THE SELECTIVE INHIBITION OF MACROPHAGE PHAGOCYTIC RECEPTORS BY ANTI-MEMBRANE ANTIBODIES*

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One of the major characteristics of macrophages is their ability to phagocytize a wide range of particulates. Recent evidence concerning the attachment and ingestion phases of phagocytosis (1) and the identification of distinct receptor sites on the plasma membrane (2-6) has emphasized the ability of these cells to distinguish between test particles. This discriminatory activity on the part of an essential phagocyte may have important implication for body economy. Unfortunately little is known about the topography of phagocytic receptors on the macrophage surface nor has their chemical nature been elucidated.

In view of the ability of anti-cellular antibodies to modify the endocytic activity of both macrophages and HeLa cells (7-10), it seemed appropriate to examine this interaction in more detail. In particular, to utilize antibodies directed against the macrophage surface as reagents for the definition of specific receptors which recognize various classes of immunoglobulins, complement, or the surface of effete cells. Furthermore, additional information was necessary concerning the specificity and mechanism of action of anti-macrophage antibody.

This report will describe the effects of rabbit anti-mouse macrophage, anti-mouse erythrocyte, and anti-mouse lysosome antibodies, all of which are in part directed against antigenic determinants on the macrophage plasma membrane, on the attachment and ingestion of particles by mouse peritoneal macrophages.

Materials and Methods

Preparation of Rabbit Anti-Mouse Erythrocyte Antiserum.—Rabbits weighing 3 kg were injected intraperitoneally with 1 ml 50% mouse erythrocytes which had been previously washed three times with large volumes of buffered saline (pH 7.2). Inoculations were once a week for 4 successive wk and the animals were bled 7 days after the fourth injection.

Preparation of Rabbit Anti-Mouse Macrophage Antiserum.—NCS mice weighing 25 g were injected intraperitoneally with 1.5 ml 10% proteose peptone. 3 days later the peritoneal cavity was lavaged with heparinized Medium 199 and the contents aspirated. The exudate

was centrifuged at 500 rpm for 5 min and resuspended in Medium 199 with 20% heat-inacti-vated fetal calf serum (HIFCS)[1] (Grand Island Biological Co., Grand Island, N.Y.) in a final concentration of 2×10^6 cells/ml. 1 ml was dispensed to 35-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) and incubated for 60 min at 37°C. At the end of this time each dish was rinsed vigorously with warm Medium 199 to remove unattached cells and reincubated with fresh Medium 199 containing 20% HIFCS for 4 days at 37°C in a 5% CO_2-air atmosphere. At 4 days the media was removed and the macrophage monolayer was rinsed twice. The adherent cells were then detached with a rubber policeman and approxi-mately 1×10^7 macrophages were incorporated into Freund's complete adjuvant (Difco Laboratories Inc., Detroit, Mich.). Rabbits weighing 3 kg each were inoculated subcutaneously twice, bi-weekly and bled 2 wk after the second injection.

Preparation of Rabbit Anti-Mouse Lysosome Antiserum.—The general method of prepara-tion was similar to that used by Trouet (11) and Tulkens et al. (12) for rat liver and will be described in detail elsewhere. In brief, 4 days after the intraperitoneal injection of Triton WR1339, the livers were homogenized and the Triton-filled lysosomes were purified by cycles of differential and discontinuous sucrose gradient centrifugation. About 58% of the total acid hydrolases were recovered in the lysosome fraction and these were enriched more than 100-fold. The lysosomes were lysed with bicarbonate, dialyzed, and the membranes discarded after ultracentrifugation. The soluble lysosome fraction was concentrated to contain 5 mg protein/ ml, mixed with complete Freund's adjuvant, and used to immunize rabbits according to the method of Trouet (11). It should be pointed out that the lysosomal material arose from both hepatocytes and Kupffer cells and may very well contain remnants of the endocytic activity of the Kupffer cells.

Separation of Immunoglobulins by $(NH_4)_2SO_4$ Precipitation.—Fresh normal rabbit serum and antisera from rabbits immunized with mouse erythrocytes, mouse peritoneal macro-phages, and mouse liver lysosomes were heat inactivated at 56°C for 30 min. Immunoglobu-lins were fractionated from the serum by precipitation with 50% $(NH_4)_2SO_4$ and the proce-dure repeated three times. The final precipitate was dissolved in nonpyrogenic isotonic saline (pH 7.2) and dialyzed exhaustively against the diluent at 4°C. The material was sterilized with a Millipore filter (Millipore Corp., Bedford, Mass.), pore size 0.45 μ, and stored at 0°C in small aliquots.

Separation of Immunoglobulins by Sephadex G-200 Filtration.—1 ml samples of $(NH_4)_2SO_4$-precipitated rabbit anti-mouse erythrocyte, rabbit anti-mouse macrophage, and rabbit anti-mouse lysosome γ-globulins were loaded on a 65×1.5 cm Sephadex G-200 column (Pharma-cia Fine Chemicals, Inc., Uppsala, Sweden) and eluted with 0.15 M phosphate-buffered saline, pH 7.4. Purity of the IgG and IgM eluted fractions was checked using duck anti-rabbit anti-serum in an immunoelectrophoretic analysis and goat anti-rabbit IgG and IgM by double diffusion in agar assay.

Hemagglutination Titrations.—Mouse blood harvested by cardiac puncture was heparinized and centrifuged at 2000 rpm. The supernatant plasma was decanted, the packed erythrocytes were washed four times with 10 volumes of buffered saline, and resuspended in phosphate-buffered saline (pH 7.4) in a concentration of 1%. Twofold dilutions of serum were prepared in plastic depression trays using buffered saline as diluent. Washed mouse erythrocytes were then added in a final concentration of 0.5% and incubated for 2 hr at room temperature and overnight at 4°C. The last dilution showing macroscopic hemagglutination was taken as the end point.

Evaluation of Phagocytosis by Mouse Macrophages.—

General procedure: The method used for collection and maintenance of mouse peritoneal

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[1] *Abbreviations used in this paper:* HIB, beef heart infusion broth; HIFCS, heat-inactivated fetal calf serum; MEM, Eagle's minimal essential medium.

macrophage cultures has been described previously (13). Macrophages were cultured on 22-mm square glass cover slips in 35-mm plastic tissue culture dishes containing 2.5 ml of Eagle's minimal essential medium (MEM) (Microbiological Associates, Bethesda, Md.) with 20% HIFCS.

The particles were added to the culture media and the plastic dish was gently agitated to allow equal dispersion. Stationary incubation was then allowed for 60 min at 37°C in a 5% CO_2-air atmosphere. At the end of the incubation period the culture medium was aspirated, cover slips were rinsed vigorously three times with isotonic saline, and immediately fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, for 10 min at 4°C. Phagocytosis was quantitated by counting the number of particles ingested per 100 macrophages (phagocytic index) and the per cent of macrophages which contained one or more particles. Results were obtained from a minimum of 200 cell counts on successive microscopic fields using a 40 X oil immersion objective and phase-contrast microscopy.

