SELECTIVE PHAGOCYTIC PARALYSIS INDUCED BY IMMATERIALIZED IMMUNE COMPLEXES*

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Selective interference with the function of receptors for Fc in mononuclear phagocytes has been obtained in two kinds of experiments. First, IgG-rich antisera that cross-reacted with macrophages reduced the uptake of erythrocytes coated with IgG antibody. This effect was obtained with concentrations of antiamacrophage serum or IgG that did not reduce or only marginally reduced the interaction of the phagocytes with complement-coated red cells (EAC)1 or with particulates recognized nonimmunologically (1, 2). Macrophages treated with the antiserum, washed, and cultivated for 24 h partially recovered the function of the Fc receptors (2). Because uptake of opsonized erythrocytes was not reduced by antiamacrophage F(ab)2, it has been postulated (C. Bianco, personal communication) that the Fc portion of the antiamacrophage IgG induced its own interiorization together with the receptors for Fc to which it bound. Recovery may have reflected the time needed to elute or to interiorize cell-bound antiamacrophage IgG as well as to replace the lost receptors for Fc. In a second type of experiment, fluid phase nonspecific IgG of the appropriate subclass has been shown to inhibit binding of IgG-coated red cells (EA) to macrophages, perhaps by competing with receptors for Fc (reviewed in reference 3). While binding of EAC (4) or ingestion of polystyrene beads (5) were not reduced by fluid phase IgG, interiorization of the Fc receptors does not seem to have been excluded by appropriate reversibility experiments.

We show here that exposure of macrophages to substrate-bound immune complexes selectively reduces the ingestion of opsonized erythrocytes. Features of the inhibition and initial studies on the restoration of the function of the receptors for Fc are also reported.

Materials and Methods

Media. Phosphate-buffered saline (PBS) of pH 7.2 contained 138 mM NaCl and 6.3 mM PO4. Bicarbonate-free powdered Dulbecco medium (Grand Island Biological Co., Grand Island, N.Y.) was reconstituted with glass-distilled water and buffered with 10 mM HEPES to pH 7.0. Fetal bovine serum (FBS) was purchased from GIBCO and heated to 56°C for 40 min.

Chemicals and Proteins. Lidocaine was a gift from Astra Pharmaceutical Products, Inc., Worcester, Mass. Subtilisin and bovine plasma albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, Mo. BSA was labeled with 125I by the method described in ref. 7. Specific activity was usually 1.5 × 106 cpm/μg protein.

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1Abbreviations used in this paper: BSA, bovine plasma albumin; DH, Dulbecco medium buffered with HEPES; E, sheep erythrocytes; EA, erythrocytes coated with IgG-rich hyperimmune rabbit anti-E serum; EAC, E coated with IgM-rich anti-E and mouse complement; FBS, fetal bovine serum; GRC, glutaraldehyde-fixed red cells.
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Antisera. Rabbit hyperimmune anti-BSA contained 27 mg antibody per ml. Purified IgG was obtained from the antiserum by chromatography on DEAE cellulose. F(ab)2 was obtained by digestion with pepsin (6) and filtration through a Sephadex G-200 column. Hyperimmune rabbit antisheep erythrocyte serum agglutinated sheep red cells to a 1:256,000 dilution. IgM-rich rabbit amboceptor was obtained from Cordis Laboratories, Miami, Fla.

Erythrocytes and Other Particulates. Sheep red cells were purchased from Animal Blood Center, Syracuse, N.Y. For the preparation of EA (19S)C (ref. 4 and C. Bianco, personal communication), sheep erythrocytes were washed three times in PBS. 0.5 ml of packed red cells were suspended in 1 ml of Dulbecco-HEPES medium (DH) containing a 1:4 dilution of IgM-rich rabbit antisheep RBC for 15 min at 37°C. The erythrocytes were washed once with DH, resuspended in 1 ml of 10% vol/vol fresh mouse serum in DH, incubated for 10 min at 37°C, washed three times in DH, and resuspended to a final concentration of 0.05% packed red cells. Erythrocytes were treated with glutaraldehyde as previously described (8). Sheep erythrocytes were labeled with 51Cr as in ref. 9. Zymosan was obtained from Fleischman Laboratories, N.Y., and polystyrene-latex beads of average diameter 2.0 μm from Dow Chemical, Midland, Mich.

