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### Research Article

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# Interferon- $\gamma$ Overcomes Glucocorticoid Suppression of Cachectin/Tumor Necrosis Factor Biosynthesis by Murine Macrophages

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## Abstract

Glucocorticoids almost completely inhibit the synthesis by isolated macrophages of cachectin/tumor necrosis factor (TNF), a cytokine implicated as a major endogenous mediator of septic shock. Despite this in vitro effectiveness, the clinical use of glucocorticoids has failed to demonstrate any clear benefit in the treatment of septic shock. In an effort to understand what other mechanisms might play a role in the patient with sepsis, we examined the effect of interferon- $\gamma$  (IFN $\gamma$ ) on the synthesis of cachectin/TNF. We show here that IFN $\gamma$ , although unable by itself to induce cachectin/TNF synthesis, enhanced the endotoxin-induced production of cachectin/TNF in vitro. Furthermore, IFN $\gamma$  overcame the inhibition of cachectin/TNF synthesis caused by the glucocorticoid, dexamethasone. These effects of IFN $\gamma$  were accounted for by increased levels of cachectin/TNF mRNA. The in vivo implications of these studies are discussed with emphasis on their relevance in human sepsis. (J. Clin. Invest. 1990; 86:1234-1240.) Key words: dexamethasone • endotoxin • lipopolysaccharide • monokine • posttranscriptional regulation • sepsis

## Introduction

Septic shock remains one of the leading causes of death in patients with infection. Individuals surviving the severe hypotension and respiratory failure typical of the acute phase of septic shock frequently progress to failure of multiple other organ systems. Appropriate treatment with antibiotics and supportive therapy is often unsuccessful at forestalling these events.

Therapies that might interfere at an early stage to prevent the development of such complications have not been forthcoming. One class of drugs, the glucocorticoids, showed initial promise. In animal models of septic shock using bacterial endotoxin (lipopolysaccharide, LPS) for the induction of shock, glucocorticoids were shown to protect against a lethal dose of LPS (1-3). Early clinical trials also seemed to support the use of glucocorticoids in septic shock (4). The issue has remained controversial however, and more recent studies concur that glucocorticoids do not improve the rate of survival in these patients (5, 6).

A preliminary report of this work has appeared in abstract form (1988. *J. Cell. Biochem.* 12A:207).

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Recent studies in our laboratory of the mechanisms of cachexia have led to the identification and purification of cachectin, a monokine produced in response to LPS (for review, see references 7 and 8). Sequencing of the purified protein showed cachectin to be identical to tumor necrosis factor (TNF),<sup>1</sup> previously purified by other groups for its antitumor activity (9, 10). Infusion of recombinant human cachectin/TNF into rats causes a syndrome resembling septic shock, both in pathophysiology and histopathology (11). Furthermore, passive immunization against cachectin/TNF increases the survival rate of mice injected with a lethal dose of LPS (12) and protects baboons injected with live *Escherichia coli* (13). These studies implicate cachectin/TNF as an essential endogenous mediator of gram-negative bacteremic shock.

Since glucocorticoids had been shown to protect against endotoxic shock in animal models, their effect on cachectin/TNF production was examined. Kawakami et al. (14) showed that dexamethasone almost completely inhibits cachectin/TNF production as measured by bioactivity. Beutler et al. (15) further examined this inhibition by looking at the cachectin/TNF mRNA and protein levels in LPS-induced peritoneal macrophages (Mφ). It was found that the rate of transcription of cachectin/TNF mRNA decreases in the presence of dexamethasone, resulting in a partial suppression of cachectin/TNF mRNA synthesis. However, there is simultaneously an almost complete suppression of the level of secreted cachectin/TNF, implicating a posttranscriptional site of action for dexamethasone. Thus, dexamethasone appears to inhibit cachectin/TNF production both transcriptionally and posttranscriptionally.

Given that dexamethasone so strongly inhibits cachectin/TNF production by isolated macrophages, it is perhaps surprising that glucocorticoids have not been more successful at preventing septic shock in the clinical setting. The discrepancy between the effects of glucocorticoids in animal models and clinical trials suggests that the models do not accurately replicate the clinical condition. Septic shock often develops after several days of illness, while, in animal models, shock is typically induced within a few hours by a single lethal bolus of endotoxin or bacteria. This endotoxin bolus causes a rapid and massive release of cachectin/TNF, but may not allow time for the production of other inflammatory mediators that may play a role in the more chronic course of sepsis in humans.

