SEGMENTAL RESPONSE OF THE MACROPHAGE PLASMA MEMBRANE TO A PHAGOCYTIC STIMULUS*

By FRANK M. GRIFFIN, JR., and SAMUEL C. SILVERSTEIN‡

(From The Rockefeller University, New York 10021)

(Received for publication 22 October 1973)

The uptake of particles by phagocytes can be separated experimentally into two steps, attachment of the particle to the cell membrane and ingestion of the particle (1). The conditions governing each step have been investigated and several factors which stimulate the ingestion of particles have been identified (1–5). There is evidence that ingestion of one type of particle enhances the rate of ingestion of a different type of particle (6, 7), but it is not known whether the stimulus to engulf one type of particle can initiate ingestion of a particle that is normally not phagocytized.

The present article describes a procedure for attaching mouse erythrocytes (RBCs)[1] to the plasma membranes of mouse peritoneal macrophages using $F(ab')_2$ immunoglobulin fragments to link the two cell types. Since immunoglobulin molecules lacking the Fc fragment do not stimulate phagocytosis (8), the immunologically attached RBCs remain stably associated with the macrophage plasma membrane for several hours. Using these RBCs as "markers" for the segments of macrophage membrane to which they are attached, we have studied the effect of phagocytosis of latex particles and opsonized pneumococci on these membrane associated RBCs. Our data show that these RBCs are attached to functionally active membrane segments since they are ingested when rabbit anti-mouse RBC IgG is added to the medium and that phagocytosis of latex and of opsonized pneumococci does not stimulate the ingestion of membrane-attached RBCs. Thus, ingestion of one particle does not trigger generalized phagocytosis of all particles attached to the cell's plasma membrane. These findings suggest that the phagocytic stimulus is confined to the segment of the cell's plasma membrane immediately adjacent to the particle being ingested.

Materials and Methods

Preparation of Immune Sera, Immunoglobulins, and $F(ab')_2$ Fragments.—

Rabbit anti-mouse macrophage $F(ab')_2$: The method of Holland et al. (9) was employed to make rabbit anti-mouse macrophage antiserum. Briefly, a rabbit was immunized with 48-h

* Supported by Grant PF-867 from the American Cancer Society and Grant AI 08697 from the U.S. Public Health Service.

‡ Established Investigator, American Heart Association.

[1] *Abbreviations used in this paper:* BHI, brain heart infusion; PBS, Dulbecco's phosphate-buffered saline; PD, solution "a" of Dulbecco's PBS; RBC, red blood cell; SAR $F(ab')_2$, sheep $F(ab')_2$ prepared against rabbit $F(ab')_2$.

explanted peritoneal macrophages from NCS mice. The animal received two biweekly injections of macrophages in Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Mich.) and was bled 2 wk after the second injection.

IgG from 10 ml of heat decomplemented (56°C for 30 min) immune serum was purified by DEAE-cellulose chromatography (10), concentrated by pervaporation, dialyzed against 0.1 M Na acetate (pH 4.25) with 0.02% Na azide, and digested for 24 h at 37°C with two additions of 4% pepsin (2,830 U/mg, Worthington Biochemical Co., Freehold, N. J.) (11). The digestion mixture was brought to pH 7 with 1 M K_2HPO_4, dialyzed against chromatography buffer [0.34 M NaCl. 0.004 M KCl, 0.01 M $NaPO_4$ (pH 7.2)] with 0.02% Na azide, and passed through a 2.5 × 110 cm Sephadex G 200 column in the same buffer. The fractions containing $F(ab')_2$ were pooled, concentrated by pervaporation against PD [solution "a" of Dulbecco's PBS (12)], sterilized by millipore filtration (0.45 μ pore size), and stored at 4°C. The yield was 33 mg of rabbit $F(ab')_2$.

Rabbit anti-mouse RBC IgG: RBCs were harvested from NCS mice by cardiac puncture, washed five times in PD, and resuspended as a 50% suspension in PD. A 3-kg rabbit was injected intraperitoneally with 1 ml of RBC suspension weekly for 4 wk. 1 wk after the fourth injection, the rabbit was bled, and 20 ml of serum was obtained and heat decomplemented at 56°C for 30 min. An IgG fraction was purified by DEAE-cellulose chromatography, as described above; concentrated by pervaporation against PD; millipore filtered (0.45 μ pore size); and stored at 4°C. The yield was 240 mg of rabbit IgG.

Sheep $F(ab')_2$ prepared against rabbit $F(ab')_2$ [SAR $F(ab')_2$]: The $F(ab')_2$ fragment of rabbit IgG (Pentex Fraction II, Pentex Biochemical, Kankakee, Ill.) was prepared and purified as described above. A sheep was repeatedly immunized with this material. Sheep IgG directed against rabbit $F(ab')_2$ was purified by affinity chromatography, using rabbit IgG linked to Sepharose 4B as an immunoadsorbent, as described by Cuatrecasas (13). The immunoadsorbent contained ~16 mg of rabbit IgG per milliliter of packed Sepharose. Sheep IgG directed against rabbit $F(ab')_2$ was eluted from the immunoadsorbent with 3 M NaSCN (freshly recrystallized) in 0.15 M NaCl and 0.05 M $NaPO_4$ (pH 6.0); dialyzed and concentrated by pervaporation against PBS (12) containing 0.01% Na azide; and sterilized by millipore filtration (0.45 μ pore size). The purified IgG was dialyzed against 0.1 M Na acetate buffer (pH 4.5) and digested with pepsin, as described for rabbit IgG. Sheep $F(ab')_2$ prepared against rabbit $F(ab')_2$ [SAR $F(ab')_2$] was purified by Sephadex G 200 chromatography, concentrated, dialyzed, and stored, as described for rabbit IgG. The yield of specific SAR $F(ab')_2$ from 40 ml of sheep serum was about 90 mg.