Animals. Female mice 20–25 g body weight, strain CFW were used.

Coating of Cover Slips or Tissue Culture Dishes. 22-mm cover glasses were precleaned with 95% ethanol, dried, and overlayed with 0.2 ml of 20 μg/ml BSA in PBS for 15 min at room temperature. The cover glasses were rinsed in saline, drained, covered with 0.2 ml of anti-BSA 1:100 in PBS for 15 min at room temperature, and rinsed. Control cover glasses were treated with BSA alone. Falcon 60-mm tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were treated similarly except that exposure to BSA and to anti-BSA was done for 30 min.

In preliminary experiments we examined the binding of [125I]BSA to cover slips. Increasing amounts of 125I-labeled BSA were applied and after 15 min at 22°C the coverslips were rinsed and the glass-associated radioactivity measured with a gammacounter. A plateau of glass-associated counts was obtained when 2 μg were applied over an area of 200 mm². Of this amount 0.45 μg BSA were bound. In other experiments, coverslip were coated with different amounts of BSA, rinsed, and treated with anti-BSA before attachment of macrophages and challenge with EA. Maximal inhibition of ingestion was obtained when more than 4 μg were applied to 320 mm².

Macrophages. Macrophage-rich peritoneal cell suspensions were obtained from normal mice killed with chloroform. Peritoneal cavities were washed with 3 ml DH. 0.2 ml of cell suspensions were applied to the coated cover slips and allowed to attach for 10 min at room temperature. The monolayers were rinsed and incubated in 1.5 ml DH for 30 min at 34°C before further treatment.

Ingestion of EA. Monolayers were incubated for 30 or 60 min at 34°C in 1.5 ml DH alone or containing 5% FBS, with the addition of 25 × 10⁶ SE per ml and 1:4,000 anti-E serum. Red cells and antisera were preincubated for 30 min. The monolayers were rinsed in DH, exposed to plain saline diluted 1:1 with distilled water for 10 sec, rinsed in 0.85% NaCl, and fixed with 2% glutaraldehyde in PBS. This osmotic lysis allows the observation of red cell ghosts attached to the macrophage surface. However, apparently only a fraction of the ghosts remain attached to the phagocytes. 200 macrophages per cover slip were scored for ingested erythrocytes. In several experiments attachment of EA to macrophages was performed for 30–60 min at 22°C and the monolayers were rinsed and fixed without the hypotonic shock.

Rosette Formation with EAC. Macrophage monolayers were overlaid with 0.2 ml of 0.05% EAC in DH and incubated for 30 min at 34°C. The monolayers were rinsed and fixed in glutaraldehyde.

Ingestion of Other Particulates. Macrophages were exposed for 30 min at 34°C to 1.5 × 10⁶ glutaraldehyde-treated E/ml, 200 μg/ml Zymosan, or 100 μg/ml polystyrene-latex. The monolayers were rinsed and fixed in glutaraldehyde.

Phagocytic Assays with 51Cr Labeled Sheep Erythrocytes (9). 9 × 22 mm cover slips were coated with BSA or with BSA anti-BSA and overlayed with 0.2 ml peritoneal cell suspensions. The monolayers were rinsed and exposed to 0.2 ml of DH 5% FBS containing 25 × 10⁶ 51Cr labeled E/ml as well as 1:4,000 anti-E serum. After 30–60 min the monolayers were rinsed and either counted directly or subjected to hypotonic lysis and counted. Radioactivity was measured in a gamma counter. Counts without lysis estimate the total interaction of EA with the macrophages while counts obtained after osmotic lysis estimate the red cells ingested by the phagocytes. Results are expressed in percent of the cpm of control monolayers plated on BSA alone.

