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Role of Cell Surface Receptors in the Regulation of Intracellular Killing of Bacteria by Murine Peritoneal Exudate Neutrophils

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The role of the Fc and third component of complement (C3) receptors on mouse neutrophils in the control of killing of *Proteus mirabilis*, opsonized in normal mouse serum (NMS) or heated immune mouse serum (HIMS), was studied. The events following incubation of neutrophils with *P. mirabilis* and the events associated with bacterial killing were assayed. The respiratory burst was quantified by chemiluminescence (CL). Levels of leukocyte-associated bacteria were determined after a 20-min ingestion period as a measure of phagocytosis. Bacterial killing was measured while ingestion was allowed to continue or as a discrete process when extracellular, noningested bacteria had been removed and neutrophils with intracellular bacteria were incubated in the presence of serum. Modification of these responses in the presence of three monoclonal antibodies (MAb), NIMP-R10 and M1/70, which bind to different epitopes of the mouse C3 receptor, and 2.4G2, which binds to the mouse Fc receptor, was investigated. MAb to the C3, but not to the Fc, receptors reduced CL, ingestion, and intracellular killing of NMS-opsonized *P. mirabilis*. MAb to the Fc receptor diminished CL to and reduced the rate of ingestion of HIMS-opsonized bacteria. The two MAb to the C3 receptor each produced a similar inhibition of ingestion and intracellular killing of HIMS-opsonized bacteria, but they only partially blocked CL. A range of MAb preparations reactive with other murine antigens did not inhibit these events, either with NMS- or HIMS-opsonized *P. mirabilis*. The results suggest that C3 receptors on mouse neutrophils played a predominant role in regulation of the killing of *P. mirabilis*. Similar results were found for *Staphylococcus aureus*. C3 receptors were necessary for maximal expression of all functions culminating in bacterial kill. That MAb to the C3 receptor inhibited phagocytosis of HIMS-opsonized bacteria in similar fashion to the effect of MAb to the Fc receptor and in contrast to the lack of effect of control MAb may reflect steric hindrance of the Fc receptor by MAb binding to the C3 receptor, or it may reflect that the receptors are linked in murine neutrophils as they are in human neutrophils.

Interaction between receptors on the plasma membrane of phagocytic cells and their respective ligands can trigger a number of important biological functions, such as immune adherence, phagocytosis, antibody-dependent cell-mediated cytotoxicity, and lysosomal enzyme release (1, 17). Of importance are the receptors for the third component of complement (C3) and for the Fc region of immunoglobulin G (IgG); both receptors have been identified on mouse neutrophils (16).

Experiments with C3- or antibody-opsonized erythrocytes, *Staphylococcus aureus*, or yeast cells (19, 25; O. Stendahl and J. Hed, RES J. Reticuloendothel. Soc., 24(Suppl.):39a, 1978) indicated that the C3 receptors of both human and mouse phagocytic cells were involved in the attachment but not the ingestion of complement-opsonized particles. In contrast, the Fc receptors of such cells bound to particles with surface-bound IgG, and engulfment ensued. However, it has also been shown that the C3 receptors of human and mouse phagocytic cells may be induced by lymphokines, fibronectin, and other inflammatory mediators to begin ingestion of C3-opsonized particles in the absence of Fc receptor involvement (23, 30–32). Griffin and Mullinax (9) used mouse cells and found that the prerequisite for particle ingestion was the ability of receptors to move within the plane of the plasma membrane of the cell.

We have found that phagocytosis and killing of *Proteus mirabilis* by murine neutrophils occurs in the presence of normal mouse serum (NMS) and in the absence of detectable specific antibody (6). The separate processes of phagocytosis and intracellular killing have also been examined. Antibody enhanced the phagocytosis of *P. mirabilis* in the presence of complement, but stimulation of intracellular killing was principally dependent on the presence of intact, extracellular complement (P. H. Hart, L. K. Spencer, N. L. Hill, P. J. McDonald, and J. J. Finlay-Jones, submitted for publication).

The present study examines more specifically the roles of the Fc and C3 receptors of mouse peritoneal exudate neutrophils in inducing an oxidative burst upon binding to opsonized *P. mirabilis* and in the phagocytosis and killing of *P. mirabilis* opsonized with NMS, heated NMS (HNMS), and heated immune mouse serum (HIMS). For this we used two independently raised rat monoclonal antibodies (MAb), NIMP-R10 (18) and M1/70 (28) which bind to different epitopes of the C3 receptor of mouse neutrophils (3, 15), as well as the rat MAb 2.4G2 which binds to the Fc receptor (30). The role of the Fc and C3 receptors in control of phagocytosis and killing of *S. aureus* was compared with that for *P. mirabilis*.

