IN VIVO ACTIVATION OF MACROPHAGE C3 RECEPTORS
FOR PHAGOCYTOSIS

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The C3 receptors of most phagocytic cells promote avid binding of particles
coated with C3 but do not promote their ingestion (1-10). We have previously
(7-12) described a unique lymphokine that activates the C3 receptors of mouse
peritoneal macrophages for phagocytosis. Generation of the lymphokine in vitro
requires an unusual series of cellular interactions. Macrophages must first phag-
ocytize IgG-containing immune complexes, thereby acquiring the ability to
trigger T lymphocytes to elaborate the lymphokine (7).

All of the elements required to generate the lymphokine—immune complexes,
macrophages, and T lymphocytes—are present at sites of immunologically me-
diated inflammation in vivo. In the present experiments we created immunologi-
cally mediated inflammation at a site in vivo, harvested macrophages from the
site, and assessed their C3 receptor activity. Our findings suggest that the
lymphokine we have generated in vitro is produced by similar mechanisms in
vivo and that it may be a critical determinant of macrophages' ability to phago-
cytize opsonized microorganisms in vivo.

Materials and Methods

Reagents and Media. Medium 199 (Microbiological Associates, Walkersville, MD);
bovine serum albumin (BSA), trypsin, lyophilized human serum albumin (HSA), and
glycine (Sigma Chemical Co., St. Louis, MO); lyophilized rabbit anti-BSA IgG and
lyophilized rabbit anti-IgM (LgM) coated with the first four complement components
(Cappel Laboratories, Cochranville, PA); sheep erythrocytes (E) (Scott Laboratories,
Inc., Fiskeville, RI); chromogranin, glutaraldehyde, and concentrated sulfuric acid
(Fisher Scientific Co., Pittsburgh, PA); and poly-L-lysine (PLL) (Miles Laboratories,
Inc., Elkhart, IN) were obtained from the manufacturers indicated.

Preparation of Immunologically Coated E. E coated with anti-E IgG (E IgG), E coated
with anti-E IgM (E IgM), and E IgM coated with the first four complement components
(E IgMC) were prepared as previously described (9).

Preparation of Soluble Immune Complexes and Aggregates. Rabbit anti-HSA antiserum
was prepared by repeatedly immunizing a rabbit with HSA. It was heat-decomplemented
(56°C for 30 min) and incubated with HSA to prepare soluble immune complexes as
previously described (9). Aggregated IgG was prepared by heating rabbit gamma globulin
at 63°C for 45 min, as previously described (9).

Animals and Cells. 20-30-g female Swiss mice (strain CD-1) were obtained from
Charles River Breeding Laboratories, Inc., Wilmington, MA; 20-30-g female athymic
BALB/c mice and their female euthymic littermates were obtained from Life Sciences,
Inc., Greenwich, CT.

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ment Award AI-00135 from the National Institutes of Health.
Peritoneal cells were harvested by lavage from noninjected mice and from mice injected intraperitoneally 4 d earlier with either 5 mg of aggregated IgG, HSA-anti-HSA complexes containing 5 mg of HSA, or a control preparation. Macrophages were purified by glass adherence and were cultivated in medium at a concentration of 4 x 10^4 per coverslip on 13-mm diam, PLL-coated glass coverslips in 35-mm petri dishes or 24-well tissue culture clusters (Costar; Belco Glass, Inc., Vineland, NJ).

C5-deficient mouse serum was obtained from 20–30-g female, C5-deficient AKR mice (The Jackson Laboratory, Bar Harbor, ME). It was adsorbed twice at 4°C for 15 min with 20% sheep E before being frozen in small aliquots at -70°C, and was thawed on ice just before use as a complement source.

**Preparation of Immobilized Immune Complexes.** Immobilized immune complexes were fixed onto 13-mm glass coverslips as previously described (11, 13).

**Preparation of Lymphokine.** Culture supernatants containing the lymphokine that activates mouse peritoneal macrophages' C3 receptors for phagocytosis were prepared and used as previously described (7).

