

## Macrophages as a Source of Tumoricidal Activity (Tumor-Necrotizing Factor)

DANIELA N. MÄNNEL,† ROBERT N. MOORE, AND STEPHAN E. MERGENHAGEN\*

Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20205

Macrophage-enriched peritoneal exudate cells from mice infected with *Mycobacterium bovis* BCG, macrophage-like tumor cells (PU 5-1.8), and peritoneal macrophages propagated in vitro with macrophage growth factor released tumoricidal activity into the culture medium within 2 to 3 h after stimulation with nanogram quantities of bacterial lipopolysaccharide. The cytotoxic activities from each of the macrophage culture supernatants eluted from diethylaminoethyl-Sephacel columns at a sodium chloride concentration of 200 mM exhibited a molecular weight of 50,000 to 60,000 as estimated by gel filtration, were stable at 56°C for 30 min, and were active at a pH range of 6 to 10. A rabbit antiserum directed against serum-derived cytotoxic activity (tumor-necrotizing factor) from BCG-infected and lipopolysaccharide-challenged mice inhibited all of the cytotoxic activities generated in vitro. This suggests that the macrophage-derived cytotoxins are identical with serum-derived cytotoxic factor, which further implies that the macrophage is the cellular source of tumor-necrotizing factor.

Activation of macrophages has been associated with the production of a number of soluble factors. One group of macrophage mediators includes substances that are cytotoxic for tumor cells (1-3, 5, 6, 8, 10, 16, 18, 19, 24, 27, 28, 31). A putative macrophage-derived factor reported to be tumoricidal in vivo as well as in vitro is tumor-necrotizing factor (TNF) (4). TNF, which is found in the serum of mice infected with *Mycobacterium bovis* BCG after injection of lipopolysaccharide (LPS), induces tumor necrosis in recipient animals (4) and is cytotoxic for a variety of tumor target cells in vitro (4, 9).

In earlier communications we described the mode of in vivo production and characterization of a cytotoxic factor for cultured tumor cells which is probably identical to TNF (13, 14). The present study was undertaken to determine the cellular source of the serum factor. Therefore, we investigated the capacity of various cell types in vitro to release cytotoxins with characteristics similar to those of the serum-derived tumor cytotoxic factor. Macrophage-enriched cell populations from BCG-infected mice, macrophage-like tumor cells (PU 5-1.8) (20), and peritoneal macrophages propagated with macrophage growth factor (30) release cytotoxic activity when stimulated in vitro with LPS. The conditions and kinetics for cytotoxin release were determined, and the cytotoxic activities obtained in culture supernatants were compared with those of the serum-derived cytotoxic factor.

† Present address: Laboratory of Immunobiology, National Cancer Institute, Bethesda, MD 20205.

### MATERIALS AND METHODS

**Mice.** Female C3H/HeN mice 6 to 12 weeks of age were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.

**LPS.** *Escherichia coli* K235 LPS was prepared by the phenol-water extraction method of McIntire et al. (15).

**Preparation of cytotoxic serum.** Mice were injected intravenously with  $2 \times 10^6$  colony-forming units of living *M. bovis* strain BCG (Phipps substrain TMC no. 1029, Trudeau Mycobacterial Collection, Saranac Lake, N.Y.) and then injected intravenously 14 days later with 10 µg of LPS. Two hours after LPS injection, animals were exsanguinated and the serum was prepared. Control serum was obtained from LPS-injected normal mice in a similar manner. All sera were stored at -20°C until used.

**Cytotoxicity assay.** Tumor cells (mouse L 929, American Type Culture Collection) at  $4 \times 10^3$  cells per 6.4-mm culture well (Costar 96, Cambridge, Mass.) were labeled in 0.1 ml of Eagle minimal essential medium (MEM) with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 5% heat-inactivated fetal calf serum (FCS), and 0.5 µCi of [<sup>3</sup>H]TdR ([*methyl*-<sup>3</sup>H]thymidine; specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) per ml for 18 to 24 h. Tumor cell monolayers were washed twice after labeling and were incubated in dilutions of cytotoxic serum or supernatants in 0.2 ml of Dulbecco MEM (GIBCO Laboratories) containing 2 g of NaHCO<sub>3</sub> per liter, 4.5 g of glucose per liter, 10% FCS, and 50 µg of gentamicin per ml. Labeled tumor cell monolayers lysed with 0.5% sodium dodecyl sulfate in water were used to estimate total incorporated [<sup>3</sup>H]TdR from labeled tumor cells in duplicate or triplicate cultures. Results are expressed as mean counts per minute ± standard deviation.

