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Generation and Characterization of a Lipopolysaccharide-Induced and Serum-Derived Cytotoxic Factor for Tumor Cells

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Serum from *Mycobacterium bovis* BCG-infected mice treated with lipopolysaccharide was cytotoxic to tumor cells in vitro. Serum-induced cytotoxicity was estimated by measuring release of [^{3}H]thymidine into culture supernatants of prelabeled tumor target cells. Serum from BCG-infected mice not treated with lipopolysaccharide or from uninfected mice treated with lipopolysaccharide was inactive. Moreover, although serum cytotoxic activity was evident with 10 syngeneic or allogeneic tumor cell lines, little or no effect was observed with normal embryonic fibroblast target cells. Maximal titers of serum cytotoxic activity were detected 14 days after BCG infection and 2 h after LPS treatment. Serum of BCG-infected, T-cell-deficient nude mice developed strong cytotoxic activity after LPS treatment; however, lipopolysaccharide-insensitive C3H/HeJ mice could produce this cytotoxic activity only after adoptive transfer with lipopolysaccharide-responsive C3H/HeN bone marrow. Physicochemical characterization of the serum cytotoxic activity revealed a heat-stable (56°C, 30 min) entity with a molecular weight of about 60,000 and an isoelectric point at pH 4.8. Biological and physicochemical characteristics of this serum cytotoxic activity as defined by an in vitro assay were very similar to characteristics of tumor necrosis factor and suggest that this molecule may be a major effector mechanism for the antitumor actions of lipopolysaccharide.

The antitumor effects of bacterial lipopolysaccharides (LPSs) have been the subject of research for almost a century (6, 23). Transplantable syngeneic tumors growing intradermally or subcutaneously can be completely cured with a single but sometimes lethal injection of LPS at a distant site (20). This effect was quite dramatic. Within 6 to 8 h of the LPS injection, erythema developed at the tumor site. By 24 h, extensive hemorrhagic necrosis was evident, and by 48 to 72 h most of the tumor mass had been sloughed (2).

Recently, Carswell et al. discovered that the tumor necrotic action of LPS was not a direct effect of LPS on tumor cells but rather was mediated by a factor present in sera of LPS-treated animals (5). A similar factor could be isolated from sera of LPS-treated mice and rabbits without tumors but previously injected with *Mycobacterium bovis* strain BCG (10, 14). The tumor necrosis activity of serum from LPS-treated animals could be separated from many of the toxic effects of LPS itself (10). Moreover, although tumor cells of different origins were inhibited or killed by this tumor necrosis factor in vitro, normal embryonic fibroblasts were unaffected under the same conditions (5, 11, 19).

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Based on these earlier observations, we have developed a sensitive and quantitative in vitro assay for cytotoxic factors in the sera of LPS-treated animals. In a previous communication we used this assay to discriminate the cytotoxic factor from lymphocyte-activating factor which appears in BCG-infected mice after LPS injection (13). In this report, conditions for optimal release of cytotoxic factors in sera of BCG-infected mice and physicochemical characterization of the active factor are presented.

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. C3H/HeJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine.

LPS. *Escherichia coli* K235 LPS was prepared by the phenol-water extraction method of McIntire et al. (16) or by the butanol extraction procedure described by Morrison (17). *Salmonella minnesota* Re mutant R595 LPS isolated by phenol-chloroform-petroleum ether method (8) was a generous gift of E. T. Rietschel, MPI, Freiburg, West Germany. Polysaccharide of *S. minnesota* (Freeman type [7]) was kindly given by C. Bona, NIH, Bethesda, Md. The phenol-water-extracted LPS of *E. coli* K235 was used for most of the studies.

