appearance of the first peak of antibody and the distribution of organisms in rats challenged with Salmonella typhimurium C5. In particular, the number of organisms detected in the blood and peritoneal cavity are affected. These results are in keeping with the data published by Blanden et al., (1966) and Collins (1969a), which show that antibody has a marked effect on organ distribution of bacteria injected by the intraperitoneal route.

There is also no apparent correlation between the presence of antibody in the serum of mice and protection against infection with Salmonella typhimurium, because although by day 6 infected mice are producing quite high titres of antibody, most of them succumb to the lethal effects of the infection. However, serum taken from normal rats or rats and mice 6 days after being injected with living Salmonella typhimurium C5 organisms can protect normal mice against intraperitoneal challenge with this organism, whereas normal mouse serum was completely ineffective in transferring any protection at all (Figs. 5.1 - 5.4). Thus it is likely that the antibody

detected in the serum of mice does exert some control on the multiplication of Salmonella typhimurium, although this control is clearly ineffective, presumably because the antibody is synthesised only at a stage when the mice already have an overwhelming infection, where death is inevitable. In the case of rats, however, it is possible that natural antibody also determines the control of multiplication of Salmonella typhimurium C5 organisms at the time of appearance of the early antibody peak. This suggestion seems warranted because there is no fall in the total number of organisms that can be recovered from rats at this time.

It is realized that other mechanisms for control of the progress of infection in rats may exist. These will be considered at a later stage in this discussion.

The difference in levels of antibody produced by rats and mice was also evident when :-

1) rats and mice were injected with numbers of Salmonella typhimurium C5 organisms which were carefully adjusted, so that at the time of the early peak of antibody production, the total numbers of those organisms that could be recovered from rats and mice were similar (Fig. 4.7),

2) rats and mice were injected with weight adjusted numbers of Salmonella typhimurium C5 (Fig. 4.8).

In order that the antigenic dose could be more accurately determined, an alcohol-killed vaccine of Salmonella typhimurium C5 was injected over a wide dose range. Again the results show that rats produce more antibody than mice, irrespective of the antigenic dose used (Figs. 4.13, 4.14, 4.15, 4.16, and 4.17). It is particularly noteworthy that rats immunised with killed vaccine did not produce a second, late peak of antibody (as measured by the in vivo bactericidal assay).

The ability of rats to produce higher titres of antibody to Salmonella typhimurium C5 than mice, may be due to an intrinsic difference in the ability of rats and mice to produce antibody to any immunogen. This seems unlikely however, since mice are known to be resistant to infection with many different organisms and this is generally accepted to indicate that mice can produce adequate antibody levels. Nevertheless, this possibility was examined by comparing the immune response elicited in rats and mice to bovine serum albumin, an antigen unrelated to Salmonella typhimurium C5. In order to obtain maximal stimulation of both rats and mice and thereby eliminate any differences which may arise due to dose dependent factors, a priming dose of this antigen was used prior to challenge with three different doses of bovine serum albumin. The results (Table 4.2) show that there is no difference in the ability of rats and mice to produce antibody to BSA. Thus it appears that the difference in antibody levels present in the serum of rats and mice immunized with the various standard doses of Salmonella typhimurium C5 used, is due to a difference in the ability of the two species of animals to respond to a particular dose of the antigen, and not due to the fact that mice are generally incapable of producing as much antibody as rats to any immunogen, even under conditions of optimal stimulation.

A number of workers have shown that immunization with killed vaccines or injection of antibody specific for a particular strain of Salmonella will protect mice against challenge with the same strain of Salmonella, when assessment of protection is made on the basis of survival rates of the animal, compared with control animals (Jenkin and Rowley, 1963 and 1965, Kenny and Herzberg, 1968). However, other workers (Hobson, 1957; Mitsuhashi, Kawakami, Yamaguchi and Nagai, 1958; Mackaness et al., 1966; Collins et al., 1966 and Blanden et al., 1966), have shown that if control of multiplication of the challenge organism is used as the

criterion for resistance, then effective protection of mice can only be achieved using living vaccines. Furthermore, using this latter criterion of resistance Collins et al., (1966) and Collins (1968), have shown that the ability of a live vaccine to protect mice against a subsequent infection with a potentially virulent strain depends on the ability of the vaccine strain to persist in the liver and spleen in appreciable numbers. On the basis of these observations, the abovementioned workers have suggested that protective mechanisms other than those relying on the presence of humoral antibody are necessary to explain host resistance to Salmonella - as determined by the ability of the host to control and eliminate the infection and not just to survive the infection.

The initial experiments of Mackaness et al., (1966) which were designed to determine what other factors may be involved, led these workers to conclude that the antibacterial mechanism involved was cellular in nature and that the cells involved were macrophages with enhanced bactericidal properties or "hyperactive macrophages". Subsequent studies (Blanden et al., and Collins et al., 1966) showed that immunity to Salmonella infection could not be passively transferred to mice by immune serum. However, the immune serum did produce a more rapid blood clearance of the challenge organism and a more effective control over the bacterial population during the initial stages of the infection, but this did not seem to materially influence the subsequent growth of organisms in the tissues. These results were confirmed by Collins and Mackaness (1968) and Collins (1969b) who concluded that immunity against Salmonella is mediated by cellular rather than humoral mechanisms. They based this conclusion on a consistent inability to demonstrate protection of mice with killed vaccines and on a lack of passive serum protection against intravenous challenge by Salmonella enteritidis or Salmonella typhimurium.

More recently, Mackaness and his colleagues have provided an explanation for the mechanism which is responsible for the appearance of phagocytic cells with enhanced bactericidal properties resulting in immunity to intracellular parasites (as determined by elimination of organisms from the tissues of an infected host). (Mackaness, 1968; Collins and Mackaness, 1970a, b, 1971; Mackaness, 1971a, b). From studies of mice infected with Listeria, Salmonella or tubercle bacilli, these workers concluded that the mechanism involved is cell mediated delayed type hypersensitivity. Their results have indicated that the antigenic stimulus provided by infection with either Listeria or tubercle bacilli evokes the formation of a large population of newly formed lymphocytes which are specifically committed to the antigen(s) that evoked their production. Cell transfer experiments into normal and irradiated recipients (Tripathy and Mackaness, 1969) clearly indicated that, while these are the cells which provide the host with immunological reactivity towards the infectious agent, they are not the effector cells which bring about the elimination of the intracellular parasites. Elimination is brought about by "activated" macrophages which arise from blood monocytes as a result of interaction of antigen and the specifically committed lymphocytes (Mackaness, 1969). Manifestation of delayed type hypersensitivity requires the participation and interaction of these same two cell types at the site where the eliciting dose of antigen is introduced (Lubaroff and Waksman, 1968).