Recovery Experiments. In these experiments macrophages plated on BSA alone or on BSA anti-
TABLE I

Ingestion of EA by Macrophages Plated on Immune Complexes

| Treatment of cover slips* | Ingestion of EA‡ | Inhibition§ |
|--------------------------|-----------------|------------|
| BSA                      | 66.7 ± 3.5 (9)  | -          |
| BSA + anti-BSA           | 8.8 ± 1.2 (9)   | 86.0 ± 2.5 (9) |

* See Materials and Methods.
‡ Percent macrophages with ingested EA ± SEM (no. experiments).
§ 100 - (% uptake experimental x 100/% uptake control).

BSA and exposed to DH for 30 min at 34°C, were further incubated in different media for periods up to 24 h before challenging with EA. In additional experiments the immune complexes were similarly treated before the layering of the exudate cell suspension, followed by the phagocytic assays.

Removal of Macrophages from Tissue Culture Dishes. 60-mm tissue culture dishes coated with BSA alone or with BSA anti-BSA and rinsed, were overlayered with about 3 x 10⁶ macrophages for 45 min at 34°C. The monolayers were rinsed and treated with 0.75 ml of 15 mM lidocaine in DH with 5% FBS at 22°C. After 10–20 min most cells could be removed by means of jets of DH 5% FBS from a syringe (10). The cell suspensions were diluted 10-fold with DH 5% FBS, centrifuged for 5 min at 1,000 rpm, resuspended in 2.0 ml DH-FBS, and plated on untreated dishes for 1 h at 34°C, at which time most macrophages reattached. They were then rinsed and tested with EA for 30 min.

Results

Paralysis of Ingestion of EA by Macrophages Plated on Cover Slips Coated with Immune Complexes. Cover slips treated with BSA alone, antiserum to BSA alone, or with BSA followed by antiserum to BSA were overlayered with peritoneal cell suspensions from normal mice and incubated for 30 min at 34°C in DH. The macrophages were then challenged for 30 min with sheep erythrocytes in the presence of IgG-rich rabbit anti-E serum, rinsed, subjected to mild hypotonic lysis, and fixed in glutaraldehyde. The percent of macrophages with ingested EA was determined microscopically. Table I and Fig. 1 A and B show that few macrophages plated on substrates coated with immune complexes ingested opsonized erythrocytes when compared to control cells plated on cover slips coated with the antigen or antiserum alone. The inhibition of ingestion persisted after macrophages on complexes were incubated in DH for 8, 16, or 24 h at 37°C. Ingestion of EA was dependent on the dilution of the antiserum used to prepare the immune complexes. In a typical experiment, 4 mg BSA were initially applied per cover slip and were followed by 0.2 ml of 1:100, 1:200, or 1:500 rabbit anti-BSA. Control cover slips were coated with BSA alone. Ingestion of EA was inhibited by 95%, 86%, or 60% respectively.

Microscopic observations of monolayers that were not subjected to hypotonic lysis indicated that the attachment of EA to macrophages was not reduced by the complexes (Fig. 2 A and B). This conclusion was supported by experiments with ²⁵Cr-labeled EA (IgG). Table II shows that while ingestion of the labeled EA was markedly inhibited, their attachment, as estimated by the radioactivity removable by mild hypotonic lysis, was not decreased by the substrate-bound complexes.

Requirement for the Fc Portion of IgG. Cover slips coated with 10 μg BSA
FIG. 1. Macrophages plated on BSA (left) or on BSA anti-BSA (right) coated cover slips. Interaction with different particulates. (A and B), EA (osmotic lysis); (C and D), polystyrene latex beads; (E and F), EAC; (G and H), aldehyde treated E. × 470.
were rinsed and incubated with DEAE cellulose-purified IgG from rabbit antiserum to BSA, or with F(ab)_2 obtained from the same IgG. Macrophages were layered on the cover slips and challenged with EA. In a typical experiment, complexes prepared with 50 or 100 μg IgG anti-BSA, inhibited ingestion of EA by 82 and 94% respectively. In contrast, no inhibition was obtained when F(ab)_2 anti-BSA was applied at 50, 100, or 150 μg/per coverslip.