MATERIALS AND METHODS

Mice. Male 4- to 12-week old BALB/c mice were used. The ethical guidelines of the National Health and Medical Research Council and the Commonwealth Scientific and

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Industrial Research Organisation of Australia were followed in all experiments.

Bacteria. The isolation of *P. mirabilis* from intraabdominal abscesses of mice has been described (20). The *S. aureus* strain was a clinical isolate. For assays in vitro, log-phase bacteria were harvested from tryptic soy broth cultures (Difco Laboratories, Detroit, Mich.) as soon as turbidity was detected (6). The viable count was assessed from predetermined standard curves by measuring absorbance at 420 nm. For enumeration of viable bacteria after incubation with cells, 0.1 or 0.05 ml of the cell-bacteria suspensions was diluted in 0.9 or 5.0 ml of 0.05% Triton X-100 in 0.9% saline at room temperature for leukocyte lysis (6). After appropriate dilution, 0.1-ml samples were spread on CLED agar (Oxoid Ltd., Basingstoke, England), and the colonies were counted after overnight aerobic incubation at 37°C.

Sera. Blood was collected from the retroorbital venous plexus of anesthetized mice, and the serum obtained was filtered and stored at −70°C (6). Negligible amounts of anti-*P. mirabilis* antibody were detected in each of seven preparations of NMS when examined by an indirect immunofluorescence assay (mean titer, 1:1.3; range, not detected in undiluted serum, 1:4). Immune serum was obtained from mice injected intraperitoneally at monthly intervals with 10^7 *P. mirabilis* organisms in a volume of 0.1 ml for a total of four injections. The mice were bled 10 days after the last injection when the anti-*P. mirabilis* titer of pooled sera was 1:512. The antibody titer of *P. mirabilis* immune serum did not change during storage at −70°C for 24 months. Heated sera (HNMS and HIMS) were prepared by incubation at 56°C for 30 min.

Opsonization of bacteria. Bacteria (10^9 cells) suspended in 1 ml of RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) were incubated with 1 ml opsonizing serum at 37°C with shaking for 30 min. The bacteria were washed two times with 0.9% saline before being mixed with phagocytic cells.

Peritoneal exudate cells. At 3.5 h before harvesting of cells, mice were injected intraperitoneally with 1 ml of brain-heart infusion broth (Oxoid) prepared with tap water. The peritoneal cavities of the mice were washed initially with 5 and then with 3 ml of cold mouse osmolality phosphate-buffered saline (MPBS) (26). Harvested cells were washed two times with MPBS before either incubation with bacteria or neutrophil enrichment on Percoll (Pharmacia, Uppsala, Sweden) gradients.

Preparation of peritoneal exudate cells with intracellular bacteria. Peritoneal exudate cells (5×10^7 per ml) which were suspended in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS; Commonwealth Serum Laboratories, Melbourne, Australia) and buffered with 10 mM HEPES to pH 7.2, were incubated with 10^8 nonpreopsonized bacteria per ml in the presence of 1% NMS for 30 min. The use of 1% NMS allowed phagocytosis to proceed with minimal intracellular killing (10). The suspensions were mixed by incubation at 37°C on a rotating platform (Nutator, Clay Adams, B-D and Co., Parsippany, N.J.) at 20 rotations per min. After 30 min, the assay tubes were placed on ice before dilution with cold HEPES-buffered HBSS. At 0 and 30 min, 0.05-ml samples were taken for the determination of viable organisms and for the preparation of cytocentrifuge smears. After 30 min of incubation of nonpreopsonized *P. mirabilis* with peritoneal exudate cells, 38 ± 12% ($n = 6$) of neutrophils were associated with a mean of 2.7 ± 0.5 intracellular bacteria.

Percoll solutions. Percoll was diluted with minimal variation to the methods previously described (10). Briefly, a stock solution labeled 100% was prepared by mixing 9 volumes of Percoll with 1 volume of 10 × HBSS. This solution was further diluted with HEPES-buffered HBSS to solutions of 81, 65, 55, and 45% Percoll with densities of 1.1002, 1.0812, 1.0693, and 1.0575 g/ml, respectively.

Discontinuous Percoll density gradient centrifugation. Preformed gradients were prepared by gentle layering of less dense Percoll solutions upon more dense solutions in polycarbonate tubes (100 by 16 mm) (Disposable Products, Adelaide, South Australia). The cells for fractionation were applied suspended in HEPES-buffered HBSS. After centrifugation at 1,600 × *g* for 30 min at 10°C (PR-6000; International Equipment Co., Div. Damon Corp., Needham Heights, Mass.), cell bands (uppermost first) were harvested with Pasteur pipettes, washed two times with MPBS, and tested for their biological activities.

