Effects of a Phagocytosis-Stimulating Factor Derived from Polymorphonuclear Neutrophils on the Functions of Macrophages

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The effects of phagocytosis-stimulating factor (PSF) derived from polymorphonuclear neutrophils on macrophage functions were studied. PSF enhanced the initial rate of phagocytosis of serum-opsonized zymosan particles by macrophages, whereas it did not affect the phagocytosis of immunoglobulin G-sensitized and inert zymosan particles. Kinetic studies showed that PSF accelerated the ingestion step, but not the attachment step, of phagocytosis by macrophages. On the other hand, PSF did not affect the other macrophage functions such as O$_2^-$ generation, chemotaxis, adherence, and enzyme release. These results suggest that PSF may specifically modulate the complement receptor function of macrophages. Immunoblot assay showed the absence of components in macrophage which reacted with purified antibodies against polymorphonuclear neutrophil-derived PSF, and an extract from phagocytosing macrophages had no phagocytosis-stimulating activity, indicating that the macrophages did not produce PSF-like substances.

We have reported that phagocytosis-stimulating factor (PSF), generated from polymorphonuclear neutrophils (PMNs) during phagocytosis, is capable of enhancing the ingestion step of complement receptor-mediated phagocytosis by PMNs specifically (6, 7). Recently, PSF has been purified to homogeneity (8), and the precursor of PSF has been identified in the granule fraction by immunoblot assay using purified anti-PSF antibodies (9).

Since macrophages as well as PMNs are well known to be professional phagocytes which play a central role in host defense against invading microorganisms, it is of interest to investigate the effect of PSF derived from PMNs on the functions of macrophages.

In this report, therefore, the effects of PSF on macrophage functions, i.e., phagocytosis, O$_2^-$ generation, chemotaxis, enzyme release, and adherence, were studied. Further, to investigate the possibility that macrophages may be capable of generating PSF-like substances, we examined whether the extract from phagocytosing macrophages had phagocytosis-stimulating activity. We also attempted by immunoblot assay, to detect macrophage components which react with purified antibodies against PMN-derived PSF.

MATERIALS AND METHODS

Animals. Hartley guinea pigs, weighing approximately 500 g, were used as the source of PMNs and macrophages.

Reagents. Glycogen, zymosan A, cytochrome c (type VI), superoxide dismutase (type I), cytochalasin B, phenolphtha-lein glucuronic acid sodium salt, N-formylthionyl-leucyl-phenylalanine, phorbol myristate acetate, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo. Micrococcus lysodeikticus was obtained from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Nitrocellulose paper (0.45-μm pore size) was purchased from Millipore Corp., Bedford, Mass. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Bio-Rad Laboratories, Richmond, Calif. 3,3′-Diaminobenzidine tetrahydrochloride was purchased from Wako Chemical Co., Ltd., Osaka, Japan.

Preparation of PMNs and macrophages. PMNs were isolated from the peritoneal cavity 13 to 15 h after an intraperitoneal injection of sterilized 0.12% glycogen in saline, as described previously (24). Resident macrophages were harvested by peritoneal lavage of untreated guinea pigs. Glycogen-induced and thioglycollate-elicited macrophages were obtained 4 days after intraperitoneal injection of sterilized 0.12% glycogen in saline and thioglycollate medium, respectively (16). The collected cells were suspended at 2 × 10$^7$ cells per ml in Hanks balanced salt solution (HBSS).

Opsonization of zymosan. Zymosan particles were opsonized with homologous fresh serum at 37°C for 30 min as described previously (1). IgG-sensitized zymosan particles were prepared by incubation of human albumin-modified zymosan particles with rabbit anti-human albumin IgG as described previously (7).

