Stimulation of Bone Marrow-Derived and Peritoneal Macrophages by a T Lymphocyte-Derived Hemopoietic Growth Factor, Persisting Cell-Stimulating Factor

By Richard M. Crapper, Gino Vairo, John A. Hamilton, Ian Clark-Lewis, and John W. Schrader

Several lines of evidence indicated that P cell-stimulating factor (PSF), a T lymphocyte-derived lymphokine known to stimulate the growth of hemopoietic stem and progenitor cells, also acted on macrophages. PSF was absorbed from medium that had been mixed for two hours at 0°C with either resident or thioglycollate-elicited peritoneal cells, suggesting the presence of receptors for PSF on cells in the population. The addition of pure PSF to populations highly enriched in either resident or elicited adherent peritoneal macrophages resulted in stimulation of macrophages with morphological changes, including increases in size, spreading, vacuolation, and the number of cytoplasmic processes, together with stimulation of proliferation and the phagocytosis of opsonized yeast. PSF also stimulated the incorporation of [3H]thymidine by bone marrow-derived adherent macrophages. Addition of pure PSF to cultures that contained only a single macrophage resulted in enhanced survival and proliferation of these isolated cells, demonstrating that the effect of PSF on macrophages was direct. These results indicate that PSF can stimulate well-differentiated functional macrophages and raise the possibility that the effects of PSF on macrophages may play a regulatory role in immune responses.

MATERIALS AND METHODS

Mice. CBA/H/Wehi mice were bred at the Hall Institute under specific pathogen-free conditions. At 6 weeks of age they entered conventional mouse rooms. C3H/Hej mice were obtained from Bar Harbor, Me., and were also bred at the Hall Institute in conventional mouse rooms. Mice were used between 7 and 10 weeks of age.

Macrophages. Resident peritoneal cells were obtained by washing out the peritoneal cavity of normal mice with 5 mL of HEPES-buffered Eagle's medium (HEM). To obtain the elicited cells, 1.5 mL of Brewer thioglycollate medium (Difco, Detroit) was injected intraperitoneally (IP) four days before harvesting. To obtain a polymorph-enriched peritoneal exudate, mice were injected IP with 2 mL of 0.2% (wt/vol) calcium caseinate 12 hours earlier.

Sources of growth factors. Medium conditioned by WEHI-3B, a myelomonocytic leukemia (W3CM), was produced and concentrated as previously described and was used as a source of PSF. WEHI-3B.S.6 is a subline of WEHI-3B, generated in this laboratory, which produces no PSF in vitro.

Conditioned medium (B.S.6 CM) was produced and concentrated as for W3CM. L cell-conditioned medium (LCCM) was produced by growing L cells to confluence in 850-cm² plastic roller bottles. The supernatant was collected, centrifuged, and used unconcentrated.

Purified PSF. PSF was purified 50,000-fold from serum-free WEHI-3B conditioned medium as described. This material contained a single N-terminal amino acid sequence, which corresponded to that predicted for the nucleotide sequence of cDNA, encoding an activity supporting the growth of lines of mast cells. PSF assay. PSF assays were performed as previously described. In brief, 8 µL of medium containing 500 cells of a cloned WEHI-3B conditioned line, R6-X, were added to 2 µL of duplicate serial twofold dilutions of W3CM. After incubation for three days, the
cells were pulsed with $[^3H]$thymidine, and after six hours the incorporated counts were determined.

Assessment of phagocytic activity. A suspension of bakers' yeast was boiled for 30 minutes and opsonized by incubation at 37 °C for 30 minutes in fresh mouse serum. Adherent macrophages on coverslips were incubated for one hour with $10^5$ yeast in 1 mL of RPMI medium at 37 °C in the 16-mm wells of Linbro trays (Flow Laboratories, McLean, Va). After this, the coverslips were removed, washed, dried, fixed in methanol, and stained with Giemsa. The ingested yeast appeared as obvious blue-staining spherical inclusions in the cytoplasm of the macrophages. The phagocytic activity is presented as yeast ingested per macrophage ± SEM.

