STUDIES ON THE MECHANISM OF PHAGOCYTOSIS

I. Requirements for Circumferential Attachment of Particle-Bound Ligands to Specific Receptors on the Macrophage Plasma Membrane*

By FRANK M. GRIFFIN, JR., JOHANNA A. GRIFFIN, JUDITH E. LEIDER, and SAMUEL C. SILVERSTEIN§

(From the Rockefeller University, New York 10021)

Phagocytosis of a variety of inert and metabolically active particulates plays an essential role in metazoan nutrition, metabolic economy, and defense against viral, bacterial, and protozoan pathogens. With the development of the lysosome concept (1–3), the processes governing the intracellular digestion of ingested particles have been clarified. In contrast, the mechanisms which regulate the ingestion phase of phagocytosis remain poorly understood. The recognition of contractile proteins in the cytoplasm of a variety of nonmuscle cells (4–7), including macrophages (4, 5, 7), has resolved one mechanism whereby chemical energy released during phagocytosis may be coupled to mechanical processes requisite for membrane movement and particle interiorization. However, no information is available which suggests how a phagocyte directs this system of contractile elements in order to engulf specific particles which are attached to its plasma membrane. That the process is indeed specific and limited to the segments of plasma membrane to which the particles to be ingested are bound has been carefully documented (8, 9).

The fastidious appetite of phagocytes is also well known. These cells avidly ingest some particles with which they come into contact while they totally ignore others (8, 9). The presence or absence of opsonins (complement and immunoglobulins) on the surface of a particle is widely recognized as the most important factor governing the fate of the particle. Particles coated with ligands such as IgG and/or complement are avidly ingested by phagocytes while the same particles lacking such ligands are neither bound to, nor ingested by, phagocytic cells.

The experiments reported in this paper were performed to examine the mechanisms which govern the vectorial and discriminatory qualities of the phagocytic process and to define the factors responsible for the segmental nature

* Supported by grants AI08697, AI07012, and AI01831 from the U. S. Public Health Service and grant PF-867 from the American Cancer Society, Inc.
† Present address: Division of Infectious Diseases, Department of Medicine, University of Alabama in Birmingham, Birmingham, Alabama 35294.
§ Established Investigator of the American Heart Association.
of the response of the macrophage plasma membrane to a phagocytic stimulus. For these purposes, we have studied the interaction of mouse peritoneal macrophages with sheep erythrocytes coated with immunoglobulins and with complement. By suitable experimental manipulations, we have produced erythrocytes which are functionally opsonized on only one "hemisphere." These erythrocytes bind to the macrophage surface but are not ingested. These findings lead us to the conclusion that phagocytosis of immunologically sensitized erythrocytes by macrophages requires the sequential and circumferential attachment of specific macrophage membrane receptors to ligands bound throughout the erythrocyte's surface. In this article we describe these experiments and propose a general hypothesis, consistent with our observations, to explain the mechanism of phagocytosis.

Materials and Methods

Chemicals. Trypsin (lot no. T-8253, twice recrystallized) was obtained from Sigma Chemical Co., St. Louis, Mo.; ovomucoid trypsin inhibitor (OTI), from Worthington Biochemical Corp., Freehold, N. J.; and sodium fluoride, from Mallinckrodt Chemical Works, New York. Brewer thioglycollate medium was obtained from Difco Laboratories, Detroit, Mich.; a 4.05% aqueous solution was prepared according to the manufacturer's directions.

Macrophages. The methods for harvesting and maintaining mouse peritoneal macrophages were essentially those of Cohn and Benson (10), modified as described previously (8). Peritoneal macrophages obtained from mice injected intraperitoneally with 1 ml of Brewer thioglycollate medium 4 days before harvest are designated activated macrophages. Peritoneal cells were harvested, and 3–4.5 x 10^5 cells were cultivated on each 13 mm diameter glass cover slip in 35 mm Petri dishes for 1–4 days in Medium 199 (Microbiological Associates, Bethesda, Md.) with 20% heat-decomplemented (56°C, 30 min) fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.), 100 U/ml of penicillin, and 100 µg/ml of streptomycin.

Erythrocytes. Sheep erythrocytes (E) in Alsever's solution (Animal Blood Center, Syracuse, N. Y.) were washed three times in solution "a" of Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} ions (11) and suspended in medium "b" or Veronal-buffered glucose containing Ca^{++}, Mg^{++}, and gelatin (VBG) (12).

Complement. Serum was prepared from C5-deficient AKR mice and stored immediately in 0.2-ml aliquots at -70°C. It served as the source of C1423 in all experiments.

Antisera. Rabbit anti-sheep E IgG (lot no. 10154), at a protein concentration of 5 mg/ml, was obtained from Cordis Laboratories, Miami, Fla. Its hemagglutination titer for E, determined as previously described (8), was 1:3,000. Rabbit anti-sheep E IgM (lot no. 80614), at a protein concentration of 350 µg/ml, was also obtained from Cordis Laboratories. It agglutinated E at a dilution of 1:320.

Rabbit Antimouse Macrophage IgG. The IgG fraction of rabbit antimouse macrophage serum was prepared as previously described (8, 13), dialyzed against PBS, concentrated by vacuum dialysis, millipore filtered (0.45 µm pore size), and stored at 4°C. The protein concentration of this IgG fraction was 12 mg/ml; it agglutinated mouse erythrocytes at a dilution of 1:500.

Rabbit Antimouse Macrophage F(ab')2. The F(ab')2 fragment of rabbit antimouse macrophage IgG was prepared by pepsin digestion, using the method of Nisonoff (14), as previously described (8). The protein concentration of this F(ab')2 fraction was 3 mg/ml; it agglutinated mouse erythrocytes at a dilution of 1:32. Rabbit antimouse C3 IgG was obtained from Microbiological Associates.

Abbreviations used in this paper: E, sheep erythrocytes; E(IgG), E coated with anti-E IgG; E(IgM), E coated with anti-E IgM; E(IgM/C), E(IgM) coated with the first four complement components; OTI, ovomucoid trypsin inhibitor; PBS, phosphate-buffered saline with Ca^{++} and Mg^{++} ions; VBG, Veronal-buffered glucose with Ca^{++}, Mg^{++}, and 0.1% gelatin.

The term "medium" as used throughout this paper refers to Medium 199.
dissolved in PBS at a concentration of 1 mg/ml, and used in experiments at a concentration of 10 μg/ml.

