Effect of Colony Stimulating Factor on Murine Macrophages

INDUCTION OF ANTITUMOR ACTIVITY

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ABSTRACT Colony stimulating factor (CSF) was assessed for its capacity to stimulate antitumor activity in macrophages. Murine peritoneal macrophages incubated with CSF for 48 h inhibited [^{3}H]thymidine (TdR) incorporation by P815 tumor cells to ~20% of control values. Inhibition of CSF-stimulated macrophages was significantly greater than inhibition by unstimulated macrophages ($P < 0.001$). CSF had little direct effect on the proliferation of either tumor cells or macrophages alone, indicating that the antitumor activity of CSF was mediated by macrophages. It is unlikely that impurities in the CSF preparations were responsible for the effect since CSF that had been purified to homogeneity was as active as crude preparations. Furthermore the activity of CSF on macrophages was blocked by addition of purified anti-CSF antibodies. In addition to being tumoristatic, CSF-stimulated macrophages were tumoricidal as determined by a tumor colony growth assay. Tumor cells that had been incubated with CSF-stimulated macrophages showed a significant reduction in tumor colony-forming units ($P < 0.01$). Thus, in addition to its effect on hemopoietic stem cells, CSF induces certain effector functions in mature macrophages that may enhance endogenous antitumor host defenses.

INTRODUCTION

Colony stimulating factor (CSF)[1] is a glycoprotein that stimulates hemopoietic stem cells to proliferate and differentiate into granulocytes and macrophages (1). For many years this stimulatory activity of CSF was assumed to be its principal biological property. Recent studies, however, indicate that CSF has other cellular effects including the capacity to directly enhance the function of mature macrophages. For example, CSF induces macrophages to secrete increased amounts of a variety of biological mediators including plasminogen activator (2), prostaglandin E (3), interferon (4), and lymphocyte-activating factor (Interleukin I) (5). In addition, one report showed that CSF caused murine macrophages to develop microbicidal activity against the protozoan *Leishmania tropica* (6). Together, these studies suggest that CSF can, at least, partially activate macrophages. An important function of activated macrophages is to inhibit and kill tumor cells. No studies to date, however, have investigated whether macrophages incubated with CSF develop antitumor activity. The experiments described in this report were designed to determine whether CSF can stimulate macrophages to inhibit tumor cell proliferation.

METHODS

Mice. Female Swiss-Webster and DBA/2 mice were purchased from Hilltop Lab Animals, Scottsdale, Pa., and Jackson Laboratories, Bar Harbor, Maine, respectively. Mice were between 8 and 16 wk old when used and were matched for age in each experiment.

Macrophages. Peritoneal exudate cells (PEC), were used as a source of macrophages. Unstimulated cells were harvested, processed, and counted as previously described (7). PEC were adjusted to a final concentration of 1 to 4.5 × 10^6/ml in medium 199 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum (M. A. Bioproducts, Walkersville, Md.) (M199-FCS). PEC were placed in either 6-mm 96 well Linbro tissue culture plates (Flow Laboratories, Inc.,

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[1] *Abbreviations used in this paper*: CSF, colony-stimulating factor; FCS, fetal calf serum; [^{3}H]TdR, tritiated thymidine; M199-FCS, Medium 199 plus penicillin (100 U/ml), streptomycin 100 μg/ml, and heat-inactivated FCS; PEC, peritoneal exudate cells.

Rockville, Md.) or 35 × 10-mm plastic tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) The PEC were allowed to adhere for 2 h and subsequently washed three times in 0.9% NaCl to remove nonadherent cells. Between 65 and 75% of cells were adherent. The adherent cells, which will be referred to as macrophages, were 99% mononuclear and ~85% phagocytic as measured by ingestion of heat-killed *Candida albicans*.

CSF. CSF was prepared from L cell-conditioned medium by methods described previously (8). Most experiments were carried out with serum-free L cell CSF concentrated 250 times by ultrafiltration using an American PM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). This preparation will be referred to as fraction 1. For certain experiments, CSF was purified to homogeneity by a series of steps including column chromatography and sucrose-density gradient centrifugation (referred to as fraction 6) (8). The endotoxin content of this preparation was reduced to <1 ng/ml (9). This material was further diluted 40-fold in sterile M199-FCS for the in vitro cultures. Thus the final endotoxin content of the purified CSF was <0.025 ng/ml. The medium plus fetal calf serum (FCS) had from <1 ng/ml to 10 ng/ml endotoxin as determined by the limulus assay. Unless stated otherwise, fraction 1 preparations were used.