It has been shown, for example, that, after bolus injection of endotoxin into baboons, interleukin 1 and interferon- $\gamma$  (IFN $\gamma$ ) are not present in significant amounts in the serum until after cachectin/TNF has reached a near-peak level, and that IFN $\gamma$  is not measurable until cachectin/TNF has almost disappeared from the serum (16). In contrast, cachectin/TNF and IFN $\gamma$  have been shown to be simultaneously elevated in some septic patients (17). Thus, the in vivo production and

1. Abbreviation used in this paper: TNF, tumor necrosis factor.

effects of cachectin/TNF in the septic patient may be complicated by the prior release of other cytokines.

IFN $\gamma$ , also called macrophage-activating factor (18–20), is produced and secreted by T lymphocytes and large granular lymphocytes in response to antigens and T cell mitogens (reviewed in reference 21). It stimulates macrophages to increase their antimicrobial activity and the secretion of various inflammatory mediators and bactericidal agents (19, 22). IFN $\gamma$  enhances cachectin/TNF production by human blood monocytes and murine peritoneal macrophages (23, 24). In addition, this lymphokine is able to override the genetic defect of endotoxin-resistant strains of mice (*lps<sup>d</sup>* phenotype), resulting in a significant increase in cachectin/TNF secretion by their macrophages when stimulated with LPS (25).

In the present study, we investigated the combined effects of IFN $\gamma$  and glucocorticoids on macrophage production of cachectin/TNF. We found that treatment with IFN $\gamma$  enabled macrophages to overcome dexamethasone-induced suppression of LPS-stimulated cachectin/TNF production.

## Methods

**Materials.** Dexamethasone was obtained from Sigma Chemical Co., St. Louis, MO. Recombinant murine IFN $\gamma$  was kindly provided by Dr. M. Palladino of Genentech Inc., South San Francisco, CA. *E. coli* (strain 0127:B8) LPS was obtained from Difco Laboratories, Detroit, MI. Polyclonal rabbit antisera raised against purified murine 17-kD cachectin were provided by Dr. B. Beutler (Howard Hughes Medical Institute, Dallas, TX) and Dr. B. Sherry (The Rockefeller University, New York).

**Cell culture.** Cells were cultured in RPMI medium 1640 (Gibco Laboratories, Grand Island, NY) containing 10% calf bovine serum supplemented with iron (HyClone Laboratories, Logan, UT). All solutions to be used on macrophages, including cell culture medium and diluents for IFN $\gamma$  and dexamethasone, were made with water of very low endotoxin content (100 pg/ml as measured by the *Limulus* amebocyte lysate assay; E-Toxate, Sigma Chemical Co.).

**Isolation of peritoneal macrophages.** Female C3H/HeN mice (20–25 g) were obtained from the Charles River Breeding Co., Wilmington, MA, and maintained at the Rockefeller Laboratory Animal Research Center before use in these experiments. The mice were given an intraperitoneal injection of 1.5 ml of sterile Brewer's thioglycolate medium (Difco Laboratories). 4 d later, macrophages were harvested by peritoneal lavage with phosphate-buffered saline, sedimented at 500 g, resuspended in RPMI medium 1640 containing 10% calf serum, and plated at 1–2  $\times$  10<sup>6</sup> cells per well in 24-well Linbro plates (Flow Laboratories, Inc., McLean, VA). After an overnight incubation, nonadherent cells were washed away with Hanks' balanced salt solution and the adherent monolayers were covered with serum-free RPMI 1640. Cells were then treated with various concentrations of dexamethasone, IFN $\gamma$ , and LPS before the analysis of cachectin/TNF RNA or protein production.

**Dot blots of cytosolic RNA.** The amount of cachectin/TNF RNA in macrophages treated with various reagents was analyzed by cytoplasmic dot hybridization (26). Cells plated as described above were placed on ice and the medium removed. The cell layer was covered with 0.3 ml of lysis buffer (0.14 M NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.6), 0.5% Nonidet P-40, 100 U/ml heparin). After 3 min on ice, the lysate was transferred to a tube and centrifuged for 3 min at 10,000 g to pellet the nuclei. The supernatant (0.25 ml) was removed and incubated at 60°C for 15 min with an equal volume of a denaturing solution containing 12 $\times$  standard saline citrate (SSC; 1 $\times$  SSC = 0.15 M NaCl/15 mM trisodium citrate) and 15% formaldehyde. The salt content of the mixture was increased by the addition of 1 vol of 20 $\times$  SSC. Samples were applied in a row to nitrocellulose filters (Schleicher &

Schuell, Inc., Keene, NH) held in a 8  $\times$  12-well dotting manifold (Schleicher & Schuell, Inc.) and serial 1:2 dilutions were run along the perpendicular axis. The filters were baked for 2 h at 80°C and hybridized with a radioactive probe as described below. Aliquots of the cell lysates were ethanol precipitated and run on an agarose gel. Ethidium bromide staining of the gel showed equivalent amounts of total RNA in the samples.