Preparation of Antibody-Coated E. 1 ml of a 5% (vol/vol) suspension of E in medium 199 was incubated with 125 μg of rabbit antisheep IgG or with 14 μg of rabbit antisheep IgM for 15 min at 37°C. These mixtures were centrifuged for 5 min at 750 g at room temperature, and the pelleted erythrocytes were resuspended in Medium 199 at a concentration of 0.5%. These preparations (0.5% E coated with anti-E IgG or anti-E IgM) are designated E(IgG) and E(IgM), respectively.

Preparation of Complement-Coated E(IgM). Freshly thawed AKR mouse serum was diluted 1:10 in VBG and mixed with an equal volume of a 5% suspension of E(IgM) in VBG. The mixtures were incubated for 10 min at 37°C and centrifuged for 5 min at 750 g. The pelleted erythrocytes were resuspended in Medium 199 at a concentration of 0.5%. This preparation [0.5% complement(C)-coated E(IgM)] is designated E(IgM)C. VBG and all erythrocyte preparations were made fresh on the day of an experiment.

Attachment of E(IgG) and E(IgM)C to Macrophages. Macrophage monolayers were washed, covered with 2 ml of Medium 199, and precooled at 4°C for 30 min. 0.2 ml of 0.5% E(IgG) or E(IgM)C, precooled at 4°C, were added to each dish, and preparations incubated at 4°C for 1 h, then washed twice with ice-cold medium. This procedure resulted in binding of E(IgG) and E(IgM)C to macrophages.

Phase-Contrast Microscopy. Cover slip cultures were fixed with glutaraldehyde and examined by phase-contrast microscopy as previously described (8).

Electron Microscopy. Cover slip cultures were processed for electron microscopy as previously described (15, 16) and examined in a Siemens Elmiskop 1A (Siemens Corp., Medical Industrial Div., Iselin, N. J.).

Miscellaneous. Protein determinations were performed by the method of Lowry et al. (17), using bovine serum albumin as a standard. VBG was prepared as described by Rapp and Borsos (12).

Results

Influence of Fc Receptor Blockade on the Uptake of E(IgG). Macrophages contain on their plasma membranes receptors for the Fc portion of IgG. These receptors mediate binding of E(IgG) to the macrophage plasma membrane (18, 19) and participate in the ingestion of these attached E(IgG) (20, 21). The experiments described below were designed to define the role of these plasma membrane receptors in the ingestion phase of phagocytosis (22). Specifically, we wished to determine whether the initial interaction of erythrocyte-bound immunoglobulin molecules [E(IgG)] with Fc receptors on the macrophage plasma membrane is sufficient to "trigger" the ingestion of E(IgG), or whether ingestion requires the interaction of erythrocyte-bound immunoglobulins with macrophage Fc receptors not involved in the initial attachment process. These two possibilities can be distinguished from one another experimentally by (a) attaching E(IgG) to macrophages via the macrophages' Fc receptors under conditions which reversibly inhibit phagocytosis, (b) blocking unoccupied Fc receptor sites by methods which do not alter the phagocytic capacity of macrophages per se, and (c) then incubating the culture under conditions which are permissive for phagocytosis. These experimental requirements have been met. The attachment and ingestion phases of phagocytosis were dissociated by incubating E(IgG) with macrophages at 4°C. At this temperature attachment proceeds normally but ingestion is inhibited.

Effect of Antimacrophage IgG on Fc Receptor-Mediated Phagocytosis. Antimacrophage IgG was used to block Fc receptors on the macrophage plasma membrane. As shown by Holland et al. (13), this immunoglobulin
preparation blocks attachment and ingestion mediated by the Fc receptor, but has little or no effect upon phagocytosis mediated by other receptors (13, 23). The Fc portion of the antimacrophage IgG molecule is required to block the Fc receptors; F(ab')2 fragments of the same immunoglobulin preparation bind to the macrophage plasma membrane (23) but have (a) no effect on the capacity of macrophages to bind C-coated erythrocytes to their surface (23), or to bind and ingest latex particles, IgG-coated erythrocytes, formaldehyde-treated erythrocytes, or opsonized pneumococci (13, 23); and (b) only minimal effect on the ability of activated macrophages to ingest C-coated erythrocytes via the macrophages' C receptors (13, 23).

E(IgG) were incubated at 4°C with 24 h explanted macrophages; after a 1 h attachment period, the nonbound E(IgG) were removed by washing the cover slip cultures. These cultures were then incubated with either antimacrophage IgG, antimacrophage F(ab')2, or medium alone for an additional 1 h at 4°C. Some preparations were fixed and examined at this point. Others were washed, incubated with fresh medium for 30 min at 37°C, then fixed and examined. The results are presented in Table I. 93% of the macrophages incubated at 4°C with E(IgG) bound an average of 14 E(IgG) each, but only 1% ingested any erythrocytes. When warmed to 37°C for 30 min, 76% of these macrophages ingested an average of eight E(IgG) each. Similarly, on warming to 37°C, 83% of macrophages which had been treated with antimacrophage F(ab')2 after E(IgG) attachment ingested an average of seven E(IgG). On the other hand, on warming to 37°C, only 8% of macrophages which had been treated with antimacrophage IgG after E(IgG) attachment ingested an average of two E(IgG) each. Antimacrophage IgG did not cause significant release of E(IgG) from the macrophage plasma membrane; an average of nine E(IgG) remained bound to each macrophage.

These results show that binding of IgG-coated erythrocytes to the macrophage plasma membrane via its Fc receptors is not sufficient to promote ingestion of these E(IgG). Rather, receptors on portions of the plasma membrane which are not involved in the initial attachment process are essential for the subsequent ingestion of the erythrocytes.

