

STUDIES ON THE MECHANISM OF PHAGOCYTOSIS  
II. The Interaction of Macrophages with Anti-Immunoglobulin  
IgG-Coated Bone Marrow-Derived Lymphocytes\*

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Phagocytic leukocytes have on their plasma membranes receptors for the Fc portion of immunoglobulin G (IgG) (1-9) and for a cleavage product of the third component of complement (C3) (3-5, 8, 10-15). Particles coated with IgG or C3 attach to the corresponding receptors on the plasma membranes of phagocytic cells. We have been studying the roles of these ligands (IgG and C3) and of their receptors in the ingestion phase of phagocytosis. We reported previously (16) that removal of ligands or blockade of receptors lying outside the zone of attachment of immunologically coated erythrocytes to the plasma membranes of mouse peritoneal macrophages prevented the ingestion of these erythrocytes. These findings led us to propose that the ingestion of a particle requires the sequential, circumferential interaction of phagocytic receptors with particle-bound ligands not involved in the initial attachment of the particle to the phagocytic cell. We termed this process the "zipper" mechanism of phagocytosis (16).

In our previous experiments, we altered ligands on the erythrocytes or blocked receptors on the macrophages. Thus, it was conceivable that our experimental procedures reduced the number of ligand-receptor bonds in the zone of attachment of the erythrocytes to the macrophages below some critical level necessary to trigger ingestion of the erythrocytes. Had this occurred, our interpretation of those experiments would be open to serious doubt. Several lines of evidence suggested that this possibility was unlikely; nevertheless, it could not be excluded. The experiments described in the present report were designed to eliminate these quantitative ambiguities and thereby to test rigorously the validity of the zipper mechanism.

In the present study we have used as phagocytic test particles bone marrow derived lymphocytes. These cells bear immunoglobulin molecules on their plasma membranes. Incubation of these lymphocytes at 25°C with anti-immunoglobulin IgG promotes capping; that is, the redistribution of nearly all surface immunoglobulin molecules from their random distribution on the lymphocyte's plasma membrane to only one arc of its circumference. We found that when

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these lymphocytes were diffusely coated with anti-immunoglobulin IgG, they were ingested by macrophages; but when the same number of anti-immunoglobulin molecules were redistributed to only one pole of the cell, the lymphocytes remained bound to the macrophages' Fc receptors but were not ingested. These findings provide unambiguous proof of the zipper mechanism of phagocytosis (16).

In addition, we have examined the fate of the immune complex caps on the lymphocyte surface and found that the macrophages phagocytize these complexes without destroying the lymphocytes. This capacity of macrophages to remove cell surface antigen-antibody complexes without destroying the cell to which they are attached may be important in understanding the effects of antigens and antibodies on cells participating in a humoral immune response, in identifying the mechanisms by which chronic viral infections are established, and in defining the roles of blocking antibodies in tumor immunology.

### Materials and Methods

*Reagents and Media.* Horse radish peroxidase (HRP)<sup>1</sup> (type II, Sigma Chemical Co., St. Louis, Mo.); trypsin (lot no. T8253, twice recrystallized, Sigma Chemical Co.); ovomucoid trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.); 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.); fetal bovine serum (FBS; Grand Island Biological Co., Grand Island, N. Y.); Medium 199 (Microbiological Associates, Bethesda, Md.); polystyrene latex beads, 1.1- $\mu$  diameter (Dow Chemical U. S. A., Midland, Mich.); antish sheep erythrocyte 7S antibodies (Cordis Laboratories, Miami, Fla.); glutaraldehyde, 50% aqueous solution (Fisher Scientific Co., Pittsburgh, Pa.); rabbit antimouse lymphocyte antiserum (lot no. 15070, Microbiological Associates); and 30% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific Co.) were obtained from the manufacturers as indicated. FBS and antilymphocyte antiserum were de complemented by heating at 56°C for 30 min before use.

Fluorescein conjugated rabbit IgG prepared from the serum of a rabbit immunized with mouse IgG was obtained from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. This IgG fraction, by virtue of its activity against  $\kappa$  and  $\lambda$  chains, is not immunoglobulin class specific and is therefore an anti-immunoglobulin IgG. It is designated Fl-anti-Ig IgG. The molar ratio of fluorescein to protein was 4:1; protein concentration was 20 mg/ml.

*Animals.* 20-30 g female mice were obtained from the outbred Swiss colony maintained at The Rockefeller University, New York, or from Charles River Breeding Laboratories, Inc., Wilmington, Mass. (strain CD-1) and served as sources of peritoneal macrophages and of mesenteric lymph node lymphocytes.

*Macrophages.* Mouse peritoneal macrophages were harvested by peritoneal lavage and cultivated on 13-mm diameter glass cover slips in 35-mm plastic Petri dishes with medium<sup>2</sup> containing 20% heat-de complemented FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, as previously described (16, 17). Macrophages used in experiments in which electron microscopy was performed were cultivated in 35-mm plastic Petri dishes without cover slips. All cultures were incubated in an atmosphere of 95% air-5% CO<sub>2</sub> at 100% relative humidity at 37°C for 24 h before use in experiments, nonadherent cells having been removed by washing after the 1st h of incubation.

*Lymphocytes.* Mesenteric lymph nodes were dissected from mice and placed immediately into ice-cold medium. The nodes were teased apart with forceps in fresh, cold medium, and heavier fragments permitted to settle at 1 g. Cells in the supernate were centrifuged at 150 *g* for 10 min at 4°C and resuspended in ice-cold medium at a concentration of  $4 \times 10^7$  cells per ml. More than 98%

<sup>1</sup> Abbreviations used in this paper: DAB, 3,3' diaminobenzidine tetrahydrochloride; E(IgG), sheep erythrocytes coated with rabbit antish sheep erythrocyte IgG; Fl-anti-Ig IgG, fluorescein-conjugated rabbit IgG directed against mouse IgG; FBS, fetal bovine serum; HRP, horse radish peroxidase; HRP-SAR, SAR-IgG coupled to HRP; PBS, phosphate-buffered saline; PD, phosphate-buffered saline without Ca<sup>++</sup> and Mg<sup>++</sup> ions; SAR-IgG, sheep IgG directed against F(ab')<sub>2</sub> fragments of rabbit IgG.

<sup>2</sup> The term "medium" as used throughout this paper refers to Medium 199.

of the cells were lymphocytes, as determined by morphology and inability to phagocytize polystyrene latex beads; greater than 95% of the cells were viable, as determined by trypan blue dye exclusion.

*Rabbit Anti-Mouse Macrophage IgG.* The IgG fraction of serum from a rabbit immunized with mouse peritoneal macrophages in complete Freund's adjuvant was prepared and isolated as previously described (18, 19).

*Sheep IgG Directed Against Rabbit F(ab')<sub>2</sub> (SAR-IgG).* The IgG fraction of serum from a sheep repeatedly immunized with the F(ab')<sub>2</sub> fragment of rabbit IgG was prepared and purified by immunoselective column chromatography as previously described (19). Immunoselection was performed using rabbit IgG coupled to Sepharose 2B by CNBr, as described by March et al. (20). The immunoselected product was released with 3 M NaSCN in 0.05 M NaPO<sub>4</sub> buffer, pH 6.15, concentrated by vacuum dialysis, and dialyzed against PD [solution "a" of Dulbecco's PBS (21)].

*HRP-Labeled SAR-IgG (HRP-SAR).* HRP was coupled to SAR-IgG, using the two-step procedure of Avrameas et al. (22). 15 mg HRP was incubated for 18 h at 25°C in 0.2 ml of 0.1 M NaPO<sub>4</sub> buffer, pH 6.8, containing 1.25% glutaraldehyde. Peroxidase was separated from nonreacted glutaraldehyde on a 2.5 × 30-cm Sephadex G25 column using 0.15 M NaCl as the eluant. The peroxidase-containing fractions were pooled and concentrated by vacuum dialysis to a volume of 1 ml. 5 mg SAR-IgG in 1 ml of 0.15 M NaCl and 0.1 ml of 1 M carbonate buffer, pH 9.5, was added, and the solution allowed to stand for 24 h at 4°C. 0.1 ml of 0.2 M lysine was added to "quench" nonreacted aldehyde groups, and the solution was permitted to stand at 4°C for 2 h, after which it was concentrated by vacuum dialysis to a protein concentration of 4 mg/ml, dialyzed against PD, and stored at 4°C until used.