**Hybridization of RNA blots.** An antisense RNA probe for cachectin/TNF mRNA was synthesized with the Gemini Riboprobe system from Promega Biotec, Madison, WI, using the method recommended by the manufacturer. RNA blots were hybridized overnight at 55°C with the probe at 2  $\times$  10<sup>5</sup> cpm/ml and washed in 0.1% SDS/0.1 $\times$  SSC at 65°C as described (27). The filters were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY). Films were quantitated by scanning with a densitometer (Helena Laboratories, Beaumont, TX).

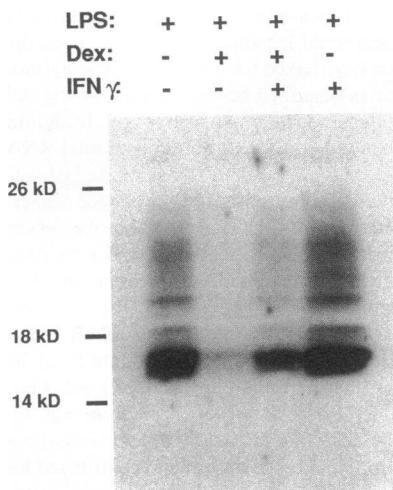
**Western immunoblot.** Macrophage-conditioned medium was adjusted to 6.5% trichloroacetic acid and 150  $\mu$ g/ml *Micrococcus lysodeikticus* (Sigma Chemical Co.), incubated on ice, and centrifuged for 10 min at 10,000 g. The precipitate was washed with acetone, resuspended in sample buffer, and electrophoresed through a 12.5% polyacrylamide gel following the method of Laemmli (28). After separation, the samples were transferred to nitrocellulose at 50 V for 4 h in a tank containing transfer buffer (0.192 M glycine/25 mM Tris-HCl/20% methanol). The nitrocellulose filter was incubated in 1% BSA in PBS for 3 h, then for 1 h with polyclonal rabbit antiserum to murine cachectin/TNF, diluted 1:500 in protein A buffer (1% BSA/0.05% Tween 20 in PBS), and finally for 1 h with <sup>125</sup>I-labeled protein A (0.2  $\mu$ Ci/ml; 8–10  $\mu$ Ci/ $\mu$ g, Amersham Corp., Arlington Heights, IL) also in protein A buffer. After each incubation, the filter was rinsed briefly with PBS/0.05% Tween 20. The rinsed filters were dried and exposed to XAR-5 film.

**TNF bioassay.** Cell culture medium was assayed for cachectin/TNF cytotoxic activity against L-929 cells according to the method of Mosmann (29). L-929 monolayers in microtiter plates were incubated with 1  $\mu$ g/ml actinomycin D (Fluka, Ronkonkoma, NY) and a series of dilutions of macrophage-conditioned medium. After 18 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co.) was added to a final concentration of 0.5 mg/ml and, 4 h later, the medium was removed. The remaining colored product was solubilized with 0.04 N HCl in isopropanol, and the plates were read in a spectrophotometer. One unit was defined as the amount of cachectin/TNF that gave 50% killing. All samples were tested in triplicate.

**Immunoprecipitation of cachectin/TNF.** Macrophage-conditioned medium was incubated overnight at 4°C with polyclonal rabbit anti-cachectin/TNF immunoglobulin conjugated, according to the method recommended by the manufacturer, to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The Sepharose was pelleted and the supernatant was analyzed in a TNF bioassay. Parallel samples were incubated with normal rabbit serum immobilized on protein A-Sepharose CL-4B (Pharmacia).

## Results

**Effect of IFN $\gamma$  on dexamethasone suppression of cachectin/TNF production.** The ability of murine peritoneal macrophages to produce cachectin/TNF in response to LPS was examined after pretreatment of the macrophages with either dexamethasone, IFN $\gamma$ , or both agents. The macrophages were incubated for 1 h with dexamethasone and/or IFN $\gamma$  before the addition of LPS. The cells continued incubating with both LPS and the pretreatment reagents for 16 h more. The medium was collected and analyzed by Western immunoblot. As has been previously described, mature secreted murine cachectin/TNF is composed of multiple forms (30). The major protein runs at 17 kD on SDS-PAGE (see Fig. 1). Bands above 18.5 kD represent variously glycosylated forms of cachectin/TNF (Sherry,