Preparation of cytotoxic serum. Mice were in-

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

Cytotoxicity assay. Tumor cells (mouse L 929, ATCC) at 4×10^4 cells per 16-mm culture well (Costar 24, Cambridge, Mass.) were labeled in 0.5 ml of Eagle minimal essential medium (EMEM) with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 5% heat-inactivated fetal calf serum (FCS), and 0.5 μCi of [^{3}H]TdR ([*methyl*-^{3}H]thymidine, specific activity, 1.9 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) for 18 to 24 h. Tumor cell monolayers were washed twice after labeling and incubated in dilutions of cytotoxic serum in 1 ml of Dulbecco MEM containing 10% FCS and 50 μg of gentamicin/ml. Labeled tumor cell monolayers lysed with 0.5% sodium dodecyl sulfate in water were used to estimate total incorporated counts per minute (cpm). For some experiments, the identical procedure was carried out in 0.4-mm flat-bottom culture wells (Costar 96, Cambridge, Ma.) with 4×10^3 cells per well in 0.2 ml of medium. Tumor cytotoxicity was estimated by measuring the release of incorporated [^{3}H]TdR from the labeled tumor cells in duplicate cultures and expressed as mean cpm ± standard deviation or as specific release according to the formula (experimental cpm − cpm of control)/(cpm of sodium dodecyl sulfate − cpm of control) × 100. Cells were counted in a Coulter Counter (model ZBI, Coulter Electronics, Inc., Hialeah, Fla.) in quadruplicate after treating adherent cells with 0.5% trypsin–0.2% ethylenediaminetetraacetic acid (EDTA) solution and suspending them in Isotone II (Coulter Electronics) containing 10% FCS.

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 (Tris), 0.1 M sodium chloride, or 1.6 M sodium chloride. All chromatography was performed at 4°C at a flow rate of approximately 12 ml/h. Up to 10 ml of either unfractionated serum or the ammonium sulfate precipitate, dialyzed 4 times against pH 7.5 Tris buffer adjusted to a final volume of 6 ml in the same buffer, was applied to the column. Fractions of approximately 3 ml were collected, and portions were sterilized by Millipore filtration before they were tested in the cytotoxicity assay. Up to 10 ml of unfractionated serum was applied on a column (1 by 30 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia) equilibrated in 0.05 M Tris buffer (pH 7.1). The column was washed with 4 column volumes of starting buffer before initiation of a linear 0 to 400 mM sodium chloride gradient (total gradient volume 280 ml). The flow rate was approximately 12 ml/h. Fractions (2 ml) were collected, and portions 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, Ma.).

A 0.5-ml amount of unfractionated BCG-LPS serum was applied to a 5-mm-thick bed of Sephadex G75 Superfine with 5% Pharmalyte (Pharmacia) (pH 4 to 6.5) and focused for 18 h at a constant power of 1 W on a flatbed apparatus (LKB 2117 Multiphor). The fractions were eluted with 6 ml of phosphate-buffered saline. After we measured the pH of individual fractions, we dialyzed the fractions against phosphate-buffered saline for 24 h, sterilized them by filtration, and assayed them for tumor cytotoxicity.

Induction of tumors and in vitro culture of tumor cells. Fibrosarcomas were induced intradermally in mice with 1 mg of 3-methylcholanthrene in 0.2 ml of trioctanoin (tricaprylin, Sigma Chemical Co., St. Louis, Mo.). For in vitro culture, solid tumor pieces were minced and a single cell suspension was obtained by enzymatic digestion (3). The cells were cultured in EMEM containing 10% FCS and 50 μg of gentamicin per ml.

Generation of chimeric mice. Bone marrow cells were obtained by gently grinding and then rinsing femura and tibiae of donor mice in cold RPMI medium (GIBCO Laboratories, Grand Island, N.Y.). Bone fragments were removed by being allowed to settle for 5 min; the cells were washed and then suspended in medium to a concentration of 10^8 cells/ml. Cell viability exceeded 90% as determined by exclusion of trypan blue dye. Mice received 850 roentgens of X-irradiation and were reconstituted within 6 h by tail vein injection of 10^7 bone marrow cells. Three days before and for 2 weeks after reconstitution, the mice were given drinking water containing 1 g each of ampicillin (Totacillin-N, Beecham Laboratories, Bristol, Tenn.), carbenicillin (Pyophen, Beecham Laboratories), and cephalothin (Keflin, Eli Lilly & Co., Indianapolis, Ind.) per liter. Four to 6 weeks after reconstitution, the mice were used in the experiments.