After application of peritoneal exudate cells (3-ml suspension of approximately 5×10^7 cells in HEPES-HBSS per gradient) to preformed Percoll gradients (3 ml of 81% Percoll solution, 3 ml of 65% Percoll solution, and 3 ml of 55% Percoll solution), leukocyte preparations of greater than 97% neutrophil purity were harvested in the band above the solution of greatest density, i.e., the harvested cells were in the density range 1.0812 to 1.1002 g/ml. The harvested cells accounted for greater than 60% of the cells applied to the gradients.

After application of peritoneal exudate cells containing intracellular bacteria (3-ml suspension of 5×10^7 cells in HEPES-HBSS) to preformed Percoll gradients (3 ml of 81% Percoll solution, 3 ml of 55% Percoll solution, and 3 ml of 45% Percoll solution), leukocyte preparations of approximately 90% neutrophil purity were harvested in the band above the most dense Percoll solution, i.e., the harvested cells were in the density range 1.0693 to 1.1002 g/ml. In this band there was a 70 ± 16% ($n = 9$) recovery of neutrophils applied to the gradient (10). Cell-associated bacterial loads remained approximately constant during fractionation on Percoll gradients at 10°C; less than 7% of the total bacteria present after Percoll fractionation and subsequent washing were extracellular as judged by [^{3}H]thymidine incorporation assays (10).

MAb. The MAb utilized were obtained as follows. (i) MAb NIMP-R10 was derived from fusion of the rat myeloma 210RCY3-Ag 123 with spleen cells from a LOU rat immunized with purified preparations of mouse eosinophils (17). Antibody-rich preparations were obtained from both the serum and ascites of rats injected intraperitoneally with the hybridoma cells. (ii) Rat MAb M1/70, which was raised against mouse macrophages (28) and was also known as anti-MAC-1 (3), was purchased as a tissue culture supernatant from Sera Lab, West Sussex, England. In addition, M1/70.15.11.5 hybridoma cells (27) were obtained from the American Type Culture Collection (ATCC TIB128), and supernatants from confluent cultures were harvested. (iii) Anti-Fc receptor MAb 2.4G2 (29) was harvested from the supernatant of rat hybridoma cells culture in vitro. This MAb binds to the macrophage Fc receptor for IgG1-IgG2b and also blocks the binding of mouse IgG1, IgG2a, and IgG2b to mouse granulocytes (14). (iv) Rat MAb produced by cell line 30-H12 (ATCC TIB107) reacts with the differentiation antigen Thy-1.2 (12). (v) Rat MAb produced by cell line 53-6.72 (ATCC TIB105) reacts with the Lyt-2 antigen (12). (vi) Rat MAb produced by cell lines 15-5-5S, 34-7-23S, and 34-4-20S (ATCC HB-24, HB-101, and HB-75, respec-

tively) produce anti-H-2 antibodies of specificities D^kK^d, D^dK^d, and D^d, respectively (21, 22).

For production of MAb iv through vi, the cells were grown in vitro, and supernatants were harvested. MAb i through iv were of the IgG2b subclass; the remaining MAb were IgG2a. MAb i through iii bound to mouse neutrophils, eosinophils, and macrophages. MAb iv and v were shown by an indirect immunofluorescence assay to bind to cells from mouse thymus and spleen, with no binding detected to neutrophils. Minimal binding of the anti-H-2 MAb vi to mouse neutrophils was detected; in contrast, binding to mononuclear cells from BALB/c mice was extensive.

All preparations of MAb in a concentrated form (1/2 to 1/5) were dialyzed extensively against MPBS before dilution with the appropriate medium and before 0.22-μm-pore-size membrane filtration. The activities of the MAb solutions were investigated when diluted 1/10, with the exception of NIMP-R10 which was used at a 1/100 dilution of ascites or at a 1/50 dilution of serum. The working concentrations of MAb i through iv were selected to give maximal levels of inhibition in the assays described. The dilutions of serum and ascites used did not influence chemiluminescence (CL) of neutrophils per se.

