TO MY FAMILY, MUM AND DAD:

FOR THEIR LOVE, UNDERSTANDING AND SUPPORT

FACTORS AFFECTING THE IN VITRO ANTI-BACTERIAL ACTION OF LEUCOCYTES

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A thesis submitted for the degree
of Doctor of Philosophy

April, 1985

Awarded Degree of Master of Science

5 May, 1986.

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SUMMARY

Comparative studies were undertaken on the *in vitro* killing of salmonellae by macrophages and neutrophils harvested from normal and Salmonella-immune mice.

The ability of normal macrophages and neutrophils to kill preopsonized salmonellae *in vitro* for maximum efficiency required the presence of factor(s) in the fresh serum. Evidence is presented that components of complement system were more important factors than immunoglobulins, acting presumably via it's receptors on the phagocytic cell surface. There was no such requirement for expression of bactericidal activity by activated macrophages and neutrophils, however, with neutrophils complement increased the rate of killing.

The relative roles of Fc and C3b receptors in the bactericidal activity of macrophages and neutrophils were also investigated. In relation to the number of both receptors, activation of macrophages resulted in increases of both bacterial killing and Fc but not C3b receptors. On the other hand, activation of neutrophils resulted in increases of both bacterial killing rates and of numbers of C3b receptors. It was concluded that bactericidal activity of both activated macrophages and neutrophils is not correlated with the numbers of C3b receptors.

Activation of macrophages and neutrophils *in vitro* for enhanced bactericidal activity was attempted. Incubation of normal macrophages and neutrophils with lymphokine-containing supernatants led to enhanced bactericidal activity against salmonellae. Supernatants derived from concanavalin A (Con A)-stimulated spleen cell cultures were also shown to affect other macrophage activities such as spreading, plasminogen activator release and non-specific esterase activity.

With regard to the current concept of macrophage heterogeneity, these studies further confirmed that not all cells in macrophage populations express effective bactericidal functions. Two subsets of macrophages with markers recognized by two monoclonals designated as M43 and M57 were shown to be especially involved in bactericidal function. There were no signs of subsets in neutrophil populations when tested by the same monoclonals. It was also shown that these two subset markers were expressed more on the surface of activated macrophages than on normal cells. The activation of macrophages resulted in increases of both bactericidal capacity and in the amount of these two markers on the macrophage subsets. This thesis, then, is particularly concerned with both the phenotypic and genetic changes in phagocytic cells which can influence their ability to kill opsonized bacteria.

STATEMENT

The material in this thesis has not been previously submitted 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.

Mustaffa Musa

ACKNOWLEDGEMENTS

I avail myself of this opportunity to record my deep and sincere gratitude to my supervisor, Professor Derrick Rowley, whose encouraging guidance and supervision has been a constant source of inspiration not only in the conducting of these studies but in building up my entire academic career. I am also thankful to Dr Bruce Reynolds for his active supervision in the early part of my work before his retirement.

I acknowledge Dr Charles Jenkin's help in English consultations. He deserves my sincere thanks for going through the first draft of my thesis.

I am also grateful to all the members of the department for their assistance, particularly Dr P. Ey for antibody purification. Special thanks to Mr John Mackrill for his help in preparing illustrations for this thesis, Mr G. Penny and Mr T. Richardson for their technical assistance.

Thanks also to Dr N.A. Locket of the Anatomy and Histology Department of this university for his consultations in Scanning Electronmicroscopy studies, Dr S. Neoh of the Clinical Immunology Department, Flinders Medical Centre for the donation of C6-deficient rabbit and

C4-deficient guinea-pig sera and Dr Lohmann-Matthes of the Max-Planck-Institut für Immunbiologie, FRG for the gift of some rat anti-mouse monoclonal antibodies.

I would like to thank Mrs Mercia Fuss for her quite efficient and dutiful typing of this thesis.

I am grateful to the Universiti Sains Malaysia, Penang for the financial support under Academic Staff under Training Scheme.

Finally, I would like to reflect my appreciation of the quiet contribution to the successful development of these studies from my loving family by allowing me to make use of the time which was conventionally theirs.

CHAPTER 1

1. INTRODUCTION

1.1 ACTIVATION OF MACROPHAGES AND NEUTROPHILS IN TERMS OF THEIR ENHANCED MICROBICIDAL ACTIVITY

1.1.1 Introduction to macrophage activation

Defence mechanisms which enable the host to combat bacterial pathogens vary. One of the most important of these is the ingestion and killing of such parasites by phagocytic cells. Metchnikoff (1905) first recognized the significance of these cells in the removal and killing of a variety of potential pathogens and stressed their importance in host defence, a view which was by no means widely accepted. However, phagocytes of the monocyte series, either fixed such as those in the liver or circulating, together with polymorphonuclear leukocytes (PMN) or neutrophils, are now recognized as playing an important protective role in host resistance to infection.

Macrophages obtained from animals which have recovered from infection with certain intracellular parasites have not only enhanced microbicidal properties, when compared with those from normal animals, but also increased rate of metabolism and levels of certain enzymes (Blanden, 1968; Dannenberg, 1968). The term 'activated macrophage' was introduced by Mackaness during the 1960s to describe such cells harvested from animals recovered from infection by such parasites. Mackaness and

colleagues, in a series of studies both *in vivo* and *in vitro* with intracellular parasites such as *Listeria monocytogenes*, *Brucella abortus* and *Salmonella typhimurium* demonstrated that the resistance of mice to these infections depended on the increased bactericidal activity of their macrophages and that antibody played an apparently minor role in this immunity (Mackaness, 1962, 1964; Khoo and Mackaness, 1964; Blanden, Mackaness and Collins, 1966; Mackaness, 1971). These studies supported some of the earlier findings of Lurie (1942) who studied the development of resistance to *Mycobacterium tuberculosis* in rabbits. He showed that lymph node macrophages from normal rabbits were unable to restrict the intracellular growth of the phagocytosed bacteria, whereas macrophages from Bacille Calmette-Guérin (BCG) vaccinated animals were able to do so. Similar *in vitro* studies, later by Suter (1953) and Berthrong and Hamilton (1959), supported these earlier findings.

Further investigations by other workers demonstrated that the increased bactericidal activity of activated macrophages was nonspecific in so far as these cells could kill bacteria antigenically unrelated to those which had induced the activation (Mackaness, 1962; Blanden *et al.*, 1966; Mackaness and Blanden, 1967; Ruskin and Remington, 1968). It was also found that the duration of this enhanced microbicidal activity was short-lived but could be regenerated rapidly if the animal were injected with the bacteria used originally to induce the 'activated' state (Mackaness, 1964). The fact that other cells were

involved in the production of activated macrophages, rather than the direct action of bacterial antigen(s) on the phagocytes, emerged from the studies of Mackaness (1969) and Lefford and McGregor (1974). They found that resistance to intracellular bacterial parasites could be effectively transferred to normal recipients by viable lymphoid cells from immune animals. Further experiments established that the lymphoid cells responsible for this phenomenon were T-cells (North, 1973; Lane and Unanue, 1972).

Later, Simon and Sheagren (1972) demonstrated that activation of macrophages could take place in vitro. In their study using a guinea-pig model, they found that incubation of normal macrophages, together with lymphocytes which had been obtained from the peritoneal cavity or lymph nodes of BCG-immunized animals in the presence of BCG, caused the macrophages to acquire a markedly enhanced listericidal activity. It had been discovered previously that when lymphocytes sensitized to specific antigens came in contact with these antigens, a soluble factor(s) was secreted into the culture medium which was able to inhibit the migration of macrophages. This factor became known as the migration inhibitory factor (MIF) (Bloom and Bennett, 1966; David, 1966). Armed with this knowledge, Fowles and co-workers (1973) made an attempt to activate normal guinea-pig peritoneal macrophages in vitro with these soluble factors. They demonstrated that normal macrophages exhibited enhanced listericidal activity following their incubation with supernatants derived from concanavalin A-

(Con A) stimulated lymph node cultures from guinea-pigs. These studies and many others (e.g., Patterson and Youmans, 1970; Krahenbuhl and Remington, 1971; Godal, Rees and Lamvik, 1971; Cole, 1975; David and Remold, 1976; Nacy and Meltzer, 1979) established beyond doubt that macrophages were activated by interacting with soluble factors released from lymphocytes under certain conditions. These soluble factors are now referred to as lymphokines, a term introduced by Dumonde and colleagues (1969). Several investigations have indicated already that the state of macrophage activation induced in vitro by lymphokines is analogous to that activation generated in vivo in actively sensitized animals (Nathan, Karnovsky and David, 1971; Hammond and Dvorak, 1972; Hammond, Selvaggio and Dvorak, 1975). Indeed, following activation with lymphokines in vitro, macrophages may destroy a variety of pathogenic micro-organisms including bacteria, fungi, protozoa and viruses. The release of these lymphokines from lymphocytes does not necessarily require the interaction of specifically sensitized lymphocytes with the sensitizing antigen but they may be generated by the interaction of lymphocytes with mitogen (Nogueira and Cohn, 1978; Buchmüller and Mauel, 1979; Maiti, Kumar and Mohapatra, 1980; Bout, Joseph, David and Capron, 1981).

It was also apparent that the state of macrophage activation induced in vitro was, as in vivo, short-lived and could only be maintained in vitro by replenishment of the lymphokine (North and Deissler, 1975). It is also interesting to note that the production of lymphokines

from Con A-stimulated lymphocytes appeared to be independent of blastogenesis (Gardner and Remington, 1978; Buchmüller and Mauel, 1979).

1.1.2 Lymphokines which alter the microbicidal capacity of macrophages (MAF)

The lymphokine which activated macrophages was initially termed macrophage activating factor (MAF) (Hibbs, Taintor, Chapman and Weinberg, 1977). It was shown to affect macrophages by means of increasing their adherence to culture dishes and by promoting their rapid spreading (Mooney and Waksman, 1970). Later, this lymphokine was also found to affect other macrophage activities such as increasing their phagocytic activity and glucose oxidation (Nathan et al., 1971). Apart from its effect on macrophage microbicidal activity (reviewed by David and Remold, 1976), this lymphokine can also augment the anti-tumour activity of these cells (Fidler, 1975; Churchill, Piessens, Sulis and David, 1975; Ruco and Meltzer, 1977, 1978).

MAF has been shown to exist in the supernatants obtained from various presensitized lymphoid populations such as, spleen cells, peritoneal exudate cells (PEC) and cortisone-resistant thymocytes, responding in vitro to the sensitizing antigen such as purified protein derivative (PPD) (Ruco and Meltzer, 1977; Weinberg and Hibbs Jr, 1979; Farr, Wechter, Kiely and Unanue, 1979). With recent advances in the production and purification of lymphokines, Kelso and MacDonald (1982) reported that in the

murine system, MAF was secreted by Lyt-2^- and Lyt-2^+ (cytolytic T lymphocyte) precursor cells. However, the greater amount of MAF was secreted by the Lyt-2^- population.

Murine MAF has now been characterized as two factors differing in molecular weight but similar with respect to their isoelectric points and instability under various denaturing conditions (Yamamoto, Leonard and Meltzer, 1983). Meltzer and colleagues, using lymphokines derived from a continuous murine T-cell line (EL-4), isolated two 'activating' factors differing in size (Meltzer, Benjamin and Farrar, 1982; Nacy et al., 1983). One factor (50,000 MW) was indistinguishable by biochemical and antigenic criteria from gamma interferon (IFN-γ) and activated macrophages such that they killed certain intracellular parasites and lysed tumour target cells. The other (23,000 MW) was distinct from IFN-γ and induced macrophages to lyse only tumour target cells. The results of a number of other investigations support the finding that IFN-γ is capable of activating macrophages (Kelso, Glasebrook, Kanagawa and Brunner, 1982; Kleinschmidt and Schultz, 1982; Roberts and Vasil, 1982; Schreiber et al., 1983; Nathan, Murray, Wiebe and Rubin, 1983; Pace et al., 1983).

1.1.3 The process of macrophage activation by MAF

To date, the exact mechanism(s) of macrophage activation by MAF is not known. However, since it is known that macrophages have surface receptors for MAF, one can postulate that the first step in the process of macrophage

activation is probably the binding of MAF to these receptors (Dy, Dimitriu, Gougerot and Hamburger, 1976; Poste, Kirsh, Fogler and Fidler, 1979a). In studies by the above investigators, it was found that treatment with trypsin and pronase abolished the ability of macrophages to respond to MAF, suggesting that these receptors are susceptible to proteolytic enzymes. It has been proposed that the receptor is a glycoprotein containing fucose. This was based on the fact that the action of MAF was inhibited by α-L-fucose or pre-treatment of the macrophage with α-1-fucosidase (Churchill and Wong, 1980; Poste et al., 1979a). However, other evidences indicate that glycolipids may also be the natural receptor for MAF (Higgins, Sabatino, Remold and David, 1978; Liu et al., 1978; Poste, Kirsh and Fidler, 1979b; Poste, Allen and Matta, 1979c).

Recent studies by Fidler and colleagues have indicated that, although MAF bound to receptors on the cell surface, activation was a result from it's action on intracellular sites (Poste et al., 1979a; Fidler et. al., 1981; Raz et. al., 1981). This was based on the fact that inactivation and/or removal of surface MAF receptors did not impair the ability of macrophages to respond to liposome-encapsulated MAF and become activated as measured by their increased tumoricidal properties. In their studies, mouse macrophages were incubated with identical doses (in terms of nanomoles/phospholipid) of liposomes of differing size which contained differing volumes of encapsulated MAF. ^{125}I-BSA was used as a surrogate marker for MAF. The amount of radioactivity associated with the

liposome-treated macrophages was shown to be due exclusively to internalized liposomes, thus providing an accurate estimation of the total volume of liposomal contents delivered intracellularly. Based on these results, they were able to calculate that internalization of 0.063µl of a MAF preparation per 10^5 macrophages was required for the development of maximum tumoricidal activity.

With regard to the time required for MAF to activate macrophages, conflicting data have been reported. This may depend on the manner in which the macrophages were elicited. For example, an 18hr incubation with MAF was required to induce maximum cytotoxic activity against tumour target cells of thioglycollate-stimulated macrophages (Fidler, Darnell and Budmen, 1976) whereas calf-serum-stimulated macrophages required only a 4hr incubation (Ruco and Meltzer, 1977, 1978). Further support for this came from a study by Nogueira and Cohn (1978), where they showed that normal macrophages required 24h longer for activation by lymphokine derived from con A-stimulated spleen cell cultures than did proteose-peptone-induced macrophages, as measured by their trypanocidal activity against Trypanosoma cruzi.

1.1.4 Activation of granulocytes

The activation of granulocytes by lymphocyte-derived soluble factors in terms of an increased microbicidal activity has not been studied as extensively as has the role of such factors in the activation of macrophages in terms of this parameter. Vadas and co-workers (1983)

showed that a supernatant containing colony-stimulating factors (CSF), prepared from human placental conditioned medium (HPCM), enhanced the antibody-dependent cell-mediated cytotoxicity of human neutrophils against P815, EL-4 and BW target cells.

More recently, in other studies using Blastomyces dermatitidis, Brummer and Stevens (1984) have reported that incubation of murine neutrophils with culture supernatants from spleen cells obtained from B. dermatitidis-immunized mice stimulated with B. dermatitidis antigens, enhanced the fungicidal activity of the phagocytes. Activation of neutrophils by the supernatants in terms of their fungicidal activity against B. dermatitidis required only a short incubation period (1h). It was also found in these studies that stimulation of normal spleen cells with Con A, resulted in the production of supernatants capable of activating neutrophils for enhanced fungicidal activity against B. dermatitidis.

Thus, these findings and others (Lomnitzer, Glover and Rabson, 1977; Cross and Lowell, 1978; Inoue and Sendo, 1983) support the concept of neutrophil 'activation'.

1.1.5 Functional heterogeneity of macrophages

At the site of an infection, large numbers of macrophages may be undergoing immunologically-mediated activation. The possibility may exist, however, that the activation is not a process which uniformly involves an entire cell population, but rather that certain cells

might undergo the activation process while others remain in a normal state, resulting in a mixed population of macrophages with diversity of functions. Moreover, functional heterogeneity may be found in sub-populations of macrophages residing in a particular tissue site and carrying out certain specialized functions. In support of this, studies on mechanisms of phagocytosis have shown already that the expression of various surface receptors is not found uniformly on all macrophages obtained from guinea-pig or rabbit (Rhodes, 1975; Walker, 1976; Silverstein and Loike, 1980). Other studies have established also that rabbit peritoneal macrophages are heterogeneous in terms of their antigen-binding capacity, and number of Fc receptors (Zembala and Asherson, 1970; Walker, 1974; Rice and Fishman, 1974; Serio, Gandour and Walker, 1979). Kavai and colleagues (1979) showed that only one fraction of the macrophages separated on a Ficoll gradient was able to fix significant amounts of monomeric radiolabelled IgG. However, it is possible that the functional heterogeneity of the macrophages found in their study was related to different stages of cellular maturation (van Furth, 1976). In 1967, McIntyre, Rowley and Jenkin demonstrated that in peritoneal cell populations derived from mice some, but not all, macrophages were able to kill opsonized bacteria which had been phagocytosed. More recently, the functional heterogeneity of macrophages has been reported also by other workers (O'Neill, Hoehn, Lesperance and Klass, 1984), who showed that normal rat alveolar macrophages, separated into four isopycnic fractions on Percoll

gradients, were functionally heterogeneous in superoxide anion release, lysosomal enzyme activity, phagocytosis and intracellular killing of Staphyloccus aureus, but not in hydrogen peroxide release.

Functional diversity of activated macrophages is also evident, with respect to their bactericidal or tumoricidal activities. Wing, Gardner, Ryning and Remington (1977) showed that these two activities could be separated. In their study, they found that macrophages from both Trichinella spiralis and Toxoplasma gondii-infected mice had the capacity to inhibit tumour cell DNA synthesis but these cells were unable to prevent the intracellular multiplication of Toxoplasma gondii. Recently, Harrington-Fowler and Wilder (1982) separated mouse peritoneal macrophages derived from Listeria-immune mice into distinct groups based on their buoyant densities on discontinuous gradients of Percoll and assayed for their ability to control an intracellular infection of L. monocytogenes. They found distinct subsets which were able to kill L. monocytogenes and to restrict intracellular growth of the bacteria whereas others did not.

Since macrophages differentiate during the process of activation, differentiation antigen(s) present on the surface of the macrophage may be a marker(s) for the stage of activation. With the use of monoclonal antibody, Kaplan and Mohanakumar (1977) have demonstrated a membrane antigen specifically associated with Corynebacterium parvum and pyran-activated macrophages. This antigen was

not expressed on glycogen or thioglycollate-elicited cells nor on normal macrophages. Studies by Sun and Lohmann-Matthes (1982) using four rat monoclonal antibodies raised against mouse macrophages showed that these antibodies reacted with membrane antigens of different subsets of macrophages. Interestingly, the subsets defined by some of these antibodies were found to have different functions. Two of the monoclonal antibodies designated as M57 and M43 eliminated macrophages that lysed antibody-coated tumour target cells from a mouse bone marrow macrophage population. However, macrophages which had been activated by lymphokine and were cytotoxic against lymphoma cells (5178Y) were eliminated only by M43.

1.2 INTRACELLULAR KILLING MECHANISMS OF MACROPHAGES AND NEUTROPHILS

1.2.1 Introduction

Macrophages as has been stated previously have been shown to be important effector cells in resistance to various bacterial infections, and different mechanisms whereby the cells kill the invading organisms have been proposed. In general, it is known that, when micro-organisms contact phagocytes such as neutrophils, eosinophils, monocytes and macrophages, phagocytosis of the micro-organisms takes place. The process of phagocytosis envelops the micro-organisms within intracellular vacuoles termed phagosomes. Within these vacuoles, following fusion with lysosomes, the micro-organisms are exposed to a variety of potentially antimicrobial agents and

degradative enzymes from which, depending on the parasite, few may survive. It has been suggested that the microbicidal mechanisms of macrophages are similar to those of the neutrophils (Nathan and Root, 1977; Johnston, 1978; Johnston, Godzik and Cohn, 1978; Johnston, Chadwick and Pabst, 1980; Sasada and Johnston, 1980).

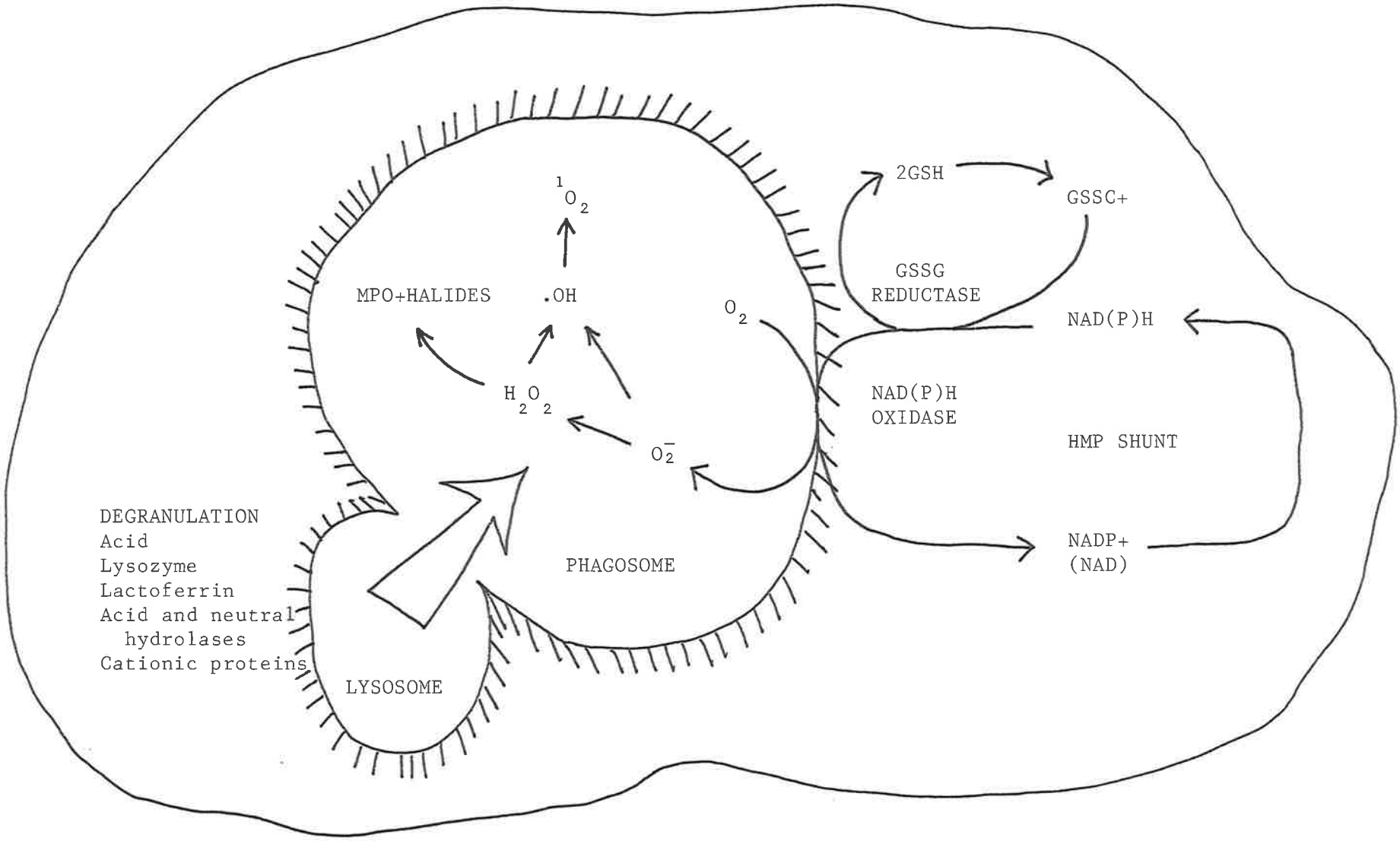
In general, during and following the ingestion of micro-organisms, metabolic and morphological changes take place in the phagocytic cell which play important roles in relation to the killing and degradation of the ingested material. These events include morphologically a degranulation response (Hirsch, 1962) and a burst of oxidative metabolism which involves the activation of a cytoplasmic NADH oxidase and stimulation of the glutathione cycle and the hexose monophosphate shunt (HMP) (Karnovsky, Simmons, Noseworthy and Glass, 1970). The intracellular killing mechanisms of phagocytes to various micro-organisms may be conveniently divided into two categories 1) oxygen-dependent and 2) oxygen-independent (Fig 1.1).

1.2.2 Oxygen-dependent killing mechanism

In response to phagocytosis or plasma membrane perturbation, the phagocyte undergoes a series of oxidative metabolic activities (Babior, 1978; Johnston, 1978; Klebanoff and Clark, 1978). This phenomenon known as the respiratory burst involves the consumption of oxygen which is converted to a number of highly reactive products such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radicals (.OH), and singlet oxygen ($^1O_2$). These

Figure 1.1

Possible intracellular killing mechanisms of macrophages and neutrophils.


$^1O_2$
MPO+HALIDES
.OH
O_2
H_2O_2
O_2^-
2GSH
GSSC+
GSSG
REDUCTASE
NAD(P)H
NAD(P)H
OXIDASE
HMP SHUNT
NADP+
(NAD)
DEGRANULATION
Acid
Lysozyme
Lactoferrin
Acid and neutral
hydrolases
Cationic proteins
LYSOSOME
PHAGOSOME

reactive oxygen metabolites have been implicated in the bactericidal activity of neutrophils and probably of monocytes as well (Johnston et al., 1973; Klebanoff, 1975; Sagone, King and Metz, 1976; Babior, 1978; Root and Cohen, 1981). In the case of macrophages recent studies (Nathan and Root, 1977; Johnston, 1978; Johnston, et al., 1978, 1980; Nathan et al., 1979a,b,c) have suggested that these cells also generate and release reactive oxygen products as a result of phagocytosis.

During phagocytosis, one of the events which occurs associated with the respiratory burst of the phagocytic cells is known as chemiluminescence first noted by Allen and colleagues (1972) using human granulocytes. It has also been shown that macrophages from various species produce chemiluminescence during phagocytosis although the signal is weaker than that from neutrophils (Nelson, Mills, Simmons and Quie, 1976; Hatch, Gardner and Menzel, 1978; MacGowan, Peterson, Keane and Quie, 1983). A possible candidate for this phenomenon was originally thought to be $^1O_2$. However, the initiator of the event appeared to be O_2^- since superoxide dismutase, which effectively removes O_2^-, virtually abolished the luminescence response by peritoneal and alveolar macrophages (reviewed in Hatch et al., 1978).

The contribution of reactive oxygen intermediates to the antimicrobial activity of macrophages has been well documented by Nathan and co-workers (1979a). Using trypomastigotes of Trypanosoma cruzi, they demonstrated

that there was a close parallel between the ability of macrophages to release H_2O_2 in response to phorbol myristate acetate (PMA) and their ability to kill intracellular trypomastigotes of T. cruzi. Sasada and Johnston (1980) in a study using Candida albicans and Candida parapsilosis showed that efficient macrophage candidacidal activity depended on the generation of reactive oxygen metabolites by these cells. Their results indicated that C. parapsilosis, the species that was killed more easily by macrophages than was C. albicans, stimulated oxygen consumption and O_2^- release by macrophages more vigorously than did C. albicans.

In another study using Toxoplasma gondii, Murray and colleagues (1979) compared normal macrophages, macrophages from chronically infected mice, and those from immune mice in their ability to kill the parasites and to generate oxygen metabolites, measured as the release of H_2O_2, when stimulated by PMA. Macrophages from normal mice released little H_2O_2 and were unable to restrict the intracellular growth of ingested parasites. Macrophages from chronically infected mice released slightly more H_2O_2 and inhibited the growth of the ingested parasites. However, macrophages from immune mice boosted by the injection of heat-killed Toxoplasma released 25 times more H_2O_2 and rapidly killed most of the ingested parasites.

Evidence for the role of reactive oxygen metabolites in the bactericidal activity of macrophages has also been provided by other workers (Johnston et al., 1975; Sagone,

King and Metz, 1976). They showed that various scavengers such as superoxide dismutase which removes O_2^-, catalase which removes H_2O_2, and benzoate which removes .OH, inhibited the killing of some bacteria such as Escherichia coli and S. aureus by human neutrophils (Johnston et al., 1975). The inhibition of the bactericidal activity by these scavengers implied the possible involvement of H_2O_2, .OH and O_2^- (probably through the formation of .OH). More recently, Repine and co-workers (1984) produced evidence which supported the involvement of .OH in the bactericidal activity of neutrophils. They examined the effect of .OH scavengers on the killing of S. aureus 502A by human neutrophils as well as the chemiluminescent responses of these cells in vitro during phagocytosis. Their results showed that .OH scavengers decreased the chemiluminescent response of neutrophils and that a close relationship existed between the .OH-dependent decreases in neutrophil chemiluminescence and their bactericidal activity. The release of .OH during the process of phagocytosis has been shown by several other investigations (Tauber and Babior, 1977; Weiss, King and Lo Buglio, 1977; Weiss, Rustagi and Lo Buglio, 1978), and the involvement of $^1O_2$ in the microbicidal processes of phagocytes indicated by other studies (Allen, Stjernholm and Steele, 1972; Krinsky, 1974; Webb, Keele and Johnston, 1974; Johnston et al., 1975).

Finally, H_2O_2, together with myeloperoxidase, and halide ions such as iodine constitute a potent microbicidal mechanism in neutrophils (Sbarra, et al., 1972; Klebanoff, 1975). However, the existence of this mechanism

in macrophages, at least mouse peritoneal and rabbit alveolar macrophages, is uncertain (Simmons and Karnovsky, 1973; Biggar, Buron and Holmes, 1976).

1.2.3 Oxygen-independent killing mechanism

Evidence that the antimicrobial killing mechanisms of macrophages, neutrophils and other phagocytes may involve oxygen-independent mechanisms is based on the finding that a number of bacteria can be effectivly killed by phagocytic cells in the absence of oxygen (Mandell, 1974). The oxygen-independent killing agents in phagocytes include the fall in pH in the phagosome, and a number of potentially antimicrobial agents. Following phagocytosis, these toxic agents are discharged from the granules either into the phagosome or extracellularly. The contents of these granules include a variety of acid hydrolases, lysozyme neutral proteinase, lactoferrin and several cationic proteins (Hirsch, 1960, 1962; Henson, 1971; Spitznagel, 1980). There are a number of reports on the antimicrobial properties of toxic proteins released from neutrophil granules. These studies (Zeya and Spitznagel, 1963; Odeberg and Olsson, 1975, 1976; Modrzakowski and Spitznagel, 1979; Modrzakowski, Goodrum and Spitznagel, 1981) established the role of lysosomal cationic proteins from human neutrophils in the bactericidal activity of these cells. Recently, Shafer, Martin and Spitznagel (1984) have isolated cationic proteins from human neutrophils and shown that these proteins exhibited antibacterial activity *in vitro* against *S. typhimurium*. Lysosomal cationic

proteins have been purified also from bovine neutrophils (Gennaro, Dolzani and Romeo, 1983), rat neutrophils (Hodinka and Modrzakowski, 1983) and rabbit granulocytes (Selsted, Szklarek and Lehrer, 1984). These highly cationic peptides of low molecular weight rich in arginine and cysteine have been shown to be toxic against a variety of fungi such as C. albicans and several species of gram-positive and gram-negative bacteria (Lehrer, Ladra and Hake, 1975; Patterson-Delafield, Martinez and Lehrer, 1980; Lehrer, Selsted, Szklarek and Fleischmann, 1983).

With regard to the oxygen-independent killing mechanism of macrophages, their lysosomal granules have been shown to contain a number of acid hydrolases (Davis, Page and Allison, 1974; Schnyder and Baggiolini, 1978). Basically, there is general agreement that the hydrolases of macrophage lysosomes are toxic too and digest certain micro-organisms. Hart (1981) has provided evidence for the importance of the contents of macrophage lysosomes to the antimicrobial activity of these cells. He showed that the inhibition of phagosome-lysosome fusion within normal mouse peritoneal macrophages by ammonium chloride reduced the rate of killing of ingested Saccharomyces cerevisiae. He suggested also that the contents of the lysosome may contribute to or enhance the oxygen-dependent killing mechanisms of macrophages by providing an acid environment. In some instances, if the phagosomes of macrophages acquire peroxidase by the ingestion of neutrophils, the enzyme together with halide and H_2O_2, may cause membrane lipid peroxidation of ingested micro-organisms (Klebanoff and Hamon, 1975; Klebanoff, 1980).

In addition to acid hydrolases, other enzymes in the cytoplasmic granules such as arginase, collagenase, neutral serine protease and elastase have been suggested to play a role in the killing mechanisms of both neutrophils and macrophages (Henson, 1971; Gordon, Unkeless and Cohn, 1974; Werb and Gordon, 1975a,b; Adams, 1980; Olds, Ellner, Kearse, Kazura and Mahmoud, 1980). Finally, the accumulation of lactic acid in the phagolysosome may account, at least in part, for the low pH. This condition is undoubtedly unfavourable and lethal for many micro-organisms (Dubos, 1953).

1.2.4 Mechanisms by which micro-organisms escape the intracellular killing mechanisms of macrophages

As mentioned earlier, reactive oxygen metabolites in conjunction with the toxic contents of lysosomal granules are themselves potentially lethal. Therefore, organisms that survive within phagocytes must have mechanisms which enable them to avoid these potentially lethal systems. One such mechanism is the ability to escape from the phagocytic vacuole. Nogueria and Cohn (1976) have found that the trypomastigotes of *T. cruzi* were able to escape from the phagocytic vacuole of normal mouse peritoneal macrophages and multiply in the cytoplasmic matrix. *Mycobacterium leprae* has also been shown to have a similar survival mechanism (Levy, Herman, Evans and Krahenbuhl, 1975). A further mechanism involves the ability of some virulent micro-organisms to inhibit the fusion of lysosomes with the phagosome and thereby are able to multiply in the vacuole. Such micro-organisms include *Toxoplasma gondii*,

Mycobacterium tuberculosis and Chlamydia psittaci (Armstrong and Hart, 1971; Friis, 1972; Jones and Hirsch, 1972). Other virulent organisms such as Listeria monocytogenes, Mycobacterium lepraemurium, Leishmania mexicana and Salmonella typhimurium are able to resist digestion by the lysosomal enzymes and are thus capable of surviving within the phagolysosomes of normal mouse macrophages (North and Mackaness, 1963; Hart et al., 1972; Alexander and Vickerman, 1975; Carrol, Jackett, Aber and Lowrie, 1979).

Another possible mechanism for the survival of intracellular pathogens is their failure to trigger the oxidative metabolism of normal macrophages. Wilson, Tsai and Remington (1980) have shown that normal mouse peritoneal macrophages, which display a respiratory burst in response to many phagocytic stimuli, failed to do so during ingestion of Toxoplasma gondii which were not antibody-coated, thus ensuring their survival. They also suggested that the membrane structure of the organism may be the cause for the failure to initiate the oxidative metabolism since antibody-coated Toxoplasma were not only phagocytosed but triggered the oxidative metabolic response of normal macrophages. Such a survival mechanism is not unique to Toxoplasma as it has been suggested that other pathogens such as Salmonella typhi and Brucella abortus survive in neutrophils by a similar mechanism (Miller, Garbus and Hornick, 1972; Kreutzer, Dreyfus and Robertson, 1979).