Thymidine incorporation by adherent macrophages. Bone marrow from CBA mice was cultured in RPMI, 15% fetal calf serum (FCS), and 20% L cell conditioned medium (LCCM) for three days in 100-mL Roux flasks. The nonadherent cells were harvested, plated out at 2.5 x $10^4$ cells per 200 μL in Linbro flat-bottom trays in medium containing FCS and 25% LCCM, and cultured for five days. The cells were then washed twice with phosphate-buffered saline (PBS), and the remaining adherent macrophages were cultured overnight in medium and FCS. The next day fresh medium containing the growth factor to be assayed and 0.5 μCi of $[^3H]$thymidine was added. The cells were cultured for another three days and then harvested, using a Skatron harvester (Flow Laboratories, Solna, Sweden), onto glass fiber filters using 0.5% Triton X-100 (Sigma, St Louis) followed by H$_2$O.

Endotoxin. Limulus amebocyte lysate (LAL) gelation test (Whittaker, M.A., Bioproducts, Walkersville, MD) was used for endotoxin measurements. Lipopolysaccharide (LPS) from Escheridria coli serotype 026:B6 (Sigma Chemical Co, St Louis) was used for calibration of the LAL test and for stimulation of macrophage phagocytic activity.

RESULTS

Absorption of PSF activity by peritoneal cells. Either resident or thioglycollate-elicited peritoneal cells, $10^7$ or $2 \times 10^7$, were mixed with 1 mL of a 1:75 dilution of W3CM, as a source of PSF, in HEM containing 3% FCS and, to inhibit proteolysis, phenoxy-methyl sulfonyl fluoride (1 mmol/L), iodoacetamide (1 mmol/L), and ethylene diamine tetra-acetic acid (1 mmol/L) were added. The cell suspensions were agitated at 0 °C for two hours, a period shown in preliminary experiments to allow maximal absorption (data not shown), and the tubes were then centrifuged. The supernatants, together with a control 1:75 dilution of W3CM, also containing the protease inhibitors, were dialyzed to remove the inhibitors and assayed for PSF activity. Figure 1 shows the thymidine incorporation at each of six serial, twofold dilutions for each supernatant.

Mixing experiments indicated that the supernatants, from a suspension of either resident or elicited peritoneal macrophages in medium alone, had no inhibitory effect on $[^3H]$thymidine incorporation by R6-X cells stimulated by a 1:75 dilution of W3CM.

A significant amount of PSF activity was removed by resident peritoneal cells, but thioglycollate-elicited cells absorbed substantially more. The observation that the elicited population absorbed more PSF suggested that the cell responsible for the removal of activity was a macrophage, as this is the predominate cell type in the mouse peritoneal cavity after thioglycollate injection. Certainly polymorphs seemed not to absorb PSF, as peritoneal cells elicited by IP injection of calcium caseinate 12 hours previously and enriched predominately for polymorphs absorbed less PSF activity than did resident peritoneal cells (data not shown).

Similar experiments using $10^7$ and $2 \times 10^7$ of the murine macrophage tumor lines J774, RAW-8, and PU-5 revealed no evidence of a significant absorption of PSF (data not shown).

Activation of peritoneal macrophages by PSF. Given the suggestion from the preceding experiments that macrophages were able to absorb PSF, we next investigated the possibility that macrophages were stimulated by PSF. Resident peritoneal cells were plated out at 500 per 10 μL of RPMI with 10% FCS in the wells of Terasaki trays (Lux Corp, Newburg Park, Calif). After 16 hours, the wells were washed twice with cold PBS to remove nonadherent cells and supplemented with fresh medium containing either $6 \times 10^7$ U/mL of pure PSF, $10%$ W3CM ($6 \times 10^4$ U/mL of PSF), or $10%$ B.S.6 CM (no detectable PSF). W3CM and B.S.6 CM, which were produced under the same conditions, were used in parallel as one approach to analyzing the possible role of endotoxin contamination in this phenomenon.

The wells were inspected by phase contrast microscopy on days 2, 4, and 6, and at all of these time points the macrophages incubated with PSF or W3CM were obviously different from those cultured with B.S.6 CM or medium alone and were more numerous, more spread out, more vacuolated, and had longer processes (Fig 2).

Stimulation of proliferation and phagocytosis by PSF. As a measure of functional activation of macrophages, we studied phagocytosis of bakers' yeast, a phenomenon thought to reflect nonspecific receptor-mediated phago-
STIMULATION OF MACROPHAGES BY PSF

Fig 2. Peritoneal macrophages are activated in vitro by PSF. Resident peritoneal cells were added to the wells of Terasaki trays. The wells were washed to remove nonadherent cells and either medium alone, purified PSF (6 x 10^5 U/mL), B.S.6 CM (10%), or W3CM (10%) was added. The trays were incubated for a further three days. (A) Medium alone. Original magnification x 500; current magnification x 385. (B) Purified PSF. Original magnification x 500; current magnification x 385.