Temperature of binding does not influence the fate of E(IgG). Incubation of macrophages at 4°C for several hours has no deleterious effect on cell viability or on the capacities of these cells to ingest E(IgG), E(IgM)C [activated macrophages (23)], or latex particles when the macrophages are rewarmed to 37°C (data not shown). However, low temperatures have many effects upon cells, one of which is to alter the physical and functional state of cell membranes. Although these changes are thought to be reversed when the cells are returned to physiological temperatures (37°C), it was possible that the interaction of the macrophage plasma membrane with antimacrophage IgG or with IgG-coated erythrocytes was qualitatively different at 4°C from the interaction at 37°C. To be certain that low temperature-induced changes were not responsible for the observed failure of E(IgG) to initiate phagocytosis, and to allow E(IgG) to attach to the macrophage plasma membrane at 37°C, we used NaF, a reversible inhibitor of phagocytosis, to temporarily block the ingestion of E(IgG).
TABLE I

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgG) attached</th>
<th>Average no. of E(IgG) attached per macrophage</th>
<th>Macrophages with E(IgG) ingested</th>
<th>Average no. of E(IgG) ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(IgG)*→4°C</td>
<td>93</td>
<td>14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E(IgG)*→37°C</td>
<td>19</td>
<td>4</td>
<td>76</td>
<td>8</td>
</tr>
<tr>
<td>E(IgG)*→antimacrophage</td>
<td>16</td>
<td>3</td>
<td>83</td>
<td>7</td>
</tr>
<tr>
<td>F(ab')2→37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E(IgG)*→antimacrophage</td>
<td>90</td>
<td>9</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

* E(IgG) were attached to macrophages at 4°C. Preparations were washed with fresh medium to remove nonattached erythrocytes.
† Each cover slip culture was covered with 0.05 ml of antimacrophage F(ab')2 (120 µg/ml) and incubated for 1 h at 4°C.
§ Each cover slip culture was covered with 0.05 ml of antimacrophage IgG (120 µg/ml) and incubated for 1 h at 4°C.

Macrophages were incubated for 1 h at 37°C in medium containing 10^{-3} M NaF. E(IgG) were added and the incubation was continued for a second hour, at which time the preparations were washed and incubated with either antimacrophage IgG, antimacrophage F(ab')2, or medium, all in the presence of 10^{-3} M NaF, for a third hour at 37°C. Some preparations were fixed and examined at this point. Others were washed, incubated in fresh medium without NaF for 30 min at 37°C, then fixed and examined. The results are presented in Table II and are similar to those described in Table I. Macrophages which were incubated with antimacrophage IgG failed to ingest bound E(IgG), while untreated macrophages and those treated with antimacrophage F(ab')2 ingested bound E(IgG) to the same extent as cells incubated for 30 min with E(IgG) in the absence of NaF.

Thus, the temperature of binding of IgG-coated erythrocytes or of antimacrophage immunoglobulins to the macrophages did not alter the experimental results. At both 4°C and 37°C, IgG-coated erythrocytes were bound to macrophages via the macrophages’ Fc receptors; addition of antimacrophage IgG blocked the subsequent ingestion of these immunologically attached erythrocytes.

EFFECTS OF ANTIMACROPHAGE IgG ON E(IgG)-Fc RECEPTOR INTERACTION. The evidence presented to this point supports the hypothesis that the functional integrity of Fc receptors lying outside the zone of attachment of E(IgG) to the macrophage surface is a necessary prerequisite for the ingestion of these IgG-coated erythrocytes. However, it is possible that antimacrophage IgG displaces erythrocyte-bound IgG molecules from Fc receptor sites within the zone of attachment of E(IgG) to the macrophage plasma membrane. Were this to occur, then the number of E(IgG)-Fc receptor bonds could be reduced below a threshold.

\[3 \text{ Under these conditions over 95% of the macrophages were viable, as judged by trypan blue exclusion and by their ability to ingest E(IgG) after removal of NaF.} \]
STUDIES ON THE MECHANISM OF PHAGOCYTOSIS

TABLE II

Fc Receptor Blockade Inhibits Ingestion of E(IgG) Previously Bound to Macrophages at 37°C in the Presence of NaF

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgG) attached</th>
<th>Average no. of E(IgG) attached per macrophage</th>
<th>Macrophages with E(IgG) ingested</th>
<th>Average no. of E(IgG) ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(IgG)→Medium with NaF</td>
<td>78</td>
<td>17</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>E(IgG)→Medium without NaF</td>
<td>9</td>
<td>4</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>E(IgG)→antimacrophage IgG→Medium without NaF</td>
<td>86</td>
<td>17</td>
<td>14</td>
<td>4</td>
</tr>
</tbody>
</table>

* E(IgG) were attached to macrophages at 37°C in the presence of NaF, as described in the text.
† Each cover slip culture was covered with 0.005 ml of antimacrophage IgG (120 µg/ml) containing 10⁻³ M NaF and incubated for 1 h at 37°C.

necessary to "trigger" phagocytosis of the bound particle. To explore this possibility, we examined in detail the effects of antimacrophage IgG on E(IgG) binding and ingestion by macrophages.

E(IgG) were attached to macrophages at 4°C. The nonattached E(IgG) were removed by washing, and the E(IgG)-macrophage complexes were incubated for 1 h at 4°C with quantities of antimacrophage IgG 2- to 10-fold greater than those necessary to block ingestion of the attached E(IgG). The cultures were then warmed to 37°C in the presence of excess antimacrophage IgG. No elution of E(IgG) from the macrophage surface occurred, and, as found previously, ingestion of E(IgG) was inhibited. Addition of up to 10 mg/ml of nonspecific (pooled) rabbit IgG to the incubation medium neither inhibited ingestion nor promoted elution of E(IgG) from the macrophage surface. Thus, neither soluble IgG nor antimacrophage IgG displaced sufficient erythrocyte-bound IgG molecules from macrophage Fc receptors to cause elution of the erythrocytes.

To determine whether antimacrophage IgG altered the interaction of E(IgG) with the macrophage surface in a structurally recognizable fashion, E(IgG)-macrophage complexes were treated with antimacrophage IgG and examined by electron microscopy. No change in the size or configuration of the E(IgG) attachment site was noted. These findings, and those outlined above, indicate that antimacrophage IgG does not cause elution of preattached E(IgG) and does not alter in a morphologically recognizable manner the attachment of E(IgG) to the macrophage plasma membrane. They do not prove, however, that antimacrophage IgG has no effect upon the interaction of erythrocyte-bound IgG with macrophage Fc receptors in the zone of initial attachment.

**Does antimacrophage IgG qualitatively alter Fc receptor function?** To further define the mechanism by which antimacrophage IgG blocks the ingestion of preattached E(IgG), we determined whether the blockade of Fc receptor-mediated phagocytosis is relative or absolute; i.e., can the capacity of antimacrophage IgG to inhibit phagocytosis of preattached E(IgG) be overcome by the addition of excess antierythrocyte IgG? E(IgG) were bound to macrophages at
4°C. Nonattached erythrocytes were removed by washing, and the E(IgG)–macrophage complexes were incubated with antimacrophage IgG, also at 4°C. The cultures were then washed and incubated in fresh medium at 37°C. Antierythrocyte IgG was added to some of these cultures. As shown in Table III, 30 μg/ml of antierythrocyte IgG overcame the antimacrophage IgG-mediated inhibition of phagocytosis and promoted the ingestion of E(IgG). Thus, the capacity of antimacrophage IgG to inhibit Fc receptor-mediated phagocytosis is relative, not absolute, and is governed by the quantity of IgG bound to the erythrocyte.