Preparation of Particles Employed in Evaluation of Phagocytosis.—

Polystyrene latex beads: Twice-washed polystyrene latex beads diameter 1.099 μ (Dow Chemical Company, Midland, Mich.) were added in a final concentration of approximately 2 X 10^7 particles/ml.

Antibody-coated sheep erythrocytes: Sheep erythrocytes obtained from Pocono Laboratory, Canadensis, Pa., were washed three times in 0.01 M ethylenediaminetetraacetate (EDTA) buffer, pH 7.4, and resuspended to a final concentration of 1 X 10^9 cells/ml. Sheep erythrocytes were routinely incubated with an equal volume of a 1/1500 dilution of rabbit anti-sheep hemolysin (Baltimore Biological Laboratories, Baltimore, Md.) for 30 min at 37°C. The sensitized cells were then washed three times with isotonic Veronal-buffered saline and resuspended in dextrose Veronal–buffered saline at a final concentration of 5 X 10^8 cells/ml. Just before evaluation of phagocytosis the sensitized sheep cells were diluted in MEM and 7.5 X 10^6 erythrocytes were added to each plastic culture dish containing the macrophage monolayer.

Formaldehyde-treated sheep erythrocytes: Sheep erythrocytes were washed five times in phosphate-buffered saline, pH 7.2. To 1 volume of packed cells was added 8 volumes cold 3% formaldehyde. The cell suspension was placed in a tightly corked flask and agitated for 24 hr at 4°C at which time 2 volumes of cold 40% formaldehyde was added and agitation continued for another 24 hr period. The cells were then centrifuged at 2000 rpm for 5 min, washed 10 times in phosphate-buffered saline, and resuspended to a final volume of 5 X 10^8 cells/ml. Approximately 7.5 X 10^6 formalinized red cells in MEM were added to each tissue culture dish containing the macrophage monolayer.

Yeast cell walls (zymosan): Zymosan particles were washed three times in phosphate-buffered saline and resuspended in MEM. Approximately 7.5 X 10^6 particles were added to each plastic culture dish containing the macrophage monolayer.

Preparation of EAC1,4,2,3: EAC1,4,2,3 were prepared by adding functionally pure C_2 in dextrose Veronal-buffered saline to the erythrocyte intermediate EAC1gp4hu (14) and incubating at 30°C for 20 min to form EAC1,4,2. Purified C3hu (15) was then added in excess and the suspension was incubated at 37°C for 30 min. The cells were washed in Veronal-buffered saline and suspended in dextrose Veronal–buffered saline to a concentration of 5 X 10^8/ml. Positive immune adherence (16) assured the attachment of C3 to the intermediate.

*Immunoglobulin Fractionation of Rabbit Anti-Sheep Hemolysin.—*Immunoglobulins in commercial rabbit anti-sheep hemolysin were precipitated with 50% $(NH_4)_2SO_4$ and repeated three times. The final precipitate was dissolved in isotonic saline and dialyzed exhaustively against the diluent. The material was sterilized using a Millipore filter, pore size 0.45 μ, and a 1.5 ml portion was loaded on a 65 X 1.5 cm Sephadex G-200 column and eluted with 0.15 M phosphate-buffered saline, pH 7.2. Hemagglutination titrations were performed on the eluted IgG and IgM peaks. Separation and purity of the IgG and IgM fractions was ascertained using duck anti-rabbit antiserum in an immunoelectrophoretic analysis and goat anti-rabbit

IgG and goat anti-rabbit IgM by double diffusion in agar assay. IgG fractions were used to coat fresh sheep erythrocytes using the previously outlined technique.

Pepsin Digestion of IgG Antibody Fractions.—Aliquots of the Sephadex G-200 column IgG eluates of rabbit anti-mouse macrophage, anti-mouse erythrocyte, and anti-mouse lysosome antibody were subjected to pepsin digestion by the method of Nisonoff (17). The IgG fractions were dialyzed against 0.07 M acetate buffer, pH 4.0, in 0.05 M sodium chloride for 12 hr at 4°C. Specimens were sterilized using a Millipore filter, pore size 0.45 μ, and divided in half. One half portion was treated with 2 mg of pepsin (twice crystallized, lyophilized, Worthington Biochemical, Freehold, N.J.) per 100 mg of protein for 18 hr at 37°C followed by dialysis against borate-buffered saline, pH 8, for 20 hr at 4°C. The remaining portion underwent the same incubation and dialysis procedures but pepsin was omitted. Both samples were evaluated for hemagglutinating activity to assure the presence of (Fab')$_2$ and intact IgG, respectively.

Mycoplasma pulmonis.—The WRAI strain of *Mycoplasma pulmonis* in the 20–26th passage was passed in beef heart infusion broth (HIB) with 20% beef heart infusion broth as previously described (18). 24 hr before use the mycoplasma broth culture was diluted 1/100 in a media containing MEM, 20% HIFCS, and 30% HIB and allowed to adhere to glass cover slips in plastic culture dishes. At the time of use the mycoplasma-coated cover slips were washed with MEM and the organisms were suspended in MEM with 20% HIFCS by scraping the cover slip with a rubber policeman. The mycoplasma suspension was overlayed on washed macrophage monolayers (macrophages explanted 40 hr previously) which had been exposed to 100 μg/ml of anti-macrophage, anti-erythrocyte, and anti-lysosome IgG antibody for 12 hr. The mycoplasma were allowed to adhere to the macrophage surface for 60 min at 37°C in a 5% CO$_2$-air atmosphere.

Ingestion of Mycoplasma pulmonis by Mouse Macrophages.—The ingestion of surface mycoplasma was determined as previously described (18) by evaluating the per cent of macrophages with surface mycoplasma remaining 1 hr after the addition of rabbit anti-mycoplasma IgG antibody (1/100). After 1 hr incubation in the presence of mycoplasma antibody, the macrophage monolayer was washed, fixed with glutaraldehyde, and examined using 100 $\times$ oil immersion phase-contrast microscopy. 100 macrophages were counted on duplicate cover slip preparations and the per cent of macrophages with surface mycoplasma was determined.

RESULTS

Anti-Membrane Activity of the Antibodies.—Although the three antisera were prepared using different immunogens, all showed certain common properties. Rabbit antibodies against mouse erythrocytes, mouse macrophages, and mouse liver lysosomes all agglutinated mouse erythrocytes to varying degrees as outlined in Table I. In addition, all antibodies gave one or more precipitin bands when reacted with a liver lysosome preparation in a double-diffusion assay. In contrast, none of the rabbit antibodies reacted positively with either purified mouse IgG or whole mouse serum.