Measurement of CL. CL was measured as previously described (7, 8) in a luminometer (model 1250; LKB Instruments, Inc., Rockville, Md.) fitted with a 37°C water-jacketed sample holder which provided low-speed rotation of the reaction vessel to maintain continuous mixing of the cell suspension. Luminol (3-aminophthalhydrazide; Koch Light Laboratories, Bucks, England) was prepared as a 0.056 M stock solution in dimethyl sulfoxide. This was diluted 1/40 in dye-free Eagle minimal essential medium (Flow Laboratories) and prewarmed to 37°C before use. Approximately 5×10^6 leukocytes (neutrophil-enriched on Percoll gradients to greater than 97% purity) in 0.05 to 0.25 ml of minimal essential medium containing NMS, such that the concentration in 1.2 ml final volume was approximately 0.5%, were added to 0.4 ml of luminol working solution before the addition of 0.4 ml of the appropriate MAb solution. CL was measured by light output integrated over 10-s intervals during the ensuing 5 min. A 0.1-ml sample of *P. mirabilis* that had been opsonized in NMS (at 2×10^8 bacteria per ml) or HIMS (at 3×10^8 bacteria per ml) was then added, and the rate of CL was measured as described above for the following 8 min. The CL response of cells to *P. mirabilis* in the absence of MAb was measured at the beginning and termination of each experiment as a check of the maintenance of neutrophil viability.

Assays of phagocytosis and killing. Several assays were used to quantify the components of neutrophil bactericidal activities: (i) Assays of phagocytosis were done, which determined two parameters of bacterial engulfment by neutrophils: one in which neutrophil-associated bacteria were determined by light microscopy and one in which extracellular (nonengulfed) bacteria were quantified by their ability to incorporate [^{3}H]thymidine. (ii) In the assay of phagocytic killing, bactericidal activity was measured, with ongoing phagocytosis possible for the duration of the assay. (iii) In the assay of intracellular killing, the bactericidal activity of neutrophils was determined after a period of phagocytosis under conditions that did not stimulate killing, followed by Percoll gradient centrifugation and washings of neutrophils to remove extracellular bacteria (10). Thus, intracellular killing could be quantified in the absence of ongoing phagocytosis.

Measurement of phagocytosis and the determination of extracellular bacteria. Approximately 5×10^6 Percoll gradient-enriched neutrophils suspended in 0.075 to 0.25 ml of RPMI 1640 were mixed with 0.35 ml of MAb solution or as a control, RPMI 1640 for 10 min at 4°C with intermittent shaking. RPMI 1640 was then added together with 0.02 ml of NMS or HIMS. To initiate the assay, 10^7 *P. mirabilis* preopsonized with NMS or HIMS were added after warming the cell suspensions to 37°C. The final volume in each tube was 1.0 ml.

After 20 min, 0.05-ml samples were withdrawn and immediately cooled before preparation of cytocentrifuge smears. After staining with Jenner-Giemsa reagents, the smears were examined by light microscopy for enumeration of the percentage of neutrophils with associated bacteria. In addition, the number of bacteria per 100 neutrophils was determined. Duplicate readings for each slide were averaged. The proportion of the initial inoculum of *P. mirabilis* that was associated with neutrophils was calculated.

For the determination of extracellular bacteria, triplicate samples of 0.2 ml were withdrawn and placed into microtiter tray wells (Linbro; Flow Laboratories, Inc., McLean, Va.), and 1.6 μCi of [^{3}H]thymidine (Amersham International, Bucks England) in 0.02 ml was added. After 30 min at 37°C, the well contents were harvested with 5% trichloroacetic acid onto glass fiber paper by using a cell harvester (Titertek; Skatron, Norway). After being washed with water and methanol, the filters were dried at 60°C, and the radioactivity was determined with ACS scintillation fluid (Amersham) and a Mark II beta scintillation counter (G. D. Searle & Co., Skokie, Ill.). The counts obtained were compared with similarly processed control suspensions of bacteria without phagocytes.

Measurement of phagocytic killing. As described for the measurement of phagocytosis, bacteria were added to cell suspensions preincubated for 10 min with MAb. Tubes were incubated at 37°C with shaking for 90 min. Samples of 0.05 ml were withdrawn at 0, 45, and 90 min for the enumeration of viable bacteria by 1/100 dilution in 0.1% Triton X-100 in saline and vortexing to lyse neutrophils and disrupt bacterial aggregates. This suspension was serially diluted in saline; 0.1-ml volumes of appropriate dilutions were spread onto CLED agar, and colonies were counted after aerobic overnight incubation at 37°C.

As previously described (6), changes in the concentration of bacteria over a time period, t, were calculated for each tube as follows: $\Delta \log_{10}$ (CFU per ml) = [$\log_{10}$ (CFU per ml) at time t] − [$\log_{10}$ (CFU per ml) at time 0].

Measurement of intracellular killing after phagocytosis. Approximately 5×10^6 Percoll gradient-enriched neutrophils, many containing intracellular bacteria but relatively free of extracellular bacteria, were mixed with MAb preparations for 10 min at 4°C as described above. RPMI 1640 and 0.1 ml of serum were then added to a final volume of 1.0 ml; the tubes were warmed to 37°C and then incubated for 90 min at 37°C on a rotating platform (Nutator) at 20 turns per min. Samples for the enumeration of viable intracellular bacteria were taken at 0, 45, and 90 min.

