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Cytokine Appearance and Effects of Anti-Tumor Necrosis Factor Alpha Antibodies in a Neonatal Rat Model of Group B Streptococcal Infection

GIUSEPPE TETI,* GIUSEPPE MANCUSO, AND FRANCESCO TOMASELLO

Istituto di Microbiologia, Facoltà di Medicina e Chirurgia, Università degli Studi di Messina, I-98122 Messina, Italy

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Cytokines are suspected of playing an important role in the pathophysiology of septic shock. This study was undertaken to determine whether tumor necrosis factor alpha (TNF-α) induces the production of other cytokines and mediates mortality in a neonatal rat model of sepsis caused by group B streptococci (GBS). We have measured TNF-α, interleukin-1α (IL-1α), interleukin-6 (IL-6), and gamma interferon (IFN-γ) levels in neonatal rats infected with different strains (H738, 259, and 90) and doses (1 50% lethal dose [LD_{50}] and 5 90% lethal doses [LD_{90}]) of type III GBS. TNF-α and IL-6 were detected by the L929 cytotoxicity and the B9 proliferation assays, respectively, in serial plasma samples. IL-1α and IFN-γ were measured in spleen homogenates by enzyme-linked immunosorbent assay kits by using antibodies raised against the corresponding mouse cytokines. Plasma TNF-α levels significantly rose above baseline values within 12 h after intraperitoneal challenge with 5 LD_{90} of GBS strain H738, corresponding to 3×10^3 CFU. A mean peak TNF-α concentration of 232 ± 124 U/ml was reached at 20 h. Peak IL-1α and IL-6 levels of 766 ± 404 U/g and 1,033 ± 520 U/ml, respectively, were reached at 24 h after bacterial challenge. Maximal spleen concentrations of IFN-γ (449 ± 283 U/g) were measured at 36 h. Concentrations of TNF-α, but not other cytokines, remained significantly elevated at 72 h, a time when mortality approached 100%. Significant correlations were found between concentrations of each of the cytokines tested and the logs of CFU concentrations in the blood. In order to ascertain whether TNF-α influenced the production of other cytokines, rat pups received two injections of anti-murine TNF-α or normal rabbit serum at 2 h before and at 26 h after challenge with live GBS. Plasma TNF-α bioactivity was undetectable in anti-TNF-α-treated animals, while IL-6 and IFN-γ, but not IL-1α, levels were significantly reduced, compared with normal serum controls. Rat pups pretreated with anti-TNF-α serum and infected with 1 and 5 LD_{90} of strains H738 and 259 showed enhanced early (48 to 72 h) survival. However, by 96 h this protection was no longer apparent.

Cytokines are low-molecular-weight proteins that can have both beneficial and detrimental effects for the host during infection. Tumor necrosis factor alpha (TNF-α), in conjunction with other inflammatory cytokines produced by mononuclear phagocytes, such as interleukin-1 (IL-1), is thought to mediate most of the pathology observed in septic shock caused by gram-negative bacteria and endotoxin shock (reviewed in reference 13).

TNF-α can induce hypotension, tissue injury, and death in animals (37). IL-1 induces fever and hypotension and potentiates the lethal effects of TNF-α (12, 29). In experimental shock induced by intravenous (i.v.) injection of *Escherichia coli* or endotoxin, death can be prevented by anti-TNF-α antibodies (6, 32, 38) or receptor antagonist for IL-1 (40). In patients with sepsis and shock caused by gram-negative bacteria, the presence of high levels of TNF-α and IL-1β is associated with a fatal outcome (10, 39).

Interleukin-6 (IL-6) is present in body fluids during bacterial infections (24) and experimental endotoxemia (15) and may also play a role in septic shock, although this issue is more controversial (13). Anti-IL-6 antibodies were found to protect against *E. coli* or TNF-α-induced mortality (33). Similarly, experimental abrogation of gamma interferon (IFN-γ) with specific antibodies was shown to prevent endotoxin-induced mortality (23).

Although signs and symptoms are identical in septic shock syndromes caused by gram-positive and gram-negative bacteria, the role of cytokines in gram-positive sepsis has been only recently addressed. Nathanson et al. have shown that the hemodynamic changes observed in human septic shock can be reproduced in dogs by challenge with not only TNF-α or endotoxin but also *Staphylococcus aureus* (26, 27). Heat-killed gram-positive bacteria are able to induce mortality in D-galactosamine-sensitized mice by a TNF-α-dependent mechanism (18). Wakabayashi et al. have shown that heat-killed *Staphylococcus epidermidis* produces hypotension, hepatic necrosis, complement activation, and elevations in circulating levels of TNF-α and IL-1β in rabbits (41).