**Concentration of antierythrocyte IgG required to overcome antimacrophage IgG-mediated Fc receptor blockade.** An agglutinating concentration of anti-E IgG is needed to promote ingestion of E(IgG) by antimacrophage IgG-treated macrophages. These agglutinated erythrocytes cannot be bound to macrophage monolayers in a reproducible fashion. For this reason, to determine the minimum quantity of antierythrocyte IgG required to initiate the ingestion of E by antimacrophage IgG-treated macrophages and to compare this quantity with the quantity of antierythrocyte IgG necessary to promote the ingestion of E by nontreated macrophages, we utilized the method outlined below.

E coated with antiE IgM and the first four C components [E(IgM)C] bind to the plasma membranes of nonactivated macrophages but are not ingested (9, 23). We incubated E(IgM)C with macrophages for 30 min at 37°C. Virtually all of the macrophages bound an average of 20 erythrocytes each; less than 5% of the macrophages ingested any erythrocytes. Some of these cultures of E(IgM)C–macrophage complexes were incubated with antimacrophage IgG; others were not. Both sets of cultures were then incubated in the presence of varying concentrations of antierythrocyte IgG for an additional 45 min at 37°C, and the number of macrophages ingesting erythrocytes was scored microscopically. The results are presented in Fig. 1. Less than 2 μg/ml of antierythrocyte IgG promoted marked ingestion (phagocytic index >250) of the attached E(IgM)C by

---

**Table III**

*Phagocytosis of Preattached E(IgG) by Antimacrophage IgG-Treated Macrophages: Effect of Excess Anti-E IgG*

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgG) attached</th>
<th>Average no. of E(IgG) attached per macrophage</th>
<th>Macrophages with E(IgG) ingested</th>
<th>Average no. of E(IgG) ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(IgG)*→medium, 4°C→37°C</td>
<td>35</td>
<td>20</td>
<td>65</td>
<td>4</td>
</tr>
<tr>
<td>E(IgG)*→antimacrophage IgG→37°C</td>
<td>83</td>
<td>13</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>E(IgG)*→antimacrophage IgG→37°C + anti-E IgG§ (30 μg/ml)</td>
<td>20</td>
<td>10</td>
<td>80</td>
<td>10</td>
</tr>
</tbody>
</table>

* As in Table I.
† E(IgG)-macrophage complexes were treated with antimacrophage IgG at 4°C, as in Table I.
§ E(IgG)-macrophage complexes, treated with antimacrophage IgG, were incubated in the presence of 30 μg/ml of anti-E IgG at 37°C for 45 min.
STUDIES ON THE MECHANISM OF PHAGOCYTOSIS

FIG. 1. The quantity of anti-E IgG necessary to promote ingestion of bound E(IgM)C by nonactivated macrophages is increased by treatment of the E(IgM)C-macrophage complexes with antimacrophage IgG. E(IgM)C were bound to nonactivated macrophages at 37°C. Some preparations were then incubated at 4°C for 1 h with antimacrophage IgG (Δ); others were not (○). Various quantities of anti-E IgG (abscissa) were then added and the cultures incubated at 37°C for 45 min. Ingestion of erythrocytes by macrophages was scored microscopically. Phagocytic index (ordinate) is the number of erythrocytes ingested per 100 macrophages.

macrophages which had not been pretreated with antimacrophage IgG. In contrast, 30 µg/ml of antiererythrocyte IgG was required to achieve a comparable phagocytic index by macrophages treated with antimacrophage IgG. Thus, the quantity of antiererythrocyte IgG necessary to overcome the phagocytosis-inhibiting effect of antimacrophage IgG is 15-fold greater than the quantity needed to promote the phagocytosis of preattached erythrocytes by nontreated macrophages.

In view of the failure of antimacrophage IgG to cause release of E(IgG) from the macrophage Fc receptors, it seems unlikely that antimacrophage IgG inhibits the ingestion of preattached E(IgG) by reducing the number of E(IgG)-Fc receptor interactions below a threshold value which must be achieved in order to trigger phagocytosis of the bound erythrocytes. Nevertheless, this possibility cannot be rigorously excluded. For this reason we chose a second system in which to test the importance of receptor-ligand interactions in the phagocytic process.

Phagocytosis of E(IgM)C Mediated by the Macrophage C Receptor. The data presented to this point are consistent with the view that receptors which do not participate in the initial binding of E(IgG) to the macrophage surface are essential for subsequent ingestion of bound E(IgG). They suggest that sequential, circumferential attachment of membrane receptors to ligands throughout the surface of the particle is necessary for phagocytosis of that particle. To further test this hypothesis, we asked whether particles containing ligands on only one hemisphere are phagocytized by macrophages. In these experiments, we used E(IgM)C and 2-4 day explanted activated macrophages. We have
Trypsin Inhibits the Ingestion of E(IgM)C by Activated Macrophages

*E(IgM)C were attached to macrophages at 4°C. Preparations were washed with fresh medium to remove nonattached erythrocytes.

† Cultures of E(IgM)C-macrophage complexes were incubated in the presence of 750 μg/ml of trypsin for 30 min at 4°C and washed twice with medium (4°C) containing 750 μg/ml of OTI and twice with medium alone.

Previously shown that macrophages harvested from the peritoneal cavities of mice injected intraperitoneally with thioglycollate medium phagocytize E(IgM)C (23). Since thioglycollate-induced peritoneal macrophages are more actively phagocytic and exhibit some of the properties of immunologically stimulated macrophages, we have called them "activated" macrophages. Phagocytosis of E(IgM)C by these thioglycollate-activated macrophages occurs independently of their Fc receptors and is mediated by the C3b receptors present on the macrophage plasma membrane (23).