Rabbit anti-CSF antibodies. Normal rabbit serum, rabbit anti-CSF serum, and purified rabbit anti-CSF IgG antibodies were prepared as previously described (10, 11). Purified anti-CSF was obtained by passing crude antiserum through an immunoadsorbent column that contained pure CSF linked covalently to cyanogen bromide-activated Sepharose 4B. The purified antibody fraction, that contained only 0.1% of the starting IgG, was selectively eluted with 2M guanidine, pH 4.0. Purified normal rabbit IgG was prepared by ammonium sulfate precipitation and ion-exchange chromatography using DEAE cellulose. In neutralization experiments a 1:20 dilution of anti-CSF serum, which was sufficient to bind all CSF, or similar quantities of purified anti-CSF IgG were added to the cultures. Antibody preparations were added to tissue cultures within 5 min of CSF and maintained throughout the entire 96 h of incubation. Normal serum and purified normal IgG were used as controls.

Tumor cells. The P815 mastocytoma cell line that is syngeneic with DBA/2 mice was used as target cells. The cells were maintained in vitro by twice weekly passage in Dulbecco's medium (M. A. Bioproducts) plus penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS and in vivo by weekly intraperitoneal passage in DBA/2 mice.

Tumor inhibition assay. The capacity of macrophages to inhibit the incorporation of tritium-labeled thymidine ([^{3}H]TdR) into tumor cells was measured as previously described (12). Briefly, macrophages derived from 9×10^5 PEC were plated in 6-mm tissue culture wells and incubated with 2×10^4 P815 tumor cells in 0.2 ml M199-FCS for 24 h. Quadruplicate wells were run for each condition. 6 h before termination of the incubation, 1.25μ Ci [^{3}H]TdR (sp act 6.7 Ci/mM; New England Nuclear, Boston, Mass.) was added to each well. The cells were aspirated onto filter paper with a MASH II harvester (M. A. Bioproducts) and washed with 0.9% NaCl. The filters were dried, placed in scintillation fluid and counted in a scintillation spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The data was expressed as a percentage of controls (tumor cells alone) calculated by the expression cpm experimental/cpm tumor cells alone ×100.

To assess the effect of CSF on macrophage function, macrophages were incubated with CSF (100–10,000 U/ml) in M199-FCS for 48 h before the addition of tumor cells. This length of time was found to be optimal for stimulation of macrophages. After 48 h the medium was aspirated from the wells; tumor cells in Dulbecco's medium plus antibiotics, FCS, and CSF were added to the monolayers for the tumor inhibition assay. Incubation with CSF did not alter the appearance of the monolayers compared with controls. In each experiment, macrophages incubated without CSF were used as controls.

Tumor colony growth assay. The number of tumor colony forming units was measured by the growth of tumor colonies in soft agar. Macrophage monolayers derived from 5×10^6 PEC were prepared in 35 × 10-mm plastic tissue culture dishes in 2.0 ml M199-FCS. CSF was added to one half of the plates, and all of the plates were incubated for 48 h at 37°C in 5% CO_2. The medium then was aspirated and 1.1×10^5 tumor cells in 2.0 ml M199-FCS with or without CSF were added to each plate. The tumor cells and macrophages were incubated together for 48 h. At the end of that time the cells were removed from the plates with a rubber policeman. In some experiments total cell counts were determined with a hemacytometer and differential counts were performed on Giemsa-stained slides. The cell suspensions were serially diluted in M199-FCS and aliquots (0.6 ml) of each dilution were mixed with 5.4 ml of 0.3% agar in McCoy's 5A medium (Gibco Laboratories, Grand Island Biological Co.). 1 ml vol of the mixture was placed in 35 × 10-ml plastic petri dishes and incubated at 37°C in a humidified atmosphere of 7.5% CO_2 for 7 d. Examination of the cultures before incubation revealed even dispersion of the cells throughout the agar medium without evidence of clumping. After 7 d of incubation, colonies of >50 cells each were scored with the aid of a dissecting microscope. Five cultures were used for each experimental point. The number of colony-forming units per plate was calculated using the linear portion of the dose-response curve. Selected colonies were shown to be composed of tumor cells by microscopic examination of Giemsa-stained slide preparations. Mature macrophages did not form colonies under these conditions.