1.2.5 Effect of activation on the microbicidal activity of macrophages and neutrophils

Resistance to infection by intracellular bacterial parasites, such as Mycobacterium tuberculosis, Listeria monocytogenes and Salmonella typhimurium, appears to depend on the activation of macrophages by lymphocytes (Mackaness, 1970). Such macrophages, in comparison with normal macrophages, exhibit morphological and physiological changes, enhanced killing of many intracellular parasites plus other manifestations of a state of activation (Karnovsky, Lazdins, Drath and Harper, 1975; Cohn, 1978; Karnovsky and Lazdins, 1978; North, 1978). With respect to the killing mechanism of macrophages, Karnovsky and colleagues (1975) demonstrated that murine peritoneal macrophages elicited either by proteose-peptone or casein or obtained from mice infected with either Listeria monocytogenes or BCG showed increased oxygen consumption or HMP shunt activation during phagocytosis compared with resident peritoneal macrophages from normal mice. Other studies have shown also that BCG-activated macrophages which have increased antimicrobial activity generate increased amounts of reactive oxygen metabolites when triggered by zymosan or PMA (Nathan and Root, 1977; Johnston et al., 1978). Similar observations were made in another study using Toxoplasma gondii (Murray, Juangbhanich, Nathan and Cohn, 1979). From this latter study it was suggested that an increased secretion of H_2O_2 might be responsible for the enhanced microbicidal power of activated macrophages. These findings together with others

have implied that the enhanced bactericidal activity of macrophages upon activation could be attributed largely to their increased ability to secrete H_2O_2 (Klebanoff and Hamon, 1975; Thomas and Aune, 1978; Nathan et al., 1979a; Murray and Cohn, 1980). A similar conclusion has been reached in other systems involving monocytes (Nakagawara, De Santis, Nogueira and Nathan, 1982; Murray and Cartelli, 1983) and granulocytes (Nathan, Brukner, Silverstein and Cohn, 1979b; Nathan, Silverstein, Brukner and Cohn, 1979c).

Considering the possibility that other antimicrobial systems could be affected during cell activation, Johnston and colleagues (1978) measured the quantity of O_2^- released from mouse macrophages elicited by either lipopolysaccharide (LPS) or thioglycollate, or from those activated by BCG. They demonstrated that such cells consistently released a much greater amount of O_2^- than did normal resident cells in response to PMA or opsonized zymosan. Similar findings were reported by Berton and Gordon (1983) with the additional observation that macrophages activated by BCG differed from thioglycollate elicited macrophages in that they released O_2^- following their adherence and spreading on plastic, whereas the latter did not.

Increased secretion of O_2^- by BCG-induced rabbit alveolar macrophages during phagocytosis has also been reported (Stokes, Davis and Sorber, 1978). Recently, Cummings, Pabst and Johnston (1980) have found that injection of muramyl dipeptide (MDP) enhanced the killing of Candida albicans and also the secretion of O_2^- by mouse

macrophages. MDP has been shown *in vitro* to enhance certain mouse macrophage functions including their cytolytic activity against tumour cells, anti-microbial activity and O_2^- generation in response to PMA (Chedid, Audibert and Johnson, 1978; Humphres, Henika, Ferraresi and Krahenbuhl, 1980; Pabst and Johnston, 1980). Recently, Wilson, Bohnsack and Weaver (1982) reported that MDP treatment of human macrophages *in vitro* enhances their response to PMA as measured by the generation of O_2^-, whereas no such enhancement was obtained using opsonized zymosan. However, such enhancement did not correlate with the anti-microbial activity of these phagocytes against *Toxoplasma gondii* or *Staphylococcus aureus*. A similar conclusion was reached in another study using peritoneal macrophages from listeria-infected mice (Drath and Karnovsky, 1975).

With respect to other possible antimicrobial systems, the levels of lysosomal enzymes in macrophages are also affected by activation. Pantalone and Page (1977) found that activation of mouse peritoneal macrophages *in vitro* with supernatants from mitogen-stimulated human peripheral blood leukocytes led to a significant production and secretion of various lysosomal acid hydrolases. Similar results have been reported following the incubation of guinea-pig macrophages with a lymphokine supernatant derived from antigen-stimulated spleen cell cultures (Poulter and Turk, 1975). However, in contrast, Remold and Mednis (1975) demonstrated that guinea-pig peritoneal macrophages incubated for 72h with a supernatant rich in macrophage-inhibitory factor (MIF) led to a decrease in

the specific activity of lysosomal enzymes which was not due to the secretion of these enzymes into the culture medium.

Activation of macrophages *in vitro* with lymphokines have been shown to enhance phagosome-lysosome fusion and subsequent killing of *Coccidioides immitis* (Beaman, Benjamini and Pappagianis, 1983). These investigators noted also that such enhancement was observed only if the macrophages had been exposed to the lymphokines before, but not after infection with the fungus. It has also been reported that the concentration of microbicidal cationic peptides in rabbit alveolar macrophages increased when these cells were elicited by complete Freund's adjuvant (Lehrer, Szklarek, Selsted and Fleischmann, 1981). With respect to neutral proteinases, macrophage activation *in vitro* with lymphokines resulted in a marked increase in collagenase production compared to normal unstimulated control cells (Wahl, Wahl, Mergenhagen and Martin, 1975). Olds *et al*. (1980) have reported a fourfold increase in the amount of arginase released from macrophages from C57BL/6J mice activated by *Corynebacterium parvum* compared with normal control macrophages.

1.2.6 The requirement of membrane stimulation for the intracellular killing of micro-organisms by macrophages and neutrophils

Over the past several years, evidence has accumulated that the bactericidal activity of phagocytes such as neutrophils may be influenced by serum proteins (Li and Mudd, 1965; Solberg and Hellum, 1973; Christie, Solberg,

Larsen, Grov and Tønder, 1976; Solberg, Christie, Larsen and Tønder, 1976; Yamamura and Valdimarsson, 1977). It has been reported that maximal ingestion and intracellular killing of micro-organisms by phagocytic cells required both IgG and C3b (Griffin, Griffin, Leider and Silverstein, 1975; Griffin, Griffin and Silverstein, 1976; Horwitz and Silverstein, 1980). Recent studies by van Furth and colleagues have helped to clarify the role of serum factors in the bactericidal activity of granulocytes, monocytes and macrophages (Leijh et al., 1979a; Leijh, van Zwet and van Furth, 1980; Leijh, van den Barselaar, Dubbeldeman-Rempt and van Furth, 1980; Leijh, van den Barselaar, van Zwet, Daha and van Furth, 1980; Leijh, van den Barselaar, Daha and van Furth, 1981; Leijh, van Zwet and van Furth, 1984). Leijh and co-workers (1981) investigated the uptake and killing of Staphylococcus aureus and Escherichia coli by normal human granulocytes. The bacteria were preopsonized with fresh or heat-inactivated human AB serum prepared from the blood of healthy donors. These studies confirmed the earlier findings, in which bacteria preopsonized with fresh serum were ingested to a higher degree than bacteria preopsonized with heat-inactivated serum. Their previous results also showed that without opsonization, ingestion did not occur (Leijh et al., 1979b). In addition, they found that preopsonization of bacteria with fresh serum not only facilitated optimal ingestion but was necessary for optimal killing. It was further shown in these studies that the presence of serum in the medium was important in obtaining this optimal

killing. Similar results were also obtained from other studies using human monocytes (Leijh et al., 1979a; van Furth and Leijh, 1980). In these investigations, the intracellular killing of preopsonized Staphylococcus aureus by monocytes was measured independently of the ingestion of the bacteria. After phagocytosis, non-phagocytosed bacteria were killed by lysostaphin. Lysostaphin-treated monocytes containing ingested bacteria were then re-incubated in the presence of normal human serum either fresh or heat-inactivated or in its absence. When 10% fresh serum was used the decrease in the number of viable intracellular bacteria amounted to 79.3% after 60 min, whereas in the presence of heat-inactivated serum only 35.6 ± 11.2% of the ingested bacteria were killed. In the absence of serum little or no killing of the phagocytosed bacteria occurred (van Furth and Leijh, 1980). These observations suggested that heat-stable serum proteins may stimulate the intracellular killing of bacteria by monocytes as well as by granulocytes to a certain degree but heat-labile serum proteins were required for maximal effect. Since all studies were performed with granulocytes and monocytes obtained from normal healthy donors, similar requirements for the intracellular bactericidal activity of 'activated' cells remain to be explored.

The stimulatory activity of heat-inactivated serum was postulated to be due to the interaction of the Fc portion of immunoglobulin IgG with the Fc receptor on the granulocyte or monocyte membrane for the following reasons (Leijh et al., 1979a, 1981). Treatment of monocytes with

sphingomyelinase, which decreased the number of Fc receptors, and neuraminidase, which increased the numbers, likewise decreased and increased the intracellular killing of Staphylococcus aureus respectively. Moreover, IgG subclasses IgG1 and IgG3 but not IgM have been shown to stimulate the intracellular killing of these bacteria by monocytes to the same degree as heat-inactivated serum.

In addition only the Fc fragment of IgG but not $(Fab')_2$ fragments stimulated the killing. The contribution of complement components in the fresh serum on the stimulation of the killing process was believed to be due to the interaction between membrane receptors and complement components most probably C3b generated by both the classical and the alternative pathways of activation. This conclusion was based on the following experiments which included the use of sera in which one or both complement pathways were blocked by ethylenediamine tetra-acetic acid (EDTA) or ethylenebis (oxyethylenenitrilo) tetra-acetic acid (EGTA), the restoration of the killing-stimulatory activity of C3-deficient serum after addition of fresh C3, and on the partial loss of the killing-stimulatory activity of fresh serum after reduction of the number of C3b receptors following treatment of granulocytes with pronase or antigranulocyte serum (Leijh et al., 1981).

Since monocytes have receptors on their membranes for various plant lectins, van Furth and Leijh (1980) studied the effect of these molecules on the bactericidal activity of these cells. They found that of five lectins tested,

only Con A, phytohaemagglutinin and soy-bean were able to stimulate the bactericidal activity of human monocytes against Staphylococcus aureus. Incubation of monocytes containing ingested bacteria with these lectins resulted in a decrease in the number of viable intracellular bacteria equal to that when fresh serum was used as the stimulatory factor. This finding implied that modulation of the monocyte membrane may determine the intracellular killing activity of these cells. Recent studies by Kasai and colleagues (1982) showed that O_2^- production from mouse peritoneal macrophages stimulated with IgG required an optimal conformational change and orientation of the Fc portion of IgG. They demonstrated that macrophages, upon stimulation with rabbit IgG adsorbed to a hydrophobic polymer polystyrene, released greater amounts of O_2^- than when incubated in the presence of soluble IgG.

A recent study by MacGowan and co-workers (1983) demonstrated also that opsonization of Listeria monocytogenes with normal fresh human serum, purified immunoglobulin IgG, or immunoglobulin IgG-deficient fresh serum promoted their phagocytosis by human neutrophils and human peritoneal macrophages. Peritoneal macrophages were obtained from peritoneal effluents of uninfected end-stage renal disease patients treated by maintenance intermittent peritoneal dialysis. Whether these cells represented a normal resident population or an activated monocyte-derived population was not known. Very few of the unopsonized bacteria or bacteria opsonized with the heat-inactivated immunoglobulin IgG-deficient serum were

phagocytosed by these cells. They also showed that freshly pooled normal human serum mediated the greatest bacterial killing whereas opsonization by purified IgG alone or immunoglobulin IgG-deficient serum produced less killing by both human neutrophils and macrophages. In addition, they found that freshly pooled normal human serum was the most effective opsonin in stimulating not only the killing activity of these cells but also the chemiluminescent responses.

In conclusion, the above studies strongly support the contention that both heat stable and heat labile components of serum enhance the bactericidal activity of phagocytic cells.

1.3 AIMS

The general aim of the studies reported in this thesis was to investigate the factors affecting the intracellular killing activity of activated macrophages.

Since the presence of serum in the culture medium has been shown to be necessary for the optimal bactericidal activity of phagocytic cells such as granulocytes, monocytes and macrophages, harvested from normal healthy animals against different strains of bacteria, it was of interest to see whether the activation of these cells by various means changes these requirements. If these initial studies showed that activation of macrophages and neutrophils did indeed negate the requirement for serum in allowing full expression of their bactericidal potential

in vitro, it would be of interest to see if this was reflected in changes in the number of Fc and C3b receptors.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 MEDIA AND BUFFERS

2.1.1 Medium for culturing macrophages

RPMI-1640 (Flow Labs), pH 7.4 was used throughout these studies. The medium contained 20mM Hepes and was buffered with 0.01M sodium bicarbonate. The medium was sterilized by filtration through a Millipore membrane (0.45 μm) and stored at 4°C in aliquots of 100ml. Prior to use, streptomycin sulphate 100 μg/ml was added.

2.1.2 Hanks' balanced salt solution for harvesting macrophages

NaCl	8 gm
KCl	0.4 gm
Na_2HPO_4	0.06 gm
KH_2PO_4	0.06 gm
Na HCO_3	0.7 gm
Glucose	1.0 gm
Phenol red	0.02 gm
Distilled water up to	1000 ml

The pH was adjusted to 7.4 with 0.5MNaOH. The medium was sterilized by filtration through a Millipore membrane (0.45 μm) and kept at 4°C in 100ml aliquots.

2.1.3 Radioimmunoassay (RIA) buffers

The RIA buffer used was Tris HCl buffer (TESA) pH 7.75, consisting of 0.5mM EDTA, 0.1% sodium azide, 0.05M Tris HCl and 132.5mM sodium chloride.

The RIA washing buffer was phosphate buffered saline (PBS) to which had been added bovine serum albumin (BSA) (Sigma) 0.1% w/v and 0.02% sodium azide. Phosphate buffered saline was prepared by adding one volume of isotonic phosphate buffer (25mM $NaH_2PO_4.2H_2O$ + 107mM Na_2HPO_4 at pH 7.4) to nine volumes of physiological saline.

2.1.4 Esterase substrate solution

The stock solution of α-naphthyl acetate consisted of 1g of α-naphthyl acetate (Sigma), 50ml of acetone and 50ml of distilled water and was stored at 4°C. The esterase substrate solution was always made up fresh by mixing 2.0ml of α-naphthyl acetate solution, 15ml of 0.1M phosphate buffer (pH 7.3), 15ml of distilled water and 20mg of fast red TR salt (4-chloro-O-toluidine diazotate, Sigma). After mixing the solution was filtered through Whatman No 1 filter paper.

2.2 BACTERIA: DESCRIPTION OF STRAINS AND CONDITIONS FOR CULTURE

The relevant characteristics of the bacterial strains used in this study are summarized in Table 2.1. All bacterial strains were obtained from the stocks of the Department of Microbiology and Immunology, University of Adelaide, and were stored as freeze-dried cultures at 4°C. Both strains of S. typhimurium used in the following studies were made streptomycin-resistant. For routine use, a freeze-dried culture was resuspended in 1ml of nutrient broth (Difco, Michigan), added to 10ml of the same and incubated at 37°C overnight. After incubation, the culture

Table 2.1: Description of bacterial strains

Strain	Reference	Serotype O-Ag	Serotype H-Ag	LD50 for mice i.v.
Salmonella typhimurium C5	Rowley and Whitby (1959)	1,4,5,12	i:1,2	< 10
S. typhimurium M206	Rowley and Whitby (1959)	1,4,5,12	i:1,2	$> 10^7$
Salmonella enteritidis 11RX	Ushiba *et al.* (1959)	rough	g,m	$\simeq 10^6$

was streaked onto nutrient agar plates containing streptomycin (100 μg/ml) or in the case of S. enteritidis 11RX onto nutrient agar plates without streptomycin. Bacterial cultures were also maintained on nutrient agar slopes, and stored at 4°C.

In all the experiments measuring phagocytosis, cultures were used in the log-phase. A few colonies from a nutrient agar plate were selected and inoculated into 10ml of nutrient broth and incubated at 37°C with shaking for 2-4 hours. Prior to use in the experiments, the log-phase cultures were washed once by centrifugation with 10ml tissue culture medium and finally resuspended in a similar volume of the same.

2.3 ANIMALS

Outbred specific pathogen-free LACA male mice were used throughout this study. They were bred and maintained at the Central Animal House, University of Adelaide. The mice were used at 6-8 weeks of age and kept in the departmental animal house. Guinea-pigs, rabbits and rats were also obtained from the above source.

2.4 COLLECTION OF MOUSE PERITONEAL CELLS

Normal resident macrophages were collected from mice which had just been received from the Cental Animal House. Activated macrophages were obtained from mice which had been injected intraperitoneally with 10^5 live S. enteritidis 11RX (La Posta, Ashley and Kotlarski, 1982), 6-8 days previously. Mice were killed by cervical dislocation and

the skin over the peritoneum reflected. Using a disposable 19G needle and a 2ml plastic syringe, 2mls of Hanks' balanced salt solution (Section 2.1.2) were injected into the peritoneal cavity. After gently massaging the abdomen, the fluid was withdrawn and placed into siliconized glass tubes. The cells from one mouse were never pooled with those from another. The tubes were then centrifuged at 400g for 5 min and the cells finally resuspended in tissue culture medium (Section 2.1.1) to a concentration of $1-2x10^6$ cells/ml and kept on ice. An aliquot of the suspension was stained with Giemsa to determine the percentage of macrophages in the population of cells. Trypan blue exclusion revealed that the viabiity of the cells was never less than 95%.

2.5 COLLECTION AND PURIFICATION OF PERIPHERAL BLOOD NEUTROPHILS FROM THE MOUSE

The neutrophils used throughout these studies were prepared from the peripheral blood of either normal mice or mice which had been previously infected intravenously with 10^5 living S. enteritidis 11RX. The neutrophils from the infected mice were harvested 6-8 days after infection since previous investigators have shown that at this time these neutrophils under appropriate conditions are able to kill the larvae of a certain metazoan parasite (Nematospiroides dubius), whereas neutrophils from normal mice are unable to do so (Penttila, Ey and Jenkin, 1984). Normal neutrophils were collected as were normal macrophages from mice which had just been received from the Central Animal House.

The method described by Pentilla, O'Keefe and Jenkin (1982) was used to purify the neutrophils from the peripheral blood. Mice were bled from the retro-orbital plexus and the blood pooled into preservative-free lithium, heparin tubes (Johns Professional Products, Australia) and kept on ice. For fractionation, a three-step density gradient was prepared as follows, using a combination of Ficoll 400 (Pharmacia), Angiograffin and Urovison (Schering Pty Ltd) and Sepalymph (Teva Pharmaceutical Industries Ltd). The density gradient was composed of:

Part A : a mixture of density 1.114g/ml was established using 10ml Angiograffin, 10ml Urovison and 58.6ml of 10% Ficoll in water.

Part B : Part A (70ml) was diluted with 7.3% Ficoll to give a density of 1.096g/ml, as measured on a hydrometer.

Part C : Sepalymph, density 1.076g/ml.

Into an 8ml siliconized glass tube, 1.3ml of Part A, then 0.9ml of part B and finally, 1.0ml of part C were gently layered. Pooled peripheral blood (3ml) was then layered gently onto the gradient and centrifuged at 500g for 40 min at room temperature. Three bands were obtained on the gradient, the cells of the bottom band (Band 3), contained predominately neutrophils. This band was removed with a Pasteur pipette and the cells were washed twice with 10ml of tissue culture medium, before finally being resuspended in the same medium at a concentration of

1-1.5x10^6 cells/ml. This cell preparation contained 90% neutrophils which were more than 95% viable as determined by trypan blue exclusion.

2.6 PREPARATION OF MOUSE MACROPHAGE MONOLAYERS

Mouse macrophage monolayers on glass coverslips were prepared by placing 1ml of the peritoneal washout cell suspension (2x10^6 cells) (Section 2.4) into Leighton tubes containing a flying glass coverslip. The tubes were incubated at 37°C in 5% CO_2 /air for 2 hours to allow the cells to settle and spread. Non-adherent cells were removed by washing the monolayers twice with 5ml tissue culture medium.

Mouse macrophage monolayers were also prepared in 96-well flat bottom micro-culture trays (Flow Labs). To ensure a confluent monolayer of adherent cells, 0.1ml of the peritoneal cell suspension (2x10^5 cells) was added to each well. The cells adhered to the bottom of the wells after incubation for 30 min at 37°C in 5% CO_2 /air. Non-adherent cells were removed by washing once with 0.3ml tissue culture medium. Fresh tissue culture medium (0.3ml) was then added to each well and the trays incubated as above for a further 30 min.

2.7 PREPARATION OF MOUSE SPLEEN CELL SUSPENSIONS

Normal or S. enteritidis 11RX-immunized mice were killed by cervical dislocation. Under semi sterile conditions, the spleens were removed and gently pressed through a sterile wire sieve into tissue culture medium in a petri

dish. All manipulations were carried out in a laminar flow chamber. The cell suspension was then filtered to remove cell clumps through sterile cotton wool, packed loosely in a 10ml plastic syringe, into sterile siliconized glass tubes. The contents of the tubes were then centrifuged at 400g for 5 min at room temperature. The cell pellets were resuspended in cold, sterile 0.84% ammonium chloride solution at pH 7.4, and kept on ice for 5 min in order to lyse the erythrocytes.

The spleen cell suspensions free of erythrocytes were then washed twice by centrifugation with 10ml tissue culture medium before finally being resuspended in the same containing 2% v/v heat-inactivated ($56^{o}C$/1 hour) foetal calf serum (HFCS, Flow Labs). The concentration of cells in the suspension was adjusted to $5-6x10^{6}$ viable cells/ml. Cell viability was determined by trypan blue exclusion and the viability of cells was never less than 95%.

2.8 STAINING FOR NON-SPECIFIC ESTERASE

Mouse macrophage monolayers, prepared on flying glass coverslips were used (Section 2.6). The monolayers were fixed for 5 min at room temperature in 1ml cold 0.5% acetone in tissue culture medium while the coverslips were still in the Leighton tubes. The monolayers were then washed once with 5ml of warm ($37^{o}C$) medium. The method described by Yam, Li and Crosby (1971) was employed to stain the macrophages for non-specific esterase using α -naphthyl acetate as a substrate (see Section 2.1.4). The

monolayers were incubated with this solution (2ml/tube) for 30 min at room temperature. The coverslips were removed, washed with excess tap water and then mounted in glycerine jelly. Finally, the slides were examined using a light microscope (Olympus Model BH-2 RFL-W) at a magnification of X400. Enzymic activity was visible as reddish granules.

2.9 PREPARATION OF A RABBIT ANTISERUM TO *S. typhimurium* $C5^{SR}$

A rabbit antiserum to *S. typhimurium* $C5^{SR}$ was raised by injecting a rabbit with a suspension of heat-killed bacteria plus complete Freund's adjuvant (CFA) as follows. Log-phase bacteria cultured in 10ml of nutrient broth were heat-killed at 56°C (water bath) for 2 hours. They were then washed three times by centrifugation on each occasion with 20ml saline and finally resuspended in saline at a concentration of $2x10^8$ bacteria/ml. An equal volume (1.5ml) of CFA was mixed with the bacterial suspension. An aliquot (0.5ml) of such a suspension was injected into a rabbit, four sites being intramuscular and two sites subcutaneous. After 10 days, the rabbit was injected intravenously with 1ml of heat-killed *S. typhimurium* $C5^{SR}$ at a concentration of $5x10^7$ bacteria/ml in saline (without CFA). This was repeated 3 days later with a similar dose of bacteria. Seven days later the rabbit was given 10^8 bacteria intravenously and at 10 days, $5x10^8$ bacteria by the same route. Blood was collected from the ear veins of the rabbit 2 weeks after the last injection. The blood was incubated at 37°C for 1 hour and then kept at 4°C for a

further 16 hours to allow for clot retraction. After incubation the serum was withdrawn with a Pasteur pipette.

The IgG immunoglobulin fraction from this serum was prepared by the method described by Ey, Prowse and Jenkin (1978). The titre of antibody in this preparation was quantitated by a passive haemagglutination assay described below. A batch of normal rabbit serum was also chromatographed on a Protein-A column, as above, in order to prepare the IgG immunoglobulin fraction to be used as a control.

2.10 QUANTITATION OF RABBIT SPECIFIC ANTIBODY TO S. typhimurium $C5^{SR}$ BY PASSIVE HAEMAGGLUTINATION

Sheep erythrocytes were washed three times by centrifugation in 10ml physiological saline and resuspended to a concentration of 2.5% v/v in saline containing 100µg/ml of S. typhimurium C5 lipopolysaccharide (LPS) (provided by Dr B. Reynolds, Department of Microbiology and Immunology, University of Adelaide). This mixture was incubated for 1 hour at 37°C and then washed again three times as above. Finally, the cells were resuspended to a concentration of 1% v/v in physiological saline.

Two-fold serial dilutions (25µl) of the IgG fraction obtained from the serum of the rabbit immunized with S. typhimurium $C5^{SR}$ were made in physiological saline in a 96-well round bottom micro-culture tray (Flow Labs) and equal volumes of lipopolysaccharide sensitized sheep erythrocytes added. The tray was then incubated at 37°C for 1 hour and finally at 4°C overnight. The last well showing

agglutination was taken as the endpoint. Based on this assay, in experiments measuring phagocytosis of bacteria, two haemagglutinating units (1/1000 dilution) were used to opsonize them.

2.11 PREPARATION OF SERUM AS A SOURCE OF COMPLEMENT

Normal rabbits, guinea-pigs, rats and mice were used as a source of complement. Blood was obtained from mice by bleeding them from the retro-orbital plexus, while guinea-pigs and rats were bled by cardiac puncture. Rabbits were bled via an ear vein. With the exception of rabbits, all animals were bled under ether anaesthesia. The blood was allowed to clot at room temperature for 1 hour and then held at $4^{o}C$ for a further 3 hours. After this, the serum was removed and stored in aliquots (0.5ml) at $-70^{o}C$.

2.12 REMOVAL OF COMPLEMENT ACTIVITY FROM NORMAL RABBIT SERUM

2.12.1 Adsorption with zymosan

A suspension of zymosan (Sigma) in saline (2mg/ml) was boiled at $100^{o}C$ for 1 hour. After three washes each in 10ml saline, the zymosan was resuspended in 1ml of fresh normal rabbit serum and incubated at room temperature for 1 hour. The zymosan was then removed by centrifuging at 1200g for 15 min at $4^{o}C$. The zymosan-adsorbed rabbit serum was stored at $-70^{o}C$. The complement activity in this serum was measured using a haemolytic assay with sheep erythrocytes as follows. Sheep erythrocytes were washed three times with physiological saline and made up to a 2.5% v/v

suspension in the same. An equal volume (1ml) of a rabbit anti-sheep erythrocyte haemolysin (Commonwealth Serum Labs), equivalent to 4 units, was then added to the erythrocyte suspension. After incubation at 37°C for 20 min, the sensitized cells were washed as before. Serial two-fold dilutions (0.1ml) of the zymosan-adsorbed rabbit serum and normal rabbit serum were prepared in a 96-well round bottom micro-culture tray (Flow Labs), after which an equal volume of the sensitized sheep erythrocytes was added. Control wells contained only saline and the sensitized sheep erythrocytes. The tray was incubated at 37°C for 1 hour.

The absence of complement activity in the zymosan-adsorbed serum was determined by a complete button shape of sheep erythrocytes at the bottom of the wells in comparison with the control wells. For the bactericidal activity (Chapter 3, Section 3.6), a 1/10 dilution of the zymosan-adsorbed rabbit serum was used which contained no complement activity.

2.12.2 Treatment of rabbit serum with aggregated rabbit IgG

An aggregated rabbit IgG suspension was prepared by heating normal rabbit serum (5ml) at 63°C for 25 min. Serial two-fold dilutions (0.1ml) of the aggregated rabbit IgG suspension was prepared in a 96-well round bottom micro-culture tray, after which an equal volume of fresh normal rabbit serum was added. The tray was incubated at 37°C for 30 min. Control wells contained fresh normal

rabbit serum without aggregated rabbit IgG. After incubation, a suspension of sensitized sheep erythrocytes (Section 2.12.1) was added to each well (0.1ml). Another control included wells containing only saline and the sensitized sheep erythrocytes. The tray was incubated at 37°C for 1 hour. From this assay one was able to determine the greatest dilution of the IgG suspension which was sufficient to remove all the complement activity from fresh normal rabbit serum.

In experiments measuring phagocytosis and killing of bacteria (Chapter 3, Section 3.6), an equal volume (2ml) of fresh normal rabbit serum in a tube was incubated at 37°C for 30 min with the correct dilution of the aggregated rabbit IgG suspension. After incubation, the tube was centrifuged at 10,000g for 30 min at 4°C to remove the aggregated rabbit IgG. The adsorbed serum was then stored at -70°C.

2.13 PURIFICATION AND IODINATION OF A SPECIFIC RABBIT ANTI-RAT IgG ANTIBODY

The rabbit anti-rat IgG serum was kindly donated by Dr Lohmann-Matthes of the Max-Planck-Institut für Immunbiologie, Freiburg, FRG. The IgG immunoglobulin fraction from this antiserum was affinity purified by Dr P. Ey of this department on a rat IgG-Sepharose column and eluted with 0.25M propionic acid. The cross-reactivity against mouse IgG in this preparation was removed by passing the eluate through a mouse-IgG Sepharose column. Finally, purified rabbit IgG anti-rat IgG antibodies were

radiolabelled with Iodine-125 (IMS:30, Amersham) using a solid-phase oxidizing agent (Iodogen) as described by Salacinski et al. (1981).

2.14 PREPARATION OF ^{125}I-LABELLED FIBRIN TRAYS

Fibrinogen (Calbiochem) was radiolabelled with Iodine-125 (IMS:30, Amersham) using the chloramine-T method of Byrt and Ada (1969). The technique used for the preparation of ^{125}I-labelled fibrin trays was that described by Gordon, Werb and Cohn (1976). To the wells of 24-well culture trays (Flow Labs), ^{125}I-labelled fibrinogen ($5x10^4$ to $1x10^5$ cpm) was added. The trays were dried at 45^{o}C, and then stored at 4^{o}C. Before use, 1ml of 10% v/v FCS in tissue culture medium was added to the wells and the trays incubated at 37^{o}C for 2 hours in order to convert fibrinogen to fibrin. The trays were washed twice with tissue culture medium (1ml/well) prior to the plasminogen activator release assay (see in detail, Chapter 5).

2.15 STATISTICAL ANALYSIS

Statistical analyses in this thesis were performed using the Student's t-test.

2.16 OTHERS

Specific procedures for some experiments are described in the relevant sections of the experimental chapters.

CHAPTER 3

3. IN VITRO BACTERICIDAL ACTIVITY OF MACROPHAGES

3.1 INTRODUCTION

Studies carried out by van Furth's group have established the participation of normal immunoglobulin IgG in enhancing the phagocytosis and intracellular killing of some bacteria such as S. aureus, Streptococcus pyogenes and E. coli by normal human monocytes and granulocytes (Leijh et al., 1979a; van Furth and Leijh, 1980; Leijh et al., 1981) and more recently by resident mouse peritoneal macrophages (Leijh, van Zwet and van Furth, 1984). They suggested that the stimulatory effect of immunoglobulin IgG was due most probably to the interaction of IgG with the Fc receptor on the membrane of the phagocyte. Studies by other investigators have supported this suggestion (Goldstein, Kaplan, Radin and Frosch, 1976; Tamoto and Koyama, 1980; Kasai, Akaike, Kunimoto and Nitta, 1982). In addition, other serum proteins such as complement components and certain plant lectins have also been shown to have a similar effect as IgG following binding to their receptors on phagocytes (van Furth and Leijh, 1980; Leijh et al., 1981). Indeed, it was shown from these studies that a complement component, most probably C3b, was required for maximum intracellular killing by phagocytes. Similar results were also reported by other workers on the enhancing effect of normal immunoglobulin IgG and complement on the bactericidal activity of human macrophages against L. monocytogenes (Mac Gowan, Peterson, Keane and Quie, 1983).

As a result of the above investigations, studies were carried out *in vitro* comparing the bactericidal activity of two populations of macrophages, normal and activated. In particular to determine if activation changed the cell's requirement for serum factors for maximal bactericidal activity. For these studies, a virulent strain of *S. typhimurium* $C5^{SR}$ was chosen as the target organism since it had been shown previously that activation of macrophages was an important prerequisite for optimal killing of this organism (Blanden, Mackaness and Collins, 1966).

3.2 THE BACTERICIDAL ACTIVITY OF NORMAL AND ACTIVATED MACROPHAGES *IN VITRO* AGAINST *S. typhimurium* $C5^{SR}$

In order to compare the bactericidal activity *in vitro* of normal, unstimulated mouse macrophages with activated macrophages, the specific pathogen free (S.P.F.) adult mice were not conventionalized. They were used immediately after being removed from the S.P.F. facilities. Activated macrophages were obtained from S.P.F. mice which had been given a sublethal intra-peritoneal injection of 10^5 living *S. enteritidis* 11RX (11RX-activated macrophages) (see Chapter 2, Section 2.4). Infection of mice with *S. enteritidis* 11RX via this route has been shown to produce a T-cell dependent activation of macrophages (La Posta, Ashley and Kotlarski, 1982). The peritoneal cells were harvested six days later using the method given in Materials and Methods, Section 2.4.

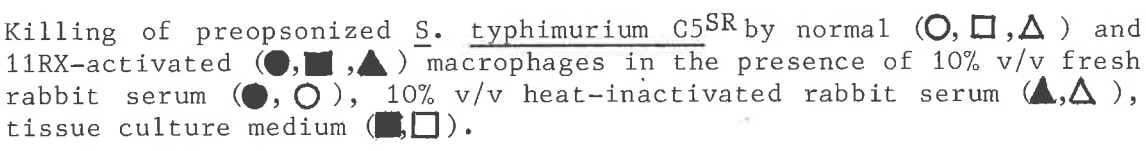
The bactericidal assays were carried out in micro-culture trays by the following method. Mouse peritoneal cells harvested from either normal or S. enteritidis 11RX-infected mice were washed once with 2ml of tissue culture medium and then resuspended in tissue culture medium at a concentration of $2x10^6$ cells/ml. The cells ($2x10^5$/well) adhered to the wells after an initial incubation of 30 min at 37°C in 5% CO_2/air. Non-adherent cells were removed by washing the wells once with 0.3ml of tissue culture medium. The monolayers were then incubated in 0.3ml of medium for a further 30 min at 37°C. The medium was removed from the monolayers prior to the addition of bacteria.

A log phase culture of S. typhimurium $C5^{SR}$ (Chapter 2, Section 2.2) at a concentration of $5x10^6$ bacteria/ml in tissue culture medium was opsonized at room temperature for 15 min with a 1/1000 final dilution of purified rabbit IgG isolated from an antiserum to S. typhimurium $C5^{SR}$ (Materials and Methods, Section 2.9). The opsonized bacteria were washed once with 10ml of medium and finally resuspended in the same at a concentration of $1-2x10^5$ bacteria/ml. The bacterial suspension was then divided into three aliquots (0.9ml/tube) and kept on ice. To the tubes, 0.1ml of either fresh or heat-inactivated (56°C/1 hour) normal rabbit serum (final concentration 10% v/v) or tissue culture medium was added. Previous studies had shown that S. typhimurium $C5^{SR}$ is not killed in the presence of specific antibody and complement (Reynolds and Pruul, 1971).