E(IgM)C were bound to thioglycollate-activated macrophages at 4°C as described in the Materials and Methods. Some preparations were then incubated with trypsin (750 μg/ml) at 4°C, washed with medium containing OTI to inhibit further proteolysis, and then fixed and examined. Other preparations were incubated for an additional 45 min at 37°C, then fixed and examined. The results are shown in Table IV. 98% of macrophages bound an average of 20 E(IgM)C each; no ingestion was noted (Table IV, line 1). Trypsin treatment at 4°C did not appreciably alter these results (Table IV, line 2). When cultures which had not been incubated with trypsin were warmed to 37°C, 88% of macrophages ingested an average of 16 E(IgM)C each (Table IV, line 3). When trypsinized cultures were warmed, however, only 9% of macrophages ingested an average of two E(IgM)C each (Table IV, line 4). Trypsin treatment at 4°C did not cause elution of E(IgM)C from the macrophage surface; 81% of these macrophages had an average of 20 E(IgM)C each still bound to their plasma membranes. Moreover, the capacity of trypsin to inhibit the ingestion of E(IgM)C was clearly related to the enzymatic and not the physical properties of this enzyme. Trypsin which had been preincubated with OTI had no inhibitory effect upon the ingestion of E(IgM)C by activated macrophages (data not shown).

**Effects of Trypsin.** There are several ways in which trypsin could have blocked the ingestion of E(IgM)C. Trypsin, at concentrations of 500–1,000 μg/ml and at 37°C, destroys the C receptor activity of the macrophage plasma membrane (19, 20). In the experiments described in this paper, trypsin was used at a

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgM)C attached</th>
<th>Average no. of E(IgM)C attached per macrophage</th>
<th>Macrophages with E(IgM)C ingested</th>
<th>Average no. of E(IgM)C ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(IgM)C*–→, 4°C</td>
<td>98</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E(IgM)C*–→trypsin†</td>
<td>96</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E(IgM)C*–→37°C</td>
<td>10</td>
<td>10</td>
<td>88</td>
<td>16</td>
</tr>
<tr>
<td>E(IgM)C*–→trypsin†–→37°C</td>
<td>81</td>
<td>20</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>
concentration of 750 μg/ml, but at 4°C. Despite this difference in temperature, it was possible that trypsinization of E(IgM)C-macrophage complexes at 4°C removed the C receptors from the macrophage surface. If this were the case, the situation resulting from the trypsinization of E(IgM)C-macrophage complexes would be analogous to that described for E(IgG)-macrophage complexes treated with antimacrophage IgG, i.e., functional removal of specific receptors from portions of the plasma membrane which are not involved in the initial erythrocyte binding. Alternatively, trypsin could have destroyed erythrocyte-bound C3b completely or converted the C3b molecule to a form of C3 not recognized by the macrophage C receptor.

**Effect of trypsin on the macrophage C receptor.** Macrophages were trypsinized (750 μg/ml) at 4°C, washed with medium containing OTI and then with fresh medium, and incubated for 45 min at 37°C with E(IgM)C. Binding and ingestion of E(IgM)C occurred to the same degree as with macrophages not pretreated with trypsin under these conditions (data not shown). Thus, it appeared that the trypsinization procedure had no deleterious effect upon the phagocytic capacity of the macrophages or upon the functional activity of their C receptors.

**Effect of trypsin on E[IgM] C.** E(IgM)C were trypsinized at 4°C, washed with medium containing OTI and then with fresh medium, and incubated for 45 min at 37°C with nonactivated or with activated macrophages. Macrophages failed to bind or ingest these trypsinized E(IgM)C (Table V, lines 2 and 5). Thus, trypsinization, under the conditions used, had no effect on the macrophage C receptor but either destroyed erythrocyte-bound C3 or converted C3b to a form of C3 not recognized by the macrophage plasma membrane.

In order to distinguish between these two possibilities, trypsinized and control E(IgM)C were reacted with anti-C3 IgG and incubated with activated or nonactivated macrophages for 45 min at 37°C. The results are presented in Table V. Nonactivated macrophages, which do not ingest via the C receptor, ingested both trypsinized anti-C3 IgG-coated E(IgM)C and control anti-C3 IgG-coated E(IgM)C. As expected, activated macrophages failed to bind trypsinized E(IgM)C, but 75% of these macrophages ingested an average of six trypsin-treated, anti-C3 IgG-coated E(IgM)C each. Thus trypsinization of E(IgM)C at 4°C did not entirely degrade or remove erythrocyte-bound C3; the trypsin treatment converted C3b on the erythrocyte surface to a form of C3 which is inactive as a ligand for the macrophage C receptor but is still recognized immunologically by anti-C3 IgG.

These data suggest that, under the conditions employed, trypsin exerts its functionally significant effects on erythrocyte-bound C3b molecules and not on erythrocyte-bound IgM or on the macrophage surface. To confirm this suggestion, and to be certain that E(IgM)C-macrophage complexes exhibit the same trypsin sensitivity as E(IgM)C and macrophages trypsinized separately, E(IgM)C-macrophage complexes were trypsinized at 4°C, treated with anti-C3 IgG at 4°C or with a C source (C5-deficient mouse serum) at 37°C, and then incubated at 37°C. 49% of macrophages in cultures treated with anti-C3 IgG and 63% of the macrophages in cultures treated with fresh C5-deficient mouse serum ingested an average of five and six E(IgM)C each, respectively, as indicated in
TABLE V

Effect of Anti-C3 IgG on the Interaction of Trypsinized and Nontrypsinized E(IgM)C with Macrophages

<table>
<thead>
<tr>
<th>Physiologic state of macrophages</th>
<th>Treatment of E(IgM)C</th>
<th>Macrophages with E(IgM)C attached per macrophage</th>
<th>Average no. of E(IgM)C ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonactivated</td>
<td>None</td>
<td>85</td>
<td>14</td>
</tr>
<tr>
<td>Nonactivated</td>
<td>Trypsin</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Nonactivated</td>
<td>Anti-C3 IgG*</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Nonactivated</td>
<td>Trypsin, then</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>anti-C3 IgG*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated</td>
<td>Trypsin</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Activated</td>
<td>Trypsin, then</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>anti-C3 IgG*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Trypsinized (750 µg/ml, 30 min, 4°C) or nontrypsinized E(IgM)C were incubated for 15 min at 37°C with 10 µg/ml of rabbit antimouse C3 IgG. Control experiments showed that anti-C3 IgG did not promote binding or ingestion of E or E(IgM) by normal or by activated macrophages.

Table VI. The capacity of added mouse C to restore erythrophagocytic activity is especially significant and confirms that the trypsin treatment does not functionally alter the C-fixing capacity of erythrocyte-bound IgM, the C-binding capacity of E, or the binding and phagocytic activities of macrophage C3b receptors.