Of more importance to this presentation was the rapid cytotoxicity of all antibody preparations against cultured monolayers of mouse macrophages. In the presence of a complement source, either 10% fresh rabbit or guinea pig serum, more than 95% of the macrophages became trypan blue positive and cytolysis ensued within 60 min.

The Influence of Heat-Inactivated Antisera and γ-Globulins on Macrophage Morphology.—In the absence of an exogenous source of complement, each of the antisera and purified γ-globulin fractions produced structural changes in

macrophages at high concentrations. Cells exposed to 600–1000 $\mu g/ml$ of anti-macrophage γ-globulin rapidly exhibited retraction of the peripheral plasma membrane and the accumulation of large phase-lucent vacuoles. Somewhat later gross "rounding up" occurred (Fig. 1 a), and was followed by the slow loss of viability. No morphologic evidence of cytotoxicity was observed with 100 $\mu g/ml$ of anti-macrophage γ-globulin after a 12 hr exposure as seen in Fig. 1 b. Similar dose-response effects were noted when anti-mouse erythrocyte and anti-mouse lysosome γ-globulins were employed (Figs. 1 c and d). All subsequent studies on phagocytic function were performed using antibodies in non-toxic concentrations.

Phagocytosis by Mouse Peritoneal Macrophages.—Initial experiments were

TABLE I

Mouse Erythrocyte Agglutinating Activity and Protein Content of Rabbit Immunoglobulin Fractions

Material	Protein	Reciprocal of HA titer
	mg/ml	
$(NH_4)_2SO_4$ fraction		
Normal rabbit γ-globulin	22	4
Rabbit anti-mouse macrophage γ-globulin	25	128
Rabbit anti-mouse erythrocyte γ-globulin	20	1024
Rabbit anti-mouse lysosome γ-globulin	26	256
Sephadex G-200 fraction		
Rabbit anti-mouse macrophage IgG	3.8	32
Rabbit anti-mouse erythrocyte IgG	4.0	256
Rabbit anti-mouse lysosome IgG	3.1	32

performed to quantitate the phagocytic activity of normal macrophages to a variety of particles. The per cent phagocytosis of each particle was determined 30 hr after macrophages were explanted on cover slips. The number of macrophages which ingested yeast cell walls (zymosan), polystyrene latex beads, formalinized sheep erythrocytes, and antibody-coated sheep erythrocytes (rabbit antisheep hemolysin, $\frac{1}{1500}$) was greater than 95%. Results are summarized in Table II.

Fig. 1. The morphology of antibody-treated macrophages. Phase-contrast microscope. $\times$ 800. (a) The cytotoxic effect of short-term exposure of cultured mouse peritoneal macrophages to 600 $\mu g/ml$ rabbit anti-mouse macrophage γ-globulin. (b) Mouse peritoneal macrophages after exposure to 100 $\mu g/ml$ rabbit anti-mouse γ-globulin for 12 hr. Cytotoxicity is not observed. (c) Characteristic appearance of cultured mouse macrophages exposed to 300 $\mu g/ml$ rabbit anti-mouse erythrocyte γ-globulin for 12 hr. (d) Mouse peritoneal macrophages after exposure to 100 $\mu g/ml$ rabbit anti-mouse erythrocyte γ-globulin for 12 hr. Plasma membranes are well spread and cytotoxicity is not observed.

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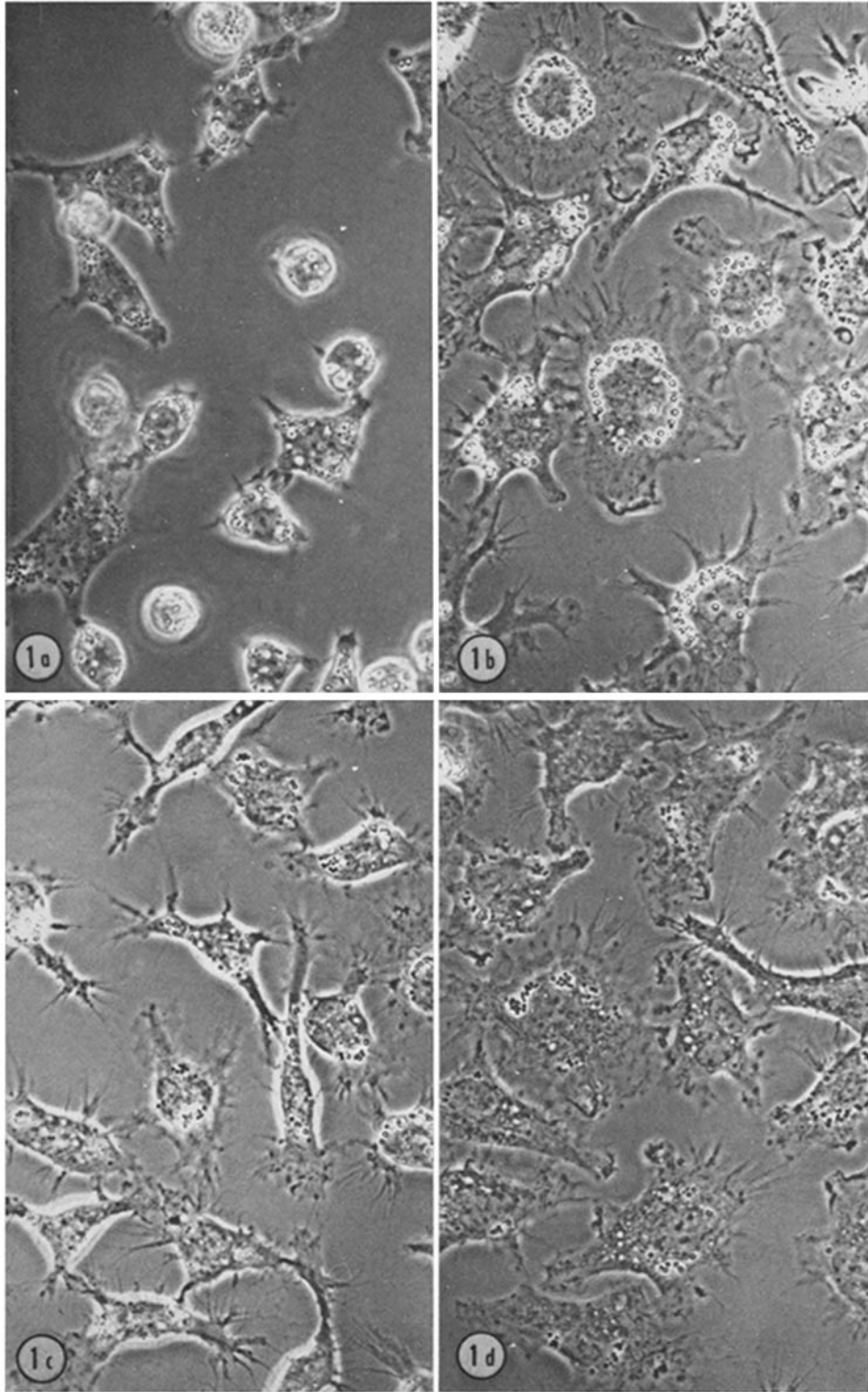
Phagocytosis by Macrophages after Exposure to Immunoglobulin Fractions.—
The exposure of macrophages for 6 hr to $(NH_4)_2SO_4$-fractionated γ-globulins
from rabbit anti-mouse macrophage, rabbit anti-mouse erythrocyte, and rabbit
anti-mouse lysosome antisera at a concentration of 175 μg/ml did not affect
phagocytosis of zymosan, latex particles, or formalinized erythrocytes. How-
ever, the ingestion of antibody-coated sheep erythrocytes was markedly
diminished. Less than 5% of the cells took up erythrocytes as compared with
more than 85% phagocytosis by control macrophages. Exposure of macro-
phages to a γ-globulin fraction from nonimmunized rabbits decreased phago-
cytosis of antibody-coated erythrocytes to only a minimal degree (Table II).
The results indicate that exposure of macrophages to anti-macrophage, anti-
erythrocyte, and anti-lysosome immunoglobulins affects the subsequent in-