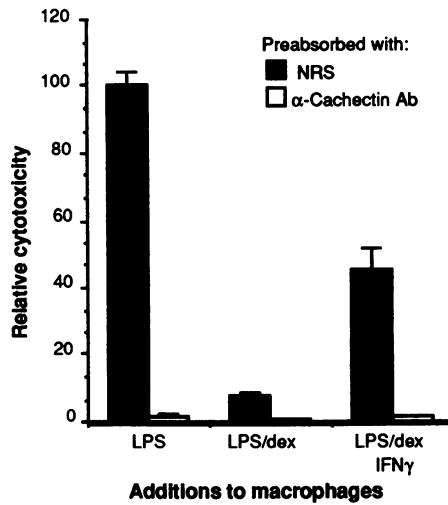


**Figure 1.** Antagonism of glucocorticoid suppression of cachectin/TNF production by IFN $\gamma$ .  $10^6$  peritoneal macrophages were preincubated for 1 h with medium, 100 nM dexamethasone, 1 U/ml IFN $\gamma$ , or both reagents, as indicated. LPS was then added to a final concentration of 100 ng/ml. The cells were incubated for 16 h more, and the medium was collected and assayed by Western blot.

B., D.-M. Jue, A. Zentella, and A. Cerami, manuscript submitted for publication). As shown in Fig. 1, cells pretreated with dexamethasone produced significantly less cachectin/TNF than cells receiving LPS alone. The simultaneous addition of 1 U/ml IFN $\gamma$  to cells treated with dexamethasone interfered with dexamethasone inhibition of LPS-induced cachectin/TNF production. In addition, pretreatment with IFN $\gamma$  alone slightly enhanced LPS-induced cachectin/TNF production. Note that all forms of cachectin/TNF decreased and increased in parallel. Thus, neither dexamethasone nor IFN $\gamma$  showed a differential effect on the glycosylated versus nonglycosylated forms of the cytokine.

To quantitate the effects of dexamethasone and IFN $\gamma$  on cachectin/TNF production, a bioassay for TNF cytotoxicity was performed. Again macrophages were pretreated with or without dexamethasone and IFN $\gamma$ . LPS was added, and after further incubation, the medium was collected and added in serial dilutions to L-929 cells in the presence of 1  $\mu$ g/ml actinomycin D. The cytotoxicity of the samples was determined after an 18-h incubation. The results are expressed in Fig. 2 as a percentage of the cytotoxic activity induced by LPS alone. At a concentration of 100 nM, dexamethasone suppressed the amount of cytotoxic activity to 7% of the LPS-induced level. Addition of 1 U/ml IFN $\gamma$  overcame the suppressive effect of dexamethasone, increasing killing activity to 45% of the LPS-induced level. When the medium was preabsorbed with immobilized anti-cachectin/TNF antibodies before addition to L-929 cells, cytotoxic activity decreased 100-fold in all samples, confirming that the bioassay was specific for cachectin/TNF under the given conditions. Preabsorption with immobilized normal rabbit serum did not affect the cytotoxicity of the samples.

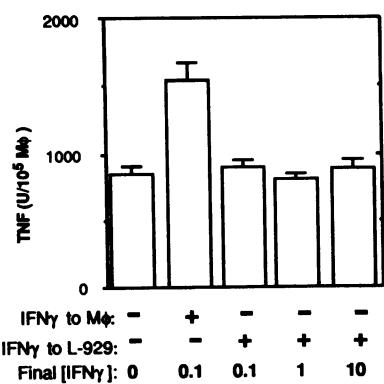
IFN $\gamma$  has been shown to exhibit cytotoxic activity toward some cell lines independent of cachectin/TNF activity (31). To affirm that the increased cytotoxicity measured in the bioassay was entirely due to increased cachectin/TNF production and not to a synergistic cytotoxic action of IFN $\gamma$  transferred with the medium, the effect of IFN $\gamma$  on the cytotoxicity assay was tested (Fig. 3). IFN $\gamma$  was added directly to the L-929 monolayer at the same time as conditioned medium from LPS-stimulated macrophages. At concentrations greater than or equaling that in the medium of macrophages that had been treated with IFN $\gamma$ , the lymphokine did not increase the cytotoxicity of



**Figure 2.** IFN $\gamma$  antagonizes glucocorticoid suppression of cachectin/TNF production. Peritoneal macrophages were incubated for 1 h with medium, 100 nM dexamethasone, or dexamethasone plus 1 U/ml IFN $\gamma$ , as indicated, before the addition of LPS to a final concentration of 100 ng/ml. 16 h after LPS addition, the medium was collected and preabsorbed with anti-cachectin/TNF antiserum or with normal rabbit serum. Aliquots were assayed for cytotoxic activity in the TNF bioassay. The data in cytotoxic units were reexpressed as the percentage of the cytotoxicity in the medium from cells treated with LPS alone. The results of multiple assays have been compiled. Error bars represent the SEM.

cachectin/TNF-containing medium. Thus, the IFN $\gamma$ -mediated enhancement of cachectin/TNF bioactivity, as measured by the L-929 cell assay, was not a result of direct action of IFN $\gamma$  on the L-929 cells, but could be attributed to an effect on the macrophages to increase cachectin/TNF production.

