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Heterogeneity of Mouse Macrophages in Terms of Their Bactericidal Activity

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Key words: Bactericidal activity, macrophage heterogeneity, macrophage activation.

ABSTRAK

Kajian ini mengesahkan bahawa tidak semua sel di dalam populasi makrofaj mempunyai fungsi-fungsi bakterisidal yang berkesan. Dua subkumpulan makrofaj dengan penanda permukaan-sel yang dikenali oleh dua antibodi monoklon, diberi nama M43 dan M57 didapati terlibat di dalam aktiviti bakterisidal satu populasi makrofaj. Dengan kaedah esei radioimuno (RIA) tidak langsung, kedua-dua penanda subkumpulan ini boleh didapati lebih banyak di atas permukaan makrofaj teraktif berbanding dengan di atas sel yang normal. Oleh itu mungkin kedua-dua penanda ini menjadi penanda untuk pengaktifan makrofaj dalam hal peningkatan aktiviti bakterisidal.

ABSTRACT

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 radioimmunoassay (RIA), it was found that these two subset markers were expressed more on the surface of activated macrophages than on normal cells. It seems possible that these two markers may serve as markers for macrophage activation in terms of enhanced bactericidal activity.

INTRODUCTION

At the site of 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 a diversity of functions. Studies on mechanisms of phagocytosis have shown that the expression of various surface receptors is not found uniformly on all macrophages obtained from guine-pig or rabbit (Rhodes 1975: Walker 1976; Silverstein and Loike 1980). Earlier studies by McIntyre *et al.* (1967) have also 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 *et al.* 1984), who showed that normal rat alveolar macrophages, separated into four isopynic fractions on Percoll gradients, were functionally heterogeneous in superoxide anion release, lysosomal enzyme activity, phagocytosis and intracellular killing of *Staphylococcus aureus,* but not in hydrogen

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peroxide release. Other studies also found distinct subsets of macrophage populations which were able to kill *Listeria monocytogenes* and to restrict intracellular growth of the bacteria whereas others did not.

It is possible that certain 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 Mohankumar (1977) have demonstrated a membrane antigen specifically associated with *Corynebacterium parvum* and pyran-activated macrophages. Later, studies by Sun and Lohman-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. These antibodies 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 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 anti-bacterial. The effect of these monoclonal antibodies plus or minus complement on the bactericidal activity of a macrophage population obtained from *Salmonella enteritidis* 11RX-infected mice was investigated in the present study. Furthermore, the expression of cell-surface markers recognized by these monoclonal antibodies, in normal and activated macrophages was also studied.

MATERIALS AND METHODS

Bacteria

For these studies, a virulent strain of *Salmonella typhimurium* C5sr (streptomycin-resistant) was chosen as the target organism since it had previously shown that activation of macrophages was an important prerequisite for optimal killing of this organism (Blanden *et al.* 1966) and that this strain is not killed in the presence of specific antibodies and complements (Reynolds and Pruul, 1971). Strain of *S.enteritidis* 11RX was used for macrophage activation. The strain were obtained from the stock of the Department of Microbiology and Immunology, University of Adelaide, Australia. Prior to use in the experiments, the log-phase cultures were washed once with 10 ml tissue culture medium RPMI-1640 (Flow Labs) and finally resuspended in a similar volume of the same at a concentration of 2×10^5 bacteria /ml. The bacteria was then opsonized at room temperature for 15 min with purified rabbit IgG isolated from an antiserum to *S. typhimurium* C5sr (donated by Dr. Reynolds of the Department).

Collection of Mouse Peritoneal Cells

Normal resident macrophages were collected from mice which had just been received from the Central Animal House, University of Adelaide. Activated macrophages were obtained from mice which had been injected intraperitoneally with 10^5 live *S. enteritidis* 11RX (La Posta, *et al.* 1982), 6 – 8 days previously and referred as 11RX-activated macrophages. Peritoneal cells, collected from the peritoneal cavity, were washed by centrifugation and finally resuspended in RPMI medium to a concentration of $2 - 3 \times 10^6$ cells/ml. An aliquot of the suspension was stained with Giemsa to determine the percentage of macrophages in the population of cells. Trypane blue exclusion revealed that the viability of the cells was never less than 95%.