RESULTS

In vitro detection of cytotoxic factors in serum of BCG-infected mice treated with LPS. Serum of BCG-infected mice treated with LPS (BCG-LPS serum) contained factors cytotoxic for tumor cells in vitro (Table 1). Cytotoxicity was detected with each of 10 different murine fibrosarcomas after treatment with 1/100 dilution of BCG-LPS serum. The same dilution of serum had little or no effect on syngeneic embryo fibroblasts. It is important to note that fibrosarcomas from lipid A-unresponsive C3H/HeJ mice were as susceptible to the active serum as fibrosarcomas from normally responsive C3H/HeN mice. That there was no difference in susceptibility between tumors of LPS-responsive and -unresponsive strains suggests that a direct effect of LPS upon target cells was unlikely. Among the tumors assayed, the C3H/HeN fibrosarcoma L 929 was the most sensitive target cell and was therefore used in all further studies. Serum from BCG-infected mice not treated i.v. with LPS, or from normal mice with or without LPS treatment, had no effect on either normal or tumor cells. LPS in medium up

to a concentration of 100 μg/ml was also without effect (data not shown).

Release of radiolabel from [^{3}H]TdR-prelabeled L 929 cells into culture supernatants at 48 h showed an inverse correlation with the number of intact cells estimated by direct cell counts (Fig. 1). Significant cytotoxicity to L 929 cells by BCG-LPS/sera could be detected with as little as a 1/1,000 dilution. Cytotoxic activity, however, even at the higher serum concentration, was dependent on continuous presence of the cytotoxic factor (Fig. 2). Target cells incubated with a 1/100 dilution of active serum for up to 8 h and then washed, released little or no radiolabel at 48 h. Even a 24-h pulse with cytotoxic factor was less efficient than continuous presence throughout the assay.

TABLE 1. *Tumor cytotoxic activity of BCG-LPS serum and normal mouse serum*[a]

Target cell	BCG-LPS serum[b]	Normal mouse serum
C3H/HeN fibrosarcoma		
L 929	55 ± 2	0 ± 1
Tumor A	19 ± 1	1 ± 0
Tumor B	19 ± 0	0 ± 1
Tumor C	13 ± 1	0 ± 0
Tumor D	12 ± 1	0 ± 0
C3H/HeJ fibrosarcoma		
Tumor A	20 ± 2	0 ± 1
Tumor B	17 ± 1	1 ± 0
Tumor C	12 ± 1	2 ± 1
Tumor D	10 ± 1	0 ± 1
Tumor E	10 ± 0	1 ± 0
Embryo fibroblasts	3 ± 0	0 ± 0

[a] Each value represents percent specific label release ± standard deviation.

[b] BCG-LPS serum and normal mouse serum were diluted 1/100.

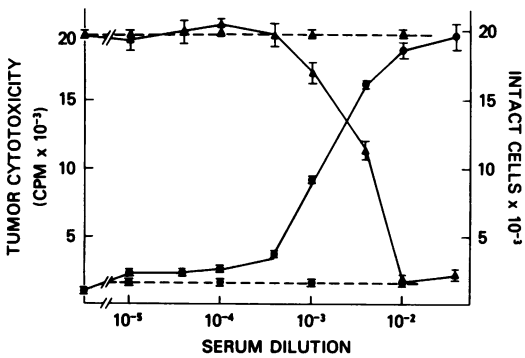

FIG. 1. *Tumor cytotoxicity dose response of BCG-LPS serum and normal mouse serum. [^{3}H]TdR-labeled or unlabeled L 929 cells were incubated in different dilutions of BCG-LPS serum (——) or normal mouse serum (- - - - -) for 48 h. Tumor cytotoxicity was estimated by [^{3}H]TdR release (total incorporated cpm = 45 × 10^3) (●) and by counting intact cells on a Coulter Counter (▲). More than 95% of the recovered cells were viable as determined by trypan blue dye exclusion.*

Optimal conditions for the in vivo production of cytotoxic factors in serum of BCG-infected mice treated with LPS. Mice were treated i.v. with LPS at various times after BCG infection (Fig. 3). Tumor cytotoxic factors were detected in the serum of BCG-LPS mice by 1 week but not 3 days after BCG infection. Maximal production was evident at 2 weeks and then progressively declined to about control levels by 8 weeks.