**Peritoneal cells.** Peritoneal exudate cells from

mice treated either 18 to 24 h previously with 1 ml of phosphate-buffered saline or 7 days previously with  $2 \times 10^6$  colony-forming units of viable BCG organisms intraperitoneally were collected after intraperitoneal injection of 8 to 10 ml of Dulbecco MEM as described elsewhere (25). This medium contained 2 g of  $\text{NaHCO}_3$  per liter, 4.5 g of glucose per liter, 10% FCS, and 50  $\mu\text{g}$  of gentamicin/ml. Peritoneal fluid was withdrawn through the anterior abdominal wall with a 19-gauge needle. Fluids from 3 to 10 mice were pooled, a sample was taken for differential and total cell counts, and the remainder was centrifuged in polypropylene tubes (no. 2074, Falcon Plastics) at  $250 \times g$  for 10 min at  $4^\circ\text{C}$ . Differential counts were made on Wright-stained cell smears prepared by cytocentrifugation (Cytospin centrifuge, Shandon Southern Instruments, Camberley, England). Washed peritoneal exudate cell suspensions from each pool of mice were adjusted to equal macrophage concentrations in Dulbecco MEM containing 2 g of  $\text{NaHCO}_3$  per liter, 4.5 g of glucose per liter, 10% FCS, and 50  $\mu\text{g}$  of gentamicin per ml.

**Spleen cells.** Spleens were aseptically removed and passed through 50-mesh stainless-steel sieves into culture medium (RPMI 1640 with 50  $\mu\text{g}$  of gentamicin per ml, GIBCO). Single-cell suspensions obtained by serial aspirations through 19- and 23-gauge needles were treated with 2:1 (vol/vol) lysing buffer (0.16 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{KHCO}_3$ ,  $10^{-4}$  M disodium ethylenediaminetetraacetate, pH 7.4) for 1 to 2 min to lyse erythrocytes. Spleen cells were centrifuged at  $250 \times g$  for 10 min at  $4^\circ\text{C}$  and resuspended in the same medium as the peritoneal exudate cells.

Adherent cell populations of either spleen or peritoneal exudate cells were obtained by allowing the cells to adhere to plastic for 2 h at  $37^\circ\text{C}$  and then removing the nonadherent cells by washing three times with medium.

**PU 5-1.8 cell line.** The PU 5-1.8 cell line derived from a spontaneous tumor in a BALB/c mouse (20) was kindly provided by R. Aksamit and S. Mizel (National Institutes of Health, Bethesda, Md.), who also provided the P 388  $\text{D}_1$  cells. The cells were grown in Eagle MEM with 10% FCS and 50  $\mu\text{g}$  of gentamicin per ml with a doubling time of 16 to 20 h. Cells were detached by scraping them off with a rubber policeman and were suspended in the same medium as the peritoneal exudate cells.

**In vitro-propagated macrophages.** L 929 cell-conditioned medium was used as a source of macrophage growth factor (colony-stimulating factor) to propagate macrophages from peritoneal exudate precursor cells (30). Peritoneal exudate cells from C3H/HeN mice taken 3 days after intraperitoneal injection of 2 ml of thioglycolate broth (Media Production Unit, National Institutes of Health, Bethesda, Md.) were suspended in enriched McCoy 5A medium (Media Production Unit) at 2,500 cells per ml. Amounts of 2 ml of cell suspension were added to 35-mm plastic tissue culture dishes (no. 3001, Falcon Plastics) and incubated at  $37^\circ\text{C}$  for 2 h. Nonadherent cells were washed off, and 2 ml of fresh medium containing 15% FCS and L-cell-derived macrophage growth factor at optimal concentrations was added. After 2 weeks of incubation, the cell number per culture had increased approximately 100-fold. As judged by microscopic examination of Wright-stained cultures, and by phago-

cytosis of latex beads and antibody-coated sheep erythrocytes, the cultured cells were greater than 99% macrophages.

**Antisera.** Female rabbits (New Zealand white) were obtained from the Division of Research Services, National Institutes of Health. The animals were injected with serum-derived tumor cytotoxic factor partially purified on Sephacryl S-200 and diethylaminoethyl-Sephacel (Pharmacia Fine Chemicals, Inc.) in complete Freund adjuvant or with equivalent fractions from serum of control mice injected intravenously with 10  $\mu\text{g}$  of LPS 2 h before exsanguination (control serum). Antigen preparations contained about 25 mg of protein per injection. The animals were boosted with the same antigen preparation subcutaneously in complete Freund adjuvant twice, after 3 weeks and after 8 weeks, and later periodically with antigen without Freund adjuvant. The rabbits were bled from the ear artery, and the serum was prepared. The sera were heat inactivated (30 min at  $56^\circ\text{C}$ ), and proteins were precipitated with 50% saturated ammonium sulfate. The precipitate was dissolved and extensively dialyzed in phosphate-buffered saline. The titer for a 50% inhibition of a 1:1,000 dilution of serum cytotoxic activity for the antiserum used in this study was 320.

