

The *In Vitro* Bactericidal Activity of Normal and Activated Mouse Macrophages against *Salmonella typhimurium* C5sr

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ABSTRAK

Perbandingan aktiviti bakterisidal *in vitro* makrofaj normal dan teraktif telah menunjukkan bahawa pembunuhan strain virulen *Salmonella typhimurium* C5sr yang beropsonin oleh makrofaj normal ditingkatkan dengan kehadiran serum segar di dalam medium kultur. Sebaliknya, kehadiran serum tersebut tidak diperlukan untuk aktiviti bakterisidal makrofaj teraktif. Pembunuhan bakteria yang meningkat oleh makrofaj teraktif diperolehi sama ada dengan kehadiran atau ketiadaan serum, dan ini tidak disebabkan oleh kewujudan imunoglobulin terikat makrofaj. Faktor di dalam serum arnab yang meningkatkan pembunuhan bakteria oleh makrofaj normal berkemungkinan besar terdiri daripada komponen-komponen sistem komplemen. Faktor-faktor tahan haba (imunoglobulin) di dalam serum arnab normal hanya berkesan jika ia imunoglobulin terikat permukaan.

ABSTRACT

A comparison of the *in vitro* bactericidal activity of normal and activated macrophages indicated that killing of the virulent strain of opsonized *Salmonella typhimurium* C5sr 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 occurred both in the presence and absence of serum, and this was not accounted for by the existence of macrophage-bound immunoglobulin. The factors in normal rabbit serum which potentiated the killing of bacteria by normal macrophages were suspected to be components of the complement system. Heat-stable factors (immunoglobulins) in normal rabbit serum were effective only if they were surface bound immunoglobulins.

INTRODUCTION

Studies have established the participation of normal immunoglobulin G (IgG) in enhancing the phagocytosis and intracellular killing of bacteria such as *Staphylococcus aureus* and *Escherichia coli* by normal human monocytes and granulocytes (Leijh *et al.* 1981; van Furth and Leijh 1980) and resident mouse peritoneal macrophages (Leijh *et al.* 1984). These studies indicated that the enhancing effect of IgG was due to the interaction of IgG with the Fc receptor on the membrane of the phagocyte. Other serum proteins such as complement components and plant lectins have been shown to have similar effect as IgG following

binding of receptors to phagocytes (Leijh *et al.* 1981). It was shown from their studies that a complement component, probably C3b, was required for maximum intracellular killing by phagocytes. Similar results were reported by other workers on the enhancing effect of normal IgG and complement on the bactericidal activity of human macrophages against *Listeria monocytogenes* (MacGowan *et al.* 1983). *In vitro* studies on the bactericidal activity of both normal and activated macrophages were carried out to determine the cell requirements for maximum bactericidal activity.

MATERIALS AND METHODS

Test Bacteria

For these studies, a virulent strain of *S. typhimurium* C5sr (streptomycin-resistant) was chosen as the test organism. It has been shown previously that activation of macrophages was an important prerequisite for optimal killing of this organism (Blanden *et al.* 1966). This strain is also not killed in the presence of specific antibody and complement (Reynolds and Pruul 1971). The strain of *S. enteritidis* 11RX was used for macrophage activation. The bacterial strains were obtained from the Department of Microbiology and Immunology, University of Adelaide, Australia. Prior to the experiments, the log-phase cultures were washed once with 10ml tissue culture medium RPMI-1640 (Flow Labs) and resuspended in a similar volume of the medium at a concentration of 2×10^5 bacterial/ml. The bacteria was then opsonized at room temperature for 15 min with purified rabbit IgG to *S. typhimurium* C5sr (Donated by Dr. Reynolds of the Department of Microbiology and Immunology, University of Adelaide). The bacterial suspension was then divided into three aliquots (or otherwise indicated), to which a final concentration 10% v/v of either fresh or heat-inactivated (56 °C/1h) normal rabbit serum or tissue culture medium was added.