Preparation of purified PSF. Purified PSF was prepared from phagocytosing PMNs as described previously (8). In brief, 10$^7$ PMNs per ml in HBSS were incubated with 1.0 mg of opsonized zymosan particles per ml at 37°C for 60 min with constant shaking, washed once, and suspended in phosphate-buffered saline to 1/10 of the original volume. Then, phagocytosing PMNs were disrupted by sonication and centrifuged at 100,000 × g for 60 min at 4°C. The resulting crude supernatant containing PSF was heated at 80°C for 30 min and then centrifuged at 100,000 × g for 60 min at 4°C to remove the denatured materials. The supernatant was chromatographed over a Sephadex G-100 column in phosphate-buffered saline (pH 7.2) at 4°C, and the fractions corresponding to approximately 16,000 daltons were collected. The resulting material was referred to as partially purified PSF. To purify PSF further, partially purified PSF was mixed with 1/2 volume of 40 mM Tris–5 mM phosphate–0.5 M NaCl buffer (pH 8.2), adjusted to pH 8.2 with 0.1 N NaOH, applied to a copper chelate affinity column, and then eluted with a linear gradient from 20 mM sodium phosphate–0.5 M NaCl (pH 7.7) to 0.1 M acetic acid–0.5 M NaCl (pH 2.8). The fractions eluted at pH 3.7 were collected.

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dialyzed against distilled water, lyophilized, stored at −60°C, and dissolved in phosphate-buffered saline before use. Purified PSF thus obtained showed an apparent homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Assay for phagocytosis.** The phagocytic activity of macrophages was assayed as described previously (6, 7). In brief, a 0.4-ml sample of macrophage suspension (10⁶ cells per ml) in HBSS supplemented with 20% heat-inactivated guinea pig serum was placed in a 35-mm plastic petri dish containing two glass cover slips (22 by 10 mm). Cells were permitted to adhere to the cover slips for 1 h at 37°C and were then washed twice with HBSS to remove nonadherent cells. Phagocytosis was initiated by the addition of 0.24 ml of opsonized zymosan suspension (10⁵ particles per ml) to the macrophage monolayer in a petri dish with or without PSF in a total volume of 2 ml. After incubation at 37°C for 10 min, the cover slips were washed with HBSS, fixed with ethanol, and stained with Wright-Giemsa, and phagocytosis was determined microscopically. The phagocytic index and the attachment index were defined as the percent positive ingestion multiplied by the average number of ingested particles per cell and the percent positive attachment multiplied by the average number of attached particles per cell, respectively (15).

**Assay for superoxide anion production.** Generation of superoxide anions by macrophages was measured on the basis of superoxide dismutase-inhibitable cytochrome c reduction (7). Macrophages (10⁵ cells) were incubated at 37°C with or without PSF (1 μg/ml) in a total volume of 1.5 ml of HBSS containing 80 μM cytochrome c with or without superoxide dismutase (30 μg/ml). At various times, each tube was placed in an ice-cold bath to stop the reaction and was then centrifuged at 1,870 × g for 5 min. The absorbance at 550 nm of each supernatant was measured with a Hitachi 200-20 spectrophotometer, and the value of cytochrome c reduction was calculated from the equation: ΔE₅₅₀ = 2.1 × 10⁴ M⁻¹ cm⁻¹ (10).

**Assay for enzyme release from macrophages.** The release of enzymes from macrophages was examined by incubating 10⁵ macrophages per ml in HBSS with or without 10⁴ opsonized zymosan particles in the presence or absence of PSF (1 μg/ml) for 0, 10, 30, and 60 min in a total volume of 2.0 ml in a shaking water bath (80 excursions per min). After incubation, each tube was placed in an ice bath and then centrifuged at 1,870 × g for 5 min. The activities of β-glucuronidase and lysozyme in the supernatant were measured. Total enzyme activities were determined in the presence of 0.1% Triton X-100 by using cell suspensions. Lysozyme activity was determined by measuring the rate of lysis of M. lysodeikicus at pH 6.2 by a turbidimetric method (20). β-Glucuronidase activity was determined by measuring phenolphthalein released from phenolphthalein glucuronate at pH 5.0 (1). Enzyme release was expressed as the percentage of the enzyme activity in the supernatant relative to the total activity.

**Assay for adherence.** Each macrophage suspension containing 2 × 10⁵ cells, with or without PSF (1 μg/ml), in 0.4 ml of HBSS was placed in a glass tube (14 by 120 mm). After incubation at 37°C for 0, 10, 30, and 60 min, tubes were washed three times with HBSS to remove the nonadherent cells, and then 2.0 ml of PBS was added. Each tube was sonicated at 168 W for 30 s, and the protein content of adherent cells was determined by the method of Lowry et al. (13). The adhesiveness of macrophages was expressed as the percentage of adherent macrophages to total macrophages before adherence in terms of cell protein concentration.