**Chromatographic procedures.** Samples were subjected to gel filtration on a Sephacryl S-200 column (2.6 by 90 cm) equilibrated in pH 7.5 buffer containing 0.05 M tris(hydroxymethyl)aminomethane and 1.6 M sodium chloride. All chromatographic procedures were performed at  $4^\circ\text{C}$  at a flow rate of approximately 12 ml/h. Samples (3 ml) of supernatants were applied to the column. Fractions of approximately 3 ml were collected, and samples were sterilized by membrane filtration before they were tested in the cytotoxicity assay. Samples (3 ml) of supernatants were applied on a column of diethylaminoethyl-Sephacel (1.5 by 30 cm) equilibrated in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.1. The column was washed with four column volumes of starting buffer prior to initiation of a linear 0 to 400 mM sodium chloride gradient (total gradient volume, 280 ml). The flow rate was approximately 13 ml/h. Fractions (3 ml) were collected, and samples were prepared for the cytotoxicity assay. The sodium chloride content of the column fractions was determined with an osmometer (model 3L, Advanced Instruments, Inc., Newton Highlands, Mass.)

**Buffers.** Buffers used for testing the pH stability of cytotoxic factors were 0.1 M glycine buffer, pH 2 and 3; 0.1 M citrate buffer, pH 4; 0.2 M acetate buffer, pH 5; 0.1 M phosphate buffer, pH 6 and 7; 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8; and 0.1 M borate buffer, pH 9 and 10. All buffers contained 0.1 M sodium chloride.

## RESULTS

**Release of tumor cytotoxic activity from macrophages obtained from BCG-infected mice.** Tumor cytotoxic activity was detected in the serum of BCG-infected mice after LPS challenge (4, 13). To determine the cellular source of this cytotoxin, we cultured cells obtained from different organs of normal and BCG-infected mice. Equal numbers of either the unfraction-

ated spleen or peritoneal exudate cell population or the adherent fraction of peritoneal exudate cells were incubated with different quantities of LPS for 2 h (Table 1). The supernatant of cells from BCG-infected mice cultured with 100  $\mu$ g or more of LPS per ml consistently contained tumoricidal activity. As demonstrated in Table 1, peritoneal exudate cells or adherent peritoneal exudate cells from BCG-infected mice required less LPS (10  $\mu$ g/ml) for liberation of cytotoxic factor than did the spleen cells. No cytotoxin release over background level was detected when the cells were cultured without LPS. Also, peritoneal exudate cells and spleen cells from normal control mice did not produce detectable levels of cytotoxic activity when cultured with 0 to 1  $\mu$ g of LPS per ml. Furthermore, supernatants containing LPS did not exert any direct cytotoxic effect on the target cells (14).

Different numbers of adherent peritoneal exudate cells and nonadherent spleen cells (less than 10% macrophages by morphology) were cultured with 10 ng of LPS per ml for 2 h to determine whether the release of the cytotoxin was dependent upon the number of adherent cells (Fig. 1A). Cytotoxic activity increased with increasing cell numbers. The influence of nonadherent cells was estimated by culturing a constant number of cells containing various percentages of adherent cells. Figure 1B shows a linear dependency of production of tumor cytotoxic activity on the number of adherent cells cultured in the presence of LPS. This suggests that the macrophages are the secretory cells for the tumoricidal activity since the nonadherent cells do not produce the cytotoxin.

Cytotoxic activity was maximal after 2 to 3 h and decayed thereafter (Fig. 2A). When the me-

TABLE 1. Tumor cytotoxic activity from different cell populations of normal and BCG-infected mice

LPS <sup>a</sup>	Spleen cells		Peritoneal exudate cells		Adherent peritoneal exudate cells	
	Normal	BCG	Normal	BCG	Normal	BCG
0	530 $\pm$ 20 <sup>b</sup>	670 $\pm$ 110	430 $\pm$ 20	740 $\pm$ 20	530 $\pm$ 80	520 $\pm$ 70
1 $\mu$ g	480 $\pm$ 10	720 $\pm$ 20	NT	900 $\pm$ 10	NT	800 $\pm$ 10
10 $\mu$ g	470 $\pm$ 70	780 $\pm$ 10	NT	1,400 $\pm$ 90	NT	1,010 $\pm$ 40
100 $\mu$ g	560 $\pm$ 70	1,150 $\pm$ 20	NT	1,400 $\pm$ 90	NT	1,750 $\pm$ 200
1 ng	630 $\pm$ 20	1,660 $\pm$ 120	660 $\pm$ 10	1,540 $\pm$ 110	660 $\pm$ 20	2,050 $\pm$ 60
10 ng	650 $\pm$ 20	1,840 $\pm$ 170	830 $\pm$ 20	1,700 $\pm$ 110	790 $\pm$ 80	1,860 $\pm$ 10
100 ng	850 $\pm$ 10	1,800 $\pm$ 100	670 $\pm$ 20	1,800 $\pm$ 70	660 $\pm$ 10	1,910 $\pm$ 10
1 $\mu$ g	800 $\pm$ 30	1,800 $\pm$ 50	640 $\pm$ 10	1,580 $\pm$ 200	750 $\pm$ 10	1,880 $\pm$ 200

<sup>a</sup> Cells ( $10^6$ /ml) from normal or BCG-infected mice were incubated in the presence of *E. coli* K235 LPS for 2 h.