**Inhibition of Ingestion as a Function of the Concentration of Anti-E Serum.** Table III shows that the inhibitory effect of immune complexes could be partially counteracted by increasing the concentration of the anti-E serum. However, significant inhibition was present even at the highest concentrations assayed.
TABLE II
Attachment and Ingestion of $^{3}$Cr-EA Added to Macrophages on Immune Complexes

| Treatment of cover slips | (A) Total counts$ | (B) Counts after lysis$ | (A - B)  |
|-------------------------|------------------|------------------------|---------|
| BSA                     | 1,134 ± 133 (3)  | 484 ± 40 (4)          | 650     |
| BSA anti-BSA            | 832 ± 41 (4)     | 87 ± 6 (4)            | 747     |

* 1 h at 22°C.
† cpm per cover slip ± SEM (no. samples), corrected for background.
These counts reflect total interaction with EA.
§ Reflect ingested EA.
| Reflect attached EA.

TABLE III
Uptake of EA by Macrophages Layered on Immune Complexes as a Function of the Dilution of the Erythrocyte-Opsonizing Antiserum

| Treatment of cover slips | Inhibition of ingestion |
|-------------------------|------------------------|
| Dilution of antiserum   | BSA* ingestion         | BSA anti-BSA ingestion$ | %     | %     | %     |
| 1:4,000                 | 88                     | 6.3                    | 93     |
| 1:2,000                 | 87.5                   | 23                     | 74     |
| 1:500                   | 88.5                   | 34                     | 62     |

Averages of triplicates.
* 4 μg per cover slip.
† 4 μg BSA per cover slip followed by 1:100 anti-BSA serum.

Role of Soluble Factors. The following results indicate that inhibition by immune complexes of the ingestion of EA is not mediated by solubilized complexes or by a soluble factor released by the cells. Small volumes of BSA solution were used to coat a known portion of the surface of a cover slip and excess BSA was removed in a way to minimize the adsorption to the remainder surface. The cover slip was then treated with antiserum to BSA, rinsed, overlaid with macrophages, and these were tested with EA as described in Materials and Methods. Thus, immune complexes were limited to a specific area of the substrate. Microscopic observation showed that ingestion was inhibited only in the area initially treated with the antigen, with a rather sharp boundary separating the macrophages on immune complexes from the nearby control cells. The former were spread and showed little interiorization of EA whereas the latter were rounded and actively phagocytic (Fig. 3). Thus, no evidence was obtained that a soluble and readily diffusible factor was released that would inhibit the ingestion of EA by macrophages situated near the immune complex-coated area.

Immune Complexes Added to the Fluid Phase. Complexes were prepared in DH with 1–4 μg/ml BSA and a range of dilutions of antiserum from 1:20 to 1:320.
The antiserum used bound at equivalency 300 μg/BSA per ml. Macrophages were plated on untreated cover slips, incubated at 34°C for 30 min with 1 ml of DH containing the complexes and then challenged with EA. No inhibition of ingestion of EA was obtained by complexes made at equivalency, at antibody or at antigen excess.

Effect of Substrate-Bound Complexes on the Interaction of Macrophages with Different Particulates. Macrophages plated on cover slips coated with BSA alone or with BSA followed by anti-BSA were incubated with EA, EAC, or with other particles recognized nonimmunologically. Whereas ingestion of EA was markedly inhibited by immune complexes, macrophages bound EAC more extensively than controls (Fig. 1 E and F). In addition, as shown in Fig. 1 and in Table IV, aldehyde-treated E, latex beads, or yeast cell walls were extensively ingested by macrophages plated on BSA anti-BSA.

Role of Macrophage Spreading in the Inhibition of Ingestion of EA. Because immune complexes are known to induce the spreading of macrophages (11, 12), it was of interest to determine whether the inhibition of phagocytosis of EA could be related to spreading. Macrophages attached to untreated cover slips were induced to spread by exposure to 50 μg/ml subtilisin (12), rinsed, and challenged with EA. Ingestion of EA was found to be unimpaired when compared to controls incubated in DH alone.