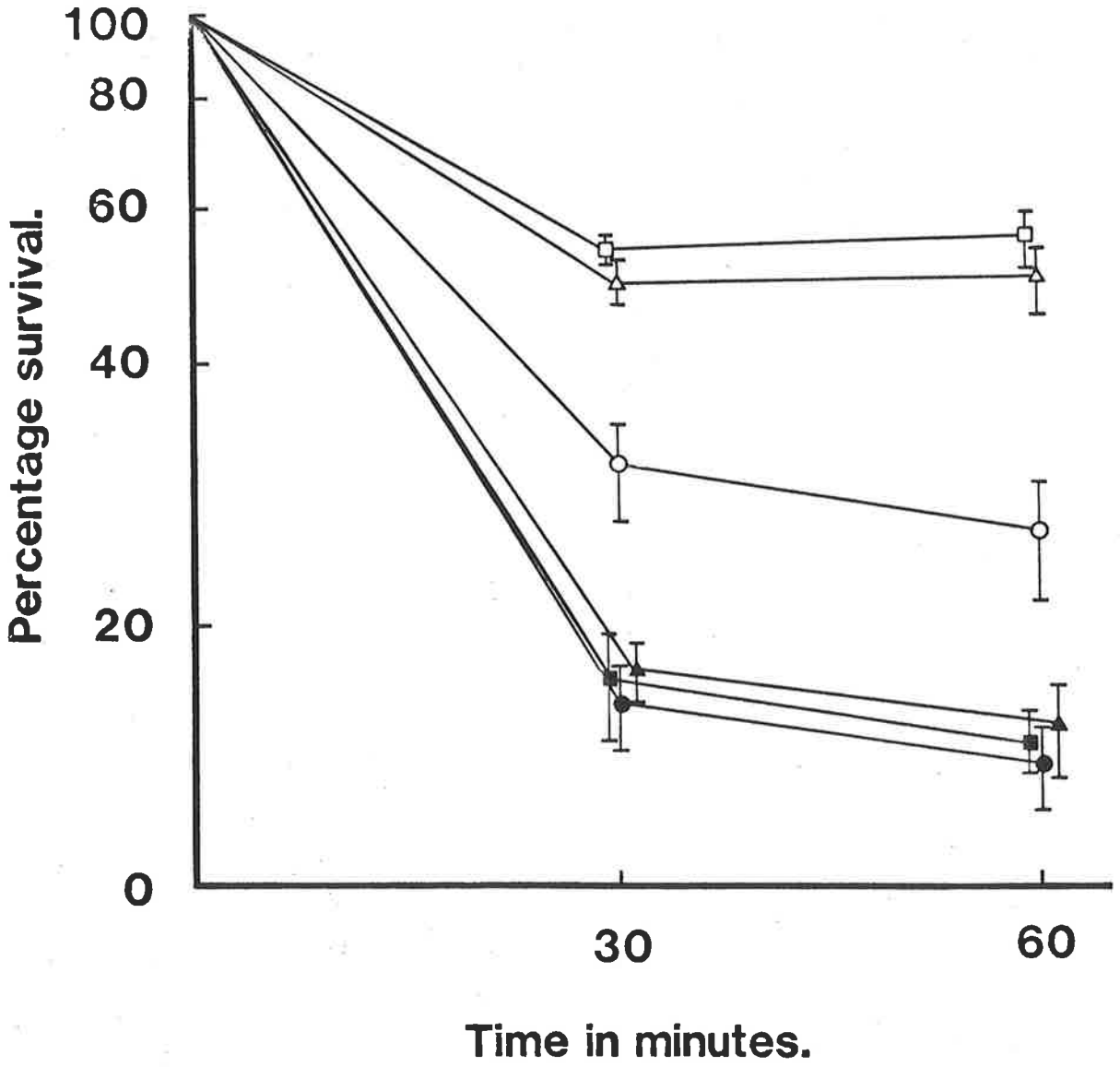
A small volume (50 µl) of the opsonized bacterial suspension from each tube (0.5-1x10^4 bacteria) was added to the monolayers. To ensure close contact between the cells and bacteria, the micro-culture trays were centrifuged at 1500g for 10 min at 4°C in a Coolspin centrifuge (PL 430, Model MSE, England). The trays were then incubated at 43°C for 2 min, followed by incubation at 37°C in 5% CO_2/air. A separate tray was set up for each time of sampling in this type of experiment and in all subsequent ones described in this thesis. At time zero (prior to incubation at 37°C) and at intervals thereafter viable counts of the bacteria were made as follows, two wells being sampled at each time point for each of the experimental conditions. To each well being sampled was added an equal volume (50 µl) of Triton-X non-ionic detergent (1% v/v in saline) in order to disrupt the macrophages. Control wells containing no macrophages but only bacteria in the presence of the three different culture media as above were always included and treated in a similar manner. The above concentration of Triton-X did not affect the viability of the bacteria. Assuming that the bacteria grew equally well in both control and experimental wells, the survival of bacteria was calculated as the number of bacteria in the experimental wells over the number of bacteria in the control wells and was expressed as a percentage.

The results given in Figure 3.1, show that the killing of the virulent strain of preopsonized S. typhimurium $C5^{SR}$ by normal macrophages was enhanced in the presence of fresh serum. In the control wells bacteria in

Figure 3.1



Each point represents the mean percentage survival ± S.D obtained from five different experiments. Variation between wells in each experiment was less that 5 per cent.


100
80
60
40
20
0
Percentage survival.
30
60
Time in minutes.

the absence of macrophages increased in number by a factor of 1.5-2.0. In contrast, 11RX-activated macrophages compared with normal macrophages showed a greatly enhanced bactericidal capacity and were able to kill opsonized S. typhimurium $C5^{SR}$ equally well in the presence and absence of serum. These experiments were repeated several times and similar results were obtained.

3.3 THE EFFECT OF THE REMOVAL OF CELL-BOUND ANTIBODY ON THE BACTERICIDAL ACTIVITY OF 11RX-ACTIVATED MACROPHAGES

It seemed possible that the enhanced bactericidal activity of 11RX-activated macrophages in the absence of serum was due to immunoglobulins bound to the cell surface which resulted in maximal stimulation thus negating the requirement for serum. Indeed, it has been shown that vaccination of mice with a living attenuated culture of S. typhimurium, results in the production of an antibody which binds to the macrophages. This cell-bound antibody may be eluted from the 'immune' macrophages (Turner, Jenkin and Rowley, 1964; Rowley, Turner and Jenkin, 1964). Despite culture in vitro this antibody may still be present on the surface of macrophages, since data given by Loor and Roelants (1974) indicated that cytophilic antibody may be detected on macrophages even after four days under in vitro conditions.

To investigate the possibility that cell-bound immunoglobulin was involved in the bactericidal activity of 11RX-activated macrophages in vitro, the following experiments were carried out. Two methods were employed to

remove possible cell-bound immunoglobulins. 11RX-activated macrophages ($1x10^6$ cells) in siliconized glass tubes were washed three times with 10.0ml aliquots of 0.005M EDTA in tissue culture medium and then once more with the same volume of medium in the absence of EDTA before being cultured and their bactericidal potential assayed as described before (Section 3.2). The second method was to treat the cells with a neutral protease (Dispase, grade II, Mannheim, Boehringer) as follows. A batch of cells ($1x10^6$) was resuspended in 1ml of tissue culture medium containing 1.5mg of Dispase and incubated at 37°C for 45 min. A similar number of macrophages was incubated with medium alone. After incubation the cells were washed once with 10ml of medium before finally being cultured as above. The bactericidal capacity of the macrophages subjected to these two treatments towards preopsonized S. typhimurium $C5^{SR}$ was measured in the presence of either 10% v/v fresh rabbit serum or tissue culture medium alone and compared with untreated macrophages under similar conditions (Section 3.2). Control wells contained no macrophages but only bacteria in the presence of the two different culture media. For each tray removed at each time point, three wells were sampled for each of the above experimental and control conditions.

The ability of 11RX-activated macrophages to kill virulent preopsonized S. typhimurium $C5^{SR}$ after the removal of possible immunoglobulins present on the cells is shown in Tables 3.1 and 3.2. In comparison with the untreated controls, the ability of 11RX-activated macrophages to

kill this organism was still retained although cell-bound proteins, if present, on their surface should have been removed by the above treatments. In addition, the expression of their enhanced bactericidal capacity was not dependent on the presence of serum in the medium.

Table 3.1

Killing of opsonized S. typhimurium $C5^{SR}$ by macrophages harvested from S. enteritidis 11RX-infected mice, before and after treatment with EDTA in the presence of 10% v/v fresh rabbit serum or tissue culture medium.

Treatment with EDTA	Serum	Percentage survival[a] 30 min	60 min
Before	+	9.1 ± 1.8	5.6 ± 1.4
	-	11.2 ± 1.9	6.1 ± 1.8
After	+	10.5 ± 1.9	6.4 ± 1.3
	-	11.5 ± 0.9	10.3 ± 1.8

a - Mean ± S.D. of triplicate cultures.

Table 3.2

Killing of opsonized S. typhimurium $C5^{SR}$ by macrophages harvested from S. enteritidis 11RX-infected mice before and after treatment with Dispase, in the presence of 10% v/v fresh rabbit serum or tissue culture medium alone.

Treatment with Dispase	Serum	Percentage survival[a] 30 min	60 min
Before	+	13.9 ± 2.4	12.0 ± 0.4
	-	16.4 ± 1.0	13.8 ± 1.0
After	+	15.3 ± 2.9	13.2 ± 2.3
	-	15.2 ± 1.2	14.5 ± 1.1

a - Mean ± S.D. of triplicate cultures.

3.4 THE SERUM REQUIREMENT FOR THE EXPRESSION OF THE BACTERICIDAL ACTIVITY OF MACROPHAGES ACTIVATED *IN VITRO* BY LYMPHOKINES

To support further the possibility that the bactericidal capacity of activated macrophages is independent of both cell-bound immunoglobulins and the presence of serum, the bactericidal activity of macrophages activated *in vitro* by lymphokines was determined. Normal macrophage monolayers ($2x10^5$ cells) were prepared in 12 wells of two 96-well micro-culture trays (Chapter 2, Section 2.6). Six monolayers in each tray were then incubated in the presence of a 30% v/v lymphokine-containing supernatant (200 µl) derived from Con A-stimulated spleen cell cultures (see Chapter 5). The other six monolayers were incubated in 200µl of a culture supernatant derived from unstimulated spleen cells to which following harvesting a similar concentration of Con A (3.0 µg/ml) had been added. The micro-culture trays were incubated at 37°C in 5% CO_2/air overnight. After incubation, the monolayers were washed once with 300 µl of tissue culture medium. The bactericidal assay was performed by adding preopsonized *S. typhimurium* $C5^{SR}$ ($1-2x10^5$ bacteria) resuspended in 1ml of 1) medium + 10% v/v fresh normal rabbit serum, 2) medium + 10% v/v heat-inactivated normal rabbit serum (56°C/1 hour), or 3) medium alone. To two monolayers for each of the above experimental conditions, an aliquot (50 µl) of opsonized bacteria was added. At time zero, one tray was removed and the other after 60 min at 37°C in 5% CO_2/air. The number of bacteria in each well was then determined as previously described (Section

3.2). Bacteria alone in the absence of macrophages were included as controls and incubated in the three experimental conditions as above.

Similar to the earlier findings the killing of virulent preopsonized S. typhimurium $C5^{SR}$ by normal control macrophages was enhanced in the presence of fresh serum (Fig 3.2.1). However, the number of bacteria killed was somewhat lower than that observed earlier. This might have been due to the length of time the macrophages had been in culture prior to the addition of the bacteria. With respect to the lymphokine-activated macrophages, the presence of serum was not necessary for them to express their bactericidal potential (Fig 3.2.2), which substantiates the earlier results indicating that cell-bound antibody was not necessary for the expression of the enhanced bactericidal capacity of activated macrophages.

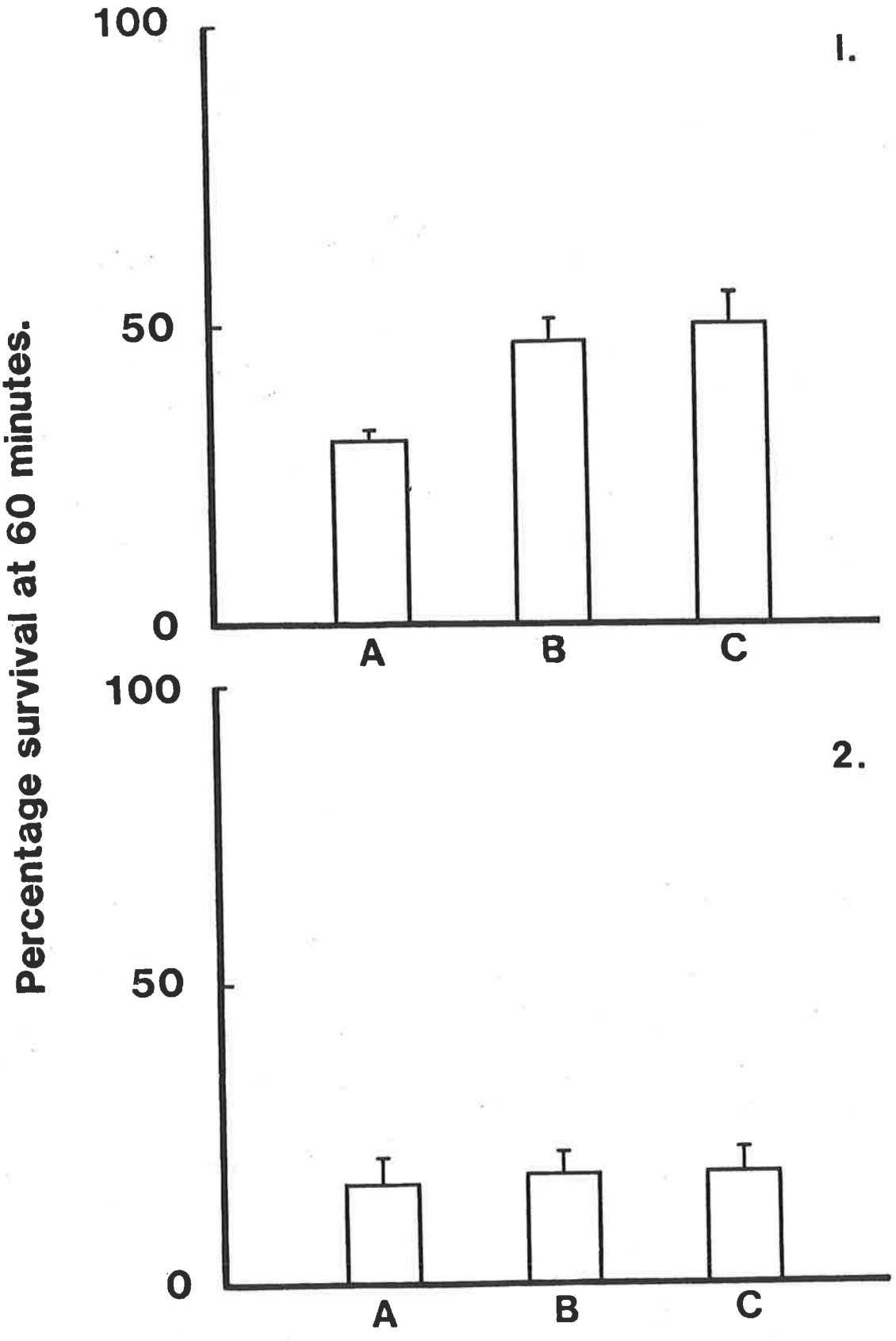
3.5 THE EFFECT OF VARIOUS DILUTIONS OF FRESH RABBIT SERUM ON THE BACTERICIDAL ACTIVITY OF NORMAL MACROPHAGES

To estimate the optimal amount of fresh serum required for the killing of virulent preopsonized S. typhimurium $C5^{SR}$ by normal macrophages, the bacteria and the cells were incubated together in the presence of various dilutions of fresh rabbit serum and the bactericidal assay performed as previously described (Section 3.2). The number of trays used and wells sampled was similar to that described in Section 3.3. It may be seen from the data (Table 3.3) that there was a correlation between the number of bacteria killed and the concentration of fresh rabbit serum in the wells. Optimal

Figure 3.2

Killing of preopsonized S. typhimurium C5 SR by (1) normal control macrophages and (2) lymphokine-activated macrophages in the presence of 10% fresh rabbit serum (A), 10% heat-inactivated rabbit serum (B) or culture medium (C).

Each histogram represents the mean percentage survival ± S.D obtained from four separate experiments. Variation between wells in each experiment was less than 5 per cent.


Percentage survival at 60 minutes.
100
50
0
A
B
C
1.
100
50
0
A
B
C
2.

killing of the preopsonized bacteria by normal macrophages was observed when 10% fresh rabbit serum was present in the medium. Concentrations greater than this did not increase significantly the number of bacteria killed.

Table 3.3

The effect of the concentration of fresh rabbit serum on the killing of opsonized S. typhimurium $C5^{SR}$ by normal macrophages.

Concentration of serum (% v/v)	Percentage survival[a]	
	30 min	60 min
0.0.	54.0 ± 1.7	61.6 ± 1.9
0.1	53.2 ± 1.0	56.4 ± 0.7
1.0	44.2 ± 0.8	47.5 ± 0.6
10.0	27.2 ± 0.9	25.2 ± 1.4
30.0	26.8 ± 1.3	22.4 ± 1.2

a - Mean ± S.D. of triplicate cultures.

3.6 THE NATURE OF THE FACTOR(S) IN FRESH SERUM INVOLVED IN STIMULATING THE BACTERICIDAL ACTIVITY OF NORMAL MACROPHAGES

As mentioned earlier, in studies using S. aureus, van Furth and Leijh (1980) concluded that the stimulatory effect of heat-inactivated serum on the intracellular killing of S. aureus by human monocytes was via the interaction of the Fc portion of the immunoglobulin IgG with its receptors on the monocyte membrane. Other studies have also shown the relative importance of this type of stimulation (McGowan, Peterson, Keane and Quie, 1983). The latter investigators have shown that the uptake of L. monocytogenes opsonized with immunoglobulin IgG-deficient human serum by both human neutrophils and peritoneal

macrophages was abolished when the serum was heated at 56°C for 30 min. In contrast, heat inactivation of normal human serum did not affect its opsonic activity. These studies together with others (Leijh et al., 1979a; van Furth and Leijh, 1980) indicated that both immunoglobulins and complement may be required to stimulate phagocytosis and killing.

In order to delineate the relative roles of immunoglobulin IgG and complement in the killing of the virulent strain of S. typhimurium $C5^{SR}$ by normal macrophages, rabbit serum lacking certain complement components and purified rabbit immunoglobulin IgG were used to stimulate the bactericidal activity of normal macrophages. The bactericidal assay was performed as previously described (Section 3.2) by adding preopsonized S. typhimurium $C5^{SR}$ ($0.5-1x10^4$ bacteria) to the normal macrophage monolayers ($2x10^5$cells) in the presence of the following sera (10% v/v) in the medium: a) zymosan-adsorbed rabbit serum; b) aggregated IgG-treated rabbit serum; c) heat-inactivated (56°C/1 hour) rabbit serum (complement activity in these sera was destroyed, see Chapter 2); d) fresh C6-deficient rabbit serum and e) fresh C4-deficient guinea-pig serum (provided by Dr S. Neoh, Department of Clinical Immunology, Flinders University of South Australia). Control wells containing only bacteria in these sera were always included. Three wells were sampled at time zero and at 60 min for each of the experimental and control conditions.

The killing of opsonized S. typhimurium $C5^{SR}$ by normal macrophages was reduced in the presence of normal rabbit serum from which more than one of the complement components had been removed (Fig 3.3). Since effective killing could still be demonstrated in the presence of serum lacking a single component such as C4 and C6, it suggested that neither of these components was involved in the stimulation of the bactericidal activity of normal macrophages.

Despite the fact that the number of bacteria killed by normal macrophages in the presence of heat-inactivated rabbit serum and tissue culture medium alone was not significantly different, the ability of these cells to kill the bacteria in the presence of purified rabbit IgG (1mg/ml) was studied (Fig 3.4). Triplicate cultures of macrophages were sampled at time zero and 60 min. Control wells contained bacteria alone in the presence of the above media. The data show that the killing of the pre-opsonized bacteria by normal macrophages in the presence of purified rabbit immunoglobulin IgG at a final concentration which was equal to that in 10% fresh rabbit serum was no different from that observed using heat-inactivated rabbit serum. In addition, no additive effect was shown when purified rabbit immunoglobulin IgG and heat-inactivated rabbit serum were present together in the culture medium.

In comparison with the number of S. typhimurium $C5^{SR}$ killed in tissue culture medium alone, it seemed clear

Figure 3.3

Killing of opsonized S. typhimurium $C5^{SR}$ by normal macrophages in the presence of various sera in the culture medium (10% v/v). (A) fresh rabbit serum, (B) fresh guinea-pig serum, (C) fresh C6-deficient rabbit serum, (D) fresh C4-deficient guinea-pig serum, (E) heat-inactivated rabbit serum, (F) zymosan-adsorbed rabbit serum, (G) aggregated IgG-treated rabbit serum, (H) tissue culture medium alone.

Each histogram represents the mean percentage survival ± S.D of three separate experiments. Variation between wells in each experiment was less than 10 per cent.

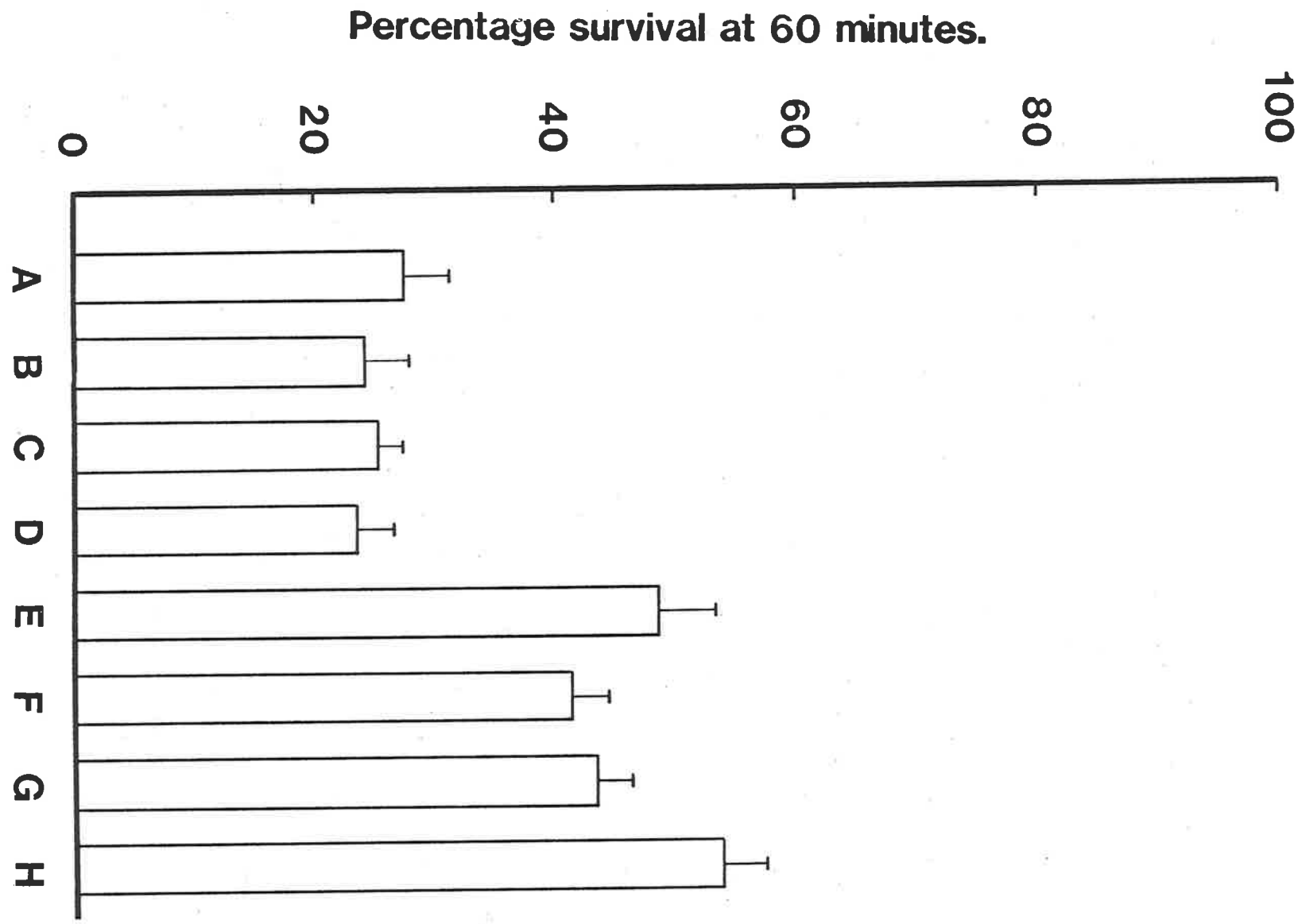
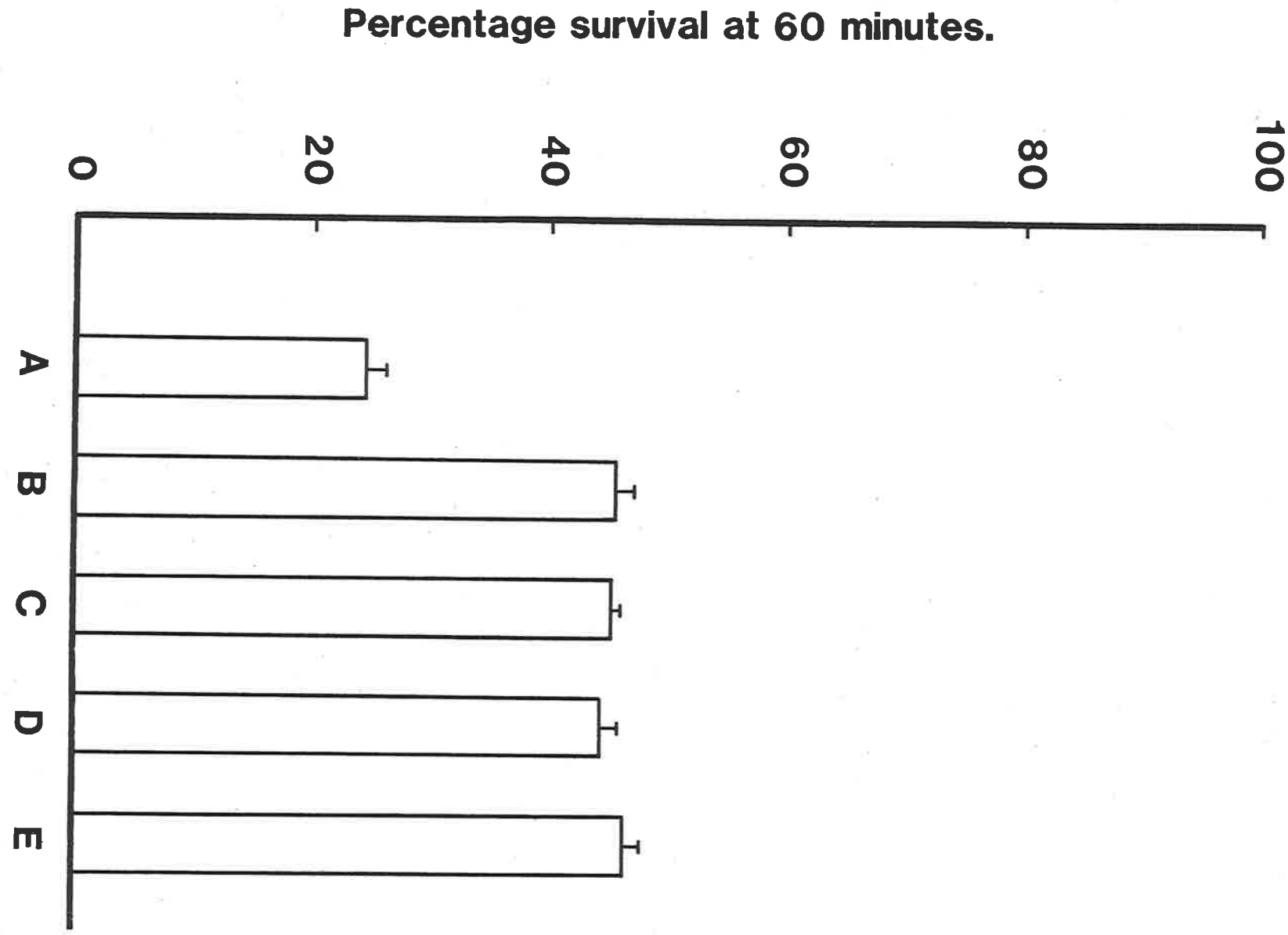

Percentage survival at 60 minutes.
0
20
40
60
80
100
A
B
C
D
E
F
G
H

Figure 3.4

The killing of preopsonized S. typhimurium $C5^{SR}$ by normal macrophages in the presence of 10% fresh rabbit serum (A), 10% heat-inactivated rabbit serum (B), purified rabbit IgG (1mg/ml) (C), 10% heat-inactivated rabbit serum + purified rabbit IgG (1mg/ml) (D), tissue culture medium (E).

Each histogram represents the mean percentage survival ± S.D. of triplicate cultures.


Percentage survival at 60 minutes.
0
20
40
60
80
100
A
B
C
D
E

that the immunoglobulin fraction (IgG) from rabbit serum did not significantly potentiate the killing of these bacteria by normal macrophages. Maximal potentiation was achieved only when heat-labile factors were present, these factors being most likely components of the complement system.

3.7 THE EFFECT OF SURFACE-BOUND IgG ON THE BACTERICIDAL ACTIVITY OF NORMAL MACROPHAGES

Normal mouse peritoneal macrophages, upon incubation with purified rabbit anti-BSA IgG, adsorbed to a hydrophobic polymer, polystyrene have been shown to generate O_2^- (Kasai et al., 1982). In these studies, it was shown also that soluble IgG did not stimulate the production of this oxygen radical by normal macrophages. From these results, it seemed possible that surface-bound IgG may be more efficient than soluble IgG in stimulating the bactericidal activity of normal macrophages. In order to test this possibility, normal macrophage monolayers ($2x10^5$ cells) were prepared either in wells of micro-culture trays as before (Chapter 2, Section 2.6) or in wells which had been pre-coated with purified rabbit IgG. The wells were coated by adding 50µl of purified rabbit IgG (1mg/ml) after which the micro-culture trays were kept at 4^oC overnight and washed once with tissue culture medium (300µl) before adding the cells. Monolayers on the uncoated wells were infected with preopsonized S. typhimurium $C5^{SR}$ ($0.5-1x10^4$ bacteria) in the presence of either 10% v/v fresh rabbit serum, purified rabbit IgG (1mg/ml) or tissue culture medium alone, whereas monolayers on the rabbit IgG-coated

wells were infected with preopsonized bacteria in the presence of tissue culture medium alone. The bactericidal assay was performed as previously described (Section 3.2). Control wells containing no macrophages but only bacteria in the presence of culture media as above were included. Two wells were sampled at time zero and at 60 min for each of the above experimental conditions. The results obtained from four separate experiments (Fig 3.5) show that purified rabbit IgG bound to the polystyrene tray enhanced the killing of virulent preopsonized S. typhimurium $C5^{SR}$ by normal macrophages to a greater extent than did soluble rabbit IgG ($p < 0.02$). However, the number of bacteria killed by normal macrophages in the presence of soluble rabbit IgG as well as 10% v/v fresh rabbit serum in these experiments was somewhat lower than that found in the previous results.

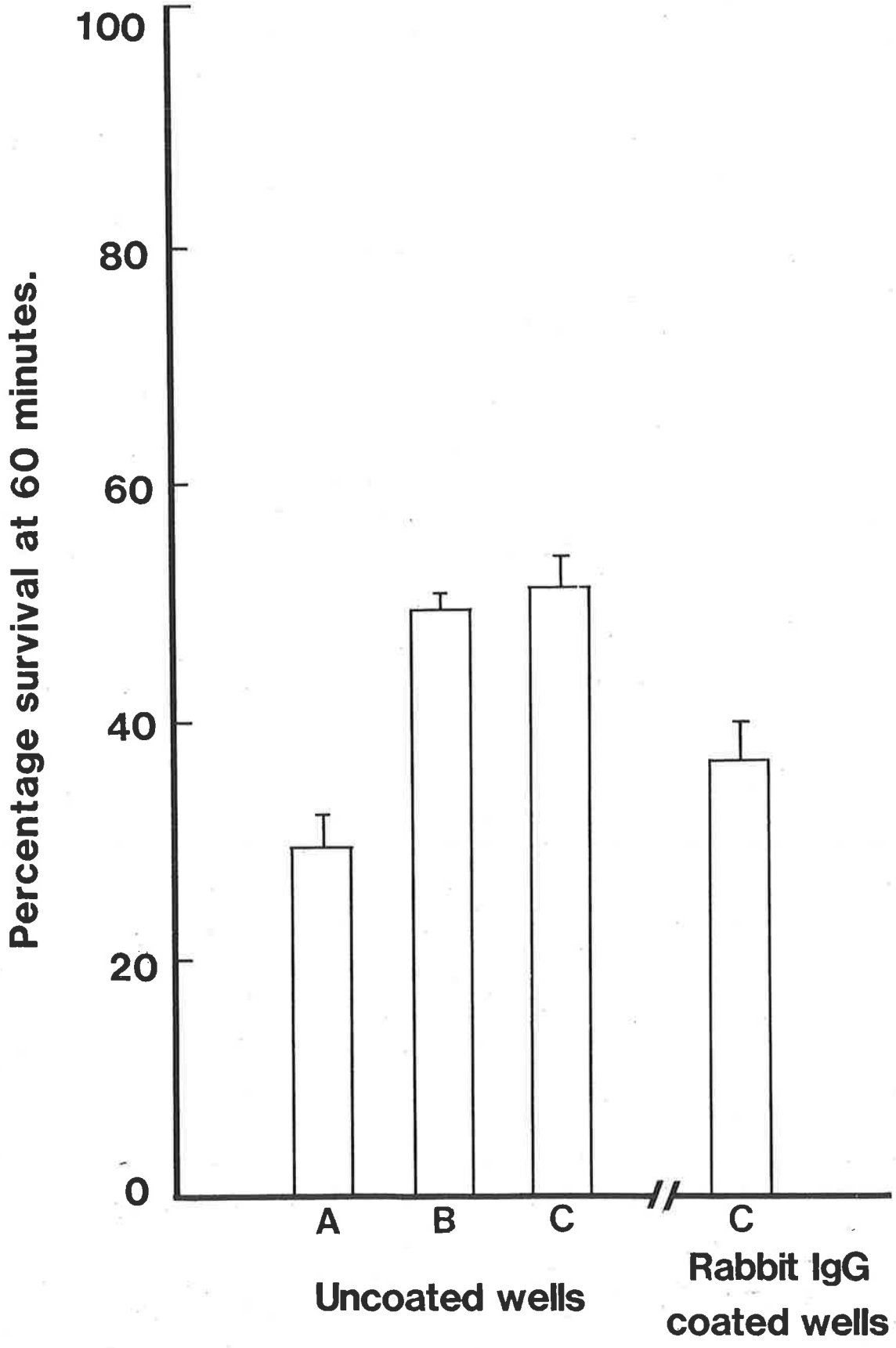
3.8 CONCLUSIONS

A comparison of the in vitro bactericidal activity of normal and activated macrophages indicated that killing of the virulent strain of opsonized S. typhimurium $C5^{SR}$ by normal macrophages was enhanced by the presence of fresh serum in the culture medium. In contrast, there was no such requirement for the bactericidal activity of activated macrophages. The enhanced killing of the bacteria by activated macrophages was obtained both in the presence and absence of serum, and this was not accounted for by the existence of cell-bound immunoglobulin.

Figure 3.5

The killing of preopsonized S. typhimurium $C5^{SR}$ by normal macrophages in the presence of 10% v/v fresh rabbit serum (A), soluble purified rabbit IgG (B), tissue culture medium alone (C).

Each point represents the mean percentage survival ± S.D. of three separate experiments. Variation between wells in each experiment was less than 5 per cent.


100
80
60
40
20
0
Percentage survival at 60 minutes.
A
B
C
C
Uncoated wells
Rabbit IgG
coated wells

The factor in normal rabbit serum which potentiated the killing of the virulent strain of opsonized S. typhimurium $C5^{SR}$ by normal macrophages was likely to be components of the complement system. Heat-stable factors (immunoglobulins) in normal rabbit serum were only effective under certain conditions.