*Light and Electron Microscopic Histochemistry.* Peroxidase was visualized by incubating glutaraldehyde-fixed preparations at 25°C for 15 min with 0.05% DAB in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% H<sub>2</sub>O<sub>2</sub>, as described by Graham and Karnovsky (23). For electron microscopy, the cultures were post-fixed in 1% osmium tetroxide (24), stained with 0.5% uranyl acetate, dehydrated with ethyl alcohol, embedded in Epon (25), and examined in a Siemens Elmiskop 1A (Siemens Corp., Iselin, N. J.), as described (19).

*Sheep Erythrocytes Coated with IgG.* Sheep erythrocytes were coated with antisheep erythrocyte IgG, and these erythrocytes [E(IgG)] were used in experiments as previously described (13).

*Preparation of IgG-Coated Lymphocytes.* 4 × 10<sup>7</sup> mouse mesenteric lymph node lymphocytes were incubated with 4 mg Fl-anti-Ig IgG in 1 ml medium for 30 min at 4°C. Some suspensions were subsequently incubated for 30 min at 25°C to permit Fl-anti-Ig IgG mediated capping of the surface immunoglobulin of the lymphocytes. All preparations were washed and resuspended in cold medium at a concentration of 4 × 10<sup>7</sup> lymphocytes per ml.

*HRP-SAR Labeling of Anti-Immunoglobulin IgG on the Lymphocyte Surface.* Preparations of both capped and diffusely coated lymphocytes, prepared as described above, were incubated at a concentration of 4 × 10<sup>7</sup> cells per ml with 400 μg/ml HRP-SAR for 30 min at 4°C. Pelleted lymphocytes were washed and resuspended in medium at a concentration of 4 × 10<sup>7</sup> cells per ml, all at 4°C.

*Phase Contrast Microscopy.* Cover slip cultures were washed once with fresh medium, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and examined by phase contrast microscopy with a ×100 objective under oil, as previously described (19).

*Fluorescence Microscopy.* Cover slip cultures were fixed with glutaraldehyde as described above and examined with a Leitz Ortholux microscope (E. Leitz, Inc., Rockleigh, N. J.) using a darkfield condenser and a mercury 200 W/Z lamp. At least 100 macrophages on each cover slip were evaluated.

*Miscellaneous.* Protein concentrations were determined by the method of Lowry et al. (26), using bovine serum albumin as a standard. Cell viability was judged by incubating cultures for 10 min at 25°C in 0.15 M NaCl containing 0.4% trypan blue dye and determining the percentage of cells excluding the dye.

## Results

*Reaction of Lymph Node Lymphocytes with Antibodies to Mouse Immunoglobulin.* To determine the percentage of mouse mesenteric lymph node lymphocytes which bind anti-Ig IgG and to evaluate the distribution of this anti-

immunoglobulin on the surface of these cells under various incubation conditions, suspensions of lymphocytes were mixed with Fl-anti-Ig IgG for 30 min at 4°C. The cells were then washed to remove noncell bound antibodies. Some preparations were further incubated for 30 min at 25°C. The cells were fixed and examined by fluorescence microscopy.

As previously found by Takahashi et al. (27), approximately 40% of mesenteric lymph node lymphocytes showed membrane fluorescence. Lymphocytes which had been labeled with Fl-anti-Ig IgG at 4°C demonstrated diffuse, circumferential fluorescence (Table I, line 1). After incubation at 25°C, 85% of these stained lymphocytes showed capping of the Fl-anti-Ig IgG (Table I, line 2).

The distribution of anti-Ig IgG on the lymphocyte surface was also determined by phase contrast and electron microscopy, using peroxidase-coupled antibodies. Lymphocytes were incubated with anti-Ig IgG as above; some preparations were warmed at 25°C to permit capping. Both capped and diffusely coated lymphocyte preparations were then incubated for 1 h at 4°C with HRP-SAR. Lymphocyte suspensions were then fixed, stained with DAB, and examined by phase contrast and electron microscopy. Approximately 40% of lymphocytes showed the brown membrane staining characteristic of the peroxidase reaction product. All labeled lymphocytes in preparations maintained at 4°C were diffusely stained (Table I, line 3), while 85% of lymphocytes which had been incubated at 25°C before labeling with HRP-SAR had peroxidase reaction product only at one pole of their surfaces (Table II, line 4). Throughout this paper preparations of lymphocytes which were incubated with anti-Ig IgG at 4°C will be referred to as diffusely coated lymphocytes, while those which were warmed at 25°C after incubation with anti-Ig IgG will be referred to as capped lymphocytes.

*Distribution of Anti-Ig IgG on the Lymphocyte Surface Determines Whether or Not Lymphocytes are Phagocytized by Macrophages.* Monolayers of mouse peritoneal macrophages were incubated at 4°C with untreated control lymphocytes or diffusely coated or capped lymphocytes. The cultures were maintained at 4°C for 10 min to allow the anti-Ig IgG-coated lymphocytes to settle and to adsorb to the macrophages. They were then incubated at 37°C for 30 min to allow further interactions of the lymphocytes with the macrophages, washed to remove lymphocytes which had not attached to the macrophages, fixed, and examined by phase contrast microscopy.

Lymphocytes which had not been treated with anti-Ig IgG were neither bound nor ingested to a significant degree by macrophages (Table II, line 1). Thus, although no attempt was made to separate lymphocytes bearing anti-immunoglobulin from other lymphocytes in the preparation, the macrophages performed the task efficiently. Diffusely coated lymphocytes were both bound to and ingested by the macrophages (Fig. 1*b* and Table II, line 2); over 99% of the macrophages bound an average of 10.3 lymphocytes each, and 71% of the macrophages ingested an average of 2.2 lymphocytes each. Capped lymphocytes, on the other hand, were avidly bound by macrophages (Fig. 1*a* and Table II, line 3); over 99% of the macrophages bound an average of 8.3 lymphocytes each, but only 5% of macrophages ingested any lymphocytes. These results demonstrate that both capped and diffusely coated lymphocytes can bind to macrophages, while only diffusely coated lymphocytes can be ingested; they suggest that both attachment and ingestion of lymphocytes are mediated by the interaction of the

TABLE I  
*Reaction of Mouse Lymph Node Lymphocytes with Antibodies to Mouse Immunoglobulin*

Incubation temperature of lymphocytes with Fl-anti-Ig IgG	Subsequent incubation with HRP-SAR	Lymphocytes stained	Distribution of stain on lymphocytes*	
			Diffuse	Capped
		%	%	%
4°C‡	No	38	>99	<1
4°C‡ → 25°C§	No	40	15	85
4°C‡	Yes	40	>99	<1
4°C‡ → 25°C§	Yes	41	15	85

\* Only stained lymphocytes are included.

‡ Mouse mesenteric lymph node lymphocytes were incubated with Fl-anti-Ig IgG for 30 min at 4°C as described in the Materials and Methods. The lymphocytes were washed and resuspended in medium at 4°C, and used at a concentration of  $4 \times 10^7$  cells/ml.

§ Subsequently incubated at 25°C for 30 min.

|| Fl-anti-Ig IgG-treated lymphocytes were subsequently incubated at a concentration of  $4 \times 10^7$  cells/ml with 400  $\mu$ g/ml of HRP-SAR for 1 h at 4°C.

Fc portion of anti-Ig IgG with Fc receptors of the macrophage plasma membrane.

To confirm that both capped and diffusely coated lymphocytes bind to the macrophage's surface via the latter's Fc receptors, we took advantage of the capacity of antimacrophage IgG to selectively block these receptors. Holland and co-workers (18) first described the effects of antimacrophage IgG on Fc-receptor function, and our findings have been essentially the same as theirs (13). Macrophages treated with antimacrophage IgG neither bind nor ingest E(IgG) (13, 18), but do ingest latex particles (18) and formaldehyde-treated sheep erythrocytes (18); furthermore, the complement receptor binding and phagocytic functions of antimacrophage IgG-treated macrophages are not impaired by this immunoglobulin preparation (13).