The time course for appearance of cytotoxic factors in serum of BCG-LPS mice is shown in Fig. 4. Cytotoxic activity was detected 30 min

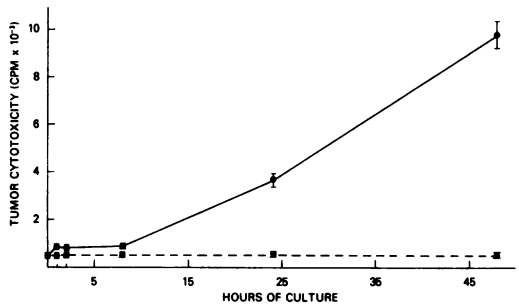

FIG. 2. *Tumor cytotoxicity after exposure to LPS-BCG serum or normal mouse serum for various times. [^{3}H]TdR-labeled L 929 cells were incubated with a 1/100 dilution of BCG-LPS serum (——) or normal mouse serum (- - - - -) for 0 to 48 h. The supernatant was replaced by fresh medium without mouse serum, and cytotoxicity was estimated by [^{3}H]TdR release at 48 h (total incorporated cpm = 40 × 10^3).*

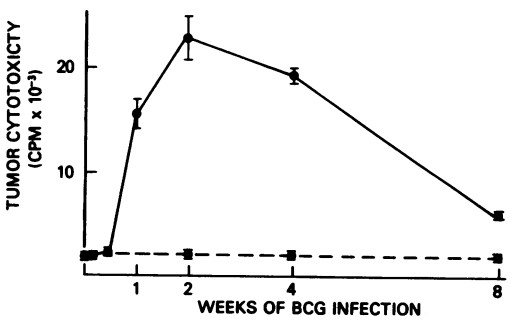

FIG. 3. *Kinetics of the appearance of cytotoxic serum activity during BCG infection. Mice were infected i.v. with 2 × 10^6 CFU of BCG and injected i.v. with 10 μg of LPS after 3 days and after 1, 2, 4, and 8 weeks. Two hours after LPS injection, the animals were bled and serum was prepared. Tumor cytotoxicity of BCG-LPS serum (——) and BCG serum (- - - - -) was estimated by [^{3}H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 60 × 10^3).*

after LPS injection, reached maximal levels by 1 to 2 h, and then progressively declined and was absent by 6 h. A second injection of LPS 6 h after the first injection failed to induce reappearance of tumor cytotoxic activity.

Cytotoxic activity in serum of BCG-LPS mice was dependent upon a sufficient dose of phenol-extracted LPS. Maximal activity occurred after 1 μg of LPS (Fig. 5). Cytotoxic activity could be detected in BCG-injected mice after treatment with polysaccharide-deficient LPS of *S. minnesota* R 595 (Re mutant), suggesting that the active principle of LPS was lipid A (Table 2). This is further confirmed by the fact that the polysaccharide part of LPS (Freeman type [7]) or a second antigenic stimulus such as purified protein derivative (PPD) was unable to induce cytotoxic serum activity in the BCG-infected mice. In addition, lipid A-unresponsive C3H/HeJ mice failed to produce serum cytotoxic factors after the BCG-LPS treatment. C3H/HeJ mice treated with BCG and butanol-extracted LPS also failed to produce serum cytotoxic activity. Butanol-extracted LPS is a strong B cell mitogen even for the lipid A-unresponsive C3H/HeJ mouse (9).

C3H/HeJ mice could produce cytotoxic factors only after adoptive transfer of lipid A-responsive C3H/HeN bone marrow (Table 3). Autologous transfer of bone marrow cells into irradiated C3H/HeJ or C3H/HeN recipient mice did not alter their responsiveness to LPS. However, irradiated C3H/HeN recipients treated

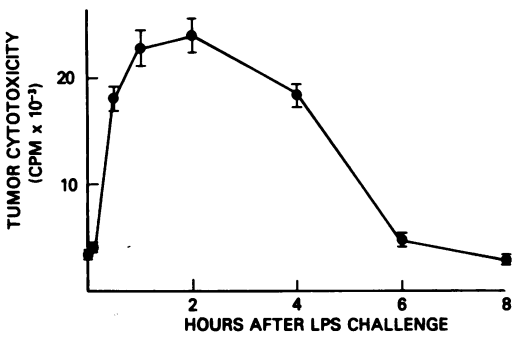

FIG. 4. *Kinetics of the appearance of cytotoxic serum activity after LPS injection of BCG-infected mice. Mice were infected i.v. with 2 × 10⁶ CFU of BCG and injected i.v. with 10 μg of LPS 2 weeks later. After 10 and 30 minutes and after 1, 2, 4, 6, and 8 h, animals were bled and serum was prepared. Tumor cytotoxicity with 1/100 dilution of the sera was estimated by [³H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 50 × 10³).*