Lymphokine-activated Macrophages

The mitogen Concanavalin (Con) A, a plant lectin, was used to prepare lymphokine-containing supernatants from spleen cells. The supernatants were prepared as follows: mouse spleen cell suspension ($5 - 6 \times 10^6$ cells/ml) in RPMI medium containing 2% v/v heat-

inactiveted FCS was dispended in 10ml aliquots into tissue culture flasks (Flow Labs). To the flasks, Concanavalin A (Con A) (Pharmacia) at a final concentration of 3.0 μg/ml or RPMI medium of similar volume was added.

After incubation for 24 hours, cell-free supernatants were obtained by centrifugation and filtration throught a millipore membrane (0.45μm). The supernatants were stored in aliquots at -70°C. Macrophage activation *in vitro* was carried out by incubating normal macrophages in 5 ml Nunc plastic tubes (Medos) in the presence of 30% v/v Con A supernatant at 37°C in 5% CO_2/air overnight. The activation state was determined by their enchanced ability to kill opsonized *S. typhimuium* C5 sr.

Bactericidal Assay

The bactericidal assay was carried out in micro-culture trays by the following method: the pre-treated cells (either normal or activated macrophages) (2×10^5 viable cells/ 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 RPMI medium, before finaly being used for the experiment. Prior to the bactericidal assay, the supernatant was removed from the monolayers prior to the addition of bacteria. A small volume (50ul) of the opsonized bacteial suspension was added to the monolayers in triplicate cultures. To ensure close contact between the cells and bacteria, the micro-culture trays were centrifuged at 1500g for 10 min at 4°C. The trays were then incubated at 43°C for 2 min, followed by incubation at 37°C in 5% CO_2/ air. Control wells containing no macrophages but only bacteria in RPMI medium were always included and treated in a similar manner. Viable counts of the bacteria on nutrient agar were made at time zero and 60 min. To each well being sampled was added an equal volume of Triton-X100 (1%v/v in saline) in order to distrupt the macrophages. The survival of bacteria was culculated as the number of bacteria in the experimental wells over the number of bacteria in the control wells and was expressed as a percentage.

Indirect Immunofluorescence Assay

To obtain good cell surface staining, the peritoneal cells in 0.1M phosphate buffer, pH 7.4 containing 0.02% sodium size (1×10^6), in 5 ml Nunc plastic tubes (Medos) were incubated for 1 hour on ice with 50 μl (0.2 μg/ml) of the monoclonal antibody. 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 400 g for 5 min with 0.1M phosphate buffer. The cells were then incubated for 1 hour on ice with a 1/50 dilution of a fluorecein-labelled rabbit anti-rat IgG (Nordic 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).

Indirect Radioimmunoassay (RIA)

The macrophage population (1×10^6 cells) in 5ml Nunc plastic tubes (Medos) were first washed once with 2ml RPMI medium containing 0.02% sodium azide by centrifugation at 400 g for 5 min at 4°C. The cells were then incubated for 90 min on ice with the monoclonal antibodies (0.2 μg IgG/ml). Control tubes contained macrophages which were incubated as above with a similar concentration of normal rat IgG. After incubation, the cells were washed three times with the RIA washing buffer (PBS + 0.1% BSA + 0.02% sodium azide). They were then incubated for a further 90 min with 50 ul RIA buffer (Tris HCl, pH 7.75, consisting of 0.5mM EDTA, 0.1% sodium azide, 0.05M Tris HCl and 132.5 mM sodium chloride) containing approximately 5×10^4 cpm ^{125}I-labelled rabbit IgG anti-rat IgG (prepared by using a solid-phase oxidizing agent as described by Salacinski *et al.* 1981). The labelled antibody was also added to 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.). The indirect RIA results were expressed as the percentage binding of radioctivity by 10^6 cells, calculated using the following formula:

$$\% \text{ specific binding} = \frac{100 \times \text{cpm experimental tube-cpm control tube}}{\text{Total cpm added}}$$

The amount of radioactivity in the control tubes containing cell incubated with normal rat IgG never exceeded 0.6% of the total counts added.

Statistical Analysis

Statistical analyses in this work were carried out using the Student's t-test.

RESULTS

Bactericidal Activity of Macrophages Following Treament with Different Monoclonal Antibodies in the Presence of Complement.