TABLE II

Influence of Immune γ-Globulins on Macrophage Phagocytosis

Material	Per cent phagocytosis			
	EA*	FRBC‡	Zymosan	Latex
Control	86	96	98	96
Norma rabbit γ-globulins	73	98	99	94
Rabbit anti-mouse macrophage γ-globulins	4	97	99	94
Rabbit anti-mouse erythrocyte γ-globulins	3	97	98	95
Rabbit anti-mouse lysosome γ-globulins	4	97	98	95

* EA, sheep erythrocytes coated with rabbit anti-sheep hemolysin ($\frac{1}{500}$).

‡ FRBC, formalinized sheep erythrocytes. γ-globulins, $(NH_4)_2SO_4$-fractionated material.

gestion of antibody-coated erythrocytes but does not affect ingestion of par-
ticulates which do not require opsonization with antibody.

The Influence of Antibodies on the Attachment of Sensitized Erythrocytes.—
The specific attachment of sensitized erythrocytes to the plasma membrane of
control macrophages and those exposed to the three globulin fractions was next
evaluated. The technique of Rabinovitch (19) for dissociating attachment and
ingestion phases of particle uptake was employed.

Macrophage monolayers explanted 40 hr previously were exposed to rabbit anti-mouse
macrophage, anti-mouse erythrocyte, and anti-mouse lysosome γ-globulins for 6 hr. The cul-
ture medium was removed and the monolayers were washed three times with warm MEM.
0.1 ml of antibody-coated erythrocytes (rabbit anti-sheep hemolysin, 1/1500) was pipetted
onto each of the macrophage monolayers and allowed to remain stationary for 15 min at 24°C.
One set of preparations were then washed twice with MEM to remove unattached cells, fixed
with glutaraldehyde, and examined by phase-contrast microscopy for rosette formation. A
second set of preparations were washed twice with MEM after the attachment time period
and incubated with MEM with 20% HIFCS at 37°C for 30 min in a 5% CO_2-air atmosphere.

The macrophage monolayer was then washed, fixed with glutaraldehyde, and examined by phase-contrast microscopy for ingestion of previously attached erythrocytes.

Exposure of macrophages to each of the γ-globulin preparations (anti-macrophage, anti-erythrocyte, and anti-lysosome) markedly reduced the attachment of antibody-coated erythrocytes. The per cent of attachment of erythrocytes to antibody-treated macrophages was less than 5% compared
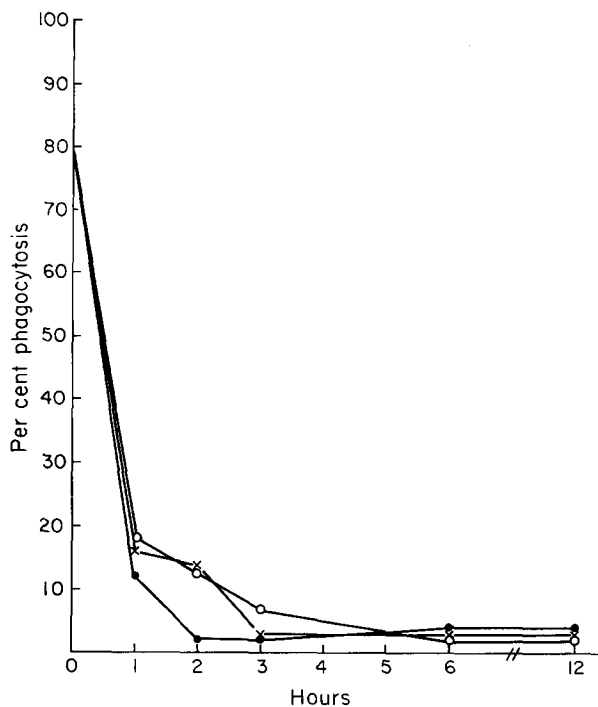

FIG. 2. Phagocytosis of sensitized sheep erythrocytes by mouse peritoneal macrophages after exposure to rabbit anti-mouse macrophage (—O—), anti-erythrocyte (—●—), and anti-lysosome (—X—) γ-globulin.

with greater than 85% rosette formation in control macrophage monolayers. Results of the ingestion assay performed by this technique were similar to the previous results and less than 5% of antibody-treated macrophages contained ingested erythrocytes.

Time Course Observations of Antibody Binding to the Macrophage Membrane.— The rate at which macrophages lost the ability to ingest sensitized erythrocytes after exposure to the three γ-globulins was next determined.

Gamma globulin fractions of each of the three antisera were added to 24-hr macrophage monolayers. Each plastic dish contained 2.5 ml of MEM with 20% HIFCS, and each cover slip monolayer contained approximately 1×10^6 macrophages. At 1, 2, 3, 6, and 12 hr inter-

vals after addition of the antibody to the media, 2.5×10^7 hemolysin (1/1500)-coated sheep erythrocytes were added and incubation continued for 45 min at 37°C in a 5% CO_2-air atmosphere. The monolayer was washed at the end of the incubation period, fixed, and examined for ingestion of erythrocytes as previously described.