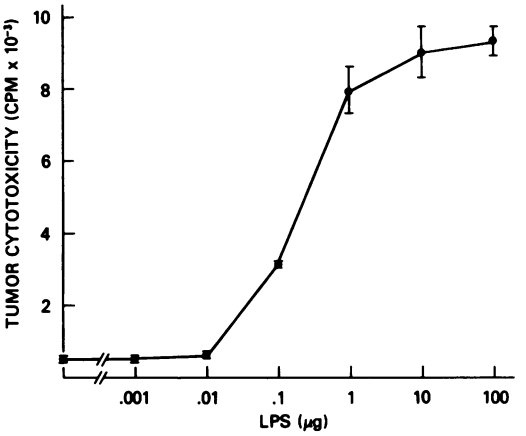

FIG. 5. *LPS dose response for induction of cytotoxic serum factor. Mice were infected i.v. with 2 × 10⁶ CFU of BCG and injected i.v. with 0.001 to 100 μg of LPS 2 weeks later. After 2 h, animals were bled and serum was prepared. Tumor cytotoxicity with a 1/100 dilution of the sera was estimated by [³H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 35 × 10³).*

TABLE 2. *Tumor cytotoxic activity in serum of untreated or BCG-infected mice challenged with different stimuli*[a]

Challenge[b]	C3H/HeN		C3H/HeJ	
	Untreated	BCG infected	Untreated	BCG infected
Ph-LPS	1,500 ± 150	28,300 ± 50	2,050 ± 50	1,850 ± 100
Bu-LPS	1,550 ± 50	26,450 ± 900	1,600 ± 100	1,550 ± 150
R 595-LPS	1,500 ± 50	25,700 ± 850	1,450 ± 50	1,850 ± 300
PS	2,050 ± 150	1,950 ± 200	1,400 ± 500	1,500 ± 50
BCG	1,850 ± 150	1,600 ± 50	1,650 ± 50	1,950 ± 100
PPD	1,650 ± 300	1,700 ± 50	1,600 ± 50	1,550 ± 100

[a] Each value represents cpm ± standard deviation.

[b] C3H/HeN or C3H/HeJ mice infected with 2 × 10⁶ CFU of BCG for 2 weeks were challenged intraperitoneally with 50 μg of phenol-extracted *E. coli* K 235 LPS (Ph-LPS), 50 μg of butanol-extracted *E. coli* K 235 LPS (Bu-LPS), 10 μg of phenol-extracted *S. minnesota* R 595 LPS, 50 μg of polysaccharide from *S. minnesota* (PS), 2 × 10⁶ CFU of BCG, or 100 μg of purified protein derivative (PPD). After 2 h, animals were bled and sera were prepared. Tumor cytotoxicity was estimated by [³H]TdR release of labeled L 929 cells after 48 h of culture in a 1/100 dilution of the sera (total incorporated cpm = 50 × 10³).

with nonresponsive C3H/HeJ bone marrow failed to produce cytotoxic serum activity after LPS challenge. Although the capacity of mice to respond to lipid A was essential for tumor cytotoxic activity in the serum, there was no apparent need for T cell participation. Serum of BCG-injected mice homozygous for the *nu* gene (nude mice) developed strong tumor cytotoxic activity after intravenous LPS treatment (Table 4). Although production of tumor cytotoxic activity in the serum of LPS-treated nude mice is at variance with an earlier observation by Old (18), a recent report by Berendt et al. showed strong LPS-induced tumor-necrotizing effects in T cell-deficient mice (4).

Biochemical characterization of the tumor cytotoxic activity from serum. Tumor cytotoxic activity was as follows. (i) Cytotoxic activity was stable at 57°C for more than 1 h, and no significant loss in activity was observed when samples were lyophilized or stored in solution at 4°C for several weeks.