<sup>b</sup> Tumor cytotoxicity of the 1:2 diluted cell-free supernatants was estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (counts per minute  $\pm$  standard deviation; total incorporated cpm =  $25 \times 10^2$ ; spontaneously released cpm =  $500 \pm 30$ ). NT, not tested.

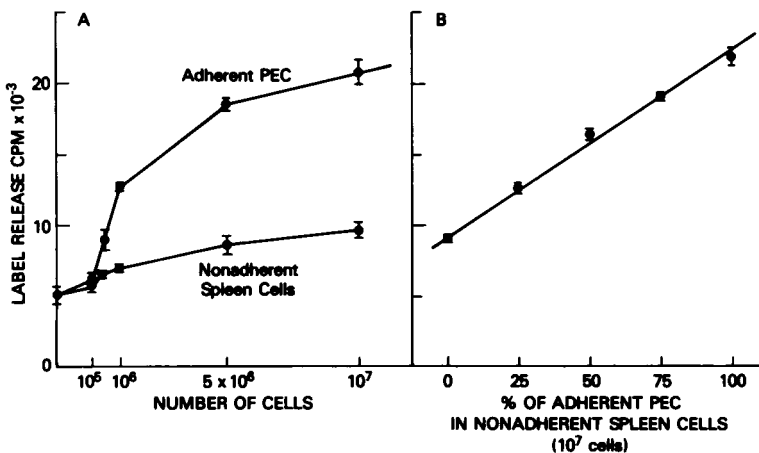


FIG. 1. Determination of tumor cytotoxic activity from LPS-challenged adherent peritoneal exudate cells (PEC) or nonadherent spleen cells from BCG-infected mice. Increasing numbers of adherent PEC or nonadherent spleen cells (A) or a mixture of both cell types (B) were cultured with *E. coli* K235 LPS (10 ng/ml) for 2 h. Cytotoxic activity of the 1:2 diluted cell-free supernatant was estimated by [<sup>3</sup>H]TdR release from labeled L 929 cells at 48 h (total incorporated cpm =  $43 \times 10^3$ ).

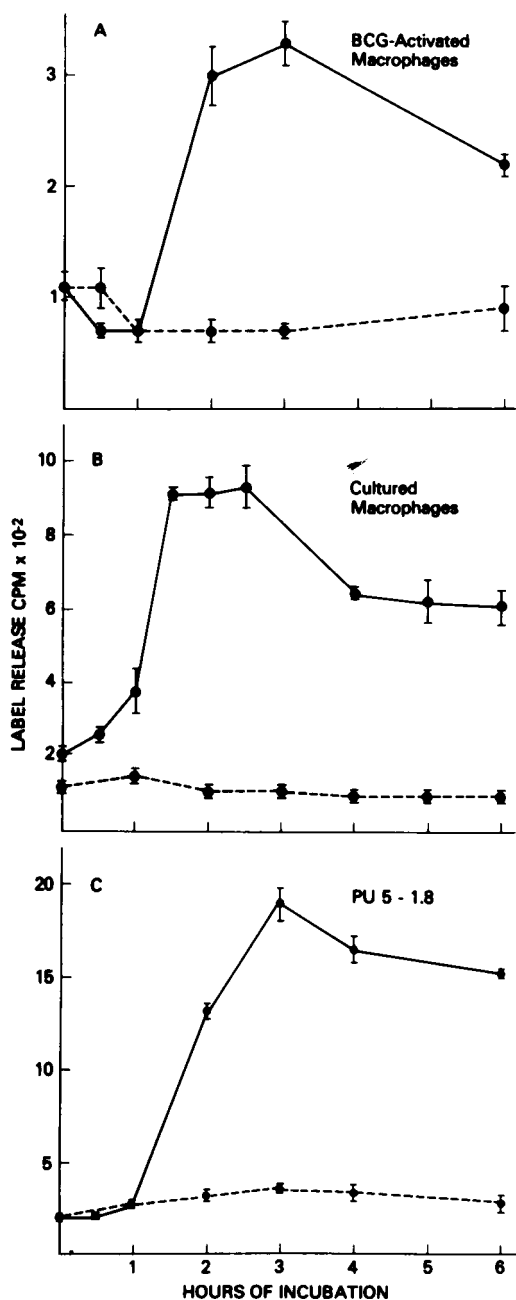


FIG. 2. Kinetics of appearance of cytotoxic activity from LPS-challenged adherent peritoneal exudate cells (PEC) of BCG-infected mice, cultured macrophages propagated in the presence of macrophage growth factor for 14 days, and PU 5-1.8 cells. Adherent PEC ( $10^6$ /ml) of BCG-infected mice (A), cultured macrophages ( $0.3 \times 10^6$ /ml) (B), and PU 5-1.8 cells ( $1.3 \times 10^6$ /ml) (C) were incubated for different periods of time with *E. coli* K235 LPS (10 ng/ml). Cytotoxic activity of the 1:2 diluted cell-free supernatants was

estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm for the experiment shown in panel A =  $12 \times 10^2$ , panel B =  $30 \times 10^2$ , and panel C =  $24 \times 10^2$ ).

medium containing LPS was replaced at hourly intervals and tested for cytotoxic activity, no significant decrease could be found after 3 h. On the other hand, supernatant from cultures stimulated with LPS 3 h earlier but collected after 21 h expressed no cytolytic activity. The supernatant obtained at this later time (pH greater than 6.8) significantly inhibited the cytotoxic activity of a 3-h supernatant in a dose-dependent fashion (50% inhibition at a 1:2 dilution of the supernatant collected at the later time). Control supernatant from unstimulated cultures, however, had the same inhibitory capacity. The cytotoxic activity was not absorbed from the medium by spleen cells, peritoneal exudate cells, or fibrosarcoma cells. The cytotoxic activity when removed from the cell culture was stable at 37°C but was lost when treated with protease, trypsin, or chymotrypsin (data not shown). This suggests that a soluble factor(s) or an enzyme(s) is released from the activated as well as the nonactivated cells after 3 h of incubation, which inactivates the cytotoxic activity.

**Release of tumor cytotoxic factor from macrophage cultures propagated with macrophage growth factor.** More homogeneous macrophage cultures were obtained by propagating thioglycolate-induced peritoneal exudate cells with mouse L 929 cell supernatant containing macrophage growth factor (11, 30). Within 13 to 14 days of culture, the cells had multiplied about 100-fold and the population consisted of over 99% macrophages, as determined by morphology and uptake of latex beads or antibody-coated sheep erythrocytes. When these cells were stimulated with different quantities of LPS, they were extremely sensitive to LPS in terms of cytotoxin release (Table 2). Picogram quantities of LPS were sufficient to induce soluble cytotoxic activity. Kinetics for detection of the cytotoxin in the supernatant (Fig. 2B) show the same pattern as for serum-derived tumor cytotoxic activity (14) or cytotoxic factor from peritoneal exudate cells of BCG-infected mice challenged in vitro with LPS (Fig. 2A).

**Release of tumor cytotoxic factor from the macrophage-like cell line PU 5-1.8.** Recently, several mouse monocyte/macrophage tumor cell lines have been described which differ in the properties they share with blood monocytes or peritoneal macrophages. The tumor line PU 5-1.8 has the ability to phagocytize, bears

estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm for the experiment shown in panel A =  $12 \times 10^2$ , panel B =  $30 \times 10^2$ , and panel C =  $24 \times 10^2$ ).

TABLE 2. Tumor cytotoxic activity from macrophage cultures propagated with macrophage growth factor<sup>a</sup>

LPS <sup>b</sup> (ng)	Label release <sup>c</sup>
0	180 ± 10
0 <sup>d</sup>	190 ± 30
0.1	1,080 ± 40
1	930 ± 10
10	970 ± 90
100	1,020 ± 170
1,000	1,000 ± 60

<sup>a</sup> Propagated macrophages were obtained by culturing peritoneal exudate cells in the presence of macrophage growth factor for 14 days.

<sup>b</sup> Propagated macrophages ( $3 \times 10^6$  cells per ml) were incubated in the presence of *E. coli* K235 LPS for 2 h.

<sup>c</sup> Tumor cytotoxicity of the 1:2 diluted cell-free supernatants was estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (counts per minute ± standard deviation; total incorporated cpm =  $30 \times 10^2$ ; spontaneously released cpm =  $70 \pm 10$ ).

<sup>d</sup> Reconstituted with 1,000 ng of LPS.

receptors for immunoglobulin and complement (22), secretes lysozyme (21, 22), and is capable of becoming cytolytic for tumor target cells in the presence of specific antisera (23). These same cells can also be activated for nonspecific tumor cell killing (M. Meltzer, personal communication). Different numbers of these PU 5-1.8 cells were incubated for 2 h with various concentrations of LPS and tested for their ability to release soluble cytotoxic activity (Fig. 3). Again, the cytotoxic activity detected was dependent on the cell number. The concentration of LPS required to induce the cytotoxin release was in the nanograms per milliliter range. The kinetics of appearance of cytotoxic activity reached maximal levels after 2 to 3 h (Fig. 2C). In contrast, cells of another macrophage-like tumor cell line, P 388 D<sub>1</sub>, did not release any cytotoxic factor when cultured with different concentrations of LPS for various periods of time (data not shown).