Lymphokine-Containing Supernatant

Ten ml of mouse spleen cell suspensions ($5-6 \times 10^6$ cells/ml) in tissue culture medium containing 2% v/v heat-inactivated foetal calf serum (FCS) was dispensed into tissue culture flasks (Flow Labs). Concanavalin A (Con A) (Pharmacia) at a final concentration of 3.0ug/ml or an equivalent volume of tissue culture medium was added to the flasks. After incubation for 24 hours, cell-free supernatants were obtained by centrifugation and filtration through a Millipore membrane (0.45µm). To the control supernatant harvested from unstimulated cell cultures, an equal concentration of Con A was added after harvesting. The supernatants were stored in aliquots at -70°C.

Removal of Complement Activity from Normal Rabbit Serum

Zymosan-adsorbed rabbit serum: Fresh rabbit serum was adsorbed with washed zymosan (Sigma) (2mg/ml) at room temperature for 1

hour. Zymosan and adsorbed material were then removed by centrifugation.

Aggregated IgG-treated rabbit serum: An aggregated rabbit IgG was prepared by heating normal rabbit serum at 63°C for 25 min. Fresh normal rabbit serum was incubated at 37°C for 30 min with the dilution (1/8) of aggregated rabbit IgG.

Collection of Mouse Peritoneal Cells

Normal resident macrophages were collected from mice immediately upon arrival from the Central Animal House, University of Adelaide. Activated macrophage referred as 11RX-activated macrophages were obtained from mice which had been previously (6-8 days) injected intraperitoneally with 10^5 live *S. enteritidis* 11RX (La Posta *et al.* 1982). Peritoneal cells obtained were washed by centrifugation and finally resuspended in tissue culture medium to a concentration of 2×10^6 cells/ml. 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 viability of the cells was more than 95%.

Bactericidal Assay

The bactericidal assay was carried out in micro-culture trays by the following steps. The cells (either normal or 11RX-activated macrophages, 2×10^5 /well) adhered to the wells after an initial incubation of 30 min at 37°C in 5% CO₂/air. Non-adherent cells were removed by washing the well once with 0.3ml RPMI-1640. The monolayers were then incubated in 0.3ml RPMI-1640 for a further 30 min. The medium was removed from the monolayers prior to the addition of bacteria. Fifty µl of the opsonized bacterial suspension was added to the monolayers in triplicate. To ensure close contact between the cell and bacteria, the micro-culture trays were centrifuged at 1500g for min at 4°C in a Coolspin centrifuge (MSE). The trays were then incubated at 43°C for 2 min, follow by incubation at 37°C in 5% CO₂/air. Control wells containing only bacteria in the presence of the three different culture media used above were included and treated in a similar manner. Viable counts of the bacteria were made on nutrient agar at time zero and 60 min. Fifty µl of Triton-X100 (1% v/v in

saline) was added to each well prior to sampling in order to disrupt the macrophages and release intracellular bacteria. The survival of bacteria was calculated by dividing the number of bacteria in the experimental wells by the number of bacteria in the control wells and was expressed as percentage.

Removal of Cell-Bound Antibody on 11RX-Activated Macrophages

Two methods were employed to remove macrophage-bound immunoglobulins. In the first, 11RX-activated macrophages in siliconized glass tubes were washed three times with 10ml aliquots of 0.005M EDTA in tissue culture medium and once with the same medium but without EDTA before being cultured and their bactericidal potency assayed as described before. The second method involved treatment of the cells with a neutral protease (Dispase, grade II, Mannheim, Boehringer) 1.5mg/ml at 37°C for 45 min. After incubation, the cells were washed once with medium before being cultured as above.

The Expression of the Bactericidal Activity of Macrophages Activated in Vitro with Lymphokines.

Normal macrophage monolayers were incubated in 30% v/v lymphokine-containing supernatant derived from Con-A-stimulated spleen cell cultures. For control, macrophage monolayers were also incubated in a culture supernatant derived from unstimulated spleen cells to which an equivalent concentration of Con-A had been added following harvesting. The cells were incubated at 37°C in 5% CO₂/air overnight. After incubation, the monolayers were washed once prior to bactericidal assay. The bactericidal assay was performed as described before in the presence of either fresh or heat-inactivated normal rabbit serum or medium alone.