Group B streptococci (GBS) are a leading cause of neonatal sepsis and meningitis. These infections are often rapidly fatal despite appropriate therapy (4). High TNF-α levels were measured in the cerebrospinal fluid of children with meningitis caused by GBS or other bacteria (2). Increased serum TNF levels in neonatal piglets at 2 and 4 h after infusion with GBS were recently reported (19).

In this study and in some of the studies mentioned above (6, 18, 32, 38, 41), high numbers of bacteria (10^9 to 10^{12} organisms per kg of body weight) or endotoxin was injected by the i.v. route. However, the site of inoculation and the compartmentalization of the inflammatory reaction can influence the levels of circulating cytokines and the response to anticytokine therapy (3, 28). Lower TNF-α levels and a lack of response to anti-TNF-α antibodies were reported in

* Corresponding author.

TABLE 1. Properties of the type III GBS strains employed in the present study

Strain	Density (g/ml)[a]	Cell-associated type-specific antigen (mg/g [dry wt] of cells)[b]	Extracellular type-specific antigen (mg/g [dry wt] of cells)[b]	LD_{50} (CFU)
H738[c]	1.045	8.9	3.3	5×10^2
259[d]	1.071	0.6	0.1	5×10^5
90[d]	1.049	3.1	0.8	2×10^3

[a] Determined by centrifugation on hypotonic Percoll gradients (21).
[b] Determined by inhibition of ELISA using purified type III antigen as a standard and monoclonal antibody P9D8 (34).
[c] Kindly provided by Bascom Anthony, National Institutes of Health, Bethesda, Md. (1).
[d] Kindly provided by Graziella Orefici, Istituto Superiore di Sanità, Rome, Italy (34, 36).

intraperitoneal (i.p.), as opposed to i.v., models of *E. coli* sepsis (3).

In a previous study, we found that GBS inoculated by either the i.p. or the i.v. route were able to induce transient increases of circulating TNF-α and IL-6 in adult mice (35). However, since GBS are scarcely virulent in adult individuals, high bacterial numbers were needed to produce mortality.

This study was undertaken to assess whether endogenous TNF-α has a role in inducing the production of other cytokines and mediating mortality in neonatal rats infected i.p. with GBS. In this model, a small number of virulent bacteria replicates locally in the peritoneal cavity and then spreads systematically, reaching very high concentrations in the blood. We have found that TNF-α, followed by IL-1α, IL-6, and INF-γ, is consistently produced and that TNF-α has a role in inducing IL-6 and IFN-γ. Elimination of circulating TNF-α with specific antibodies moderately increased survival time.

MATERIALS AND METHODS

Bacterial strains. The three strains used in the present study were c-protein-negative type III GBS isolated from septic neonates. As shown in Table 1, these strains had different degrees of virulence for neonatal rats. Since the type-specific capsular polysaccharide is the main virulence factor of GBS, the strains were also characterized for the amount of type III polysaccharide produced and buoyant density, an estimation of capsule size (Table 1).

ELISA inhibition. The amount of type-specific antigen produced by GBS strains was quantitated by inhibition of a previously described enzyme-linked immunosorbent assay (ELISA) using the type-specific monoclonal antibody P9D8 (34). Briefly, GBS were grown to the early stationary phase in a chemically defined medium (11) and centrifuged. The pellet was washed once in H_2O, lyophilized, and weighed. The supernatant was precipitated by overnight treatment with 80% ethanol at 4°C. The type-specific antigen was extracted from the pellet by incubation (16 h at 37°C) with 1 mg of mutanolysin (Sigma Chemical Co.; distributed by Mascia Brunelli, Milan, Italy) per ml in 30 mM potassium phosphate buffer, pH 7, containing 10 mM $MgCl_2$.

Mutanolysin extracts and precipitates from supernatants (inhibitors) were mixed with equal volumes of P9D8 (type-specific antibody) and added to microtiter wells coated with tyrosylated type-specific polysaccharide (34). The ELISA was then completed exactly as described previously (34). The amount of type-specific antigen contained in samples was estimated from a standard curve obtained by plotting inhibition of absorbance against known concentrations of purified type-specific polysaccharide (34).