The results are shown in Fig. 2. At the 1 hr time point, macrophage ingestion of antibody-coated erythrocytes had been reduced to 20% and at 3 hr less than 10% of the macrophages were capable of interiorizing erythrocytes. Minimal variation in inhibition of macrophage phagocytosis was observed between anti-macrophage, anti-erythrocyte, and anti-lysosome γ-globulin fractions at the early time points. After 6 and 12 hr exposure all three γ-globulin fractions
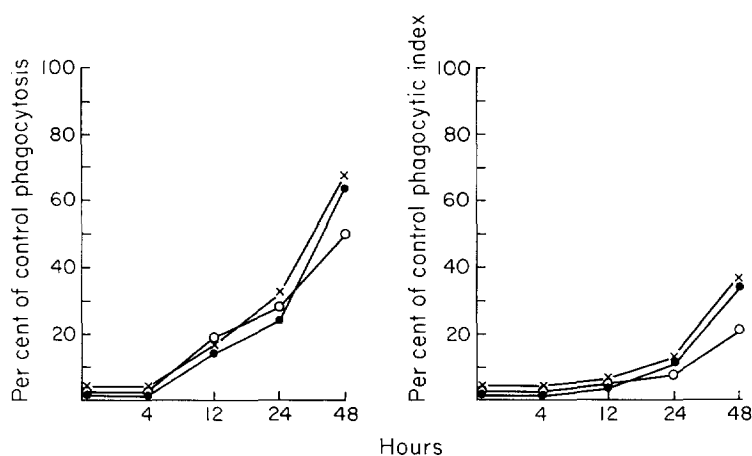

Fig. 3. The phagocytic recovery rate of mouse peritoneal macrophages after removal of anti-membrane antibodies. Rabbit anti-mouse macrophage γ-globulin (—O—); rabbit anti-mouse erythrocyte γ-globulin (—●—); rabbit anti-mouse lysosome γ-globulin (—X—).

had reduced the phagocytosis of antibody-coated erythrocytes to less than 5%. Throughout this period, the uptake of zymosan, formalinized erythrocytes, and polystyrene beads remained within the control range.

Recovery of Phagocytic Activity by Antibody-Coated Macrophages.—The recovery of phagocytic activity after the removal of the three antibodies was evaluated.

Macrophages harvested and explanted 24 hr previously were exposed to the anti-macrophage, anti-erythrocyte, and anti-lysosome γ-globulin fractions for 6 hr. The monolayers were then washed three times with warm MEM and fresh medium (2.5 ml of MEM with 20% HIFCS) was added. At 4, 12, 24, and 48 hr after the medium change, antibody-coated erythrocytes were added to the culture dish and phagocytosis was evaluated.

The results are shown in Fig. 3. 4 hr after rinsing the macrophage monolayer and addition of antibody-free media, phagocytosis of antibody-coated

erythrocytes remained below 5%. At 12, 24, and 48 hr thereafter the per cent of macrophages ingesting antibody-coated erythrocytes gradually increased but did not return to control values during the time periods studied. The number of ingested erythrocytes per 100 macrophages remained significantly below control values at all of the time periods studied. Phagocytosis of zymosan and formalinized erythrocytes did not differ from control values during the same periods. The gradual return toward normal phagocytic activity may be explained by subsequent elution of the antibody from the macrophage membrane and/or interiorization of the plasma membrane to which the antibody is bound.

Effect of Immune IgG on Macrophage Phagocytosis.—In order to further characterize the antibody involved in inhibiting attachment and ingestion of hemolysin-coated erythrocytes, the $(NH_4)_2SO_4$ γ-globulins of anti-macrophage,

TABLE III

Influence of Membrane-Bound Immune IgG on Macrophage Phagocytosis

Material	Per cent phagocytosis			
	EA*	FRBC‡	Zymosan	Latex
Control	85	96	97	95
Rabbit anti-mouse macrophage IgG	4	97	97	96
Rabbit anti-mouse erythrocyte IgG	2	98	98	97
Rabbit anti-mouse lysosome IgG	4	98	95	97

EA, rabbit anti-sheep hemolysin ($\frac{1}{1500}$)-coated sheep erythrocytes.

‡ FRBC, formalinized sheep erythrocytes.

anti-erythrocyte, and anti-lysosome antisera were separated by Sephadex G-200 column fractionation.

The IgG fraction was assayed for purity using double diffusion in agar and immunoelectrophoresis. No IgM contamination was identified. The IgG antibody was added to 38-hr-old macrophage monolayers for 6 hr and phagocytic assays performed using zymosan, polystyrene beads, formalinized sheep erythrocytes, and hemolysin (1/1500)-coated sheep erythrocytes.

Results are summarized in Table III. Phagocytosis of antibody-coated erythrocytes was less than 5% in macrophages exposed to anti-macrophage IgG, anti-erythrocyte IgG, and anti-lysosome IgG as compared with control macrophage phagocytosis of 85%. No difference in phagocytosis of zymosan, polystyrene beads, or formalinized erythrocytes was observed between macrophages exposed to immune IgG and control monolayers.

A control macrophage monolayer after 1 hr incubation with hemolysin-coated sheep erythrocytes is seen in Fig1 4 *a*. A macrophage monolayer previously exposed to 100 μg/ml anti-macrophage IgG for 6 hr followed by addition of hemolysin-coated sheep erythrocytes is seen in Fig. 4 *b*. Normal ingestion of