**Assessment of Phagocytosis via Macrophages' C3 Receptors and Fc Receptors.** After a 1 h incubation, macrophage monolayers were washed, overlaid with fresh medium, and incubated for an additional 3 h at 37°C. Some cells were then overlaid with lymphokine-containing supernatant, others with fresh medium. 0.2 ml of 0.5% E, IgG, IgM, or IgMG were added to each 35-mm petri dish, and cultures were incubated for 30 min at 37°C. Cultures were then washed and fixed with glutaraldehyde, and E attachment and ingestion were scored by phase contrast microscopy. Because in all of these assays all macrophages avidly bound IgMG and none bound or ingested either E or IgM, individual attachment and ingestion data obtained using these particles are not reported.

**Assessment of Macrophage C3 Receptor Mobility.** Freshly harvested macrophages were plated on coverslips coated with C3-containing immune complexes. After a 1 h incubation to allow macrophages to adhere, coverslips were washed and were overlaid with lymphokine-containing supernatant or fresh medium. After a 15 min incubation at 37°C, cultures were washed and covered with 1 ml of fresh medium. 0.1 ml of 0.5% IgMG was added to each well and cultures were incubated for 1 h at 37°C, washed, and fixed with glutaraldehyde. E attachment was scored by phase contrast microscopy.

In this assay, C3 receptors that are mobile become trapped on the immobilized immune complexes when they diffuse randomly from the nonadherent to the adherent macrophage surface; macrophages with mobile receptors therefore lose the ability to bind IgMG. In contrast, C3 receptors that are immobile are unable to diffuse within the macrophage plasma membrane and remain in place even when macrophages are plated on immobilized immune complexes; these macrophages therefore retain the ability to bind IgMG.

**Presentation of Results.** Unless otherwise indicated, each result presented is the average of at least three separate determinations, each performed in duplicate. The attachment index and the phagocytic index are the number of E attached or ingested by 100 macrophages and were obtained by multiplying the percent of macrophages that had attached or ingested any E by the average number of E attached or ingested per macrophage. Each result is presented as mean ± SEM.

**Results**

Outbred Swiss mice were injected intraperitoneally with either soluble immune complexes or aggregated IgG. 4 d later, peritoneal macrophages were harvested, plated as described in Materials and Methods, and assayed for their ability to phagocytize via their Fc receptors and C3 receptors. Macrophages from mice that had received no injection avidly ingested IgG but failed to ingest IgMG (Table I, line 1), as expected. Macrophages from mice injected with either nonaggregated IgG or "sham" immune complexes composed of HSA and non-immune rabbit serum behaved identically to those from mice that had received no injections (data not shown). In contrast, macrophages from mice that had
TABLE I
Effects of In Vivo Administration of Immune Complexes and Aggregates on the Fc Receptor and C3 Receptor Function of Macrophages from Euthymic and Athymic Mice

| Mice             | Intraperitoneal injection | Phagocytic index |
|------------------|---------------------------|------------------|
|                  |                           | IgG              | IgMC             |
| Swiss            | None                      | 461 ± 26         | 0                |
|                  | HSA-anti-HSA              | 47 ± 3           | 79 ± 9           |
|                  | Aggregated IgG            | 89 ± 10          | 91 ± 24          |
| Euthymic littermates | None                      | 254*             | 0                |
|                  | HSA-anti-HSA              | 36*              | 138 ± 13         |
|                  | Aggregated IgG            | 48*              | 88 ± 11          |
| Athymic          | None                      | 465*             | 0                |
|                  | HSA-anti-HSA              | 113*             | 4 ± 4            |
|                  | Aggregated IgG            | 49*              | 6 ± 3            |

* Single determination.