Buoyant density. GBS were grown to the early stationary phase in a chemically defined medium (11) and washed once in H_2O. Bacterial suspensions were centrifuged on preformed hypotonic Percoll gradients (Pharmacia S.p.A., Cologno Monzese, Italy) exactly as described previously (21). Bacterial density was estimated by using marker beads of known density (Pharmacia).

Bacterial inocula. For inoculation of experimental animals, GBS were grown in Todd-Hewitt broth (Difco, Direct International Distributors, Milan, Italy) to the late stationary phase, washed, and resuspended in phosphate-buffered saline (pH 7.2; 0.01 M phosphate, 0.15 M NaCl) (PBS). In preliminary experiments, it was found that the bacterial growth phase did not influence the amount of cytokines produced in infected animals.

The number of bacteria in these suspensions was determined photometrically by using a previously constructed curve correlating A_{570} with CFU. Levels of endotoxin in all GBS suspensions were <0.5 ng/ml, as determined by an E-Toxate *Limulus* amebocyte lysate assay kit (Sigma) (see below).

Neonatal rat model. Neonatal (≤48 h old) Sprague-Dawley rats were employed. Adult rats of both sexes were obtained from Charles River Italia (Calco, Italy) and mated at our institution. Pups were injected i.p. with PBS suspensions of GBS or with plain PBS (50 μl). GBS-infected rat pups generally died within 96 h.

For cytokine kinetics studies, groups of five animals were sacrificed by decapitation at different times after injection with GBS. Blood was collected in heparinized containers and centrifuged, after 10 μl were saved for colony counts. Plasma (0.25 to 0.35 ml) was stored at −70°C until assayed for cytokines. The spleens were removed immediately after death, weighed, mixed with cold PBS, and homogenized in the cold. Centrifuged supernatants were sterile filtered and stored at −70°C. The number of CFU in blood samples was determined by standard pour plate methods in tryptic soy agar (Difco). The volumes of the samples collected from each animal were always sufficient to perform all the assays described.

TNF-α assay. TNF-α was measured in plasma samples by cytotoxicity in L929 murine fibroblasts. Briefly, after L929 cells were cultured in microtiter plates for 20 h, the culture medium was removed and diluted plasma samples or standards were added in duplicate with actinomycin D (1 μg/ml). Eight serial twofold dilutions (from 1:8 to 1:1,024) were tested for each plasma sample. After a 24-h incubation, the cells were stained with 0.2% crystal violet in methanol-H_2O (1:4), washed, and lysed with 1% sodium dodecyl sulfate in H_2O. A microtiter plate reader was used to measure A_{492}. TNF activity was expressed in units per milliliter, 1 U being defined as the amount of TNF causing 50% lysis of L929 cells. The assay was calibrated by using murine recombinant TNF-α (specific activity of 20 U/ng) (Genzyme; distributed by Omnia Res., Cinisello Balsamo, Italy) as a standard. TNF activity in selected plasma samples was totally inhibited by a 1:100 dilution of rabbit anti-TNF-α serum (Genzyme) but not by normal rabbit serum. Table 2 shows the sensitivity and the reproducibility of this assay.

IL-1α assay. In preliminary experiments, we had difficulties in measuring circulating IL-1 bioactivity in rats or mice. In addition, immunological reagents or kits for the detection of IL-1β, the predominant form of circulating IL-1, were not

TABLE 2. Reproducibility and sensitivity of the cytokine assays employed in the present study

Assay	Lower limit of detection (U/ml)[a]	Interassay coefficient of variation (%)[b]	Intraassay coefficient of variation (%)[c]
TNF-α (plasma)	8	13	6
IL-6 (plasma)	10	15	10
IL-1α (spleen)	2.5	13	4
IFN-γ (plasma)	4	10	7
IFN-γ (spleen)	2	9	8

[a] Obtained by multiplying the reciprocal of the lowest sample dilution tested by the detection limit observed with standards. Recovery of standards in spiked samples from normal animals was always >90%.

[b] Determined by assaying the same samples five times on different days with different reagents.

