

Inhibition of Nonspecific Tumoricidal Activity by Activated Macrophages with Antiserum Against a Soluble Cytotoxic Factor

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Mouse peritoneal macrophages activated for tumor cytotoxicity by any of several *in vivo* or *in vitro* treatments released a soluble cytotoxin into culture fluids only after exposure to small amounts of bacterial lipopolysaccharides. This cytotoxic factor was physicochemically similar to the cytotoxic factor (tumor necrosis factor) in sera of BCG-infected mice injected with lipopolysaccharide. A rabbit antiserum against partially purified serum cytotoxic factor also inhibited the activity of macrophage-derived cytotoxic factor. Of special interest was the observation that rabbit anti-cytotoxic factor inhibited the cytotoxic activity of macrophages both in the presence and in the absence of exogenously added lipopolysaccharide. Inhibition was not complete but was consistent in all experiments. Thus, cytotoxic factor was implicated as a possible effector molecule in the nonspecific tumoricidal activity of activated macrophages.

The tumor necrotic effects of bacterial lipopolysaccharides (LPS) have been known for several decades (2, 23). Recent findings by Carswell et al. showed that this tumor necrosis activity is mediated by a factor found in sera of LPS-treated tumor-bearing animals (1). This factor was also found in sera of BCG-infected mice hours after LPS injection (BCG-LPS serum) (1). That the serum factor also has cytotoxic activity against tumor cells *in vitro* (but not against normal embryo cells) provides a relatively simple and quantitative assay (1, 5, 8, 12, 20).

We have previously shown that a cytotoxic factor (CF) physicochemically similar to that found in BCG-LPS sera can be detected in cultures of LPS-treated macrophages from BCG-infected mice (9, 10). This observation suggested that CF may contribute to the cytotoxic activity of activated macrophages (9).

We have explored this possibility in this report by using the immunoglobulin G (IgG) fraction from a rabbit antiserum raised against serum-derived CF to define a possible role for this molecule in nonspecific macrophage-mediated tumor cytotoxicity.

MATERIALS AND METHODS

Mice. Male and female C3H/HeN and C57BL/6N mice, 6 to 12 weeks of age, were obtained from the Division of Research Services, National Institutes of Health.

LPS. LPS from *Escherichia coli* K235 was prepared

by the phenol-water extraction method of McIntire et al. (13).

Lymphokine supernatant. C3H/HeN mice were immunized intradermally with 5×10^6 viable *Mycobacterium bovis*, strain BCG (Phipps substrain, TMC 1029, Trudeau Mycobacterial Collection, Saranac Lake, N.Y.) (22). Animals were sacrificed 3 to 6 weeks after BCG immunization. Spleens were aseptically removed and passed through no. 50 mesh stainless-steel sieves into tissue culture medium (RPMI 1640 with antibiotics; GIBCO Laboratories, Grand Island, N.Y.). Single-cell suspensions, obtained by serial aspirations through 19- and 23-gauge needles, were treated with tris(hydroxymethyl)aminomethane-buffered NH_4Cl solution to lyse erythrocytes. Spleen cells were centrifuged at $250 \times g$ for 10 min at 4°C and suspended to a concentration of 5×10^6 viable cells/ml in medium with 5% heat-inactivated fetal calf serum (GIBCO). Twenty-milliliter portions of the spleen cell suspension with 50 to 100 μg of purified protein derivative (Connaught Medical Research Laboratories, Toronto, Canada) per ml were incubated in upright 75-cm² plastic tissue culture flasks (no. 3023; Falcon Plastics, Oxnard, Calif.) for 48 to 60 h at 37°C . Supernatant fluids were pooled, centrifuged at $450 \times g$ for 15 min at 4°C , and stored at -20°C until used.

Preparation of cytotoxic serum. Mice were injected intravenously with 2×10^6 viable BCG and injected intravenously 14 days later with 10 μg of LPS. Two hours after LPS injection, animals were exsanguinated, and the serum (BCG-LPS serum) was prepared (8). Control serum was obtained from LPS-injected normal mice in a similar manner. All sera were stored at -20°C until used.

Antisera. Female rabbits (New Zealand white)

were obtained from the Division of Research Services, National Institutes of Health. Animals were immunized with BCG-LPS serum-derived tumor CF partially purified by Sephacryl S200 and diethylaminoethyl-Sepharcel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) chromatography or with an equivalent fraction from control serum. Rabbits were injected subcutaneously in complete Freund adjuvant as described (8, 9). Each immunizing preparation contained about 25 mg of protein per injection. The animals were boosted with the same antigen preparations subcutaneously in complete Freund adjuvant twice at 3 and 8 weeks and again at periodic intervals without Freund adjuvant. Rabbits were bled from the ear artery, and serum was prepared. All antisera were heat inactivated (30 min at 56°C), and the proteins were precipitated with 50% saturated ammonium sulfate. Precipitates were dissolved and extensively dialyzed against phosphate-buffered saline (PBS). Heat-inactivated serum was applied on protein A-Sepharose CL-4B (Pharmacia) at pH 7, and IgG was eluted with glycine buffer at pH 3. This IgG fraction was extensively dialyzed against Dulbecco minimal essential medium (DMEM). Normal rabbit IgG was obtained from Cappel Laboratories, Inc., Downingtown, Pa.