Coating of Tray with Purified Rabbit IgG

Purified rabbit IgG was prepared according to the method described by Ey *et al.* (1978). The wells were coated by adding 50µl of purified rabbit IgG (1mg/ml) after which the micro-culture trays were kept at 4°C overnight and washed once with medium prior to use.

Statistical Analysis

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

RESULTS

The Bactericidal Activity of Normal and 11RX-Activated Macrophages

Results (Figure 1) show that killing of the virulent strain of preopsonized *S. typhimurium* C5sr by normal macrophages was enhanced in the presence of fresh serum. Bacteria incubated in control wells in the absence of macrophages increased in number by a factor of 1.5 to 2. The 11RX-activated macrophages showed an enhanced bactericidal capacity compared to normal macrophages, and were able to kill opsonized bacteria equally well in the presence or absence of serum.

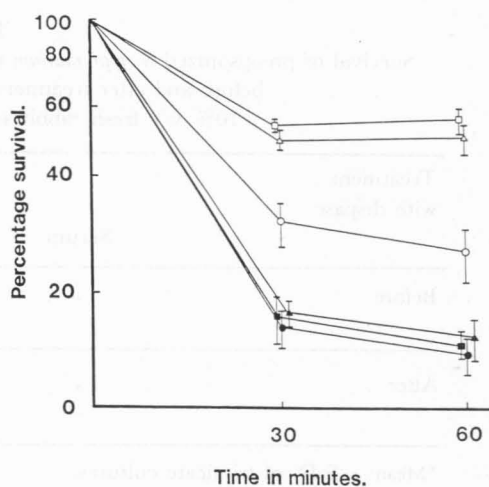


Fig 1 Survival of preopsonized *S. typhimurium* C5sr incubated with normal (○, □, △) and 11RX-activated (●, ■, ▲) macrophages in the presence of 10% v/v fresh rabbit serum (○, ●), 10% v/v heat-inactivated rabbit serum (△, ▲), or tissue culture medium (□, ■). Each point represents the mean percentage survival \pm S.D. obtained from five replicate experiments.

The Effect of the Removal of Cell-Bound Antibody on the Bactericidal Activity of 11RX-Activated Macrophages

The enhanced bactericidal activity of 11RX-activated macrophages may be due to immunoglobulins already bound to the cell surface which result in maximal stimulation in the absence of added serum. To investigate this possibility, the bactericidal activity of 11RX-activated macrophages was determined after removal of the possible

TABLE 1
Survival of preopsonized *S. typhimurium* C5sr incubated with 11RX-macrophages before and after treatment with EDTA, in the presence of 10% v/v rabbit serum or culture medium alone.

Treatment with EDTA	Serum	Percentage survival*	
		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

*Mean ± S.D. of triplicate cultures.

TABLE 2
Survival of preopsonized *S. typhimurium* C5sr incubated with 11RX-activated macrophages before and after treatment with Dispase, in the presence of 10% v/v fresh rabbit serum or culture medium alone.

Treatment with dispase	Serum	Percentage survival*	
		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

*Mean ± S.D. of triplicate cultures.

cell-bound immunoglobulins and addition of either fresh rabbit serum or medium alone. This activity was compared with that of untreated macrophage cultures under similar conditions (Tables 1 and 2). The ability of 11RX-activated macrophages to kill the bacteria was still retained although cell-bound immunoglobulins on their surface had been removed. In addition, the expression of enhanced bactericidal capacity was not dependent on the presence of serum in the medium.

The Serum Requirement for Expression of the Bactericidal Activity of Macrophages Activated In Vitro with 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. As in the earlier studies, the killing of the bacteria by normal macrophages was enhanced in the presence of fresh serum (Fig. 2.1). However, for lymphokine-activated macrophages, the presence of serum was not necessary for expression of bactericidal potential (Fig. 2.2). These findings substantiate earlier results indicating that cell-bound antibody was not necessary for expression of enhanced bactericidal capacity in activated macrophages.