Rabbit antiserum against type 1 pneumococci: The rabbit atntiserum against type 1 pneumococci was a gift of Dr. Robert Austrian of the University of Pennsylvania Medical Center, Philadelphia. It contained 1.8 mg of protein per milliliter of serum, and was dialyzed against Medium 199 (Microbiological Associates, Bethesda, Md.) before use.

Macrophages.—The methods for harvesting and maintaining mouse peritoneal macrophages were those of Cohn and Benson (14) with the following modifications: 2.5 ml of a suspension of 6 × 10^5 peritoneal cells per milliliter in Medium 199 containing 20% heat-decomplemented (56°C for 30 min) fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N. Y.) was placed into a 35 mm plastic tissue culture dish (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing three 13-mm diameter glass cover slips. Cells were permitted to adhere to the cover slips for 1 h at 37°C in 5% CO_2-air mixture, washed twice with Medium 199 to remove nonadherent cells, overlaid with 2.5 ml of Medium 199 containing 20% FBS, and incubated for 48 h at 37°C.

Particles.—

Latex particles: Latex particles, 1.1 μ in diameter (Dow Chemical Co., Midland, Mich.), were washed twice in PD, suspended in Medium 199 at a concentration of 4 × 10^9 particles per milliliter, and stored at 4°C until used.

Pneumococci:—Diplococcus pneumoniae, type 1, strain SV-1, was kindly provided by Dr. Robert Austrian. To ensure that all pneumococci were of the smooth, encapsulated form, the bacteria were passed through mice. NCS mice weighing ~25 g were injected intraperitoneally with 0.5 ml of a suspension of pneumococci grown overnight in brain heart infusion (BHI) broth. When the mice died, 2–6 days after inoculation, they were frozen at −20°C until used. After thawing, the heart was removed aseptically from the mouse and cultured overnight in BHI broth at 37°C in a candle jar.

The organisms were washed twice by centrifugation in Medium 199 at 850 *g* for 15 min at room temperature and resuspended in Medium 199 at a concentration of 1–2 × 10^8 organisms per milliliter. Bacteria were counted in a Petrof-Hauser chamber.

Pneumococci were opsonized by incubating 1–2 × 10^8 pneumococci with antiserum (18 μg protein) in 1 ml of Medium 199 at 4°C for 30 min. The suspension was added directly to test dishes.

Mouse erythrocytes: RBCs were harvested from NCS mice by cardiac puncture on the day of an experiment, washed three times in Medium 199, and suspended in Medium 199 at a concentration 5 × 10^8 per milliliter.

Phase Contrast and Electron Microscopy.—Cover slip cultures were fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2) for 10 min at 4°C. Preparations were examined by phase contrast microscopy with a 100 × objective under oil. The percentage of macrophages with RBCs attached and with particles ingested and the number of RBCs and particles per macrophage were obtained by counting a minimum of 100 macrophages.

Cells were processed for electron microscopy as described (15, 16) and examined in a Siemens Elmiskop 1 A.

Miscellaneous.—Crystalline colchicine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in Medium 199 at a concentration of 10^{-4} M immediately before use. Protein concentration was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

RESULTS

Hemagglutination Titers of $F(ab')_2$ Immunoglobulin Fragments and of Rabbit Anti-Mouse RBC IgG.—The hemagglutination titers and protein concentrations of the $F(ab')_2$ fragments and of rabbit anti-mouse RBC IgG are given in Table I. Rabbit anti-mouse RBC IgG agglutinated mouse RBCs as expected. Rabbit anti-mouse macrophage $F(ab')_2$ also agglutinated mouse RBCs, indicating that at least one membrane antigen is shared by the RBC and the macrophage and that the $F(ab')_2$ preparation could be employed to coat mouse RBCs.

When mouse RBCs were coated with a subagglutinating titer of rabbit anti-mouse macrophage $F(ab')_2$, SAR $F(ab')_2$ agglutinated the RBCs. These results suggested that a similar system could be used to attach mouse RBCs to the surface of mouse macrophages.

Immunological Attachment[2] *of RBCs to Macrophages.*—Several methods of attaching RBCs to macrophages were evaluated. Melsom and Seljelid (18) reported attachment of unmodified mouse RBCs to mouse macrophages. In our observations this method resulted in a low degree of attachment. 22% of macrophages attached an average of 2.5 RBCs per macrophage. Efforts to cross agglutinate mouse RBCs and mouse macrophages using rabbit anti-mouse

[2] The term "immunological attachment" is used to denote attachment of mouse RBCs to mouse macrophages by the procedure described in this section.

