Production of a Factor (CIF) from Normal Fibroblast Cells Inhibiting Tumor Necrosis Factor/Cachectin Production (42939)

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Abstract. **Murine embryonic fibroblast cells produce a factor designated cytotoxin-inhibiting factor (CIF) which inhibits tumor necrosis factor (TNF) and interleukin 1 production as well as tumoricidal activity by lipopolysaccharide-activated macrophages. This study determines the physiologic conditions of CIF production in serum-free medium. CIF production was largely dependent upon the presence of lipopolysaccharide. A quantitative correlation between fibroblast cell number, lipopolysaccharide concentration, and incubation time was established. Evidence is presented that CIF inhibited the production or release of TNF. CIF did not destroy TNF after production and release nor did it sequester secreted TNF. The supernatant fluids which inhibited TNF production did not suppress the capability of resting macrophages to phagocytize opsonized sheep erythrocytes, suggesting that only functions expressed in the activated state are inhibited.** [P.S.E.B.M. 1989, Vol 191]

Activated cytotoxic macrophages are thought to play a role in the body's defense against neoplasia and infections. An efficient way to render macrophages cytotoxic is by the addition of lipopolysaccharide (LPS). Such activated macrophages kill certain tumor cells and secrete a soluble factor, tumor necrosis factor (TNF), which lyses certain tumor cells very efficiently (reviewed in 1). We have described previously a cytotoxin inhibiting factor (CIF), produced by normal mouse embryo cells, which could inhibit both TNF and interleukin 1 production and tumoricidal activity by such activated macrophages (2, 3). We now show that little or no CIF is produced in the absence of bovine serum. However, following addition of LPS, CIF production by murine embryonic fibroblast and human foreskin fibroblast cells is markedly enhanced. This study includes the determination of conditions which maximize CIF production by such cultures.

Materials and Methods

TNF Assay. We assay TNF essentially as described previously (4). Briefly, L-929, HeLa, or other susceptible cells are established as monolayers by seeding 96-well flat-bottomed microtiter trays with 30,000 cells/0.1 ml and incubating in an humidified atmosphere of 5% CO_2 at 37°C for 15 to 18 hr. Medium is usually Eagle's minimal essential medium (MEM) with 10% bovine fetal calf serum. Two-fold serial dilutions of TNF-containing fluids are then made across the plate using a hand-held Titertek pipetter which permits the simultaneous dilution of 8–12 samples. Actinomycin D at 5 μg/ml is then added in 0.1 ml. Cell killing by TNF is evaluated by staining the cells with crystal violet after 19 hr of additional incubation. Destruction of the monolayer is quantitated by the use of a Titertek Multiscan apparatus fitted with a 595-nm filter. Control wells, without TNF, are used for 100% survival and wells with maximal killing are used for 0% survival. These data are plotted with a computer program and dose-response curves generated. A unit of TNF activity is defined as the reciprocal of the dilution which results in 50% destruction of the monolayer.

CIF Assays. We assay CIF as previously described (2, 3) except that we now determine units of CIF as follows: dilutions of CIF-containing supernatants are placed on macrophage cultures in 96-well flat-bottomed microtiter trays. Usually, 3-fold dilutions are made and no serum is present, unless otherwise indicated. Following 1.5 hr of incubation, LPS is then added and the plates are incubated for an additional 19 hr. TNF yields are then determined as described for TNF assays and

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compared with yields in control wells with LPS but without CIF. Plotting of the percentage of inhibition of TNF against the log dilution of CIF produces a sigmoid dose-response curve. We refer to a unit of CIF as the reciprocal of the dilution which inhibits TNF production by 50%.

Concentration of CIF. CIF was concentrated by ultrafiltration using Amicon stirred ultrafiltration cells and PM10 and PM30 Diaflo ultrafilters (Amicon Corporation, Danvers, MA).

Embryonic Fibroblast Cultures. The procedure for cultivation of embryonic fibroblast cells from mice has been described (3).