Peritoneal cells. Mice were treated intraperitoneally (i.p.) 1 day previously with 1 ml of PBS or 7 days previously with: (i) 2×10^6 viable BCG; (ii) 500 μ g of pyran copolymer (National Cancer Institute, Bethesda, Md.); (iii) 1.4 mg of killed *Corynebacterium parvum* (Wellcome Research Laboratories, Beckenham, England); (iv) 2% starch suspension (Connought Medical Research Laboratories); or (v) 1:1,000 suspension of latex beads (1- μ m polystyrene beads; Dow Chemical Co., Midland, Mich.) in 0.5 ml of PBS each. Peritoneal exudate cells (PEC) were collected after i.p. injection of 8 to 10 ml of DMEM as described (21). This medium contained 2 g of NaHCO_3 and 4.5 g of glucose per liter, 10% fetal calf serum, and 50 μ g of gentamicin per ml. Peritoneal fluid was withdrawn through the anterior wall through 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°C. Differential counts were made on Wright-stained cell smears prepared by cytocentrifugation (Cytospin centrifuge; Shandon Southern Instruments, Camberley, England). Washed PEC suspensions from each pool of mice were adjusted to equal macrophage concentrations in medium used for cell collection. Macrophages were exposed to dilutions of lymphokine supernatants as adherent PEC for 6 h at 37°C in 5% CO_2 in moist air.

CF assay. Tumor cells (mouse L929, American Type Culture Collection, Rockville, Md., unless indicated otherwise) at 4×10^3 cells per 6.4-mm culture well (Costar 96, Cambridge, Mass.) were labeled in 0.1 ml of Eagle minimal essential medium (EMEM) with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 5% heat-inactivated fetal calf serum, and 0.5 μ Ci of [methyl- ^3H]thymidine per ml (^3H]TdR; specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 18 to 24 h at 37°C in 5% CO_2 in moist air (7). Tumor cell monolayers

were washed twice after labeling and incubated with dilutions of cytotoxic samples in 0.2 ml of DMEM. After 48 h, labeled tumor cell monolayers lysed with 0.5% sodium dodecyl sulfate (SDS) in water were used to estimate total incorporated [^3H]TdR. Cytotoxicity was estimated by measuring release of [^3H]TdR from prelabeled tumor cells in duplicate cultures and expressed as mean counts per minute \pm standard error of the mean (SEM) or as percentage of SDS total counts.

Macrophage cytotoxicity assay. A modified protocol of the cytotoxicity assay described by Meltzer et al. (15) was used. Tumor 1023 was induced in a C3H/HeNir male mouse with pellets of 1% 3-methylcholanthrene in paraffin. Target cell lines were established from enzyme-digested tumor fragments and were maintained in culture with EMEM supplemented with 10% fetal calf serum. [^3H]TdR-prelabeled tumor cells were obtained from washed and trypsin-digested monolayers (2×10^6 to 3×10^6 viable cells/75-cm 2 culture flask in 20 ml of EMEM with 25 mM HEPES buffer, 5% fetal calf serum, and 1 μ Ci of [^3H]TdR per ml for 18 to 24 h at 37°C in 5% CO_2 in moist air). Six thousand tumor cells were added to 6.4-mm culture wells (Costar 96) with in vivo- or in vitro-activated adherent PEC in a total volume of 0.2 ml of DMEM. After 48 h, labeled tumor cell monolayers lysed with 0.5% SDS in water were used to estimate total incorporated counts. Cytotoxicity was estimated by measurement of release of [^3H]TdR from prelabeled tumor cells in duplicate cultures and expressed as mean counts per minute \pm SEM or percentage of SDS total counts.

RESULTS

Correlation between macrophage tumoricidal activity and release of CF. A soluble CF physicochemically similar to that found in BCG-LPS sera is detected in culture fluids of LPS-treated macrophages from BCG-infected animals (9, 10). To determine whether CF plays a role in the nonspecific tumoricidal activity of activated macrophages in vitro, we attempted to correlate levels of macrophage tumoricidal activity with release of CF into culture fluids (Table 1). Macrophages from mice treated i.p. with viable BCG, killed *C. parvum*, or pyran copolymer were strongly cytotoxic to tumor cells in vitro; cells from mice treated with PBS, starch, or latex beads had little or no cytotoxic activity. Levels of CF released by any of these cytotoxic or noncytotoxic macrophages, however, were negligible. These observations confirm those in many previous reports which failed to detect soluble cytotoxins in cultures of activated macrophages (see reference 6 for review).