**Physicochemical characteristics of tumor cytotoxic activities from LPS-stimulated cultures.** The physicochemical characteristics of the cytotoxic activities induced by LPS in macrophage-enriched or macrophage-like cultures were compared with those of the serum-derived tumor cytotoxic factor. When applied to diethylaminoethyl-Sephacel columns at pH 7.1, the *in vitro*-generated cytotoxins from all three sources were retained; they eluted in a sodium chloride salt gradient at 200 mM sodium chloride. Figure 4 shows a representative elution profile obtained with the cytotoxic activity from LPS-treated and BCG-activated macrophages.

The apparent molecular weights estimated by gel filtration on Sephacryl S-200 were similar for all three cytotoxic factors. The activity eluted in a broad peak in the molecular weight range of 50,000 to 60,000 (Fig. 5). The cytotoxins were stable for 30 min at 56°C but completely destroyed after 10 min at 100°C (Table 3). The pH sensitivity of cytotoxic factor from serum was compared with that of cytotoxic factor from BCG-activated macrophages stimulated *in vitro* with LPS (Table 4). In both cases cytotoxic activity was stable at pH 6 to 10 but was lost when held below pH 6.

A rabbit antiserum raised against serum-derived cytotoxic factor inhibited the cytotoxic activities derived from all macrophage sources (Table 5). Control serum exhibited only a slight inhibitory effect which was not observed when purified immunoglobulin G was used (data not shown).

## DISCUSSION

Macrophages secrete a variety of products that lyse tumor target cells (1-3, 5, 6, 8, 10, 16, 18, 19, 24, 27, 28, 31). In some cases the chemical nature of these cytotoxins has been characterized. Arginase (6), hydrogen peroxide (18), or serine proteases (1) are released by properly activated macrophages and induce tumor cell death. None of the substances mentioned above appears to be the active principle in the cyto-

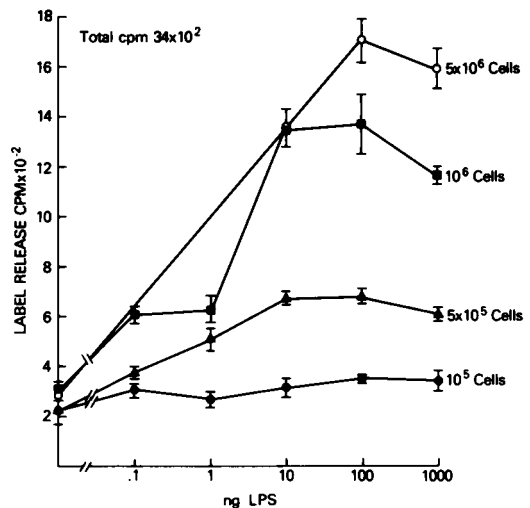


FIG. 3. Determination of tumor cytotoxic activity from LPS-challenged PU 5-1.8 cells. Different numbers of PU 5-1.8 cells were incubated in the presence of *E. coli* K235 LPS for 2 h. Cytotoxic activity of the 1:2 diluted cell-free supernatants was estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm =  $34 \times 10^2$ ).

toxic factor described in this communication. The activity of the cytotoxin was lost after treatment with protease, trypsin, and chymotrypsin,