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* C5sr 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 was performed as previously described. It may be seen from the

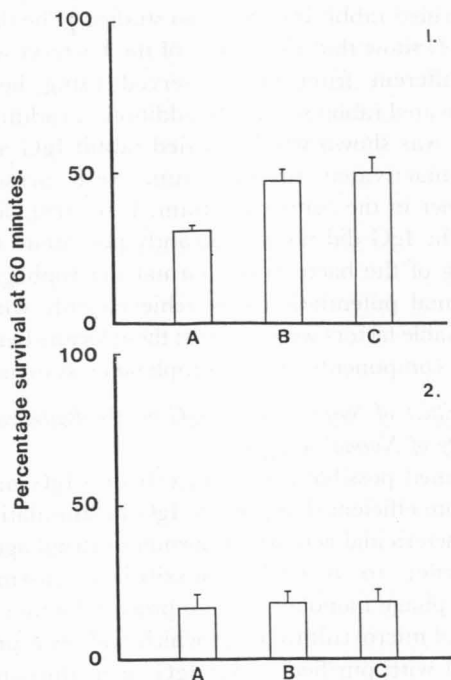


Fig 2 Survival of preopsonize *S. typhimurium* C5sr incubated with (1) normal control macrophages or (2) lymphokine-activated macrophages in the presence of 10% v/v fresh rabbit serum (A), 10% v/v heat-inactivated rabbit serum (B) or culture medium alone (C). Each histogram represents the mean percentage survival \pm S.D obtained from four separate experiments.

data (Table 3) that there was a correlation ($r = 0.96$ for serum concentration 0-10%) between the number of bacteria killed and the concentration of fresh rabbit serum in the wells. Optimal killing of the bacteria by normal macrophages was observed when 10% fresh rabbit serum was present in the medium. Concentrations greater than this did not increase significantly ($P < 0.001$) the number of bacteria killed.

The Nature of the Factor(s) in Fresh Serum Involved in Stimulating the Bactericidal Activity of Normal Macrophages

In order to delineate the relative roles of IgG and complement in the killing of the virulent strain of *S. typhimurium* C5sr by normal macrophages, rabbit serum lacking certain complement compo-

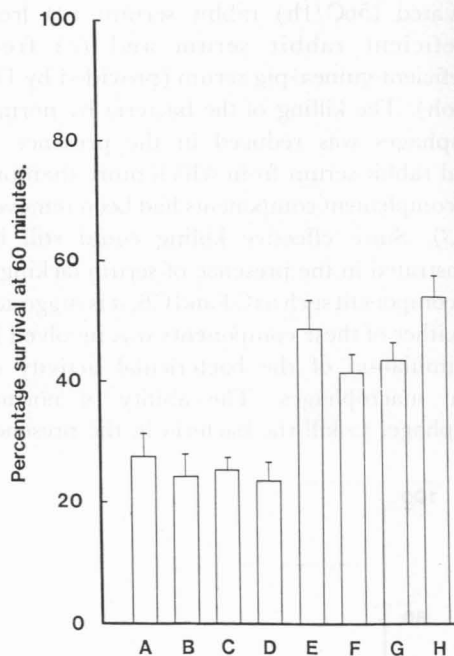


Fig 3 Survival of preopsonized *S. typhimurium* C5sr incubated with normal macrophages in the presence of various sera in the culture medium (10% v/v). (A) fresh rabbit serum, (B) fresh guinea-pig serum, (C) fresh C6-deficient rabbit serum, (D) fresh C4-deficient guinea-pig serum, (E) heat-inactivated 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 \pm S.D. of three separate experiments.

TABLE 3
The effect of the concentration of fresh rabbit serum on the killing of preopsonized *S. typhimurium* C5sr by normal macrophages.