Macrophage Cultures. We employed macrophages from two sources. Usually, peritoneal exudate macrophages from various strains of mice (BALB/c, C57BL/6, or outbreds) are harvested by lavage from the peritoneal cavity 4–5 days after intraperitoneal injection of 3 ml of 3% thioglycollate broth. We have also employed the cloned J774.1 cell line which was originally obtained from a tumor in a BALB/c mouse (5). The cloned cell line is useful since we can be assured that accessory cells are not present. Macrophages from all sources produced TNF upon stimulation with LPS and also kill tumor cells. In addition, all respond to CIF and no longer kill tumor cells or produce TNF. Macrophages from all sources are plated at 100,000 cells in 0.1 ml of medium in 96-well flat-bottomed microtiter trays. Just prior to use, these cultures are washed twice with serum-free fortified MEM since maximal yields of TNF are produced in serum-free media (6). Fortified MEM is MEM with double concentrations of vitamins and essential amino acids and with the addition of nonessential amino acids and sodium pyruvate.

Phagocytosis. In these experiments we used ^{51}Cr-labeled opsonized sheep erythrocytes according to the method of Vogel *et al.* (7). Sheep erythrocytes, freshly harvested, were washed and labeled with 0.2 mCi of ^{51}Cr/ml in the presence of antibody to sheep erythrocytes. After additional washing to remove unincorporated ^{51}Cr, 1×10^8 erythrocytes were added to 24-well trays containing thioglycollate-stimulated peritoneal macrophage from C57BL/6 mice (2×10^5 cells/well). Macrophages were preincubated with the indicated units of CIF for 2 hr and then washed before the addition of erythrocytes. Following incubation of macrophages for 1 hr with erythrocytes, they were washed to remove nonphagocytosed erythrocytes, treated for 3 min with erythrocyte lysing buffer, washed again twice, and then the macrophages lysed with 0.5% sodium dodecyl sulfate. Aliquots of lysed macrophages were counted in a Beckman Gamma DP 5500 counting system.

Results

Effect of LPS and Macrophages on the Generation of CIF. Variations in the yields of different CIF preparations led us to investigate conditions responsible. The first possibilities considered were whether variable levels of endotoxin contaminations in our media, presence of macrophages, or variable fibroblast cell density might be responsible for the variations. Accordingly, various numbers of secondary mouse embryo cells were plated in 1.0 ml of MEM with 10% bovine serum in 24-well plastic trays. Following overnight incubation, wells were washed three times with serum-free fortified MEM and then 1.0 ml of the same medium, with and without 1 μg/ml LPS and with and without 500,000 BALB/c peritoneal macrophages, was added to appropriate wells and incubated for 18 hr. TNF was then assayed as described. Resultant yields of TNF are shown in Table I and demonstrate that (i) the presence of macrophages during the generation of CIF had little effect on the yield of the inhibitor, (ii) the addition of LPS greatly increased the yield of CIF, and (iii) apparent yield of CIF was dependent upon the number of mouse embryo fibroblasts.

When CIF was concentrated by ultrafiltration through PM10 or PM30 ultrafiltration membranes, some activity (from 10 to 50% of the starting activity) flowed through but there was also a considerable concentration of activity in the retentate. We considered that we had at least two separate activities; one with a M_r of less than 10,000 and the other with a M_r of greater than 30,000. When CIF was prepared in the presence of indomethacin (1×10^{-4} *M*), the smaller molecular weight component was no longer found and indicated that it may have been a prostaglandin. All preparations of CIF have subsequently been prepared in the presence of indomethacin.

Effect of Concentration of LPS on Yield of CIF. Realizing that LPS greatly enhanced the apparent yield of CIF as shown in Table I, we then began to strictly

Table I. Inhibition of TNF Production by Macrophages by Supernatant from Variable Numbers of Mouse Embryo Cells Generated in the Presence or Absence of LPS and/or Macrophages