*IFN $\gamma$  modulation of LPS-induced cachectin/TNF production.* The range of IFN $\gamma$  concentrations effective at overcoming dexamethasone suppression was examined next. Macrophages were treated with dexamethasone, LPS, and various concentrations of IFN $\gamma$ , and 16 h later, the medium was tested for cytotoxic activity. As shown in Fig. 4, as little as 0.1 U/ml IFN $\gamma$  caused a significant increase in cachectin/TNF levels over those reached in the absence of IFN $\gamma$ . The effect reached



**Figure 3.** IFN $\gamma$  has no direct effect on L-929 cells. Peritoneal macrophages were incubated with 100 ng/ml LPS with or without 1 U/ml IFN $\gamma$  for 16 h before collection of the medium. No dexamethasone was used in this assay. The medium was assayed for cytotoxic activity with or without the addition of IFN $\gamma$  directly to the L-929 cells. The final concentration of IFN $\gamma$  (in U/ml) on the L-929 cells is indicated in each case in the last row (Final [IFN $\gamma$ ]). Results are expressed in units of cytotoxicity  $\pm$  SEM.

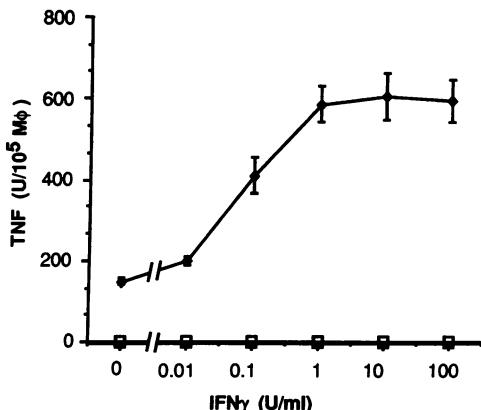


Figure 4. Dose-dependent effect of IFN $\gamma$  on glucocorticoid suppression. Peritoneal macrophages were preincubated for 1 h with 100 nM dexamethasone and various concentrations of IFN $\gamma$ . LPS was added to a concentration of 100 ng/ml (solid symbols), and the medium was collected 16 h later to be tested for cytotoxic activity. Error bars represent the SEM. Control macrophages were treated identically with dexamethasone and IFN $\gamma$  but without the addition of LPS (open symbols). Results are expressed as cytotoxic units $\pm$ SEM.

a maximum at 1 U/ml IFN $\gamma$ . No further reversal of dexamethasone suppression was achieved by increasing the concentration to 100 U/ml IFN $\gamma$ .

When macrophages were treated with IFN $\gamma$ , in the absence of LPS and dexamethasone, no cytotoxic activity was detected (Fig. 4). This was true throughout the range of IFN $\gamma$  concentrations tested, from 0.01 to 100 U/ml. In other experiments, 1,000 U/ml IFN $\gamma$  was also unable to stimulate cachectin/TNF production in the absence of LPS (data not shown). Thus, IFN $\gamma$  by itself was not able to induce cachectin/TNF synthesis.

Since IFN $\gamma$  is known to increase the sensitivity of macrophages to LPS, a concentration course of LPS was performed. Macrophages were treated with medium, dexamethasone, IFN $\gamma$ , or both reagents, before the addition of various concentrations of LPS. The resultant cytotoxic activity in medium showed a dose-dependent increase in response to LPS (Fig. 5). In the absence of LPS, no cachectin/TNF activity was detected, no matter what pretreatment the cells had received. The presence of IFN $\gamma$  increased the cytotoxicity at all concentrations of LPS. The maximum level of cytotoxic activity was raised about 1.5-fold by IFN $\gamma$ . Dexamethasone had the opposite effect as IFN $\gamma$  on the concentration course, causing a decrease in the cytotoxicity at each LPS concentration and in the maximum level attainable. When pretreated with both IFN $\gamma$  and dexamethasone, LPS-induced macrophages secreted an amount of cytotoxic activity intermediate to that in response to either agent alone. Thus IFN $\gamma$  and glucocorticoids each independently exerted an effect on the level of the cachectin/TNF response to LPS.