Macrophage cultures were incubated at 37°C in medium containing antimacrophage IgG. The cultures were then washed, incubated at 37°C with suspensions of either capped or diffusely coated anti-Ig IgG-treated lymphocytes, washed again to remove nonattached lymphocytes, fixed, and examined by phase contrast microscopy. Macrophages which had been pretreated with antimacrophage IgG, and which therefore had their Fc receptors blocked, neither bound nor ingested capped or diffusely coated lymphocytes (Table II, lines 4 and 5). Thus, both the attachment and the subsequent ingestion of anti-Ig IgG-treated lymphocytes by macrophages are mediated by the binding of the Fc portion of anti-Ig IgG to Fc receptors on the macrophage plasma membrane.

*Can Capped Lymphocytes Be Ingested by Macrophages?* It was possible that capping rendered lymphocytes noningestible under any circumstances. Alternatively, antigen-antibody complexes present on the lymphocyte cap might have altered the distribution or functional activity of the macrophage's Fc receptors so that the macrophage could not ingest these IgG-coated lymphocytes. To examine these possibilities, capped lymphocytes were incubated with macrophages for 10 min at 4°C, then for 30 min at 37°C. An average of eight lymphocytes was bound per macrophage. Rabbit antimouse lymphocyte antise-

TABLE II  
*Effect of the Distribution of Anti-Ig IgG on the Lymphocyte Surface on the Interaction of Lymphocytes with Macrophages*

Pretreatment of lymphocytes	Pretreatment of macrophages	Macrophages with attached lymphocytes	Lymphocytes attached per macrophage (Avg. no.)	Macrophages with ingested lymphocytes	Lymphocytes ingested per macrophage (Avg. no.)
		%		%	
None	None	18	1.2	3	1
Anti-Ig IgG 4°C*	None	>99	10.3	71	2.2‡
Anti-Ig IgG 4°C* → 25°C§	None	>99	8.3	5	1
Anti-Ig IgG 4°C*	Antimacrophage IgG	9	2.3	1	1
Anti-Ig IgG 4°C* → 25°C§	Antimacrophage IgG	4	1	0	0

Monolayers of 24-h explanted mouse peritoneal macrophages were washed twice with medium and incubated in medium at 4°C or 37°C as described in the footnotes below. Each value is the average of three separate experiments.

\* Mouse mesenteric lymph node lymphocytes were incubated with F1-anti-Ig IgG for 30 min at 4°C as described in the Materials and Methods. The lymphocytes were washed and resuspended in medium at 4°C.  $4 \times 10^7$  of these cells were incubated with macrophage monolayers for 10 min at 4°C.

‡ The number of these lymphocytes ingested per macrophage is substantially lower than the number of IgG-coated erythrocytes ingested under similar conditions. Microscopic examination of these preparations revealed that nearly all lymphocytes which remained attached to macrophages had capped at the site of attachment. Many of the lymphocytes which were diffusely coated with anti-immunoglobulin IgG when attached to macrophages at 4°C probably capped at the beginning of the 37°C incubation period and were therefore not ingested.

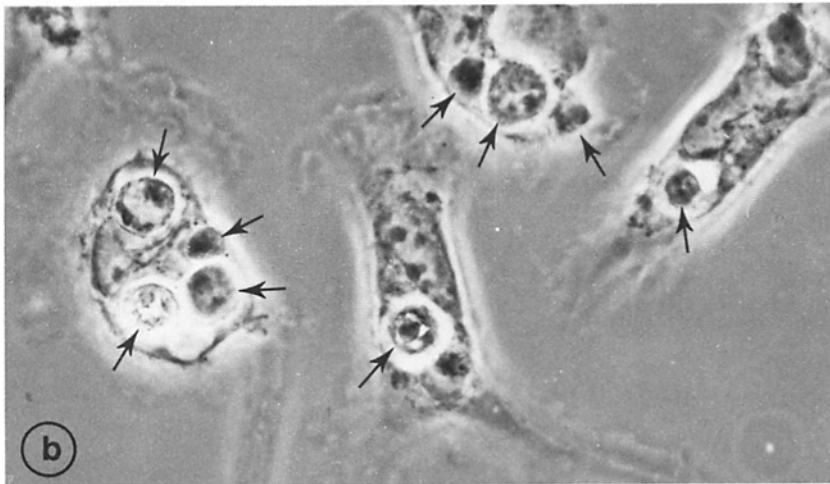
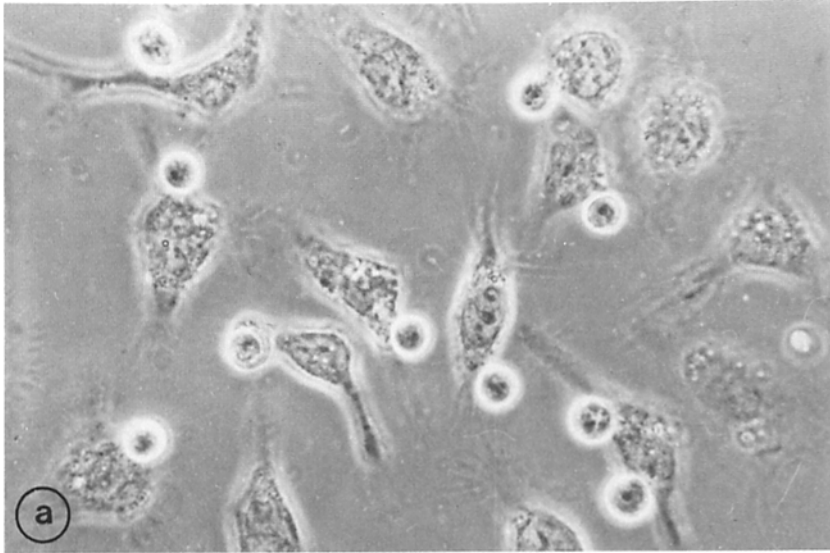
§ Subsequently incubated at 25°C for 30 min.

|| Macrophage monolayers were preincubated for 1 h at 37°C in medium containing 340 µg/ml of antimacrophage IgG.

rum (1:10 dilution in medium) was added to the cultures, and the incubation was continued for 30 min at 37°C. The cultures were then washed, fixed, and examined by phase contrast microscopy. 70% of macrophages in cultures, to which antilymphocyte antiserum had been added, ingested an average of two capped lymphocytes each (Fig. 1c). Macrophages in control cultures, treated exactly as in the experimental cultures except that antilymphocyte antiserum was not present in the final 30 min of incubation, did not ingest capped lymphocytes.

Two conclusions may be drawn from these results: (a) the alterations produced in the lymphocyte surface by the capping reaction do not render these lymphocytes noningestible per se; (b) the interaction of capped lymphocytes with the macrophage surface does not paralyze the segment of macrophage plasma membrane to which these lymphocytes are attached.

*Does the Interaction of Macrophages with Capped Lymphocytes Alter the Functional Activity of Macrophage Fc Receptors Lying Outside the Zone of Lymphocyte Attachment?* In previous work we reported that blockade of Fc



receptors outside the zone of attachment of IgG-coated erythrocytes to macrophages inhibited the ingestion of these erythrocytes (16). These findings led us to suggest that ingestion of a particle requires the participation of macrophage plasma membrane receptors lying outside the zone of particle attachment. At about the same time, Rabinovitch et al. (28) reported that macrophages plated on antigen-antibody complexes bound but did not ingest IgG-coated erythrocytes. Their findings indicated that the interaction of macrophages with immune complexes at one site on the cells' plasma membranes can alter the functional activity of Fc receptors lying elsewhere on the macrophage surface. Thus it seemed possible that antigen-antibody complexes in the lymphocyte cap might have caused a redistribution of Fc receptors in the macrophage plasma membrane, leading to the removal of these receptors from segments of the macrophage plasma membrane lying outside the zone of lymphocyte attachment.