The pattern of CF release by macrophage cultures incubated with small amounts of LPS, however, was entirely different from that described above. Cultures of activated macrophages from three different sources (BCG-, *C.*

TABLE 1. Correlation between macrophage tumoricidal activity and release of cytotoxic factor^a

Macrophages from mice treated with:	Macrophage tumoricidal activity	Release of CF ^b	
		-LPS	+LPS
BCG	2,240 ± 130 (26)	470 ± 20 (11)	1,410 ± 60 (33)
Pyran	2,380 ± 250 (27)	640 ± 10 (15)	1,600 ± 110 (38)
<i>C. parvum</i>	2,020 ± 130 (23)	560 ± 20 (13)	1,640 ± 80 (39)
PBS	770 ± 70 (9)	340 ± 30 (8)	480 ± 60 (11)
Starch	620 ± 20 (7)	360 ± 80 (8)	360 ± 60 (8)
Latex	790 ± 10 (9)	340 ± 40 (8)	400 ± 60 (9)
No macrophages	680 ± 70 (8)	220 ± 70 (5)	
SDS total counts	8,740 ± 700 (100)	4,250 ± 170 (100)	

^a Adherent PEC from mice treated i.p. with 2×10^6 viable BCG, 500 μ g of pyran, 1.4 mg of *C. parvum*, 2% starch in water, or latex beads 7 days previously or with PBS 1 day previously were incubated with [³H]TdR-prelabeled tumor cells. Cytotoxicity (released [³H]TdR) was estimated at 48 h and expressed as mean counts per minute \pm SEM for duplicate cultures and as percentage of SDS total counts (in parentheses).

^b For release of CF, the cytotoxic activity of 2-h supernatants from 10^6 adherent PEC was tested on [³H]TdR-prelabeled L929 cells. Cytotoxicity (released [³H]TdR) was estimated at 48 h and expressed as mean counts per minute \pm SEM for duplicate cultures and as percentage of SDS total counts (in parentheses).

parvum-, or pyran-treated mice) incubated with 10 ng of LPS per ml released significant levels of CF. LPS alone (data not shown) or culture fluids from LPS-treated inflammatory macrophages had little or no cytotoxic activity (Table 1).

Even though detectable levels of CF were evident only in culture fluids of activated macrophages treated with an extraordinary stimulus such as LPS, CF could still contribute to the cytotoxic effects of these cells in the absence of LPS. For example, CF bound to macrophages or released only in the macrophage-tumor cell microenvironment would not be detected in culture fluids. To examine this alternative, we attempted to inhibit CF activity with a partially purified and specific rabbit antiserum.

Inhibition of the cytotoxic activity in BCG-LPS sera by rabbit antiserum against serum-derived CF. CF in sera of BCG-infected mice treated intravenously with LPS was partially purified by ammonium sulfate precipitation, diethylaminoethyl ion exchange, and Sephacryl S200 chromatography as previously described (8). Sera of normal mice treated with LPS (control sera) were fractionated by an identical protocol. Rabbits were immunized with purified CF or control sera and bled periodically. The ammonium sulfate precipitate of serum from rabbits immunized with CF inhibited the cytotoxic activity in BCG-LPS sera: 50% inhibition was evident at a 1:640 dilution of antiserum; inhibition was complete at 1:160 (Fig. 1). In contrast, control serum even at the highest concentration (1:40) effected only a slight inhibition of cytotoxic activity.

The inhibitory activity of rabbit anti-CF was isolated in the IgG fraction (Table 2). IgG from rabbit anti-CF purified by elution from protein A-Sepharose strongly inhibited the cytotoxic ac-