Expression of results. Mean values are given ± one standard deviation. The significance of differences between means was determined with Student's t test.

RESULTS

CL. Incubation of the anti-Fc-receptor MAb 2.4G2 with mouse neutrophils did not stimulate CL. In contrast, incubation with both M1/70 and NIMP-R10 resulted in a burst of

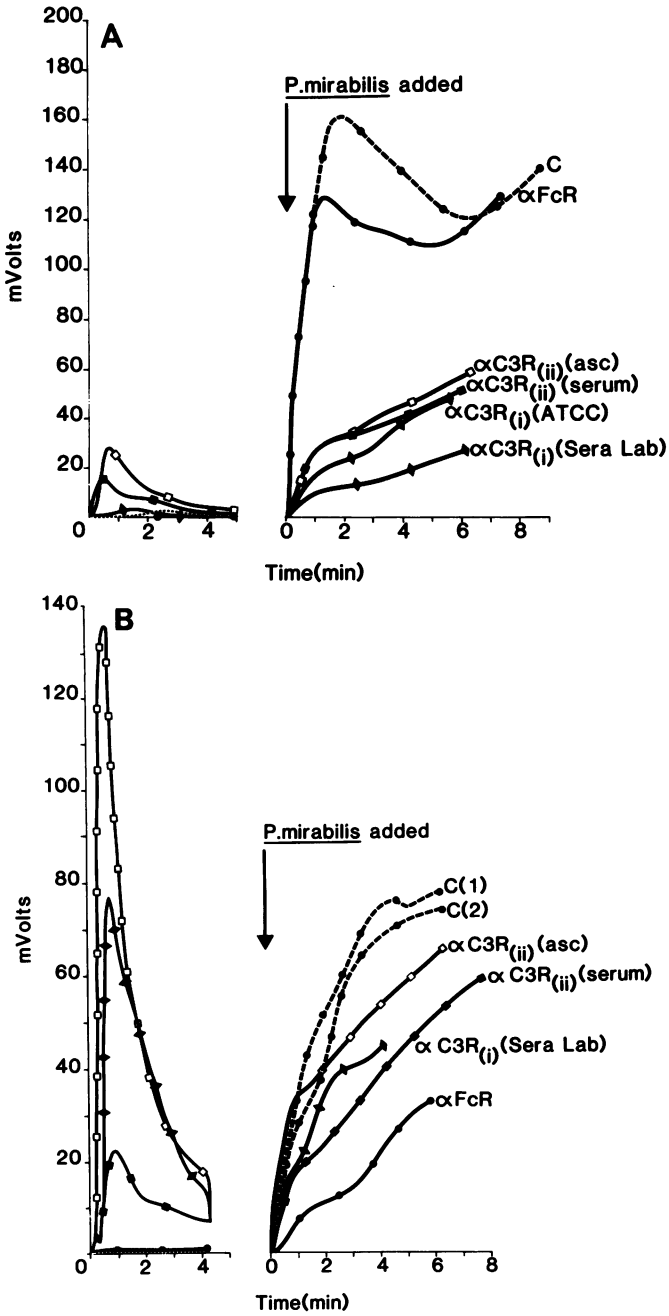

FIG. 1. Effect of MAb on the CL response by neutrophil-enriched leukocytes. (A) Light output (0 to 200 mV) integrated over 10-s intervals upon subsequent addition of 2×10^7 *P. mirabilis* opsonized with NMS. (B) Light output (0 to 140 mV) integrated over 10-s intervals upon subsequent addition of 3×10^7 *P. mirabilis* opsonized with HIMS. Abbreviations: C, control; αFcR, 2.4G2; $\alpha C3R_{(i)}$, M1/70; $\alpha C3R_{(ii)}$, NIMP-R10.

light production; a response which returned to close to basal levels after 5 min (Fig. 1).

The addition of 2×10^7 NMS-preopsonized *P. mirabilis* to neutrophils resulted in a strong CL response which was decreased by pretreatment with anti-C3 receptor MAb but not with anti-Fc receptor MAb (Fig. 1A). This experiment was repeated with similar findings. In contrast, the CL response to 3×10^7 HIMS-preopsonized *P. mirabilis* was reduced by pretreatment with the anti-Fc receptor MAb and to a lesser extent with the anti-C3 receptor antibodies (Fig. 1B).

Phagocytosis of *P. mirabilis*. Phagocytosis of HIMS-opsonized but not NMS-opsonized *P. mirabilis* was inhibited by pretreatment with the anti-Fc receptor MAb 2.4G2 (Table 1). Anti-C3 receptor MAb reduced phagocytosis of both NMS- and HIMS-opsonized bacteria (Table 1). Incubation of neutrophils with MAb reactive with H-2, Lyt-2, or Thy-1.2 antigens did not reduce the number of *P. mirabilis* phagocytosed (Table 1).