TABLE II
Effect of Trypsin Treatment on Macrophage Fc Receptor Function

| Intraperitoneal injection | Phagocytic index of IgG by macrophages |
|---------------------------|----------------------------------------|
|                           | Nontreated | Trypsin-treated |
| None                      | 461 ± 26   | 424 ± 18        |
| HSA-anti-HSA              | 47 ± 3     | 284 ± 7         |
| Aggregated IgG            | 89 ± 10    | 309 ± 16        |

been injected with either immune complexes or aggregates ingested ElgG poorly but were able to ingest ElgMC (Table I, lines 2 and 3). The defective uptake of ElgG by macrophages from mice that had received injections of immune complexes appeared to be due to blockade of the cells' Fc receptors by the complexes because treatment with 1 mg/ml of trypsin for 30 min at 37°C, a procedure that releases immune complexes from cells' Fc receptors (14), enabled macrophages to phagocytize ElgG efficiently (Table II).

To determine whether or not T lymphocytes were required for in vivo activation of macrophages' C3 receptors, we performed experiments identical to those reported in Table I, lines 1-3, using athymic mice and their euthymic, congeneric littermates. The macrophages of the euthymic littermates behaved identically to those of outbred Swiss mice (Table I, lines 4-6); however, macrophages from athymic mice were unable to phagocytize ElgMC (Table I, lines 7-9). Macrophages from athymic mice could, after immune complex uptake, signal T lymphocytes from other mice to elaborate the lymphokine (data not shown), and these animals' macrophages could respond to the lymphokine in vitro (Table III), indicating that the failure of their C3 receptors to be activated in vivo was not due to an intrinsic defect in the macrophages' ability to participate in the generation of the lymphokine or in their ability to respond to it, but rather was due to the absence of T lymphocytes. Thus, activation of macrophages' C3 receptors in vivo requires T lymphocytes.

The C3 receptors of mouse peritoneal macrophages are normally immobilized and unable to diffuse within the cell's plasma membrane (11, 15). Activation of macrophages' C3 receptors for phagocytosis by the lymphokine in vitro is accompanied by the acquisition of receptor mobility (11). We found that receptor
TABLE III
Response of Macrophages from Athymic Mice to Lymphokine Treatment In Vitro

| Intraperitoneal injection | Phagocytic index of EIgMC |
|---------------------------|---------------------------|
|                           | Medium | Lymphokine |
| None                      | 0      | 92 ± 1     |
| HSA-anti-HSA              | 4 ± 4  | 110 ± 6    |
| Aggregated IgG            | 6 ± 3  | 101 ± 6    |

TABLE IV
Effect of In Vivo Administration of Immune Complexes and Aggregates on Macrophages' C3 Receptor Mobility

| Mice              | Intraperitoneal injection | Attachment index of EIgMC |
|-------------------|---------------------------|---------------------------|
|                   |                           |                           |
| Swiss             | None                      | 1,224 ± 28                |
|                   | HSA-anti-HSA              | 108 ± 16                  |
|                   | Aggregated IgG            | 115 ± 26                  |
| Euthymic Liquidmates | None                      | 1,074 ± 24                |
|                   | HSA-anti-HSA              | 83 ± 16                   |
|                   | Aggregated IgG            | 83 ± 22                   |
| Athymic           | None                      | 692 ± 76                  |
|                   | HSA-anti-HSA              | 795 ± 35                  |
|                   | Aggregated IgG            | 762 ± 68                  |

mobility accompanied in vivo activation of macrophages' C3 receptors as well (Table IV). C3 receptors of macrophages from all mice were intrinsically immobile (Table IV, lines 1, 4, and 7) and unable to promote phagocytosis (Table I, lines 1, 4, and 7). C3 receptors of macrophages from euthymic mice that had received intraperitoneal injections of immune complexes or aggregates were both mobile (Table IV, lines 2, 3, 5, and 6) and able to promote phagocytosis (Table I, lines 2, 3, 5, and 6). In contrast, C3 receptors of macrophages from athymic mice that had received injections of immune complexes or aggregates were both immobile (Table IV, lines 8 and 9) and unable to promote phagocytosis (Table I, lines 8 and 9). These results support our hypothesis that the lymphokine activates macrophages' C3 receptors for phagocytosis by freeing the receptors from their anchors and allowing them lateral mobility.

Discussion

In the present experiments we created an immunologically mediated inflammatory focus in the mouse peritoneal cavity by injecting either soluble immune complexes or aggregated IgG. We found that macrophages present at this inflammatory site were markedly impaired in their ability to ingest via their Fc receptors but had acquired the ability to phagocytize via their C3 receptors.