Number of macrophages /cm^2	Number of mouse embryo cells/cm^2	S_{50} units of TNF[a] (% inhibition)	
		+1 μg/ml LPS	No LPS
0	400,000	8 (99.4)	660 (55)
0	200,000	125 (91.4)	710 (51)
0	100,000	140 (90.3)	1000 (31)
0	50,000	165 (89.6)	1000 (31)
0	25,000	275 (81.0)	1600 (0)
500,000	400,000	7 (99.5)	1050 (28)
500,000	200,000	37 (97.4)	1000 (31)
500,000	100,000	69 (95.3)	1000 (31)
500,000	50,000	210 (86.5)	1300 (10)
500,000	25,000	426 (71.0)	2000 (0)

[a] Control 774.1 macrophages produced 1450 ± 175 S_{50} units of TNF. Supernatants were tested (quadruplicate) at 1/4 dilution for effects on TNF production.

control possible LPS contamination in reagents and on glassware. The serum used for fibroblast proliferation was certified to contain less than 0.03 ng/ml LPS and plastic ware was generally employed. When glassware was used, it was heated at 210°C for 4 hr to destroy any LPS that may have been present since it is known to survive autoclaving temperatures. Maximal LPS in the medium was less than 0.003 ng/ml as measured by the Limulus amoebocyte assay. We then determined the effect of various concentrations of LPS on the subsequent yield of CIF. As represented in Figure 1, the yield of CIF was greatly dependent upon the addition of LPS and a maximum yield was achieved at 1.23 μg/ml or greater of LPS. Little, if any, CIF was produced by these mouse embryo cultures when added LPS was used at 0.015 μg/ml or less. For future studies, we employed 4 μg/ml LPS.

Effect of Cell Numbers on LPS-Induced CIF Production. We next studied the effect of cell numbers on CIF production after LPS addition. Secondary cultures of mouse embryo cells were prepared in 24-well trays. Following plating of the cells on MEM with 10% bovine serum and allowing attachment of cells for 6 hr, cultures were washed twice with serum-free fortified MEM and then 1 ml of the same medium was added to each well with 4 μg/ml LPS. The removal of the serum prevented the growth of the cells; they remained attached and well maintained as observed visually with a microscope.

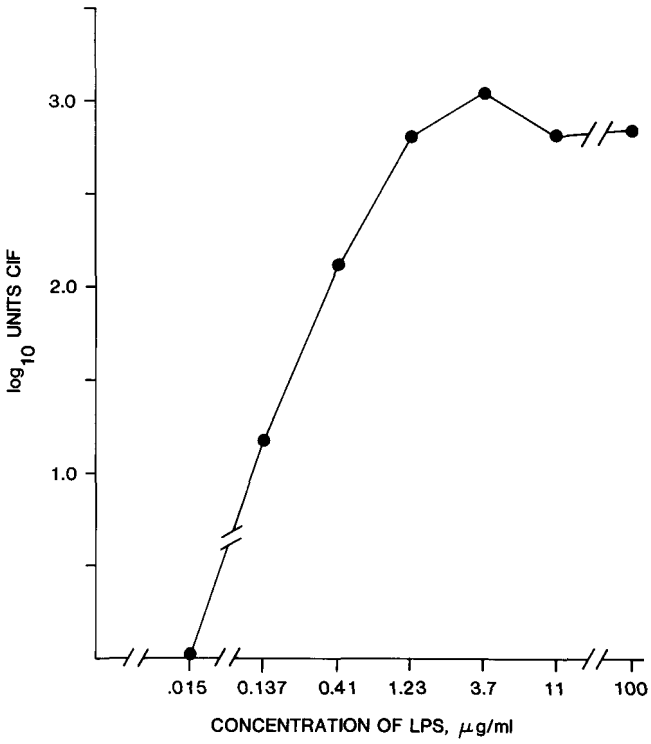

Figure 1. Effect of LPS concentration upon the yield of CIF. LPS was added to (C57BL/6) mouse embryo cultures (cells/cm^2/ml) in serum-free medium and incubated for 24 hr. Titers of CIF were then determined on macrophages in the presence of LPS (1 μg/ml). Macrophages were obtained from the peritoneal cavity of C57BL/6 mice and were elicited with thioglycollate broth.