CHAPTER 4

4. THE BACTERICIDAL ACTIVITY OF MACROPHAGES AND NEUTROPHILS IN VITRO: CORRELATION WITH Fc AND C3b RECEPTORS

4.1 INTRODUCTION

Mechanisms whereby macrophages and neutrophils eliminate certain foreign particles, such as bacteria, involve the processes of recognition, phagocytosis and killing. Although the killing process is the final step in the elimination of bacteria by these cells, it will obviously depend on earlier steps such as binding of the bacteria to the membrane of the phagocyte, followed by ingestion.

Of the known mechanisms, in this regard, the most widely studied and probably of greatest biologic significance is the Fc and C3b receptor-mediated phagocytosis by these cells. Both macrophages and neutrophils have been shown to express on their surface, Fc and C3b receptors (Berken and Benacerraf, 1966; Lay and Nussenzweig, 1968; Henson, 1972; Mantovani, Rabinovitch and Nussenzweig, 1972; Mantovani, 1975; Scribner and Fahrney, 1976; Unkeless, 1977; Diamond, Bloom and Scharff, 1978; Diamond and Scharff, 1980; Diamond and Yelton, 1981). Several studies have indicated that C3b receptors are involved under appropriate conditions in the binding of bacteria to the membrane of the phagocytic cell but unlike binding, via the Fc receptor, the bacteria were not ingested (Mantovani *et al.*, 1972; Reynolds, Atkinson, Newball and Frank, 1974; Griffin, Bianco and Silverstein, 1975;

Scribner and Fahrney, 1976). There is also some evidence that specific antibody and complement bound to bacteria influence the bactericidal activity of human granulocytes (Solberg and Hellum, 1973; Solberg, Christie, Larsen and Tønder, 1976). Other studies have also suggested that specific antibody not only enhances the phagocytosis of bacteria by macrophages but also plays some role in determining the fate of the ingested bacteria (Rowley, 1958; Jenkin and Benacerraf, 1960; Rowley and Jenkin, 1962). Investigations by van Furth and his colleagues have shown that the stimulatory effect of serum factors such as IgG and the C3b complement component on the bactericidal activity of phagocytes was mediated by an interaction of these with their specific cell receptors (Leijh et al., 1979a, 1981).

Investigations by Arend and Mannik (1973) have indicated that, in relation to the number of Fc receptors, rabbit alveolar macrophages harvested from animals stimulated a number of times with CFA possessed $2.16 \pm 0.34 \times 10^6$ receptor sites for IgG per cell, whilst minimally stimulated cells, obtained after a single injection of adjuvant possessed only $1.21 \pm 0.23 \times 10^6$ receptor sites. However, it is not known from these studies whether an increase in the number of IgG receptors on the cell membrane is related to an increase in the bactericidal capacity of the macrophages. With regard to the C3b receptor, it has been shown that the activation of macrophages resulted in their ability, unlike normal resident macrophages, to phagocytose C3b-coated particles such as

erythrocytes in the absence of IgG (Bianco, Griffin and Silverstein, 1975; Mørland and Kaplan, 1977; Griffin and Griffin, 1979). As yet, it is not clear whether this functional change in the C3b receptor of the activated macrophages represents a quantitative change, involving the number of C3b receptors, or only a qualitative change in the nature of the receptor.

The following experiments were designed to investigate whether there was a change in the number of Fc and C3b receptors following activation of both macrophages and neutrophils and whether this could be correlated with their bactericidal activity.

4.2 THE BACTERICIDAL ACTIVITY OF NEUTROPHILS FROM NORMAL AND SALMONELLA-INFECTED MICE IN THE PRESENCE AND ABSENCE OF SERUM

In the previous chapter, it was shown that the ability of normal macrophages to kill S. typhimurium $C5^{SR}$ in vitro was enhanced in the presence of fresh serum in the culture medium, whereas no such requirement was necessary for macrophages from S. enteritidis 11RX-infected mice. In view of this, it was decided to extend the observation to neutrophils. Initial experiments in the present chapter, compare the in vitro bactericidal activity of neutrophils obtained from both normal mice and S. enteritidis 11RX-infected mice in the presence and absence of serum. To exaggerate the possible difference in the bactericidal activity of neutrophils harvested from normal mice compared with those harvested from S. enteritidis 11RX-infected mice, an avirulent S. typhimurium $M206^{SR}$ was used.

Log-phase bacteria were opsonized by incubating $5x10^6$ bacteria/ml, in tissue culture medium with an IgG fraction from a rabbit anti-S. typhimurium $C5^{SR}$ serum at a final dilution of 1/1000 for 15 min at room temperature (see Chapter 2). They were then washed once with tissue culture medium (10ml) and finally resuspended in the same medium at a concentration of $2-3x10^5$ bacteria/ml. In Nunc plastic tubes (Medos), neutrophils ($1-1.5x10^6$ cells) obtained either from normal mice or S. enteritidis 11RX-infected mice in 0.45ml of medium (Chapter 2, Section 2.5) were mixed with an equal volume of preopsonized S. typhimurium $M206^{SR}$ ($1x10^5$ bacteria). To the tubes which were kept on ice, either 0.1ml of fresh C6-deficient rabbit serum (10% v/v) (kindly provided by Dr S. Neoh of the Clinical Immunology Department, Flinders Medical Centre, Adelaide) or tissue culture medium was added. C6-deficient rabbit serum was used in these experiments, as the source for factors in serum enhancing phagocytosis since S. typhimurium $M206^{SR}$ is sensitive to the bactericidal activity of complement in the presence of specific antibody. For the purpose of comparison, the ability of macrophages obtained from normal mice and S. enteritidis 11RX-infected mice to kill S. typhimurium $M206^{SR}$ was also determined in a similar manner. Controls included bacteria (without cells), suspended in either fresh C6-deficient rabbit serum (10% v/v) or tissue culture medium alone. The mixture (130µl) from each tube was transferred to two 96-well micro-culture trays, three wells in each tray. The trays were centrifuged at 1500g for 10 min as before (Section 3.2).

They were then incubated for 2 min at 43°C before finally being incubated at 30°C in 5% CO_2/air. The bactericidal assay was performed at 30°C due to the fact that preliminary experiments indicated that at 37°C, the bacteria were killed equally rapidly by neutrophils from normal mice and those infected with S. enteritidis 11RX. Three wells from each tray were sampled for the respective experimental conditions, one tray at time zero and the other at 60 min in the following manner. To each well was added an equal volume (130 µl) of 1% Triton-X which lysed the cells releasing any viable bacteria. The percentage survival of bacteria was calculated as previously described (Chapter 3, Section 3.2).

As found previously (Chapter 3) using a virulent strain of S. typhimurium $C5^{SR}$, the killing of the avirulent strain of opsonized S. typhimurium $M206^{SR}$ by normal macrophages was enhanced in the presence of fresh serum in the culture medium (Table 4.1). A similar requirement was also found for normal neutrophils (Table 4.2). Bacteria in the absence of phagocytic cells grew equally well in both culture media and increased in number by a factor of 1.5-2.0. Macrophages from S. enteritidis 11RX-infected mice killed equally well in the presence or absence of serum. In contrast, the killing of the bacteria by neutrophils from these mice was greater ($p < 0.001$) in the presence of fresh serum than in tissue culture medium alone, although the killing of the bacteria in medium was very effective. These results confirmed the previous data (Chapter 3) that the addition of serum to the culture

medium seems to enhance the bactericidal activity of both macrophages and neutrophils obtained from normal mice.

Table 4.1

Killing of an avirulent strain of opsonized S. typhimurium M206^{SR} by macrophages obtained from normal mice and S. enteritidis 11RX-infected mice, in the presence of either fresh C6-deficient rabbit serum (10% v/v) or tissue culture medium alone.

Source of macrophages	Serum	Percentage survival at 60 min	
		Experiment I	Experiment II
Normal mice	+	27.0 ± 0.7[a]	24.7 ± 1.3
	-	51.1 ± 1.0	47.5 ± 1.2
S. enteritidis 11RX-infected mice	+	4.3 ± 0.3	4.0 ± 0.4
	-	4.8 ± 0.5	5.2 ± 0.7

a - Mean ± S.D of triplicate cultures.

Table 4.2

Killing of an avirulent strain of opsonized S. typhimurium M206^{SR} by neutrophils obtained from normal mice and S. enteritidis 11RX-infected mice, in the presence of either fresh C6-deficient rabbit serum (10% v/v) or tissue culture medium alone.

Source of neutrophils	Serum	Percentage survival at 60 min	
		Experiment I	Experiment II
Normal mice	+	29.2 ± 0.9[a]	32.1 ± 0.8
	-	60.2 ± 1.0	54.8 ± 0.7
S. enteritidis 11RX-infected mice	+	5.0 ± 0.4	4.6 ± 0.3
	-	16.3 ± 0.8	17.8 ± 1.2

a - Mean ± S.D of triplicate cultures.

4.3 QUANTITATION OF Fc and C3b RECEPTORS ON THE SURFACE OF MACROPHAGES BY AN INDIRECT RADIOIMMUNOASSAY (RIA)

In view of the previous findings that macrophages from S. enteritidis 11RX-infected mice do not require further stimulation by serum factors in the culture medium, to kill the opsonized bacteria, it was of interest to see whether this could be accounted for by a change in the number of Fc and C3b receptors on the cell surface. The expression of these two receptors on the macrophages was measured in the following manner.

An indirect RIA was performed using a rat monoclonal antibody against the mouse C3b receptor. This monoclonal antibody, designated MAS-034 or anti-Mac 1 antibody (Sera-Lab, Sussex), has been shown to recognize the C3b receptor on murine macrophages and human neutrophils (Beller, Springer and Schreiber, 1982). A rat monoclonal antibody against mouse Fc receptor was provided by Dr A. Lopez of the Walter Eliza Hall, Melbourne. The indirect RIA to detect Fc and C3b receptors was carried out on macrophages obtained from both normal mice and S. enteritidis 11RX-infected mice. Macrophages activated in vitro with lymphokines were also included. The activation of macrophages by lymphokines was carried out as follows. Normal macrophages ($1x10^6$ cells) were incubated with a Con A supernatant (see Chapter 5) at a concentration of 30% v/v in 1ml of tissue culture medium in seven nunc plastic tubes (Medos) at 37^oC in 5% CO_2/air overnight. After overnight incubation the cells were washed once with tissue culture medium (2ml). The ability of the above three populations of macrophages to kill opsonized S. typhimurium $C5^{SR}$ was

then determined. The bactericidal assays were performed in 96-well micro-culture trays as described previously (Chapter 3, Section 3.2) by adding preopsonized S. typhimurium $C5^{SR}$ ($0.5-1x10^4$ bacteria) to the macrophages ($2x10^5$ cells) in the presence of tissue culture medium alone. Control wells contained bacteria in the presence of medium alone. Three wells were sampled at time zero and 60min. In parallel with these tests, indirect RIA were carried out as follows for Fc and C3b receptors. Macrophages representing the above three populations ($1x10^6$ cells) in nunc plastic tubes were first washed once with 2ml of tissue culture medium containing 0.02% sodium azide by centrifugation at 400g for 5 min at 4^oC. The cells were then incubated for 90 min on ice with the monoclonal antibodies (0.2µg IgG/ml) (duplicate tubes). Control tubes contained macrophages which were incubated as above with normal rat IgG. After incubation, the cells were washed three times with the RIA washing buffer (Chapter 2) (2ml). They were then incubated for a further 90 min with 50µl RIA buffer (Chapter 2) containing approximately $5x10^4$ cpm ^{125}I-labelled rabbit IgG anti-rat IgG. The labelled antibody was also added to two tubes with no cells in order to monitor the radioactivity remaining in the tubes after washing. After this period of incubation, any unbound radioactivity was removed by washing the cells three times with the RIA washing buffer as before. The amount of radioactivity associated with the cells was then assayed in a Packard gamma counter (Packard Instrument Co. Inc.). In control tubes containing no cells to which the labelled antibody

had been added less that 50 cpm of the added counts remained after washing. The indirect RIA results were expressed as the percentage binding of radioactivity by 10^6 cells, calculated using the following formula:

$$\% \text{ specific binding} = 100 \times \frac{\text{cpm experimental tube} - \text{cpm control macrophages with normal rat IgG}}{\text{Total cpm added}}$$

The amount of radioactivity in the control tubes containing cells incubated with normal rat IgG never exceeded 0.6% of the total counts added. As shown in Table 4.3, a population of macrophages obtained from S. enteritidis 11RX-infected mice appeared to express more Fc receptors than a normal macrophage population ($p<0.05$). Macrophages activated in vitro with a lymphokine-containing supernatant expressed an intermediate value. However, this value obtained from three separate experiments was not significantly different from the value obtained using normal macrophages ($0.05 < p < 0.1$).

Table 4.3

Quantitation of Fc and C3b receptors on the surface of macrophages of different populations by indirect RIA.

	% Radioactive binding /10^6cells[a]		
	Normal macrophages	11RX-activated macrophages	Lymphokine-activated macrophages
rat anti-mouse Fc receptor	1.3 ± 0.3	2.4 ± 0.6	1.6 ± 0.2
rat anti-mouse C3b receptor (MAS-034)	4.8 ± 0.6	3.6 ± 0.3	3.3 ± 0.5
Bactericidal[b] capacity	49 ± 7.1	6.1 ± 4.5	19.8 ± 7.8

a - Mean ± S.D from three separate experiments. Variation between tubes in each experiment was less than 5 per cent.

b - Percentage survival of S. typhimurium $C5^{SR}$ in the presence of culture medium alone at 60 min.

With respect to the C3b receptor, unlike the Fc receptor, their number appeared to be expressed more on a normal macrophage population than that obtained either from S. enteritidis 11RX-infected mice ($p < 0.05$) or a population that had been activated in vitro by a lymphokine-containing supernatant ($p < 0.05$). The indirect immunofluorescence (IF) staining using MAS-034 monoclonal antibody was also performed in conjunction with the indirect RIA on macrophages harvested from both normal and S. enteritidis 11RX-infected mice.

To obtain good cell surface staining, the peritoneal cells in 0.1M phosphate buffer, pH 7.4 containing 0.02% sodium azide ($1x10^6$), in nunc plastic tubes (two tubes for each population) were incubated for 1 hour on ice with 50 µl (0.2µg/ml) of the monoclonal antibody (MAS-034). Normal

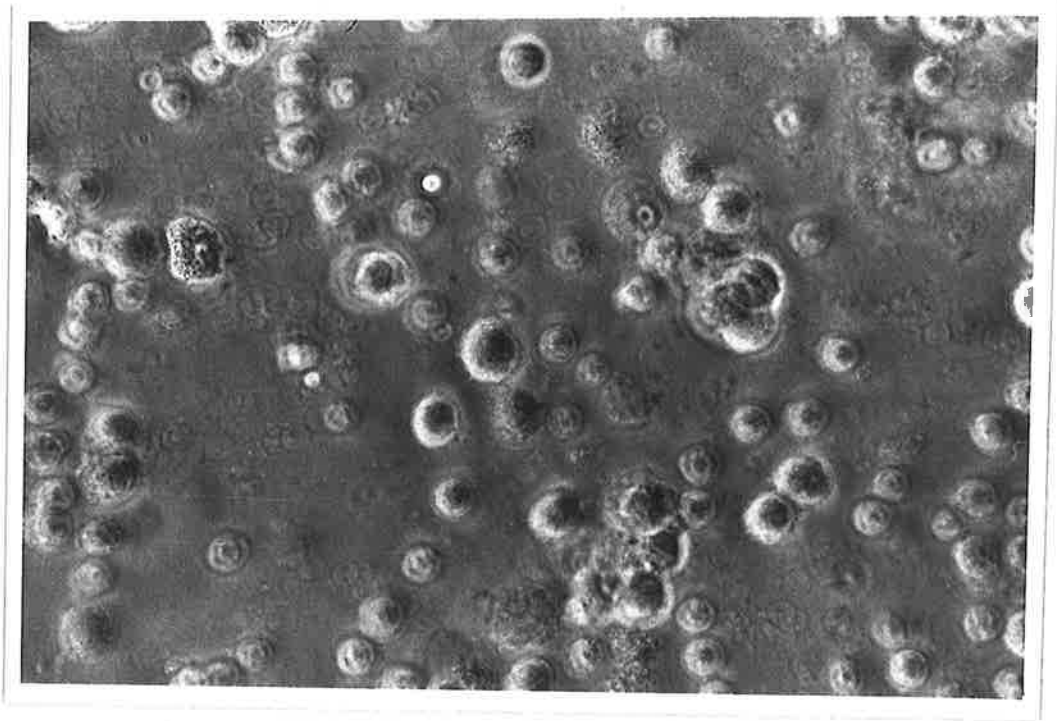
rat IgG added to one of the tubes was included as a control for non-specific binding. After incubation, the cells were washed three times by centrifugation at 400g for 5 min with 0.1M phosphate buffer, pH 7.4 containing 0.02% sodium azide (2ml). The cells were then incubated for 1 hour on ice with 0.1ml of a 1/50 dilution of a fluorescein-labelled rabbit anti-rat IgG (Nordi c Labs). Finally, the cells were washed as before and a drop of the cell suspension examined at a magnification of X400 using an ultra violet microscope (Olympus, Model BH-2 RFL-W). The IF staining demonstrated that all the macrophages in the S. enteritidis 11RX-activated cell population were stained less brightly than macrophages obtained from normal mice (Figs 4.1 and 4.2). This experiment was repeated twice and similar results were obtained.

The expression of C3b receptors on macrophages harvested from both normal and S. enteritidis 11RX-infected mice was also determined by an indirect RIA using another rat monoclonal antibody. This rat anti-mouse C3b receptor antibody, designated as NIMP-R10 was provided by Dr A. Lopez of Walter Eliza Hall, Melbourne. The indirect RIA using NIMP-R10 (0.2 µg/ml) was performed exactly as above. As was found before using MAS-034 monoclonal antibody, the NIMP-R10 monoclonal antibody showed that more C3b receptors were expressed on macrophages from normal mice than those obtained from 11RX-infected animals (Table 4.4) ($p < 0.001$).

Figure 4.1

The immunofluorescence staining of normal macrophages with rat anti-mouse C3b receptors monoclonal antibody (MAS-034). The same field was examined under ordinary light (A) and uv light (B) (X400).

A



B

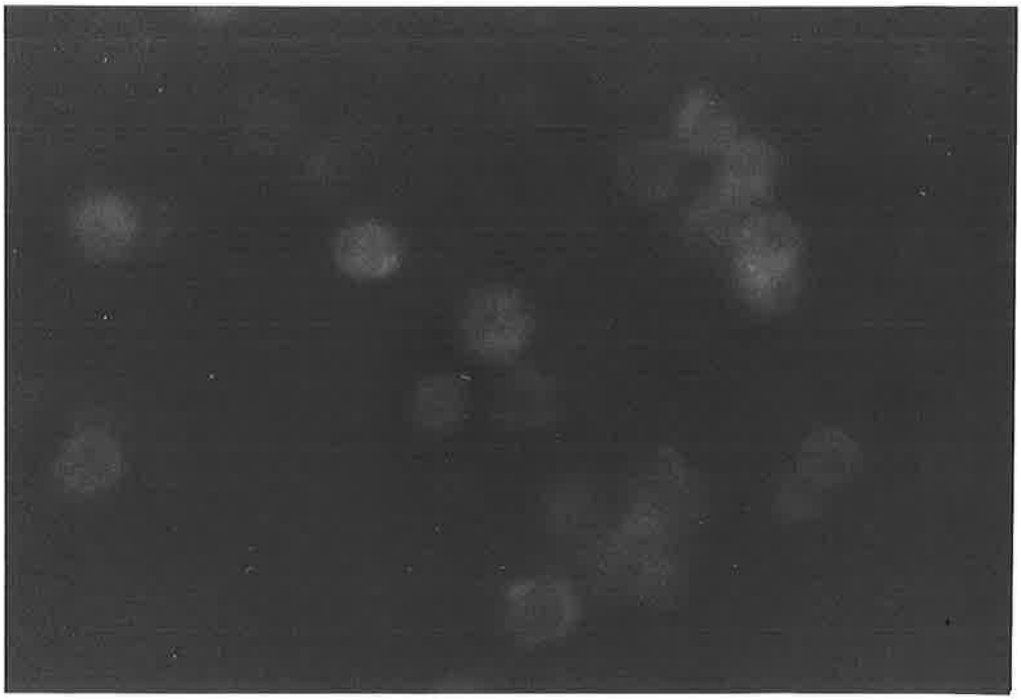
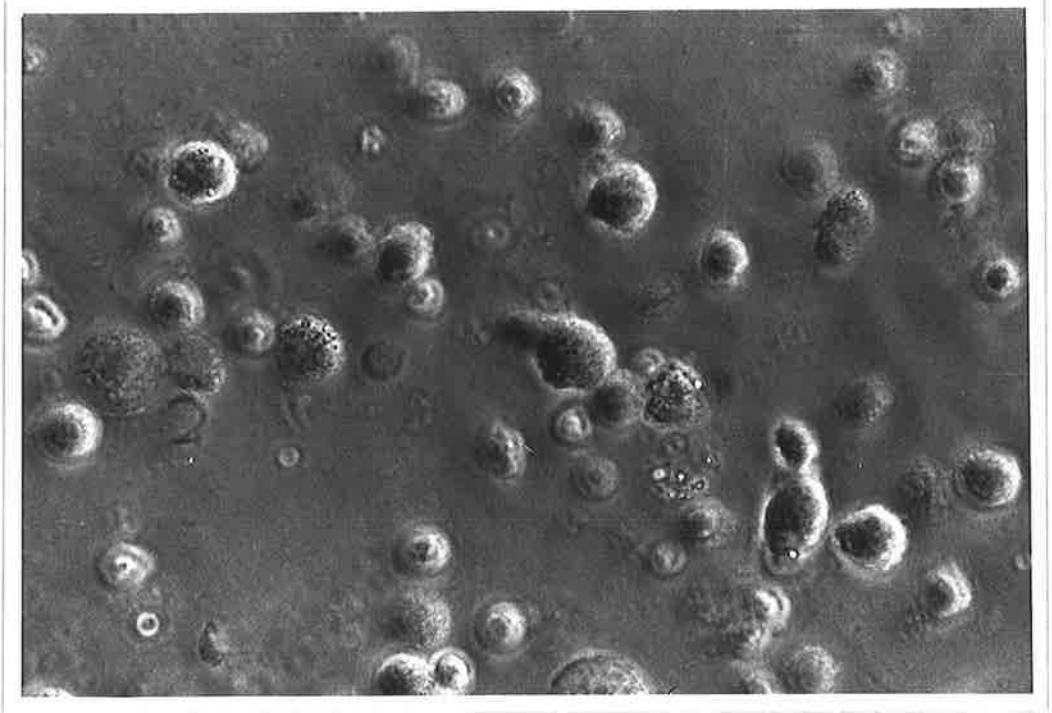


Figure 4.2

The immunofluorescence staining of macrophages from S. enteritidis 11RX-infected mice with a rat anti-mouse C3b receptors monoclonal antibody (MAS-034). The same field was examined under ordinary light (A) and under uv light (B) (X400).

A



B

Table 4.4

Quantitation of C3b receptor on the surface of macrophages harvested from both normal and S. enteritidis 11RX-infected mice using NIMP-R10 monoclonal antibody by indirect RIA.

Source of macrophages	% Radioactive binding /10^6cells[a]
Normal mice	4.0 ± 0.20
S. enteritidis 11RX-infected mice	2.23 ± 0.15

a - Mean ± S.D from three tubes.

These data show that the activation of macrophages results not only in an increase in their bactericidal activity but also in the number of Fc receptors. The finding that the number of C3b receptors do not increase suggests that these are not important for the expression of the enhanced bactericidal activity of these phagocytes.

4.4 QUANTITATION OF C3b RECEPTORS ON THE SURFACE OF NEUTROPHILS BY INDIRECT RIA

The previous observations on the enhancing effect of fresh serum on the bactericidal activity of neutrophils harvested from S. enteritidis 11RX-infected mice, endorsed the belief that they might express different numbers of C3b receptors than those from normal mice.

The expression of C3b receptors on neutrophils harvested from both normal and S. enteritidis 11RX-infected mice was measured by the indirect RIA as previously described (Section 4.3) using anti-Mac 1 antibody (MAS-034). The percentage binding of radioactivity per 10^6 neutrophils is shown in Table 4.5. In contrast to macrophages (Table 4.3), the expression of C3b receptors was

increased in the neutrophil population derived from mice infected with S. enteritidis 11RX ($p<0.001$), in comparison with the population obtained from normal mice.

Table 4.5

Quantitation by indirect RIA of C3b receptors on the surface of neutrophils harvested from both normal and S. enteritidis 11RX-infected mice using MAS-034 monoclonal antibody.

Source of neutrophils	% Radioactive binding /10^6cells[a]
Normal mice	2.86 ± 0.45
S. enteritidis 11RX-infected mice	6.13 ± 0.35

a - Mean ± S.D from three tubes.

4.5 THE EFFECT OF PRE-TREATMENT OF NEUTROPHILS FROM BOTH NORMAL AND S. enteritidis 11RX-INFECTED MICE WITH EITHER MAS-034 OR NIMP-R10 MONOCLONAL ANTIBODY ON THEIR BACTERICIDAL ACTIVITY

In view of the above observations (Table 4.5) that neutrophils obtained from normal mice and S. enteritidis 11RX-infected mice, expressed different numbers of C3b receptors on their membranes it was decided to study in more detail the role of this receptor in the bactericidal activity of these cells. Both NIMP-R10 and MAS-034 monoclonal antibodies were tested for their ability to block the bactericidal activity of the neutrophils.

Neutrophils (1-1.5x10^6 cells), in nunc plastic tubes obtained from either normal mice or S. enteritidis 11RX-infected mice (two tubes per population) were incubated for 90 min on ice with 0.5ml of either NIMP-R10 or MAS-034 monoclonal antibodies (0.2 µg/ml). Controls included the above cells incubated with normal rat IgG. After incub-

ation, the cells were washed once with tissue culture medium (2ml) and resuspended in the same medium (0.45ml). The ability of these treated cells to kill opsonized S. typhimurium M206^{SR} was then determined as previously described (Section 4.2). The cells which had been incubated with either MAS-034 and NIMP-R10 monoclonal antibodies were mixed with an equal volume (0.45ml) of opsonized S. ty phimurium M206^{SR} ($1x10^5$ bacteria) in the presence of 10% v/v fresh C6-deficient rabbit serum. The control cells were mixed with the bacteria as above in the presence of either fresh C6-deficient rabbit serum (10% v/v) or tissue culture medium. Further controls for bacterial growth were also included in which the bacteria alone were suspended in either fresh C6-deficient rabbit serum (10% v/v) or tissue culture medium. The bactericidal assay was performed in 96-well micro-culture trays (two trays) as described previously (Section 4.2). Three wells were sampled for each of the experimental conditions at time zero and at 60 min. As shown in Table 4.6, the number of bacteria killed, in the presence of fresh C6-deficient serum, by neutrophils from normal mice which had been preincubated with MAS-034 and NIMP-R10 monoclonal antibodies was much lower than those of control neutrophils in the presence of the same serum. It appeared from these results that both MAS-034 and NIMP-R10 monoclonal antibodies blocked the C3b receptor function. The monoclonal antibodies had a similar effect on neutrophils from S. enteritidis 11RX-infected mice, although the treated neutrophils were still highly efficient in killing the bacteria.

Table 4.6

The effect of pretreatment of neutrophils from normal mice and S. enteritidis 11RX-infected mice with MAS-034 and NIMP-R10 monoclonal antibodies on their bactericidal activity.

Source of neutrophils	Pretreatment	serum C6-deficient	Percentage survival of S. typhimurium M206^{SR} at 60 min	
			Experiment I	Experiment II
Normal mice	Normal rat IgG	+	29.8 ± 1.2[a]	32.7 ± 1.4
		-	51.3 ± 1.2	56.4 ± 0.7
	MAS-034	+	44.8 ± 0.8	49.1 ± 0.7
	NIMP-R10	+	41.1 ± 0.3	44.3 ± 0.6
S. enteritidis 11RX-infected mice	Normal rat IgG	+	3.5 ± 0.2	3.8 ± 0.1
		-	19.3 ± 0.3	21.2 ± 0.7
	MAS-034	+	15.5 ± 0.4	17.0 ± 0.5
	NIMP-R10	+	19.5 ± 0.4	22.2 ± 0.8

a - Mean ± S.D of triplicate cultures.

The results imply that the enhanced ability to kill bacteria by these cells does not correlate well with their number of C3b receptors.

4.6 INCUBATION OF MACROPHAGES WITH NIMP-R10 AND THEN WITH MAS-034 MONOCLONAL ANTIBODIES

Since the two rat monoclonal antibodies (NIMP-R10 and MAS-034) used in these studies were directed against mouse C3b receptors, it was of interest to know whether or not both of them bind to the same receptor. In order to determine this, experiments were carried out in which normal macrophages were incubated at first with NIMP-R10 and then with MAS-034 monoclonal antibodies as follows. Macrophages from normal mice ($1x10^6$ cells) in two nunc plastic tubes were first incubated for 90 min on ice with 0.5ml of NIMP-R10 monoclonal antibody (0.2μg/ml). Similar numbers of normal macrophages were also incubated as above with 0.5ml (0.2μg/ml) of either normal rat IgG (two tubes) or MAS-034 monoclonal antibody (one tube). After incubation, the cells were washed three times with 2ml of the RIA washing buffer (Chapter 2). After washing, the macrophages which had been incubated with normal rat IgG were then treated as follows. One tube was reincubated with either tissue culture medium (RPMI), whilst the other with a similar concentration of normal rat IgG (0.2μg/ml). The macrophages which had been incubated with MAS-034, were reincubated with tissue culture (RPMI) whilst the macrophages which had been incubated with NIMP-R10, were reincubated with either tissue culture medium (RPMI) or MAS-034. After incubation for 90 min on ice, the cells

were washed as before. Finally, they were incubated with ^{125}I-labelled rabbit IgG anti-rat IgG, washed and assayed for the binding of radioactivity as described before (Section 4.3).

The results obtained from three separate experiments (Table 4.7) show that incubating macrophages with NIMP-R10 and then with MAS-034 did not result in an increased amount of binding as detected using the labelled anti IgG. It seems, therefore, that both NIMP-R10 and MAS-034 monoclonal antibodies recognize the same receptor, although a greater proportion of MAS-034 monoclonal antibody binds to the receptor compared with the NIMP-R10 monoclonal antibody.

Table 4.7

Effect of incubation of normal macrophages with NIMP-R10 followed by incubation with MAS-034 monoclonal antibodies (the indirect RIA), on the total amount of monoclonal antibody bound.

Treatment of cells		% Radioactive binding /10^6cells[a]
First incubation	Second incubation	
Normal rat IgG	RPMI[b]	0.3 ± 0.1
Normal rat IgG	Normal rat IgG	0.35 ± 0.1
MAS-034	RPMI	6.4 ± 0.7
NIMP-R10	RPMI	3.4 ± 0.1
NIMP-R10	MAS-034	7.0 ± 0.7

a - Mean ± S.D of three separate experiments. Variation between tubes in each experiment was less than 5 per cent.

b - Medium for culturing macrophages (Chapter 2).

4.7 THE EFFECT OF VARIOUS DILUTIONS OF OPSONIZING SPECIFIC ANTIBODY ON THE BACTERICIDAL ACTIVITY OF MACROPHAGES AND NEUTROPHILS OBTAINED FROM *S. enteritidis* 11RX-INFECTED MICE

The finding that macrophages from *S. enteritidis* 11RX-infected mice possessed more Fc receptors than did normal macrophages, led to an investigation of the role of this receptor in the bactericidal activity of these cells. Changes in the density and avidity of Fc receptors on activated macrophages have been reported by Rhodes (1975). These studies suggested that activated cells might require lower levels of opsonizing antibodies than normal cells for phagocytosis. It was of interest to investigate whether or not macrophages from *S. enteritidis* 11RX-infected mice require lower levels of opsonizing antibodies to kill salmonellae than do neutrophils from the same mice.

The ability of the above cells to kill *S. typhimurium* $M206^{SR}$ in the presence of various dilutions of opsonizing specific antibody was determined as follows. Log-phase *S. typhimurium* $M206^{SR}$ was opsonized by incubating 5.0×10^6 bacteria/ml, in tissue culture medium with an IgG fraction from a rabbit anti-*S. typhimurium* $C5^{SR}$ serum (Chapter 2) at a final dilution of 1/1000, 1/5000 and 1/10,000 for 15 min at room temperature as previously described (Section 4.2). The bacteria were then washed once with tissue culture medium (10ml) and finally resuspended in the same medium at a concentration of $2\text{-}3 \times 10^5$ bacteria/ml. In nunc plastic tubes, 0.45ml of neutrophils or macrophages ($1\text{-}1.5 \times 10^6$ cells), obtained from *S. enteritidis* 11RX-infected mice

(Chapter 2) were mixed with 0.45ml of the opsonized bacteria ($1x10^5$ bacteria) as above in the presence of 0.1ml of 10% v/v fresh C6-deficient rabbit serum. The mixture (130µl) from each tube was transferred into three wells of three 96-well micro-culture trays (Flow Labs). Controls included bacteria alone without cells, incubated in fresh C6-deficient rabbit serum (10% v/v). The bactericidal assay was carried out as previously described (Section 4.2). Three wells were sampled at each time point. The results illustrated in Table 4.8 did not indicate that macrophages harvested from S. enteritidis 11RX-infected mice required lower levels of opsonizing antibody to kill S. typhimurium than did neutrophils harvested from the same mice.

Table 4.8

Effect of various dilutions of opsonizing specific antibody on the killing of S. typhimurium $M206^{SR}$ by macrophages and neutrophils harvested from S. enteritidis 11RX-infected mice, in the presence of fresh C6-deficient rabbit serum (10% v/v).

Dilutions of specific antibody	Type of cells	Percentage survival[a]	
		30 min	60 min
1/1000	macrophages	11.5 ± 1.9	4.0 ± 1.3
	neutrophils	13.0 ± 1.1	5.8 ± 0.7
1/5000	macrophages	29.2 ± 1.5	18.2 ± 1.7
	neutrophils	33.6 ± 2.1	21.1 ± 1.5
1/10,000	macrophages	34.3 ± 1.3	36.7 ± 1.2
	neutrophils	36.9 ± 0.7	40.3 ± 1.7

a - Mean ± S.D of triplicate cultures.

4.8 SCANNING ELECTRON MICROSCOPE (SEM) STUDIES OF NEUTROPHILS HARVESTED FROM BOTH NORMAL AND *S. enteritidis* 11RX-INFECTED MICE

Morphological studies using SEM on mouse macrophages activated by a supernatant from a mixed lymphocyte culture (MLC) have shown that these treated cells exhibited an increase in membrane activity, more numerous surface formations covering the entire surface, prominent ruffled, ridgelike profiles, fingerlike processes, and short, swollen microvilli which sometimes formed spherules, whereas normal macrophages showed little membrane activity, a relatively low number of ridgelike profiles, short ruffles, and microvilli which were confined to a central zone and did not extend over the entire surface (Nabarra, Cavelier, Dy and Dimitriu, 1978). These findings are similar to the previous observations by Mørland and Kaplan (1977).

Nabarra and colleagues (1978) have shown also that the surface morphology of the macrophage appears to play an important role in the destruction of target cells *in vitro*. In these studies, they observed strong contacts between activated macrophages and mastocytoma cells. In contrast, the attachment of normal macrophages with target cells was not very strong, and that normal washing was sufficient to separate them.