Statistical evaluation. Student's *t* test was used to evaluate the data.

RESULTS

Effect of CSF on macrophage tumoristatic activity. Experiments were performed to determine whether CSF enhanced the capacity of macrophages to inhibit the incorporation of [^{3}H]TdR by P815 tumor cells. Preliminary experiments showed that macrophages required stimulation by CSF for 48 h to become tumoristatic; incubation for 24 h had little effect. In addition CSF had to be present during the tumoristatic assay. A series of seven separate experiments were completed using a concentration of 1,000 U/ml CSF (Table I). These results showed that unstimulated macrophages suppressed [^{3}H]TdR uptake to a mean of 90±6.8% (±SEM) of controls whereas macrophages stimulated by 1,000 U/ml CSF suppressed uptake to 23±6.0% of controls ($P < 0.001$). Macrophages alone and macrophages stimulated by CSF incorporated negligible amounts of [^{3}H]TdR. CSF alone had little effect on tumor cell incorporation; further experi-

TABLE I
Effect of CSF on Macrophage Tumoristatic Activity

Exp. No.	Percentage of tumor cell uptake				
	TC + M	TC + M +CSF	M	M + CSF	TC + CSF
1	110	22	3.4	1.5	ND
2	63	18	ND	ND	ND
3	83	45	7	4.2	83
4	84	5	0.8	ND	98
5	77	8	1.1	ND	106
6	112	18	ND	ND	108
7	98	45	3.3	1.9	99
	90±6.8	23±6.0	3.1±1.1	2.5±0.9	99±4.4

P815 tumor cells (TC) and macrophages (M) were incubated either alone or with CSF (1,000 U/ml). [^{3}H]TdR incorporation was determined as a measure of proliferation. The results are expressed as the percentage of tumor cell uptake alone. Values are mean±1 SEM; ND, not done.

ments showed that CSF doses as high as 4,000 U/ml were not directly tumoristatic.

Fig. 1 shows the effect of different concentrations of CSF. In this experiment unstimulated macrophages suppressed tumor cell incorporation to 63% of control values. Macrophages stimulated by 400 U/ml of CSF or more suppressed tumor cell [^{3}H]TdR uptake to <20% of controls. Little additional effect was noted when doses >400 U/ml CSF were used.

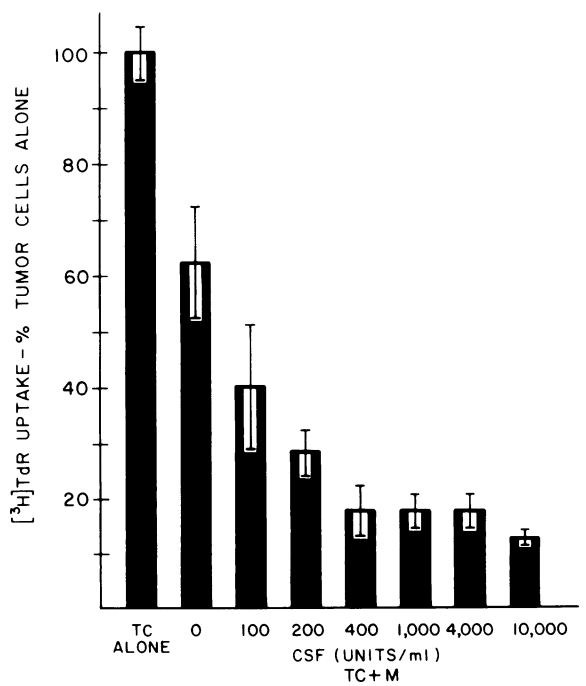

FIGURE 1 Dose response to CSF. P815 tumor cells (TC) were incubated alone or with macrophages (M) stimulated by different concentrations of CSF. [^{3}H]TdR uptake was determined as a measure of tumor cell proliferation.