The effect of three different rat monoclonal antibodies, designated M43, M57, and M143 (provided by Dr. Lohmann-Matthes, The Max-Planck Institute fur Immunbiologie, Postfach 1169, D-7800, Freiburg, FRG), in the presence of complement on the bactericidal avtivity of 11RX-activated macrophages was first investigated. In 5 ml Nunc plastic tubes (Medos), 2 – 3 × 10^6 macrophages from *S. enteritidis* 11RX- infected mice, in RPMI medium were incubated for 90 min on ice in the presence of the monoclonal antibodies at a concentration 0.2µg/ml. Controls included normal and 11RX-activated macrophages incubated as above with a similar concentration of normal rat IgG. After incubation, the cells were washed once with RPMI medium and reincubated for a further 5 min with 100µl of a 1/30 dilution of a rabbit anti-rat IgG serum, as a facilitating antibody (Sun and Lohmann-Matthes, 1982) (provided by Dr Lohman-Matthes). The cells were washed again as before and 0.5 ml 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 RPMI medium and their viability was determined by trypan blue exclusion. Following treatment with either M43 of M57 monoclonal antibody plus complement, 60% of the macrophages remained viable while with M143, 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. typhimuriam* C5 sr was tested using a similar number of viable macrophages (2 × 10^5 cells) added to the wells. The results from three separate experiments are shown in *Fig. 1*. The data show that pretreatment of a 11RX-activated macrophage population with M43 and M57 monoclonal antibodies plus complement,

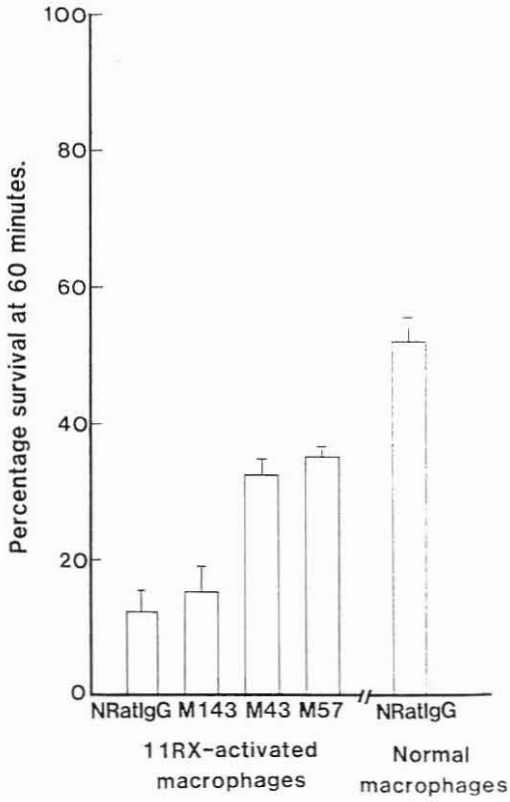

Fig. 1: The killing of virulent opsonized S. typhimurium C5 sr by macrophages from S enteritidis 11RX-infected mice, which had been pre-treated with various rat monoclonal antibodies to mouse macrophages in the presence of complement. Controls included macrophages from both normal and infected mice pretreated 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.

resulted in a decrease ($p < 0.05$) in the number of opsonized *S. typhimurium* 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.

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 then to determine the distribution of these markers in normal and 11RX-activated macrophage populations, first qualitatively by indirect immunofluorescence (IF). Results from these experiments revealed that only a proportion of the cells (20–30%, figures not shown) from both a normal and 11RX-activated population of macrophages were stained positively with M43 monoclonal antibody. Similar results were also observed with M57, but only 10–20% of the total cell population were stained with M143 monoclonal antibody. It was also noted 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 harvested from normal mice.

TABLE 1
Cytotoxicity of rat anti-macrophage monoclonal antibodies plus complement (C) for 11RX-activated macrophages

Tretment 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.

In conjuction with these studies, the release of ^{51} Chromium (Cr) from prelebelled macrophages was followed after addition of the monoclonal antibodies and complement. The ^{51} Cr release assay was performed according to the method described previously by Sun and Lohmann-Matthes (1982). The data of these assays, shown in Table 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 used.

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, 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 then carried out as described before which included a population of 11RX-activated macrophages 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. The bactericidal assays were then performed using a similar number of viable pre-treared macrophages.