This is one of the main reasons why Mackaness and his colleagues propose that immunity to intracellular parasites and delayed hypersensitivity are different manifestations of a common immunological mechanism. It is also consistent with most of the findings on infections with intracellular parasites that Mackaness and his colleagues have reported. Their data indicates that development of delayed hypersensitivity generally coincides with the onset of acquired immunity (Mackaness and

Blanden, 1967; Mackaness, 1968; Collins and Mackaness, 1968) and that delayed hypersensitivity is only induced when live vaccines, which are more effective in eliciting protection than killed ones, are used (Mackaness et al., 1966; Collins et al., 1966; Blanden et al., 1966; Collins, 1968). This model also provides an explanation for the observation that once immunity to an intracellular parasite has been elicited, it is non-specific, in the sense that the immunity is manifested to other, antigenically unrelated intracellular parasites, but that when it has diminished, it can only be recalled by challenge with the homologous strain (Mackaness, 1964; Blanden et al., 1966; Collins and Mackaness, 1970b).

While this is a most attractive proposal to explain the mechanisms of immunity to intracellular parasites, unfortunately there are some observations which cannot be readily explained using this model. Mackaness himself points out that while this mechanism provides a very good explanation for the elimination of living organisms present in infective foci scattered throughout various tissues of the infected host, it is not easy to provide an explanation for the wide-spread activation of phagocyte cells in areas quite remote from the infecting foci which also occurs (Mackaness, 1971a).

More puzzling is the fact that other workers in this field have obtained results which are not consistent with those of Mackaness and his colleagues. For example, if immunity to intracellular parasites is due solely to activated macrophages, it would seem likely that when immunity to an intracellular parasite has been elicited, then, at the height of resistance any intracellular parasite would be inactivated equally effectively. This has been found not to be so. Coppel and Youmans (1969) have shown that mice immunized and boosted with either Listeria or tubercle bacilli eliminate the homologous challenge organism more effectively than the heterologous one. These workers also found that while a living attenuated vaccine of the H37Ra strain of Myco. tuberculosis was much more effective than

killed vaccine of the same strain in immunizing mice to infection with tubercle bacilli, this was not apparent when Listeria organisms were used to challenge the immunized mice. In this latter instance, both types of vaccine were equally effective. In addition Youmans and Youmans (1965 and 1966a, b) reported that the immunological potency of a ribosomal subfraction of the H37Ra strain of Myco. tuberculosis is, on a weight basis, equivalent to that of whole viable cells, when determined by protection against tuberculous infection. However, the immunity induced by the ribosomal subfraction was specific for the tubercle bacillus, and no delayed hypersensitivity could be demonstrated in these animals. (Coppel and Youmans, 1969; Youmans and Youmans, 1969a). Collins and Mackaness (1970a) have suggested that when immunity can be demonstrated in vaccinated animals which have negligible or very low levels of demonstrable tuberculin sensitivity this is due to the presence of memory (sensitized lymphocytes) without a high level of immunological reactivity. Immunity is manifested on challenge due to a prompt response to the infection. They based their suggestion on the finding that reinfection of previously vaccinated animals (with low levels of tuberculin sensitivity) with tubercle bacilli is accompanied by a rapid recall of hypersensitivity.

Unfortunately, this explanation does not adequately explain the findings of Youmans and Youmans (1969) because they observed that when mice vaccinated with the ribosomal subfraction of Myco. tuberculosis were injected with an intravenous injection of a booster dose of viable virulent or avirulent mycobacterial cells, no anamnestic response was found in terms of appearance of hypersensitivity to tuberculin and immunity to challenge with a heterologous intracellular parasite (Listeria).

Equally puzzling are the results of Raffel (1948 and 1950), who found that a high degree of hypersensitivity to tuberculin can be induced in guinea pigs, without a concomitant induction of immunity to tubercle bacilli.

A possible explanation for these contradictory findings is that different groups of workers have used different experimental designs in their studies. Nevertheless, this type of explanation is unsatisfactory, if for no other reason that it resolves nothing. A more satisfactory explanation, although again not necessarily an adequate or correct one is that proposed by Coppel and Youmans (1969) who suggest that there are two mechanisms involved in acquired immunity to facultative intracellular parasites. One, compatible with the findings of Mackaness and his colleagues, is non-specific and is mediated by activated macrophages, and the other is specific and is mediated by a mechanism which is not yet understood, but possibly involves specific antibody. It certainly seems necessary to consider seriously this possibility of a second, specific mechanism of immunity, because recent experiments by other workers suggest that the findings of Youmans and his colleagues with tubercle bacilli are not unique and restricted to this particular intracellular parasite. Independent observations by Eisenstein, Winston and Berry (1968), Venneman, Bigley, Klun, Zachary and Dodd (1968) and Venneman and Berry (1971), have indicated that the ribosomal fraction of Salmonella typhimurium is highly immunogenic and protective for mice.

The experimental results reported in this thesis obviously do not help to resolve the controversy that exists, since no attempt was made to study and correlate the presence of delayed hypersensitivity and the fate of Salmonella typhimurium in rats and mice challenged with this organism. However, some discussion of the results obtained in terms of the proposed mechanisms of immunity outlined above is perhaps warranted.

In agreement with other workers, normal Swiss white mice clearly lack any kind of effective defense mechanism against Salmonella typhimurium because they are unable to control multiplication of even very

low doses of this organism and usually die of the infection. Rats, on the other hand, do possess the capacity to control the multiplication of Salmonella typhimurium, but it is difficult to argue that the mechanism responsible for this involves "activated" macrophages, at least during the early stages of infection. Although in vitro phagocytic experiments indicated that rat and mouse macrophages do not differ in their ability to phagocytose and kill opsonised Salmonella typhimurium organisms, studies of the fate of this organism injected intraperitoneally into normal rats indicated that no increase above inoculum numbers could be observed at any stage after injection. Thus rats do not appear to have to rely on any mechanism acquired as a result of challenge with Salmonella typhimurium to control this infection. This leads to the inevitable conclusion that rats are able to control the multiplication of Salmonella typhimurium because they possess specific natural antibody in their serum. It is of course possible that the functional activity of the phagocytic cells of rats increases very early in the course of infection - that is, rats may behave in a manner similar to those prestimulated mice in which Collins (1968) demonstrated a recall of immunity. However, if this were so, one might expect a much more rapid elimination of organisms as observed by Collins (approximately 8 days), rather than a minimum of 21 days, which is the time taken by rats to completely eliminate the infecting organism. Furthermore, it is interesting to note that although a marked increase in reticulo-endothelial cell activity was demonstrated in rats 6 and 12 days after infection, this appeared to have no effect on the total number of Salmonella typhimurium C5 organisms that could be recovered from rats at those times.