TABLE I

Hemagglutination Titers of F(ab')$_2$ Fragments and of Rabbit Anti-Mouse RBC IgG

Test material	Mouse RBCs*	Mouse RBCs coated with rabbit anti-mouse macrophage F(ab')$_2$‡	
		60 μg	7.5 μg
		Reciprocal of hemagglutination titer	
Rabbit anti-mouse RBC IgG (50 mg/ml)	2000	—	—
Rabbit anti-mouse macrophage F(ab')$_2$ (3 mg/ml)	32	—	—
SAR F(ab')$_2$ (10 mg/ml)	—	>12,800	2,400

* 2×10^7 freshly harvested mouse RBCs in 0.2 ml of PD were incubated with twofold dilutions of test substance in an equal volume of PD for 3 h at 4°C. The lowest dilution demonstrating macroscopic agglutination was taken as the end point.

‡ 1×10^8 freshly harvested mouse RBCs in 1 ml of Medium 199 were incubated for 30 min at 37°C with either 60 μg or 7.5 μg of rabbit anti-mouse macrophage F(ab')$_2$. The cells were washed and suspended at a concentration of 1×10^8 F(ab')$_2$ coated RBCs per milliliter in PD. The agglutination test was performed as above.

macrophage F(ab')$_2$ were generally unsuccessful. Mixed agglutination of mouse RBCs and mouse macrophages, both of which had been coated with rabbit anti-mouse macrophage F(ab')$_2$, was achieved reproducibly and uniformly upon addition of SAR F(ab')$_2$ to the system.

1×10^8 mouse RBCs were incubated with a subagglutinating quantity (60 μg protein) of rabbit anti-mouse macrophage F(ab')$_2$ in 1 ml of Medium 199 for 30 min at 37°C. The RBCs were washed, further incubated with SAR F(ab')$_2$ (16 μg protein) in the same medium for 30 min at 37°C, washed twice again and resuspended in Medium 199 at a concentration of 1×10^8 RBCs per milliliter.

Macrophage monolayers were washed twice with Medium 199 and incubated for 2 h at 37°C with 0.05 ml of rabbit anti-mouse macrophage F(ab')$_2$ (6 μg protein) in Medium 199. The cover slips were washed twice with Medium 199 to remove excess F(ab')$_2$, and incubated at 37°C for 1 h with 5×10^6 RBCs which had been coated with rabbit anti-mouse macrophage F(ab')$_2$ and SAR F(ab')$_2$. Cover slips were washed gently to remove excess unbound RBCs and then fixed and examined by phase contrast microscopy, as described in Materials and Methods.

As shown in Table II and Fig. 1, *a*, 98% of macrophages attached an average of 11 RBCs per cell. The attachment was stable for at least 4 h; less than 0.1% of macrophages ingested RBCs during this interval. This procedure was employed throughout the remaining experiments to attach RBCs to macrophages.

Effect of Rabbit Anti-Mouse Macrophage F(ab')$_2$ on the Ingestion of Latex Particles and Opsonized Pneumococci.—The experiments of Carey et al. (19) showed that antibody directed against HeLa cell membranes inhibited the phagocytic uptake of colloidal gold and staphylococci. However, Holland et al. (9) found that neither rabbit anti-mouse macrophage IgG nor anti-mouse

TABLE II

*Attachment of Mouse RBCs to Mouse Macrophages**

Cells	Macrophages with RBCs attached	Average number of RBCs attached per macrophage	Number of RBCs attached per 100 macrophages	Macrophages ingesting RBCs
	%			%
RBCs and macrophages	22.5	2.5	56	<0.1
$F(ab')_2$-coated RBCs linked to $F(ab')_2$- coated macrophages by SAR $F(ab')_2$	98	11	1078	<0.1

* Each value represents the average of four experiments.

macrophage $F(ab')_2$ adversely affected the ability of macrophages to ingest latex particles or zymosan. To be certain that our preparation of anti-mouse macrophage $F(ab')_2$ did not alter the rate or extent of phagocytosis of latex particles or opsonized pneumococci, we examined the uptake of these "test" particles by macrophages. Latex was employed as a test particle which is ingested via a nonspecific phagocytic receptor site; opsonized pneumococcus as one whose ingestion is dependent upon the interaction of the Fc portion of the opsonizing immunoglobulin with the Fc receptor site on the macrophage surface. To be certain that ingestion of the pneumococci was dependent upon the Fc receptor site, we used nonopsonized pneumococci as a control. The numbers of particles added per 35-mm Petri dish were as follows: Latex particles—6×10^8; opsonized or nonopsonized pneumococci—$1\text{–}2 \times 10^8$. Macrophages were coated with rabbit anti-mouse macrophage $F(ab')_2$, as described above, and incubated for 1 h at 37°C with one of these test particles suspended in 2.5 ml of Medium 199.