To examine this possibility, we attached capped lymphocytes to macrophages as described in Table II, line 3. We then incubated these lymphocyte-macrophage complexes with E(IgG) at 37°C for 30 min. The preparations were washed, fixed, and examined by phase contrast microscopy. 62% of macrophages with capped lymphocytes attached ingested an average of seven E(IgG) each. The capped lymphocytes remained attached to the macrophage plasma membrane and were not ingested. Thus segments of the macrophage plasma membrane not in contact with the capped lymphocytes retain Fc receptors which are functionally intact.

*Visualization of Anti-Ig IgG on Bound and Ingested Lymphocytes.* Implicit in our discussion to this point has been the assumption that capped lymphocytes are bound to the macrophage surface via the region of the anti-immunoglobulin cap. To test this assumption, we used fluorescein and peroxidase-labeled antibodies to visualize the location of these ligands on capped lymphocytes which had been bound to macrophages and on diffusely coated lymphocytes which had been ingested by macrophages.

Lymphocytes were incubated with F1-anti-Ig IgG at 4°C as previously described; some preparations were then permitted to cap by incubating them at 25°C. Where indicated, the diffusely coated or capped lymphocytes were further incubated for 1 h at 4°C with HRP-SAR. The lymphocytes were then incubated with macrophages for 10 min at 4°C, followed by 30 min at 37°C. The cultures were fixed, stained as appropriate, and examined by phase contrast, fluorescence, and electron microscopy. To avoid sampling errors in experiments which

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FIG. 1. Phase contrast micrographs of whole mounts of macrophages incubated with antibody-coated mouse lymphocytes. (a) Lymphocytes were incubated for 30 min at 4°C with anti-Ig IgG and further incubated for 30 min at 25°C. These capped lymphocytes were then incubated with macrophages for 10 min at 4°C and for an additional 30 min at 37°C. Lymphocytes that were not attached to macrophages were removed by washing. Most of the macrophages have lymphocytes attached to their plasma membranes.  $\times \sim 500$ . (b) Lymphocytes were incubated for 30 min at 4°C with anti-Ig IgG. These diffusely coated lymphocytes were then incubated with macrophages as described in Fig. 1a. Most of the macrophages have ingested one or more lymphocytes (arrows).  $\times 1,000$ . (c) Capped lymphocyte-macrophage complexes, prepared as in Fig. 1a, were incubated at 37°C with antimouse lymphocyte serum. Many of the attached lymphocytes have been ingested (arrows).  $\times 1,200$ .



were studied by electron microscopy, parallel cultures were studied by phase contrast microscopy.

By phase contrast microscopy, capped lymphocytes appeared to be bound to macrophages by the DAB-stained, HRP-SAR-labeled cap, while all ingested lymphocytes whose staining could be evaluated appeared diffusely stained (Fig. 6*b*). Similar results were obtained when the interaction of Fl-anti-Ig IgG labeled lymphocytes with macrophages was examined by fluorescence microscopy.

By electron microscopy the DAB-stained, HRP-SAR-labeled cap was easily visualized. The macrophage plasma membrane adhered to the lymphocyte surface only in the region of the cap and did not advance around the lymphocyte beyond the region of the cap (Figs. 2-4). Occasionally, lymphocytes were found closely apposed to macrophages in the absence of DAB staining. Serial sections through one of these areas showed that DAB stain was present in some portions of the attachment zone (Fig. 5*a-d*). Thus, failure to find DAB-stained material in selected thin sections does not reflect absence of an HRP-labeled anti-immunoglobulin cap binding the lymphocyte to the macrophage.

We noted considerable variation (300-2,500 Å) in the apparent thickness of the DAB-stained material in the space between a macrophage and its attached lymphocyte. However, in sections in which DAB staining was minimal, the contact zone extended for several microns, and the plasma membranes of the macrophage and the lymphocyte were closely and uniformly apposed throughout the zone and were separated by a distance of approximately 100-200 Å. These anatomical features were identical to those noted in the contact zone between IgG-coated erythrocytes and macrophages (Fig. 7).

Electron microscopy confirmed that lymphocytes which were ingested were diffusely stained (Fig. 6). These lymphocytes were rapidly degraded within phagocytic vacuoles, and the distribution of DAB stain after intracellular degradation of these lymphocytes varied from diffuse to particulate within the vacuolar system.

These results demonstrate directly that capped lymphocytes are bound to the macrophage plasma membrane by the immunoglobulin cap and that only the diffusely coated lymphocytes are ingested.

*Evaluation of Fluorescein and DAB-Stained Material within Macrophages.* Vacuoles within both the capped lymphocytes and the macrophages (Figs. 2-4) appear to contain peroxidase-labeled antibodies derived from lymphocyte caps. Ingestion of anti-immunoglobulin caps by lymphocytes has been described previously (29). In contrast, the removal of antibodies directed against cell surface antigens by macrophages has not been observed previously and is of considerable interest.

Examination of a large number of thin sections by electron microscopy showed that most of the DAB-stained material is within vacuoles in the macrophage cytoplasm, and not on invaginations of the macrophage plasma membranes (cf. Fig. 2 with Figs. 3 and 4). Although these results confirm the intracellular location of the fluorescein and peroxidase-labeled antibodies, they do not provide information regarding the mechanism by which these labeled antibodies were removed from the surfaces of the capped lymphocytes and ingested by the macrophages. Portions of the lymphocyte cap may have been shed into medium

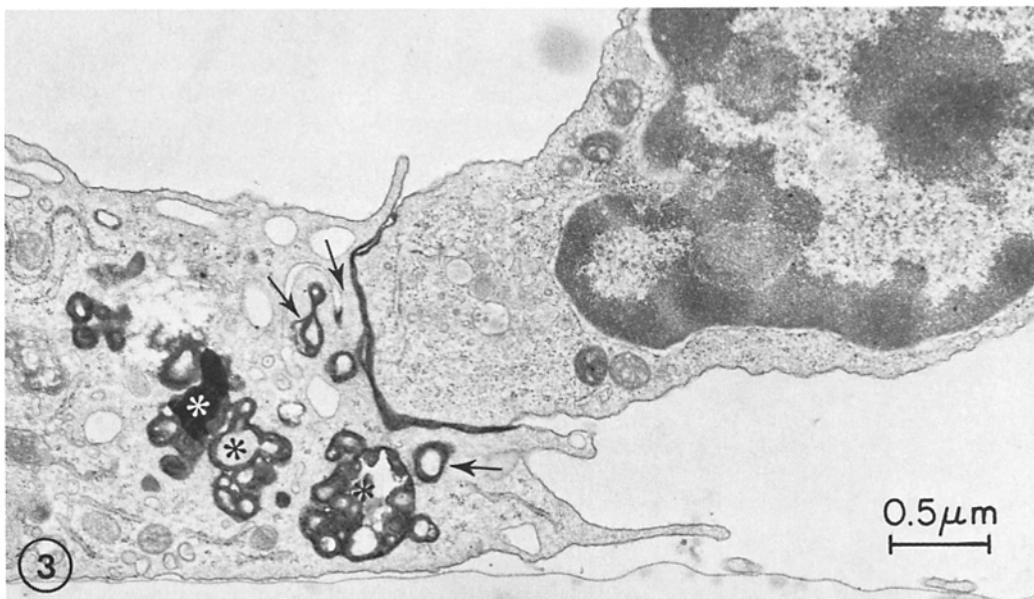
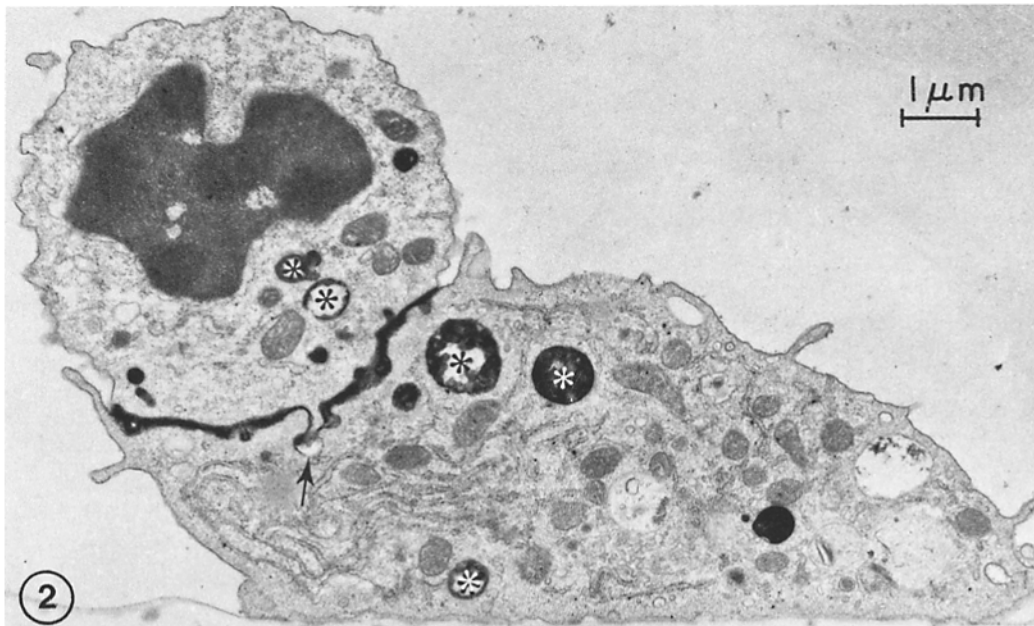