These findings do not, of course, deny the possibility that the complete elimination of Salmonella typhimurium in rats is due to the mechanism proposed by Mackaness and his colleagues. It is, however, worth noting firstly that on day 21 after infection, when a superinfecting dose of

Salmonella typhimurium is cleared from the circulation of infected rats much more rapidly than on day 6 and 12, the activity of the reticulo-endothelial system, as determined by the clearance of an antigenically unrelated organism, has returned to normal. Secondly the late antibody peak detected in the serum of infected rats coincides with the time during which the Salmonella typhimurium C5 organisms are being eliminated. Whether there is any significance in this correlation has to await further experimental work.

The studies of Rowley et al., (1968) showed that 10 days after being injected with Salmonella enteritidis 11RX mice had an increased activity of their reticulo-endothelial system and were also immune to challenge with Salmonella typhimurium C5. These findings are obviously consistent with those of Mackaness and his colleagues (Blanden et al., 1966) and support the proposal that immunity to intracellular parasites is due to the presence of "activated" macrophages. However, the results presented in Chapter V show that mice injected with Salmonella enteritidis 11RX are as resistant to challenge with Salmonella typhimurium after the carrier state is lost, when their reticulo-endothelial system is no longer hyper-active, as they are 10 days after injection with Salmonella enteritidis 11RX, when the carrier state is present. Although the results indicate that this resistance to challenge can be correlated with the presence of levels of antibody opsonic for Salmonella typhimurium C5 in the serum of these 11RX recovered mice, this observation acquires an added significance in the light of the proposal of Mackaness and his colleagues. Because Salmonella enteritidis 11RX and Salmonella typhimurium C5 clearly share cross-reacting antigenic determinants, the control of multiplication and the elimination of Salmonella typhimurium from 11RX-recovered mice may be brought about by a prompt recall of immunity due to activated macrophages, since these animals are likely to show a delayed-type hypersensitivity response to Salmonella typhimurium antigens.

The only finding which is not consistent with this possibility is the demonstration that 11RX-recovered mice become susceptible to Salmonella typhimurium C5 following the injection of C5 polysaccharide or O-acetylated galactan. From the data reported by Ackerman (Ph. D. thesis), who studied the resistance of mice to Listeria, this type of treatment probably led to desensitization or loss of ability to show a typical delayed hypersensitivity reaction, but it should not result in increased susceptibility to infection. In fact Ackerman found that immunized desensitized mice were slightly more resistant to challenge with Listeria, than immunized mice which showed strong delayed hypersensitivity reactions to Listeria antigens injected into their footpads. Because injection of either C5 polysaccharide or O-acetylated galactan into 11RX-recovered mice led to depletion of antibody levels specific for Salmonella typhimurium and resulted in the susceptibility of these animals to challenge with this organism, it does seem that the presence of specific antibody in 11RX-recovered mice plays a significant role in control of infection to Salmonella typhimurium. Again, it should be emphasized that this conclusion is based only on the data available, which do not provide absolutely conclusive proof that this is the case.

It is, however, tempting to suggest that an analogy can be drawn between 11RX-recovered mice and normal rats, since normal rats can also be made susceptible to Salmonella typhimurium C5 infection as a result of injection of C5 polysaccharide, a treatment which decreases the level of natural antibody for Salmonella typhimurium normally present in normal rat serum (Figs. 5.12, 5.13 and 5.14). The observation that when rats are injected with Methotrexate they become susceptible to Salmonella typhimurium infection also provides circumstantial evidence that antibody plays a role in the control of infection in rats, since this treatment leads to reduction in levels of natural antibody and suppression of antibody synthesis, without impairment of phagocytic cell function. This evidence can only be regarded as circumstantial because Methotrexate may be

increasing the susceptibility of rats due to its effect on production of monocytes which are required for the production of "activated" macrophages. In fact, McGregor and Koster have shown that cyclophosphamide prevents adoptive immunization of mice against Listeria because it interferes with monocyte production [cited by Mackaness (1971b)].

The data using X-irradiated rats can also not be regarded as proving unequivocally that antibody plays the major role in immunity to Salmonella typhimurium C5 infection. Experiments need to be carried out to determine whether 1) normal rats possess any lymphocytes specifically sensitized to Salmonella typhimurium, which are capable of transferring immunity and delayed hypersensitivity to susceptible animals (this was tried, unsuccessfully in X-irradiated mice); 2) the dose of X-irradiation given to rats destroys the sensitized lymphocytes, if they are present; 3) tissue obtained from the femora and tibiae of normal adult rats contains any such sensitized cells, and 4) the interaction of specifically sensitized lymphocytes and antigen leads to the production of "activated" macrophages when a heterologous species of animal is the source of monocytes or monocyte precursors.

Because only the intraperitoneal challenge route was used in the present series of experiments, it is relevant to point out that in 1966 Blanden et al., recommended that the intravenous challenge route should be used when infections with intracellular parasites were being studied, in order to clearly dissociate the cellular and humoral components of the resistance mechanism. This same conclusion was reached by Collins (1968 and 1969b) after carrying out experiments which compared the intravenous, intraperitoneal and subcutaneous routes of infection of mice with Salmonella enteritidis.

Collins found that when mice, immunized with killed vaccines of Salmonella enteritidis, were challenged with living organisms of the same strain, more significant immunity, as measured by survival rates, were observed when the intraperitoneal as compared to the intravenous challenge route was used. Studies of the fate of the organisms in the peritoneal cavity, liver, spleen and blood of these challenged animals indicated that the reason for the protective effect was that antibody could reduce the size of the initial viable population injected only if the peritoneal route was used and that it was also responsible for slowing the rate of dissemination of the remaining organisms to the liver, spleen and blood. Antibody did not have any effect on the rate of multiplication of any organisms present in the liver, spleen or blood, hence the lower rate of survival if the intravenous challenge route was used.

In the present study, however, the levels of natural antibody present in rats appear to be much more effective in controlling Salmonella typhimurium infection than the experiments reported with mice would lead one to expect, because not only is there an initial reduction of the inoculum dose observed, there is also a complete lack of multiplication above the original inoculum level in spite of the fact that the organisms localize in the liver and spleen within 2 hours of injection. This does not necessarily mean that different results would not be obtained if the intravenous challenge route is used and experiments carried out using this challenge route may lead to a better understanding of any cellular mechanisms involved in the immunity of rats to Salmonella typhimurium.