Effect of purified CSF (fraction 6) on tumoristatic activity. To exclude the possibility that the tumoristatic effect resulted from impurities in the CSF preparation, such as endotoxin, further studies were conducted using highly purified CSF. Results are shown in Table II. The purified CSF (1,000 U/ml) induced macrophages to suppress tumor cell [^{3}H]TdR incorporation to 5–28% of controls. In several of these experiments, unstimulated macrophages had modest inhibitory effects. The purified CSF stimulated macrophages to inhibit tumor cells to an equal or greater degree than did the unpurified preparation. In the absence of macrophages, CSF did not significantly inhibit [^{3}H]TdR uptake.

Effect of anti-CSF antibodies. Anti-CSF serum was used in additional experiments to assure that CSF was responsible for the inhibitory activity. In initial experiments, it was found that unfractionated rabbit serum had independent stimulatory effects on macrophages as judged by a decrease in [^{3}H]TdR incorporation by tumor cells. Although the antiserum partially blocked the CSF-mediated tumor inhibition, the nonspecific stimulation of macrophages tended to obscure this effect. Further experiments were carried out using purified rabbit anti-CSF IgG preparations. In the study shown in Fig. 2, normal IgG had little effect whereas anti-CSF IgG almost totally blocked the tumor inhibitory activity of CSF. Normal IgG and anti-CSF IgG preparations did not stimulate macrophages.

Other studies on the tumoristatic activity of CSF. To determine whether CSF could stimulate tumori-

TABLE II

Effect of Purified CSF (Fraction 6) on Macrophage Tumoristatic Capacity

Exp. No.	Percentage of tumor cell uptake			
	TC + M	CSF	TC + M +CSF	TC + CSF
		U/ml		
1	48±1	1,000	24±8	74±14
2	81±3	1,000	17±4	96±22
3	56±19	200	40±4	92±16
		1,000	28±16	80±23
		4,000	22±9	81±16
4	51±12	100	14±1	ND*
		200	11±1	ND
		400	8±3	ND
		1,000	5±1	106±7
		4,000	5±1	105±10
		10,000	5±1	103±6

P815 tumor cells (TC) were incubated with or without macrophages (M) and CSF. [^{3}H]TdR incorporation was determined and results expressed as the percentage of tumor cell uptake alone. Quadruplicate cultures were used for each determination.

* Not done.

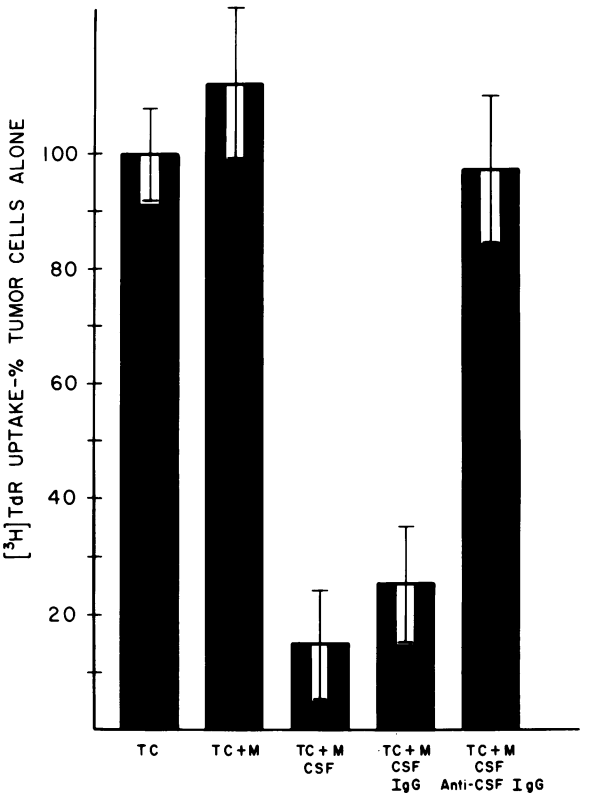

FIGURE 2 Effect of anti-CSF IgG on the stimulation by CSF. Purified normal IgG and anti-CSF IgG were added along with CSF to cultures of macrophages (M) and tumor cells (TC). [^{3}H]TdR uptake was determined as a measure of tumor cell proliferation.

static activity in lymphocyte populations, 9×10^5 spleen cells were incubated with 1,000 U/ml CSF for 48 h. Tumor cells (2×10^4) were then added for 24 h and [^{3}H]TdR incorporation was determined. CSF-stimulated spleen cells did not inhibit [^{3}H]TdR uptake by tumor cells.