**Time course of the IFN $\gamma$  effect.** Fig. 6 shows a time course of the IFN $\gamma$  effect. Cells were incubated with IFN $\gamma$  for 0–48 h before the addition of dexamethasone and LPS. Although 12–18 h of preincubation was required for demonstration of the full magnitude of the effect, the ability of IFN $\gamma$  to partially reverse dexamethasone suppression was clear even without preincubation.

**IFN $\gamma$  and glucocorticoid effects on cachectin/TNF mRNA level.** Glucocorticoids are known to affect cachectin/TNF syn-

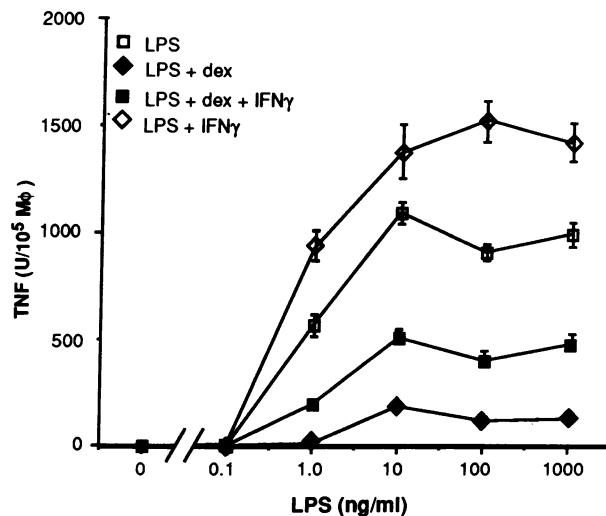


Figure 5. Combined effect of IFN $\gamma$  and glucocorticoids on cachectin/TNF production. Peritoneal macrophages were incubated for 1 h with medium, 100 nM dexamethasone, 1 U/ml IFN $\gamma$ , or both reagents. LPS was then added to achieve the indicated final concentrations, and the cells were incubated for an additional 16 h before collection of the medium for measurement of cytotoxic activity. Results are expressed as cytotoxic units $\pm$ SEM.

thesis at two different levels (15). They cause a decrease in the steady-state level of cachectin/TNF mRNA, and in addition, they appear to inhibit a later posttranslational step in the production of mature cachectin/TNF (Luedke, C., D.-M. Jue, and A. Cerami, manuscript in preparation). To determine the level at which IFN $\gamma$  acts to overcome dexamethasone suppression, cachectin/TNF mRNA levels were quantitated. Fig. 7 shows a representative experiment to examine the combined effect of IFN $\gamma$  and dexamethasone on cachectin/TNF mRNA levels. Macrophages were treated with LPS and increasing concentrations of dexamethasone with or without IFN $\gamma$ . Cytosolic RNA was harvested, dot blotted, and probed with radiolabeled antisense cachectin mRNA (Fig. 7 A). The results of densitometric scanning of the blot's autoradiograph are displayed in Fig. 7 B. Dexamethasone and IFN $\gamma$  each independently modulated the mRNA level. As has been previously described elsewhere, dexamethasone suppressed steady-state cachectin/TNF mRNA levels to one third of the LPS-induced level (15). In the absence of dexamethasone, IFN $\gamma$  raised the cachectin/TNF

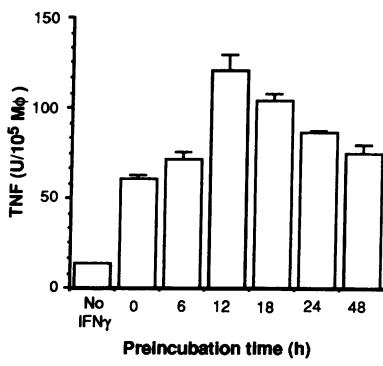
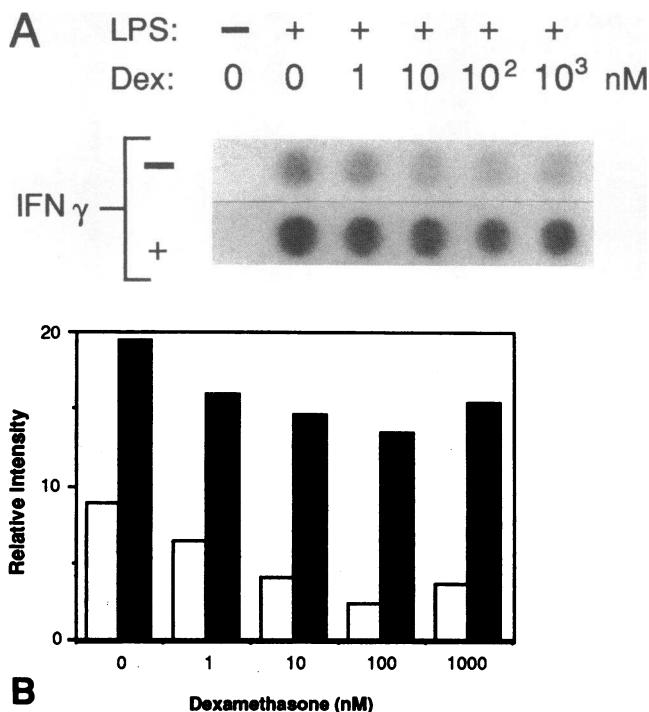