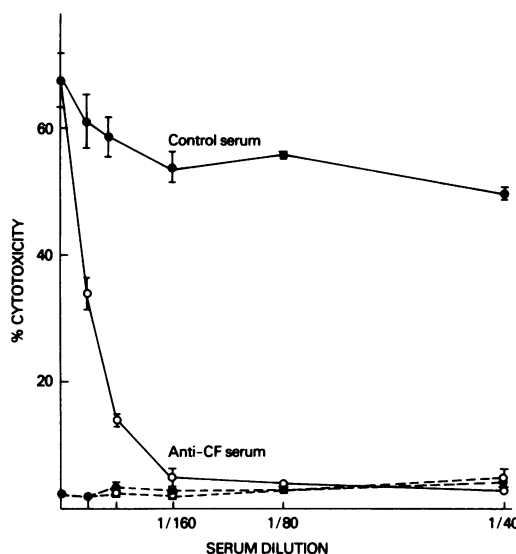


FIG. 1. Inhibition of the cytotoxic activity in BCG-LPS sera by rabbit antisera directed against serum-derived CF. [³H]TdR-labeled L929 cells were incubated with 1:1,000-diluted BCG-LPS serum (—) or medium (---). Dilutions of ammonium sulfate precipitates of heat-inactivated anti-CF (○) or control serum (●) were added. Cytotoxicity was estimated by measurement of [³H]TdR release at 48 h and expressed as percentage of SDS total counts (total incorporated counts per minute = 3×10^3).

tivity of BCG-LPS sera on both L929 and 1023 tumor target cells; IgG from control sera had little or no effect. That inhibition of cytotoxic activity in BCG-LPS sera by IgG from rabbit anti-CF was due to specific interactions with CF in BCG-LPS sera and not to "nonspecific" protective effects on target cells was demonstrated by the following experiment. Protein A-Sepha-

TABLE 2. Inhibition of cytotoxic activity in BCG-LPS sera by IgG from rabbit anti-CF

IgG dilution	Tumor cytotoxicity ^a with given target cells									
	L929					1023				
	BCG-LPS serum		Medium			BCG-LPS serum		Medium		
	Anti-CF	Control	Anti-CF	Control		Anti-CF	Control	Anti-CF	Control	
1:40	460 ± 70 (12)	2,400 ± 200 (63)	340 ± 30 (9)	380 ± 70 (10)		1,070 ± 40 (20)	2,570 ± 100 (48)	680 ± 100 (13)	800 ± 80 (15)	
1:80	620 ± 50 (16)	2,500 ± 40 (66)	320 ± 30 (8)	360 ± 60 (9)		1,120 ± 60 (21)	2,710 ± 10 (51)	690 ± 100 (13)	810 ± 100 (15)	
1:160	910 ± 50 (24)	2,350 ± 40 (62)	340 ± 50 (9)	380 ± 10 (10)		1,260 ± 60 (24)	2,560 ± 70 (48)	680 ± 60 (13)	760 ± 100 (14)	
1:320	1,360 ± 30 (36)	2,500 ± 200 (66)	380 ± 70 (10)	390 ± 80 (10)						
1:640	1,710 ± 70 (45)	2,330 ± 70 (61)	410 ± 40 (11)	390 ± 50 (10)						
No IgG				360 ± 50 (9)		2,670 ± 50 (50)		660 ± 10 (13)		
SDS total counts							5,320 ± 300 (100)			

^a [³H]TdR-labeled L929 cells were incubated with medium or 1:1,000-diluted BCG-LPS serum. [³H]TdR-labeled 1023 cells were incubated with medium or 1:100-diluted BCG-LPS serum. Different concentrations of IgG equivalent to whole serum dilutions were added. Cytotoxicity was estimated by measurement of [³H]TdR release at 48 h and expressed as mean counts per minute ± SEM for duplicate cultures and as percentage of SDS total counts (in parentheses).

rose columns were exposed to equal concentrations of IgG from anti-CF and control sera. When BCG-LPS serum was passed over the anti-CF column, all cytotoxic activity was retained. In contrast, the titer of cytotoxic activity in BCG-LPS serum was unchanged after passage over the control serum column (Table 3).

Effect of rabbit anti-CF on the tumoricidal activity of activated macrophages. Macrophages activated *in vivo* by the effects of BCG infection or activated *in vitro* by lymphokines were cultured with tumor target cells in the presence of IgG from sera of immunized (anti-CF), control (control sera), and untreated rabbits (Fig. 2). Anti-CF exerted a significant inhibitory effect on macrophage-mediated cytotoxicity with both BCG- and lymphokine-activated cells. Inhibition was most evident at the lower effector/target cell ratios. IgG from sera of untreated rabbits had no effect on macrophage tumoricidal activity. In contrast, IgG from sera of control rabbits (animals immunized with sera from normal mice injected with LPS) actually enhanced tumor cytotoxicity. Since both target cells and mouse serum were of C3H/HeN origin, enhancing activity in control serum could have been mediated by rabbit anti-C3H/HeN antibodies not present in sera of untreated rabbits. In fact, absorption of all rabbit sera with 1023 target cells reduced this enhancement without affecting the inhibitory effects of anti-CF (Fig. 3).