Restoration of the Ingestion of EA. Macrophages plated on complexes of BSA anti-BSA and incubated at 34°C in medium enriched with serum recovered
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Table IV

Ingestion of Different Particulates by Macrophages Plated on Immune Complexes

| Particles            | Treatment of cover slips | BSA | BSA anti-BSA |
|----------------------|--------------------------|-----|--------------|
| Polyvinyltoluene latex |                          | 91  | 92           |
| Sheep GRC*            |                          | 93  | 96           |
| Zymosan               |                          | 71  | 81           |

* 5 x 10⁶/dish.
‡ Percent of macrophages with ingested particles. Averages of duplicates.

The ability to ingest EA (Fig. 4). Recovery increased with time and with the serum concentration in the medium (Table V).

The effect of serum could not be ascribed to its content of homologous antigen because (a) in experiments similar to those shown in Table V, incubation of macrophages for 4 or 8 h in DH with 1% BSA failed to induce recovery of the ingestion of EA; (b) heat-inactivated (56°C, 30 min) mouse serum was as or more effective than fetal bovine serum; and (c) ingestion was restored in experiments in which cover slips were coated with BSA and mouse anti-BSA and the monolayers incubated in DH with 33% heat-inactivated mouse serum (not shown).

Serum-mediated restoration of ingestion of EA could be explained by removal or masking of substrate or cell-bound complexes, by an effect on the macrophages or by a combination of these mechanisms. That removal or masking of the cell-bound complexes may account for the recovery of ingestion is shown by the following experiments. Glass-bound complexes, in the absence of macrophages were treated for 4 h with DH alone or with DH with 20 or 50% FBS. The cover slips were rinsed, overlayers with macrophages, and these were challenged with EA. In a typical experiment complexes treated with DH were inhibitory of ingestion (7% phagocytosis), while phagocytosis by macrophages on complexes that had been treated with 20 or 50% serum was respectively, 72 and 93% of controls (cover slips coated with BSA alone).

Restoration of ingestion was also obtained when macrophages plated on BSA or on BSA anti-BSA-coated tissue culture dishes were removed with lidocaine, replated on untreated dishes, and tested with EA. Parent cultures on BSA and on complexes were also tested. Averages ± standard errors of five separate experiments were as follows. Phagocytosis by parent cultures on BSA and on complexes were, respectively, 76.6 ± 3.5 and 9.9 ± 2.1%. Macrophages from BSA-coated dishes replated on untreated substrate phagocytosed like the initial population (72.4 ± 4.1). Cells removed from complexes-coated dishes and replated showed clear although incomplete recovery (57.7 ± 4.2).

Discussion

We have examined the phagocytic recognition displayed by macrophages
Fig. 4. Serum-mediated recovery of ingestion of EA (osmotic lysis). (A), macrophages plated on BSA; (B) and (C), macrophages plated on BSA anti-BSA complexes. A and B incubated in DH for 4 h; C incubated in DH with 50% FBS for 4 h. Partial recovery of ingestion of EA in C. × 470.
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TABLE V

*Ingestion - SEM (no. experiments) by macrophages on BSA anti-BSA in percent of the ingestion by macrophages plated on BSA alone.

| Incubation medium | Incubation period |
|-------------------|-------------------|
|                   | 4 h               | 8 h               |
| Dulbecco HEPES    | 9 ± 1.2 (13)*     | 8 ± 1.9 (4)       |
| DH 20% FBS        | 20 ± 5.7 (3)      | 56.5 (2)          |
| DH 50% FBS        | 66.3 ± 3.5 (14)   | 94.3 ± 0.5 (4)    |

* Ingestion ± SEM (no. experiments) by macrophages on BSA anti-BSA plated on glass coated with immune complexes of BSA and anti-BSA. The main observations can be summarized as follows. (a) Ingestion of EA was inhibited by the complexes but attachment of the red cells to the phagocytes was not. (b) Neither binding of EAC nor ingestion of particulates recognized nonimmunologically were inhibited by substrate-bound complexes. (c) Inhibition of ingestion of EA was Fc-dependent, did not appear to depend on solubilized immune complexes or on some cell-derived inhibitor; and was not a consequence of macrophage spreading. (d) Uptake of EA by the macrophages could be restored by incubation of the phagocytes on complexes with serum-containing media or by removing the macrophages from complexes-coated dishes followed by replating of the cells on uncoated substrate.