It would appear that, no ultrastructural study has been reported comparing the surface morphology of normal and 'activated' neutrophils. As a result, SEM studies were carried out on neutrophils harvested from both normal and

S. enteritidis 11RX-infected mice. The neutrophils ($1.5x10^6$ cells) were allowed to attach to poly-L-Lysine-coated aluminium slips (1 mg/ml) for 1 hour at room temperature. They were fixed, dried and coated for scanning electron microscopy using the method described by Gamliel, et al. (1983). Finally, they were examined at a magnification $6x10^3$ using an electron microscope with SEM mode (ETEC Autoscan), located at the Electron Optical Centre, The University of Adelaide. Macrophages obtained from both normal and S. enteritidis 11RX-infected mice were included for the purpose of comparison. The results confirmed the finding that activated macrophages were morphologically different from normal macrophages (Fig 4.3). Macrophages from S. enteritidis 11RX-infected mice showed more numerous formations covering the cell surface and more swollen microvilli. With respect to neutrophils, the SEM micrograph showed that there was not much difference between the surface of normal and 11RX-activated neutrophils (Fig 4.4).

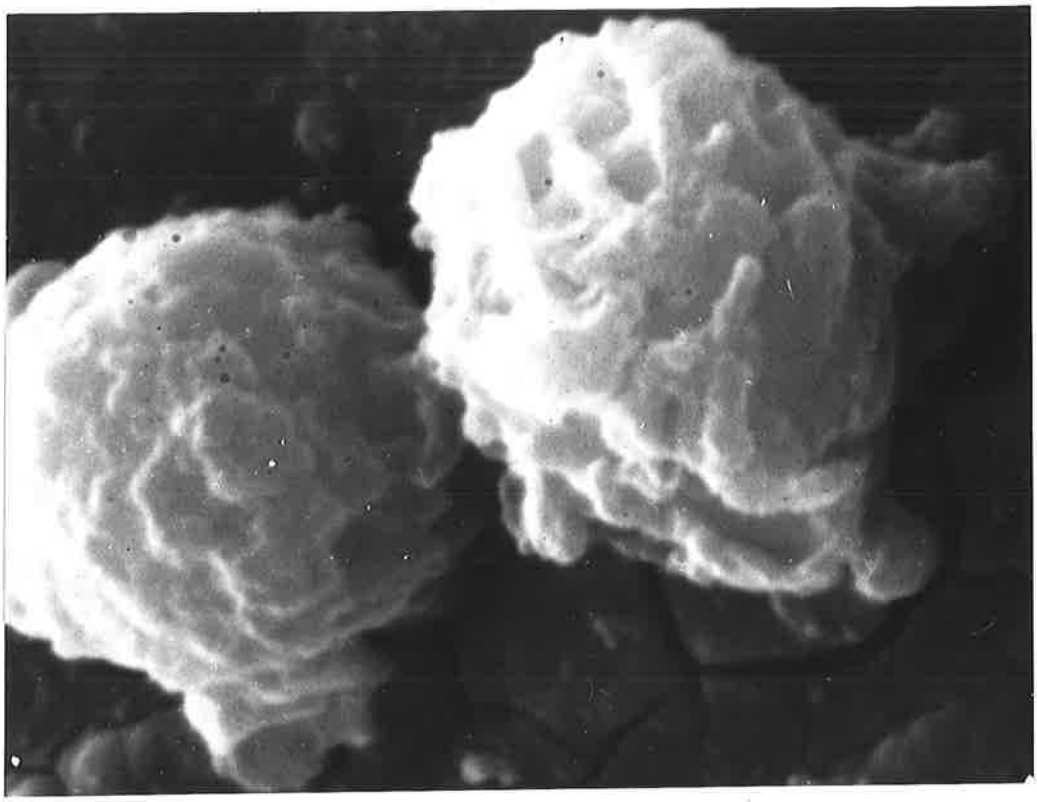
4.9 CONCLUSIONS

The important findings of these studies are that Fc and C3b receptor stimulation mediated by serum appeared to be important for the killing of salmonellae in vitro by both macrophages and neutrophils harvested from normal mice. Such stimulation was not required for the bactericidal activity of macrophages harvested from S. enteritidis 11RX-infected mice. However, with neutrophils obtained from the same infected mice, it appeared to enhance their

Figure 4.3

Scanning electron micrograph of macrophages from normal mice (A), and S. enteritidis 11RX-infected mice (B) (X6000).

A



B

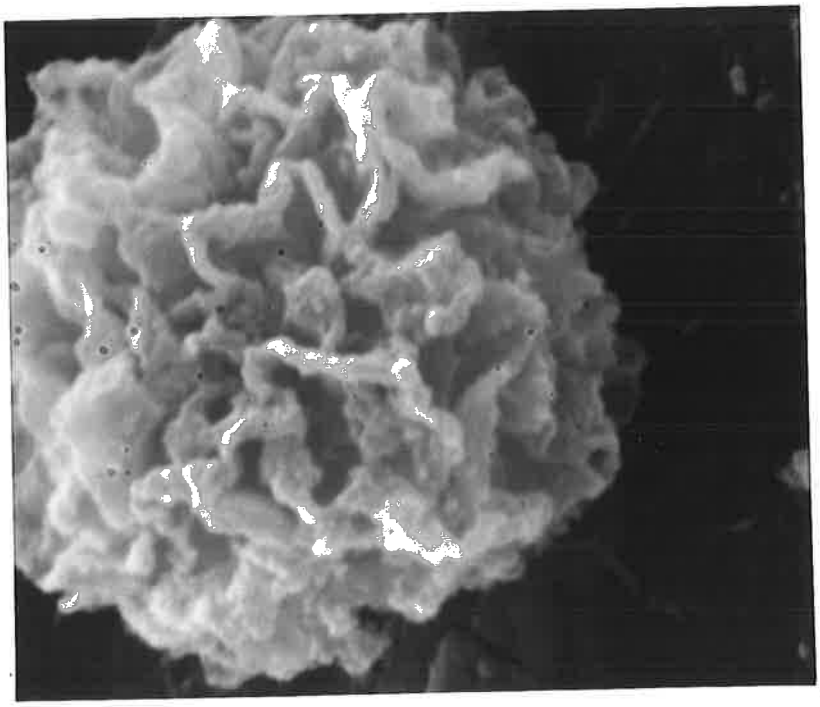
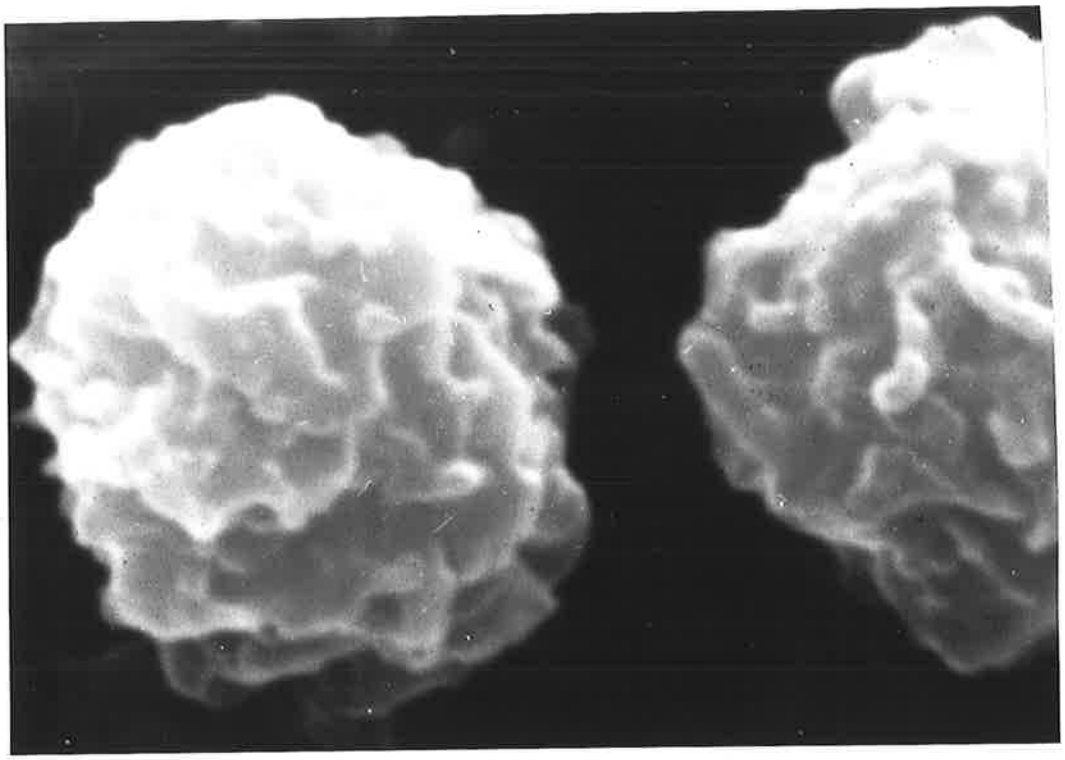


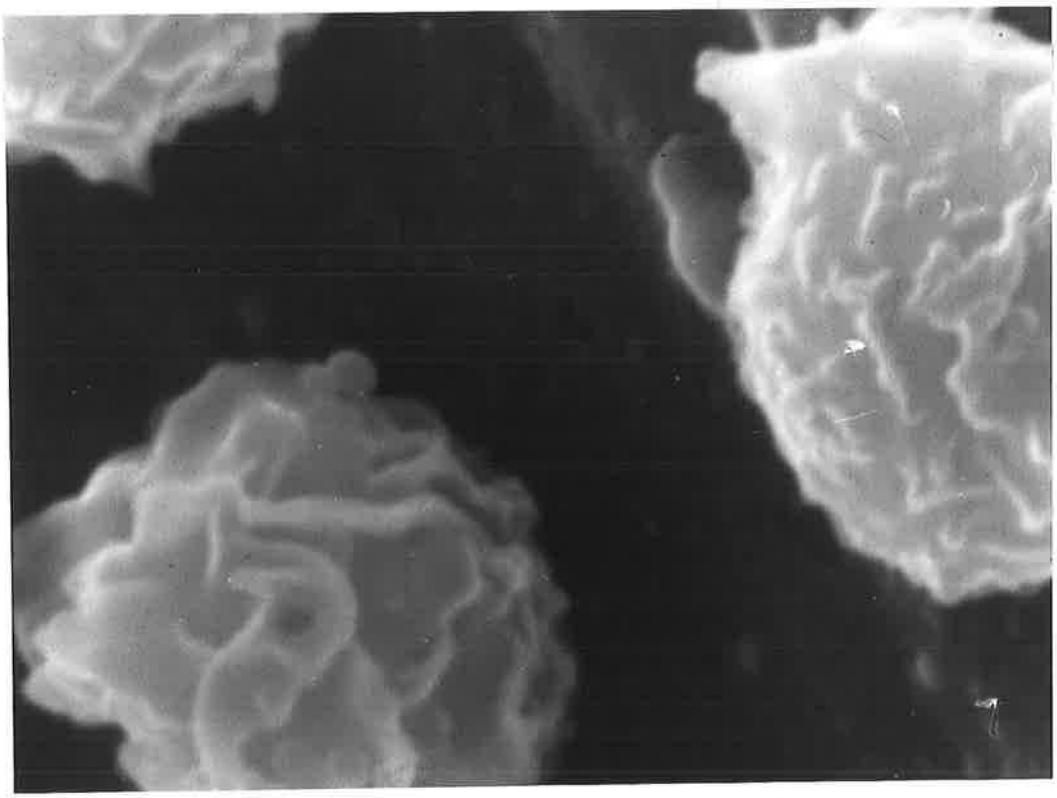
Figure 4.4

Scanning electron micrograph of neutrophils from normal mice (A), and S. enteritidis 11RX-infected mice (B) (X6000).

A



B



bactericidal properties, although the bactericidal capacity of these cells in the presence or absence of serum was very effective and greater than that of normal neutrophils.

Macrophages obtained from S. enteritidis 11RX-infected mice showed an increase in both bactericidal activity and number of Fc receptors but not C3b receptors. In contrast, neutrophils obtained from the same infected mice expressed an increase in the number of C3b receptors.

Scanning electron microscope studies showed that neutrophils obtained from normal mice and S. enteritidis 11RX-infected mice, unlike macrophages, are not morphologically different.

CHAPTER 5

5. THE ACTIVATION OF MACROPHAGES AND NEUTROPHILS BY LYMPHOKINES AS EXPRESSED BY THEIR ENHANCED BACTERICIDAL PROPERTIES

As described earlier (Chapter 1, p.3), Simon and Sheagren (1972) demonstrated that pre-incubation of normal macrophages with lymphocytes obtained from the peritoneal cavity or lymph nodes of BCG-immunized guinea-pigs, in the presence of BCG greatly enhanced the bactericidal activity of the macrophages against L. monocytogenes.

In the following experiment, an attempt was made to generate in vitro, a population of activated macrophages. A spleen cell suspension was prepared by the method described previously (Chapter 2) from mice which had been injected intravenously with 10^5 living S. enteritidis 11RX, two weeks earlier. The cell suspension was passed through a sterile nylon-wool column to remove adherent cells and the cells passing through the column were washed once with tissue culture medium (10ml) and finally resuspended in a similar volume. Monolayers of normal macrophages ($2x10^5$ cells) were prepared in two 96-well micro-culture trays (see Chapter 2). The 11RX-primed lymphocytes were then co-cultured with monolayers of normal macrophages (triplicate cultures in each tray) with or without 11RX antigens (10µg/ml) in a ratio of approximately 10 lymphocytes to 1 macrophage. The source of 11RX antigens was a protein extract, prepared from S. enteritidis 11RX by a technique previously described by Ashley, Kotlarski and

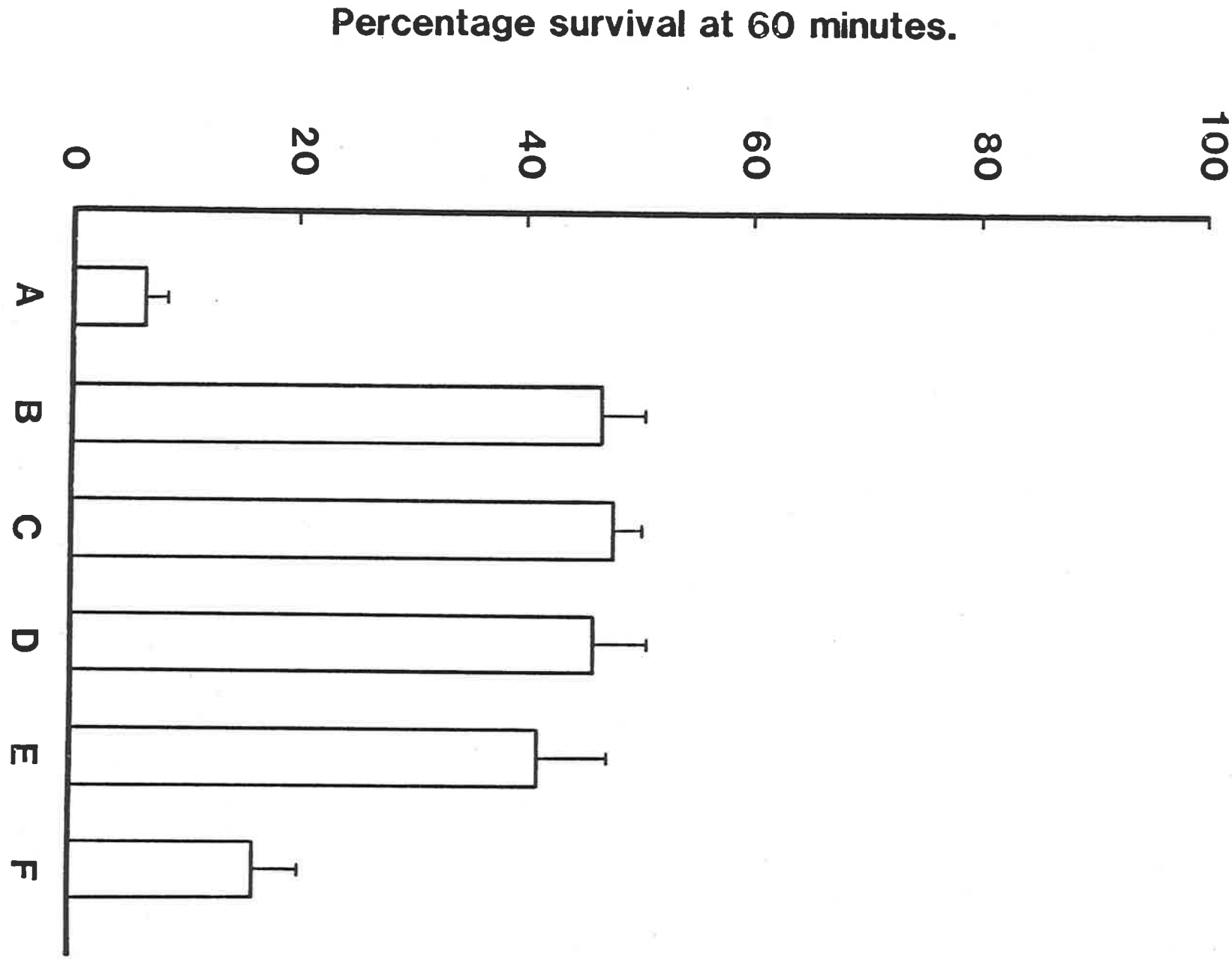
Hardy (1974) (kindly provided by Dr I. Kotlarski of this department). The cell cultures were then incubated at 37°C in 5% CO_2/air for 24 hours and finally washed three times with tissue culture medium (0.3ml) prior to assaying the bactericidal properties of the macrophages. Other controls included normal macrophage monolayers (triplicate wells in each tray) which were incubated as above with 1) lymphocytes from normal mice with the protein extract, 2) protein extract alone, and 3) tissue culture medium alone. Macrophages obtained from S. enteritidis 11RX-infected mice were also included in these experiments and incubated in the presence of tissue culture medium alone. After washing the cells, the bactericidal assay was carried out as previously described (Chapter 3, Section 3.2) by adding preopsonized S. typhimurium $C5^{SR}$ ($0.5-1x10^4$ bacteria) to the macrophages in the presence of tissue culture medium alone. Control wells containing bacteria in medium in the absence of macrophages were always included.

As shown in Figure 5.1, incubation of normal macrophages with lymphocytes obtained from S. enteritidis 11RX-infected mice following exposure to 11RX antigens, enhanced the ability of the phagocytes to kill virulent S. typhimurium $C5^{SR}$. However, the number of bacteria killed was lower than that obtained with macrophages activated in vivo by injecting the mice with S. enteritidis 11RX. Normal macrophages which had been exposed to 1) lymphocytes obtained from normal mice in the presence of 11RX-antigens, 2) 11RX-primed lymphocytes in the absence of 11RX-antigens, 3) 11RX-antigens alone, 4) tissue culture

Figure 5.1

The bactericidal activity of macrophages from S. enteritidis 11RX-infected mice towards S. typhimurium $C5^{SR}$ after preincubation with tissue culture medium (A), compared with macrophages from normal mice following preincubation with either tissue culture medium (B), 11RX antigens alone (10µg/ml) (C), normal lymphocytes + 11RX antigens (D), 11RX-primed lymphocytes without 11RX antigens (E), 11RX-primed lymphocytes with 11RX antigens (F).

Each histogram represents the mean percentage survival ± S.D of three separate experiments. Variation between wells in each experiment was less than 5 per cent.


Percentage survival at 60 minutes.
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A
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C
D
E
F

medium alone were much less efficient with regard to their bactericidal properties. These results demonstrated that mouse macrophages can be activated *in vitro* in terms of their ability to kill *S. typhimurium* $C5^{SR}$ by co-culturing them with antigen-primed lymphocytes derived from *S. enteritidis* 11RX-infected mice together with a protein extract from the same organism.

In view of these results, attempts were made to activate mouse macrophages and neutrophils using supernatants containing lymphokines.

5.1 PREPARATION AND DETERMINATION OF LYMPHOKINE ACTIVITY IN CON A-STIMULATED SPLEEN CELL CULTURE SUPERNATANTS

The mitogen Con A, a plant lectin, has been widely used to prepare lymphokine-containing supernatants from spleen cells (Nogueira and Cohn, 1978; Büchmuller and Mauel, 1979; Haidaris and Bonventre, 1981; Brummer and Stevens, 1984). In the present investigation, supernatants containing lymphokines were prepared as follows. A spleen cell suspension ($5-6x10^6$ cells/ml) was prepared as previously described (see Chapter 2). The cell suspension was dispensed in 10ml aliquots into six plastic tissue culture flasks (Flow Labs) and incubated at 37^oC in 5% CO_2/air. To three of the flasks Con A (Pharmacia) at a concentration of 3.0 µg/ml was added. After incubation for 24 hours, cell-free supernatants were obtained by centrifuging the pooled cultures from three flasks at 400*g* for 10 min at room temperature. After filtration through a Millipore membrane (0.45 µm), the supernatants were stored in

aliquots (1ml) at -70°C. To the control supernatants harvested from unstimulated spleen cell cultures (three flasks), a similar concentration of Con A was added after harvesting.

5.1.1 The effect of supernatants from Con A-stimulated spleen cells on macrophage spreading

One of the characteristics of activated macrophages is that, compared with normal macrophages, they have an enhanced ability to spread on glass (Blanden, Lefford and Mackaness, 1969; Mackaness, 1970). This criterion was selected at first to measure the activating properties of the Con A supernatants.

Normal mouse peritoneal cells (Chapter 2) containing $4x10^5$ macrophages (1ml) were cultured in a 24-well culture tray (Flow Labs) in the presence of either a control supernatant (30% v/v) or a Con A supernatant (three wells each). The tray was incubated at 37°C in 5% CO_2/air, after which non-adherent cells were removed by washing the wells three times with tissue culture medium (1ml). After washing a similar volume of either a control or a Con A supernatant (30% v/v) was added to the remaining cells. The cultures were further incubated at 37°C in 5% CO_2/air and examined (x100) at hourly intervals using an inverted microscope (Olympus, Model CK). In another experiment, for the purpose of comparison, peritoneal cells obtained from normal mice and S. enteritidis 11RX-infected mice were incubated in the presence of tissue culture medium alone. In contrast to macrophages from normal mice, macrophages

from S. enteritidis 11RX-infected mice tended to spread, more extensively over the wells of the culture tray (Fig 5.2). This difference was noticeable as early as 1 hour. After incubating macrophages from normal mice for six hours with a Con A supernatant, the cells had spread more uniformly and more extensively than those which had been incubated with the control supernatant (Fig 5.3). These findings indicated that Con A supernatants contained lymphokines that changed macrophages into a morphologically activated state. As a result of these observations, a more quantitative assay was used to measure the lymphokine activity in the Con A supernatants.

5.1.2 The effect of a Con A supernatant on the release of a plasminogen activator from mouse macrophages

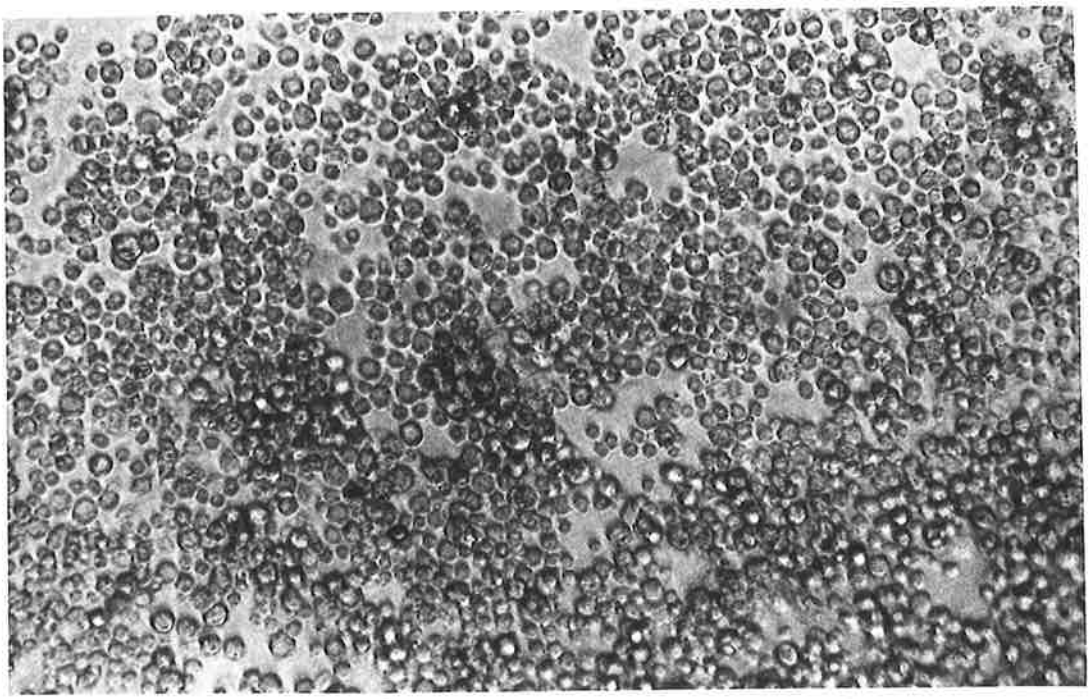
A plasminogen activator is one of the enzymes synthesized and secreted by macrophages. This neutral proteinase catalyzes the conversion of plasminogen to plasmin, a potent fibrinolytic enzyme (Unkeless, Gordon and Reich, 1974). A sensitive assay based on the degradation of ^{125}I-labelled fibrin has been developed to measure the release of plasminogen activator from macrophages (Gordon, Werb and Cohn, 1976).

Plasminogen activator has been shown to increase when exudates containing macrophages are induced by various inflammatory agents such as thioglycollate broth, or after infection by intracellular parasites such as BCG and subsequent challenge either in vivo or in vitro with specific antigen (Unkeless, Gordon and Reich, 1974; Gordon and

Figure 5.2

Three hour cultures of macrophages obtained from A) normal mice, and B) S. enteritidis 11RX-infected mice incubated in the presence of tissue culture medium alone (X100).

A



B

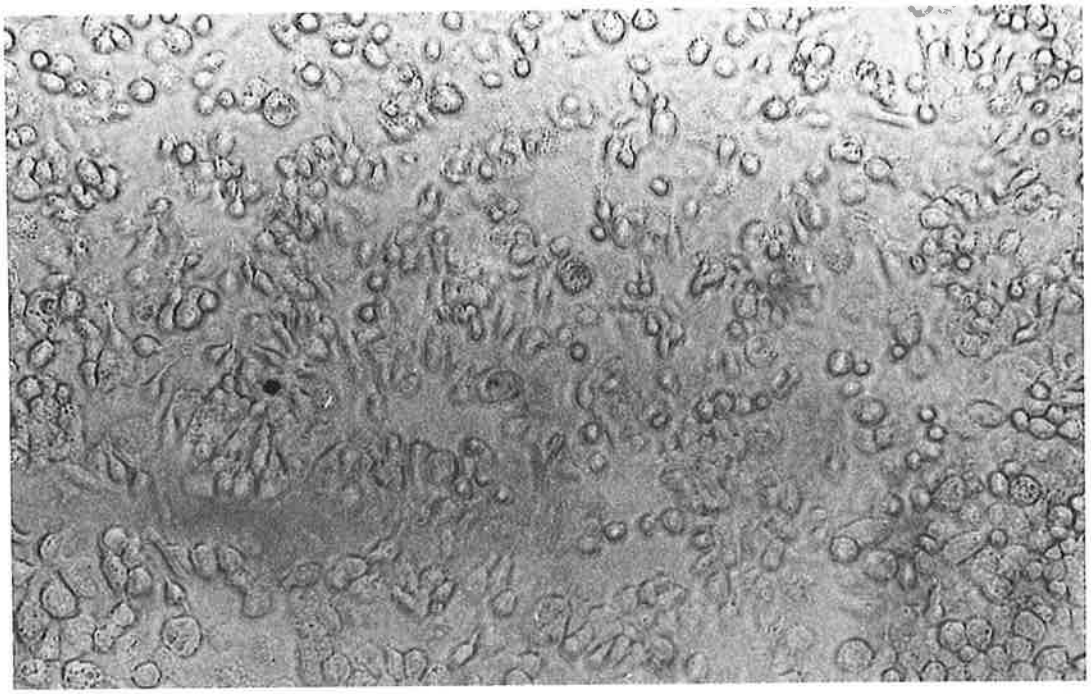
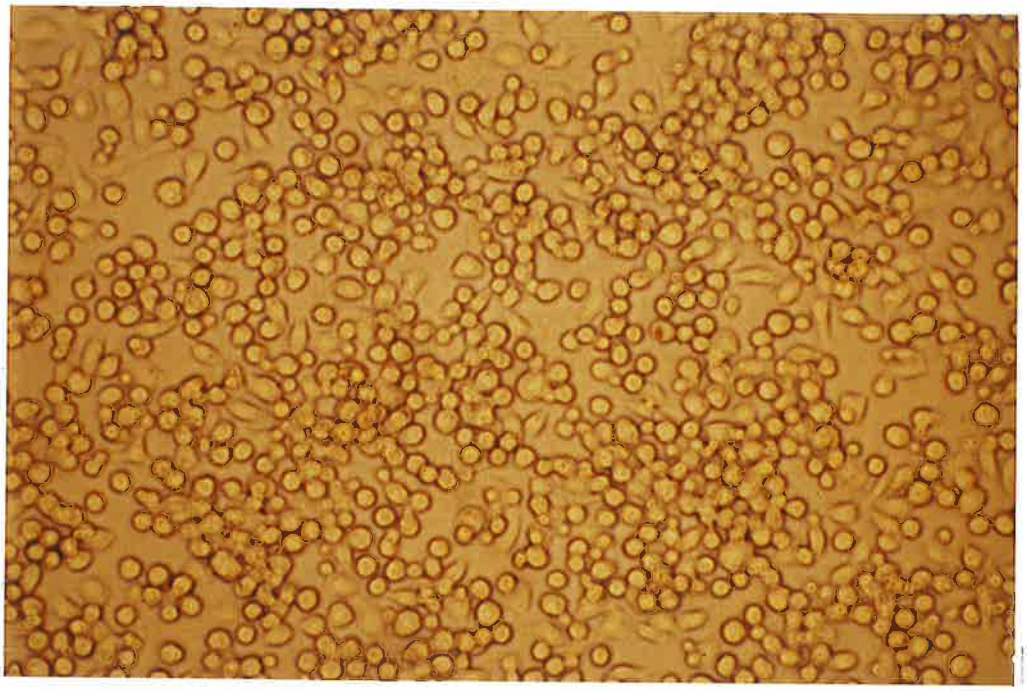


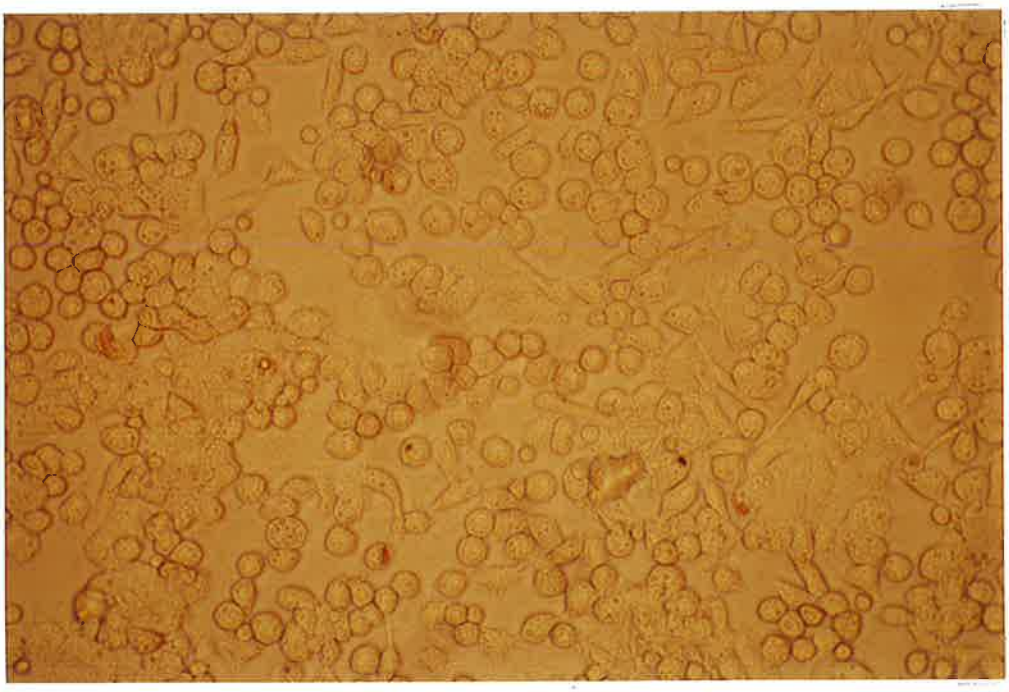
Figure 5.3

Six hour cultures of macrophages from normal mice incubated in the presence of A) a control supernatant, and B) a Con A supernatant (X100).

A



B



Cohn, 1978). Interestingly, supernatants derived from either Con A or antigen-stimulated spleen cell cultures have also been reported to induce the release in vitro of plasminogen activator from resident mouse macrophages (Vassalli and Reich, 1977; Klimatzek and Sorg, 1977; Gordon and Cohn, 1978). Since the assay for the plasminogen-dependent fibrinolytic activity is sensitive, quantitative and a good criterion for macrophage activation, it was selected as the method to determine the lymphokine activity of the Con A supernatants.

The assay was similar to that described by Gordon and Cohn (1978). ^{125}I-fibrin-coated trays were prepared by the method given in Chapter 2. A number of wells containing $4x10^5$ normal macrophages (0.5ml) in tissue culture medium supplemented with FCS (2% v/v) and soybean trypsin inhibitor (STI) (60µg/ml, Sigma) was set up. STI was used in order to prevent immediate fibrinolysis during culture. In six wells serving as controls (with no macrophages), tissue culture medium (0.5ml) was added. After incubation at 37°C in 5% CO_2/air for 30 min, all wells were washed twice with the medium (1ml). The macrophages were finally incubated again at 37°C in 5% CO_2/air for 24 hours in the presence of either a control supernatant or a Con A supernatant (three wells per supernatant) at a final concentration of 30% v/v in tissue culture medium containing STI (60 µg/ml). The six control wells (with no macrophages) were incubated as above with medium alone.