The results are shown in Table III. Latex particles were ingested avidly by $F(ab')_2$-coated macrophages. 93% of macrophages ingested an average of about 20 particles each. Nonopsonized pneumococci were not ingested. Opsonized pneumococci, however, were ingested as avidly as latex, 94% of macrophages ingesting an average of about 20 organisms each. These results are essentially the same as those obtained when we used macrophages which had not been coated with rabbit anti-mouse macrophage $F(ab')_2$.

Effect of Test Particle Ingestion on the Fate of Immunologically Attached RBCs.—To determine whether ingestion of one particle initiated ingestion of a different type of particle which had been previously attached to the macrophage plasma membrane, RBCs were attached to macrophages using SAR $F(ab')_2$ as a link. Then either latex particles or opsonized pneumococci were added to the medium and incubation was continued at 37°C for 1 h. Both latex particles and opsonized pneumococci were ingested as avidly as previously (compare Tables III and IV). RBCs remained attached to the phagocytes (Fig. 1, *b* and *c*),

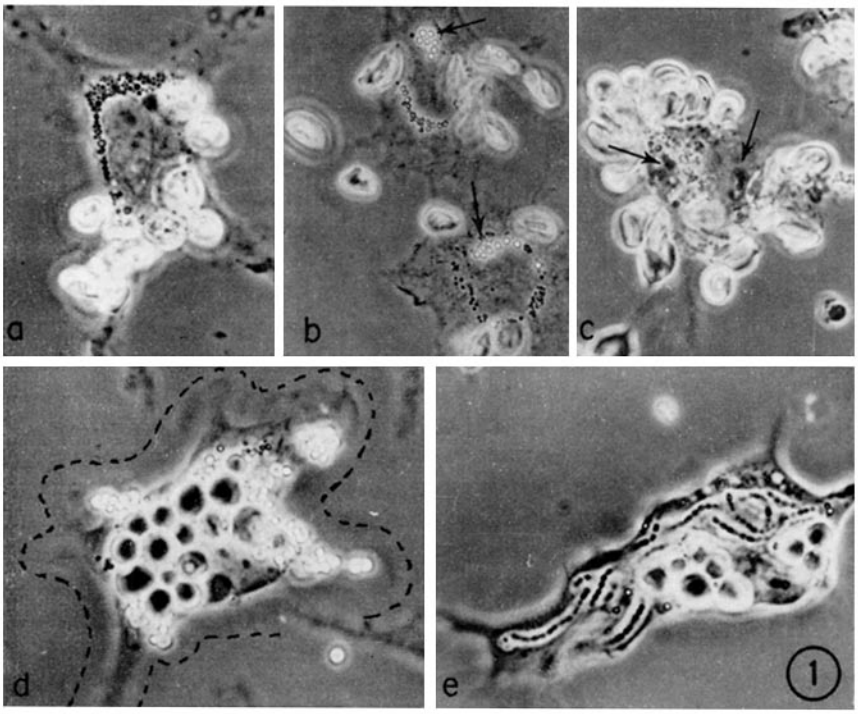

FIG. 1. Macrophage interaction with RBCs and test particles. Phase contrast microscopy. (× 1000). (*a*) Immunologic attachment of mouse RBCs to mouse macrophages. (*b*) Same as (*a*) after a 1-h incubation with latex particles. Note that latex particles (arrows) are within the macrophage while RBCs remain attached to the macrophage plasma membrane. The phase dense perinuclear bodies are lipid droplets. (*c*) Same as (*a*) after a 1-h incubation with opsonized pneumococci. Opsonized pneumococci (arrows) are within the macrophage while RBCs remain attached to the macrophage plasma membrane. (*d*) Same as (*b*) after a 1-h incubation with rabbit anti-mouse RBC IgG. The macrophage plasma membrane has been outlined. Note the difference in phase density between the interiorized RBCs here and attached RBCs in (*a*), (*b*), and (*c*). Many small, refractile latex particles are seen within the macrophage, while one is clearly extracellular. (*e*) Same as (*c*) after a 1-h incubation with rabbit anti-mouse RBC IgG. Again note the difference in phase density between interiorized RBCs (*d* and *e*) and attached RBCs (*a*, *b*, and *c*). Several extracellular pneumococci are also seen.

with $<0.1\%$ of macrophages ingesting an average of one RBC per macrophage. Electron microscopy confirmed the ingestion of test particles by macrophages and the exclusion of RBCs from the interior of macrophages (Fig. 2).

Effect of Rabbit Anti-Mouse RBC IgG on the Ingestion by Macrophages of Immunologically Attached RBCs.—The interpretation of the experiment reported in the above section would be altered significantly if immunological attachment of RBCs to the macrophage led to "paralysis" of the segment of

TABLE III

*Ingestion of Latex Particles and Pneumococci by F(ab')$_2$-Coated Macrophages**

Test particle	Macrophages ingesting test particle	Average number of particles ingested per macrophage
	%	
Latex particles	93	~20
Nonopsonized pneumococci	0	0
Opsonized pneumococci	94	~20

* Each value represents the average of four experiments.