A comparison of the immune response of rats and mice to the antigens of Salmonella typhimurium C5 show that mouse lymphoid tissues respond poorly and produce an inadequate immune response. The reason for this could be, as Jenkin (1962) suggested, that mouse tissues may share antigenic determinants with this organism. However, if this is so, it does

not mean that mice cannot respond at all, because it was shown in Chapter V that mice do produce some protective antibody when challenged with Salmonella typhimurium C5 and that serum from these animals could be used to passively transfer protection to other mice.

The studies reported in this thesis provide some information on the mechanism of resistance of two species of animals to infection with Salmonella typhimurium C5. However, it is obvious that many questions remain unanswered.

There is need for a biological assay technique with which to quantitate the various antibodies which may have different biological activities. Furthermore, one should be able to use such an assay to determine the specificity of these antibodies produced in terms of the different O-somatic antigens that Salmonella organisms possess. In this way one could not only determine the quantities of a particular antibody that may be essential to protect an animal against infection, but also it would make possible the direct comparison of the antibody requirements for protection of animals of different strains within the same species and even of animals of different species.

The origin of the antibody detected in mice recovered from infection with Salmonella enteritidis 11RX and which may be responsible for protection against infection with Salmonella typhimurium C5 can only be speculated upon. The two alternatives seem to be :-

(1) Mice possess a very low level of this antibody which cannot be detected by the methods available and infection with Salmonella enteritidis 11RX provides no more than an adjuvant action which results in increased levels of this antibody.

(2) The antibody which is detected in mice that have recovered from infection with Salmonella enteritidis 11RX is antibody directed against a component other than lipopolysaccharide, such as protein.

The fate of gram negative organisms injected into the rats or mice used in this study has not been precisely determined. One would like to know whether - (a) bacterial multiplication and killing occur simultaneously in both resistant and susceptible animals or, (b) whether the rate of multiplication in resistant animals is negligible or just less than that in susceptible animals, and (c) whether there is any bacterial killing at all in very susceptible animals. Furthermore, one would like to know whether the fate of bacteria is similar in different organs and at different stages of the infection.

The answers to these questions would shed some light on the question of effective antigenic doses resulting from the injection of a living as opposed to a killed vaccine into resistant and susceptible animals.

In the situation where an organism is multiplying within an animal and at the same time is being killed, one would like some information regarding the amount of antibody produced which is being adsorbed by bacteria. If this adsorption does occur it will result in errors when quantitation studies are being carried out.

Many experiments carried out in the past have utilized killed vaccines to induce immune sera for use in passive transfer studies, or to prime animals. However, as shown by the present studies, a second antibody peak is detected in rats only when living vaccines are used. Therefore, it is possible that the use of killed vaccines may not present a complete picture of the role of induced antibody in immunity to Salmonella infections.

Furthermore, there is an obvious need for more thorough studies of the cellular response of rats to Salmonella infections. These factors should be examined closely at regular intervals throughout the infection, beginning immediately prior to and after challenge and extending to the period following the elimination of the infecting organism.

The question of passive transfer of both cells and serum must also be reviewed. An attempt should be made to correlate antibody levels with the time lymphoid cells used to transfer protection are harvested. Furthermore, in the case of intracellular parasites, the method of raising immune or hyperimmune serum for passive transfer studies should be accurately defined and following from this the determination of number and timing of doses of passively transferred serum necessary to effectively protect the recipient animal.

These are but a few of the problems facing students of host-parasite relationships and species susceptibility to infection.

Summary

Some of the factors which determine the resistance of rats and the susceptibility of mice to infection with Salmonella typhimurium C5 have been investigated. From the results presented and discussed it is apparent that natural antibody appears to play a decisive role in the protective mechanism of rats. Rats possess natural antibody in their serum prior to challenge and after challenge are able to produce antibody at a rate and in quantities which suggests that the rats have already been immunized against infection with this organism. That is, they appear to produce a secondary rather than a primary immune response. Furthermore, rats are able to control the multiplication of Salmonella typhimurium C5 organisms, and eventually eliminate them completely. In contrast, mice produce much lower levels of antibody and at a rate which appears to have no apparent controlling effect on the multiplication of Salmonella typhimurium C5 organisms.

Whilst the results presented in this thesis support the hypothesis that antibody plays an important part in the protection of rats against Salmonella infection, there is no evidence presented which would refute the argument that cellular factors also play an important part. The experiments described were not designed to investigate the role of

cellular factors in the immune response of rats or mice. However, it can be concluded that in the model studied, the immunity of rats to infection with Salmonella typhimurium C5 organisms is unique in that :-

(1) rats do possess natural antibody in their serum,

(2) rats produce a second peak of antibody in response to challenge with the living organism

and

(3) there is no detectable hyperactivity of the reticulo-endothelial system at the time of occurrence of the second peak of antibody, when the Salmonella typhimurium C5 infection is eliminated completely.

BIBLIOGRAPHY

AMBROSE, C.T. and COONS, A.H. (1963). J. exp. Med., 117, 1075.

ASKONAS, B.A. and RHODES, J.M., (1965). Nature (Lond.), 205, 470.

AUZINS, I. (1968). Aust. J. exp. Biol. med. Sci., 46, 93.

AUZINS, I. and ROWLEY, D. (1962). Aust. J. exp. Biol. med. Sci., 40, 283.

AUZINS, I. and ROWLEY, D. (1963). Aust. J. exp. Biol. med. Sci., 41, 539.

AUZINS, I. and ROWLEY, D. (1969). Immunology, 17, 579.

AXELROD, A.E., CARTER, B.B., McCOY, R.H. and GEISINGER, R. (1947). Proc. Soc. exp. Biol. (N.Y.), 66, 137.

BAILEY, C.E. (1923). Amer. J. Hyg., 3, 370.

BANG, F.B. and WARWICK, A. (1960). Proc. nat. Acad. Sci. (Wash.) 46, 1065.

BEHRING, E.A. von and KITISATO, S. (1890). Dtsch. med. Wschr., 16, 1113.

BEKKUM, D.W. van and VOS. O., (1957). J. cell. comp. Physiol., 50, (Suppl.1), 139.

BENACERAFF, B., SEBESTYEN, M.M. and SCHLOSSMAN, S. (1959). J. exp. Med., 110, 27.

BEN-EFRAIM, S., FUCHS, S. and SELA, M. (1967). Immunology, 12, 573.

BEN-HAREL, S. (1951). Amer. J. Hyg., 53, 1.

BERNSTEIN, F. (1930). Z. Immun. - Forsch., 54, 400.

BIOZZI, G., BENACERRAF, B. and HALPERN, B.N. (1953). Brit. J. exp. Path., 34, 441.

BIOZZI, G., STIFFEL, C., MOUTON, D., LIACOPOULOS-BRIOT, M., DECREUSEFOND, C. and BOUTHILLIER, Y. (1966). Ann. Inst. Pasteur, 110, (suppl. 3), 7.