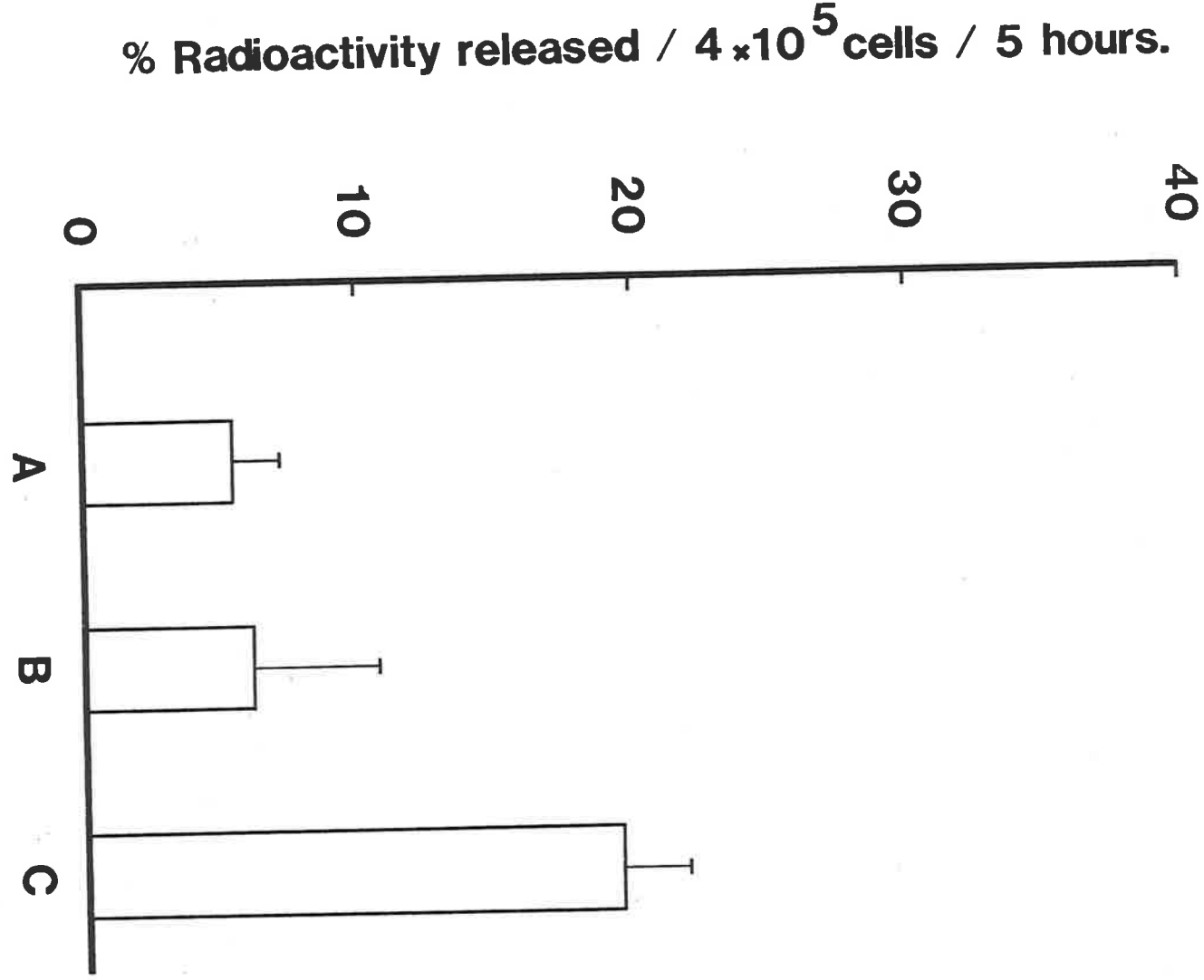
Fibrinolytic assays were initiated by first washing the macrophages and three of the control wells three times, each with 1ml of tissue culture medium and then adding 0.5ml of medium (without STI) containing 5% v/v acid-treated FCS (AT-FCS). AT-FCS, devoid of inhibitors was prepared by adding 1N HCl to 10ml of fresh FCS until the pH reached 3.2. After 2 hours at room temperature, the pH was adjusted back to 7.4 with 1N NaOH and the serum filtered through a Millipore filter (0.45 µm). A further three control wells containing no macrophages were also washed and then incubated with trypsin (0.02%) to measure the total radioactivity released following proteolysis. Aliquots of medium (100 µl) were withdrawn after incubation at 37°C in 5% CO_2 /air for 5 hours and assayed for radioactivity in a Packard gamma counter (Packard Instrument Co. Inc.). Under these conditions, fibrinolysis of ^{125}I-fibrin-coated trays is dependent only on the presence of plasminogen which is due to the release of a plasminogen activator from macrophages. The results expressed as a percentage were calculated on the amount of radioactivity released into the culture medium of the experimental wells over the total radioactivity released by trypsin digestion.

As seen in Figure 5.4, following incubation of normal macrophages with a Con A supernatant for 24 hours, there was enhanced fibrinolysis compared with the degree of fibrinolysis obtained in macrophage cultures exposed to a control supernatant ($p < 0.05$). These results indicated that the Con A supernatants contained lymphokines which

Figure 5.4

Fibrinolysis by macrophages cultured on ^{125}I-fibrin-coated trays. (A) control wells with no macrophages, (B) normal macrophages preincubated with a control supernatant, (C) normal macrophages preincubated with a Con A supernatant.

Each histogram represents the mean percentage of radioactivity released ± S.D of three separate experiments. Variation between wells in each experiment was less than 5 per cent.


% Radioactivity released / 4 x 10 5 cells / 5 hours.
0
10
20
30
40
A
B
C

caused the release of a plasminogen activator from the macrophages.

5.1.3 The effect of a Con A supernatant on the non-specific esterase activity of macrophages and neutrophils

Activation of macrophages leads to increased levels of certain intracellular enzymes such as peroxidase, esterase and hydrolases. There are, however, controversial reports on the levels of some of these enzymes synthesized by normal, compared with activated cells. For example, non-specific esterases have been found to be increased in activated macrophages when compared with normal cells (Hard, 1970), although Karnovsky, Drath and Lazdins (1976) reported that there was no difference. In view of these reports, the effect of Con A supernatants on the non-specific esterase activity of mouse macrophages was investigated. For comparative purposes the non-specific esterase activity of macrophages from S. enteritidis 11RX-infected mice was also studied. Similar experiments were carried out on neutrophils obtained from both normal and S. enteritidis 11RX-infected mice.

Normal macrophage monolayers (two for each experimental protocol), prepared on glass coverslips (see Chapter 2) were used. The monolayers were then incubated at 37^{o}C in 5% CO_2 /air for 24 hours with 1ml of tissue culture medium containing either a Con A supernatant, a control supernatant (30% v/v) or tissue medium alone. As mentioned above, other controls included 11RX-activated macrophages which were incubated in just medium. After incubation, the

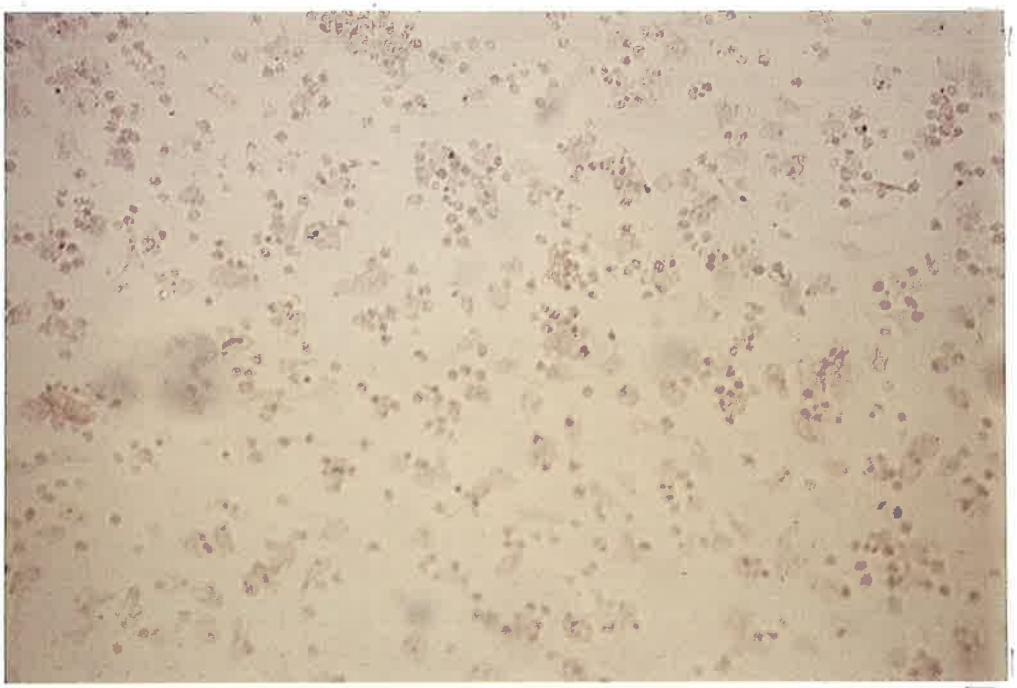
monolayers were washed twice with medium (1ml). In the case of neutrophils, the cells ($1x10^6$) obtained and purified from the peripheral blood of normal and S. enteritidis 11RX-infected mice (see Section 2.5) were cultured on glass coverslips (two coverslips for each population), which had been coated previously with poly-L-lysine (1mg/ml, Sigma), according to the method described by Mazia, Sale and Schatten (1974) for 1 hour at 37^oC in 5% CO_2 /air. After incubation, the cells were washed once with tissue culture medium (1ml) and then subjected to the same treatments as for macrophages. All the cultures were then fixed and stained for non-specific esterase activity as described in Materials and Methods, Section 2.8 and examined using a light microscope (Olympus, BH2) at a magnification of X400.

In comparison with normal macrophages, 11RX-activated macrophages exhibited a stronger and more diffused non-specific esterase activity (Fig 5.5). With respect to neutrophils, neither cells from normal nor S. enteritidis 11RX-infected mice displayed any sign of non-specific esterase activity (Fig 5.6). Interestingly, incubation of normal macrophages with the Con A supernatant resulted in an increase in the non-specific esterase, in contrast with those incubated with the control supernatant (Fig 5.7). This observation, together with the previous ones, implied that the Con A supernatants activated the macrophages. To support further these observations, the following experiments were carried out.

Figure 5.5

Staining of macrophages from normal mice (A), and S. enteritidis 11RX-infected mice (B) for non-specific esterase (X400).

A



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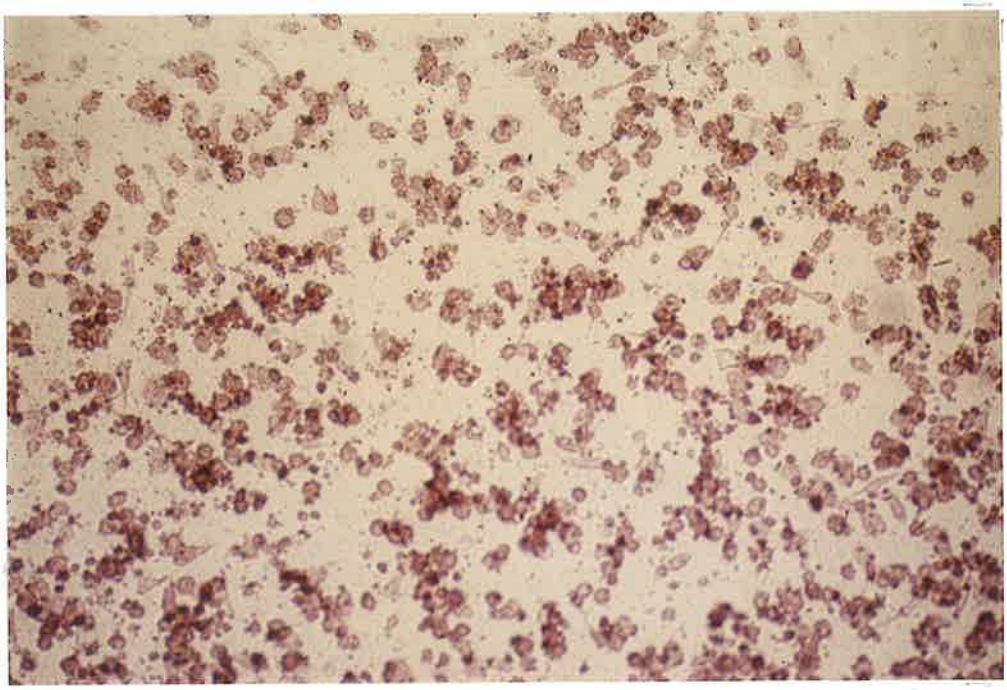
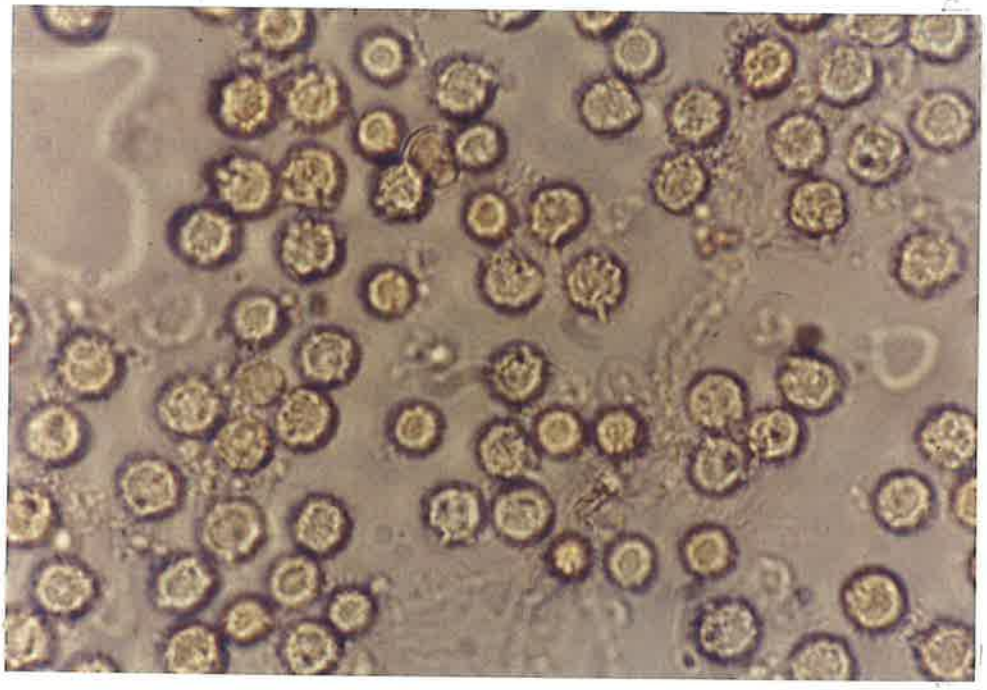


Figure 5.6

Staining of neutrophils from normal mice (A), and S. enteritidis 11RX-infected mice (B) for non-specific esterase (X1000).

A



B

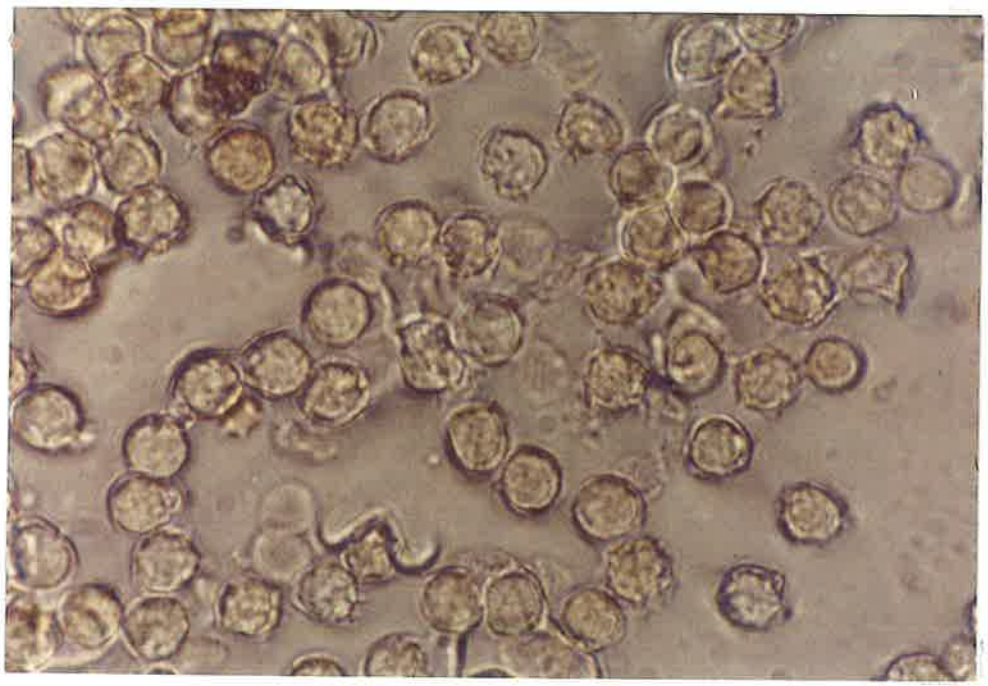
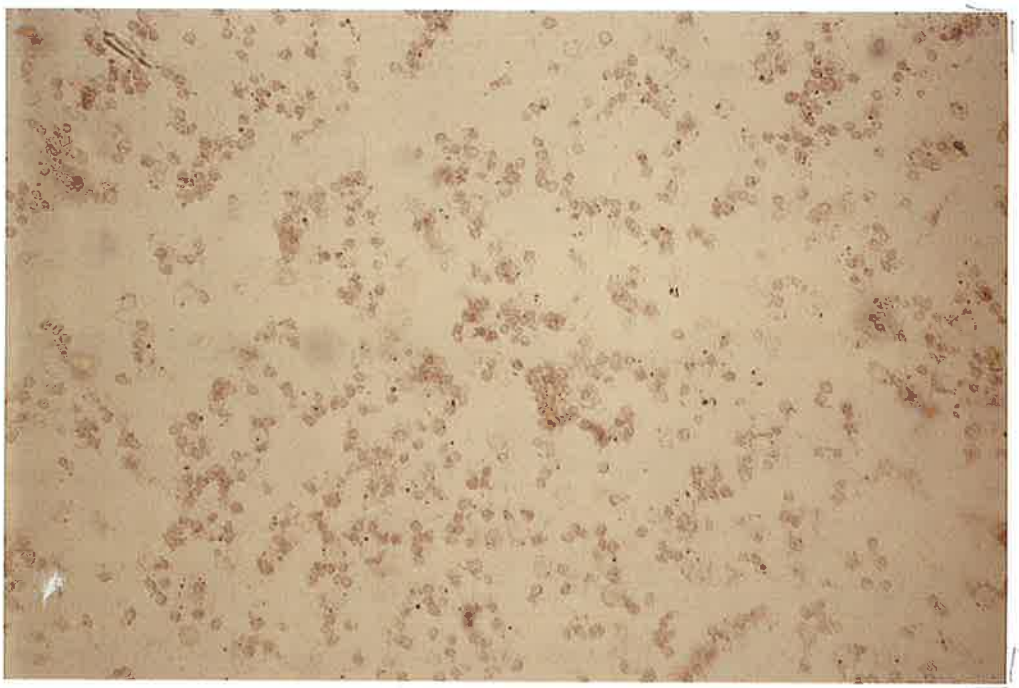


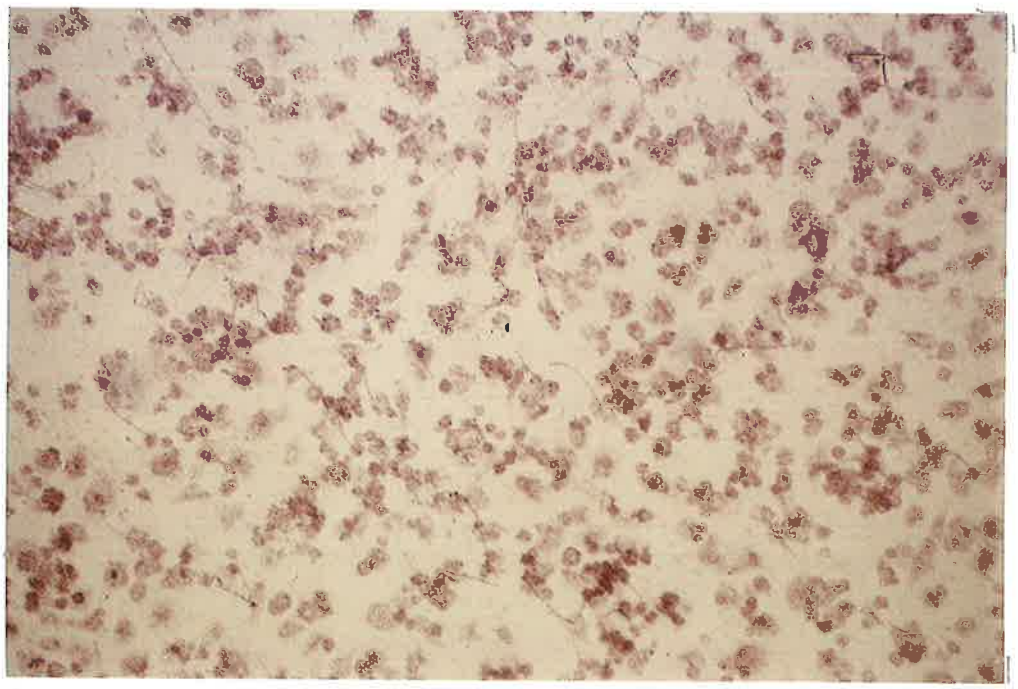
Figure 5.7

Staining of normal macrophages which had been pre incubated with a control supernatant (A), and a Con A supernatant (B) for non-specific esterase (X400).

A



B



5.2 THE EFFECT OF CON A SUPERNATANTS ON THE BACTERICIDAL ACTIVITY OF MOUSE MACROPHAGES

Previous data (Chapters 3 and 4) indicated that under certain conditions, macrophages obtained from salmonella-infected mice showed an enhanced ability to kill opsonized salmonellae *in vitro*, whereas macrophages obtained from normal, unstimulated mice were less able to do so. The greatest difference was observed when the cells were allowed to ingest the bacteria in serum-free medium. In view of these results it was of interest to see whether normal macrophages, following treatment with a Con A supernatant, had enhanced bactericidal properties as well as an alteration in enzyme levels reported above.

Normal macrophage monolayers were prepared in two 96-well micro-culture trays (Flow Labs) as described previously (Chapter 2). The monolayers ($2x10^5$ cells) were incubated at 37°C in 5% CO_2/air overnight in the presence of 0.3ml of either 1) tissue culture, 2) tissue culture medium containing 30% v/v of a control or 3) a Con A supernatant (three wells in each tray for each experimental condition). Monolayers of macrophages obtained from *S. enteritidis* 11RX-infected mice were also prepared and incubated as above in the presence of just medium. After incubation overnight, the monolayers were washed twice with medium (0.3ml) before starting the bactericidal assay. The bactericidal assay was performed as previously described (Chapter 3, Section 3.2) by adding to the monolayers, preopsonized *S. typhimurium* $C5^{SR}$ ($0.5-1x10^4$ bacteria) in the presence of tissue culture medium. Control wells

containing bacteria in medium alone were always included. The results of five separate experiments using different batches of Con A supernatant are displayed (Fig 5.8). Consistent with earlier findings 11RX-activated macrophages, in contrast to normal macrophages, showed an enhanced ability to kill opsonized S. typhimurium $C5^{SR}$ in the serum-free medium. The data also show that incubation of normal macrophages with a Con A supernatant, in comparison with a control supernatant, greatly alters their bactericidal potential. Indeed, such treated cells are able to kill bacteria as efficiently as those macrophages obtained from mice infected with S. enteritidis 11RX.

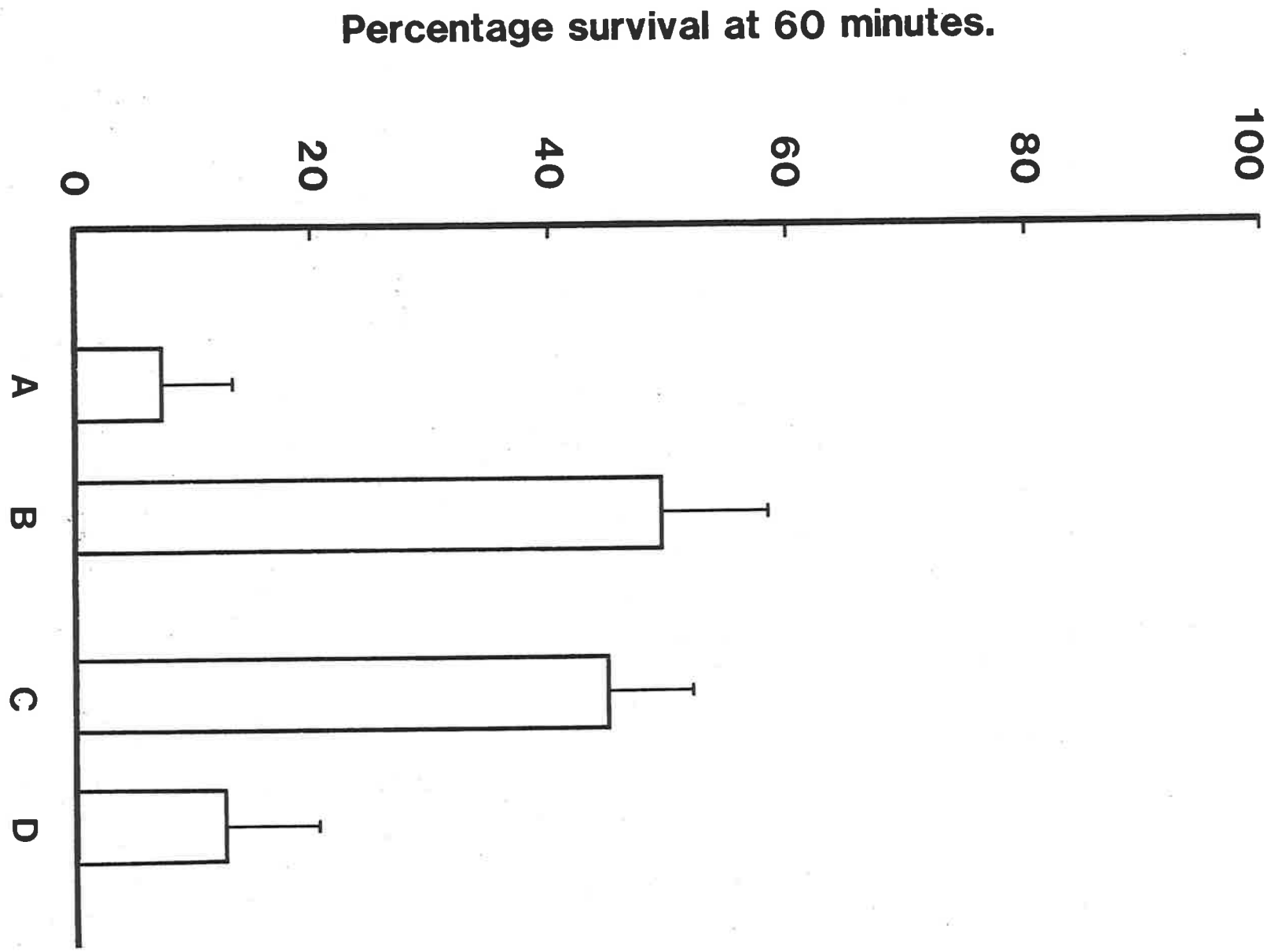
5.3 THE EFFECT OF A CON A SUPERNATANT ON THE BACTERICIDAL ACTIVITY OF MOUSE NEUTROPHILS

Since the previous experiments showed that a Con A supernatant increased the bactericidal activity of mouse macrophages, it was of interest to see whether or not mouse neutrophils could be altered in a similar fashion. Neutrophils were obtained and purified from the peripheral blood of both normal and S. enteritidis 11RX-infected mice as previously described in Materials and Methods, Section 2.5. Neutrophils ($1-1.5 \times 10^6$ cells) from normal mice were incubated in a nunc plastic tube (Medos) at $37^{o}C$ in 5% CO_2 /air for 3 hours in the presence of either tissue culture medium (0.5ml) or a similar volume of medium containing 30% v/v of either a control supernatant or a Con A supernatant. An incubation period of three hours was chosen based on a previous report that incubation of

Figure 5.8

The killing of opsonized S. ty phimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice, preincubated with tissue culture medium (A), compared with macrophages from normal mice preincubated with 1) tissue culture medium (B), 2) a control supernatant (C), or 3) a Con A supernatant (D).

Each histogram represents the mean percentage survival ± S.D of five separate experiments. Variation between wells in each experiment was less than 10 per cent.


Percentage survival at 60 minutes.
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A
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murine neutrophils, with a Con A supernatant for as little as 1 hour, enhanced their fungicidal activity against B. dermatitidis (Brummer and Stevens, 1984). A similar number of neutrophils from S. enteritidis 11RX-infected mice was incubated as above in the presence of tissue culture medium. After incubation, the cells were washed once by centrifugation with medium (2ml) and resuspended in the same (0.45ml). A log-phase culture of S. typhimurium $M206^{SR}$ was used in these experiments for a similar reason as mentioned in Chapter 4 (Section 4.2). Preopsonized S. typhimurium $M206^{SR}$ ($1x10^5$), suspended in 0.55ml of tissue culture medium containing 10% v/v fresh C6-deficient rabbit serum were added to 1) neutrophils obtained from S. enteritidis 11RX-infected mice, 2) neutrophils obtained from normal mice which had been preincubated with either a control supernatant or 3) a Con A supernatant. Neutrophils from normal mice which had been preincubated with tissue culture medium were infected with opsonized bacteria in the presence of medium alone. Controls included bacteria alone, suspended in either tissue culture medium or the same medium containing fresh C6-deficient rabbit serum (10% v/v). The contents from each tube were transferred to two 96-well micro-culture trays (130µl/well). The bactericidal assay was carried out as previously described in Chapter 4, Section 4.2. Three wells for each experimental and control protocol were sampled at time zero and at 60 min, one tray being used for each sampling time.

The results of two separate experiments are shown in Table 5.1. Normal neutrophils which had been exposed to a Con A supernatant exhibited an increased ability to kill opsonized S. typhimurium $M206^{SR}$, in comparison with those cells which had been exposed to a control supernatant. However, the number of bacteria killed was slightly lower than that observed using neutrophils obtained from S. enteritidis 11RX-infected mice.

Whether the same factor in the Con A supernatant was responsible for the alteration of both neutrophils and macrophages with regard to their bactericidal activity is not known.

5.4 THE EFFECT OF A SUPERNATANT FROM ANTIGEN-STIMULATED SPLEEN CELL CULTURES ON THE BACTERICIDAL ACTIVITY OF MACROPHAGES, COMPARED WITH THAT FROM SPLEEN CELLS STIMULATED BY CON A

Previous results (Fig 5.1) showed that co-culture of normal macrophages with splenic lymphocytes obtained from S. enteritidis 11RX-infected mice, in the presence of specific antigens, enhanced the ability of the macrophages to kill opsonized S. typhimurium $C5^{SR}$. In view of these results, it was of interest to compare supernatants from antigen-stimulated 11RX-primed spleen cell cultures with those from cultures stimulated with Con A, with regard to their capacity to enhance the bactericidal activity of macrophages.

Spleen cell suspensions were prepared by the method previously described (Chapter 2) from S. enteritidis 11RX-infected mice. The spleen cells ($5x10^5$) in tissue culture

Table 5.1

Killing of opsonized *S. ty phimurium* M206^{SR} by neutrophils obtained from *S. enteritidis* 11RX-infected mice, preincubated for 3 hours at 37°C in tissue culture medium, compared with neutrophils obtained from normal mice, preincubated with either a Con A supernatant, a control supernatant or tissue culture medium alone (RPMI).

Source of neutrophils	Preincubation (3h/37°C) with	C6-deficient rabbit serum	Percentage survival at 60 min	
			Experiment I	Experiment II
S. enteritidis 11RX-infected mice	RPMI	+	7.5 ± 0.5[a]	8.3 ± 0.4
Normal mice	a Con A supernatant	+	14.3 ± 0.4	13.1 ± 0.7
Normal mice	a control supernatant	+	31.3 ± 1.3	29.1 ± 0.9
Normal mice	RPMI	-	58.6 ± 0.8	53.8 ± 0.6

a - Mean ± S.D of triplicate cultures.

medium (0.1ml) containing 2% v/v HFCS were incubated at 37°C in 5% CO_2/air for 24 hours with or without 11RX antigens (see Section 5.0), in a 96-well micro-culture tray (Flow Labs). Cell-free supernatants were collected by centrifuging at 400g for 10 min at room temperature pooled cultures from 30 wells. After centrifuging, the supernatants were filtered through a Millipore membrane (0.45 µm). To the control supernatant, derived from cells incubated in the absence of 11RX antigens, was added the antigens at a concentration of 10µg/ml. The Con A supernatant and its control supernatant were prepared as previously described (Section 5.1).

The ability of these supernatants to enhance the bactericidal potential of normal macrophages against S. typhimurium $C5^{SR}$was then determined as before (Section 5.2). Normal macrophage monolayers ($2x10^5$ cells) were prepared in two 96-well micro-culture trays (Chapter 2). They were then incubated at 37°C in 5% CO_2/air overnight in the presence of either 1) the control supernatants (30% v/v) to which either 11RX antigens or Con A had been added, 2) the supernatant from Con A-stimulated or 3) a spleen cell culture stimulated with 11RX antigens and finally, 4) tissue culture medium alone. A further control included macrophages obtained from S. enteritidis 11RX-infected mice, incubated as above in the presence of just medium. Three monolayers in each tray were used for each of the above protocols. After overnight incubation, the monolayers were washed twice with tissue culture medium (0.3ml) and the bactericidal assay performed as previously

described (Chapter 3, Section 3.2) by adding to the monolayers, preopsonized S. typhimurium $C5^{SR}$ (0.5-1x10^4 bacteria) in the presence of medium. Control wells with bacteria in medium alone were included. One tray was sampled at time zero and the other at 60 min.

The results showed that incubation of normal macrophages with either a Con A supernatant or with a supernatant derived from antigen-stimulated spleen cells, increased the ability of macrophages to kill opsonized S. typhimurium $C5^{SR}$ to a similar extent (Fig 5.9). In contrast, the control supernatants, which were derived from unstimulated cultures of spleen cells to which either 11RX antigens or Con A had been added, had no stimulatory effect. The number of bacteria killed by macrophages which had been exposed to both a Con A- and an antigen-stimulated spleen cell culture supernatant was similar to that observed with macrophages from S. enteritidis 11RX-infected mice.

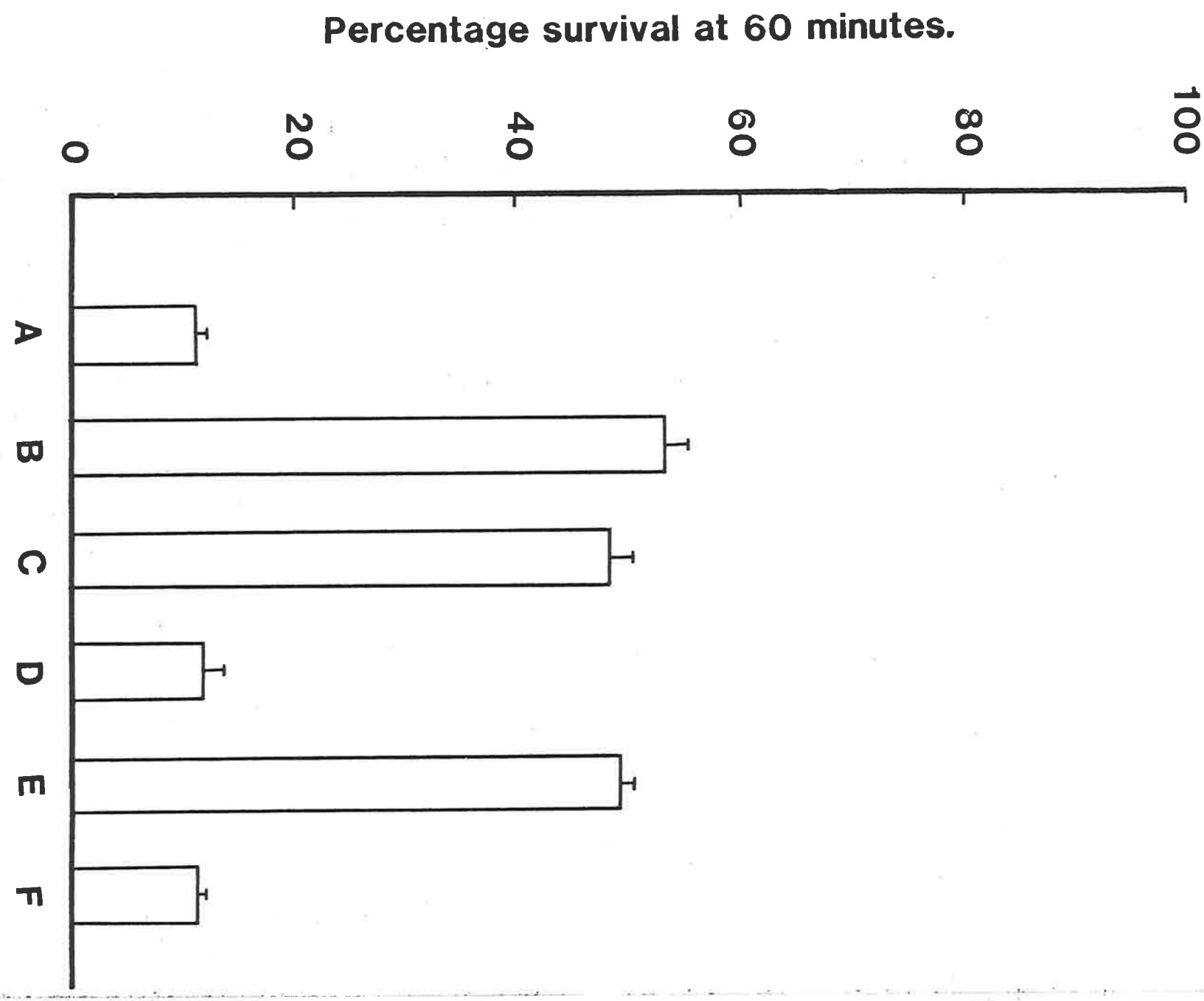
5.5 THE TIME REQUIRED FOR THE RELEASE OF LYMPHOKINES FROM CON A-STIMULATED SPLEEN CELL CULTURES AS MEASURED BY THE ABILITY OF THE SUPERNATANTS TO ALTER THE BACTERICIDAL PROPERTIES OF MACROPHAGES

For the purpose of this experiment, a spleen cell suspension (5-6x10^6 cells/ml) was prepared as previously described (see Chapter 2). The cell suspension was dispensed in 5ml aliquots into 20 plastic tissue culture flasks (Flow Labs) and incubated at 37°C in 5% CO_2/air, ten in the presence of Con A (3.0µg/ml) and another 10 in it's absence. After incubation for 12, 18, 24, 48 or 72 hours,

Figure 5.9

The killing of opsonized S. ty phimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice, preincubated with tissue culture medium (A) compared with macrophages from normal mice, preincubated with either 1) tissue culture medium (B), 2) a supernatant derived from unstimulated spleen cells to which Con A had been added (C), 3) a Con A supernatant (D), 4) a supernatant derived from unstimulated spleen cells to which 11RX antigens had been added (E), 5) an 11RX-stimulated spleen cell culture supernatant (F).