Preliminary experiments (data not shown) investigating phagocytic activity after 1, 3, or 5 days of incubation with PSF had indicated that maximal activity was achieved after three days; this time interval was used in future experiments dealing with phagocytosis by peritoneal macrophages.

Resident peritoneal cells (2 x 10^5) were plated out onto glass coverslips in 16-mm wells of Linbro trays and incubated in RPMI medium containing 10% FCS for 16 hours at 37 °C. The coverslips were then removed and washed twice in cold PBS to remove all nonadherent cells and were placed in wells containing either medium alone or medium supplemented with pure PSF, W3CM, or B.S.6 CM. Results of assays of cell proliferation and phagocytic activity are presented in Table 1. B.S.6 supernatant had no effect on the number or activity of the resident macrophages over control, whereas both proliferation and phagocytosis were stimulated by pure PSF in a dose-dependent manner.

Stimulation of phagocytic activity by PSF is not due to LPS. As endotoxin is a potent macrophage activator, experiments were performed to exclude it as a contaminant in the activation observed by PSF. Preparations of purified PSF and LPS were heated to 85 °C for 30 minutes, a procedure known to destroy the biological activity of PSF but not LPS. As shown in Table 2, this treatment eliminated the stimulatory effect of PSF on macrophage activation but had no effect on stimulation by LPS, thus establishing conclusively that the macrophage activation observed with PSF was, in fact, due to PSF.

Furthermore, in separate experiments (data not shown) using the LAL assay system, we determined that our media, the FCS, and the purified PSF all contained less than 1 ng/mL of endotoxin. As shown in Table 2, this amount is less than that required to activate resident peritoneal macrophages.

Table 1. Purified PSF Stimulates the Proliferation and Phagocytic Activity of Resident Peritoneal Macrophages

<table>
<thead>
<tr>
<th>Additive</th>
<th>PSF Activity (U/mL)</th>
<th>No. of Macrophages</th>
<th>Yeast Ingested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12 ± 3</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>Pure PSF 6 x 10^5</td>
<td></td>
<td>39 ± 5*</td>
<td>10.9 ± 1.1†</td>
</tr>
<tr>
<td>Pure PSF 1.8 x 10^5</td>
<td></td>
<td>27 ± 3*</td>
<td>8.5 ± 0.7†</td>
</tr>
<tr>
<td>Pure PSF 6 x 10^4</td>
<td></td>
<td>18 ± 2†</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>B.S.6 CM 10%</td>
<td></td>
<td>14 ± 2</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>W3CM 10%</td>
<td>6 x 10^5</td>
<td>48 ± 4*</td>
<td>11.2 ± 0.9†</td>
</tr>
</tbody>
</table>

Two hundred resident peritoneal cells were plated out onto glass coverslips and allowed to adhere. The coverslips were then washed and placed in wells containing the growth factor as indicated. Counting indicated that the wells contained 17 ± 2 (SEM) macrophages. On day 4 (three days later), the numbers of macrophages were counted again, and phagocytic activity, scoring 50 macrophages for each point, was determined for each macrophage. Results of one representative experiment are presented as means ± SEM.

*P < .001 in comparison to the mean number of macrophages in wells containing B.S.6 CM or medium alone.
†P < .05 in comparison to B.S.6 CM or medium alone.
‡P < .001 in comparison to the mean number of yeast ingested by macrophages cultured in either medium alone or B.S.6 CM.
Cells were harvested using Triton X-100, and results shown are means ± SEM for each determination, and results presented are means (± SEM) for each macrophage.

**PSF stimulates thymidine incorporation by bone marrow-derived macrophages.** Adherent bone marrow-derived macrophages were cultured for three days either in medium alone, pure PSF, LCCM, W3CM, or B.S.6 CM, all in medium containing 0.5 µCi[^3H]thymidine per well. Cells were harvested using Triton X-100, and incorporated thymidine counts were determined. Results are presented in Table 3. It is apparent that substantial activity was incorporated using LCCM and less but still significant activity was incorporated with PSF or W3CM. In contrast, only control levels of[^3H]thymidine incorporation occurred in cultures supplemented with B.S.6 CM.

**PSF stimulates proliferation of single peritoneal macrophages.** A potential problem with the data was that despite the use of low cell numbers and the fact that the wells were washed to remove nonadherent cells, the cell population present was still probably heterogeneous. For example, cells such as fibroblasts, which have been shown to produce CSF-1,[^27] could still be present. To counter this problem, experiments were performed in which each culture well contained only a single adherent macrophage.