**Assay for chemotaxis.** Macrophage migration was assayed by employing a modified Boyden chamber with Sartorius membrane filters (pore size, 5 μm) to separate the chamber into compartments as described previously (24). Test solution (200 μl) was placed in the lower compartment, and 300 μl of macrophage suspension (3 × 10⁶ cells per ml) with or without PSF (1 μg/ml) was introduced into the upper compartment. After incubation at 37°C for 60 min, the filter was fixed and stained. The distance from the top of the filter to the furthest two cells in the same focal plane was measured microscopically by the method of Zigmund and Hirsch (25).

**Polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was carried out in 15% sodium dodecyl sulfate according to the method of Laemmli and Favre (12) with a slight modification (5). A slab gel (60 by 87 by 1 mm) with a linear 7.5 to 20% gradient of polyacrylamide was used. Electrophoresis was carried out for about 2 h at 15 mA.

**Blotting procedure.** Electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose paper was carried out at 20 V for 1 h in transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% ethanol (pH 8.3), according to the method of Towbin et al. (21).

**Preparation of purified anti-PSF antibodies.** Antiserum against purified PSF was raised in rabbits as described previously (9). Purified anti-PSF antibodies were obtained from anti-PSF serum by the affinity purification method of Olmsted (17) with a slight modification (9).

**Immunoblot assay.** Immunodetection of protein on nitrocellulose was carried out as described previously (4). The nitrocellulose paper containing the transferred proteins was soaked in 10% normal goat serum in Tris-buffered saline (TBS) for 30 min at room temperature and then incubated with purified anti-PSF antibodies (1:10 dilution) in TBS containing 10% normal goat serum overnight at 4°C. The nitrocellulose paper was rinsed five times with TBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution) in TBS containing 1% bovine serum albumin for 1 h at room temperature, and then rinsed five times with TBS containing 0.05% Tween 20. To develop the staining, the nitrocellulose paper was exposed to TBS containing 0.5 mg of 3,3′-diaminobenzidine tetrahydrochloride per ml and 0.05% H₂O₂. The reaction was terminated after 30 min by washing with cold water.

**RESULTS**

Effect of PSF on phagocytosis by macrophages. Table 1 shows that PSF enhanced the phagocytosis of complement-opsonized zymosan particles by resident macrophages, but had no effect on the phagocytosis of IgG-sensitized zymosan particles. PSF only slightly affected the phagocytosis of inert zymosan particles by macrophages over the range of a macrophage-to-particle ratio of 1:60. Since, in mouse macrophages, it has been reported that the complement receptor-mediated phagocytic activity of glycogen-induced and thiglycolate-elicited macrophages is higher than that of resident macrophages (2, 14), we also examined the effect of PSF on phagocytosis of complement-opsonized zymosan particles by glycogen-induced and thiglycolate-elicited macrophages. In guinea pig macrophages, the phagocytic activity of glycogen-induced and thiglyco-
late-elicited macrophages was similar to that of resident macrophages, whereas PSF showed the same phagocytosis-stimulating effect on these macrophages as on resident macrophages (data not shown). Thus, the following experiments were carried out using glycogen-induced macrophages because of the advantage of obtaining a large number of cells.

**Effect of PSF concentration on phagocytosis of complement-opsonized zymosan particles by macrophages.** The dose dependence of PSF on complement receptor-mediated phagocytosis by macrophages is shown in Fig. 1. The phagocytosis-stimulating activity of PSF approached a plateau at a concentration of 0.3 μg/ml, at which point phagocytic activity was enhanced approximately 1.7-fold.

**Kinetics of stimulation of phagocytosis by macrophages.** It is well known that the process of phagocytosis consists of two steps, attachment of particles to the cell membrane and ingestion of the particles (18). To determine which step of phagocytosis by macrophages PSF acts on, we investigated the time course of ingestion and attachment of complement-opsonized zymosan particles by macrophages in the presence or absence of PSF (Fig. 2). Macrophages ingested the complement-opsonized zymosan particles almost linearly with incubation time until 20 min, and the phagocytic index at 10 min was 63.3 ± 2.52. The ingestion of complement-opsonized zymosan particles by macrophages was accelerated in the presence of PSF (1.0 μg/ml), and the phagocytic index at 10 min was 100.4 ± 5.22, indicating that PSF causes an increase of ingestion at the initial rate. On the other hand, the attachment index was not much affected by the addition of PSF. To confirm the effect of PSF on the attachment step, macrophages were exposed to complement-opsonized zymosan particles in the presence of cytochalasin B (5.0 μg/ml), which inhibits ingestion but does not affect attachment of particles to the plasma membrane (26). The ingestion was greatly impaired (>95%), but the number of particles attached to cytochalasin B-treated macrophage surface was the same in the presence and in the absence of PSF (data not shown). These results indicate that the PSF acts on the ingestion step, but not on the attachment step, in the process of phagocytosis by macrophages.