TABLE 3. *Demonstration of tumor cytotoxic factor in serum after LPS treatment of BCG-infected mice*

Donor	Recipient[a]	No. of mice with cytotoxic serum factor per no. of mice tested
C3H/HeN	C3H/HeN$_x$	13/14
C3H/HeJ	C3H/HeN$_x$	0/18
C3H/HeJ	C3H/HeJ$_x$	0/15
C3H/HeN	C3H/HeJ$_x$	6/8

[a] Recipient animals received 850 roentgens of X-irradiation before reconstitution with 10^7 bone marrow cells. Six weeks after reconstitution, animals were infected i.v. with 2×10^6 CFU of BCG, and 2 weeks later they were injected i.v. with 10 μg of LPS. After 2 h, mice were bled, and tumor cytotoxicity was estimated by [^{3}H]TdR release of labeled L 929 cells after 48 h of culture in a 1/100 dilution of the sera.

TABLE 4. *Tumor cytotoxic activity of sera from BCG-infected C3H/HeN and nude mice challenged with LPS*[a]

Challenge (μg of LPS)	C3H/HeN[b]	C3H/HeN nu/nu
0	3,700 ± 100	2,900 ± 350
0.5	11,200 ± 550	15,700 ± 50
5	23,800 ± 850	29,800 ± 950
50	29,800 ± 700	28,500 ± 950

[a] Each value represents cpm ± standard deviation.
[b] C3H/HeN or C3H/HeN nu/nu mice infected i.v. with 2×10^6 CFU of BCG for 2 weeks were injected i.v. with 0, 0.5, 5, and 50 μg of LPS and bled 2 h later. Tumor cytotoxic activity was estimated by [^{3}H]TdR release of labeled L 929 cells after 48 h of culture in a 1/100 dilution of the sera (total incorporated cpm = 56×10^3).

(ii) BCG-LPS and control sera were applied to Sephacryl S-200 columns and eluted with Tris buffer containing 0.1 M sodium chloride. The elution profile showed one major peak; cytotoxic activity was found at an apparent molecular weight of 150,000. Control serum fractions had little or no cytotoxic activity. Most of the cytotoxic activity in serum was found in the precipitate of a 40 to 60% saturated ammonium sulfate solution. The ammonium sulfate precipitate was chromatographed as described above (Fig. 6A). Again, major cytotoxic activity was found in the immunoglobulin G range; however, a second peak with an apparent molecular weight of about 55,000 to 60,000 was also detected. Fractions of LPS-induced control serum were inactive. Rechromatography of the same ammonium sulfate-

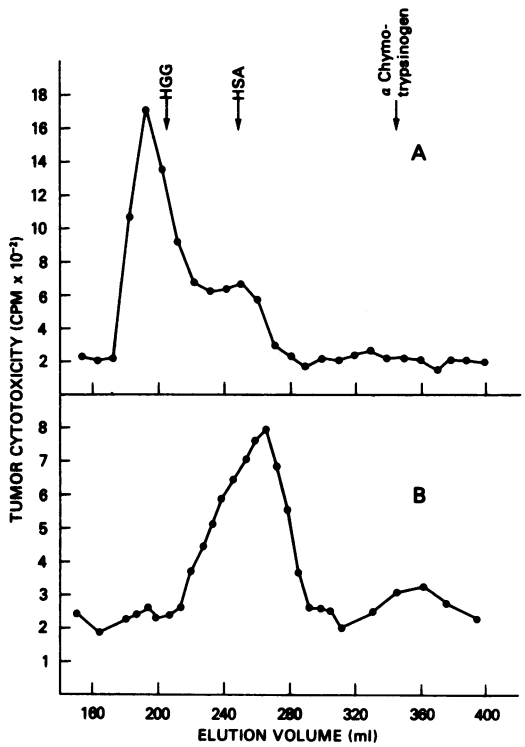

FIG. 6. *Sephacryl S-200 chromatography of ammonium sulfate-precipitated cytotoxic activity from BCG-LPS serum. Material precipitated with 40 to 60% ammonium sulfate saturation from 2 ml of BCG-LPS serum was subjected to Sephacryl S-200 columns. The columns were 2.6 by 90 cm, and the flow rate was approximately 12 ml/h. The columns were equilibrated and eluted with 0.05 M Tris buffer (pH 7.5) containing 0.1 M sodium chloride (A) or 1.6 M sodium chloride (B). Tumor cytotoxicity of a 1/50 dilution of the 3-ml sterile filtered fractions was estimated by [^{3}H]TdR release of labeled L 929 cells at 48 h. Total incorporated cpm = 35×10^2 (A), 30×10^2 (B).*