[c] Determined by assaying the same sample in 15 replicate wells.

available to us. It was recently shown that marked and sustained elevations of IL-1α levels can be measured in spleen homogenates of mice injected with endotoxin (30). For these reasons, spleen homogenates from septic rat pups were tested by using an ELISA kit specific for mouse IL-1α (InterTest-1αX; Genzyme). This assay makes use of the multiple antibody sandwich principle. In preliminary experiments, we found that the antibodies contained in this kit cross-react with rat IL-1α to a degree sufficient to allow its quantitation. The assay was performed exactly as described by the manufacturer except that rat IL-1α was used as a standard. Undiluted and diluted (1:10) homogenates were tested in duplicate. Upon dilution, IL-1α immunoreactivity declined in parallel to the standard curve. This was still linear at the highest standard concentration tested (85 U/ml). Table 2 shows the sensitivity and the reproducibility of this assay.

The rat IL-1α standard we used was obtained from the culture supernatants of spleen macrophages stimulated with 1 μg of *E. coli* O55:B5 lipopolysaccharide (Difco) per ml by using Sepharose-bound anti-IL-1α. This was prepared by coupling activated Sepharose beads (Pharmacia) with an immunoglobulin fraction of rabbit anti-mouse IL-1α serum (Genzyme) obtained by ammonium sulfate precipitation. Sepharose–anti-IL-1α beads were incubated with the macrophage supernatants, washed, and eluted with 0.05 M glycine buffer, pH 2.5. After neutralization, the material was dialyzed against RPMI 1640 and sterile filtered. This preparation contained 85 U of IL-1 activity per ml, as determined by the D10 G.4.1 proliferation assay (20). This bioassay was calibrated by using mouse recombinant IL-1α as a standard. Bioactivity of our rat IL-1α preparation was >75% inhibited by rabbit anti-mouse IL-1α serum.

IL-6 assay. IL-6 was measured by using the IL-6-dependent B9 hybridoma proliferation assay (8). Briefly, standards or diluted plasma samples were mixed with B9 cells (2×10^4 cells per ml) and cultured for 3 days in microtiter plates. Nine serial twofold dilutions were tested for each plasma sample. Final dilutions ranged from 1:10 to 1:2,560. Cell proliferation was assessed by a colorimetric method using 100 μg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) per well. After a 3-h incubation, the reduced dye was solubilized by the addition of a mixture of 20% sodium dodecyl sulfate and 50% dimethylformamide in H_2O. A microtiter plate reader was used to measure A_{570}. One unit of IL-6 was defined as the amount that induced 50% maximal proliferation. The assay was calibrated by using murine recombinant IL-6 (specific activity of 400 U/ng; Genzyme) as a standard. Table 2 shows the sensitivity and the reproducibility of this assay.

IFN-γ assay. IFN-γ was measured in plasma and spleen homogenates by the InterTest-γ ELISA kit (Genzyme). Like the abovementioned InterTest-1αX assay, this kit employs antibodies raised against mouse IFN-γ cross-reacting with rat IFN-γ. The assay was performed as recommended by the manufacturer except that rat recombinant IFN-γ was used as a standard. Purified rat recombinant IFN-γ (a generous gift of Peter H. van der Meide, TNO Health Research, Rijswijk, The Netherlands) had a specific antiviral activity of 7×10^9 U/g. Table 2 shows the sensitivity and the reproducibility of this assay. The upper limit of the assay (i.e., the point at which the standard curve deviated from linearity) was 400 U/ml. Plasma samples were diluted twofold and tested in duplicate. Undiluted and diluted (1:5) homogenates were also tested in duplicate. Upon dilution, IFN-γ immunoreactivity declined in parallel to the standard curve.

Detection of endotoxin. Selected plasma samples or bacterial suspensions were tested for the presence of endotoxin by a *Limulus* amebocyte lysate assay kit (E-Toxate; Sigma). To remove lysate inhibitors, plasma samples (0.1 ml) were extracted with an equal volume of chloroform before the assay. To remove lysate inhibitors occasionally found in bacterial suspensions, these were diluted 1:10 in endotoxin-free water and heated for 5 min at 75°C before the assay.

Effects of anti-TNF-α. In order to assess the role of TNF-α in inducing the production of other cytokines, rat pups were treated with rabbit anti-murine TNF-α serum (Genzyme) or normal rabbit serum before infection. The anti-TNF-α serum preparations used in this study were capable of blocking the cytotoxic activity of 10 U of murine recombinant TNF-α per ml when diluted 64,000-fold.