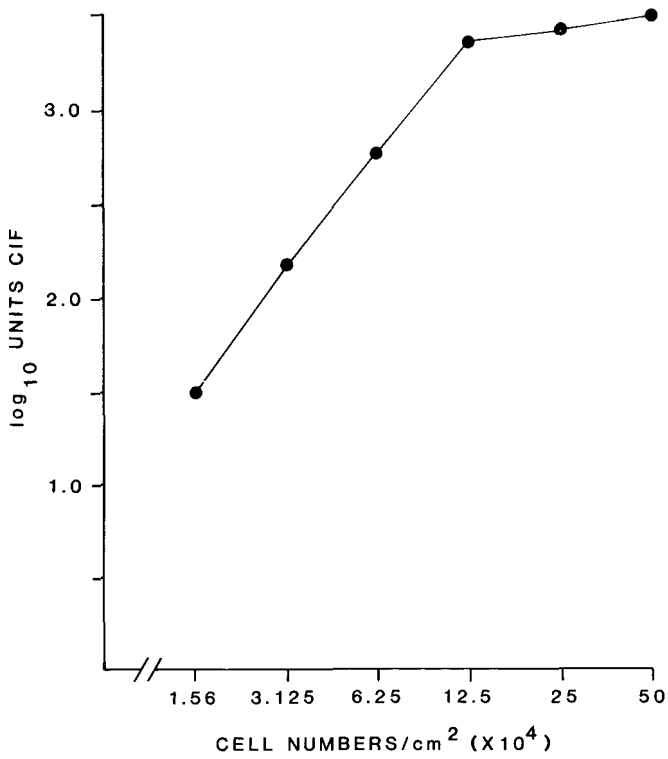

Figure 2. Effect of cell numbers on the yield of CIF. Cells were plated at the indicated densities and LPS was added at 4 μg/ml. Supernatant fluids were harvested 24 hr later and assayed for CIF on C57BL/6 macrophages (peritoneal).

Supernatant fluids were harvested 24 hr later and units of CIF determined. As shown in Figure 2, the yield of CIF was greatly dependent on cell numbers and the yield reached a maximum at about 125,000 cells/cm^2 which is near a monolayer.

Time Course of Production of CIF. Secondary mouse embryo cell monolayers were established, washed free of serum, and LPS was added at 4 μg/ml in serum-free medium. Supernatants were harvested at various periods of time and assayed for CIF yield. As can be seen from Figure 3, CIF production was apparent by 5 hr, reaching a maximum at about 2 days. Other studies have indicated that the production of CIF is detectable within 2 hr after the addition of LPS. Cells, again by microscope evaluation and trypsin blue staining, appeared to be healthy at the end of 3 days.

Sequential Production of CIF. Since the failure to continue to produce CIF after 2 days may have been due to a depletion of some essential nutrient for CIF production, we determined how long mouse embryo cell cultures could produce CIF if given a medium change and fresh LPS every 24 hr. Flask cultures of mouse embryo cells were washed free of serum and placed in serum-free medium with 4 μg/ml LPS. The medium was replaced daily with fresh medium and LPS. CIF yields were then determined and the results are shown in Table II. Rather constant yields were found up to 7 days and then slowly declined thereafter. Light microscopic observations indicated that the cells