BLANDEN, R.V., MACKANESS, G.B. and COLLINS, F.M. (1966). J. exp. Med., 124, 585.

BOEHME, D.H. and DUBOS, R.J. (1958). J. exp. Med., 107, 523.

BOEHME, D.H., SCHNEIDER, H.A. and LEE, J.M. (1959). J. exp. Med., 110, 9.

BOWEN, S.T., GOWEN, J.W. and TAUBER, O.E. (1957). Proc. Soc. exp. Biol. (N.Y.) 94, 479.

BOYCOTT, A.E. and PRICE-JONES, C. (1926). J. Path. Bact., 29, 87.

BOYDEN, S. (1960). Nature (Lond.)., 185, 724.

BOYDEN, S. (1966). Advanc. Immunol., 5, 1.

BUCHNER, H. (1889). Zbl. Bakt. 1. Abt. Orig., 5, 817.

BULL, C.G. and BARTUAL, L. (1920). J. exp. Med., 31, 233.

BULLOCK, W.E. and CRAMER, W. (1919). Cited by Topley W.W.C. and Wilson, G.S. (1964). "Principles of Bacteriology and Immunity" Vol. II 5th ed. Wilson, G.S. and Miles, A.A., Edward Arnold (Publishers) Ltd., P.1491.

BURGI, E. (1907). Arch. Hyg., 62, 239.

BUTLER, W.T. (1961). Fed. Proc., 20, 27.

BUTLER, W.T. and COONS, A.H. (1964). J. exp. Med., 120, 1051.

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. and SUSSDORF, D.H. (1964). In "Methods in Immunology" Publ. W.A. Benjamin Inc. (N.Y.) p. 149.

CARLINFANTI, E. (1947). J. Immunol. 59, 1.

CLARK, E.R. and CLARK, E.L. (1928). Amer. J. Anat., 41, 227.

COHN, Z.A. and MORSE, S.I., (1959). J. exp. Med., 110, 419.

COLLINS F.M., MACKANESS, G.B. and BLANDEN, R.V. (1966). J. exp. Med., 124, 601.

CONGDON, C.C. and URSO, I.S. (1957). Amer. J. Path., 33, 749.

CRAIG, C.P. and SUTER, E. (1966). J. Immunol., 97, 287.

CRUCHARD, A. and COONS A.H. (1964). J. exp. Med., 120, 1061.

CRUMPTON, M.J., DAVIES D.A.L. and HUTCHINSON, A.M., (1958). J. gen. Microbiol., 18, 129.

DAVIDSOHN, I. and STERN, K., (1949). Proc. Soc. exp. Biol. (N.Y.), 70, 142.

DAVIDSOHN, I. and STERN, K. (1949b). Cancer Res., 9, 426.

DAVIDSOHN, I. and STERN, K. (1954). J. Immunol. 72, 216.

DIXON, F.J., JACOT-GUILLARMOD, H. and McCONAHEY, P.J. (1967). J. exp. Med., 125, 1119.

DOWNIE, A.W. and DUMBELL, K.R. (1956). Ann. Rev. Microbiol. 10, 237.

DUTTA, N.K. and HABBU, M.K. (1955). Brit. J. Pharmacol., 10, 153.

EDSALL, G., GAINES, S., LANDY, M., TIGGERT, W.D., SPRINZ, H., TRAPANI, R.J., MANDEL, A.D. and BENENSON, A.S. (1960). J. exp., Med., 112, 143.

ELBERG, S.S. (1960). Bact. Rev., 24, 67.

ELBERG, S.S., SCHNEIDER, P. and FONG, J. (1957).
J. exp. Med., 106, 545.

FENN, W.O. (1921). J. gen. Physiol., 3, 575.

FENNER, F., (1959). Brit. Med. Bull., 15, 240.

FENNER, F. and RATCLIFFE, F.N. (1965). "Myxomatosis"
University Press, Cambridge.

FINK, M.A. and QUINN, V.A. (1953). J. Immunol., 70, 61.

FINKELSTEIN, M.H. (1953). J. Path. Bact., 37, 359.

FINKELSTEIN, M.H. and UHR, J.W. (1964). Science, 146, 67.

FISHMAN, M., and ADLER, F.L. (1963). J. exp. Med., 117, 595.

FOLEY, G.E. and AYCOCK, W.L., (1944). Endocrinology, 35, 139.

FOLEY, G.E. and AYCOCK, W.L. (1945). Endocrinology, 37, 245.

FONG, J., CHIN, D., AKIYAMA, H.J. and ELBERG, S.S. (1959).
J. exp. Med., 109, 523.

FORD, C.E., ILBERY, P.L.T. and LOUTIT, J.F. (1957).
J. cell comp., Physiol., 50, 109.

FRIEDMAN R.M. and BUCKLER, C.E. (1963). J. Immunol. 91, 846.

FURNESS, G. and FERREIRA, I. (1959). J. infect. Dis., 104, 203.

FURNESS, G. and ROWLEY, D. (1956). J. gen. Microbiol. 15, 140.

GARDNER, K.U. (1920). Amer. Rev. Tuberc. 4, 734.

GIBSON, H.J. (1930). J. Hyg. (Camb.)., 30, 337.

GIBSON, H.J. (1932). J. Immunol., 22, 211.

GOLD, E.R. and FUNDENBERG, H.H. (1967). J. Immunol.,
99, 859.

GORER, P.A. and SCHUTZE, H. (1938). Amer. J. Hyg. 38, 647.

GOWEN, J.W. (1960). Bact. Rev., 24, 192.

GOWEN, J.W. and SCHOTT, R.G. (1933a). Amer. J. Hyg., 18, 674.

GOWEN, J.W. and SCHOTT, R.G. (1933b). Amer. J. Hyg. 18, 688.

GRAY, D.F. and CHEERS, C. (1967). Aust. J. exp. Biol. med. Sci., 45, 417.

GREENE, M.R. (1933). Amer. J. Hyg. 17, 60.

GUGGENHEIM, K., BOECHLER-CZACZKES, E. and HALEVY, S. (1951). J. infect. Dis., 88, 105.

HAHON, N. and WILSON, B.J. (1960). Amer. J. Hyg., 71, 69.

HARDY, D. and ROWLEY, D. (1968). Immunology, 14, 401.

HARRIS, R.J.C. (1956). Proc. Roy. Soc., B., 146, 59.

HENRY, C. and JERNE, N.K. (1968). J. exp. Med., 128, 133.

HOBSON, D. (1956). Brit. J. exp. Path., 37, 20.

HOBSON, D. (1957). J. Hyg. (Camb). 55, 322.