In order to further exclude the possibility that the effects of the PSF preparation were in fact due to contamination by endotoxin, we repeated experiments using cells from endotoxin-hypo-responsive C3H/HeJ mice.[^28] Both resident (means of 1.3 x 10^6 cells per mouse) and thioglycollate-elicited (9 x 10^6 cells per mouse) cells from C3H/HeJ mice were plated out at limiting dilution in Terasaki wells and allowed to adhere. After washing out nonadherent cells, wells containing single macrophages were identified as described. These wells were supplemented with either pure PSF (6 x 10^8 units), W3CM (10% containing 6 x 10^8 units of PSF), an optimal concentration of LCCM, B.S.6 CM, or medium alone.

Results of experiments with both resident and elicited macrophages are shown in Fig 3. In wells that were not supplied with purified PSF, W3CM, or LCCM, virtually all the macrophages from the resident peritoneal cell population and approximately half of the macrophages from the elicited peritoneal cell population were dead by day 6. In contrast, in the wells supplemented with pure PSF, LCCM, or W3CM, there was both enhanced survival and evidence of proliferation of macrophages. As beforehand, the macrophages in the wells containing a source of PSF or CSF-1 appeared more

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**Table 2. Effect of PSF and LPS on Macrophage Phagocytic Activity**

<table>
<thead>
<tr>
<th>Additive</th>
<th>Yeast Ingested (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PSF 6 x 10^8 U/mL</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>PSF 1.8 x 10^8 U/mL</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>PSF 6 x 10^7 U/mL</td>
<td>8.6 ± 0.4</td>
</tr>
<tr>
<td>LPS 0.1 ng/mL</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>LPS 1.0 ng/mL</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>LPS 10 ng/mL</td>
<td>9.3 ± 0.3</td>
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</tbody>
</table>

Phagocytic assays were performed as described after three days' incubation with the indicated additive. Preparation of PSF and LPS were heated to 85 °C for 30 minutes. Thirty macrophages were scored for phagocytic activity.

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**Table 3. Stimulation of Bone Marrow-derived Macrophages by PSF**

<table>
<thead>
<tr>
<th>Additive</th>
<th>[^3H]Thymidine Incorporation (Mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>—</td>
<td>321 ± 5</td>
</tr>
<tr>
<td>Purified PSF 6 x 10^8 U/mL</td>
<td>1.634 ± 164*</td>
</tr>
<tr>
<td>Purified PSF 6 x 10^7 U/mL</td>
<td>894 ± 91*</td>
</tr>
<tr>
<td>B.S.6 CM 10%</td>
<td>445 ± 8</td>
</tr>
<tr>
<td>W3CM 10%</td>
<td>1.104 ± 131†</td>
</tr>
<tr>
<td>W3CM 1%</td>
<td>920 ± 109†</td>
</tr>
<tr>
<td>LCCM 10%</td>
<td>5.457 ± 518</td>
</tr>
<tr>
<td>LCCM 2.5%</td>
<td>2.778 ± 618</td>
</tr>
</tbody>
</table>

Adherent bone marrow-derived macrophages were cultured for three days in the presence of the indicated growth factor and [^3H]thymidine. Cells were harvested using Triton X-100, and results shown are means (± SEM) of triplicate determinations from one representative experiment.

[^3H]thymidine: *P < .01 v mean of medium alone.†P < .01 v mean of B.S.6 CM.‡P < .05 v mean of B.S.6 CM.
activated, being larger and exhibiting greater adherence and spreading (Fig 4).

PSF increases phagocytic activity of J774. J774 is a macrophage tumor cell line that has receptors for and is stimulated by CSF-1, the lymphokine stimulating macrophage procoagulant activity and retinoic acid. Despite being unable to detect absorption of PSF activity by any of the tumor cell lines, experiments were performed with J774 to determine whether there was any response to PSF in the phagocytosis assay. As shown in Table 4, a substantial (twofold to threefold) increase in the phagocytic activity of the J774 cells was seen after incubation with PSF.

DISCUSSION

In this report we have demonstrated that purified PSF, a potent lymphokine released by activated T cells, stimulates both the activation, in terms of morphology and phagocytic capacity, and the proliferation of well-differentiated macrophages. The direct action of PSF on normal macrophages was established conclusively by showing effects on single isolated peritoneal macrophages in the wells of Terasaki trays, both resident and elicited peritoneal macrophages responding with increased size, spreading, number and length of processes, and vacuolation. Whereas both macrophage types responded to PSF with increased survival and evidence of activation, the proliferation response was far greater with the elicited macrophages. The stimulatory effect of PSF on phagocytosis was also evident using the cloned macrophage tumor line J774, a further piece of evidence that PSF can act directly on macrophages.