The inhibitory effects of anti-CF on tumor cytotoxicity by activated macrophages was independent of mouse strain and activation stimulus (Table 4). Effects of anti-CF were, however, dependent upon the effector/target cell ratio and the amount of anti-CF added during the cytotoxicity assay. Inhibition of macrophage tumoricidal activity was not evident at effector/target cell ratios greater than or equal to 15:1 to 20:1 or with less than 0.25 µg of anti-CF IgG per ml (data not shown). This latter effect was not due to loss by absorption or destruction of antibody activity during the 48-h cytotoxicity assay. The titer of anti-CF in fluids of BCG-activated macrophage-tumor cell cultures (at 20:1 and 7:1 effector/target cell ratios) remained unchanged through 48 h.

Detectable levels of CF in fluids of activated macrophage cultures are found only after exposure of cells to small amounts of LPS. CF is released within 2 h of LPS treatment. If LPS-treated macrophages are washed after this 2-h interval, the cells remain cytotoxic but CF cannot be detected in culture fluids again through 48 h (8). It is possible that anti-CF inhibits only the initial, LPS-dependent event. Later cyto-

TABLE 3. Absorption of cytotoxic activity from BCG-LPS sera by rabbit anti-CF bound to protein A-Sepharose

Eluate ^a	Cytotoxicity		
	Anti-CF IgG	Protein A coated with control IgG	Buffer
1:4,000	590 ± 60 (13)	770 ± 190 (17)	790 ± 60 (18)
1:2,000	590 ± 60 (13)	920 ± 160 (21)	910 ± 130 (20)
1:1,000	650 ± 60 (14)	1,240 ± 210 (28)	1,210 ± 40 (27)
1:500	610 ± 50 (14)	1,580 ± 50 (35)	1,680 ± 130 (37)
Spontaneous release			600 ± 40 (13)
SDS total counts			4,480 ± 240 (100)

^a One milliliter samples of protein A-Sepharose CL-4B were each coated with buffer, 5 mg of anti-CF IgG, or 5 mg of control IgG. Samples of 10 µl of BCG-LPS serum were applied to the columns, and 5 ml of the eluate was collected. [³H]TdR-labeled L929 cells were incubated with different dilutions of the eluates. Cytotoxicity was estimated by [³H]TdR release at 48 h and expressed as mean counts per minute ± SEM for duplicate cultures and as percentage of SDS total counts (in parentheses). More than 50% of the cytotoxic activity from BCG-LPS serum applied was recovered from the uncoated column (buffer).

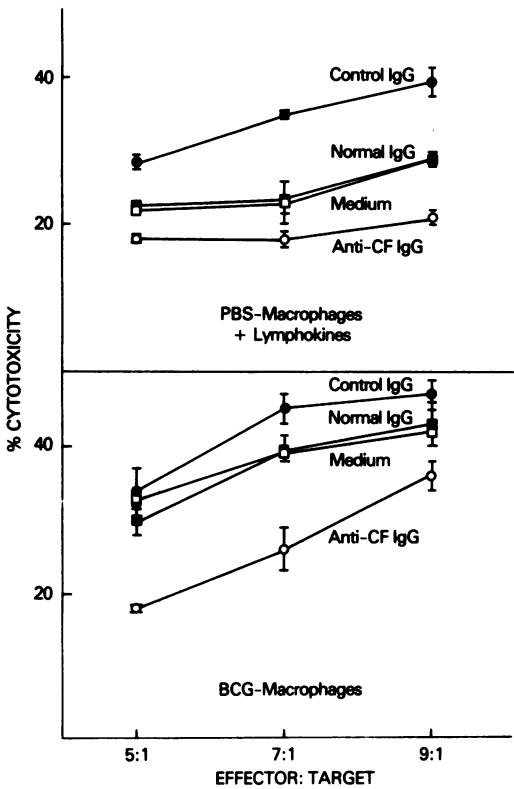


FIG. 2. Inhibition of macrophage tumoricidal activity by IgG from rabbit anti-CF. Adherent PEC from mice treated i.p. with PBS 1 day previously and treated in vitro with lymphokines (1:5 diluted in medium) for 6 h or adherent PEC from mice infected i.p. with 2×10^6 viable BCG 7 days previously were incubated at different effector/target ratios with [³H]TdR-labeled tumor cells. The cells were cultured in medium (□), rabbit IgG (■), anti-CF IgG (○), or control IgG (●). Final IgG concentration was 0.5 mg/ml. Cytotoxicity was estimated by [³H]TdR release at

toxic mechanisms may be unrelated to this early event and thus may not be inhibited by anti-CF. To test this possibility, we examined effects of anti-CF on tumor cytotoxicity by BCG-activated macrophages cultured with and without LPS for 2 h. All cultures were then washed and incubated with anti-CF and target cells (Fig. 4). Anti-CF inhibited the cytotoxic activity of both LPS-treated and nontreated BCG-activated macrophages. The degrees of inhibition were comparable.