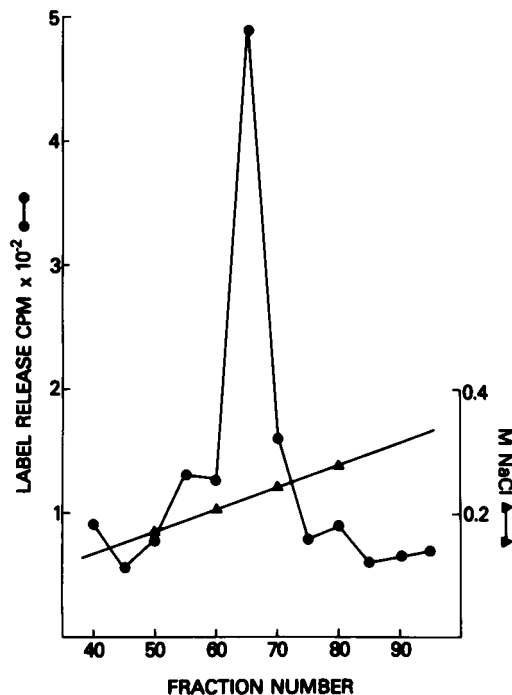


FIG. 4. Diethylaminoethyl-Sephacel chromatography of tumor cytotoxic supernatant from BCG-activated peritoneal exudate cells. A 3-ml amount of supernatant from BCG-activated adherent peritoneal exudate cells ( $10^6$ /ml) incubated in the presence of *E. coli* K235 LPS (10 ng/ml) for 2 h was applied on a column of DEAE-Sephacel (1.5 by 30 cm) equilibrated in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.5). The column was washed with four column volumes of starting buffer prior to initiation of a 0 to 400 mM sodium chloride gradient (total gradient volume, 280 ml). The flow rate was approximately 13 ml/h. Fractions (3 ml) were collected and sterile filtered. Tumor cytotoxic activity of a 1:4 dilution of the fractions was estimated by [ $^3$ H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm =  $25 \times 10^3$ ). The molarity of the fractions was determined with an osmometer.

but was unaltered by the serine esterase inhibitor diisopropyl fluorophosphate (data not presented).

The soluble cytotoxic activities obtained from adherent peritoneal exudate cells of BCG-infected mice, from in vitro-propagated macrophages, or from PU 5-1.8 cells share many characteristics with the previously described serum-derived cytotoxic factor (14). In all four systems, the inducing stimulus, LPS, was effective in minute (nanogram) quantities. Optimal levels of cytotoxicity were detected after 2 to 3 h. The macrophages were stimulated by BCG, by cultivation in macrophage growth factor-containing medium, or as a result of characteristics of the tumor cell line. Behavior on ion-exchange and gel filtration columns and heat stability of the in vitro-generated cytotoxins were very similar to

TABLE 4. pH sensitivity of tumor cytotoxic activities from different sources

pH	Cytotoxic serum <sup>a</sup>	Propagated macrophages <sup>a</sup>
2	130 ± 70 <sup>b</sup>	320 ± 20
3	100 ± 10	330 ± 20
4	170 ± 10	330 ± 10
5	220 ± 30	450 ± 10
6	1,940 ± 190	1,120 ± 40
7	1,710 ± 200	1,180 ± 90
8	1,890 ± 130	1,230 ± 30
9	1,960 ± 60	1,080 ± 50
10	1,940 ± 80	860 ± 20

<sup>a</sup> Samples of pooled serum-derived cytotoxic activity chromatographed on a Sephacryl S-200 column (1:100 dilutions) or samples of cytotoxic supernatant (1:4 diluted) from macrophages propagated in the presence of macrophage growth factor for 14 days were dialyzed in buffers of different pH levels for 18 h and then dialyzed in phosphate-buffered saline until the pH was stable.

<sup>b</sup> Tumor cytotoxicity of the serum-derived samples and of supernatant samples was estimated by [ $^3$ H]-TdR release of labeled L 929 cells at 48 h (counts per minute ± standard deviation; for cytotoxic serum, total incorporated cpm =  $35 \times 10^2$  and spontaneously released cpm =  $160 \pm 10$ ; for cultured macrophage supernatant, total incorporated cpm =  $20 \times 10^2$  and spontaneously released cpm =  $310 \pm 60$ ).

TABLE 3. Heat sensitivity of tumor cytotoxic activities from different sources

Treatment	Cytotoxic serum	BCG-activated peritoneal exudate cells	PU 5-1.8	Propagated macrophages <sup>a</sup>
None	1,800 ± 50 <sup>b</sup>	900 ± 100	700 ± 10	1,200 ± 10
30 min, 56°C	1,700 ± 50	750 ± 80	400 ± 20	1,000 ± 100
10 min, 100°C	100 ± 30	150 ± 10	150 ± 10	150 ± 20

<sup>a</sup> Propagated macrophages were obtained by culturing peritoneal exudate cells in the presence of macrophage growth factor for 14 days.

<sup>b</sup> Tumor cytotoxicity of 1:100 diluted cytotoxic serum or 1:2 diluted supernatants was estimated by [ $^3$ H]TdR release of labeled L 929 cells at 48 h (counts per minute ± standard deviation; total incorporated cpm =  $30 \times 10^2$ ; spontaneously released cpm =  $100 \pm 30$ ).

TABLE 5. Cross-reactivity of tumor cytotoxic activities from different sources with a rabbit antiserum

Treatment	Cytotoxic serum	BCG-activated peritoneal exudate cells	PU 5-1.8	Propagated macrophages <sup>a</sup>
None	2,100 ± 100 <sup>b</sup>	2,400 ± 100	1,100 ± 10	1,600 ± 50
Control serum <sup>c</sup>	1,400 ± 100	2,200 ± 100	800 ± 50	1,300 ± 30
Antiserum <sup>c</sup>	200 ± 30	300 ± 10	100 ± 30	500 ± 20

<sup>a</sup> Propagated macrophages were obtained by culturing peritoneal exudate cells in the presence of macrophage growth factor for 14 days.