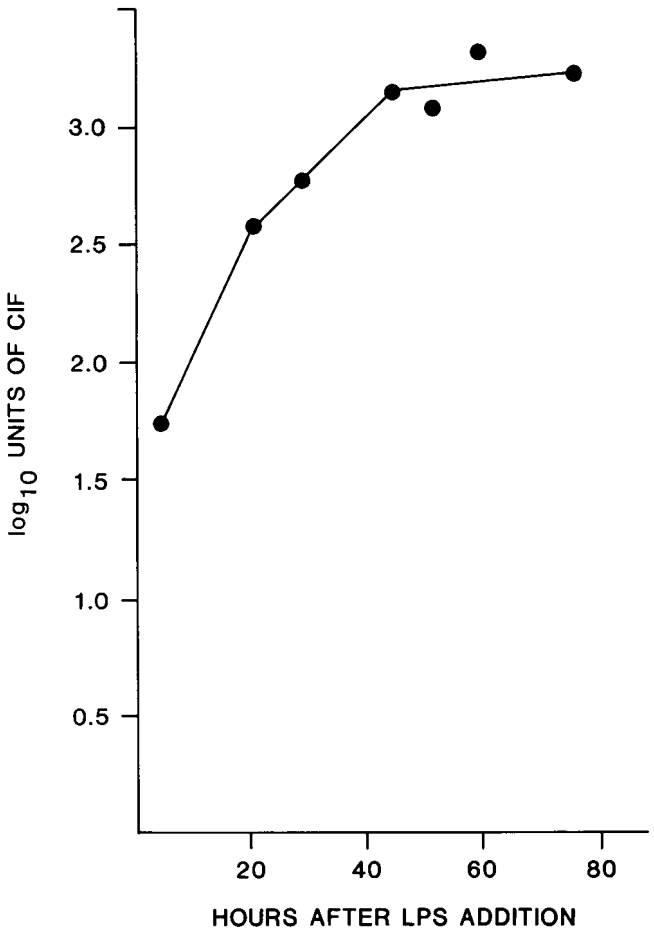

Figure 3. Time course of production of CIF after LPS addition to mouse embryo cell cultures. LPS was added at 4 μg/ml. CIF was assayed on C57BL/6 macrophages harvested from the peritoneal cavity after thioglycollate broth elicitation.

began to deteriorate at about the time the yields of CIF began to decline. Cells began to round up and become granular and some were detached. The reason for the relatively low yield of CIF on Day 1 is this study is unknown and higher yields often occur.

Production of CIF by Other Cells. Human foreskin cultures in serum-free media in the presence of LPS have also been shown to produce significant amounts of CIF (200–400 units in 24 hr) when measured on mouse macrophages, indicating that the factor is not species specific. We have not yet measured CIF on human macrophages so we do not know if there is a species preference as there is for TNF. Maximum yields of human CIF seems to require a higher concentration of LPS (>75 μg/ml). We have also shown that certain mouse tumor cells lines produce CIF (Loewenstein, Gifford, and Gallily, submitted for publication).

Effect of CIF on Phagocytosis. So far we have shown that CIF inhibits macrophage functions induced by LPS activation. We therefore studied its effect on functions carried out by the macrophage in its resting state. As shown in Table III, we determined the effect of CIF on macrophage phagocytosis of ^{51}Cr-labeled opsonized sheep erythrocytes. The results obtained

Table II. Daily (Sequential) Production of CIF by Mouse C57BL/6 Embryo Cultures[a]

Day	Units of CIF
1	80
2	410
3	440
4	460
5	360
6	430
7	470
8	330
9	200
10	230

[a] Flask cultures of mouse embryo cells were washed free of growth medium and placed in serum-free medium with 4 μg/ml LPS. Medium was replaced daily with fresh serum-free medium with 4 μg/ml LPS. CIF units were then determined by assay on C57BL/6 macrophages harvested from the peritoneal cavity of thioglycollate broth-injected mice (4 days prior to harvesting).

Table III. Effect of CIF on Phagocytosis of Opsonized ^{51}Cr-Labeled Sheep Erythrocytes by C57BL/6 Macrophages[a]

Units of CIF	cpm ± SD[b]	% control
None	5725 ± 647	—
160	7748 ± 562	135
48	7666 ± 681	134
16	7364 ± 270	129

[a] Sheep erythrocytes were washed and labeled with 0.2 mCi ^{51}Cr/ml in the presence of antibody to sheep erythrocytes. After additional washing to remove unincorporated ^{51}Cr, 1 × 10^8 erythrocytes were added to 24-well trays containing thioglycollate-stimulated peritoneal macrophages from C57BL/6 mice (2 × 10^5 cells/well). Macrophages were preincubated with the indicated units of CIF for 2 hr and then washed before the addition of erythrocytes. Following incubation of macrophages for 1 hr with erythrocytes, they were washed to remove nonphagocytosed erythrocytes, treated for 3 min with erythrocyte lysing buffer, washed again twice, and then the macrophages lysed with 0.5% sodium dodecyl sulfate. Aliquots of lysed macrophages were counted in a Beckman Gamma DP 5500 counting system.
[b] Standard deviation; three replicate samples.

clearly indicate that CIF employed at 160 units did not have any inhibitory effect on phagocytosis via the Fc receptors. Indeed, some stimulation of phagocytosis was apparent.