Effect of Trypsin on the E(IgM)C-MACROPHAGE-BINDING SITE. In order to be certain that E(IgM)C remained bound to the macrophage by the interaction of erythrocyte-bound C3b with the macrophage's C3b receptors in cultures subjected to trypsin treatment, trypsinized E(IgM)C-macrophage complexes were incubated at 37°C for 30 min with heat-inactivated C5-deficient mouse serum, a source of C3b inactivator. C3b inactivator is a heat-stable serum protein which cleaves C3b into two fragments, erythrocyte-bound C3d and fluid-phase C3c (24-26). Since the plasma membrane of the mouse peritoneal macrophage does not recognize C3d (9), treatment of these preparations with C3b inactivator should result in release of the erythrocytes from the surface of the macrophage. As shown in Table VII, the majority of bound E(IgM)C were eluted from macrophages by this procedure. These results indicate that, in trypsinized preparations, E(IgM)C remained bound to the macrophage surface by C3b-C3b receptor bonds.

While the above experiment demonstrated that E(IgM)C remained attached to macrophages by the macrophages' C3b receptors after trypsin treatment, it was possible that trypsin destroyed some C3b-C3b receptor bonds in the attachment zone. If this were the case, then the number of these bonds remaining could have been reduced to a level insufficient to trigger erythrocyte ingestion but still sufficient to maintain erythrocyte attachment to the macrophage surface. We reasoned that if there is some threshold number of C3b-C3b receptor interactions which is sufficient to mediate E(IgM)C binding but insufficient to trigger E(IgM)C ingestion, then we should be able to produce erythrocytes...
Anti-C3 IgG or Fresh Mouse Serum Promote Ingestion of Trypsinized E(IgM)C Previously Bound to Activated Macrophages

Cover slip cultures containing trypsinized (750 μg/ml, 4°C, 30 min) E(IgM)C-macrophage complexes were incubated for 15 min at 4°C in medium containing 10 μg/ml of rabbit antimouse C3 IgG. The cells were then washed, incubated in fresh medium at 37°C for 30 min, and then fixed and examined.

Cover slip cultures were trypsinized at 4°C as described above, washed, and then incubated at 37°C for 30 min in medium containing 30% fresh mouse serum.

**TABLE VI**

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgM)C attached</th>
<th>Average no. of E(IgM)C attached per macrophage</th>
<th>Macrophages with E(IgM)C ingested</th>
<th>Average no. of E(IgM)C ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→trypsin→37°C</td>
<td>89</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→trypsin→anti-C3 IgG→37°C</td>
<td>51</td>
<td>10</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→trypsin→37°C + fresh mouse serum</td>
<td>ND</td>
<td>ND</td>
<td>63</td>
<td>6</td>
</tr>
</tbody>
</table>

* Cover slip cultures containing trypsinized (750 μg/ml, 4°C, 30 min) E(IgM)C-macrophage complexes were incubated for 15 min at 4°C in medium containing 10 μg/ml of rabbit antimouse C3 IgG. The cells were then washed, incubated in fresh medium at 37°C for 30 min, and then fixed and examined.

**TABLE VII**

Heat-Inactivated Mouse Serum Causes Elution of E(IgM)C from the Macrophage Surface

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Macrophages with E(IgM)C attached</th>
<th>Average no. of E(IgM)C attached per macrophage</th>
<th>Macrophages with E(IgM)C ingested</th>
<th>Average no. of E(IgM)C ingested per macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→medium→37°C</td>
<td>15</td>
<td>18</td>
<td>75</td>
<td>14</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→trypsin→37°C</td>
<td>74</td>
<td>19</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>E(IgM)C, 4°C→trypsin→37°C + heat-inactivated mouse serum</td>
<td>15</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

* E(IgM)C-macrophage complexes were incubated for 30 min at 4°C with 750 μg/ml of trypsin, washed (4°C) twice with OTI, and twice with fresh medium.

† Cover slip cultures were trypsinized at 4°C as described above, washed, and then incubated at 37°C for 30 min in medium containing 30% heat-inactivated (56°C, 30 min) mouse serum, a source of C3b inactivator.

Macrophages Average no. Macrophages Average no.

Coated with limiting dilutions of C3b, which bind to the macrophage surface but are not ingested.

E(IgM) were incubated for 10 min at 37°C with various dilutions of C5-deficient mouse serum. These E(IgM)C, with different quantities of C3b on their surfaces, were then incubated at 4°C with activated macrophages. Cover slip cultures were washed to remove nonbound erythrocytes and then incubated for 45 min at 37°C. Attachment and ingestion were scored microscopically. As the
FIG. 2. Influence of the concentration of C5-deficient mouse serum used to prepare E(IgM)C on the interaction of E(IgM)C with activated macrophages. E(IgM) were incubated with various dilutions of C5-deficient mouse serum (abscissa). The resulting E(IgM)C were then incubated at 37°C for 45 min with activated macrophages. Attachment (○) and ingestion (□) were scored microscopically and are plotted as percent of maximum attachment or ingestion (ordinate).

concentration of mouse serum used to prepare E(IgM)C was diminished, both attachment and ingestion fell in parallel (Fig. 2). We could find no concentration of erythrocyte-bound C3b which mediated predominantly binding but not ingestion of E(IgM)C by macrophages. Therefore, disruption by trypsin of some C3b-C3b receptor bonds in the zone of attachment of E(IgM)C to macrophages is not sufficient to account for the failure of macrophages to ingest bound E(IgM)C.

Discussion

The phagocytosis of particulate materials by cells can be separated experimentally into two steps: (a) attachment of the particle to the cells' surface and (b) ingestion of the particle (22). The former occurs in the cold or in the absence of the generation of metabolic energy, while the latter is highly temperature dependent and requires active cellular metabolism. We have taken advantage of these differences to dissociate the attachment and ingestion phases of phagocytosis from one another. E(IgG) and E(IgM)C were attached to macrophages at 4°C. Macrophages incubated at this temperature remained viable and, upon warming to 37°C, exhibited unimpaired phagocytic capacities as measured by their ingestion of latex particles (data not presented), E(IgG) (Table I) and E(IgM)C (Table IV). Similar results were obtained when erythrocytes were attached to macrophages at 37°C in the presence of NaF. These erythrocytes were promptly ingested upon removal of this metabolic inhibitor. By these methods we have achieved a system which is highly synchronous, thereby facilitating analysis of the ingestion phase of phagocytosis.