Each histogram represents the mean survival ± S.D of triplicate cultures.


Percentage survival at 60 minutes.
0
20
40
60
80
100
A
B
C
D
E
F

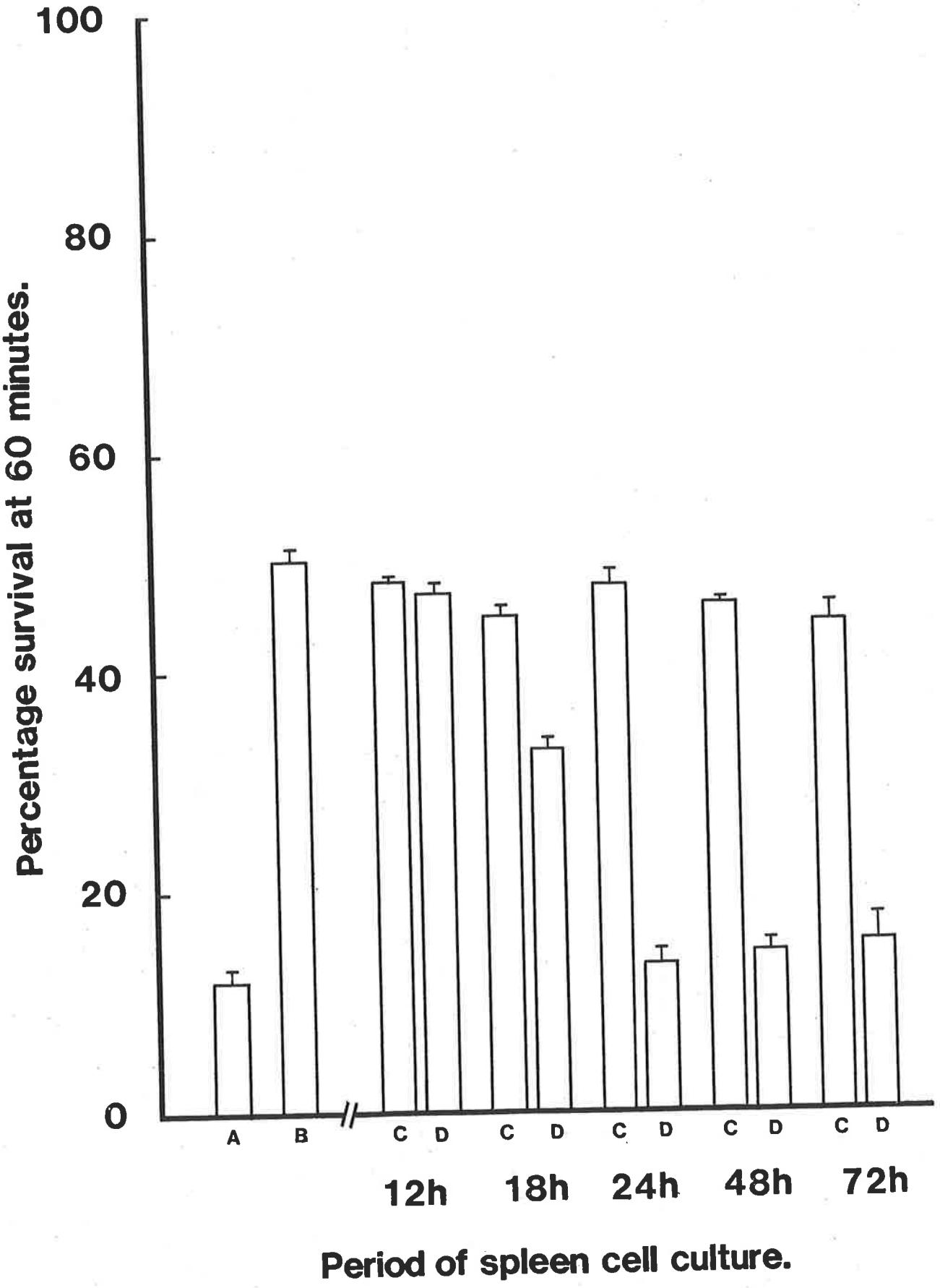
two flasks from each of the experimental and control series were removed at each time and cell-free supernatants from Con A-stimulated and unstimulated cultures were collected by centrifuging the pooled cultures from two flasks at 400g for 10 min at room temperature. The supernatants were then filtered through a 0.45μm membrane. After filtration a similar concentration of Con A was added to the unstimulated culture supernatants.

The ability of these supernatants, which had been collected at various times, to enhance the bactericidal activity of normal macrophages S. typhimurium $C5^{SR}$ was then determined. Normal macrophage monolayers ($2x10^5$ cells) were prepared in two 96-well micro-culture trays (Chapter 2). The monolayers (three wells per supernatant in each tray) were incubated at 37°C in 5% CO_2 /air for 24 hours. The volume of supernatant added was 0.3ml. Other controls included normal macrophages and macrophages obtained from S. enteritidis 11RX-infected mice, which were also incubated for the same period, in the presence of just medium. After incubation, the monolayers were washed twice with tissue culture medium (0.3ml). The bactericidal assay was performed as previously described (Chapter 3, Section 3.2). Control wells contained bacteria, incubated in medium alone. One tray was sampled at time zero the other at 60 min. As shown in Figure 5.10, supernatants derived from cultures stimulated with Con A for periods of time less than 18 hours were unable to enhance the bactericidal properties of the macrophages. Enhancement was noted with an 18-hour culture supernatant, and appeared to be maximal

Figure 5.10

The killing of opsonized S. ty phimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice, preincubated with tissue culture medium (A), and macrophages from normal mice, preincubated with either 1) tissue culture medium (B), 2) a control supernatant (C), 3) a Con A supernatant (D), harvested from spleen cell cultures at various times.

Each histogram represents the mean percentage survival ± S.D of triplicate cultures.


100
80
60
40
20
0
Percentage survival at 60 minutes.
A
B
C
D
C
D
C
D
C
D
C
D
12h
18h
24h
48h
72h
Period of spleen cell culture.

using supernatants following stimulation of the spleen cells for 24 hours or longer.

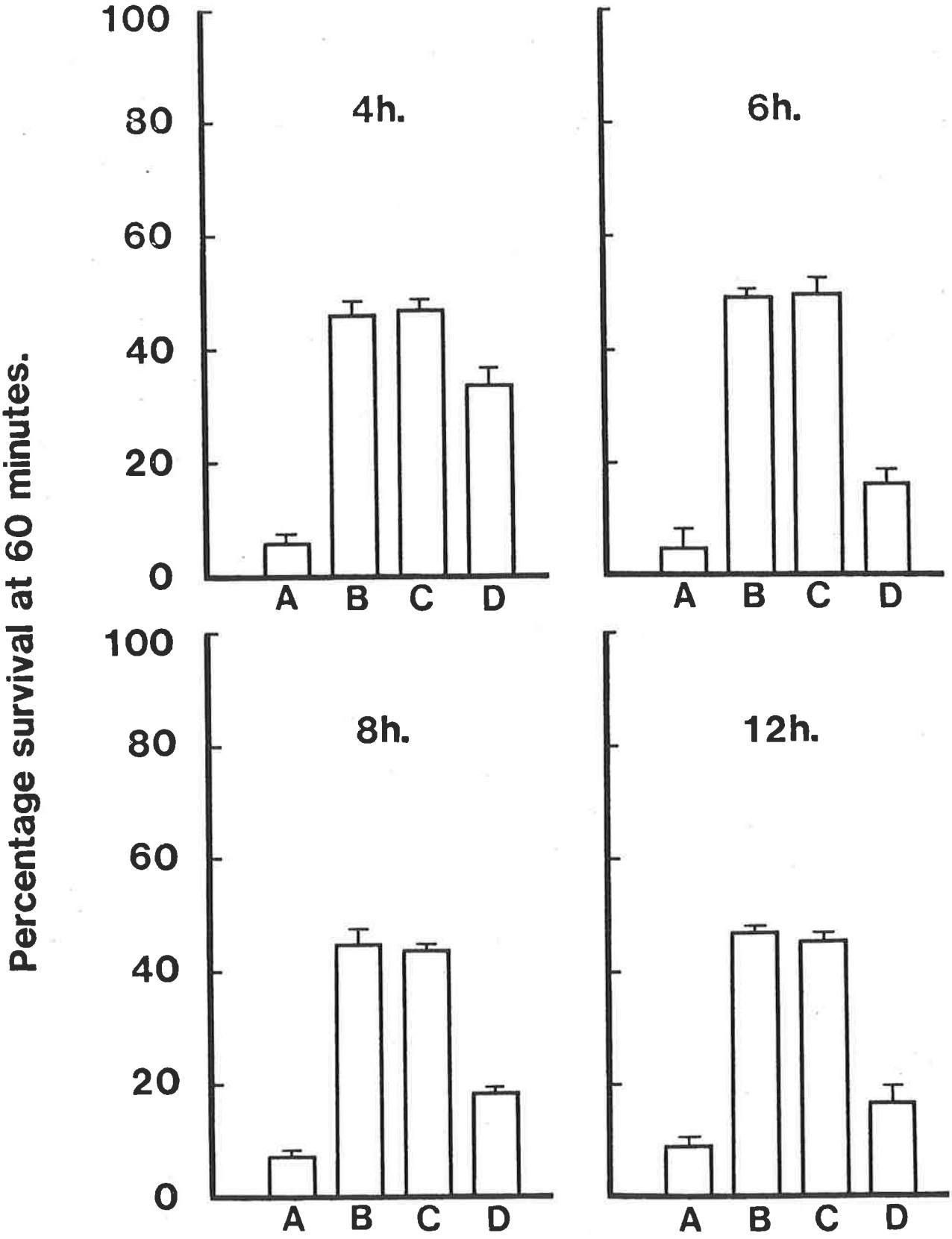
5.6 THE TIME REQUIRED FOR A CON A SUPERNATANT TO ACTIVATE MACROPHAGES AS MEASURED BY THEIR ENHANCED BACTERICIDAL ACTIVITY

The time required for a Con A supernatant to activate macrophages, as measured by an increase in their bactericidal activity, was determined as follows. A Con A supernatant and a control supernatant were harvested from a 24-hour spleen cell culture as before. Normal macrophage monolayers ($2x10^5$ cells) were prepared in 96-well microculture trays (Chapter 2). They were incubated at 37^oC in 5% CO_2/air for 4, 6, 8 or 12 hours in the presence of either a control or a Con A supernatant (0.3ml). Other controls included normal macrophages and macrophages obtained from S. enteritidis 11RX-infected mice, which were also incubated as above, in the presence of tissue culture medium alone. Three monolayers per tray were used for each experimental protocol. After incubation for the above intervals of time, the monolayers were washed twice with tissue culture medium (0.3ml) and the bactericidal assays performed as previously described (Chapter 3, Section 3.2). Control wells contained bacteria in medium. The data presented in Figure 5.11 show that in comparison with macrophages, which had been preincubated with either tissue culture medium or a control supernatant, macrophages incubated for a period as short as 4 hours in a Con A supernatant possessed enhanced bactericidal properties. These enhanced properties appeared to be maximal after the

Figure 5.11

The killing of opsonized S. typhimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice, preincubated with tissue culture medium (A), and macrophages from normal mice, preincubated either with tissue culture medium (B), a control supernatant (C), or with a Con A supernatant (D), for 4, 6, 8 and 12 hours.

Each histogram represents the mean percentage survival ± S.D of triplicate cultures.


Percentage survival at 60 minutes.
100
80
60
40
20
0
4h.
6h.
8h.
12h.
A
B
C
D

macrophages had been exposed to the supernatant for 6 hours or longer.

5.7 CONCLUSIONS

A supernatant derived from Con A-stimulated spleen cell cultures was shown to activate macrophages as measured by their enhanced bactericidal activity, degree of spreading, plasminogen activator release, and non-specific esterase activity. Such supernatants were also able to alter the microbicidal activity of neutrophils against S. typhimurium.

CHAPTER 6

6. HETEROGENEITY OF MOUSE MACROPHAGES IN TERMS OF THEIR BACTERICIDAL ACTIVITY

6.1 INTRODUCTION

Various studies involving monoclonal antibodies have shown that a population of macrophages is not homogeneous with regard to function (Chapter 1, Section 1.1.5). Sun and Lohmann-Matthes (1982) have found, for example, that four monoclonal antibodies, derived from the fusion of spleen cells from a rat immunized against mouse macrophages were cytotoxic, in the presence of complement to a proportion of the cells and capable of eliminating certain specific functions which the population exhibited. Two of the monoclonal antibodies designated M43 and M57 destroyed subsets of macrophages which killed antibody-coated tumour targets while M102 eliminated natural killer cells. A subset of macrophages recognized by M143, has not as yet been assigned a particular function. In view of these observations, it was of interest to see if some of these monoclonal antibodies were capable of pin-pointing a subset of macrophages, one of whose functions was antibacterial. The effect of these monoclonal antibodies (kindly provided by Dr Lohmann-Matthes, the Max-Planck-Institut für Immunbiologie, Postfach 1169, D-7800, Freiburg, FRG) plus complement on the bactericidal activity of a macrophage population obtained from S. enteritidis 11RX-infected mice was investigated in the following manner.

6.2 BACTERICIDAL ACTIVITY OF MACROPHAGES FOLLOWING TREATMENT WITH DIFFERENT MONOCLONAL ANTIBODIES IN THE PRESENCE OF COMPLEMENT

In nunc plastic tubes (Medos), $2x10^6$ macrophages from S. enteritidis 11RX-infected mice, suspended in 0.5ml of medium were incubated for 90 min on ice in the presence of the monoclonal antibodies at a concentration of 0.2µg/ml. Two tubes were used for each monoclonal antibody. Controls included macrophages from both normal and S. enteritidis 11RX-infected mice incubated as above with a similar concentration of rat IgG. After incubation, the cells were washed once with tissue culture medium (2ml) and re-incubated for a further 5 min with 100 µl, of a 1/30 dilution of a rabbit anti-rat IgG serum, as facilitating antibody (Sun and Lohmann-Matthes, 1982). The cells were washed again as before and 0.5ml of a 1/6 dilution of rabbit serum added as a source of complement. They were then incubated at 37°C in 5% CO_2/air for 90 min. Finally, the cells were washed twice with tissue culture medium (2ml) and their viability determined by trypan blue exclusion. Following treatment with either M43 or M57 monoclonal antibody plus complement, 60% of the macrophages remained viable while with M143 monoclonal antibody, the viability was around 70%. Approximately 85% of the control cells incubated with normal rat IgG were viable after the above treatment.

The ability of the remaining cells to kill opsonized S. typhimurium $C5^{SR}$ was then tested in 96-well microculture trays. A similar number of viable macrophages

($2x10^5$) from each tube was added to the wells. The bactericidal assay was carried out as previously described (Chapter 3, Section 3.2) by adding to the macrophages, preopsonized S. ty phimurium $C5^{SR}$ ($0.5-1x10^4$ bacteria) in the presence of tissue culture medium alone. Control wells contained bacteria in the absence of macrophages. For each protocol two wells were sampled at time zero and at 60 min. The results from three separate experiments are shown in Figure 6.1. The data show that pre-treatment of a 11RX-activated macrophage population with M43 and M57 monoclonal antibodies plus complement, resulted in a decrease ($p<0.05$) in the number of opsonized S. ty phimurium $C5^{SR}$ killed by these cells, compared with those treated with normal rat IgG, whereas M143 monoclonal antibody had no effect. These data indicate that there are subsets of cells in the macrophage population which are in part responsible for the bactericidal activity displayed by that population.

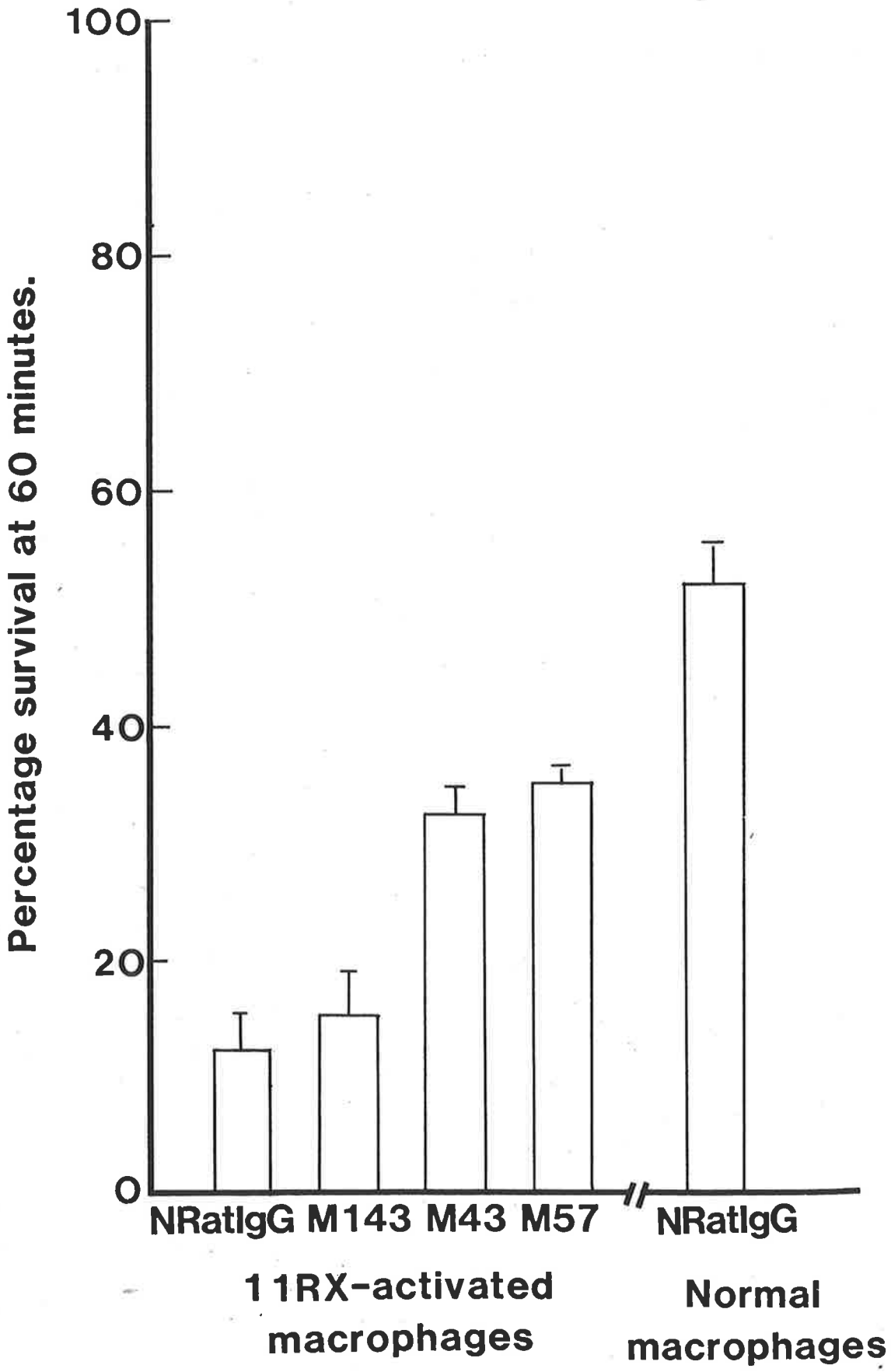
6.3 EXPRESSION OF CELL-SURFACE MARKERS IN NORMAL AND 11RX-ACTIVATED MACROPHAGE POPULATIONS RECOGNIZED BY MONOCLONAL ANTIBODIES

In view of the above results, it was of interest to determine the distribution of these markers in normal and 11RX-activated macrophage populations by indirect immunofluorescence (IF). This was carried out as described earlier (Chapter 4, p.68). Results from these experiments revealed that only a proportion of the cells (20-30%) from both a normal and 11RX-activated population of macrophages was stained positively with M43 monoclonal antibody (Figs.

Figure 6.1

The killing of virulent opsonized S. typhimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice pre-treated with various rat monoclonal antibodies to mouse macrophages in the presence of complement. Controls included macrophages from both normal and infected mice pre-treated with normal rat IgG.

Each histogram represents the mean percentage survival ± S.D of three separate experiments. Variation between wells in each experiment was less than 5 per cent.


100
80
60
40
20
0
Percentage survival at 60 minutes.
NRatIgG
M143
M43
M57
NRatIgG
11RX-activated macrophages
Normal macrophages

6.2 and 6.3). Similar results were also observed with M57, but only 10-20% of the total cell population were stained with M143 monoclonal antibody (data not shown).

It was also clear from these studies that those cells staining in the population of macrophages obtained from S. enteritidis 11RX-infected mice were more intensely stained than those present in the population recovered from normal animals.

In conjunction with these studies, the release of ^{51}Cr from prelabelled macrophages was followed after addition of the monoclonal antibodies and complement. The method followed was that described by Sun and Lohmann-Matthes (1982). Macrophages from S. enteritidis 11RX-infected mice in a nunc plastic tube were first labelled with ^{51}Cr by incubating $2x10^7$ cells for 40 min at 37°C in 1.0ml of tissue culture medium plus 10% FCS containing 500 µCi sodium chromate (^{51}Cr) (Amersham). The cells were washed three times with PBS and adjusted to a concentration of $3x10^6$ cells/ml in tissue culture medium. An aliquot (100µl) of the cell suspension containing $3x10^5$ labelled macrophages was dispensed into 18 wells of a 96-well microculture tray. The tray was then incubated at 37°C in 5% $CO/_2$ air for 30 min. After incubation, the monolayers were washed once with tissue culture medium (0.3ml). The monolayers were then incubated with 50µl of medium containing 0.2µg/ml of the monoclonal antibodies (three wells for each monoclonal antibody). Controls included monolayers (three wells) incubated with a similar concentration of

Figure 6.2

The immunofluorescence staining of macrophages from normal mice with M43 monoclonal antibody, examined under a microscope using ordinary light (A) and uv light (B) (X400).

A

B

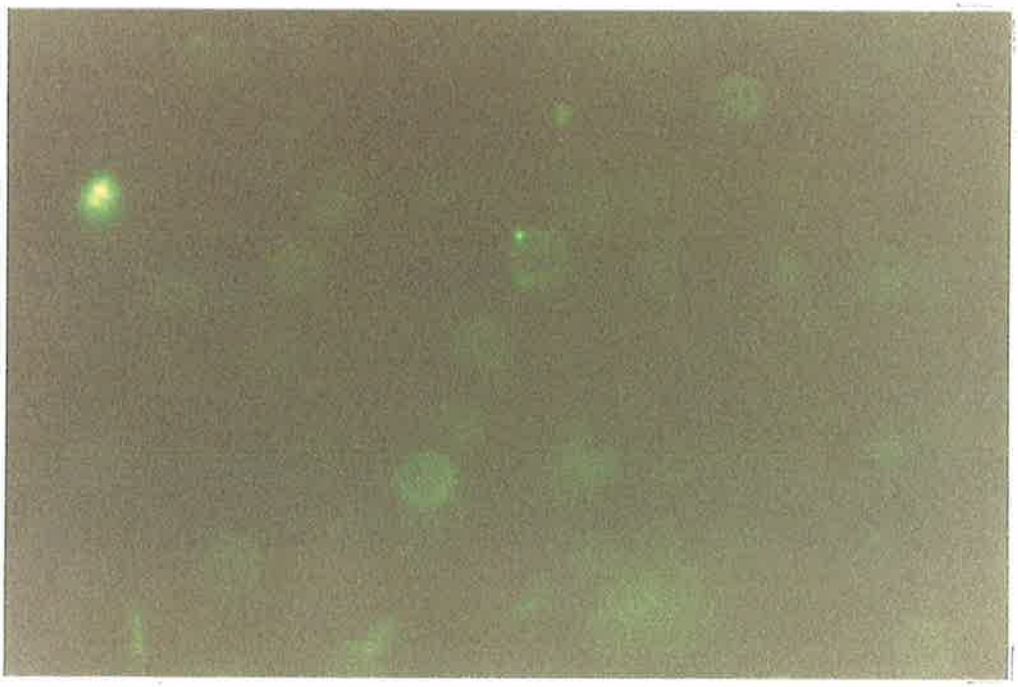
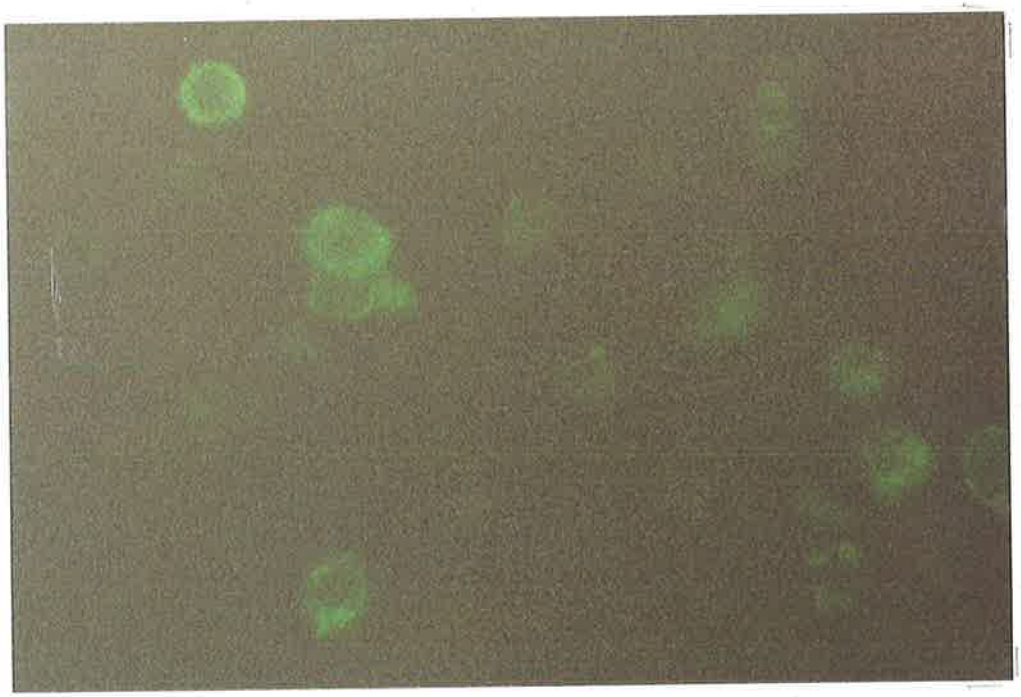


Figure 6.3

The immunofluorescence staining of macrophages from S. enteritidis 11RX-infected mice with M43 monoclonal antibody, examined under a microscope using ordinary light (A) and uv light (B) (X400).

A

B



normal rat IgG and monolayers (six wells) incubated with tissue culture medium alone. The tray was incubated for 40 min at 37°C in 5% CO_2/air. After this period of time the monolayers were washed twice with tissue culture medium (0.3ml) and incubated with 50µl of a 1/30 dilution of a rabbit anti-rat IgG serum for a further 5 min. The monolayers were then washed twice as before and 100µl of a 1/6 dilution of fresh rabbit serum added as a source of complement. The tray was then incubated once more for 90 min at 37°C in 5% CO_2/air. After incubation one-half of the cell-free supernatant (50 µl) was removed from each well and the radioactivity assayed in a Packard gamma counter (Packard Instrument Co.). To determine the total amount of radioactivity that could be released, control monolayers which had been incubated with medium alone (three wells) were treated with 100µl of trypsin (0.02%). Percentage cytotoxicity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = \frac{100 \times \text{amount of radioactivity (cpm) in the experimental supernatants}}{\text{Amount of radioactivity (cpm) in supernatants from control wells treated with trypsin}}$$

The data from three separate experiments, shown in Table 6.1 substantiate the previous findings that only a proportion of cells in the macrophage population expressed the cell-surface markers recognized by the monoclonal antibodies.

Table 6.1

Cytotoxicity of rat anti-macrophage monoclonal antibodies plus complement (c) for 11RX-activated macrophages.

Treatment of cells	% ^{51}Cr release[a]
Normal rat IgG + C	17.1 ± 2.9
Rabbit anti-rat IgG + C	18.7 ± 2.4
M57 + C	49.7 ± 5.8
M43 + C	41.7 ± 5.6
M143 + C	32.3 ± 4.5

a - Mean ± S.D of three experiments. Variation between well in each experiment was less than 10 per cent.

6.4 THE EFFECT OF TREATING 11RX-ACTIVATED MACROPHAGES WITH BOTH M43 AND M57 MONOCLONAL ANTIBODIES IN THE PRESENCE OF COMPLEMENT ON THEIR BACTERICIDAL ACTIVITY

Since treatment of 11RX-activated macrophages with either M43 or M57 monoclonal antibody alone did not completely abolish the bactericidal activity of the cell population (Fig. 6.1), it seemed possible that the M43 and M57 cell-surface markers were being expressed on two separate subsets of cells. In view of this possibility experiments were carried out as described before (Section 6.2) which included a population of 11RX-activated macrophages ($2x10^6$ cells) which had been treated with both M43 and M57 monoclonal antibodies plus complement. The treatment of the macrophages with these monoclonal antibodies was carried out sequentially. After exposure to the first monoclonal antibody (M43), complement was added. The cells were then washed and the process repeated with the second monoclonal antibody (M57). Following this, the viable macrophages ($2x10^5$ cells) from each tube were added to the wells and assayed for their bactericidal activity as

described in Chapter 3. Three wells were sampled at time zero and at 60 min. The percentage viability of macrophages after exposure to both M43 and M57 monoclonal antibodies plus complement was approximately 40% compared with the 85% viability of cells exposed to normal rat IgG in the presence of complement.

It was found that treating the 11RX-activated macrophage population to both M43 and M57 monoclonal antibodies plus complement reduced the ability of these cells to kill opsonized S. typhimurium $C5^{SR}$ to that observed when macrophages from normal animals were treated with normal rat IgG plus complement (Fig. 6.4). These data suggest that the two monoclonal antibodies detect two different subsets of cells, both of which display bactericidal activity. Indirect IF was also performed as previously described (Chapter 4, p.68) on cells which had been treated as above and the results supported the suggestion of the existence of two different subsets. In normal and 11RX-activated macrophage populations, 50-60% of the cells were positive when stained with both M43 and M57 monoclonal antibodies (Figs. 6.5, 6.6 and Table 6.2) compared with the percentage previously described when using either M43 or M57 alone.

Figure 6.4

The killing of virulent opsonized S. typhimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice pre-treated with various rat monoclonal antibodies to mouse macrophages in the presence of complement. Controls included macrophages from both normal and infected mice pre-treated with normal rat IgG.

Each histogram represents the mean percentage survival ± S.D of triplicate cultures.