**Effect of PSF on various macrophage functions other than phagocytosis.** We first examined the effect of PSF on superoxide anion generation associated with phagocytosis by macrophages (Fig. 3). When complement-opsonized zymosan particles (cell-to-particle ratio, 1:1) were added to the macrophages, O$_2^-$ generation from control macrophages increased rapidly with increasing incubation time and reached a plateau within 7.5 min. Addition of PSF to the reaction mixture had no effect on the phagocytosis-induced O$_2^-$ generation. The effect of PSF on soluble stimuli-induced O$_2^-$ generation from macrophages was also examined. Rates of O$_2^-$ generation from macrophages stimulated with phorbol myristate acetate (50 ng/ml), N-formylmethionyl leucyl phenylalanine (10$^{-7}$ M), and concanavalin A (50 μg/ml) were 27.91 ± 0.46, 1.70 ± 0.10, and 6.09 ± 0.33 nmol per 2 × 10$^6$ cells per min, respectively. PSF (1 μg/ml) showed no effect on O$_2^-$ generation by macrophages in response to these soluble stimuli.

Next, we examined the effect of PSF on chemotaxis of macrophages.

**TABLE 1. Effect of PSF on phagocytosis by macrophages**

<table>
<thead>
<tr>
<th>Particle Description</th>
<th>Cell/particle ratio</th>
<th>Phagocytic index (Control)</th>
<th>Phagocytic index (PSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement-opsonized zymosan</td>
<td>1:6</td>
<td>64.9 ± 1.82</td>
<td>108.8 ± 6.76</td>
</tr>
<tr>
<td>IgG-sensitized zymosan</td>
<td>1:6</td>
<td>43.2 ± 1.31</td>
<td>40.4 ± 1.91</td>
</tr>
<tr>
<td>Inert zymosan</td>
<td>1:6</td>
<td>7.2 ± 0.76</td>
<td>7.0 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>12.9 ± 1.27</td>
<td>11.5 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>1:60</td>
<td>18.2 ± 0.45</td>
<td>17.1 ± 0.91</td>
</tr>
</tbody>
</table>

* Macrophage monolayers (4 × 10$^5$ cells per dish) were incubated with complement-opsonized, IgG-sensitized, or inert zymosan particles in the presence or absence of PSF (1.0 μg/ml) in a total volume of 2.0 ml at 37°C for 10 min. After incubation, phagocytosis was assayed microscopically. The values represent the means ± standard errors of three experiments.
macrophages, PSF did not significantly affect macrophage chemotaxis in response to zymosan-activated serum and Escherichia coli extract. PSF did not exhibit chemotactic and chemokinetic activities (data not shown).

The effect of PSF on enzyme release and adhesiveness of macrophages was also studied. During phagocytosis of opsonized zymosan particles, 10.30 ± 0.30% lysozyme, a specific granule constituent of macrophages, and 5.59 ± 1.55% β-glucuronidase, an azurophil granule constituent, were released from macrophages within 10 min. PSF (1 μg/ml) did not cause any effect on enzyme release.

We found that 58.5 ± 1.3% of macrophages adhered to a glass tube in 10 min, and PSF showed no effect on adhesiveness.

Possibility of the generation of PSF-like substances from macrophages. Since it was reported that both PMNs and macrophages could produce endogenous pyrogen during phagocytosis (3), it is possible that macrophages are capable of generating PSF-like substances during phagocytosis. We determined whether macrophages possess a component with an antigenic determinant common to PMN-derived PSF by immunoblot assay using purified anti-PSF antibodies. A protein with a molecular weight of 36,000, the precursor of PSF (9), was detected in PMNs (Fig. 4), whereas resident and glycogen-induced macrophages did not possess any components which reacted with purified anti-PSF antibodies. Various reports have demonstrated that thioglycolate-elicited macrophages but not resident macrophages could produce some enzymes such as plasminogen activator and collagenase (11, 22). However, no protein band that reacted with purified anti-PSF antibodies was detected even in the thioglycolate-elicited macrophages. In addition, we examined whether the extract from phagocytosing macrophages had a phagocytosis-stimulating activity, but such an activity was not found (data not shown). These results indicate that macrophages do not generate PSF-like substances.