HOLLAND, J.J. and PICKETT, M.J. (1956). Proc. Soc. exp. Biol. (N.Y.) 93, 476.

HOLLAND, J.J. and PICKETT, M.J. (1958). J. exp. Med., 108, 343.

HOWARD, J.G., (1961). Nature (Lond.). 191, 87.

HOWARD, J.G. and WARDLAW, A.C. (1958). Immunology, 1, 338.

HUTT, F.B. and CRAWFORD, R.D. (1960). Canad. J. Genet. Cytol., 2, 357.

IRWIN, M.R. (1929). Genetics, 14, 337.

IRWIN, M.R. (1933). J. Immunol., 24, 285.

JACKSON, G.D.F. and JENKIN, C.R. (1969). Aust. J. exp. Biol. med. Sci., 47, 91.

JANEWAY, C.A. and HUMPHREY, J.H. (1968). Immunology, 14, 225.

JANEWAY, C.A. and SELA, M. (1967). Immunology, 13, 29.

JENKIN, C.R. (1962). J. exp. Med., 115, 731.

JENKIN, C.R. (1963). Brit. J. exp. Path., 44, 47.

JENKIN, C.R. and BENACERRAF, B. (1960). J. exp. Med., 112, 403.

JENKIN, C.R., KARNOVSKY, M.L. and ROWLEY, D. (1967). Immunology, 13, 335.

JENKIN, C.R. and PALMER, D.L. (1960). J. exp. Med., 112, 419.

JENKIN, C.R. and ROWLEY, D. (1959). Nature, (Lond.). 184, 474.

JENKIN, C.R. and ROWLEY, D. (1959a). Brit. J. exp. Path., 40, 474.

JENKIN, C.R. and ROWLEY, D. (1961). J. exp. Med., 114, 363.

JENKIN, C.R. and ROWLEY, D. (1963). Bact. Rev. 27, 391.

JENKIN, C.R. and ROWLEY, D. (1963a). Collogues Internationaux du Centre National de la Recherche Scientifique, 115, 291.

JENKIN, C.R. and ROWLEY, D. (1965). Aust. J. exp. Biol. med. Sci. 43, 65.

JENKIN, C.R., ROWLEY D. and AUZINS, I. (1964). Aust. J. exp. Biol. med. Sci., 42, 215.

JENSEN, K.A. (1929). Z Immun-Forsch., 63, 298.

JERNE, N.K. (1955). Proc. nat. Acad. Sci. (Wash.) 41, 849.

JERNE, N.K. (1960). Ann. Rev. Microbiol. 14, 341.

JERNE, N.K. and NORDIN, A.A. (1963). Science, 140, 405.

JERNE, N.K., NORDIN, A.A. and HENRY, C. (1963). In "Cell-bound antibodies" ed. B. Amos and H. Koprowski. Wistar Inst. Press (Philadelphia). p.109.

JETER, W.S., McKEE, A.P. and MASON, R.J. (1961). J. Immunol. 86, 386.

KABAT, E.A. and MAYER, M.M. (1961a). "Experimental Immunochemistry" 2nd Ed. Charles C. Thomas. Publisher Springfield, Illinois. p.149.

KABAT, E.A. and MAYER, M.M. (1961b). ibid. p. 150.

KABAT, E.A. and MAYER, M.M. (1961c). ibid, p.556.

KANTOCK, M., WARWICK, A. and BANG, F.B. (1963). J. exp. Med., 117, 781.

KANTOR, F.S., OJEDA, A. and BENACERRAF, B. (1963). J. exp. Med., 117, 55.

KAUFFMANN, F. (1966). "The Bacteriology of Enterobacteriace" Munksgaard - Copenhagen. p. 227.

KENDALL, F.E. (1938). cited in "Methods in Immunology and Immunochemistry" vol. 1 (1967). Ed. Williams, C.A., and Chase, M.W. p.319. Academic Press, New York and London.

KLIGLER, I.J., GUGGENHEIM, K. and HERNHEISER, H. (1946). J. infect. Dis., 78, 60.

KOTELKO, K., STAUB, A.M., and TINELLI, R. (1961). Ann. Inst. Pasteur, 100, 618.

LANDSTEINER K. and POPPER, E. (1909). Z Immun-Forsch., 2, 377.

LANDY, M., MICHAEL, J.G. and WHITBY, J.L. (1962). J. Bact., 83, 631.

LEVINE, B.B., OJEDA A. and BENACERRAF, B. (1963a). J. exp. Med., 118, 953.

LEVINE, B.B., OJEDA A. and BENACERRAF, B. (1963b). Nature (Lond.). 200, 544.

LEVINSON S.O., MILZER, A. and LEWIN, P. (1945). Amer. J. Hyg. 42, 204.

LINDSLEY, D.L., ODELL, T.T. and TAUSCHE, F.G. (1955). Proc. Soc. exp. Biol. (N.Y.) 90, 512.

LOVELL, R. (1951). Lancet, ii, 1097.

LURIE, M.B. (1933). J. exp. Med., 57, 181.

LURIE, M.B. (1939). J. exp. Med., 69, 579.

LURIE, M.B. (1942). J. exp. Med., 75, 247.

MACKANESS, G.B. (1954). Amer. Rev. Tuberc., 69, 495.

MACKANESS, G.B. (1960). J. exp. Med., 112, 35.

MACKANESS G.B. (1962). J. exp. Med., 116, 381.

MACKANESS G.B. (1964). J. exp. Med., 120, 105.

MACKANESS G.B., BLANDEN R.V. and COLLINS F.M. (1966). J. exp. Med., 124, 573.

MACKIE, T.J. and FINKELSTEIN, M.H. (1930). J. Hyg. (Camb). 30, 1.

MACKIE T.J. and FINKELSTEIN, M.H. (1931). J. Hyg. (Camb). 31, 35.

MACKIE, T.J. and FINKELSTEIN, M.H. (1932). J. Hyg. (Camb)., 32, 1.

MAKINODAN T. (1956). Proc. Soc. exp. Biol. (N.Y.) 92, 174.

MAKINODAN T. and ANDERSON, N.G. (1957). Blood, 12, 984.

MAKINODAN T., GENGOZIAN N. and CONGDON, C.C. (1956). J. Immunol., 77, 250.

MATHOT, C., ROTHEN, A. and SCHER, S. (1966). Experientia (Basel), 22, 833.

MAURER, P.H. (1963). Proc. Soc. exp. Biol. (N.Y.) 113, 553.

MAURER, P.H. (1964). Progr. Allergy, 8, 1.

MAURER, P.H. (1965). J. exp. Med., 121, 339.

McDEVITT, H.O. and SELA, M. (1965). J. exp. Med., 122, 517.