Rat pups from each litter were randomly assigned to control or experimental groups (five animals per group) and kept with the mother. Experimental animals received two intracardiac injections of 50 μl of rabbit anti-TNF-α serum separated by a 28-h interval. Control animals were treated identically but received normal rabbit serum instead of anti-TNF-α. Two hours after the first intracardiac injection, the animals were inoculated i.p. with 3×10^3 CFU (5 90% lethal doses [LD_{90}]) of strain H738. Pups were sacrificed at 24 and 48 h after bacterial challenge, and CFU and cytokine levels were assayed as described above.

In preliminary experiments, it was determined that intracardiac injections were well tolerated by rat pups. Moreover, normal rabbit serum (plain or inactivated at 56°C for 30 min) had no effect on GBS-induced lethality. In further experiments, we assessed the effect of anti-TNF-α antibodies on mortality. Rat pups were treated with anti-TNF-α or normal rabbit serum as described above and inoculated with 1 or 5 LD_{90} of GBS strains H738 or 259. Mortality was observed every 12 h for 5 days.

Data expression and statistical analysis. Data on cytokine concentrations are expressed as means ± standard deviations of five observations. Each observation was performed on a different animal and is the average of two replicate measurements. To calculate mean values, results below the detection levels were assigned a theoretical value of half the detection level. Statistical analysis was performed with StatView II software (Abacus Concepts, Inc., Calabasas, Calif.) on a Macintosh computer (Apple Computer, Inc., Cupertino, Calif.). Differences in cytokine concentrations were evaluated by one-factor analysis of variance (ANOVA) for repeated measures (differences over time) or factorial

models (differences between experimental groups and controls). With both types of analysis, significance was assessed by Fisher's protected least significant difference (PLSD) test.

Correlations between cytokine and CFU concentrations were assessed by linear regression analysis. Mortality data were analyzed by the χ^2 test.

RESULTS

Cytokine kinetics after infection with 5 LD_{90}. Figure 1 shows CFU and cytokine levels in serial samples taken from neonatal rats after i.p. injection with 3×10^3 CFU (5 LD_{90}) of strain H738. Bacteria were detected in the blood within 4 h after injection. Their numbers increased exponentially over the following 48 h, reaching 6×10^7 to 6.4×10^8 CFU/ml before the death of the animals (Fig. 1A).

TNF-α was undetectable (<8 U/ml) in normal plasma samples obtained from five untreated animals (baseline or 0-h controls). TNF-α activity increased significantly ($P < 0.05$ compared with baseline values) to 55 ± 27 U/ml at 12 h postinfection and reached peak levels of 232 ± 124 U/ml at 20 h (Fig. 1A). TNF-α activity remained at significantly elevated levels of 96 ± 12 U/ml ($P < 0.01$) at 72 h after challenge.

No detectable IL-1α was found in normal spleen homogenates. IL-1α increased to significant ($P < 0.01$) levels of 748 ± 322 U/g at 20 h and reached a maximal level of 766 ± 404 U/g at 24 h (Fig. 1B). IL-1α values declined slowly thereafter and were no longer significantly elevated at 72 h.

Low levels (≤25 U/ml) of IL-6 activity were measured in normal plasma (Fig. 1B). IL-6 rose concomitantly with IL-1α. Plasma IL-6 activity significantly increased over baseline values at 20 h (569 ± 173 U/ml; $P < 0.01$) and reached peak levels of 1,033 ± 520 U/ml at 24 h post infection. IL-6 activity also declined and was no longer significantly elevated at the end of the experiment.

Though never detectable in normal plasma, IFN-γ was measurable in 22 of 48 plasma samples taken between 4 and 72 h after infection. Plasma IFN-γ elevations were highly variable but reached statistical significance at 16 ($P < 0.01$) and 36 h ($P < 0.05$) (Fig. 1C). No correlation could be found between plasma IFN-γ values and cytokine concentrations (including spleen IFN-γ) or CFU in individual samples (data not shown).

Significantly increased IFN-γ activity ($P < 0.01$) was measured in spleen homogenates at 20 h postinfection with maximal values of 449 ± 283 U/g being reached at 36 h. Spleen IFN-γ values at 72 h were not significantly higher than preinfection values.

Correlation between cytokine levels and CFU. There was no significant correlation between the number of CFU in the blood and cytokine levels. However, after logarithmic transformation, CFU values were found to significantly correlate with concentrations of each cytokine (Fig. 2). Figure 2A also shows that concentrations of $\geq 10^6$ CFU/ml were always associated with plasma TNF-α values of ≥70 U/ml and vice versa.