It is important to note that the measurement of macrophage-mediated tumor cytotoxicity by release of [³H]TdR used in these studies underestimates the extent of macrophage-tumor cell interaction. Macrophage-mediated cytostatic effects are not measured by this assay. Moreover, dead tumor cells that have not yet autolyzed and released nuclear [³H]TdR by 48 h are likewise not measured (15). Culture morphology (Fig. 5) demonstrates the striking effects of anti-CF on activated macrophage-tumor cell interactions. Cultures incubated in medium alone showed the strong cytopathic effects of activated macrophages on tumor cells (bottom). The cell density did not increase over 48 h. Morphology of cultures incubated with IgG from control or normal rabbit sera was comparable to that of cultures incubated in medium alone (middle). In contrast, cell density of cultures in the presence of anti-CF was very high (top) and similar to the cell density obtained when noncytotoxic resident macrophages were cultured with tumor cells. Since only tumor cells are able to grow under these conditions, increased cell density can be attributed only to tumor cell growth.

48 h and expressed as percentage of SDS total counts (total incorporated counts per minute = 6×10^3 ; spontaneous release = 7%).

DISCUSSION

Data presented in this report demonstrate for a variety of in vivo and in vitro treatments that macrophages activated for nonspecific tumor cytotoxicity are also able to release soluble cytotoxic factor after exposure to small amounts (nanograms) of LPS. Activation in vivo or in vitro, however, was not sufficient for CF release.

Over a broad range of experimental conditions, LPS was always necessary. Moreover, after the initial LPS-induced release of CF from activated macrophages, further release of CF with or without LPS was not evident (8).

These observations suggest that release of CF from activated macrophages is yet another example of macrophage effector function regulated by two stages: cells must be primed by one stimulus to enter into an inactive but receptive stage in which they can then respond or be

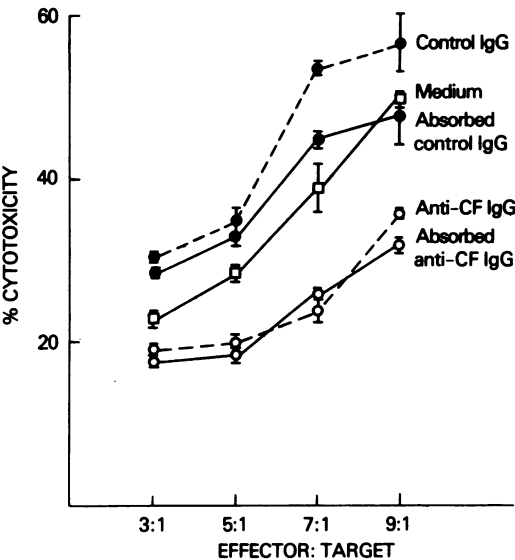


FIG. 3. Inhibition of macrophage tumoricidal activity by anti-CF from rabbit sera absorbed with 1023 fibrosarcoma cells. Two milliliters of IgG solutions (1 mg/ml) was absorbed three times with 25×10^6 1023 cells each for 30 min at 0°C. Adherent PEC from mice treated i.p. with 2×10^6 viable BCG 7 days previously were incubated at different effector/target ratios with [3 H]TdR-labeled tumor cells. The cells were cultured in medium (□), unabsorbed (---) or absorbed (—) anti-CF IgG (○), or control IgG (●). Final IgG concentration was 0.5 mg/ml. Cytotoxicity was estimated by [3 H]TdR release at 48 h and expressed as percentage of SDS total counts (total incorporated counts per minute = 3.5×10^3 ; spontaneous release = 14%).