<sup>b</sup> Tumor cytotoxicity of 1:1,000 diluted cytotoxic serum or 1:2 diluted supernatants was estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (counts per minute ± standard deviation; total incorporated cpm = 30 × 10<sup>2</sup>; spontaneously released cpm = 80 ± 10).

<sup>c</sup> Rabbit sera were heat activated (30 min at 56°C), precipitated with ammonium sulfate (50% saturation) and added to the cytotoxicity assay in a final concentration equivalent to 1/80 of the original serum.

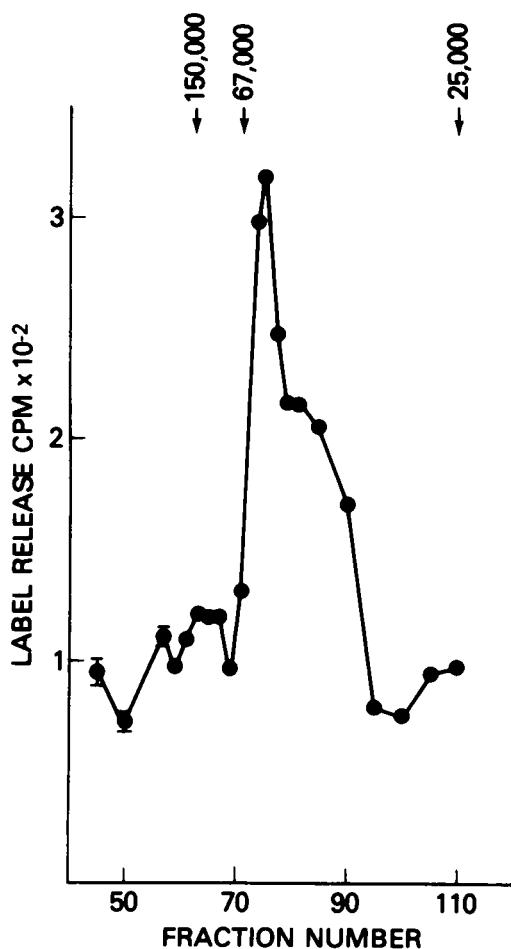


FIG. 5. Sephacryl S-200 chromatography of tumor cytotoxic supernatant from BCG-activated peritoneal exudate cells. A 3-ml amount of supernatant from BCG-activated adherent peritoneal exudate cells (10<sup>6</sup>/ml) incubated in the presence of *E. coli* K235 LPS (10 ng/ml) for 2 h was applied to a Sephacryl S-200 column. The column was 2.6 by 90 cm, and the flow rate was approximately 12 ml/h. The column was equilibrated and eluted with 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) containing 0.1 M sodium chloride. Tumor cytotoxic

those of serum cytotoxin. Inhibition of the cytotoxic activities was achieved with a rabbit antiserum directed against the serum cytotoxic factor. The cytotoxic activity found in serum of BCG-infected mice after LPS injection was unstable below pH 6, as was the cytotoxin from BCG-activated macrophages. If, indeed, the serum-derived cytotoxic factor which we previously studied (14) is identical to TNF, then the tumoricidal factor from cultured macrophages is probably TNF. Matthews described the spontaneous release of TNF from rabbit blood monocytes *in vitro* (12). This cytotoxic activity clearly differs in mode of production and physicochemical properties from the murine cytotoxin dealt with in the present report. However, obtaining direct proof that the *in vitro*-generated cytotoxic activity has tumor-necrotizing activity may not as yet be feasible because the *in vivo* assay requires large volumes of TNF-containing serum (4).

Recent studies have demonstrated that the development of tumoricidal macrophages requires several activation signals (25, 26, 32). In a similar manner, release of the soluble cytotoxin from macrophages also required more than one stimulus, which suggests that this cytotoxin may be involved in the mechanism of macrophage tumor cell destruction. The lack of LPS dose-response effect in eliciting soluble cytotoxic activity (Table 2) implies that LPS acts in a trigger mode rather than as a primary stimulus on properly activated macrophages.

Several studies demonstrate that macrophage tumor cell killing is dependent upon effector-target cell contact (7, 17, 29). It can be hypothesized that the cytotoxin herein described is labile in the presence of proteases and consequently that high concentrations are only obtained within the region of cell-cell contact. Activated macrophages could carry this cytotoxin

activity of 1:4 dilutions of the 3-ml sterile filtered fractions was estimated by [<sup>3</sup>H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 14 × 10<sup>2</sup>).

inserted in the membrane and might liberate it into the medium only when stimulated with inflammatory substances such as LPS. In fact, preliminary results show that an antiserum directed against the cytotoxic factor partially inhibits tumor cell killing by BCG-activated macrophages cultured with LPS. This supports the concept that this cytotoxin plays a central role in tumor cell killing by these cells.

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#### LITERATURE CITED

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