TABLE IV

*Effect of Test Particle Ingestion on the Phagocytosis of Immunologically Attached RBCs by Macrophages**

Test particle	Macrophages with RBCs attached	Average number of attached RBCs per macrophage	Macrophages ingesting test particle	Average number of test particles ingested per macrophage	Macrophages with RBCs ingested
	%		%		%
None	98	11	—	—	<0.1
Latex	98.5	10	94	~20	<0.1
Opsonized pneumococci	97.5	11.5	93	~20	<0.1

* Each value represents the average of four experiments.

macrophage plasma membrane adjacent to the RBC attachment site. To examine this possibility, the following experiments were performed.

RBCs were immunologically attached to macrophages and the monolayers overlaid with either Medium 199 or test particles in Medium 199. After a 1 h incubation at 37°C, the excess particles were removed by washing, and 0.05 ml of Medium 199 containing rabbit anti-mouse RBC IgG (24 μg protein) was placed onto each cover slip culture. The cover slips were incubated for 1 h at 37°C, and then fixed and examined by phase contrast microscopy.

More than 98% of the macrophages ingested an average of seven RBCs per macrophage whether or not a test particle had been previously ingested (Fig. 1, *d* and *e*). These results indicated that the macrophage was capable of ingesting opsonized RBCs, but they did not prove that the RBCs ingested were those previously attached to the macrophages. In fact, despite washing a substantial number of "free floating," nonattached RBCs was present in all preparations. Thus the ingested RBCs could have been derived from this free-floating population.

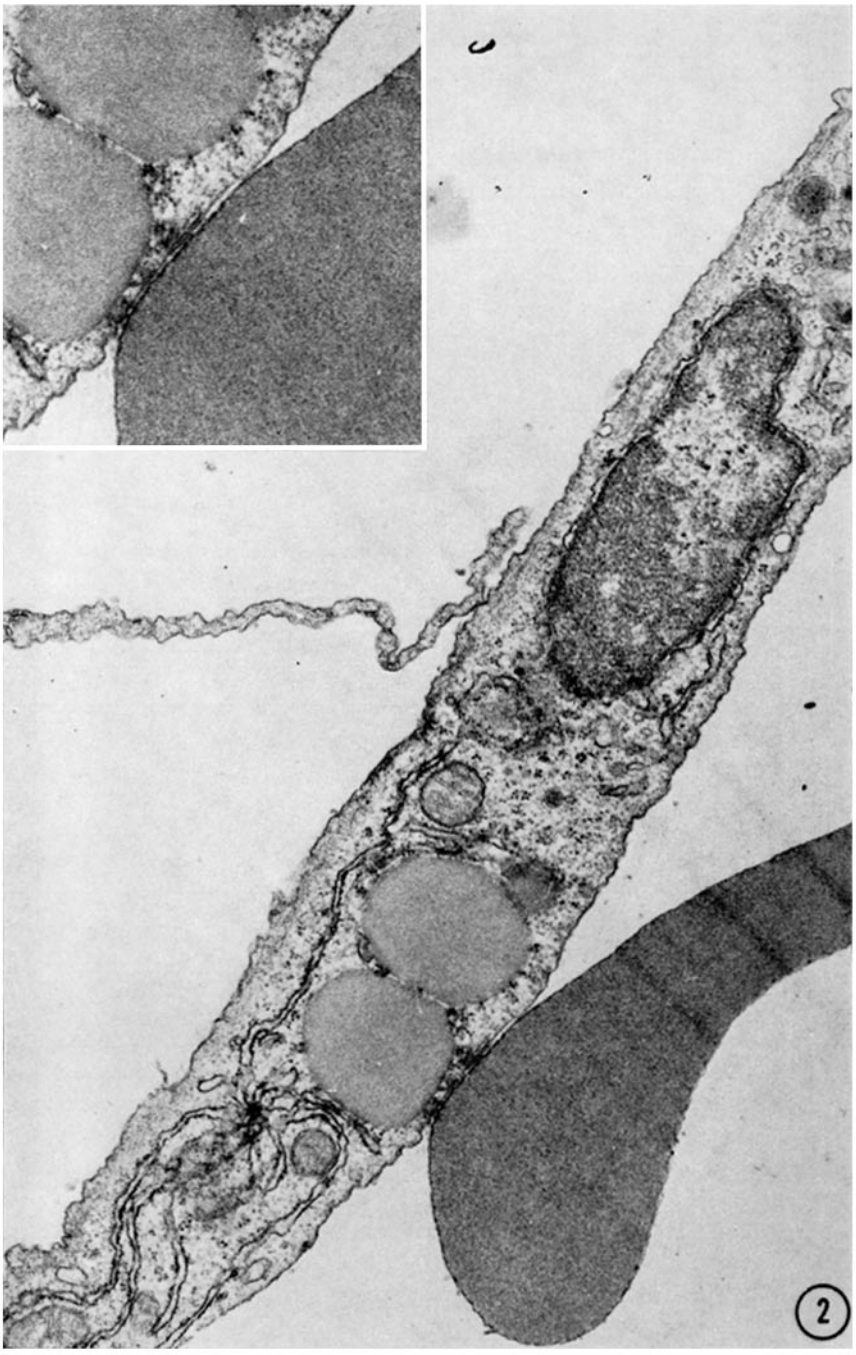

FIG. 2. Electron micrograph of a macrophage treated as in Figure 1, *c*. RBCs were immunologically attached to the macrophage plasma membrane. Opsonized pneumococci were then added and the preparations incubated for 1 h at 37°C. The pneumococci have been phagocytized while the RBC remains attached to the macrophage plasma membrane. The insert (upper left) is a fivefold enlargement of the RBC-macrophage attachment site. Identical results were obtained when latex particles served as test particles. (× 14,000).