"Trigger" vs. "Zipper" Mechanism of Phagocytosis. Previous studies (8, 9), have shown that the stimulus to phagocytize one particle does not prompt the ingestion of other particles attached to the macrophage surface. Rather, the
response of the macrophage plasma membrane to a phagocytic stimulus is confined to the segment of membrane immediately adjacent to the particle initiating that stimulus (8, 9). From the segmental nature of the response, we infer that the signal to phagocytize a particle is initiated by that particle and that, if any membrane-bound or soluble intracellular factors are activated by this signal, their effects are restricted to the segment of membrane adjacent to the particle. Implicit in this statement is the concept of a signal triggering ingestion of a membrane-bound particle. However, there is no compelling evidence that there is a signal which triggers ingestion. There is no minimum number of particles which must be bound before the macrophage begins to ingest the particles. Moreover, there is no size which distinguishes particles large enough to be ingested by macrophages from those too small to be phagocytized. Bacteria, latex particles 1.1–14 μm in diameter, and a variety of mammalian and avian erythrocytes are ingested by macrophages. These particles are contained within vacuoles, the sizes of which vary with the diameter of the particle being ingested, and the membranes of which are generally closely apposed to the entire surface of the phagocytized particle.

Thus the magnitude and quality of the response of the macrophage plasma membrane to a phagocytic stimulus appears to be governed by the number, size, and shape of the particles attached to it. The observed capacity of macrophages to ingest particles of a variety of sizes indicates that there is no "threshold" size or level of stimulation which a particle must achieve to initiate a phagocytic response. Rather, these data suggest that the macrophage plasma membrane is capable of a phagocytic response which is exactly proportional to the size and number of particles presented to it. In this way, as in the segmental nature of the membrane response, phagocytosis differs in a qualitative sense from the "all or none" response of neurons to an electrical stimulus (27). In the case of the neuron, propagation of an action potential is a quantitatively invariant cell-wide membrane response triggered by stimuli of greater than a given threshold in magnitude. Stimuli of less than this threshold value may alter the membrane potential but they do not trigger propagation of an action potential. In contrast, the macrophage plasma membrane responds segmentally and in exact proportion to the size of the phagocytic stimulus.

This capacity of the macrophage to modulate its response to a phagocytic stimulus suggested to us that the response of the cell’s plasma membrane is regulated directly by the particle being ingested and that it is not triggered by a specific stimulus of set magnitude. Rather, we considered that the step by step interaction of membrane receptors with particle-bound ligands may result in a zipper-like movement of the membrane around the particle. These two possible mechanisms, triggering and zippering, can be distinguished experimentally as illustrated in Figs. 3 and 4. A trigger mechanism assumes that once a given

---

4 We use the term trigger to mean a signal which, above a set threshold, precipitates a cellular response whose magnitude is independent of the signal's intensity.

5 We use the term zipper to describe ligand-mediated circumferential spreading of the plasma membrane around a particle. Although a zipper joins only edges to one another, we use this term here to convey the idea of sequential receptor-ligand interactions which lead to the close apposition of two surfaces.
FIG. 3. Mechanisms of phagocytosis of E(IgM): alterations of the particle surface. (a) Erythrocytes coated with IgM and C (i) bind to the macrophage plasma membrane via receptors (ii) for C3b. (b) C3b molecules (ligands) outside the attachment zone are cleaved by mild tryptic proteolysis. (c) Interaction of particle-bound ligands with membrane receptors triggers phagocytosis. (d) Receptors bind to ligands on the lower hemisphere of the particle, but closure of the phagocytic vacuole around the particle does not ensue because the upper hemisphere of the particle lacks ligands which can bind (zipper) to membrane receptors on the advancing pseudopods.

FIG. 4. Mechanisms of phagocytosis of E(IgG): alterations of cell membrane receptors. (a) IgG (γ) coated erythrocytes bind to the macrophage plasma membrane via receptors (i,ii) for the Fc portion of IgG. (b) Unoccupied membrane Fc receptors are blocked experimentally and the E(IgG)-macrophage complexes are incubated under conditions favorable for phagocytosis. (c) IgG binding to membrane receptors triggers phagocytosis. (d) Closure of the phagocytic vacuole does not occur because membrane receptors cannot attach to particle-bound IgG and the advancing membrane pseudopods cannot zipper together.

number of particle-bound ligands attaches to membrane receptors, a stimulus will be initiated which predestines the particle for ingestion. The zipper mechanism, on the other hand, suggests that phagocytosis will occur only if macrophage membrane receptors can bind to ligands throughout the surface of the particle.
If particles coated with appropriate ligands such as IgG or C3b, are ingested by a trigger mechanism initiated by their attachment to receptors on the macrophage plasma membrane, then they should be ingested even when ligands are removed from the nonattached surfaces of the particles (Fig. 3c) or when the receptors that are not involved in the initial binding process are blocked (Fig. 4c). If, on the other hand, ingestion of bound particles requires the stepwise binding of ligands to appropriate membrane receptors of macrophages, then ingestion should not proceed if ligands are removed from the nonattached surface of the particle (Fig. 3d) or if membrane receptors not involved in the initial attachment process are blocked (Fig. 4d). In a functional sense, we have constructed erythrocyte-macrophage complexes analogous to the situations depicted in Figs. 3b and 4b and find that ingestion of the particles behaves as predicted by the zipper mechanism.

Phagocytosis Mediated by the C Receptors. Phagocytosis of E(IgM)C by "activated" macrophages occurs independently of their Fc receptors and is mediated by C3b receptors present on the macrophage plasma membrane (23). E(IgM)C were bound to C3b receptors of activated macrophages at 4°C, and the E(IgM)C-macrophage complexes were incubated with trypsin at 4°C (Figs. 3a and b). From the data presented (Fig. 2, and Tables V and VII), it seems likely that the C3b molecules which bound the erythrocytes to the macrophage surface were protected from tryptic proteolysis by their interaction with the macrophage's C3b receptors. C3b molecules not so protected were cleaved to a form of C3 not recognized by the macrophage C receptors (Table VI). Thus trypsinization provided an efficient means of obtaining "partially opsonized" particles, i.e., erythrocytes bearing C3b ligands only on the portions of their surfaces which were attached to corresponding receptors on the macrophage surface. Although these trypsinized E(IgM)C retained functional ligands (C3b molecules) in the zone of attachment to the macrophage surface, they were not ingested when the macrophages were warmed to 37°C (Table IV). These findings support the zipper mechanism described in Fig. 3d.