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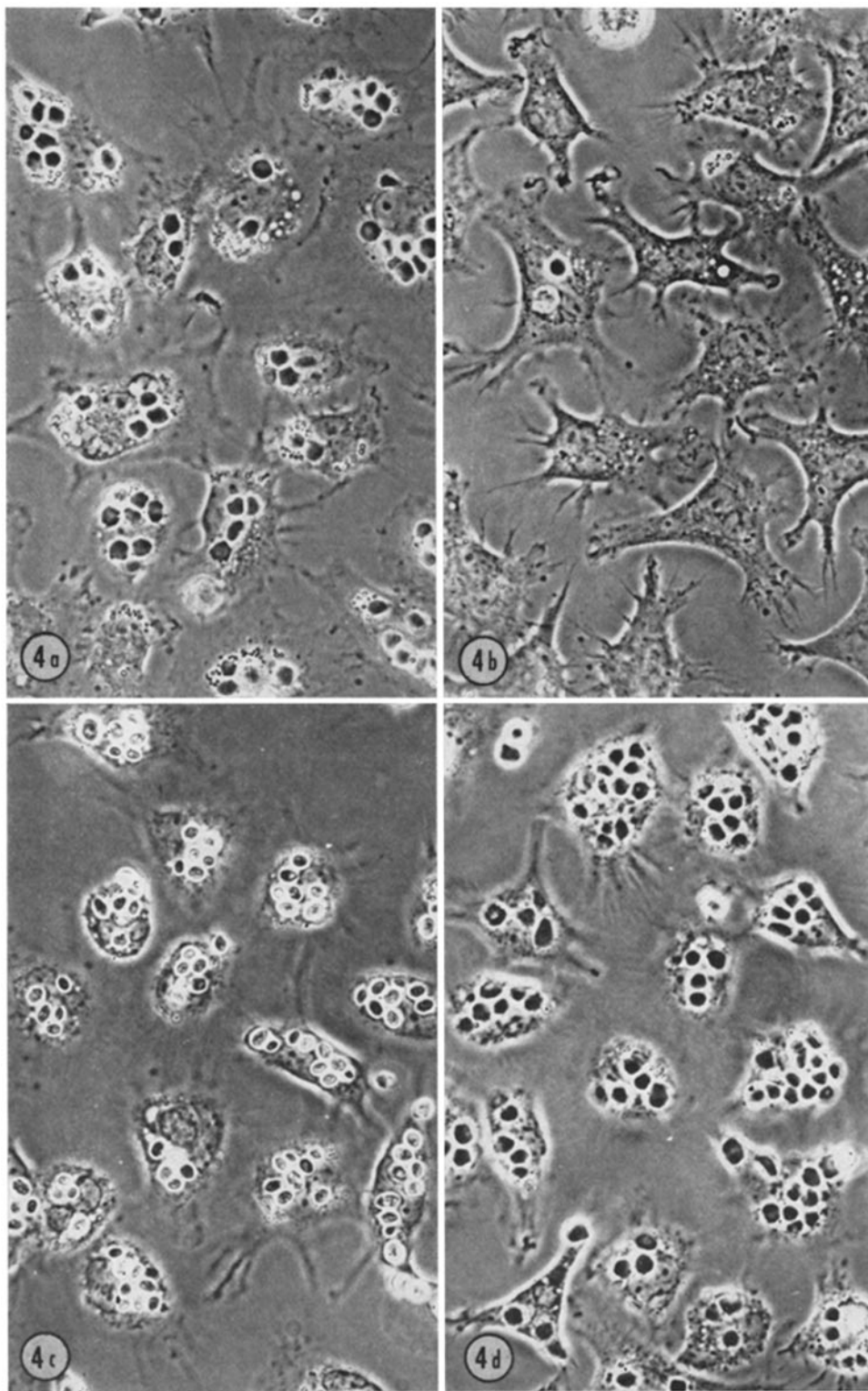
zymosan and formalinized sheep erythrocytes by macrophages previously exposed to anti-macrophage IgG is shown in Figs. 4 c and d.

Macrophage Phagocytosis after Exposure to Pepsin-Treated Immune IgG.—Anti-macrophage IgG, anti-erythrocyte IgG, and anti-lysosome IgG was subjected to pepsin digestion for 20 hr at 37°C using the method of Nisonoff (17). Nondigested IgG incubated and dialyzed by the same procedure was employed as a control. Hemagglutination titers of pepsin-digested IgG and nondigested IgG were identical and it is assumed that (Fab′)$_2$ fragments are present in the pepsin-digested material. However, further separation of the 5S (Fab′)$_2$ fragment was not performed. Macrophages were exposed to the pepsin-digested IgG fractions and to control IgG fractions for 6 hr and the phagocytosis of hemolysin-coated sheep erythrocytes evaluated.

TABLE IV

Influence of Pepsin-Digested Immune IgG on Macrophage Phagocytosis of Antibody-Coated Erythrocytes

Material	Per cent phagocytosis	No. EA*/100 macrophages
Control	92	440
Rabbit anti-mouse macrophage (Fab′)$_2$ fragment	88	410
Rabbit anti-mouse erythrocyte (Fab′)$_2$ fragment	90	384
Rabbit anti-mouse lysosome (Fab′)$_2$ fragment	90	430
Rabbit anti-mouse macrophage IgG (control)	4	8
Rabbit anti-mouse erythrocyte IgG (control)	2	2
Rabbit anti-mouse lysosome IgG (control)	2	2

* EA, rabbit anti-sheep hemolysin ($\frac{1}{1500}$)-coated sheep erythrocytes.

The results are shown in Table IV. Less than 5% phagocytosis of antibody-coated erythrocytes was observed in macrophages exposed to the nondigested IgG's. In contrast, 88–90% of the macrophages ingested antibody-coated erythrocytes when exposed to the pepsin-digested IgG antibody from anti-macrophage, antierythrocyte, and anti-lysosome antisera. 92% of control macrophages ingested red cells in these experiments. The results suggest that the receptor site on the macrophage membrane for attachment and subsequent ingestion of antibody-coated erythrocytes is blocked by the intact IgG molecule but not by the pepsin digestion products of IgG.

Fig. 4. Phagocytosis by macrophages. Phase-contrast microscope. × 800. (*a*) Phagocytosis of sensitized sheep erythrocytes by cultured mouse peritoneal macrophages. (*b*) Markedly decreased phagocytosis of sensitized sheep erythrocytes by cultured mouse peritoneal macrophages after exposure to 100 μg/ml rabbit anti-mouse macrophage IgG. (*c*) Phagocytosis of zymosan by cultured mouse peritoneal macrophages after exposure to 100 μg/ml rabbit anti-mouse macrophage IgG. (*d*) Phagocytosis of formalinized sheep erythrocytes by cultured mouse peritoneal macrophages after exposure to 100 μg/ml rabbit anti-mouse macrophage IgG.

Evaluation of Attachment of Mouse and Rabbit Erythrocytes to Macrophages with Membrane-Bound IgG Antibody.—The mechanism by which the antibodies attach to the macrophage membrane was evaluated with fresh mouse and rabbit erythrocytes.

Macrophages explanted 24 hr previously were exposed for 6 hr to anti-macrophage IgG, anti-erythrocyte IgG, and anti-lysosome IgG. The culture media was removed and the macrophage monolayer was washed three times with warm MEM. 0.1 ml of a washed suspension of fresh mouse erythrocytes was layered over one set of antibody exposed macrophages and 0.1 ml of a washed suspension of fresh sheep erythrocytes was pipetted onto a second set of antibody-exposed cover slip preparations. Incubation was allowed for 15 min at 24°C. The monolayers were washed with MEM to remove unattached erythrocytes and evaluated for rosette formation as previously described.

Attachment of mouse and rabbit erythrocytes to macrophages was observed in less than 5% of the IgG-coated cells. These observations suggest binding of the IgG molecule by its Fab portion to the macrophage membrane, as might be expected. If the Fc fragment of the immune IgG attached to the Fc receptor site on the macrophage membrane, the exposed Fab portion would bind erythrocytes and significant attachment would have been expected.

Phagocytosis of IgG-Coated Sheep Erythrocytes.—In order to examine the specificity of phagocytic inhibition of antibody-coated erythrocytes by the macrophage-bound IgG antibody, phagocytosis of erythrocytes coated with IgG was studied.