Induction of cytokines by 1 LD_{50}. It was of interest to determine whether lower doses of GBS also induced increased cytokine production. In addition, it was necessary to rule out the possibility that strain H738 was not typical of type III GBS in its ability to stimulate cytokine production. For these reasons, in further experiments rat pups were injected with 1 50% lethal dose (LD_{50}) of each of the three GBS strains listed in Table 1. Animals were sacrificed at 12, 24, 48, and 72 h after challenge, and cytokine levels were compared with those of animals receiving i.p. injections of PBS (Table 3). As shown in Table 3, there was considerable variation in CFU and cytokine levels within each experimental group. However, the concentrations of each cytokine were significantly elevated at 24 and/or 48 h in pups challenged with any of the type III strains (Table 3). Significantly

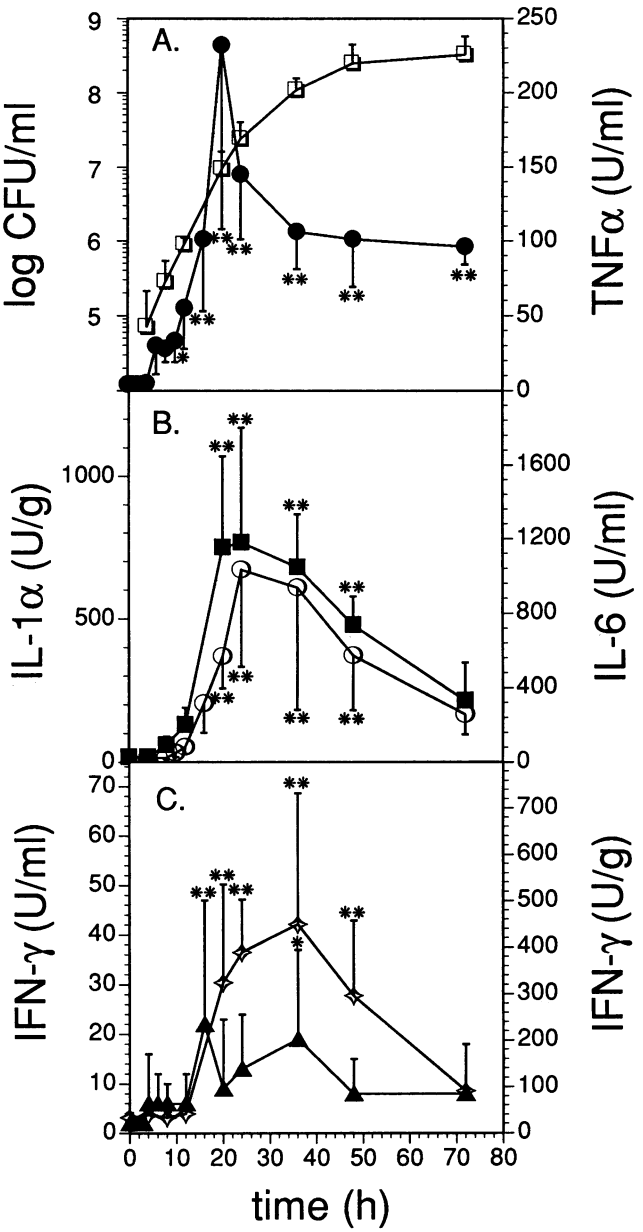

FIG. 1. Cytokine kinetics in neonatal rats infected with 5 LD_{90} of strain H738. Rat pups (five per group) were sacrificed at different times after i.p. challenge with 3×10^3 CFU. (A) Blood CFU (□) and plasma TNF-α (●) units; (B) IL-1α units in spleen homogenates (■) and IL-6 units in plasma (○); (C) IFN-γ units in plasma (▲) and spleen homogenates (◇). Points and bars, means ± standard deviations of five observations, each conducted on a different animal; *, $P < 0.05$ versus 0 h; **, $P < 0.01$ versus 0 h, as determined by Fisher's PLSD test by using one-factor ANOVA for repeated-measure models.