precipitated material on an identical column but with a buffer of higher sodium chloride content (1.6 M) shifted the elution of cytotoxic activity to a single peak at 55,000 to 60,000 (Fig. 6B). In a previous study, we reported these dissociating conditions in more detail, and the effect has been used to separate lymphocyte-activating factor from cytotoxic activity (13).

(iii) Tumor cytotoxic serum or control serum was applied to diethylaminoethyl-Sephacel columns and washed with Tris buffer (pH 7.1). A gradient was started from 0 to 0.4 M sodium chloride. Cytotoxic activity in BCG-LPS serum eluted from the column at a 0.17 to 0.2 M sodium chloride concentration (Fig. 7). No cytotoxic activity was detected in LPS-induced control serum fractions. Pooled fractions containing cytotoxic factor were then applied to a Sephacryl S-200 column after extensive dialysis and lyophilization and chromatographed in Tris buffer containing 0.1 M sodium chloride. Cytotoxic activity eluted as a single peak at an apparent molecular weight of 55,000 to 60,000.

(iv) A 0.5-ml amount of unfractionated BCG-LPS serum was subjected to flatbed electrofocusing in a pH gradient from 4 to 6.5. Cytotoxic activity was recovered from the fraction eluates in a single peak together with serum albumin at about pH 4.8 (Fig. 8); yield was less than 5% of that applied, explanable in part by the instability of activity below pH 6 (data not shown).

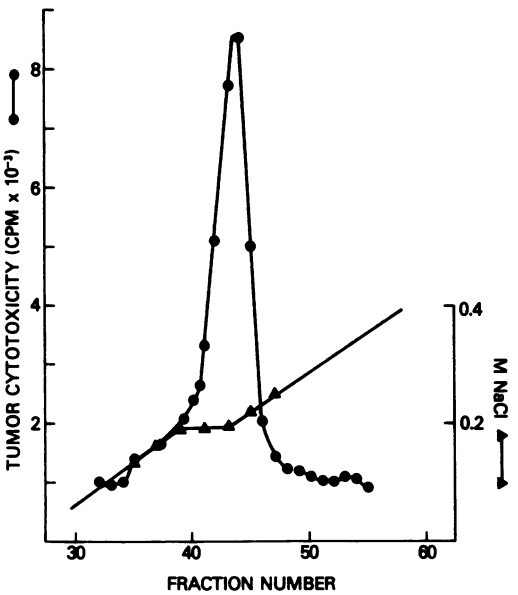

FIG. 7. *DEAE-Sephacel chromatography of BCG-LPS serum. A 2-ml amount of unfractionated serum was applied on a column (1.5 by 30 cm) of DEAE-Sephacel equilibrated in 0.5 M Tris buffer (pH 7.5). The column was washed with 4 column volumes of starting buffer before initiation of a 0 to 400 mM sodium chloride gradient (total gradient volume was 280 ml). The flow rate was approximately 12 ml/h. Fractions (2 ml each) were collected and sterile filtered. Tumor cytotoxicity of a 1/10 dilution of the fractions was estimated by [^{3}H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 50 × 10^3). The molarity of the fractions was determined with an osmometer.*

DISCUSSION

Discovery of soluble mediators in sera of BCG-infected mice treated with microgram quantities of LPS that reproduced tumor necrotic actions of milligram quantities of LPS in noninfected animals was an important advance in the analysis of the antitumor properties of bacterial endotoxin (5).

Characterization of these soluble mediators (tumor necrosis factors) promised the opportunity of separating, for the first time, the therapeutic action of LPS from the toxic and often lethal side effects. Fulfillment of this promise, however, was impeded by a difficult and imprecise in vivo assay.

Serum-induced necrosis of an intradermal tumor transplant required large volumes of active serum (0.5 ml per mouse), and the extent of the tumor necrosis endpoint was dependent upon location, size, and vascularization of the tumor (18).