To examine this possibility, cover slip cultures were inverted over a well containing 0.05 ml of rabbit anti-mouse RBC IgG and incubated for 1 h at 37°C. They were then fixed and examined by phase contrast microscopy. Under these circumstances, any free-floating RBCs and any RBCs that detached from the macrophages would fall to the bottom of the well, away from the monolayers, and would, therefore, be unavailable for ingestion. Nearly all macrophages ingested approximately the same number of RBCs as when the cover slips were incubated upright with IgG overlying the monolayers (see Table V). Thus, the RBCs ingested were derived from the attached population. To further confirm this observation, cover slips were inverted over wells containing rabbit anti-mouse RBC IgG, placed onto a warm stage, and observed continuously for 45 min by phase contrast microscopy. Only those RBCs already attached to the macrophage plasma membrane were ingested.

TABLE V

Effect of Rabbit Anti-Mouse RBC IgG on the Phagocytosis of Immunologically Attached RBCs by Macrophages

Test particle previously ingested	*RBC-associated macrophages ingesting RBCs	Average number of RBCs ingested per macrophage
	%	
None	94	6–7
Latex	93	6–7
Opsonized pneumococci	90	5–6

* More than 90% of macrophages were RBC-associated, i.e., had RBCs either attached or ingested, or both.

Effect of Colchicine on Attachment and Ingestion.—Studies of rabbit alveolar macrophages (20) and of *Acanthamoeba* (21) have demonstrated that the protein and lipid composition of phagosomal membranes is very similar to that of plasma membranes. Tsan and Berlin (22), however, demonstrated that the activities of adenine, adenosine, and lysine transport systems of intact polymorphonuclear leukocytes and macrophages were not diminished when the cells interiorized large amounts of membrane during the ingestion of latex particles. Ukena and Berlin (23), however, found that in the presence of colchicine the activities of adenine and lysine transport systems of polymorphonuclear leukocytes decreased after phagocytosis of latex. These observations suggested to these authors that the polymorphonuclear leukocyte and the macrophage are able to selectively exclude from phagosomes certain constituents of their plasma membranes and that colchicine abolishes the polymorphonuclear leukocyte's capacity for such selectivity. Using immunologically attached RBCs as a marker, we tested the effect of colchicine on the capacity of the

macrophage to selectively ingest portions of its plasma membrane during phagocytosis of latex particles.

Macrophages were preincubated for 2 h with 10^{-6} M colchicine in Medium 199; the same concentration of the drug was maintained throughout the RBC attachment and test particle ingestion procedures. This concentration of colchicine disrupts microtubules and alters the spreading characteristics of mouse peritoneal macrophages (Z. Cohn, unpublished observation). Colchicine did not alter the percentage of macrophages with RBCs attached or the number of RBCs attached per macrophage. The number of latex particles ingested per macrophage and the percentage of macrophages ingesting latex were likewise not affected by the drug. When macrophages coated with immunologically attached RBCs ingested latex particles, no RBCs were ingested. Thus, under these circumstances, colchicine did not impair the ability of the macrophage to selectively interiorize portions of its membrane during phagocytosis.

DISCUSSION

Several methods of attaching particles to the plasma membranes of macrophages have been described. Melsom and Seljelid (18) found that mouse RBCs attach spontaneously to mouse macrophages under rigorously controlled conditions of osmolarity and serum concentration. In the absence of serum, attachment of a few RBCs to a few macrophages occurs in our system, but the degree of attachment is quite low when compared with that obtained by immunologic linking.

Allen et al. (24) attached bacteria to macrophages with concanavalin A. Under these circumstances, the bacteria were not phagocytized. Jones and Hirsch (4) found that *Mycoplasma pulmonis* attach spontaneously to mouse macrophages and are not ingested unless antimycoplasma antibody is added. Thus, the failure of the macrophage to ingest RBCs attached to its plasma membrane is neither surprising nor unique.

The influence of phagocytosis of one particle on the ingestion of a different particle by phagocytes has also been examined previously. Cohn and Morse (6) demonstrated that ingestion of heat-killed staphylococci enhanced the subsequent uptake and killing of *Staphylococcus albus* by polymorphonuclear leukocytes. Since the marker particle, live *S. albus*, was ingested under all experimental conditions, prior ingestion of heat-killed organisms presumaly increased the rate of uptake of the marker particle but did not initiate its ingestion. That ingestion of one particle may not initiate ingestion of another particle was suggested by their observation that ingestion of live *Staphylococcus aureus*, an organism which is not phagocytized under their experimental conditions, was not stimulated by the prior ingestion of heat-killed organisms.