McDEVITT, H.O. and SELA, M. (1967). J. exp. Med., 126, 969.

McDEVITT, H,O. and TYAN, M.L. (1968). J. exp. Med., 128, 1.

McINTYRE J., ROWLEY, D. and JENKIN, C.R. (1967). Aust. J. exp. Biol. med. Sci., 45, 675.

METCHNIKOFF, E. (1905). "Immunity in Infective Diseases" (Translated from French by F.G. Binnie). Cambridge University Press, London.

MICHAEL, J.G. and ROSEN, F.S. (1963). J. exp. Med. 118, 619.

MILNER, K.C., JELLISON, W.L. and SMITH, B. (1957). J. infect. Dis., 101, 181.

MITCHISON, N.A. (1964). Proc. roy. Soc. B., 161, 275.

MITSUHASHI, S., HASHIMOTO, H. and KAWAKAMI, M. (1960). Jap. J. exp. Med., 30, 375.

MITSUHASHI, S., SAITO, K., OSAWA, N. and KURISHIGE, S. (1967). J. Bact., 94, 907.

MITSUHASHI, S., SATO, I. and TANAKA, T., (1961). J. Bact., 81, 863.

MELLER, G. and WIGZELL, H. (1965). J. exp. Med., 121, 969.

MURRAY, I.M. (1963). J. exp. Med., 117, 139.

NELSON, R.A. and LEBRUN, J. (1956). J. Hyg. (Camb)., 54, 8.

NOWELL, R.C., COLE L.J., HABERMEYER, J.G. and ROAN P.L. (1956). Cancer Res., 16, 258.

NUTTALL G.H.P. (1888) Z. Hyg. 4, 353.

OUCHTERLONY, O. (1958). Progr. Allergy, 5, 1.

PADGETT, G.A., REIQUAM, C.W., HENSON, J.B. and GORHAM, J.R., (1968). J. Path. Bact., 95, 509.

PARKER R.C. (1961). "Methods of Tissue Culture", 3rd ed. Paul B. Hoeber, Inc. p. 55.

PEARLMAN D.S. (1967). J. exp. Med., 126, 127.

POTTER, E.V. and STOLLERMAN, G.H. (1961). J. Immunol., 87, 110.

QUAN, S.F., WHEELER, C.M., GOLDENBERG M.I. and KARTMAN, L. (1965). Amer. J. trop. Med. Hyg., 14, 638.

RAFFEL, S. (1949). Ann. Rev. Microbiol., 3, 221.

READE, P.C., TURNER, K.J. and JENKIN, C.R. (1965). Immunology, 9, 75.

REED, L.J. and MUENCH, H. (1938). Amer. J. Hyg. 27, 493.

RIBI, E., ANACKER, R.L., BREHMER, W., GOODE G. LARSON, C.L., LIST, R.H., MILNER, K.C. and WICHT, W.C. (1966). J. Bact. 92, 869.

RIVAROLA, A., FRIEDMAN, M. and LAWRENCE, W. (1967). Transplantation, 5, 1223.

ROBBINS, J.B., KENNY, K. and SUTER, E. (1965). J. exp. Med., 122, 385.

ROBERTSON, O.H. and SIA R.H.P. (1927). J. exp. Med., 46, 239.

RODEY, G.E., PARK, B.H. WINDHORST, D.B. and GOOD, R.A. (1969). Blood, 33, 813.

ROWLEY D. (1954). Brit. J. exp. Path. 35, 528.

ROWLEY, D. (1958). Nature (Lond.)., 181, 1738.

ROWLEY, D. (1959). J. exp. Med., 110, 27.

ROWLEY D. (1960). J. exp. Med., 111, 137.

ROWLEY, D. (1968). J. Bact., 95, 1647.

ROWLEY D., AUZINS, I. and JENKIN, C.R. (1968). Aust. J. exp. Biol. med. Sci. 46, 447.

ROWLEY, D. and JENKIN, C.R. (1962). Nature, (Lond.) 193, 151.

ROWLEY, D. and TURNER, K.J. (1966). Nature (Lond.). 210, 496.

ROWLEY, D., TURNER, K.J. and JENKIN, C.R. (1964). Aust. J. exp. Biol. med. Sci, 42, 237.

ROWLEY, D. and WHITBY, J.L. (1959). Brit. J. exp. Path., 40, 507.

ROWLEY, D.A. and FITCH, F.W. (1961). J. exp. Med., 120, 987.

SABIN, A.B. (1952). Proc. nat. Acad. Sci. (Wash.) 38, 540.

SAITO, K., NAKANO, M., AKIYAMA, T. and USHIBA, D., (1962). J. Bact., 84, 500.

SAITO, K. AKIYAMA, T., NAKANO, M. and USHIBA, D. (1960) Jap. J. Microbiol., 4, 395.

SALT, G. (1956). Proc. roy. Soc. B., 146, 93.

SANTOS, G.W. and OWENS, A.H. (1964). Johns Hopkins Med. J. 114, 384.

SCHAEDLER, R.W. and DUBOS, R.J. (1956). J. exp. Med., 104, 67.

SCHELL, K. (1960a). Aust. J. exp. Biol. med. Sci. 38, 271.

SCHELL, K. (1960b). ibid. p. 289.

SCHNITZER, R.J. (1943). J. Immunol. 46, 143.

SCHOTT, R.G. (1932). Genetics 17, 203.

SCHWARTZ, R., EISNER, A. and DAMESHEK, W. (1959). J. clin. Invest., 38, 1394.

SEGRE, D. and KAEBERLE, M.L. (1962). J. Immunol., 89, 782.

SHELLAM, G.R. and NOSSAL, G.J.V. (1968). Immunology, 14, 273.

SHILO, M. (1959). Ann. Rev. Microbiol. 13, 255.

SILVERS, W.K. and BILLINGHAM, R.E. (1958). Cited in "Methodology in Mammalian Genetics" ed. William J. Burdette. p. 109.

SKARNES, R.C. and WATSON, D.W. (1957). Bact. Rev., 21, 273.

SOBEY, W.R., MAGRATH, J.M. and REISNER, A.H. (1966). Immunology, 11, 511.

SOKHEY, S.S. and CHITRE R.B.G.D. (1937). Bull off. int. Hyg. publ., 29, 2093.

SPRINGER, G.F., HORTON, R.E. and FORBES, M. (1959). Ann. N.Y. Acad. Sci., 78, 272.

STAUB, A.M. (1965). In "Methods in Carbohydrate Chemistry" 5, Ed. Roy L. Whistler. Acad. Press N.Y. and London, p. 94.

STERN, K. and DAVIDSOHN, I. (1954). J. Immunol., 72, 209.