It was found that treating the 11RX-activated macrophages 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 mice were treated with normal rat IgG plus complement (*Fig. 2*). These data suggest that the two monoclonal antitbodies detect two different subsets of cells, both of which display bactericidal activity. Indirect IF was also perfomed as previously described on cells which had been treated as above and the results support the suggestion of the existence of the two different subsets (Table 2).

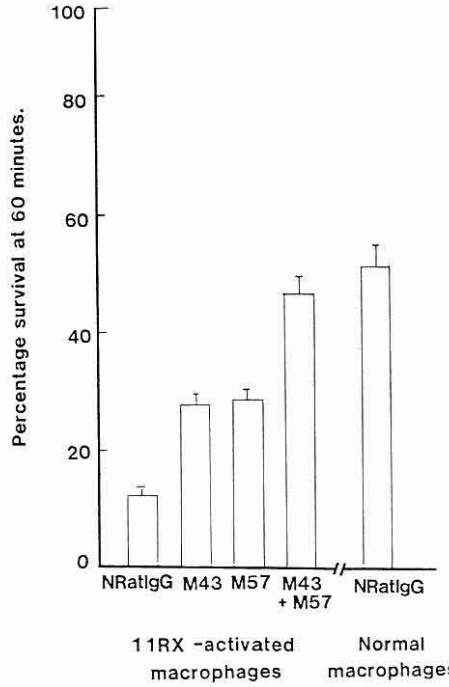

Fig. 2: The killing of virulent opsonized S. typhimurin *C5 sr by macrophages from* S. enteritidis *11RX-infected mice, which had been pre-treated with various rat monoclonal antibodies to mouse macrophages in the presence of complement. Controls included macrophages from both normal and infected mice pre-treated with normal rat IgG. Each histogram represents the mean percentage survival ± S.D of triplicate cultures.*

TABLE 2
Percentage of cells in the macrophage population from normal mice and *S. enteritidis* 11RX-infected mice stained with rat anti-mouse monoclonal antibody as detected by indirect immunofluorescence (IF).

	% positive cells[a]	
	Macrophage population	
Rat anti-mouse monoclonal antibody	Normal	11RX-activated
M43	19.7 ± 2.5	24.0 ± 1.7
M57	28.2 ± 4.8	25.0 ± 2.9
M43 + M57	52.6 ± 0.2	56.2 ± 6.5

a - mean ± counts from three fields (total of 200 cells).

Detection of Cells-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 cells show enhanced bactericidal properties. Activation of macrophages *in vitro* with lymphokines should, therefore, likewise increase the proportion of these markers. The expression of these cell-surface markers on normal, 11RX-activated and lympokine-activated macrophage populations was measured by an indirect RIA. The monoclonal antibody (M143) was included in these experiments, although the macrophage function associated with this marker is not yet known. The ability of these cell populations to kill opsonized *S. typhimurium* C5 sr was also determined.

The indirect RIA shows that normal and 11RX-activated macrophage populations express cell-surface markers recognized by M57, M43 and M143 monoclonal antibodies but in different amounts (Table 3). Macrophages from *S. enteritidis* 11RX-infected mice had more M143 ($p < 0.001$), M43 ($p < 0.001$) and M57 ($p < 0.02$) cell-surface markers than did macrophages from normal mice. Macrophages activated *in vitro* with lymphokiness, in comparison with normal macrophages, also displayed more M43 ($p < 0.05$) and and M57 ($p < 0.05$) cell-surface markers, but the number of those recognized by M143 was not significantly different (Table 3). This finding was in agreement with the immunofluorescence result (data not shown).

These result 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 markers on their cell surface.

TABLE 3
Detection of cell-surface markers on normal macrophages, 11RX-activated macrophages and macrophages activated *in vitro* with lymphokine by indirect RIA.

Rat anti-mouse monoclonal antibody	% radioactive binding /10^6 cells		
	Normal[a] macrophages	11RX-activated[a] macrophages	Lymphokine-activated macrophages[b]
M43	1.06 ± 0.24	2.66 ± 0.33	1.63 ± 0.23
M57	1.13 ± 0.17	1.65 ± 0.24	1.57 ± 0.32
M143	1.25 ± 0.4	4.0 ± 1.0	1.77 ± 0.06
Bactercidal[c] capacity	51.0 ± 7.7	10.9 ± 3.5	19.8 ± 7.8

a - Mean ± S.D of six separate experiments.
b - Mean ± of there separate experiments.
c - Percentage survival of *S.typhimurium* C5 sr in the presence of RPMI medium at 60 min.