Rabinovitch and Gary (7) attached glutaraldehyde-treated RBCs to mouse peritoneal macrophages. Ingestion of the RBCs was prevented by employing a very brief incubation time and low temperature. When macrophages, with

glutaraldehyde-treated RBCs attached to their plasma membranes, were incubated with heat-killed staphylococci for 20 min at 34°C, ingestion of RBCs was stimulated in those macrophages that had ingested staphylococci. But since the glutaraldehyde-treated RBC is an ingestable particle, these investigators could not determine whether it was the initiation of RBC phagocytosis or the enhancement of the rate of RBC ingestion that was prompted by uptake of the organisms.

In order to dissociate the attachment and ingestion phases of phagocytosis, we have immunologically linked mouse RBCs to mouse peritoneal macrophages, using the $F(ab')_2$ fragments of rabbit and sheep immunoglobulins. RBCs which are linked to the macrophage plasma membrane in this way are not ingested and remain stably associated with the macrophage surface for over 4 h at 37°C (Table II). These mouse RBCs provide easily indentifiable markers for the segments of plasma membrane to which they are attached. We have used this system to determine whether or not the phagocytosis of latex particles or of opsonized pneumococci (the test particles) enhances the ingestion of all particles which are attached to the macrophage plasma membrane. One of two clearly distinguishable results, which are illustrated diagrammatically in Fig. 3, should ensue from such an experiment. If the phagocytosis of these test particles causes generalized activation of the macrophage plasma membrane, then ingestion of the marker RBCs should be stimulated in rate and/or quantity (Fig. 3, *a*). If, on the other hand, phagocytosis of the test particles is initiated by a stimulus which is confined to the segment of plasma membrane immediately adjacent to the test particle, then the marker RBCs should not be ingested, but should remain attached to the cell's surface (Fig. 3, *b*). Our results are in agreement with the latter model (Fig. 3, *b*), indicating that the phagocytic stimulus is confined to the segment of plasma membrane adjacent to the test particle. Furthermore, the segmental nature of the membrane response is not dependent upon whether a nonspecific receptor site (latex) or the Fc receptor site (opsonized pneumococcus) is stimulated.

The failure of immunologically attached RBCs to be ingested by the macrophages could have resulted from "paralysis" of the plasma membrane's phagocytic capacity, as suggested by the experiments of Carey et al. (19). In our experiments, immunologically attached RBCs were readily engulfed when anti-RBC IgG was added, indicating that cross-linking of RBCs to macrophages by $F(ab')_2$ fragments does not inhibit phagocytosis.

Treatment of cells with colchicine has several effects, one of which may be to cause disorganization, or random distribution, of cell surface components (23, 25). It was, therefore, of interest to determine whether the segmental response to a phagocytic stimulus is maintained in the presence of the drug. Colchicine (10^{-6}M) did not alter the number or distribution of RBCs which could be immunologically attached to macrophages and did not stimulate the ingestion of these RBCs. In the presence of colchicine, latex particles were actively phago-

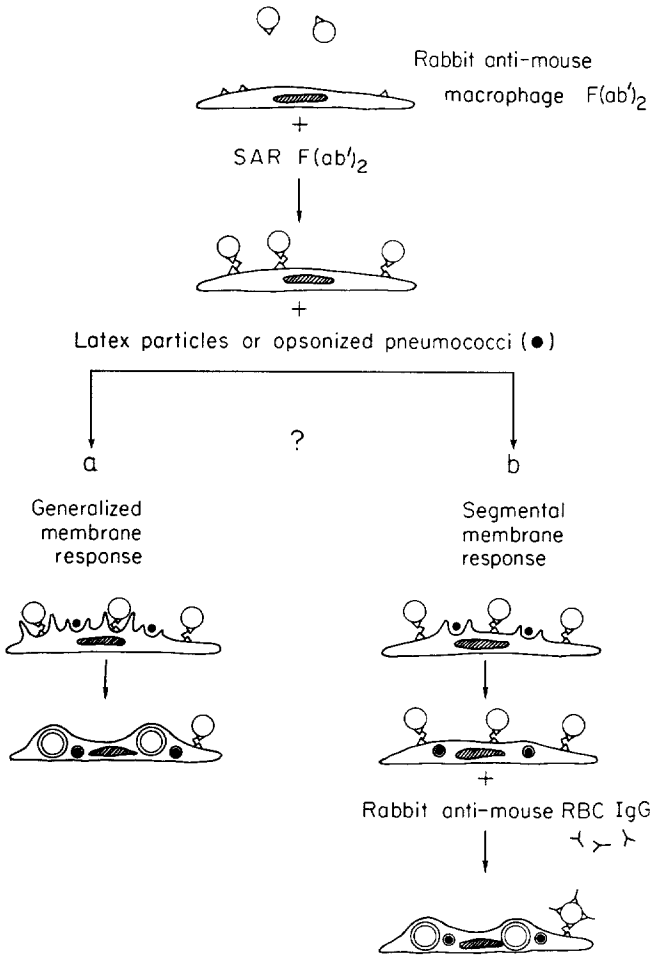

FIG. 3. Models for the response of the macrophage to a phagocytic stimulus. (*a*) If phagocytosis of a particle causes generalized activation of the macrophage plasma membrane, then ingestion of the test particle (latex, opsonized pneumococcus) triggers phagocytosis of immunologically attached RBCs. (*b*) If the stimulus to ingest a particle elicits a segmental membrane response, then test particles are ingested while immunologically attached RBCs remain bound to the macrophage plasma membrane.

cytized, while immunologically attached RBCs remained on the cell surface Thus, colchicine does not alter the segmental response of the macrophage membrane to a phagocytic stimulus.