In each of the preceding experiments, macrophages were harvested from several outbred Swiss-Webster mice and pooled, thus allowing mixing of allogeneic cells. To eliminate the possibility that the inhibitory effect was due to the interaction of allogeneic cells, experiments were performed using macrophages from inbred mice. When macrophages from DBA/2 mice were stimulated with CSF, tumor inhibitory activity was similar to that observed with Swiss-Webster mice.

Determination of cell numbers. To confirm the results of the tumor inhibition assay, cell counts were determined in plates containing tumor cells or tumor cells and macrophages. The results indicate that the number of tumor cells increased markedly (more than 20-fold) in cultures with unstimulated macrophages but did not change in cultures with CSF-stimulated macrophages (Table III). Tumor cells cultured alone increased approximately threefold. Similar differences were noted in the tumor colony assay (see below). Furthermore, the numbers of macrophages in cultures with or without CSF were not statistically different at 48 or 96 h ($P > 0.05$).

Inhibition of tumor colony growth. The tumor inhibition assay described above does not directly measure the effect of macrophages on tumor cell growth. Therefore, experiments were conducted to determine if CSF-stimulated macrophages would either permanently inhibit or kill tumor cells. The tumoricidal effect was measured by the suppression of tumor cell colony growth in soft agar. The results of four consecutive experiments are shown in Table IV. The total number of tumor colonies was enhanced by incubation of tumor cells with normal macrophages, thus indicating a discrepancy between the [^{3}H]TdR incorporation and the colony growth assays. Tumor colonies were markedly reduced in cultures that contained CSF-stimulated macrophages compared with those that contained unstimulated macrophages or tumor cells alone ($P < 0.01$). Similar results were noted with macrophages from Swiss-Webster (experiments 1–3) and DBA/2 (experiment 4) mice.

DISCUSSION

The experiments described in this report demonstrate that CSF can induce antitumor activity in macro-

TABLE III
Effect of CSF on Macrophages and Tumor Cells

Cells in culture	48 h		96 h	
	Macrophages × 10^5	Viability	Macrophages × 10^5	Tumor cells × 10^5
		%		
Unstimulated-Mϕ + TC	20.4±4.0	89±5.0	7.8±1.2	25.8±5.1
CSF-stimulated-Mϕ + TC	18.7±2.1	87±4.2	12.4±5.1	0.85±0.2
TC	—	—	—	3.5±1.6

Peritoneal macrophages from 5 × 10^6 PEC were plated in 35 × 10-mm tissue culture dishes with or without CSF (1,000 U/ml). After 48 h, cells were removed from certain dishes with a rubber policeman for determination of cell counts and viability. 1.1 × 10^5 P815 tumor cells were added to the remaining plates and the incubation was continued for an additional 48 h. Total cell numbers were then counted and the relative numbers of macrophages and tumor cells were determined by examination of Giemsa-stained slides. Each value represents the mean±1 SD derived from four separate plates (at 48 h) and three separate plates (at 96 h). There were no significant differences between the numbers of macrophages in cultures with or without CSF after 48 or 96 h of incubation. TC, tumor cells; Mϕ, adherent cells.

phages. Since tumoricidal activity is a principal characteristic of activated macrophages, these data suggest that CSF can serve to activate these cells. Unlike certain lymphokines (13), a prolonged exposure (48 h) and inclusion during the tumoristatic assay were necessary for induction of tumoristatic activity. Shorter exposures to CSF were ineffective.

Endotoxin and other lymphokines, which may contaminate unfractionated CSF preparations, can activate macrophages. In these studies two series of experiments were performed to determine if CSF was essential for the induction of macrophage antitumor activity. First CSF, which was purified to homogeneity (fraction 6) and largely devoid of contaminating substances (9), was used in place of relatively impure preparations (fraction 1). Fraction 6 CSF had macrophage-stimulatory activity similar to or greater than the impure preparations. Second, purified anti-CSF IgG antibodies were used to neutralize the activity of CSF. These antibody preparations, which have previously been shown to inhibit in vitro growth of granulocyte-macrophage colonies (10, 11), effectively blocked the macrophage activating effects of CSF. In addition, CSF did not markedly affect the proliferation of either tumor cells or macrophages. Taken together these control experiments show that CSF was required for the stimulation of tumoristatic activity in macrophages. However, since culture media and FCS contain modest quantities of endotoxin, it is possible that the macrophage-stimulating effect results