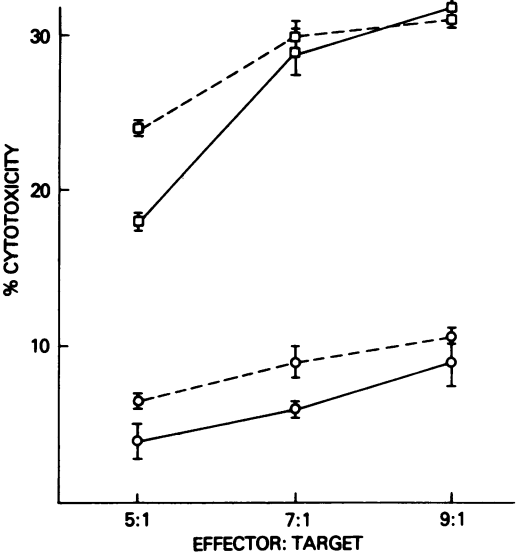
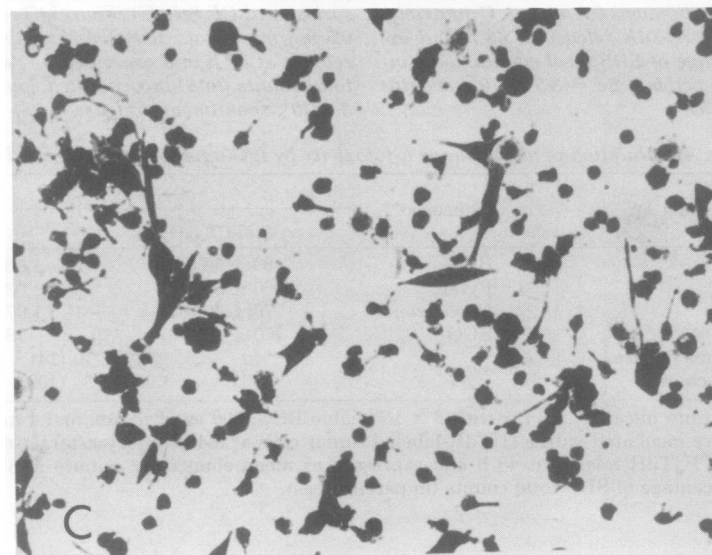
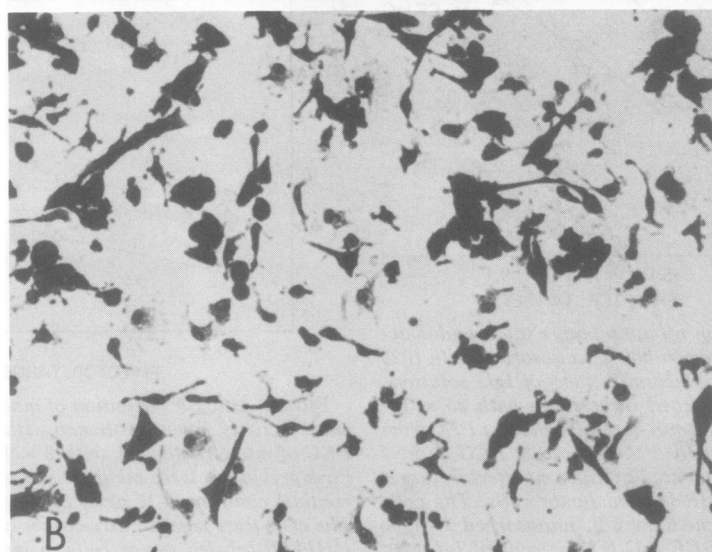
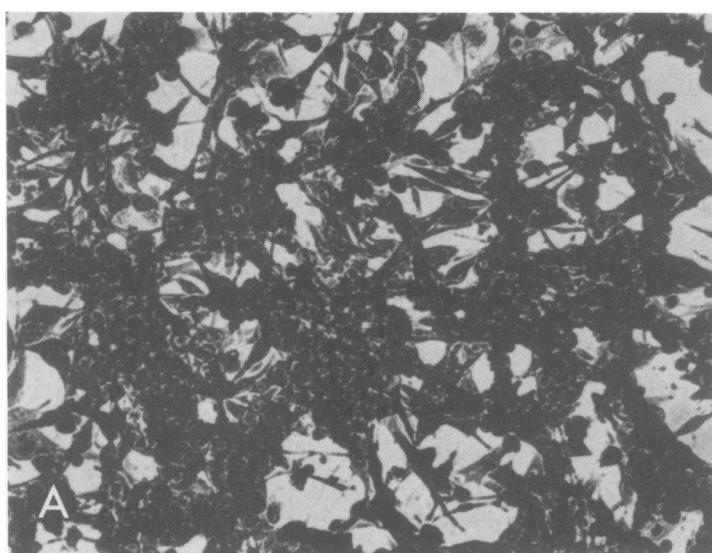


FIG. 4. Anti-CF inhibition of macrophage tumoricidal activity after treatment with LPS. Adherent PEC of mice treated i.p. with 2×10^6 viable BCG 7 days previously were incubated in medium (—) or in medium containing 10 ng of LPS per ml (---) for 2 h. The cells were washed three times and cultured with [3 H]TdR-labeled tumor cells in medium (□) or absorbed anti-CF IgG (○). Final IgG concentration was 0.5 mg/ml. Cytotoxicity was estimated by [3 H]TdR release at 48 h and expressed as percentage of SDS total counts (total incorporated counts per minute = 3×10^3 ; spontaneous release = 5%).