Commercial rabbit anti-sheep hemolysin was layered over a Sephadex G-200 column and eluted with 0.15 M phosphate-buffered saline, pH 7.4. Purity of the IgG peak was evaluated and confirmed using double diffusion in agar and immunoelectrophoretic techniques. The reciprocal of the hemagglutinating titer of the IgG fraction was 64. Fresh sheep erythrocytes were reacted with the IgG fraction (1/20) by the methods previously described for preparation of antibody-coated sheep erythrocytes. Macrophages explanted 40–42 hr previously were exposed to anti-macrophage IgG, anti-erythrocyte IgG, and anti-lysosome IgG for 6 hr and phagocytosis of IgG-coated erythrocytes evaluated.

Results are summarized in Table V. The macrophage surface–bound IgG antibody markedly inhibited ingestion of IgG-coated sheep erythrocytes. The concentration of each of the three IgG antibodies to which the macrophage monolayers were exposed was 100 μg/ml in these experiments. The per cent of macrophage phagocytosis in the presence of surfcace-bound anti-macrophage IgG, anti-erythrocyte IgG, and anti-lysosome IgG was 5, 4, and 11%, respectively, as compared with 95% of IgG-coated erythrocytes in control monolayers. These findings indicate that the macrophage-bound IgG antibody is selective in inhibiting attachment and ingestion of IgG-coated erythrocytes.

Phagocytosis of EAC1,4,2,3 by Mouse Macrophages.—In order to evaluate the effect of macrophage surface–bound immune IgG on phagocytosis of EAC1, 4,2,3, the following experiments were performed.

EAC1,4,2,3 prepared as previously outlined were diluted in MEM and 7.5 $\times$ 10^6 cells

were added to culture dishes containing 46-hr-old macrophages previously exposed for 6 hr to 100 μg/ml of anti-macrophage, anti-erythrocyte, and anti-lysosome IgG. Incubation was allowed for 30 min at 37°C in a 5% CO_2-air atmosphere and at the end of the incubation period the per cent phagocytosis of EAC1,4,2,3 was enumerated using phase-contrast microscopy.

The results are summarized in Table VI. Ingestion of EAC1,4,2,3 by macrophages exposed to the three immune IgG fractions ranged from 31 to 43% as

TABLE V

Influence of Macrophage Membrane–Bound IgG Antibody on Phagocytosis of IgG-Coated Sheep Erythrocytes

Material	IgG-coated SRBC*	
	Per cent phagocytosis	No./100 macrophages
	%	
Control	97	588
Rabbit anti-mouse macrophage IgG	5	24
Rabbit anti-mouse erythrocyte IgG	4	16
Rabbit anti-mouse lysosome IgG	11	18

* IgG-coated SRBC, rabbit anti-sheep hemolysin IgG ($\frac{1}{2}0$) coating sheep erythrocytes.

TABLE VI

Influence of Macrophage Membrane–Bound IgG Antibody on Phagocytosis of EAC1,4,2,3

Material	EAC1,4,2,3	
	Per cent phagocytosis	No./100 macrophages
	%	
Control	85	330
Rabbit anti-mouse macrophage IgG	43	88
Rabbit anti-mouse erythrocyte IgG	31	63
Rabbit anti-mouse lysosome IgG	35	75

compared with control macrophage phagocytosis of 85%. The results indicate that receptor sites for EAC1,4,2,3 are present on the mouse peritoneal macrophage surface and that phagocytosis of C3-coated erythrocytes is affected by the presence of surface-bound immunoglobulins. The impairment in phagocytosis of C3-coated erythrocytes was however less than that previously observed with IgG-coated erythrocytes.

In control macrophages, essentially all receptor activity for EAC1,4,2,3 cells can be removed by brief trypsinization as initially described by Lay and Nussenzweig (6). In macrophages coated with IgG molecules, and which express about 50% of control activity, only minimal further reduction occurs after

trypsinization. This suggests that the remaining complement sites are "protected" by the presence of IgG on the membrane.

Evaluation of Ingestion of Mycoplasma pulmonis by Macrophages.—In order to define the effect of macrophage-bound IgG antibody on ingestion of another antibody coated particle, *Mycoplasma pulmonis* coated with anti-mycoplasma antibody was studied.

During 1 hr exposure of macrophages to a mycoplasma suspension, large numbers of mycoplasma attached to the macrophage surface but were not ingested. 100% of macrophages had surface mycoplasma and each macrophage had 10–100 mycoplasma adherent to its surface. No difference in the number of attached mycoplasma was observed between macrophage monolayers exposed for 12 hr to 100 μg/ml of anti-macrophage, anti-erythrocyte, and anti-lysosome IgG antibody and unexposed macrophages.

TABLE VII

Influence of Macrophage Membrane–Bound Antibody on Ingestion of Opsonized Mycoplasma pulmonis

Material	Particle added	Per cent macrophages ingesting surface mycoplasma
Control	Mycoplasma	0
Control	Mycoplasma + anti-mycoplasma IgG	99
Rabbit anti-mouse macrophage IgG	Mycoplasma + anti-mycoplasma IgG	1
Rabbit anti-mouse erythrocyte IgG	Mycoplasma + anti-mycoplasma IgG	0
Rabbit anti-mouse lysosome IgG	Mycoplasma + anti-mycoplasma IgG	1
Normal rabbit γ-globulin	Mycoplasma + anti-mycoplasma IgG	0

Upon the addition of anti-mycoplasma antibody, prompt phagocytosis of the attached organisms took place by control macrophages. Only 1% of control macrophages had mycoplasma remaining on the surface 1 hour after coating the mycoplasma with anti-mycoplasma antibody. In contrast, monolayers previously exposed to anti-macrophage, anti-erythrocyte, and anti-lysosome IgG antibody, exhibited no phagocytosis and 99% of macrophages still had mycoplasma remaining on their surface Table VII. The results indicate that normal ingestion of opsonized mycoplasma by macrophages is inhibited by the presence on the macrophage surface of immune IgG molecules.

DISCUSSION

These studies suggest that the mouse erythrocyte and macrophage share common surface antigens and that antibodies produced against either are capable of interacting with external plasma membrane constituents of the other. The antigens of lysosomes may originate either from liver macrophage mem-

branes or from red cells interiorized by the Kupffer cells. Hemagglutinating activity is present in antibodies prepared against macrophages, liver lysosomes, and erythrocytes and all three immunoglobulin preparations rapidly lyse macrophages and erythrocytes in the presence of complement. These phenomena apparently result from the binding of immunoglobulin molecules to the macrophage and erythrocyte membrane. Although extensive studies were not performed, we were unable to completely dissociate hemagglutinating and anti-macrophage activity from anti-macrophage γ-globulins by absorption with macrophages. Further work is therefore needed in identifying specific antigenic components of the macrophage plasma membrane.