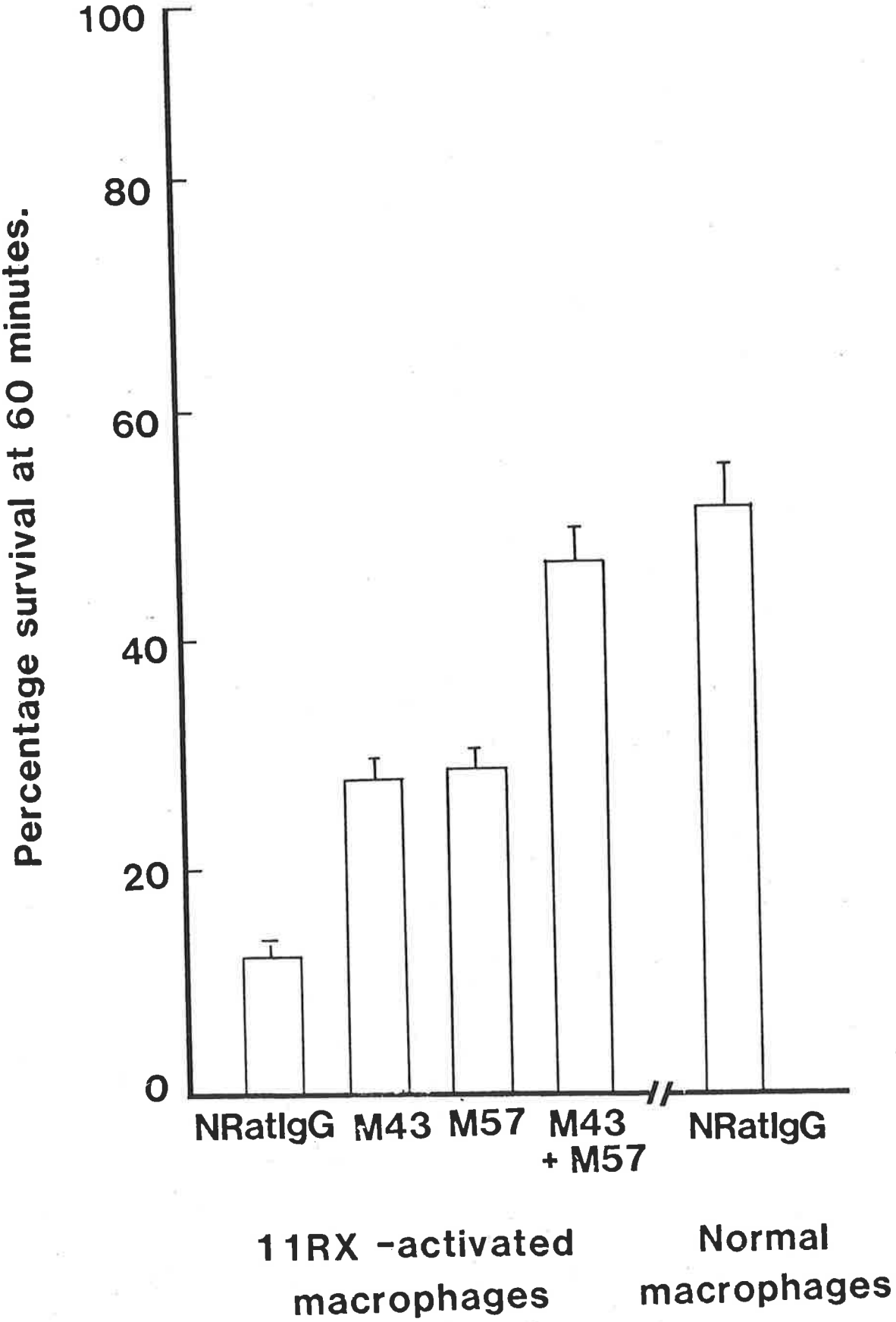
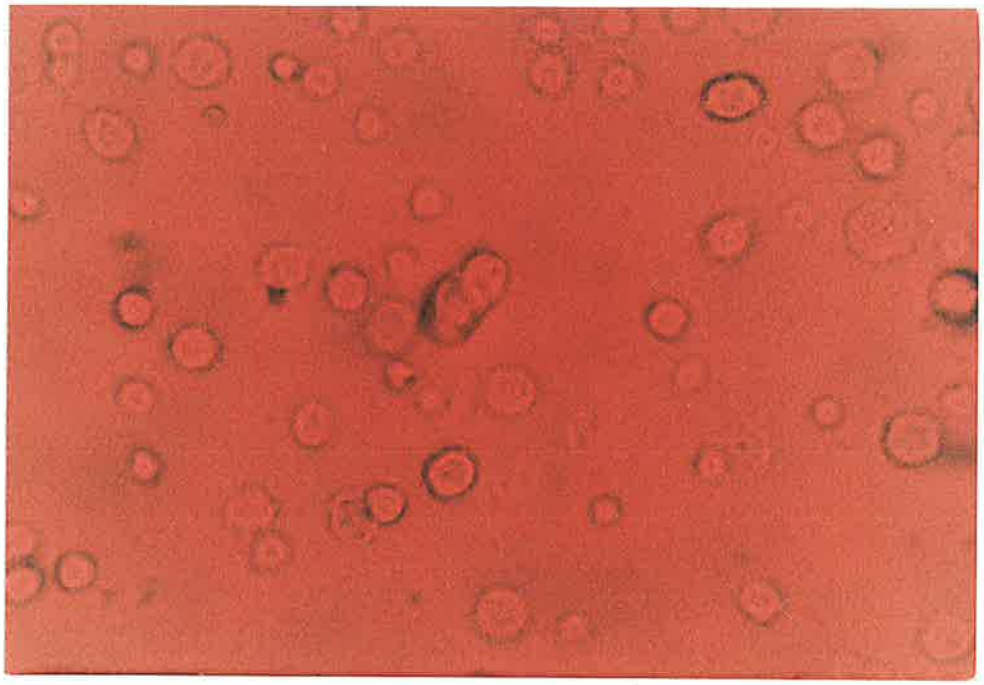

100
80
60
40
20
0
Percentage survival at 60 minutes.
NRatIgG
M43
M57
M43 + M57
NRatIgG
11RX -activated macrophages
Normal macrophages

Figure 6.5

The immunofluroescence staining of normal macrophages with both M43 and M57 monoclonal antibodies, examined under a microscope using ordinary light (A) and uv light (B) (X400).

A



B

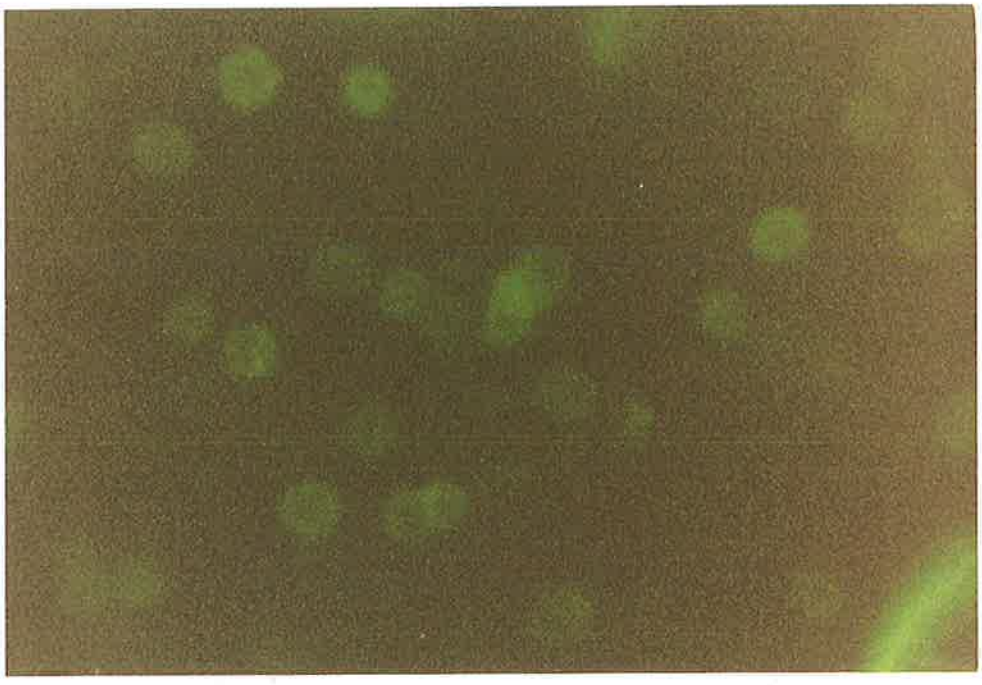
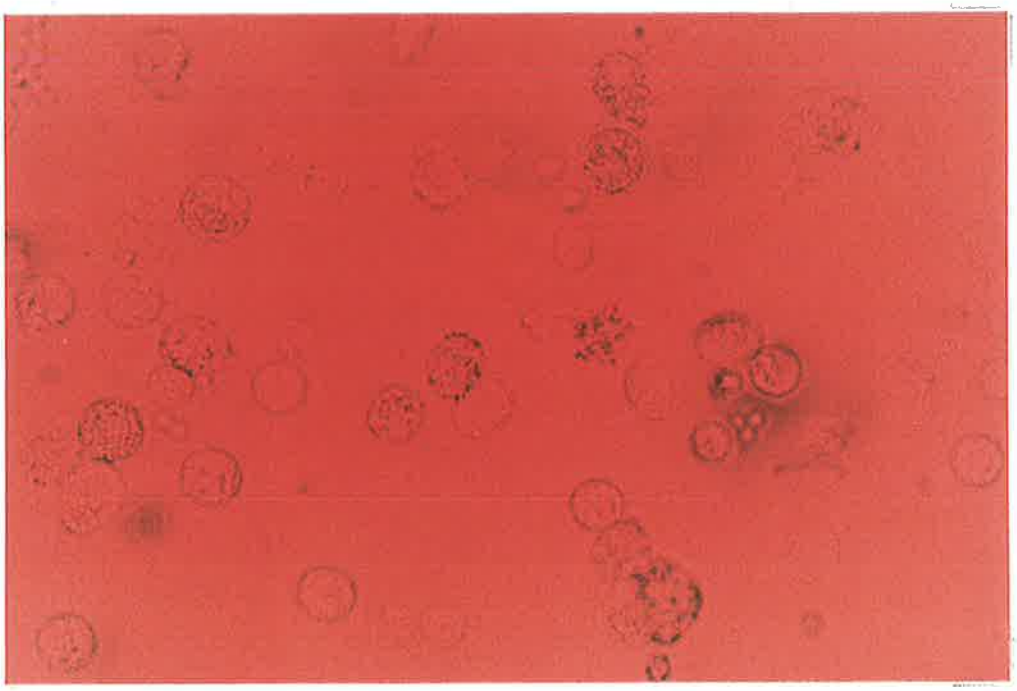


Figure 6.6

The immunofluorescence staining of macrophages from S. enteritidis 11RX-infected mice with both M43 and M57 monoclonal antibodies, examined under a microscope using ordinary light (A) and uv light (B) (X400).

A



B

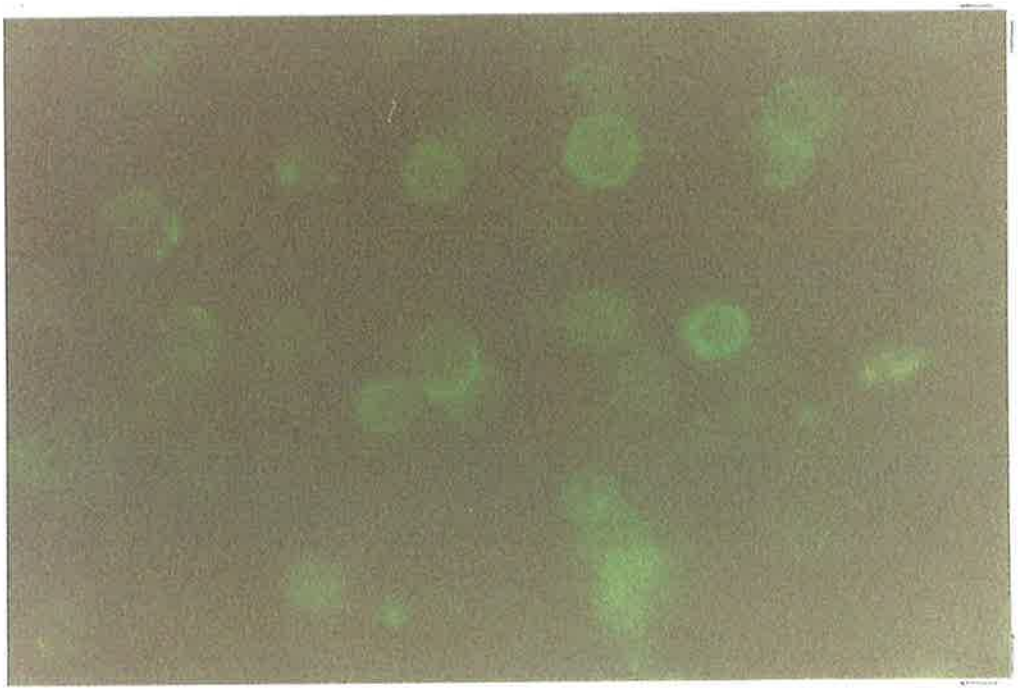


Table 6.2

Percentage of cells in the macrophage populations from normal mice and S. enteritidis 11RX-infected mice stained with rat anti-mouse monoclonal antibody as detected by indirect immunofluorescence.

	% Positive cells[a]	
	Macrophage population	
Rat anti-mouse monoclonal antibody	Normal mice	S. enteritidis 11RX-infected mice
M43	19.7 ± 2.5	24 ± 1.7
M57	28.2 ± 4.8	25.9 ± 2.9
M43 + M57	52.6 ± 0.2	56.2 ± 6.5

a - Mean ± S.D counts from three fields (total of 200 cells).

6.5 DETECTION OF CELL-SURFACE MARKERS ON MACROPHAGES RECOGNIZED BY RAT ANTI-MACROPHAGE MONOCLONAL ANTIBODIES BY INDIRECT RIA

In view of the previous results indicating that certain cell-surface markers occurring on different macrophage subsets may be involved in their bactericidal activity, the same markers might be expected to be more common on an activated macrophage population since these show enhanced bactericidal properties. Activation of macrophages *in vitro* with lymphokines should, therefore, likewise increase the proportion of these receptors. The expression of these cell-surface markers on both normal and 11RX-activated macrophage populations was measured by an indirect RIA. In some of the experiments, the indirect RIA was also performed on macrophages activated *in vitro* with a lymphokine-containing supernatant.

Macrophage activation *in vitro* was carried out by incubating normal macrophages ($1x10^6$ cells) in nunc plastic

tubes (nine tubes) with a Con A supernatant (see Chapters 4 and 5) at a concentration of 30% v/v in 1ml of tissue culture medium overnight at 37°C in 5% CO_2/air. After incubation, the cells were washed once with tissue culture medium (2ml). The ability of these cells and macrophages obtained from normal mice and S. enteritidis 11RX-infected mice to kill opsonized S. ty phimurium $C5^{SR}$ in the presence of tissue culture medium alone was determined as previously described (Sections 3.2 and 4.3). One tube of macrophages which had been incubated with a Con A supernatant (above) was used for this purpose. In conjunction with these tests, an indirect RIA was carried out in nunc plastic tubes using the monoclonal antibodies (two tubes for each monoclonal antibody) by the method described in Chapter 4, Section 4.3. Controls included macrophages from each population incubated with a similar concentration of normal rat IgG (0.2µg/ml), and two tubes being used also for this purpose. The monoclonal antibody (M143) was included in these experiments, although the macrophage function associated with this marker is not known. The indirect RIA from six separate experimental results (Table 6.3) show that macrophage populations from normal mice and S. enteritidis 11RX-infected mice express cell-surface markers recognized by M57, M43 and M143 monoclonal antibodies but in differing amounts. Macrophages from S. enteritidis 11RX-infected mice had more M143 ($p < 0.001$), M43 ($p < 0.001$) and M57 ($p < 0.02$) cell-surface receptors than did macrophages from normal mice. Macrophages activated in vitro with lymphokines, in comparison with

normal macrophages, also displayed more M43 ($p < 0.05$) and M57 ($p < 0.05$) cell-surface markers, but the number of those recognized by M143 was not significantly different (Table 6.4). These results indicate with respect to the M43 and M57 cell-surface markers, that the expression of enhanced bactericidal activity of activated macrophages may be accounted for by subsets of cells with greater numbers of these on their cell surface. This finding was in agreement with the immunofluorescence results.

The expression of the receptors recognized by M143, M43 and M57 on neutrophils from both normal and 11RX-activated cell populations was also studied. The indirect RIA results (data not shown) indicated that very few or none of these markers were found on neutrophils. Results from the indirect immunofluoresence staining confirmed these findings.

Table 6.3

Detection of cell-surface markers on macrophages from normal mice and S. enteritidis 11RX-infected mice with rat anti-mouse monoclonal antibodies by the indirect RIA.

	% Radioactive binding / 10^6 cells[a]	
	Source of macrophages	
Rat anti-mouse monoclonal antibody	Normal mice	S. enteritidis 11RX-infected mice
M43	1.06 ± 0.24	2.66 ± 0.33
M57	1.13 ± 0.17	1.65 ± 0.24
M143	1.25 ± 0.4	4.0 ± 1.0
Bactericidal capacity[b]	51.0 ± 7.7	10.9 ± 3.5

a – Mean ± S.D of six separate experiments. Variation between tubes in each experiment was less than 5 per cent.

b – Percentage survival of S. typhimurium $C5^{SR}$ in the presence of tissue culture medium alone at 60 min.

Table 6.4

Detection of cell-surface markers on normal macrophages and macrophages activated *in vitro* with lymphokines by indirect RIA.

	% Radioactive binding / 10^6 cells[a]	
Rat anti-mouse monoclonal antibody	Normal macrophages	Lymphokine-activated macrophages
M43	1.23 ± 0.06	1.63 ± 0.23
M57	1.0 ± 0.1	1.57 ± 0.32
M143	1.43 ± 0.42	1.77 ± 0.06
Bactericidal[b] capacity	49.0 ± 7.1	19.8 ± 7.8

a - Mean ± S.D of three separate experiments. Variation between tubes in each experiment was less than 5 per cent.

b - Percentage survival of *S. tymphimurium* $C5^{SR}$ in the presence of tissue culture medium alone at 60 min.

6.6 THE EFFECT OF PRE-TREATING 11RX-ACTIVATED MACROPHAGES WITH RAT ANTI-MACROPHAGE MONOCLONAL ANTIBODY IN THE PRESENCE AND ABSENCE OF COMPLEMENT ON THEIR BACTERICIDAL ACTIVITY

Studies by Sun and Lohmann-Matthes (1982) have demonstrated that M43 and M57 monoclonal antibodies eliminate the antibody dependent cell cytotoxic (ADCC) activity of macrophages. However, the inhibition of the killing of antibody-coated tumour targets was only observed when the subsets of cells recognized by the above antibodies were lysed by treating them with antibody and complement. Treatment of macrophages with the monoclonal antibodies alone did not inhibit this activity. In view of these studies, the ability of M43 and M57 monoclonal antibodies to eliminate the bactericidal activity of 11RX-activated macrophages following the exposure of the cells to these antibodies in the presence or absence of complement was investigated.

Experiments were performed as previously described (Section 6.2) with the exception that fresh rabbit serum and heat-inactivated rabbit serum (56°C/1 hour) were used. In nunc plastic tubes (one tube per experimental protocol), 11RX-activated macrophages ($2x10^6$) were incubated for 90 min on ice with 0.5ml medium containing 0.2 µg/ml of either M43 or M57 monoclonal antibody. Control 11RX-activated macrophages were incubated with normal rat IgG at a similar concentration as above. After incubation, the cells were washed once with tissue culture medium (2ml) and re-incubated for a further 5 min with 100µl of a 1/30 dilution of a rabbit anti-rat IgG serum. After washing as before, the cells were incubated again for 90 min at 37°C in 5% CO_2/air with 0.5ml of a 1/6 dilution of either fresh or heat-inactivated rabbit serum. Finally, the cells were washed twice with tissue culture medium (2ml) and their viability determined by trypan blue exclusion. The percentage viability of the cells after treatment with the monoclonal antibodies in the presence of fresh rabbit serum was similar to that obtained from the previous experiments mentioned earlier (Section 6.2). In the presence of heat-inactivated rabbit serum, the percentage viability of the cells was similar to those treated with normal rat IgG. The ability of the remaining viable macrophages to kill opsonized S. typhimurium $C5^{SR}$ was then tested as before (Chapter 3, Section 3.2). Similar numbers of viable macrophages ($2x10^5$) from each tube were added to the wells in the presence of tissue culture medium alone. Control wells containing no macro-

phages but only bacteria were always included. Two wells were sampled at time zero and at 60 min.

The results from three separate experiments are shown in Figure 6.7. The partial inhibition of the bactericidal activity of the 11RX-macrophage population by the M43 monoclonal antibody required the removal of a subset of cells from the cell population. Treatment of this cell population with the antibody alone, in the absence of complement, had no effect. In contrast, treatment of the cells with M57 monoclonal antibody, both in the presence and absence of complement, inhibited to a degree the killing of S. tymphimurium $C5^{SR}$ by these activated phagocytes. It appeared, therefore, that the reduction in the amount of bacteria killed by macrophages treated with M57 monoclonal antibody alone may be due these antibodies preventing the binding of opsonized bacteria to the cell membrane.

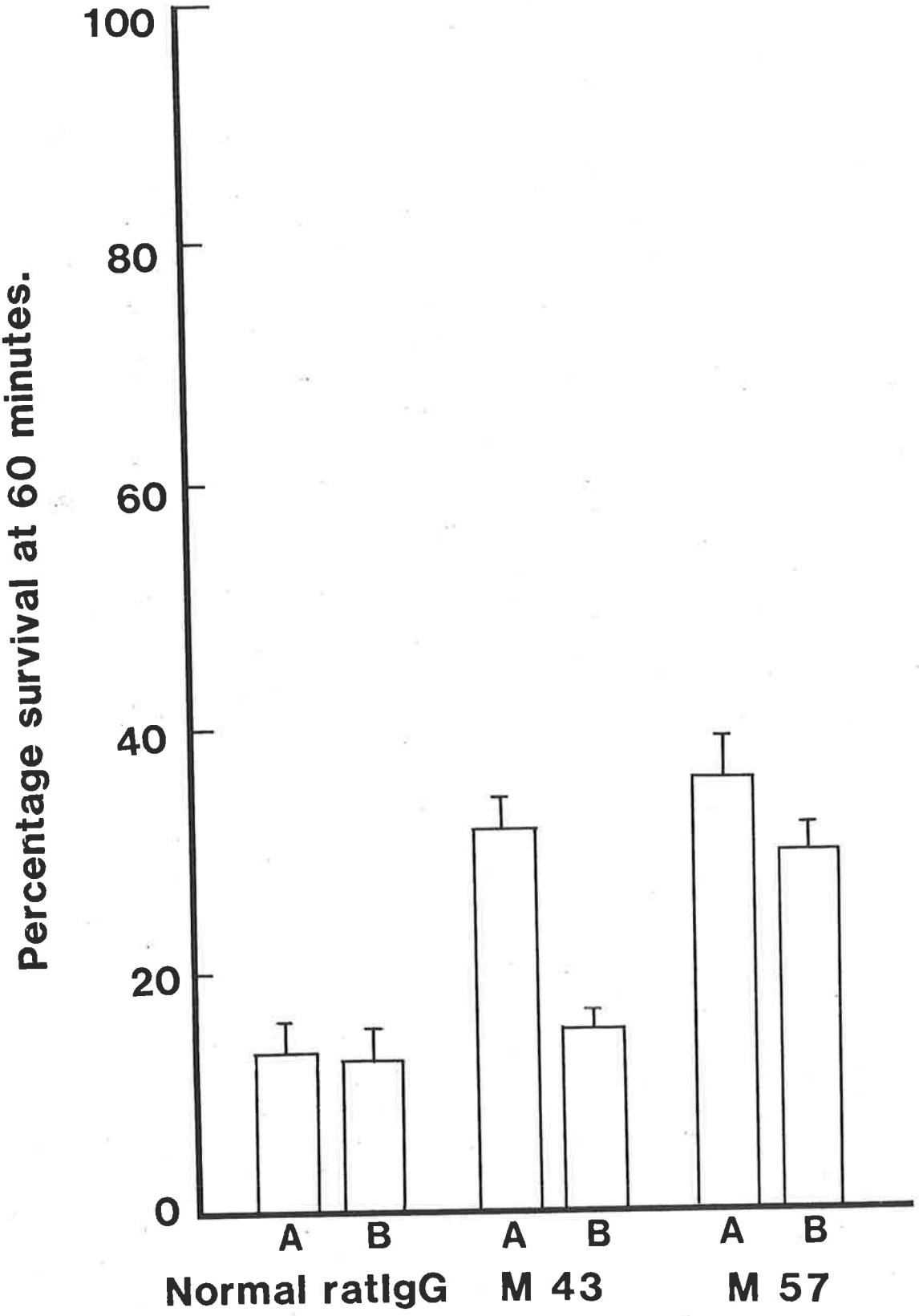
6.7 CONCLUSIONS

These studies confirm that not all cells in a macrophage population express effective bactericidal functions. Two subsets of macrophages with cell-surface markers recognized by two monoclonal antibodies, designated M43 and M57 were shown to be involved in the bactericidal activity of a macrophage population. By indirect RIA, it was shown that these monoclonal antibodies did not react with neutrophils obtained from either normal or S. enteritidis 11RX-infected mice. It was also found that these two subset markers were expressed more on the surface of activated macrophages than on normal cells.

Figure 6.7

The killing of virulent opsonized S. typhimurium $C5^{SR}$ by macrophages from S. enteritidis 11RX-infected mice which had been pre-treated with various rat anti-macrophage monoclonal antibodies either in the presence (A) or absence (B) of complement. Control 11RX-activated macrophages were pre-treated with normal rat IgG.

Each histogram represents the mean percentage survival ± S.D of three separate experiments. Variation between wells in each experiment was less than 5 per cent.


Percentage survival at 60 minutes.
100
80
60
40
20
0
A
B
A
B
A
B
Normal ratIgG
M 43
M 57

CHAPTER 7

7. DISCUSSION

The resistance of mice to Salmonella infections appears to depend on a cell mediated type of immunity in which macrophages become activated and are able to eliminate these intracellular parasites (Hobson, 1957; Ushiba et al., 1959; Howard, 1961; Blanden et al., 1966; Collins, Mackaness and Blanden, 1966). However, despite the requirement for macrophage activation in the expression of this immunity, specific antibody appeared to be an important prerequisite before the bactericidal potential of these phagocytes could be displayed (Jenkin and Benacerraf, 1960; Jenkin, 1963; Malendez et al., 1978; Cooper et al., 1983).

It is clear also from a series of studies carried out in vitro by Leijh and van Furth's group together with others that factors in normal serum influenced the bactericidal activity of phagocytic cells such as granulocytes, monocytes and macrophages (Li and Mudd, 1965; Solberg and Hellum, 1973; Solberg et al., 1976; Leijh et al., 1979a, 1981, 1984). Leijh and van Furth found that bacteria were not ingested by these cells unless they had been pre-opsonized with normal human serum. Moreover, opsonization with fresh serum led to more efficient phagocytosis than did opsonization with heat inactivated serum (Leijh et al., 1979a, 1981). A similar conclusion was reached by other workers (MacGowan et al., 1983). Further evidence provided by Leijh and van Furth's group showed that these

serum factors not only enhanced phagocytosis but were also necessary to allow the cells to exhibit their maximal bactericidal potential. It would appear that the expression of this potential required that serum be present in the medium throughout the time that the bactericidal activity of the cells was being studied (van Furth and Leijh, 1980; Leijh et al., 1981).

In view of these results, initial studies reported in this thesis were designed to determine whether or not the bactericidal activity of macrophages and neutrophils from both normal and S. enteritidis 11RX-infected mice was enhanced in the presence of serum. The reason for using phagocytes from S. enteritidis 11RX-infected mice was that previous studies have shown that both macrophages and neutrophils from such stimulated animals have altered microbicidal properties (Cooper et al., 1983; Penttila, Ey and Jenkin, 1984). It seemed important to compare these cells with those from normal mice with regard to their bactericidal activity in the presence and absence of serum since the data reported by previous investigators regarding this phenomenon had been obtained using phagocytes from normal individuals.

The results of these studies using preopsonized strains of S. typhimurium are given in Chapters 3 and 4. The data show that the bactericidal activity of macrophages and neutrophils from normal animals is enhanced in the presence of serum in the medium. Thus, one may conclude that despite the fact that the bacteria were

preopsonized, their intracellular fate was also determined to some extent by the continual presence of serum in the extracellular environment. This fact is borne out by the data given in Table 3.3, where the number of bacteria killed in the time period under study correlated well with the concentrations of fresh rabbit serum in the medium. It should be pointed out that this enhancing property of rabbit serum was not species specific since similar results were obtained using guinea-pig serum or rat serum (data not shown). In contrast with a previous report by Leijh *et al*. (1984), neither foetal calf serum nor mouse serum showed any enhancing properties.

With regard to the *in vitro* killing activity of macrophages and neutrophils, obtained from Salmonella-infected mice, the data presented in this thesis (Chapters 3 and 4) indicate that the presence of serum extracellularly was not required by macrophages. The opsonized bacteria were killed by these cells equally well both in the presence or absence of serum. However, in the case of neutrophils, although the numbers of opsonized bacteria killed were great in the absence of serum, more were killed in it's presence.

In view of the finding that the enhanced bactericidal activity of activated macrophages was independent of serum, it was interesting to know whether this could be accounted for by immunoglobulins bound to the surface of these cells. Other studies have previously shown that following infection of mice with salmonellae, the macro-

phages from these animals possessed cytophilic antibodies (Turner, et al., 1964; Rowley et al., 1964; Marecki, Hsu and Mayo, 1975). The data obtained from the present investigation show that attempts to remove surface immunoglobulins by two different methods from macrophages harvested from S. enteritidis 11RX-infected mice did not alter their ability to kill S. ty phimurium in the absence of serum. Their bactericidal activity was equally good in it's presence and absence. These data were confirmed using macrophages activated by a supernatant obtained from Con A-stimulated spleen cells. Again the enhanced bactericidal activity was serum independent (Fig 3.2). With regard to neutrophils obtained from S. enteritidis 11RX-infected mice there did appear to be some enhancement of their bactericidal activity in the presence of serum.

The results presented in the above studies (Chapter 3) are in agreement with those previously reported by other workers (Mitsuhashi, Sato and Tanaka, 1961; Blanden et al., 1966; Blanden, 1968) in so far as that macrophages obtained from Salmonella-infected mice exhibited a greater capacity to kill phagocytosed opsonized salmonellae than did normal macrophages.

From the investigations already described in detail (Chapter 1, Section 1.2.6), it seemed possible that the factors in serum which enhanced the bactericidal activity of normal macrophages against S. typhimurium were immunoglobulins together with the complement component C3b. However, results obtained in the present study indicate

that complement components are the essential factors, since the bactericidal activity of normal macrophages was not enhanced in the presence of heat inactivated serum. The importance of complement was strengthened further by the observation (Fig 3.4), that the presence of IgG in the culture medium alone was not sufficient to stimulate the microbicidal activity of normal macrophages against S. typhimurium. However, some stimulation was observed if the macrophages were in contact with surface bound immunoglobulin IgG (Fig 3.5). These findings are in agreement with those reported by Kasai et al. (1982) who showed that IgG adsorbed to a hydrophobic polymer, polystyrene or protein A-stimulated mouse macrophages to generate O_2^- whereas soluble IgG did not. This may be due to the stimulation of Fc receptors by, in the former case, suitably orientated immunoglobulin molecules. The importance of complement was substantiated when it was found that it's removal by adsorption with zymosan or aggregated IgG abolished the enhancing effect of the serum. The use of C6 and C4 deficient sera which were able to enhance the bactericidal capacity of normal macrophages indicated that these components were not responsible for this effect. Although the above work has not shown definitely that the component responsible is C3b, data by other workers suggest that this might be so. Schorlemmer and Allison (1976) have shown, for example, that C3b binding to it's receptor on the surface membrane of mouse macrophages affects the secretion of certain lysosomal enzymes.

It has been suggested (Chapter 1, Section 1.2.6) that the binding of immunoglobulin and C3b to their respective receptors on the membrane of the phagocyte influences, in some way as yet unknown, the bactericidal capacity of these cells. In view of this one might expect that activated cells may express more of these receptors. The data given in Chapter 4 showed that activation of macrophages resulted in an increase in number of their Fc receptors which appeared to correlate with the enhanced capacity of these cells to kill ingested S. ty phimurium. In contrast, the number of C3b receptors did not increase, but decreased. These findings are supported by the observations of Arend and Mannik (1973), Rhodes (1975) with respect to the Fc receptor and results of Ezekowitz, Austyn, Stahl and Gordon (1981) regarding the C3b receptor. The latter investigators found, using a monoclonal antibody (anti-Mac 1) directed against the C3b receptor, that macrophages obtained from BCG-stimulated mice had a reduced number of these receptors. Similar results were also found in another study using macrophages from trypanosome-infected mice (Grosskinsky et al., 1983). Thus, it would appear that the enhanced ability of activated macrophages to kill bacteria does not correlate with an increase in the number of C3b receptors. Neutrophils, however, with enhanced bactericidal activity obtained from S. enteritidis 11RX-infected mice did have an increased number of C3b receptors when compared with those harvested from normal animals. However, the results indicate (Table 4.2) that the enhanced bactericidal

activity of these cells was largely independent of the presence of serum, and so it is difficult from these studies to associate an altered bactericidal capacity with changes in the number of C3b receptors. Furthermore, blocking the C3b receptors with monoclonal specific antibody diminished but did not abolish the ability of the cells to kill opsonized S. typhimurium.

In view of the fact that earlier results (Fig 3.2) showed that macrophages could be activated by supernatants from Con A-stimulated spleen cells as measured by their ability to kill a larger number of ingested bacteria than could normal cells, it was decided to investigate in more detail some other parameters which may change as a result of this activation. In particular, it was also of interest to determine if neutrophils from normal animals could be stimulated in vitro by such supernatants as measured by this particular function. It was found (Chapter 5) that supernatants obtained from Con A-stimulated spleen cells not only induced in normal macrophages an enhanced bactericidal capacity but also induced changes in certain other parameters indicative of activation. These parameters were 1) an increased rate of spreading on surfaces, 2) plasminogen activator release, and 3) an increase in non-specific esterase activity. A number of reports in the literature have already demonstrated the ability of Con A supernatants to affect various macrophage functions such as spreading, plasminogen activator release, collagenase production and microbicidal as well as tumoricidal activity (Fowles et al., 1973; Wahl et al., 1975; Vassalli

and Reich, 1977; Nogueira and Cohn, 1978; Buchmuller and Mauel, 1979; Bout et al., 1981; Haidaris and Bonventre, 1981; Kniep et al., 1981). Furthermore, it was found that one needed to treat the spleen cells at least for 18 hours with such supernatants before they were able to alter the bactericidal properties of normal macrophages. Normal macrophages needed to be in contact with these lymphokine-containing supernatants for only a relatively short period of time of four hours for such a change to take place. This agrees with previous investigations that have shown that the continuous presence of lymphokines was not required for macrophages to express the various properties of activated cells (Manheimer and Pick, 1973; Buchmuller and Mauel, 1979).

Comparative studies showed that the supernatants from Con A-stimulated spleen cells had the same ability to enhance the bactericidal capacity of normal macrophages as did supernatants obtained from spleen cells from mice sensitized to S. enteritidis 11RX, incubated in the presence of a protein extract from 11RX. It was also found that the bactericidal properties of neutrophils from normal mice were also enhanced following the incubation of these phagocytes for a period of three hours in the presence of a Con A supernatant. Whether or not the same factor is involved in activating both macrophages and neutrophils under these circumstances is not clear.

It has been suggested that certain functions of a population of macrophages may be carried out by subsets of

cells within that population. For instance, Ia positive macrophages are necessary for antigen presentation (Beller, Kiely and Unanue, 1980) and only certain sub-populations can perform macrophage-mediated cytotoxicity (ADCC) (Sun and Lohmann-Matthes, 1982). Macrophage heterogeneity has been observed in other functions and characteristics such as phagocytosis (Zembala and Asherson, 1970; Rice and Fishman, 1974), numbers of Fc receptors (Walker, 1974; Serio, Gandour and Walker, 1979) and enzyme content (Fishman and Winberg, 1979; Picker, Raff, Goldyn and Stobo, 1980).

With respect to their bactericidal activity earlier studies have shown that following phagocytosis some bacteria were able to survive and multiply within certain macrophages (Jenkin and Benacerraf, 1960; Mackaness, 1960). These earlier indications of macrophage heterogeneity in terms of their bactericidal function are supported by the data shown in Chapter 6. Two subsets of macrophages with cell surface markers recognized by two rat anti-mouse monoclonal antibodies, designated M43 and M57 were shown to be involved in the bactericidal activity expressed by a population of macrophages. This was based on the finding that removal of these subsets of cells from the cell population by treatment with the monoclonal antibodies and complement reduced the ability of the cell population to kill virulent opsonized S. typhimurium $C5^{SR}$ (Figs 6.1 and 6.4). The data also show that unlike the subset bearing the M43 marker, the bactericidal activity of that bearing the M57 marker could be abolished in the

presence of the monoclonal antibody alone (Fig 6.7). To abolish the activity of the former subset one had to treat the cells with both antibody and complement which resulted in their lysis.

Since subsets of cells bearing M43 and M57 markers were shown to be involved in the bactericidal activity expressed by a population of normal macrophages, it was of interest to know whether these markers were more common in an activated macrophage population. The results of these experiments (Table 6.3) show that the markers recognized by these monoclonal antibodies are expressed more in an activated macrophage population than in a normal one as was a further marker identified with a monoclonal antibody M143. Treatment of macrophages with this latter monoclonal antibody did not diminish the ability of the population to kill S. typhimurium. By immunofluorescence, it was found that in both a normal and activated population of macrophages about 20-30% of the cells displayed these markers but they appeared to be more dense on the surface of the activated macrophages. Similar results were obtained with macrophages activated by incubation in vitro with Con A supernatants (Table 6.4). These latter data indicate further the use of monoclonal antibodies in defining subsets of macrophages and the possibility of assigning a particular function to a particular subset. However, more work needs to done before one can say that the increased density of the markers recognized by the monoclonal antibodies M43 and M57 on the activated cell population can be directly linked to their enhanced bactericidal properties.

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