Mixed agglutination of cells having similar or identical antigenic determinants has been demonstrated repeatedly, and labeled anti-immunoglobulin reagents have found wide use in biology and medicine. The methods we describe here are sutiable for linking a variety of different cells types to one another and may

prove generally useful in studies requiring prolonged contact between different cell types and in dissecting further the attachment and ingestion phases of phagocytosis.

SUMMARY

A method of attaching mouse RBCs to mouse macrophages is described. Both cell types were coated with rabbit anti-mouse macrophage $F(ab')_2$, and cross-linkage of cells was effected with sheep $F(ab')_2$ directed against rabbit $F(ab')_2$. 98% of macrophages attached an average of 11 RBCs each. Attachment occurred at 37°C and was stable for at least 4 h. Less than 0.1% of macrophages ingested RBCs under these conditions.

Latex particles and opsonized pneumococci were ingested as avidly by RBC-coated macrophages as by native macrophages. Ingestion of these particles did not prompt ingestion of attached RBCs. When anti-RBC IgG was added, however, over 90% of macrophages ingested an average of six RBCs each. Thus, ingestion of one particle does not trigger generalized phagocytosis of all particles attached to the cell's plasma membrane, and the phagocytic stimulus is confined to the segment of the cell's plasma membrane immediately adjacent to the particle being ingested.

We would like to acknowledge the excellent technical assistance of Ms. B. Fried and Ms. J. Adams, and to thank Dr. Z. Cohn for helpful discussions.

BIBLIOGRAPHY

1. Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell Res.* **46:**19.
2. Rabinovitch, M. 1967. The role of antibodies in the ingestion of aldehyde-treated erythrocytes attached to macrophages. *J. Immunol.* **99:**232.
3. Rabinovitch, M. 1967. Studies on the immunoglobulins which stimulate the ingestion of glutaraldehyde-treated red cells attached to macrophages. *J. Immunol.* **99:**1115.
4. 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.
5. Jones, T. C., S. Yeh, and J. G. Hirsch. 1972. Studies on attachment and ingestion phases of phagocytosis of *Mycoplasma pulmonis* by mouse peritoneal macrophages. *Proc. Soc. Exp. Biol. Med.* **139:**464.
6. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. I. Observations on the requirements and consequences of particle ingestion. *J. Exp. Med.* **111:**667.
7. Rabinovitch, M., and P. P. Gary. 1968. Effect of the uptake of staphylococci on the ingestion of glutaraldehyde-treated red cells attached to macrophages. *Exp. Cell Res.* **52:**363.
8. Huber, H., and H. H. Fudenberg. 1968. Receptor sites of human monocytes for IgG. *Int. Arch. Allergy Appl. Immunol.* **34:**18.
9. Holland, P., N. H. Holland, and Z. A. Cohn. 1972. The selective inhibition of

macrophage phagocytic receptors by anti-membrane antibodies. *J. Exp. Med.* **135**:458.

10. Williams, C. A., and M. W. Chase. 1967. Methods in Immunology and Immunochemistry. Academic Press, New York. **1**:322.
11. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestive products. *Methods Med. Res.* **10**:134.
12. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**:167.
13. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* **245**:3059.
14. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121**:153.
15. Luft, J. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
16. Hirsch, J. G., and M. E. Fedorko. 1968. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "post-fixation" in uranyl acetate. *J. Cell Biol.* **38**:615.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
18. Melsom, H., and R. Seljelid. 1973. The cytotoxic effect of mouse macrophages on syngeneic and allogeneic erythrocytes. *J. Exp. Med.* **137**:807.
19. 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.
20. Nachman, R. L., B. Ferris, and J. G. Hirsch. 1971. Macrophage plasma membranes. I. Isolation and studies on protein components. *J. Exp. Med.* **133**:785.
21. Weisman, R. A., and E. D. Korn. 1967. Phagocytosis of latex beads by *Acanthamoeba*. I. Biochemical properties. *Biochemistry*. **6**:485.
22. Tsan, M., and R. D. Berlin. 1971. Effect of phagocytosis on membrane transport of nonelectrolytes. *J. Exp. Med.* **134**:1016.
23. Ukena, T. E., and R. D. Berlin. 1972. Effect of colchicine and vinblastine on the topographical separation of membrane functions. *J. Exp. Med.* **136**:1.
24. Allen, J. M., G. M. W. Cook, and A. R. Poole. 1971. Action of concanavalin A on the attachment stage of phagocytosis by macrophages. *Exp. Cell Res.* **68**:466.
25. Berlin, R. D., and T. E. Ukena. 1972. Effect of colchicine and vinblastine on the agglutination of polymorphonuclear leukocytes by concanavalin A. *Nat. New Biol.* **238**:120.