The Effect of Pre-treating 11RX-activated Macrophages with Rat Anti-Macrophage 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 cytotoxity (ADCC) activity of macrophages. However, the inhibition of the killing of antibody coated tumor targets was only observed when the subsets of cells recognized by the above antibodies were lysed by treating them with the 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 with the exception that fresh rabbit serum and heat-inactivated rabbit serum (56°C/1 hour) were used. After such treatment, the bactericidal assay was carried out as before using a similar number of viable macrophages.

The results are shown in *Fig. 3*. The partial inhibition of the bactericidal activity of the 11RX-activated macrophages population by the

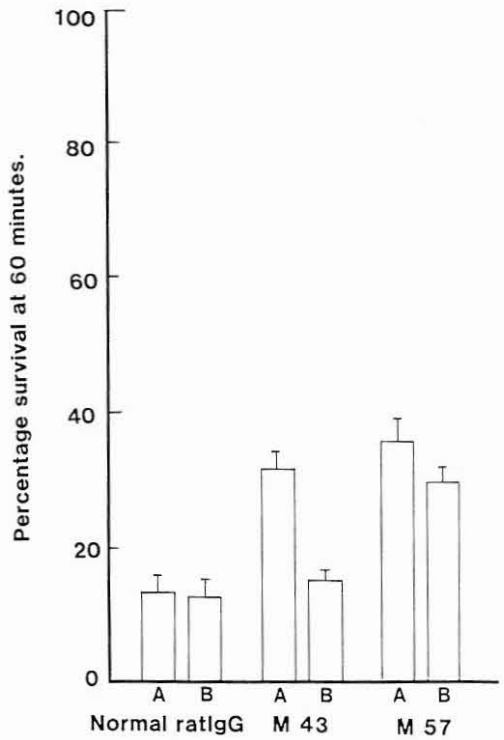

Fig.3: The killing of virulent opsonized S.typhimurium *C5 sr by macrophages from* S.enteritidis *11RX-infected mice which had been pre-treated with various rat monoclonal antibodies to mouse macrophages either in the presence (A) or absence (B) of complement. Control included 11Rx-activated macrophages pre-treated with normal rat IgG. Each histogram represents the mean percentage survival ± S.D of three experiments.*

M43 monoclonal antibody required the removal of a subset of cells from the cell population. Treatment of this cell population with the monoclonal 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.typhimurium* 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 could be related to an inhibition of phagocytosis.

DISCUSSION

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 marophages are necessary for antigen presentation (Beller *et al.* 1980) and only certain subpopulations can perform macrophage-mediated cytotoxicity (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 *et al.* 1979) and enzyme content (Fishman and Winberg 1979).

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; Mackeness 1960). These earlier indications of macrophage heterogeneity in terms of their bactericidal function are supported by data from the present studies. 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. The data also show that unlike the subset bearing the M43 marker, the bactericidal activity of that bearing the M57 could be abolished in the presence of the monoclonal antibody alone. It is possible that this antibody may interfere with the function of the relevent surface marker which could be directly involved in the bactericidal mechanism of macrophages such as phagocytosis. To abolish the activity of the former subset (M43), 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 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. However, treatment of macrophages with this latter monoclonal antibody did not diminish the ability of the population to kill *S.typhimurium* C5 sr. By immunofluorescence,it was found that in both normal and activated populations 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. These data together with data from indirect RIA clearly show that the suface markers recognized by the above monoclonal antibodies are not specific markers for activated macrophages. Similar results were obtained with macrophages activated by incubation *in vitro* with Con A supernatants. However, the amounts of the markers on lymphokine-activated macrophages were less than those on 11RX-activated macrophages. This may reflect the stage of macrophage activation of the two populations as expressed by their enhanced ability to kill the target bacteria. However, more work needs to be done before one can be sure 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 or as indicator for macrophage activation.

In conclusion, the present studies indicate further the use of monoclonal antibodies in defining subsets of macrophages and the possibility of assigning a particular function to a particular subset.

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