FIG. 2. Capped lymphocytes were incubated at 4°C with HRP-SAR, washed, and further incubated with macrophages at 37°C as described in the text. The plasma membrane of the macrophage adheres to the lymphocyte surface only in the region of the DAB-stained cap. Both the macrophage and the lymphocyte have cytoplasmic vacuoles containing peroxidase-labeled material (asterisks). Note the DAB-stained invagination of the macrophage plasma membrane which appears to be forming directly below the center of the lymphocyte cap.

FIG. 3. Lymphocyte-macrophage complexes were formed and incubated as in Fig. 2. The lymphocyte is bound to the macrophage plasma membrane only in the region of the lymphocyte's DAB-stained uropod. Peroxidase-labeled antibodies are also seen in vacuoles in the macrophage cytoplasm (asterisks) and on tubular invaginations (arrows) of the macrophage plasma membrane. Rough endoplasmic reticulum and mitochondria are excluded from the portion of macrophage cytoplasm adjacent to the capped lymphocyte. This part of the macrophage contains many randomly arranged microfilaments.

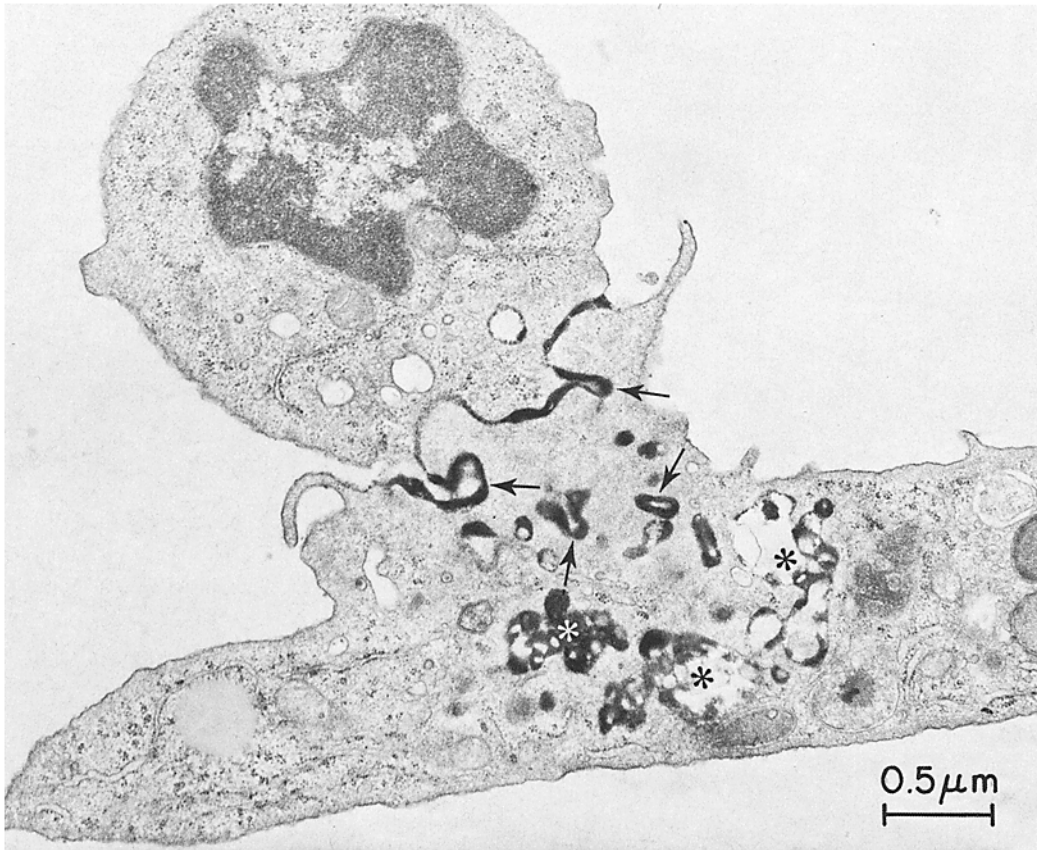


FIG. 4. Lymphocyte-macrophage complexes were formed and incubated at 37°C as in Fig. 2. In this section there is a massive accumulation of randomly arranged microfilaments in the macrophage cytoplasm beneath the attached lymphocyte. These microfilaments form a continuous band which extends from the segment of plasma membrane in contact with the DAB-stained lymphocyte cap to the portion of macrophage plasma membrane in contact with the surface of the culture dish. As in Fig. 3, rough endoplasmic reticulum and mitochondria are almost entirely excluded from this part of the macrophage cytoplasm. DAB-stained material is present within vacuoles (asterisks) of the macrophage, and on the tubular invaginations (arrows) of the macrophage plasma membrane.

from the lymphocyte surface and subsequently ingested by the macrophages. To examine this possibility we performed two different types of experiments:

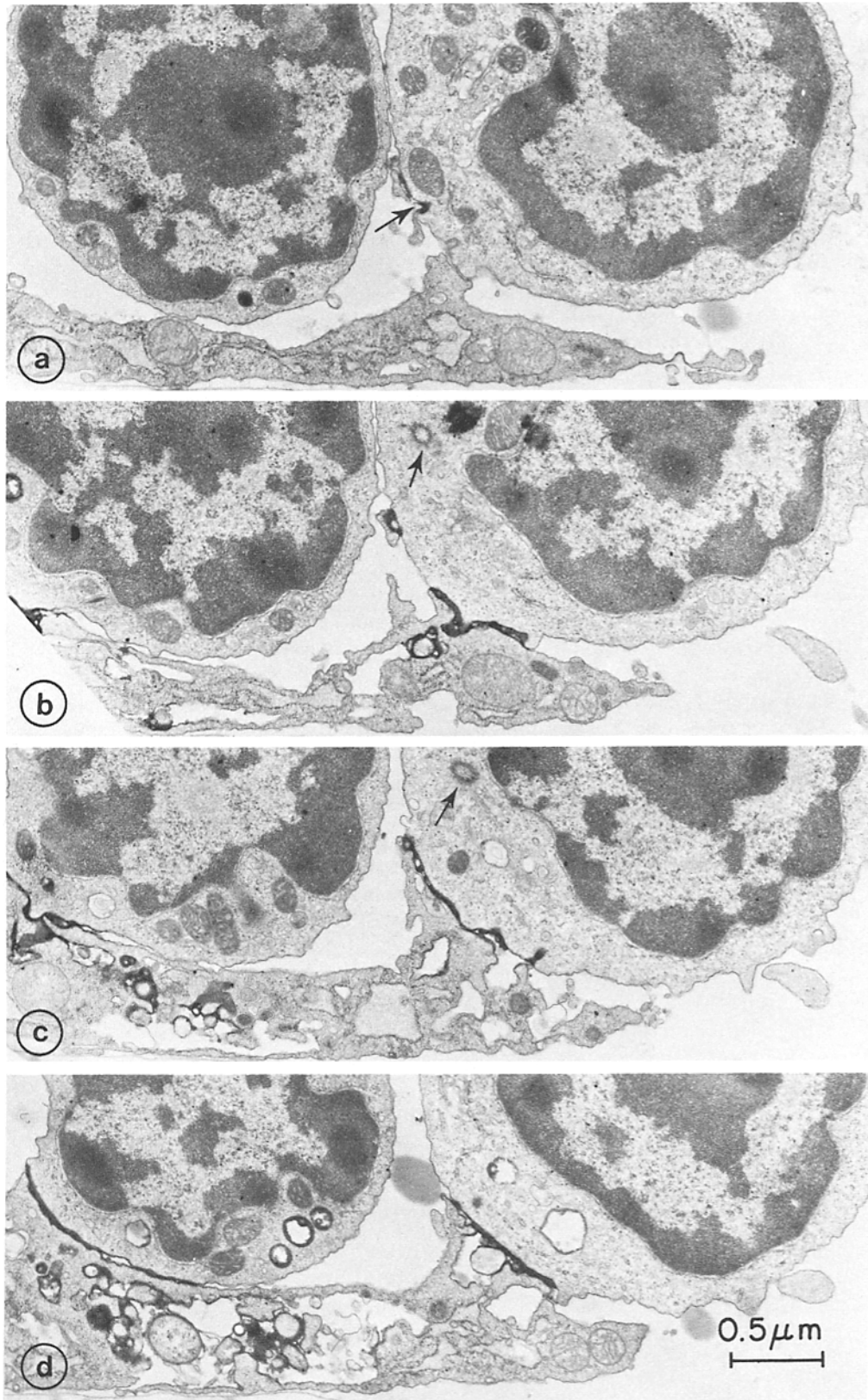
I. Lymphocytes were capped with F1-anti-Ig IgG, and some were further incubated for 1 h at 4°C with HRP-SAR. The cells were then washed to remove noncell associated antibodies, incubated in medium for 30 min at 37°C to allow possible shedding of surface-bound immune complexes, and pelleted by centrifugation at 150 *g* for 10 min. The medium was carefully removed and incubated with macrophages for 30 min at 37°C, after which the macrophages were washed, fixed, stained with DAB where appropriate, and examined by fluorescence and phase contrast microscopy. No fluorescein or DAB-stained material was found within the macrophages.

II. Lymphocytes were capped with F1-anti-Ig IgG; some cells were further

incubated for 1 h at 4°C with HRP-SAR. The lymphocytes were then washed and used as follows: (a) Lymphocytes were incubated for 1 h at 4°C and then for 30 min at 37°C with macrophages whose Fc receptors had been blocked by pretreating the macrophages for 1 h at 4°C with 1.7 mg/ml antimacrophage IgG. The cultures were then washed to remove nonattached lymphocytes, fixed, stained with DAB where appropriate, and examined by fluorescence and phase contrast microscopy. Less than 14% of macrophages which had been pretreated with antimacrophage IgG bound any lymphocytes, and only 5% of all macrophages in these cultures contained fluorescein or DAB-labeled immunoglobulins. (b) Capped lymphocytes were incubated with macrophages for 1 h at 4°C. An average of eight lymphocytes attached to each macrophage. These cultures were washed at 4°C to remove nonattached lymphocytes and further incubated for 1 h at 4°C with 1.7 mg/ml antimacrophage IgG. [This procedure blocks Fc receptors on the macrophage surface lying outside the zone of attachment of IgG-coated particles to the macrophage; it does not interfere with already established bonds between particle-bound immunoglobulins and Fc receptors (16).] 2 ml medium was then added to the culture, and it was incubated for an additional 30 min at 37°C. The cells were then fixed, stained with DAB where appropriate, and examined by fluorescence and phase contrast microscopy. Nearly all macrophages which were treated with antimacrophage IgG after lymphocyte attachment had lymphocytes bound to their plasma membranes, and 55% of these macrophages contained fluorescein or DAB-labeled immunoglobulins within cytoplasmic vacuoles. These results show that the fluorescein-and/or DAB-stained immunoglobulins seen within macrophage vacuoles in these and preceding experiments were interiorized via the macrophages' Fc receptors and that ingestion of the labeled immunoglobulins by macrophages occurred only at the sites of lymphocyte-macrophage attachment.

*Fate of Capped Lymphocytes Immunologically Bound to Macrophages.* It was of interest to determine whether or not lymphocytes remained viable on the macrophage surface after macrophages had ingested the immune complex cap from the lymphocyte surface. Capped lymphocytes were prepared and incubated with macrophages as described above; more than 99% of macrophages bound an average of eight lymphocytes each, more than 99% of which were viable, as judged by trypan blue dye exclusion. Parallel cultures were further incubated at 37°C in fresh medium containing 5% FBS and tested for trypan blue dye exclusion after further incubation for various periods up to 6 h. Less than 50% of lymphocytes eluted from the macrophages during this time, and 97, 93, and 87% of attached lymphocytes remained viable at 1, 3, and 6 h, respectively.

*Microfilaments in the Macrophage Cytoplasm.* Many of the electron micrographs of macrophages ingesting peroxidase-labeled immunoglobulins from the surfaces of capped lymphocytes show a zone of microfilaments in the portion of macrophage cytoplasm adjacent to the lymphocyte cap (Figs. 2-4). With the exception of some smooth membrane-bound vacuoles, virtually all other organelles are absent from this portion of the cytoplasm. To determine whether the presence of this zone of microfilaments is associated with the peculiar experimental situation being examined here or whether it appears during the phagocytosis of other IgG-coated particles, we studied the ingestion of E(IgG). As shown in Fig. 7, a zone of microfilaments measuring roughly  $1 \times 1 \mu\text{m}$  is present



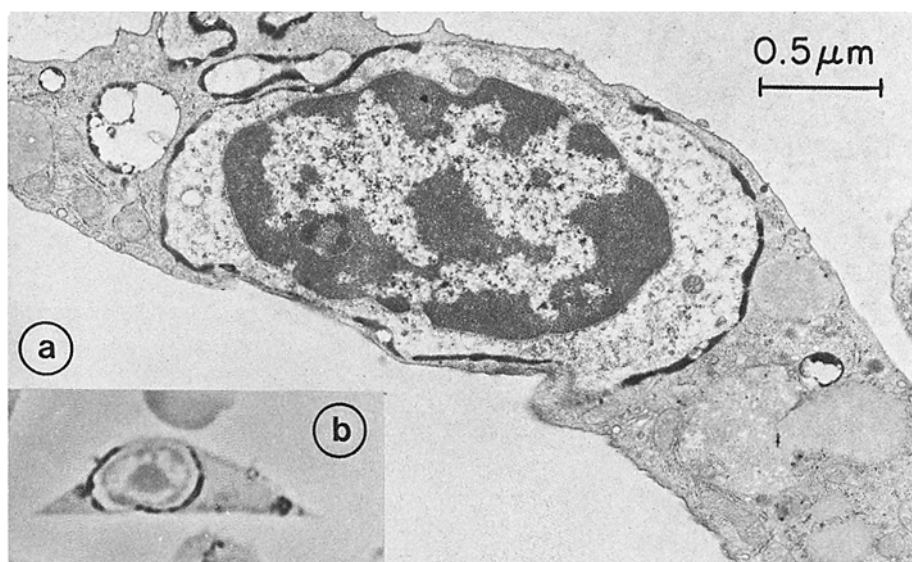


FIG. 6. Lymphocytes were incubated with rabbit anti-Ig IgG and subsequently with HRP-SAR, all at 4°C. These diffusely coated cells were washed and incubated with macrophages for 10 min at 4°C and for 20 min at 37°C. (a) Electron micrograph of a lymphocyte diffusely stained with DAB within a macrophage phagocytic vacuole. (b) Insert. Phase micrograph of a diffusely stained lymphocyte which has been ingested by a macrophage. Thick section of Epon embedded cell. Unstained.  $\times 700$ .

in the macrophage cytoplasm directly adjacent to an erythrocyte being ingested. These microfilamentous arrays are not present around phagocytic vacuoles containing erythrocytes which have been completely internalized, nor do they extend to segments of plasma membrane outside the zone of erythrophagocytosis. Thus the presence of microfilamentous arrays in the portion of macrophage cytoplasm adjacent to the lymphocyte cap probably reflects the ongoing phagocytosis of the cap.