The mechanism through which immune complexes inhibit the ingestion of EA by macrophages could involve, (a) the loss of effective receptors for Fc, either because they are blocked by the immune complexes or removed from the macrophage surface by interiorization (13, 14). Function of the receptors could also be affected by the immune complexes induced fusion of lysosomes with the plasma membrane, leading to intercalation of lysosomal membranes in the plasma membrane (15, 16). Because binding of EA is less affected, the implication is that attachment requires fewer Fc receptors than ingestion. Ingestion may require multiple and sequential interactions between the Fc receptors and Fc domains of the erythrocyte bound IgG (17). These interactions could trigger the metabolically dependent spreading of the phagocyte plasma membrane and underlying cell cortex over the surface of the attached erythrocyte (18–20). With fewer effective receptors available, this "zippering" process would be interrupted and therefore ingestion would be inhibited. If this hypothesis is correct, scanning electron microscopy should reveal partially or incompletely interiorized EA. Alternatively, the membrane triggering of ingestion could depend on simultaneous or sequential multiple cross-linking of Fc receptors such as postulated (21–23) for reaginic-mediated histamine release from mast cells (bridging hypothesis). Again, this bridging would not occur if fewer Fc receptors are available on the phagocyte surface. Another explanation of the inhibition of ingestion of EA could be, (b) failure of the transmission of the signal for particle ingestion. Glass-immobilized complexes could induce "paralysis" or "refractoriness" of the macrophage receptors. Because uptake of other particles such as latex beads is not affected by the complexes, it would be necessary to postulate a
specialized and separate machinery for the interiorization of particles opsonized by IgG.

A choice between these mechanisms has to await the improvement of the methodology to measure the number of Fc receptors per cell. This would allow the determination of the number of these receptors before and after exposure to glass-absorbed complexes. Whatever the mechanism, our findings clearly show that it is possible to selectively inhibit the phagocytic functions of the Fc receptors of macrophages, although contact with the opsonized particle is not affected. This phagocytic paralysis may be of relevance to the understanding of the behavior of macrophages and other cells bearing receptors for Fc when they come into close contact in vivo with antibody coated target cells which they cannot ingest or with immobilized complexes such as those found on basal membranes (24–26).

Summary

The phagocytic recognition by peritoneal macrophages plated on glass- or plastic-bound immune complexes of bovine plasma albumin (BSA) and anti-BSA was examined. Ingestion but not the attachment of erythrocytes opsonized with an IgG rich antiserum (EA) was markedly inhibited. In contrast, macrophage interactions with complement-coated (EAC) red cells, or ingestion of latex particles, yeast cell walls or glutaraldehyde-treated erythrocytes was not inhibited. Complexes prepared with pepsin-treated anti-BSA IgG were ineffective indicating a requirement for the Fc region. Inhibition of ingestion of EA was not a consequence of macrophage spreading and did not appear to be mediated by solubilized complexes or by cell-derived inhibitors of phagocytosis. Significant restoration of the ability to ingest EA was obtained when macrophages on complex-coated substrates were incubated for 4–8 h in medium enriched with mouse or fetal bovine serum. Restoration was also attained by removing macrophages from complex-coated dishes and replating onto uncoated dishes. The selective inhibition of ingestion of EA may be due to blocking of Fc receptors by the complexes but depletion of receptors by endocytosis of complexes cannot be ruled out. Alternatively, the complexes may have induced selective failure of the interiorization mechanism.

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