The attachment of anti-macrophage, anti-erythrocyte, and anti-lysosome IgG to the macrophage membrane occurs by means of its Fab fragment and effectively blocks the functional properties of the Fc phagocytic receptor. This interaction severely curtails both the attachment and subsequent engulfment of IgG-coated sheep erythrocytes and mycoplasma but does not affect the capacity of the cell to interiorize yeast cell walls, polystyrene spheres, or formalinized erythrocytes, particles which do not require immunoglobulins for efficient uptake. This data reemphasizes the previous observations that separate recognition sites are involved for the engulfment of immunologically and nonimmunologically dependent particles by the macrophage surface membrane.

The plasma membrane antigens with which these antibodies interact are as yet unknown. For the effective inhibition of the Fc receptor the intact IgG molecule appears to be required. Treatment of cells with pepsin-digested globulin does not block phagocytosis although it retains hemagglutinating activity. Since the anti-membrane antibodies attach by means of their Fab region, it suggests a separate role for the Fc piece. This may combine with the Fc receptor producing a second attachment site of the molecule to the membrane. The inability of (Fab')$_2$ to block phagocytosis also suggests that the antibody is not directed toward the Fc receptor per se but to an adjacent antigen.

Anti-membrane antibodies also inhibit the expression of the complement receptor and reduce the ingestion of EAC1,4,2,3 by approximately 50%. This may indicate a greater degree of heterogeneity of the complement-dependent sites on the membrane. An alternative explanation would depend on the spatial separation of the phagocytic receptors, the complement receptor being located at a point more distant from the attached IgG molecule than the Fc receptor. In this context it would be of interest to examine the selective influence of IgM molecules of similar specificity.

These observations on the in vitro influence of anti-membrane antibodies may facilitate the interpretation of the in vivo responses to anti-macrophage serum. Efficient binding of anti-macrophage serum to both macrophages and erythrocytes would be expected in the whole animal. Both the route of administration

and antibody concentration would then play a significant role. Intraperitoneal injection would influence local macrophage function and viability whereas the intravenous route might result in the sequelae of an antibody-erythrocyte interaction. This may explain the toxicity of anti-macrophage serum given by the intravenous route (9) and its lack of effect on reticuloendothelial system elements of liver and spleen. Finally, it is conceivable that the presence of antibodies directed against erythrocyte surface antigens, as seen in various human disease states, may influence the functional properties of macrophages and other members of the mononuclear phagocyte series.

SUMMARY

Rabbit antibodies were prepared against purified mouse macrophages, erythrocytes, and liver lysosomes. In the presence of complement each of these reagents was capable of lysing mouse erythrocytes and macrophages. In the absence of complement, all antisera agglutinated mouse erythrocytes and at high concentration produced a cytotoxic effect on macrophages. At IgG concentrations of 100 $\mu g/ml$, no morphological evidence of cytotoxicity was evident. These data suggest the presence of common antigens on the erythrocyte and macrophage plasma membrane.

Anti-macrophage, anti-erythrocyte, and anti-lysosomal γ-globulins and IgG, employed at subtoxic concentrations, all inhibited the attachment and ingestion of opsonized erythrocytes and mycoplasma. This occurred without significant reduction in the phagocytosis of polystyrene particles, formalinized erythrocytes, and yeast cell walls. Each of the anti-membrane IgG antibodies was capable of blocking the Fc receptor on the macrophage plasma membrane. Attachment to the macrophage membrane occurred by means of the Fab region. However, a role for the Fc portion of the molecule was suggested since pepsin-digested IgG was unable to block the receptor.

Each of the IgG antibodies produced a partial blockade of the complement receptor and reduced the ingestion of EAC1,4,2,3 by approximately 50%.

REFERENCES

1. Rabinovitch, M. 1968. Phagocytosis: the engulfment stage. *Seminars Hematol.* **5**:134.
2. Berken, A., and B. Benacerraf. 1966. Properties of antibodies cytophilic for macrophages. *J. Exp. Med.* **123**:119.
3. LoBuglio, A. F., R. S. Cotran, and J. H. Jandl. 1967. Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. *Science (Washington).* **158**:1582.
4. Huber, H., M. J. Polley, W. D. Linscott, H. H. Fudenberg, and Muller-Eberhard. 1968. Human monocytes. Distinct receptor sites for the third component of complement and for immunoglobulin G. *Science (Washington).* **162**:1281.
5. Quie, P. G., R. P. Messner, and R. C. Williams, Jr. 1968. Phagocytosis in subacute

bacterial endocarditis. Localization of the primary opsonic site to Fc fragment. *J. Exp. Med.* **128**:553.

6. Lay, W. H., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. *J. Exp. Med.* **128**:991.

7. Cohn, Z. A., and E. Parks. 1967. The regulation of pinocytosis in mouse macrophages. IV. The immunological induction of pinocytic vesicles, secondary lysosomes, and hydrolytic enzymes. *J. Exp. Med.* **125**:1091.

8. Jennings. J. F., and L. A. Hughes. 1969. Inhibition of phagocytosis by anti-macrophage antibodies. *Nature (London).* **221**:79.

9. Unanue, E. R. 1968. Properties and some uses of anti-macrophage antibodies. *Nature (London).* **218**:36.

10. Carey, F. J., N. O. Kuhn, and C. G. Harford. 1965. Effects of anticellular serum on phagocytosis and the uptake of tritiated thymidine and uridine by HeLa cells. *J. Exp. Med.* **121**:991.

11. Trouet, A. 1970. Characteristiques et proprietes antigeingues des lysosomes du foie. Doctorate Thesis. Universite Catholique De Louvain, Belgium.

12. Tulkens, P., A. Trouet, and F. Van Hoof. Immunological inhibition of lysosome function. *Nature (London).* **228**:1282.

13. Cohn, Z. A., and B. Benson. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121**:153.

14. Borsos, T., and H. J. Rapp. 1967. Immune hemolysis. A simplified method for the preparation of EAC 4 with guinea pig or with human complement. *J. Immunol.* **99**:263.

15. Müller-Eberhard, H. J., U. Nilsson, and T. Aronsson. 1960. Isolation and characterization of two β₁-glycoproteins of human serum. *J. Exp. Med.* **111**:201.

16. Nishioka, K 1963. Measurements of complement by agglutination of human erythrocytes reacting in immune-adherence. *J. Immunol.* **90**:86.

17. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. H. N. Eisen, editor. *Methods Med. Res.* **10**:134.

18. Jones, T. C., and J. G. Hirsch. 1971. The interaction in vitro of *Mycoplasma pulmonis* with mouse peritoneal macrophages and L-cells. *J. Exp. Med.* **133**:231.

19. Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell Res.* **46**:19.