### Discussion

The interactions of particle-bound ligands, such as IgG or the opsonic fragment of C3, with their corresponding receptors on the macrophage plasma membrane presumably initiate signals which, when transmitted to the macrophage cytoplasm, regulate the phagocytic process. We have previously shown that the effects of signals which initiate phagocytosis of one particle are not transmitted throughout the macrophage plasma membrane but are restricted to

FIG. 5. Consecutive sections showing the contact zone between two capped lymphocytes and a macrophage's plasma membrane. (a) Peroxidase reaction product (arrow) is seen between a single macrophage pseudopod and the surface of one lymphocyte. (b) The area of contact between the macrophage and the lymphocyte on the right is increased and corresponds to the DAB-stained segment of the lymphocyte's plasma membrane. Note centriole (arrow) in this lymphocyte's cytoplasm. (c) Both lymphocytes show peroxidase-labeled caps in this section. The centriole seen in the previous section is also present here (arrow). (d) There are extensive areas of contact between the macrophage and the DAB-stained immunoglobulin caps on both lymphocytes.

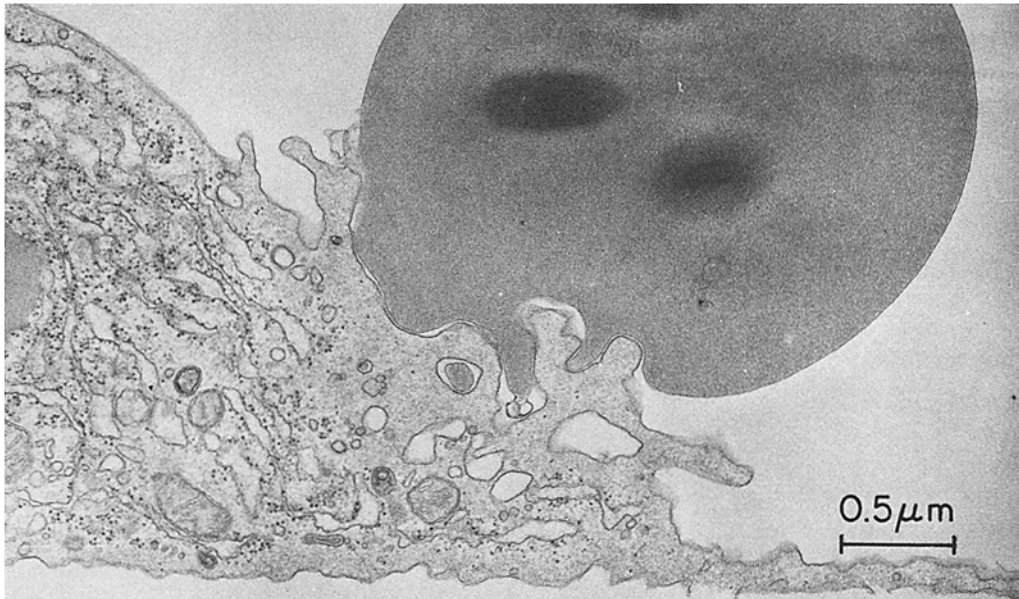


FIG. 7. IgG-coated sheep erythrocytes were incubated with macrophages for 30 min at 4°C. The nonattached erythrocytes were removed by washing, and the culture was incubated at 37°C for 10 min, at which time it was fixed and processed for electron microscopy. A dense accumulation of microfilaments, similar to that seen in Figs. 3 and 4, is found in the portion of macrophage cytoplasm directly adjacent to the IgG-coated erythrocyte. Other cellular organelles, which are clearly evident on the left side of the micrograph, are excluded from the microfilament-containing part of the macrophage cytoplasm.

the segment of plasma membrane immediately adjacent to the particle initiating the signal (19). Moreover, a signal initiating ingestion by one type of receptor (e.g., the Fc receptor) is not transmitted to other potentially phagocytosis-promoting receptors (e.g., the complement receptors) on the surface of the same cell. Our finding that ingestion of E(IgG) does not stimulate ingestion of capped lymphocytes bound to the macrophages' Fc receptors confirms this conclusion. In addition, it demonstrates that signals generated by the interaction of Fc receptors with IgG molecules on one particle [E(IgG)] are not transmitted to other, presumably identical, Fc receptors complexed with IgG molecules on different particles (capped lymphocytes) residing elsewhere on the macrophage plasma membrane. We derive four general conclusions from these facts: (a) the signal to phagocytize a particle is initiated by the particle; (b) the response to that signal is confined to the segment of macrophage plasma membrane adjacent to the particle initiating the signal; (c) a signal initiating ingestion via any given phagocytic receptor is not transmitted to other phagocytic receptors on the macrophage plasma membrane; and (d) if any diffusible intracytoplasmic factors are formed as a result of these signals, the effects of these factors must be confined to the segments of plasma membrane adjacent to the particle being ingested.

*Confirmation of the "Zipper" Hypothesis.* In a previous paper (16) we presented evidence that explains the mechanism underlying the localized response of the macrophage plasma membrane to a phagocytic signal. We found that

ligands on the particle and receptors on the plasma membrane of the phagocytic cell are required in both the attachment and the ingestion phases of phagocytosis. When ligand-coated particles were bound to their corresponding macrophage surface receptors, the particles were not ingested if either the ligands or the receptors were removed from surfaces lying outside the attachment zone. These findings indicated that the initial attachment of particle-bound ligands to plasma membrane receptors is not sufficient to "trigger" particle ingestion. We suggested that phagocytosis of a particle requires the sequential, circumferential interaction of plasma membrane receptors with particle-bound ligands not involved in the initial attachment process, and termed this proposal the zipper hypothesis. Although the experimental evidence supporting this hypothesis was substantial, it did not constitute unambiguous proof of the mechanisms proposed.

In the present experiments we have shown that the same number of ligands (anti-Ig IgG) that are sufficient to promote lymphocyte ingestion when they are diffusely distributed over the lymphocyte's surface do not promote ingestion when these ligands are concentrated at one pole of the lymphocyte. Thus, there is no absolute number of receptor-ligand interactions which can "trigger" phagocytosis of a particle. Ligands must be present over the entire particle surface in order for ingestion to occur. In short, the zipper mechanism is confirmed.

*Receptor-Ligand Interaction and the Role of Contractile Proteins.* Although the zipper hypothesis explains the vectorial orientation of the macrophage plasma membrane with respect to its intended particulate meal, it does not explain the mechanisms by which pseudopods advance around a particle. Actin and myosin have been found in the cytoplasm of a variety of nonmuscle cells, including phagocytic cells (30-33); and an actin-binding protein (34-37), polymerized actin (30), and microfilament bundles (36, 38, 39) have been identified in association with the plasma membranes of phagocytizing macrophages (38) and phagocytizing thyroid epithelial cells (39). Cytochalasin B, a compound which impairs actin gelation (40-42) and microfilament function (43), inhibits phagocytosis. Actin isolated from polymorphonuclear leukocytes of a patient with recurrent infections and marked defects in neutrophil motility and phagocytosis exhibits defective gelation (32). Thus there are abundant structural and functional data linking these contractile proteins and the phagocytic process. Our electron micrographs show a large zone of aggregated microfilaments confined to the region directly adjacent to the immune complexes (Figs. 3 and 4) or erythrocyte (Fig. 7) being ingested. Extensive microfilament aggregation is not observed beyond the attachment zone of the IgG-coated particle to the macrophage surface.