Effect of CIF on TNF Activity. We considered the possibility that CIF destroyed TNF after production and release or sequestered secreted TNF so that it would not be able to adsorb to receptors on susceptible target cells. To test this possibility, we incubated CIF (or diluent) with various dilutions of TNF, incubated the mixtures at 37°C for 90 min, and then assayed the mixture for biologic activity of TNF. As seen in Table IV, no significant loss of TNF activity resulted.

Discussion

The results presented in this article show that normal mouse embryo cells and human foreskin fibroblasts produce a factor which can dramatically inhibit macrophage production of TNF. Our data further show that

Table IV. Effect of CIF on TNF Activity

Dilution of TNF	S_{50} units of TNF activity[a]	
	Without CIF	With CIF[b]
1/10	3300 ± 180	3380 ± 320
1/40	930 ± 50	1060 ± 100
1/80	410 ± 10	420 ± 30
1/160	210 ± 3	220 ± 7
1/320	120 ± 10	120 ± 20
1/640	77 ± 10	68 ± 15
1/1280	43 ± 5	45 ± 4

[a] ±SEM.
[b] Eight-hundred units of CIF (or diluent) added to TNF dilution and incubated for 90 min and the mixture was then assayed on L-929 cells for residual TNF activity.

production of CIF is an inducible process. LPS is an efficient inducing agent. Our previous observations of the production of CIF in cultures grown in the presence of serum may have been due to the presence of low levels of LPS which often is present in sera and other reagents. These lots of sera are no longer available so we cannot test them for LPS content. Nevertheless, we now employ serum certified to contain less than 0.03 ng/ml and since serum is employed at 10% final concentration, the resultant medium contains less than 0.003 ng/ml LPS. This amount of LPS is much less than that required for CIF production (Fig. 1). There may be other inducers in addition to LPS but we have not made a study of substances for this activity.

It is noteworthy that LPS induces a short-term and almost immediate production of TNF *in vivo* (8) and by macrophages *in vitro* (9) and, as shown in this study, a long-term production of CIF by normal fibroblast cells. As far as we are aware, this is the first report of an LPS-induced factor from fibroblasts, that in turn plays an important regulating role on some cells of the immune system. The production of this inhibitor may explain why mice become tolerant to subsequent injection of LPS and cannot produce TNF again, at least for as long as 5 days (10). This "tolerance" is also seen in macrophage cultures. Nondividing macrophages produce TNF for only a short time after the addition of LPS and cannot be induced to produce it when LPS is again added (6). However, C1.26 macrophages could be restimulated following 1 week of proliferation (about three generations) (3). We have not found CIF activity in macrophage cultures so CIF does not seem to be directly responsible for the *in vitro* tolerance. It is also interesting that there is a strict requirement for the continual presence of LPS for TNF production. Removal of LPS at any time during the production period will cause the immediate cessation of further TNF production (6). The evaluation and understanding of the regulation of TNF production is extremely important since TNF seems to be the mediator for many deleterious effects of LPS *in vivo* (11). Thus, natural CIF production may play an important role in gram-negative bacterial infections and in endotoxemia. Administration of exogenous CIF to patients with gram-negative bacterial infections may have a beneficial effect by preventing TNF production and, therefore, the deleterious effects of TNF during the infection.

We have now demonstrated that CIF inhibits or suppresses a variety of functions displayed by macrophages as a result of activation by LPS (2, 3). These functions include in addition to TNF production, interleukin 1 secretion and tumoricidal activity. The absence of any direct effect of CIF on TNF activity indicates that the effect of CIF is on production or release of TNF. Absence of a significant effect on overall protein synthesis (3) indicated that CIF does not act through general paralysis of the macrophages. This conclusion is supported here by the lack of any CIF-mediated inhibitory effect on phagocytosis carried out in the absence of LPS. These data indicate that CIF affects the activated but not the resting state of the macrophage.

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