TABLE 4. Inhibition of macrophage cytotoxicity by IgG against serum-derived CF^a

Mouse strain	Stimulus	Cytotoxicity	
		Anti-CF IgG	Medium
C3H/HeN	BCG	480 ± 50 (40)	1,060 ± 40 (89)
	Pyran	560 ± 30 (47)	1,030 ± 90 (86)
	<i>C. parvum</i>	770 ± 30 (64)	1,070 ± 80 (90)
C57BL/6N	BCG	470 ± 40 (40)	780 ± 100 (65)
Spontaneous release		170 ± 10 (14)	
SDS total counts		1,200 ± 90 (100)	

^a Adherent PEC from mice treated i.p. with 2×10^6 viable BCG, 500 µg of pyran, or 1.4 mg of *C. parvum* 7 days previously were incubated with [3 H]TdR-labeled tumor cells at a 10:1 effector/target ratio. Cytotoxicity was estimated by [3 H]TdR release at 48 h and expressed as mean counts per minute ± SEM for duplicate cultures and as percentage of SDS total counts (in parentheses).



triggered by another stimulus for full functional activity. Other seemingly unrelated examples of this two-stage regulation include: secretion and release of plasminogen activator (3), of prostaglandin E_2 (16), and of lymphocyte-activating factor (IL1; 17); elicitation of the H_2O_2 burst (18, 19); and C_3b receptor-mediated phagocytosis (4).

The exact role of CF in nonspecific tumoricidal activity of activated macrophages is not yet defined. Data presented in this report show that the cytotoxic activity of activated macrophages from a variety of sources can be partially but reproducibly inhibited by a purified rabbit anti-CF. Inhibition by anti-CF was effected even with activated macrophages that released no detectable CF in culture and also with activated macrophages that had previously released CF in a response to an LPS trigger and were incapable of further release. The inhibitory effect of anti-CF was not due to functional damage of the macrophage effector cell: (i) antibody-mediated cytotoxicity by BCG-activated macrophages for ^{51}Cr -labeled TNP modified chicken erythrocytes was unaffected by anti-CF (D. Männel, unpublished data); and (ii) activated macrophages incubated with anti-CF for up to 2 h remained cytotoxic after removal of antibody by washing.

In fact, we could not demonstrate binding of anti-CF to the activated cytotoxic macrophage by any of several techniques: the anti-CF titer in fluids of activated macrophage cultures was unchanged through 48 h even though the cytotoxic activity of these activated cells was strongly inhibited. Moreover, use of fluorescein-labeled anti-rabbit antibody to demonstrate binding of rabbit anti-CF to cytotoxic cells or direct attempts to show binding with iodinated Fab fragments of anti-CF were unsuccessful (Männel, unpublished data). CF may be stored within the macrophage and secreted or exposed on the cell membrane only after LPS treatment or perhaps after tumor cell contact.

Complete or near complete inhibition of macrophage cytotoxic activity by anti-CF was evident only at low effector/target cell ratios. At ratios greater or equal to 15:1 to 20:1, macrophage tumoricidal activity was unaffected even by the highest concentration of antibody. Inhibition was not complete at any level of cytotoxicity (range, 33 to 89%) but was consistent in all experiments. Cinemicrographic and microscopic studies of activated macrophages and tumor cells show that tumor cell destruction involves

close contact between effector and target cells (11, 14). In fact, recent studies suggest that binding of tumor cells to macrophages may be a prerequisite event for cytolysis (11). One could assume that the failure to detect anti-CF inhibition at high macrophage/tumor cell ratios reflects steric exclusion of the antibody from the presumed site of killing, the area of contact between macrophage and tumor cell. Alternatively, cytotoxic mechanisms of nonspecifically activated macrophages may vary with the microenvironment. CF may play a major role in cytotoxicity at low cell densities, a hypothesis consistent with the inhibitory effects of anti-CF. Cytotoxicity at higher cell densities, however, might be mediated by multiple and overlapping mechanisms so that elimination of CF-mediated events would be compensated.

In any event, the data presented in this report strongly implicate CF as at least one of the effector molecules in activated macrophage-mediated tumor cytotoxicity. The interesting findings by Kull and Cuatrecasas that CF must be internalized by the target cell to obtain cytolysis (C. F. Kull, Jr., and P. Cuatrecasas, *Fed. Proc.* **39**: 478, 1980) and the observations in this paper suggest a series of future experiments that could clarify the mechanisms of macrophage tumoricidal activity.

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Fig. 5. Morphology of macrophage-tumor cell cultures after incubation in rabbit anti-CF IgG. 1023 tumor cells were cultured with adherent PEC from mice treated i.p. with 2×10^6 viable BCG 7 days previously at an effector/target ratio of 7:1. Cultures were incubated in medium (C), 0.5 mg of 1023-absorbed control IgG per ml (B), or 0.5 mg of 1023-absorbed anti-CF IgG per ml (A) for 48 h and then fixed and stained. Magnification = $\times 100$.

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