Phagocytic killing of bacteria. Neutrophils that had been preincubated with MAb were added to complement- (NMS) or antibody- (HIMS) preopsonized *P. mirabilis*. The anti-C3 receptor MAb significantly reduced the ability of neutrophils to phagocytose and kill NMS-opsonized *P. mirabilis* (Table 2). Killing of HIMS-opsonized bacteria was insufficient to allow significant differences to be discerned (Table 2). However, it should be noted that antibody was significantly less effective than complement in stimulating bacterial killing in this assay.

The MAb to the C3 receptor had a similar effect on the phagocytic killing of *S. aureus* in NMS. The decrease ($\bar{x} \pm$ standard deviation) in the viability of *S. aureus* over 90 min was 1.3 ± 0.2 $\log_{10}$ (CFU per ml) in control assays, compared with 1.3 ± 0.2 (pretreatment with 2.4G2) and -0.1 ± 0.1 (pretreatment with NIMP-R10).

Intracellular killing of bacteria. The continuing presence of 10% NMS was necessary for significant killing of intracellular *P. mirabilis* (Table 3); the anti-Fc receptor MAb did not significantly reduce the rate of bacterial killing in the presence of NMS. Anti-C3 receptor MAb reduced NMS-stimulated intracellular killing of *P. mirabilis*, but to a level that was still greater than the kill measured in the absence of serum.

Intracellular killing in the presence of 10% HIMS or 10% HNMS was similar to that measured in the presence of NMS together with anti-C3 receptor MAb. Anti-C3 and anti-Fc receptor MAb had no significant effects on the rates of bacterial killing in the presence of heated sera.

Similar results were obtained with *S. aureus*. Extracellular 10% NMS stimulated the intracellular killing of *S. aureus* (Δ $\log_{10}$ CFU per ml $= -1.0 \pm 0.2$ over 90 min). This was significantly diminished with anti-C3 receptor MAb (Δ $\log_{10}$ CFU per ml $= -0.4 \pm 0.3$). No significant change was found when the neutrophils were treated with the MAb to the Fc receptor before addition to the assay containing 10% NMS (Δ $\log_{10}$ CFU per ml $= -0.9 \pm 0.1$).

DISCUSSION

These studies have highlighted the importance of the C3 receptors of neutrophils in their interactions with and their subsequent biological responses to *P. mirabilis* in the presence of NMS. Binding of heat-labile components of mouse serum to C3 receptors was necessary for maximal CL (Fig. 1), maximal phagocytosis (Table 1), maximal rates of killing with ongoing phagocytosis (Table 2), and once ingestion was complete, intracellular killing (Table 3). These findings were supported by similar results obtained for the intracellular killing of *S. aureus*.

The interaction between particles and phagocytic cells activates a membrane oxidase which in turn triggers the respiratory burst. Chemically reactive molecules, e.g., superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals are produced by the partial reduction of oxygen (2). The generation of a respiratory burst was inves-

TABLE 1. Effect of MAb on phagocytosis of *P. mirabilis*[a]