TABLE IV
*Inhibition of Tumor Cell Colony-Forming Units by CSF-stimulated Macrophages**

Cells in culture	Mean number colonies/plate±SD			
	Experiment number			
	1‡	2‡	3‡	4§
Tumor cells alone	37,500±3,940	27,230±13,490	25,920±4,510	31,810±3,500
Tumor cells + unstimulated macrophages	174,850±22,890	180,990±7,660	63,230±8,180	169,980±41,420
Tumor cells + CSF-stimulated macrophages	1,160±630‖	2,900±380‖	3,060±880‖	9,090±1,530‖

* The mean number of tumor colonies was determined from five plates after tumor cells had been incubated either alone, with unstimulated macrophages, or with CSF-stimulated macrophages.
‡ Swiss-Webster mice.
§ DBA/2 mice.
‖ $P < 0.01$ compared with tumor cells alone or tumor cells plus unstimulated macrophages.

from a combined action of this lipopolysaccharide with CSF.

The mechanism of macrophage activation by CSF remains speculative. The relatively long incubation period that is necessary for activation suggests that intermediate steps are required. CSF has been reported to cause release of interferon by macrophages (4). Furthermore, interferon stimulates murine macrophages and human monocytes to lyse tumor cells (14, 15). While such a mechanism for activation is attractive, other soluble mediators are also released by macrophages in response to CSF (2–5). Detailed experiments will be required to clarify these possibilities.

Tumoristasis, as measured by [^{3}H]TdR incorporation, is a known property of activated macrophages (16–18); however, this assay system has potential artifacts. Media exhaustion, the presence of cold thymidine, and nonspecific inhibitors may suppress tumor cell proliferation. Therefore, inhibition of tumor cell proliferation may not always correlate with tumoricidal activity. For these reasons, a tumor cell colony growth assay was used to determine whether CSF-stimulated macrophages killed or permanently inhibited proliferation of tumor cells. The results showed that the number of surviving tumor cells (as defined by the number of colony-forming units) was markedly decreased by CSF-stimulated macrophages, suggesting a tumoricidal effect. Determination of cell counts yielded results similar to the colony assay. Thus, all three assays showed that CSF-stimulated macrophages inhibited and/or killed tumor cells.

It is noteworthy that unstimulated macrophages appeared to enhance tumor growth as measured by cell counts and the colony assay but had a negligible effect in the [^{3}H]TdR incorporation assay. These differences, as yet unexplained, may have been due to differences in the assay conditions such as time of incubation or to differences in cell density in the two culture systems.

Other potential cytolytic cells may have been present in the adherent cell populations in these assays. However, it is doubtful whether these cells had a significant role in the antitumor effect induced by CSF. The vast majority of cells in the monolayers were macrophages, i.e., mononuclear, phagocytic cells. Thus, the effector to target cell ratio for other potential cytolytic cells was low. In addition, natural killer cells were unlikely to have caused the effect because P815 tumor cells are resistant to natural killer cell activity (19). Separate studies suggested that cytotoxic T lymphocytes were not responsible for our results. In these experiments spleen cell populations, which contain potential cytotoxic T lymphocytes, did not have antitumor activity when stimulated by CSF.

Tumoristatic and tumoricidal activity have been used as markers for activated macrophages. The data in this report, therefore, indicate that macrophages are activated by exposure to CSF. However, not all populations of activated macrophages have the same functional properties (20, 21). Preliminary studies from our laboratory indicate that CSF does not induce macrophage microbicidal activity against *Toxoplasma gondii* (unpublished results). This suggests that CSF may not stimulate all functions attributed to activated macrophages. These results contrast with those published by Handman and Burgess (6), and may be due to differences in the CSF preparation, assay system, or pathogen used.

The data presented in this report have two important implications. The first is that CSF, which is believed to be a normal biologic regulator, may have a role in endogenous antitumor host defense mechanisms. The second is that CSF has the potential for use as a therapeutic agent. This compound, which represents one of a new class of endogenously produced antitumor substances, may act more specifically and with greater safety than previously used immunologically active agents. Future in vivo studies with various tumor systems should help to define the physiological and therapeutic roles for CSF.

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