Phagocytosis Mediated by the Fc Receptor. E(IgG)-macrophage complexes, formed at 4°C, were further incubated at this temperature with antimacrophage IgG to block Fc receptors. When these cultures were subsequently incubated at 37°C under conditions optimal for particle ingestion, phagocytosis of the IgG-coated erythrocytes did not occur. Failure of the bound E(IgG) to be ingested was not a result of inhibition of the cells' phagocytic capacity, but rather of specific Fc receptor blockade, for this antimacrophage IgG fraction does not block the processes of particle attachment and ingestion in general (13, 23). Moreover, F(ab')2 fragments of this same antimacrophage IgG did not inhibit the ingestion of E(IgG) (Table I).

Low temperature-induced alterations of the macrophage plasma membrane were not responsible for the failure of antimacrophage IgG-treated cells to ingest attached E(IgG), since similar results were obtained when E(IgG) binding and Fc receptor blockade were accomplished at 37°C in the presence of NaF (Table II). Under these conditions as well, macrophages whose Fc receptors had been blocked failed to ingest bound E(IgG), while those which had not been treated with antimacrophage IgG ingested bound E(IgG) normally.
The mechanism by which antimacrophage IgG inhibits Fc receptor-mediated phagocytosis was explored in detail. Antimacrophage IgG treatment of E(IgG)-macrophage complexes did not cause elution of the attached erythrocytes and did not alter the size or configuration of the E(IgG) attachment site as monitored by electron microscopy. These findings suggest that antimacrophage IgG acts by blocking Fc receptor sites outside the zone of initial interaction of E-bound IgG molecules with macrophage membrane Fc receptors; they are consistent with a zipper mechanism of phagocytosis as illustrated in Fig. 4d. However, additional experiments (Table III) showed that antimacrophage IgG blockade of Fc receptor function can be overcome by excess anti-E IgG. The quantity of anti-E IgG necessary to overcome the effects of antimacrophage IgG is 15-fold greater than the amount of anti-E IgG needed to promote erythrophagocytosis in macrophages which have not been treated with antimacrophage IgG (Fig. 1). Because of these quantitative considerations, we cannot rigorously exclude the possibility that antimacrophage IgG reduces the interaction of E-bound IgG with macrophage Fc receptors below a threshold necessary to trigger phagocytosis (Fig. 4c).

Recently, we have obtained additional evidence in support of the zipper mechanism of phagocytosis. Mouse B lymphocytes were incubated with anti-mouse immunoglobulin IgG at 4°C. These lymphocytes, with IgG coating their entire surfaces, were readily ingested when incubated with macrophage monolayers at 37°C for 30 min. Other preparations of IgG-coated lymphocytes were warmed to room temperature for 20 min to permit immunoglobulin capping before being incubated with macrophages. These capped lymphocytes, with anti-immunoglobulin IgG located on only one pole of the cells, bound to the macrophage plasma membrane via their immunoglobulin caps but were not ingested. Details of these experiments will be the subject of a future communication.6

Requirement for Metabolic Energy. Although the zipper hypothesis accounts for the selectivity and specificity of the phagocytic process, it does not directly explain its dependence upon metabolic energy. Presumably metabolic energy is needed to move the plasma membrane into apposition with the particle surface, to effect possible translational movements of receptors in the plane of the membrane, and to fuse the membranes of advancing pseudopods as they meet at the apex of the particle being ingested. The mechanisms by which the pseudopods are extended are not known, but roles for cytoplasmic contractile elements (actin and myosin) have been proposed (4–7). Indeed we have observed a massive zone of polymerized microfilaments which is confined to the portion of cytoplasm immediately subjacent to the particle being ingested.7 It seems likely, therefore, that cytoplasmic contractile elements provide the locomotive force for the advancing pseudopods. The interaction of membrane receptors with ligands on the surface of the particle determines the spacial orientation of these pseudopods and may regulate the polymerization of their underlying contractile elements.

The zipper mechanism is consistent with North's (28) suggestion that spreading of cells on surfaces reflects their attempt to phagocytize a particle of infinite diameter. Moreover, oriented cell movement, as in chemotaxis, also may be regulated by a zipper-like mechanism, as suggested by Carter (29, 30). Gradients of surface-bound, as opposed to fluid-phase, ligands may facilitate the movement of cell membrane pseudopods in the direction of increasing ligand density. The net result is the movement of the cell toward the region of greatest ligand density.

Summary

These experiments were designed to evaluate the role of macrophage plasma membrane receptors for the third component of complement (C) and for the Fc portion of IgG in the ingestion phase of phagocytosis. Sheep erythrocytes (E) were coated with anti-E IgG [E(IgG)]; these E(IgG) were then attached to cultivated monolayers of mouse peritoneal macrophages under conditions which reversibly inhibit ingestion of E(IgG). The E(IgG)-macrophage complexes were further incubated under similar conditions with an antimacrophage IgG fraction which blocks Fc receptor-mediated ingestion but has no effect upon ingestion mediated by other phagocytic receptors. When these cultures were subsequently incubated under conditions optimal for particle ingestion, phagocytosis of the IgG-coated erythrocytes did not occur; the erythrocytes remained bound to the Fc receptors of the macrophage plasma membrane.

To determine whether ligands must cover the entire surface of an attached particle to permit ingestion of that particle, C-coated E [E(IgM)C] were bound to the C receptors of thioglycollate-induced (activated) macrophages at 4°C. E(IgM)C-macrophage complexes were then trypsinized at 4°C, a procedure which resulted in cleavage of erythrocyte-bound C3b molecules to a form of C3 not recognized by the macrophage receptors for C3b. Under the conditions used, trypsin did not affect the attachment of E(IgM)C to the macrophage surface or the macrophage receptors for C3b. When these trypsin treated E(IgM)C-macrophage complexes were incubated at 37°C, the bound E(IgM)C were not ingested; the erythrocytes remained attached to the macrophage plasma membrane via the macrophage's C receptors.

These results indicate that attachment of a particle to specific receptors on the macrophage plasma membrane is not sufficient to trigger ingestion of that particle. Rather, ingestion requires the sequential, circumferential interaction of particle-bound ligands with specific plasma membrane receptors not involved in the initial attachment process.

We are grateful to Doctors Celso Bianco, Zanvil Cohn, and James Hirsch for many stimulating discussions and for critical readings of this manuscript.

Received for publication 28 July 1975.

References