Table 4. PSF Stimulates Phagocytosis of Yeast by J774

<table>
<thead>
<tr>
<th>Additive</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast Ingested</td>
<td>Yeast Ingested</td>
</tr>
<tr>
<td>Purified PSF 6 x 10^5 U/mL</td>
<td>3.8 ± 0.4</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>B.S.6 CM 10%</td>
<td>6.4 ± 0.3*</td>
<td>1.90 ± 0.15*</td>
</tr>
<tr>
<td>W3CM 10%</td>
<td>4.0 ± 0.3</td>
<td>0.65 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>6.3 ± 0.5*</td>
<td>1.68 ± 0.16*</td>
</tr>
</tbody>
</table>

J774 cells (5 x 10^4) were cultured for 48 hours on glass coverslips in medium containing 10% FCS and the indicated growth factor. The coverslips were washed, and phagocytic activity was determined. Results presented are means ± SEM (for each cell) from two separate experiments. *P < .001 vs mean phagocytosis using B.S.6 CM or medium alone.

Four lines of evidence argued against the possibility that these results were due to endotoxin contamination. First, supernatants harvested from parallel cultures of a variant of WEHI-3, WEHI-3.B.S.6, that did not produce PSF, failed to activate macrophages. Second, C3H/HeJ macrophages, which are hyporesponsive to endotoxin, also responded to purified PSF and to W3CM. Third, PSF that had been heat-inactivated failed to stimulate macrophages, whereas in parallel experiments using LPS, the same heat treatment did not reduce the stimulation of phagocytosis. Finally, in separate experiments, it was established that the concentrations of LPS in the media, the FCS, and preparations of purified PSF were less than that required to stimulate phagocytosis by peritoneal macrophages.

It has been suggested that PSF affects mainly immature cells, whereas the other hemopoietic growth factors affect more differentiated mature cells. However, based on our data, PSF can now be added to the list of cytokines, such as CSF-1, GM-CSF, and interferon-γ, known to stimulate mature macrophages. Although the preparation of pure PSF was derived from a myelomonocytic leukemia, WEHI-3, PSF from this source has similar biochemical and biological properties to that derived from the physiological source, the activated T cell, and comparison of cDNA clones generated from WEHI-3 or a C57BL/6 T cell line showed only a single nucleotide difference.

The present findings are consistent with the report that the production of prostaglandin E by macrophages was stimulated by a factor in WEHI-3B conditioned medium that could not be separated from the factor stimulating the growth of granulocyte-macrophage colonies in agar. These effects were probably due to PSF because the only hemopoietic growth factor produced by WEHI-3B is PSF. Similarly, concanavalin A-stimulated spleen cell supernatants were reported to contain an activity that stimulated release of lymphocyte-activating factor by macrophages and that coeluted on gel filtration with colony-stimulating factor. Because T cells produce two hemopoietic growth factors that are active on the macrophage lineage and coelute on gel filtration, PSF and a distinct T cell granulocyte-macrophage CSF, it remains to be determined whether these molecules stimulate LAF release. Gel filtration of phorbol myristate acetate-stimulated EL-4 supernatants has identified two factors activating macrophages in a nonspecific cytotoxicity assay. One of these was interferon-γ, the
other factor had a mol wt of 23,000 and could have been either PSF or GM-CSF or both.

Purified IL 3 was reported to augment the primary cytolytic T lymphocyte response to allogeneic tumor cells. The present experiments raise the possibility that this effect of IL 3 resulted from its effects on macrophages or related accessory cells, an important point in view of the claim that IL 3 acts on helper T cells.

Our data suggest that PSF is an important activator of macrophages in in vivo inflammatory responses. Certainly PSF is released from lymph node cells and spleen cells during immunological reactions, in amounts that would be sufficient to activate macrophages in vitro. It will thus be important to investigate the effect of PSF on parameters of potential immunological significance in the macrophage, such as the expression of Ia antigens and production of IL 1. Because PSF is normally released from T cells together with two other regulators of macrophage function, interferon-γ and GM-CSF, and as there is evidence that in some situations, lymphokines may have opposing effects, it will also be important to investigate interactions between PSF and other T cell lymphokines.

REFERENCES

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