DISCUSSION

We have reported that PSF, generated from phagocytosing PMNs, enhances the ingestion step of complement receptor-mediated phagocytosis by PMNs specifically. The present study showed that PSF enhanced complement receptor-mediated phagocytosis by macrophages, whereas neither IgG-mediated nor nonspecific phagocytosis was affected by PSF. Kinetic studies of phagocytosis revealed that PSF did not affect the attachment step, but enhanced the ingestion step, in the process of complement receptor-mediated phagocytosis by macrophages. These results clearly indicate that the mode of action of PSF on phagocytosis by macrophages is similar to that of PMNs (7). Recently, it has been recognized that neutrophils and macrophages have at least two distinct complement receptors, i.e., type 1 (CR1) and type 3 (CR3) (19). It still remains to be elucidated which type of receptor is affected by PSF.

We previously demonstrated that PSF did not affect the other PMN functions such as chemotaxis, enzyme release, and adherence (7). Although phagocytosis-associated O₂⁻ generation from PMNs was slightly increased by PSF treatment, this stimulation was thought to be due to the stimulation of phagocytosis by PSF rather than to the direct stimulation of the O₂⁻-forming system by PSF, because resting PMNs generated little O₂⁻ in response to PSF treatment. In the case of macrophages, no significant enhancement of O₂⁻ generation by PSF was observed, which is presumably due to the generation of much O₂⁻ in the initial period of phagocytosis under our conditions. We also found that PSF affected macrophage functions such as chemotaxis,

![FIG. 3. Effect of PSF on O₂⁻ generation from macrophages. Macrophages (10⁷ cells) were incubated with 10⁷ opsonized zymosan particles in the presence (○) or absence (●) of PSF (1.0 μg/ml) in a total volume of 1.5 ml of HBSS containing 80 μM cytochrome c. with or without 30 μg of superoxide dismutase per ml, at 37°C with constant shaking. The values represent the means ± standard errors of three experiments.](http://iai.asm.org/)

![FIG. 4. Detection of proteins which react with purified anti-PSF antibodies. Samples (20 μl each) of PMNs, resident macrophages, glycogen-induced macrophages, and thioglycolate-elicited macrophages (10⁷ cells per ml) were subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and then blotted onto nitrocellulose paper. The nitrocellulose paper was treated with 10% normal goat serum and then incubated with purified anti-PSF antibodies (1:10) in TBS containing 10% normal goat serum overnight at 4°C. After being washed with TBS, the nitrocellulose paper was treated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000) in TBS containing 1% bovine serum albumin for 1 h at room temperature and then stained by means of peroxidase activity. (A) Coomassie blue-stained polyacrylamide gel. (B) Immunoblotted nitrocellulose paper. Lanes (both panels): 1, PMNs; 2, resident macrophages; 3, glycogen-induced macrophages; 4, thioglycolate-elicited macrophages. Protein standards used were phospholipase b (94,000 [94K]), albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20K), and α-lactoalbumin (14K).](http://iai.asm.org/)
enzyme release, and adherence only slightly. These results indicate that the effect of PSF is specific for the phagocytic function of macrophages as well as that of PMNs.

In the inflammatory process the rapid accumulation of PMNs is observed, followed by the prolonged accumulation of macrophages at the inflammatory sites. It is well known that PMNs act only as professional phagocytes, whereas macrophages act not only as immunocompetent cells but also as professional phagocytes. Recently, Wright et al. (23) have reported the presence of a lymphokine which enhances the complement receptor-mediated phagocytosis by macrophages. However, this lymphokine is not utilized in the early stage of inflammation, at which point a specific immune response is not yet induced. As shown in this communication, macrophages do not generate PSF-like substances, but PSF, generated from phagocytosing PMNs, efficiently enhances the phagocytic activity not only of PMNs but also of macrophages. Therefore, PSF seems to have an important role in the host defense mechanism in the early stage of inflammation.

LITERATURE CITED