MAb	Cell-associated bacteria				Extracellular bacteria	
	% Neutrophils with cell-associated bacteria	Mean no. of cell-associated bacteria	Total no. of cell-associated bacteria[b]	*P* vs no MAb[c]	[^{3}H]thymidine incorporated (10^3 cpm)	*P* vs no MAb[d]
NMS-opsonized *P. mirabilis*						
	57.0 ± 3.5	2.1 ± 0.2	59.9 ± 10.0		2.2 ± 0.2	
2.4G2	51.0 ± 7.8	1.8 ± 0.0	46.0 ± 6.5	NS[e]	2.7 ± 0.2	NS
M1/70	43.8 ± 7.1	1.8 ± 0.1	39.3 ± 8.2[f]	<0.05	12.0 ± 8.3[f]	<0.01
NIMP-R10	41.4 ± 6.4	1.6 ± 0.3	32.0 ± 4.2[f]	<0.01	18.7 ± 2.5[f]	<0.001
αLyt-2	56.3 ± 4.6	1.9 ± 0.1	52.5 ± 1.9	NS	2.1 ± 0.2	NS
αThy-1.2	52.8 ± 1.8	1.9 ± 0.3	50.1 ± 8.2	NS	2.3 ± 0.1	NS
HIMS-opsonized *P. mirabilis*						
	24.2 ± 3.0	4.7 ± 0.6	55.9 ± 7.4		13.5 ± 1.4	
2.4G2	14.9 ± 2.1	5.1 ± 0.8	38.3 ± 8.6	<0.05	19.9 ± 1.6	<0.001
M1/70	15.0 ± 4.2	4.0 ± 0.5	30.4 ± 10.8[f]	<0.01	17.2 ± 2.3[f]	<0.001
NIMP-R10	14.0 ± 2.2	3.2 ± 0.3	23.0 ± 5.9[f]	<0.001	20.1 ± 1.3[f]	<0.001
αLyt-2	22.8 ± 0.4	5.2 ± 0.3	59.6 ± 4.8	NS	14.1 ± 1.1	NS
αThy-1.2	18.9 ± 1.1	5.0 ± 0.0	46.8 ± 2.5	NS	12.1 ± 3.2	NS
HIMS-opsonized *P. mirabilis*						
	39.2 ± 6.4	3.9 ± 0.4	36.5 ± 4.3		ND[g]	
2.4G2	20.5 ± 0.8	2.6 ± 0.6	17.1 ± 3.2	<0.01	ND	
M1/70	24.3 ± 5.7	2.9 ± 0.4	22.4 ± 4.3	<0.01	ND	
NIMP-R10	20.4 ± 4.3	2.2 ± 0.6	15.9 ± 7.1[h]	<0.001	ND	
αH-2 (D^kK^d)	40.8 ± 3.6	3.6 ± 0.1	52.3 ± 2.1	<0.01	ND	
αH-2 (D^dK^d)	49.0 ± 3.3	2.4 ± 0.2	40.1 ± 7.1	NS	ND	
αH-2 (D^d)	35.3 ± 1.6	2.5 ± 0.1	35.5 ± 0.7	NS	ND	

[a] Data are mean plus or minus standard deviation (n = 3 for each preparation of MAb).
[b] Expressed as a percentage of the total *P. mirabilis* added to the assay.
[c] *P* expresses the significance of the difference of means for the total cell-associated bacteria for test versus control groups.
[d] *P* expresses the significance of the difference of means for [^{3}H]thymidine incorporated for test versus control groups.
[e] NS, Not significant.
[f] Mean of results with two preparations of the same MAb, each in triplicate.
[g] ND, Not done.

tigated with the chemical substrate luminol, which is oxidized to the luminescent phthalate ion by a variety of oxygen metabolites, and hence light is emitted. This widely used method (7, 8) provided a useful assay for the production of reactive oxygen species.

Wright and Silverstein (31) reported that stimulation of the C3 receptors of human polymorphonuclear cells could not trigger the release of H_2O_2, even under conditions in which they readily promoted phagocytosis. Analogous results of a dissociation of phagocytosis from stimulation of the oxidative metabolic burst have been reported for murine macrophages (32). In the present experiments with mouse neutrophils, the addition of the anti-C3 receptor MAb stimulated a CL response. Subsequent incubation with NMS-opsonized *P. mirabilis* generated a further response. Thus, although phagocytosis of complement-opsonized *P. mirabilis* via C3 receptors may not have been directly related to generation of the CL response, they both occurred simultaneously.

Greater phagocytosis (Table 1) and killing (Table 2) of bacteria by neutrophils was seen when NMS rather than HIMS was included in the preopsonizing and assay mix-

TABLE 2. Effect of MAb on the phagocytic killing of serum-opsonized *P. mirabilis* by peritoneal exudate neutrophils

Group	Opsonizing serum	Serum in phagocytic assay	MAb	Δ $\log_{10}$ (CFU/ml)[a]
A	50% NMS	2% NMS		−2.8 ± 0.5
			2.4G2	−2.6 ± 1.4
			M1/70	−0.8 ± 0.4[b]
			NIMP-R10	−0.9 ± 0.5[b]
B	50% HIMS	2% HIMS	—	−0.7 ± 0.1
			2.4G2	−0.6 ± 0.1
			M1/70	−0.4 ± 0.2
			NIMP-R10	−0.2 ± 0.3

[a] Change in the viability ($\log_{10}$ CFU per ml) of *P. mirabilis* after 90 min: $\bar{x}$ ± standard deviation for (group A) five experiments or (group B) three experiments.
[b] Significantly different from control (no MAb treatment), $P < 0.001$ (Student's t test).