Figure 6. Time course of IFN $\gamma$  effect. Peritoneal macrophages were incubated for the indicated lengths of time with 1 U/ml IFN $\gamma$  before the addition of 100 nM dexamethasone. 1 h after dexamethasone addition, 100 ng/ml LPS was added to a final concentration of 100 ng/ml, and the cells were incubated for a further 16 h before collection of the medium for measurement of cytotoxic activity. Results are expressed as cytotoxic units $\pm$ SEM.



**Figure 7.** IFN $\gamma$  antagonizes glucocorticoid suppression at the RNA level. (A) Dot blot of cytosolic RNA.  $10^6$  peritoneal macrophages were preincubated for 1 h with the indicated concentrations of dexamethasone, with or without 1 U/ml IFN $\gamma$ . 100 ng/ml LPS was added, and the cells were incubated for 2 h more before lysis and harvest of the cytosolic RNA. (B) Quantitation of dot blot results by densitometric scan. The bars represent the relative intensity of the autoradiograph in A: with IFN $\gamma$  (solid bars) or without IFN $\gamma$  (open bars).

message level above that induced by LPS alone, and this increased level was mildly suppressed by pretreatment of the macrophages with dexamethasone. Thus, neither reagent was able to completely override the effect of the other. In the absence of LPS, no cachectin/TNF mRNA was detected.

In Fig. 8, the combined effects of dexamethasone and IFN $\gamma$  on both cachectin/TNF mRNA and protein levels are compiled for comparison. The results are expressed relative to the mRNA and protein level induced by LPS in the absence of either reagent. While dexamethasone decreased the steady-state level of cachectin/TNF mRNA about threefold, another effect was exerted by this hormone at a posttranscriptional site, such that the level of secreted protein was decreased about 20-fold. IFN $\gamma$  alone caused an increase in both the mRNA and protein levels induced by LPS. In the presence of dexamethasone, IFN $\gamma$  still caused an increase in the mRNA level, but the protein level did not rise in parallel and, in fact, decreased to about half of the LPS-induced level. Thus, while counteracting glucocorticoids' effect on the mRNA level, IFN $\gamma$  did not appear to strongly reverse glucocorticoid suppression at the second, posttranscriptional site of action on cachectin/TNF production.

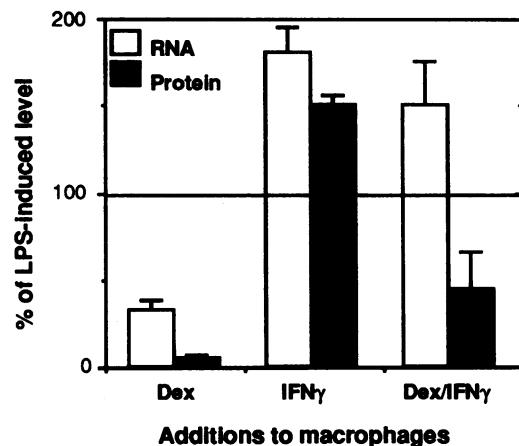
## Discussion

By measurement of both immunoreactive protein and bioactivity, IFN $\gamma$  has been shown here to counteract glucocorticoid

suppression of cachectin/TNF production. In addition, IFN $\gamma$  enhanced the level of cachectin/TNF produced by macrophages stimulated with LPS in the absence of glucocorticoids. As with many of IFN $\gamma$ 's actions on macrophages, such as priming for tumoricidal activity, the enhancement of cachectin/TNF production required the presence of LPS. No cachectin/TNF was detectable when IFN $\gamma$  was added by itself. Note that LPS is a ubiquitous contaminant of glassware and tap water and that rigorous exclusion of it from all solutions must be practiced to prevent macrophage activation by "non-LPS"-containing solutions.