SUTER, E. (1953). J. exp. Med., 97, 235.

SUTER E. (1956). Bact. Rev., 20, 94.

SVEHAG, S.E. (1964). J. exp. Med., 119, 517.

TALIAFERRO W.H. and TALIAFERRO L.G. (1951). J. Immunol., 66, 181.

THOMAS, E.D., BAKER J.A. and FERREBEE, J.W. (1963). J. Immunol., 90, 324.

TOPLEY, W.W.C. and WILSON, G.S. (1964a). "Principles of Bacteriology and Immunity". 5th Ed., G.S. Wilson and A.A. Miles. Edward Arnold (Publishers). Ltd., London 1, p.556.

TOPLEY, W.W.C. and WILSON, G.C. (1964b). ibid, 1, 889.

TOPLEY, W.W.C. and WILSON, G.S. (1964c). ibid, 2, 1496.

TOPLEY, W.W.C. and WILSON, G.S. (1964d). ibid 2, 1508.

TOPLEY, W.W.C. and WILSON, G.S. (1964e). ibid, 2, 1723.

TRENTIN, J.J. (1956). Proc. Soc. exp. Biol. (N.Y.) 92, 688.

TRENTIN, J.J. (1957). Proc. Soc. exp. Biol., (N.Y.) 96, 139.

TCHISTOVITCH, N. (1890). Ann. Inst. Pasteur, 4, 285.

TURNER, K.J., JENKIN, C.R. and ROWLEY, D. (1964). Aust. J. exp. Biol. med., Sci., 42, 229.

TURNER K.J. and ROWLEY, D. (1963). Aust. J. exp. Biol. med. Sci. 41, 595.

UHR, J.W. and BAUMANN, J.B. (1961). J. exp. Med., 113, 935.

UHR, J.W. and WEISSMAN C. (1965). J. Immunol. 94, 544.

USHIBA, D., SAITO, K., AKIYAMA, T., NAKANO, M., SUGIYAMA, T. and SHIRONO, S. (1959). Jap. J. Microbiol., 3, 231.

USHIBA, D., SAITO, K., AKIYAMA, T. and SUGIYAMA T. (1959). 11th Symposium, Kanto Branch Meeting of the Japan Microbiological Association, Tokyo, p.81.

UPHOFF, D.E. (1957). J. Nat. Cancer Inst. 19, 123.

UPHOFF, D.E. (1958). Proc. Soc. exp. Biol. (N.Y.) 99, 651.

UPHOFF, D.E. (1961). Transplant. Bull., 28, 12.

WASHBURN, T.C., MEDEARIS D.N. and CHILDS, B (1965). Pediatrics, 35, 57.

WEBSTER, L.T. (1937). J. exp. Med., 65, 261.

WEIGLE, W.O. (1961). Advanc. Immunol., 1, 283.

WELLER, T.H. ENDERS J.F., ROBBINS, F.C. and STODDARD, M.B., (1952). J. Immunol., 69, 645.

WELLMAN, G., LIEBKE, H., and ENGEL H. (1962). cited by Topley, W.W.C. and Wilson, G.S. (1964). "Principles of Bacteriology and Immunity" Vol. II 5th ed. Wilson G.S. and Miles A.A., Edward Arnold (publishers) Ltd., p. 1338.

WESTPHAL, O., LUDERITZ, O. and BISTER, F. (1952). Z. Naturf, 73, 148.

WHITBY, J.L. and ROWLEY, D. (1959). Brit. J. exp. Path. 40, 358.

WRIGHT, A.E. and DOUGLAS, S.R. (1903). Proc. roy. Soc. B., 72, 357.

WU, W.G. and MARCUS, S. (1964). J. Immunol., 92, 397.

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ACKERMAN, V.P. (1964). Ph.D. Thesis.

BEERNINK, K.D. and STEWARD, J.P. (1967). Immunol., 12, 207.

BEERNINK, K.D. and STEWARD, J.P. (1968). Immunol., 15, 319.

COLLINS, F.M. (1968). J. Bacteriol., 95, 1343.

COLLINS, F.M. (1969a). J. Bacteriol., 97, 667.

COLLINS, F.M. (1969b). J. Bacteriol., 97, 676.

COLLINS, F.M. and MACKANESS, G.B. (1968). J. Immunol., 101, 830.

COLLINS, F.M. and MACKANESS, G.B. (1970a). Cell. Immunol., 1, 253.

COLLINS, F.M. and MACKANESS, G.B. (1970b). Cell. Immunol., 1, 266.

COPPEL, S. and YOUMANS, G.P. (1969). J. Bacteriol., 97, 127.

EISENSTEIN, T.K., WINSTON, S.H. and BERRY, L.J. (1968). Bact. Proc., p.87.

HOBSON, D. (1957). J. Hyg. (Camb)., 55, 334.

KENNY, K. and HERZBERG, M. (1968). J. Bacteriol., 95, 406.

LUBAROFF, D.M. and WAKSMAN, B.H. (1968). J. exp. Med., 128, 1425.

MACKANESS, G.B. (1968). Amer. Rev. Resp. Dis., 97, 337.

MACKANESS, G.B. (1969). J. exp. Med., 129, 973.

MACKANESS, G.B. (1971a). Ann. Inst. Pasteur, 120, 428.

MACKANESS, G.B. (1971b). J. Inf. Dis., 123, 439.

MACKANESS, G.B. and BLANDEN, R.V. (1967). Prog. Allergy, 11, 89.

MITSUHASHI, S., KAWAKAMI, M., YAMAGUCHI, Y. and NAGAI, N. (1958). Jap. J. exp. Med., 124, 573.

RAFFEL, S. (1948). J. Inf. Dis., 82, 267.

RAFFEL, S. (1950). Experientia, 6, 410.

READE, P.C. and JENKIN, C.R. (1964). Proceedings ivth. Int. Symp. R.E.S., p.288.

TRIPATHY, S.P. and MACKANESS, G.B. (1969). J. exp. Med., 130, 17.

VENNEMAN, M.R. and BERRY, L.J. (1971). J. Res. Soc., 9, 491.

VENNEMAN, M.R., BIGLEY, N.I., KLUN, C.L., ZACHARY, A. and DODD, M.C. (1968). Fed. Proc., p.429.

YOUMANS, A.S. and YOUMANS, G.P. (1965). J. Bact., 89, 1291.

YOUMANS, A.S. and YOUMANS, G.P. (1966a). J. Bact., 91, 2139.

YOUMANS, A.S. and YOUMANS, G.P. (1966b). J. Bact., 91, 2145.

YOUMANS, G.P. and YOUMANS, A.S. (1969). J. Bact., 97, 134.

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