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

*Mean \pm S.D. of triplicate cultures.

nents and purified rabbit IgG were used to stimulate the bactericidal activity of normal macrophages. The bactericidal assay was performed as previously described by adding preopsonized bacteria to the normal macrophage monolayers in the presence of the following sera (10% v/v): (a) zymosan-adsorbed rabbit serum; (b) aggregated IgG-treated rabbit serum; (c) heat-inactivated (56C/1h) rabbit serum; (d) fresh C6-deficient rabbit serum and (e) fresh C4-deficient guinea-pig serum (provided by Dr. S. Neoh). The killing of the bacteria 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). Since effective killing could still be demonstrated in the presence of serum lacking a single component such as C4 and C6, it is suggested that neither of these components was involved in the stimulation of the bactericidal activity of normal macrophages. The ability of normal macrophages to kill the bacteria in the presence

of purified rabbit IgG was also studied. The data (Fig. 4) show that the killing of the bacteria was no different from that observed using heat-inactivated rabbit serum. In addition, no additive effect was shown when purified rabbit IgG and heat-inactivated rabbit serum were present together in the culture medium. It seemed clear that the IgG did not significantly potentiate the killing of the bacteria by normal macrophages. Maximal potentiation was achieved only when heat-labile factors were present; these factors being likely components of the complement system.

The Effect of Surface-Bound IgG on the Bactericidal Activity of Normal Macrophages

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 were prepared either in wells of micro-culture trays which had been pre-coated with purified rabbit IgG or in uncoated wells. Monolayers on the uncoated wells were

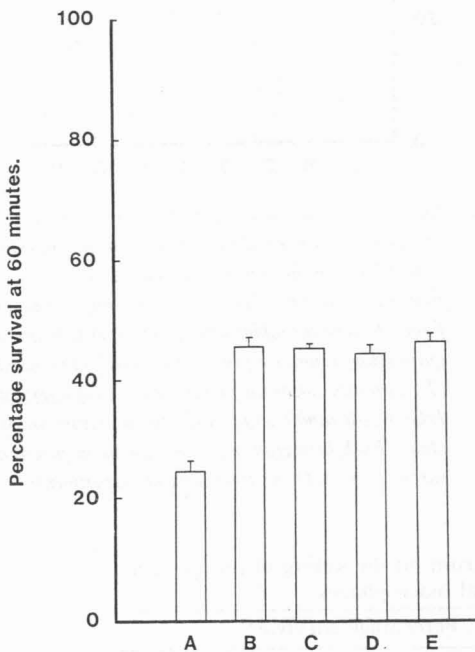


Fig 4 Survival of preopsonized *S. typhimurium* C5sr incubated with normal macrophages in the presence of 10% v/v fresh rabbit serum (A), 10% v/v heat-inactivated rabbit serum (B), purified rabbit IgG (1mg/ml) (C), 10% v/v heat-inactivated rabbit serum + purified rabbit IgG (1mg/ml) (D), tissue culture medium (E). Each histogram represents the mean percentage survival \pm S.D. of triplicate cultures.

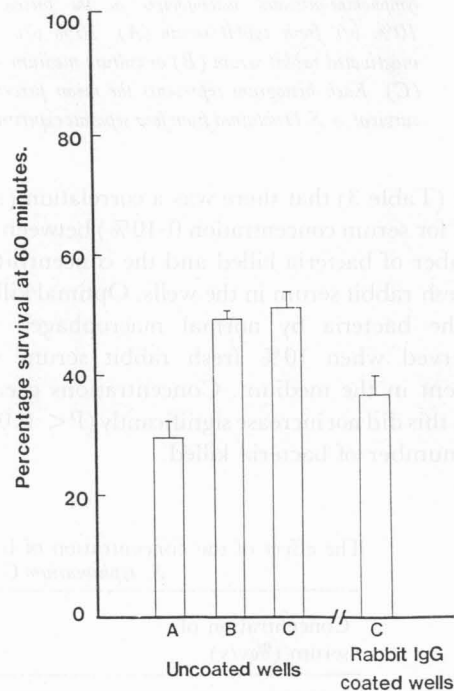


Fig 5 Survival of preopsonized *S. typhimurium* C5sr incubated with normal macrophages in the presence of 10% v/v fresh rabbit serum (A), soluble purified IgG (B), tissue culture medium alone (C). Each histogram represents the mean percentage survival \pm S.D of three separate experiments.