IFN $\gamma$  caused a significant reversal of glucocorticoid suppression at a concentration of 0.1 U/ml with the maximal effect at 1 U/ml. These concentrations are comparable to those required for other effects of IFN $\gamma$  on both murine and human macrophages (19, 32). Preincubation of macrophages with IFN $\gamma$  was not necessary for reversal of glucocorticoid suppression. However, 12–18 h of preincubation significantly amplified the effect. These kinetics agree with those found for activation of human monocytes and macrophages, which typically require 12–24 h of in vitro exposure to IFN $\gamma$  to exhibit a fully activated state (33, 34).

Thus the concentration and time courses for the IFN $\gamma$ -mediated antagonism of glucocorticoid suppression agree well with the dosage and kinetics described for other effects of this lymphokine on macrophages. Furthermore, the IFN $\gamma$  concentration required for this in vitro effect on peritoneal macrophages is similar to levels observed in humans in vivo: the serum level of IFN $\gamma$  is reported to reach 1–10 U/ml in gram-negative septicemia due to *Neisseria* infection (17), well above the minimum concentration shown in the present study to antagonize dexamethasone suppression. The comparison of the in vitro concentration of IFN $\gamma$  in medium with the serum level of this lymphokine may be justified by the observation that monocytes, isolated from the blood of patients with serum levels of IFN $\gamma$  ranging from 2 to 19 U/ml, demonstrate activated phenotypes in various assays (35). This suggests that at the serum levels measured in septic patients, IFN $\gamma$  could counteract the glucocorticoid suppression of cachectin/TNF production.



**Figure 8.** Comparison of dexamethasone suppression of cachectin/TNF RNA and protein levels in the absence and presence of IFN $\gamma$ . The results are compiled from multiple assays and expressed as percentage of the LPS-induced level. Error bars represent the SEM.

Comparison of IFN $\gamma$  levels in experimental bacteremia in animals with those in human sepsis suggests that the clinical failure of glucocorticoids might be attributable to a difference in the degree of activation of the immune system. When the serum concentrations of various cytokines are measured in baboons, after the infusion of a lethal dose of live *Escherichia coli*, the animals achieve peak serum cachectin/TNF levels 90 min after the bacterial infusion (16). IFN $\gamma$  is not detectable in the serum until 2 h after the infusion and does not peak until 8 h. Thus, IFN $\gamma$  is not present at detectable levels during the first 90 min after the challenge, the period during which the lethal dose of cachectin/TNF is being produced. The protocol used here is typical of most of the animal studies of gram-negative shock, in that a single lethal dose of LPS or bacteria is given, causing the rapid onset of septic shock. It may be concluded that, in these studies, no IFN $\gamma$  is present to counteract glucocorticoid suppression of cachectin/TNF production. Thus in the animal models, glucocorticoids can effectively decrease cachectin/TNF production and its subsequent lethal effects.

In contrast, elevated serum levels of IFN $\gamma$  are observed in septic patients. High serum levels are found even in the absence of shock, that is, before the achievement of lethal levels of cachectin/TNF (17). It was shown in that study that the levels of cachectin/TNF and IFN $\gamma$  each correlated positively with the severity of the disease. Although it is difficult to differentiate cause and effect, this correlation implies that blood monocytes are exposed to high IFN $\gamma$  concentrations during the peak period of cachectin/TNF synthesis. Thus, IFN $\gamma$  is likely to be present before or at the time that glucocorticoid therapy is begun and can interfere with glucocorticoid suppression of cachectin/TNF production.

The ability of IFN $\gamma$  to overcome glucocorticoid suppression of cachectin/TNF production in peritoneal macrophages in vitro was observed when IFN $\gamma$  was added any time from 2 d before to the same time as the glucocorticoid. Furthermore, it has been shown by others that fresh peripheral blood monocytes tend to be even more responsive to IFN $\gamma$  than are macrophages. Exposure of these blood monocytes to IFN $\gamma$  for as little as 6 h may result in an activated state lasting as long as 5–7 d after the removal of the lymphokine (35, 36). Thus, even if the IFN $\gamma$  levels were elevated several hours before the administration of glucocorticoids, efficient glucocorticoid-induced suppression of cachectin/TNF production might not be possible.

Based on these various lines of evidence, combining the results of in vitro studies of murine peritoneal macrophages with in vitro and clinical observations of the human system, we postulate that the presence of IFN $\gamma$  in the serum of septic patients prevents efficient glucocorticoid suppression of cachectin/TNF production. The chronicity of clinical sepsis, unlike the animal models, allows for the presence of IFN $\gamma$  during the early period of cachectin/TNF synthesis, thereby possibly affecting both the production and effects of this monokine. Since IFN $\gamma$  interferes with the suppressive action of glucocorticoids, this may partially explain the failure of glucocorticoids to prevent the development of shock in clinical trials.

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