We have described in this report a sensitive and quantitative in vitro assay to detect cytotoxic mediators in sera of LPS-treated, BCG-

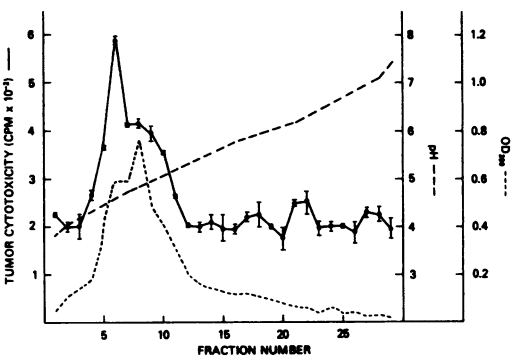

FIG. 8. *Electrofocusing of BCG-LPS serum. A 0.5-ml amount of unfractionated BCG-LPS serum was focused on a flatbed of Ultrodex with 5% Pharmalyte (pH 4 to 6.5) for 18 h. Fractions were removed and eluted with 6 ml of phosphate-buffered saline and dialyzed, and the tumor cytotoxicity of a 1/20 dilution of the fractions was estimated by [^{3}H]TdR release of labeled L 929 cells at 48 h (total incorporated cpm = 35 × 10^2).*

infected mice; cytotoxic activity in serum can be reproducibly detected through a 1/1,000 dilution, and the cytotoxic endpoint is now defined by release of [^{3}H]TdR from prelabeled target cells instead of subjective grading for tumor necrosis.

Properties of cytotoxic factors in serum by the in vitro assay were very similar to those of tumor necrosis factor as defined by the in vivo assay.

(i) The time course for optimal production of tumor necrosis factor and for cytotoxic serum activity (intervals between BCG infection and LPS treatment and between LPS treatment and serum collection) was identical (5).

(ii) Production of tumor necrosis factor and cytotoxic serum activity were both dependent upon normal LPS responsiveness; LPS-insensitive C3H/HeJ mice or X-irradiated LPS-sensitive C3H/HeN chimeric mice reconstituted with C3H/HeJ bone marrow failed to produce either activity after LPS challenge (13a).

(iii) The in vivo tumor necrotic action of LPS was evident in mice homozygous for the *nu* gene (data not shown); serum from LPS-treated, BCG-infected nude mice was also cytotoxic to tumor target cells in vitro.

(iv) Physicochemical characterization of mouse tumor necrosis factor reveals a heat-stable activity with an apparent molecular weight of 150,000 (10). A recent report of a similar tumor necrotic factor of rabbit origin, however, described activity with an apparent molecular weight of about 50,000 (14). This conflict in apparent molecular weight of mouse and rabbit tumor necrosis factor(s) could be explained by species differences. Our results, however, suggest an alternative explanation; mouse tumor necrosis factor may exist as an aggregate in serum. Cytotoxic activity of active serum, as previously described by Carswell et al. (10), eluted from Sephacryl S-200 in the 125,000 to 150,000 molecular weight region. Ammonium sulfate precipitation of this same serum followed by gel filtration in high ionic strength buffer of the precipitate (conditions favoring separation of aggregates) led to elution of cytotoxic activity at 55,000 to 60,000 daltons.

Several investigators have suggested that the cellular source of tumor necrosis factor may be the macrophage. Indeed, soluble cytotoxic factors have been isolated from macrophage cultures after a variety of in vitro treatments (1, 12, 21, 24). Matthews described a soluble cytotoxic factor from rabbit monocytes which resembled the rabbit serum-derived tumor necrosis factor (15). Preliminary results from our laboratory confirm and extend this observation. Macrophages from BCG-infected mice treated in vitro with LPS release soluble cytotoxic factors within 2 h of treatment. Physicochemical characteristics of this macrophage-derived cytotoxin were very similar to those of the serum factor described in this report. Cytotoxic factors were not detected in fluids of LPS-treated control macrophage cultures. These results suggest that the LPS-induced release of this cytotoxin may depend upon the level of macrophage activation. Recent observations by Russel and co-workers indicate that macrophages within growing tumors are either activated or can be activated with very small quantities of LPS (22). The tumor necrotic action of LPS could certainly be mediated by soluble factors released by these intratumor-activated macrophages.

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