infected with preopsonized bacteria in the presence of either 10% v/v fresh rabbit serum, purified rabbit IgG (1 mg/ml) or medium alone. Whereas monolayers on the rabbit IgG-coated wells were infected with preopsonized bacteria in the presence of medium alone. The bactericidal assay was performed as previously described. Normal mouse peritoneal macrophages, upon incubation with purified rabbit anti-BSA IgG, adsorbed to a hydrophobic polymer, polystyrene have been shown to generate superoxide anion (Kasai *et al.* 1982). In this studies, it was also shown 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. Our results (Fig. 5) show that purified rabbit IgG bound to the polystyrene tray enhanced the killing of the bacteria by normal macrophages to a greater extent than did soluble rabbit IgG ($p < 0.02$).

DISCUSSION

Studies carried out *in vitro* by Leijh and others showed that factors in normal serum influenced the bactericidal activity of normal phagocytic cells such as granulocytes, monocytes and macrophages (Solberg and Hellum 1973; Leijh *et al.* 1979, 1980, 1984; MacGowan *et al.* 1983). These serum factors not only enhanced phagocytosis but were also necessary to allow the cells to exhibit their maximal bactericidal potential. We decided to investigate whether the bactericidal activity of macrophages from both normal and *S. enteritidis* 11RX-infected mice was enhanced in the presence of serum, and our data show that the bactericidal activity of macrophages from normal animals is enhanced. Thus, 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 observation was confirmed by our findings that 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 the enhancing property of rabbit serum was not species specific since similar results were obtained using guinea-pig or rat serum (data not shown). With

regard to the *in vitro* killing activity of macrophages obtained from Salmonella-infected mice, our data indicate that the presence of serum extracellularly was not required. The opsonized bacteria were killed by these cells equally well both in the presence or absence of serum. In view of the finding that the enhanced bactericidal activity of activated macrophages was independent of serum, we investigated whether this could be accounted for by immunoglobulins bound to the surface of these cells.

Other studies have shown that macrophages from mice previously infected with salmonellae possessed cytophilic antibodies (Turner *et al.* 1964; Rowley *et al.* 1964). The present investigation showed that removal of surface immunoglobulins from macrophages using two different methods did not alter their ability to kill bacteria in the absence of serum. 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. It seemed possible that the factor in serum which enhanced the bactericidal activity of normal macrophages was immunoglobulins together with the complement component C3b (Leijh *et al.* 1984). 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 that the presence of IgG in the culture medium alone was not sufficient to stimulate the bactericidal activity of normal macrophages. However, some stimulation was observed if the macrophages were in contact with surface-bound IgG. 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 superoxide anion whereas soluble IgG did not. This may be due to the binding of Fc receptors by, in the former case, suitably orientated immunoglobulin molecules. The importance of complement was substantiated when it was found that its removal by adsorption with zymosan or aggregated IgG abolished the enhancing effect of the serum. Both C6 and C4 deficient sera were

able to enhance the bactericidal capacity of normal macrophages indicating that these components were not necessary for this effect. Absence of C4 prevents classical (immunoglobulin mediated) complement activation. A C6 deficiency abrogates formation of the terminal membrane attack complex of complement. Thus activation of complement must have occurred by the alternate pathway, and the component most likely to be involved in macrophages stimulation is C3b. Although our results have not shown definitively that the component responsible is C3b, data by other workers suggests that this might be so. Schorlemmer and Allison (1976) have shown, for example, that C3b binding to its receptor on the surface membrane of mouse macrophages effects the secretion of certain lysosomal enzymes.

In conclusion, the present study strongly supports the contention that both heat stable and heat labile components of serum enhance the bactericidal activity of normal macrophages. Activation of these cells either *in vivo*, or *in vitro*, however, negate the requirement for such serum components in allowing full expression of their bactericidal potential and it would be of interest to see if this reflect changes in the number of Fc and C3b receptors on their membrane.

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