Based upon these findings, we propose the following model for immune ligand-mediated phagocytosis (Fig. 8). Ligands bind particles to specific receptors on the plasma membrane of the phagocytic cell (Fig. 8*a*). The initial ligand-receptor interaction generates a signal that initiates the aggregation of contractile proteins and leads to the extension of pseudopods in the area of the attached particle (Fig. 8*b*). Receptors on the plasma membranes of the advancing pseudopods bind to additional ligands on the particle's surface, resulting in the generation of additional signals, continued aggregation of cytoplasmic contractile proteins, and further extension of pseudopods (Fig. 8*c*). The process repeats



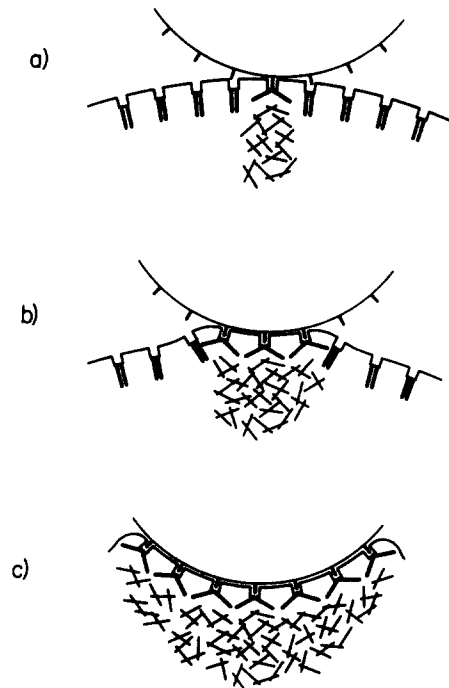


FIG. 8. Model of proposed phagocytic mechanism. ( ' ) IgG molecules or other ligands; ( Y ) Fc receptors or other membrane receptors in an inactive state; ( Y ) Fc receptors or other membrane receptors in an activated state; ( X ) cytoplasmic contractile proteins.

many times until the plasma membranes of the advancing pseudopods meet and fuse with one another to form the phagocytic vacuole.

*Effects of Macrophages upon Capped Lymphocytes.* In addition to defining the roles of particle-bound ligands and membrane receptors in the ingestion phase of phagocytosis, our studies of the interaction of capped lymphocytes with macrophages have led to several novel and unexpected observations.

**MACROPHAGES REMOVE THE CAP FROM THE LYMPHOCYTE'S SURFACE.** Pretreatment of macrophages with antimacrophage IgG prevents binding of capped lymphocytes to macrophages and ingestion of lymphocyte-bound anti-immunoglobulins by these phagocytes. Thus the capped lymphocytes attach to the macrophages via the latter's Fc receptors, and the functional integrity of these receptors is required for macrophage ingestion of lymphocyte-bound anti-immunoglobulins. Several lines of evidence indicate that the macrophage ingests the cap directly from the surface of the lymphocyte. (a) Bright field and fluorescence microscopy showed that macrophages that failed to bind capped lymphocytes did not contain labeled immunoglobulins. Electron microscopy showed that invaginations of the macrophage membrane containing peroxidase-labeled immunoglobulins lie within the zone of attachment of the lymphocytes to the macrophages (Figs. 3 and 4). Moreover, DAB-stained material within the macrophages was found in vacuoles located predominantly in the region of the macrophage cytoplasm adjacent to the capped lymphocyte. DAB-stained material was never observed on the macrophage surface outside the zone binding the

lymphocyte to the macrophage. (b) Examination of medium in which capped lymphocytes had been incubated showed that insufficient lymphocyte-bound immunoglobulin was shed into this medium to account for the uptake of labeled immunoglobulin by the macrophages. (c) Treatment of capped lymphocyte-macrophage complexes with antimacrophage IgG did not inhibit ingestion of lymphocyte-bound anti-immunoglobulins by the macrophages. These data indicate that Fc receptors lying outside the zone of lymphocyte attachment do not participate in the removal of labeled anti-immunoglobulins from the cap.

**CAPPED LYMPHOCYTES DO NOT ELUTE FROM THE MACROPHAGE SURFACE.** Most of the labeled anti-immunoglobulin in the cap was ingested by the macrophages within 30 min of 37°C incubation. However, the majority of lymphocytes remained bound to the macrophages for several more hours. We cannot explain this continued attachment of lymphocytes to macrophages in the absence of demonstrable ligands, but similar observations have been made by Rutishauser and Sachs (44). They noted that cells aggregated with concanavalin A became increasingly resistant to a disaggregation by  $\alpha$ -methyl mannoside, and they suggested that irreversible bonds may form between cells after ligand-induced receptor alignment and multipoint bridging occurs.

**CAPPED LYMPHOCYTES REMAIN VIABLE ON THE MACROPHAGE SURFACE.** Capped lymphocytes remained viable despite their attachment to the macrophages' Fc receptors and the removal of their anti-immunoglobulin caps by these phagocytic cells. The macrophages used in these experiments were derived from peritoneal cavities of nonstimulated mice. Perhaps, as suggested by the work of Evans et al. (45), the macrophages must first be "armed" or "activated" to exert cytotoxic effects on antibody-coated target cells. The system we have described here should prove useful in studying the possible cytotoxic activities of such activated macrophages.

*Physiological Significance of Ingestion of the Cap by Macrophages.* In our experiments, the marker being examined is labeled anti-immunoglobulin. Ingestion of these anti-immunoglobulin molecules implies that the antigens to which they are bound (i.e., lymphocyte plasma membrane immunoglobulins) are concurrently removed from the lymphocyte surface. It should be noted that we have no direct evidence that plasma membrane immunoglobulins are removed from the lymphocyte surface together with the labeled anti-immunoglobulins. Assuming that lymphocyte plasma membrane immunoglobulin-anti-immunoglobulin complexes are removed by the macrophages, the capacity of mononuclear phagocytes in concert with specific antibodies to alter cell surfaces may have broad physiological consequences.

Capping of surface moieties may be a protective mechanism for lymphocytes. Lymphocytes bearing surface immunoglobulins might become targets for phagocytic cells if antigens bound to these surface immunoglobulins were themselves then coated by antigen-specific IgG. Such lymphocytes would be diffusely coated with IgG and could be ingested by macrophages. Capping of these surface immunoglobulin-antigen-IgG complexes would still permit binding of the lymphocytes to macrophages but would prevent the lymphocytes' ingestion and destruction. Likewise, binding of an antigen to surface immunoglobulins over the entire lymphocyte surface could render the lymphocyte ingestible if the

macrophage surface were able to recognize this antigen. The capacity of polyvalent antigens to cap surface immunoglobulins on antigen-responsive cells (46-49) would prevent the destruction of these cells by macrophages.

Virus-infected, virus-transformed, and neoplastic cells of many species bear on their surfaces antigens not present on the surfaces of their normal counterparts (50, 51). Antibodies to these antigens are frequently produced by the host and can bind specifically to these antigenic determinants. Some of these immune complexes have been shown to cap on the cell's surface (52, 53). Capping of these antigens could prevent ingestion and destruction of the abnormal cells by macrophages, but the macrophage should be able to bind the cells by the immune complex caps. Our finding that antibodies complexed to cell surface antigens are removed by macrophages from the plasma membranes of viable cells raises the possibility that phagocytic cells may clear abnormal surface determinants from these cells, leaving them no longer recognizable as abnormal and thus able to continue to proliferate within the host.

### Summary

We have examined the effect of the distribution of anti-immunoglobulin IgG molecules on the surface of bone marrow-derived lymphocytes upon the interaction of these cells with macrophages. Lymphocytes which were diffusely coated with antibodies to surface immunoglobulin were ingested by macrophages. Lymphocytes which had the same number of anti-immunoglobulin IgG molecules redistributed to one pole of the surface bound to the macrophages' Fc receptors but were not ingested. These results confirm our previous hypothesis that ingestion of an immunologically coated particle requires the sequential, circumferential binding of specific receptors on the plasma membrane of a phagocytic cell to immunologic ligands distributed over the entire particle surface.

Macrophages which had bound capped lymphocytes by the macrophages' Fc receptors removed the immune complex caps from the lymphocyte surface without destroying the lymphocytes. These lymphocytes remained attached to the macrophage surface. The finding that macrophages can phagocytize immune complexes from the surface of a cell without destroying the cell to which these complexes are attached may be important in understanding the effects of antigens and antibodies on cells participating in a humoral immune response, in identifying the mechanisms by which chronic viral infections are established, and in defining the roles of blocking antibodies in tumor immunity.

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