We have previously (9, 10) found that macrophages' Fc receptors could be blocked in vitro by microgram quantities of immune complexes. Our finding that macrophages obtained from an in vivo milieu containing immune complexes were defective in their ability to phagocytize IgG-coated particles strongly suggests that immune complexes can block the cells' Fc receptors in vivo as well. Receptor blockade was apparently achieved by occupancy of receptors by the complexes rather than by receptor internalization or destruction, because trypsin
treatment promptly restored Fc receptor activity. The apparent ease with which
macrophages' Fc receptors can be blocked by immune complexes in vivo strongly
suggests that these receptors may be unable to promote uptake of opsonized
microorganisms at sites of infection in an immune host.

Macrophages' C3 receptors are not normally phagocytic (1-10). We have
previously found (7-12) that a unique lymphokine can activate these receptors
so that they can promote phagocytosis. For T lymphocytes to elaborate the
lymphokine, they must receive a signal from macrophages that have ingested
IgG-containing immune complexes (7). We therefore determined the ability to
phagocytize via their C3 receptors of macrophages obtained from an inflamma-
tory focus containing macrophages, immune complexes, and T lymphocytes.
These macrophages were able to phagocytize C3-coated particles, indicating that
their C3 receptors had been activated in vivo. The requirement for both T
lymphocytes and immune complexes for activation of macrophages' C3 receptors
in vivo is identical to the requirements for activation of macrophages' C3
receptors in vitro, suggesting that the mechanisms we have identified for activa-
tion of these receptors in vitro are the same mechanisms by which the receptors
are activated for phagocytosis in vivo.

Our findings support the following model for the ingestion of opsonized
microorganisms in vivo. Invading microorganisms become coated with both IgG
and C3 in the immune host. Many pathogens shed antigens that become bound
by host antibody, and soluble immune complexes composed of shed microbial
antigen and host antibody are probably plentiful at sites of infection. Macro-
phages' Fc receptors are easily blocked when they ingest the immune complex
debris, thereby impairing these receptors' ability to clear IgG-coated microor-
ganisms. Uptake of these soluble complexes triggers macrophages to signal T
lymphocytes to elaborate the lymphokine that activates macrophages' C3 recep-
tors for phagocytosis. Unlike their Fc receptors, macrophages' C3 receptors are
not very susceptible to blockade by immune complexes, even when the complexes
contain complement components (9). These receptors are therefore able to
promote phagocytosis of the opsonized microorganisms. Note that our model
proposes a most unusual type of receptor cooperation in which ingestion of
immune complex debris by macrophages' Fc receptors initiates a series of cellular
and molecular events culminating in activation of the cells' C3 receptors and that
it is the macrophages' C3 receptors, not their Fc receptors, that are primarily
responsible for promoting phagocytosis of opsonized microorganisms.

Summary

We assessed the effects of exposure to immune complexes in vivo on macro-
phages' Fc receptor function and C3 receptor function. Peritoneal macrophages
from mice injected intraperitoneally with immune complexes were markedly
impaired in their ability to phagocytize via their Fc receptors but had acquired
the ability to phagocytize via their C3 receptors. In vivo activation of macro-
phages' C3 receptors for phagocytosis required T lymphocytes, because macro-
phages from athymic mice could not be activated by injection of immune
complexes.

The requirement for both T lymphocytes and immune complexes for activa-
tion of macrophages' C3 receptors in vivo is identical to the requirements for
activation of macrophages' C3 receptors in vitro, suggesting that the mechanisms
we have identified for activation of these receptors in vitro are the same mechanisms by which the receptors are activated for phagocytosis in vivo. The susceptibility of macrophages’ Fc receptors to blockade by immune complexes and the activation of their C3 receptors for phagocytosis in a milieu containing immune complexes suggest that it may be macrophages’ C3 receptors, not their Fc receptors, that are primarily responsible for promoting phagocytosis of opsonized microorganisms in immune hosts.

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