TABLE 3. Effect of MAb on the intracellular killing of *P. mirabilis* by peritoneal exudate neutrophils[a]

Extracellular serum	No. of expts	Intracellular killing[b] after treatment with:			
		No MAb	2.4G2	M1/70	NIMP-R10
10% NMS	6	−2.0 ± 0.6	−1.6 ± 0.6	−0.7 ± 0.3[c]	−0.5 ± 0.2[c]
10% HIMS	2	−0.5 ± 0.1	−0.5 ± 0.1	−0.4 ± 0.1	−0.3 ± 0.1
10% HNMS	4	−0.4 ± 0.2	−0.5 ± 0.2	−0.4 ± 0.2	−0.3 ± 0.2
No serum	4	+0.1 ± 0.2	ND[d]	ND	ND

[a] *P. mirabilis* was phagocytosed in the presence of 1% NMS. Extracellular bacteria were removed before pretreatment of neutrophils with MAb and transfer of neutrophils to medium with extracellular serum supplements.
[b] Change in the viability ($\log_{10}$ CFU per ml) of *P. mirabilis* after 90 min: $\bar{x}$ ± standard deviation.
[c] Significantly different from control (no MAb treatment), $P < 0.001$ (Student's t test).
[d] ND, Not done.

tures. This indicated that antibody was less efficient than complement in the opsonization of *P. mirabilis* and subsequent stimulation of neutrophil function. We have nevertheless found a cumulative effect of antibody and complement together in the stimulation of microbial ingestion but not of intracellular killing (data not shown). From previous reports that the Fc receptor was involved in the overall control of ingestion (4) and killing (13) of bacteria by neutrophils, one would have predicted that incubation with the anti-Fc receptor MAb may have inhibited the stimulation of leukocyte activity by bacteria opsonized in NMS; however, such an effect was not observed. Nevertheless, the anti-Fc receptor MAb did inhibit the CL response to (Fig. 1B) and phagocytosis of (Table 1) HIMS-opsonized *P. mirabilis*.

An unexpected finding was that the pretreatment of neutrophils with anti-C3 receptor MAb inhibited phagocytosis of *P. mirabilis* that had been opsonized in HIMS (Table 1). This may have been partly caused by physical interference by anti-C3 receptor MAb of antibody-opsonized bacteria reaching and interacting with unoccupied Fc receptors. Alternatively, the Fc portion of the anti-C3 MAb may have bound nonspecifically to the neutrophil Fc receptors. However, this latter possibility was not supported by the finding that phagocytosis of *P. mirabilis* opsonized with HIMS by neutrophils pretreated with MAb reactive with H-2 antigens on mouse neutrophils or with mouse Lyt-2 or Thy-1.2 antigens, all of which may have bound by their Fc portions to the Fc receptors, was not significantly less than that measured when cells were pretreated with medium alone. It has also been shown with human neutrophils that anti-C3 receptor antibody can inhibit ingestion of IgG-opsonized erythrocytes (24).

The components of heated sera which stimulated intracellular killing of *P. mirabilis* by mouse neutrophils to an extent greater than was measured in the absence of serum remain unidentified. Leijh et al. (13) concluded that nonspecific immunoglobulin of heated, nonimmune serum stimulated intracellular killing of *Escherichia coli* by human phagocytic cells. In addition, they showed that IgG1 and IgG3 as well as Fc fragments of IgG, but not $F(ab')_2$ fragments of IgG, stimulated killing of intracellular *E. coli* to the same degree as heated serum. In the present experiments with mouse neutrophils incubated in HNMS, pretreatment with the anti-Fc receptor MAb did not reduce the rate of intracellular killing of *P. mirabilis* compared with that by neutrophils not treated with MAb.

These studies do not appear to support the role of the neutrophil C3 receptor as one to solely promote binding of a particle to a phagocyte, with engulfment being dependent on involvement of the Fc receptor (4, 19, 25; Stendahl and Hed, RES J. Reticuloendothelial. Soc., 1978). Because the antibacterial response in the presence of normal serum is equivalent in the presence or absence of anti-Fc receptor MAb, the C3 receptor on mouse neutrophils appears to be able to act alone in the stimulation of ingestion and killing of *P. mirabilis* and *S. aureus* by NMS. However, this result may reflect the activation of complement receptors of neutrophils upon elicitation into the peritoneal cavity by an acute inflammatory agent (30, 31). The mechanism whereby MAb to the complement receptor influences Fc receptor-mediated phagocytosis and killing is not clear. It has been reported that the human neutrophil responds to polyvalent cross-linking of C3b receptors by a cytoskeletal-dependent rearrangement of both the C3b and Fc receptors (11). This redistribution occurs bidirectionally and results in an overlapping topographical distribution of the receptors (11). A similar cooperative interaction between the Fc and C3 receptors on mouse neutrophils may explain the results reported herein. It is proposed that in future experiments the effects of both monovalent and bivalent anti-C3 receptor MAb [i.e., Fab and $F(ab')_2$ fragments] on Fc receptor-mediated functions of mouse neutrophils should be examined as a means of distinguishing the effects attributable to blocking only from those that may result from blocking and cross-linking receptors.

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