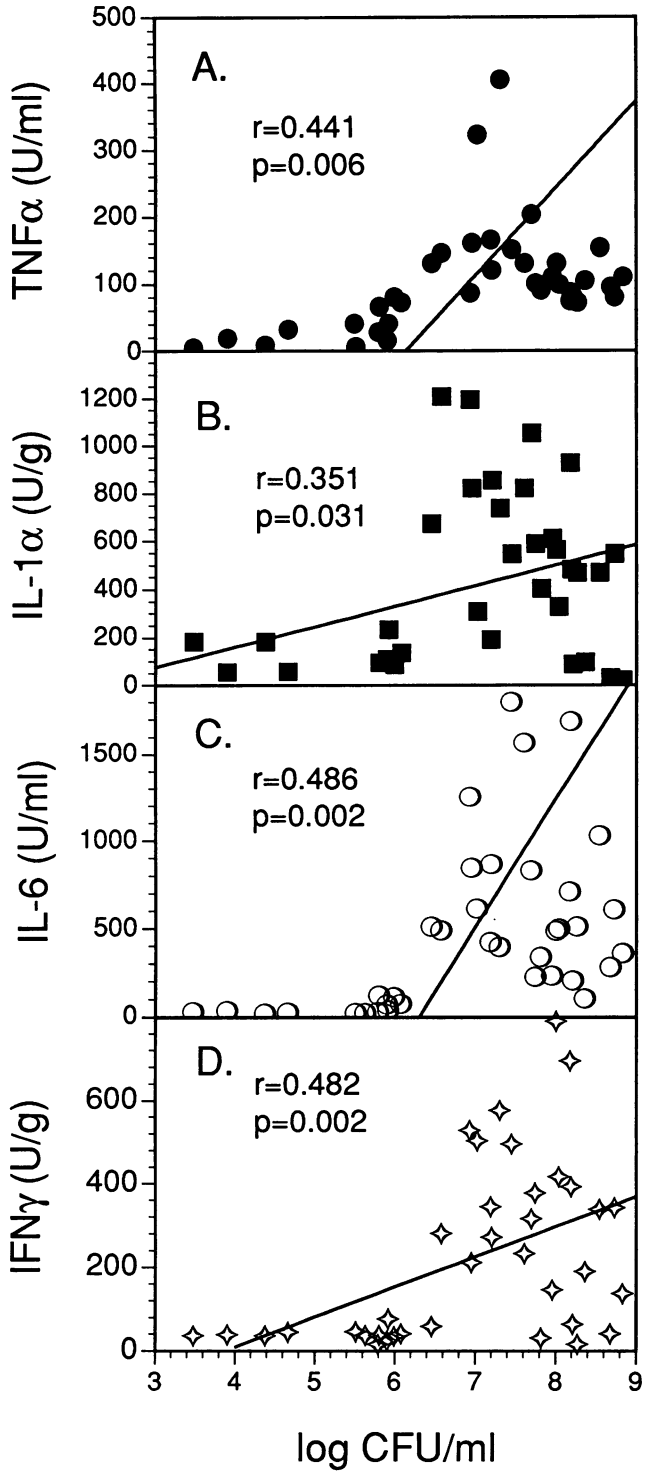

FIG. 2. Correlation between cytokine concentrations and blood CFU in neonatal rats infected with 5 LD_{90} of strain H738. Shown are individual data from the same experiments whose results are reported in Fig. 1. TNF-α and IL-6 levels were measured in plasma, while IL-1α and IFN-γ levels were measured in spleen homogenates. *r* and *P* values were calculated by linear regression analysis.

elevated TNF-α levels were measured at 72 h in pups injected with strains H738 and 90.

Absence of endotoxin in plasma. Since, theoretically, i.p. infection might result in alterations of the intestinal barrier to endogenous bacteria and their products, we sought to rule out the possibility that the observed cytokine changes were due to penetration of endotoxin from intestinal bacteria into the circulation. For this reason, aliquots of plasma samples obtained during the experiments reported in Table 3 (two randomly selected samples for each experimental group) were tested for the presence of endotoxin. Endotoxin concentrations were below the limit of detection (0.05 ng/ml) of the assay in each of the 32 samples tested (data not shown).

Effect of anti-TNF-α on cytokine production and mortality. In order to assess whether TNF-α was responsible for the production of other cytokines or mediated GBS-induced mortality, groups of rat pups were injected with rabbit anti-TNF-α or normal serum. This treatment was able to completely prevent the TNF-α response, as shown by the absence of plasma TNF-α activity in anti-TNF-α-treated pups at 24 and 48 h after infection (Fig. 3). Moreover, these plasma samples contained significant residual anti-TNF-α activity, being still capable of blocking in vitro the cytotoxicity of rat TNF-α (10 U/ml) when diluted 160- to 640-fold (data not shown). In contrast, TNF-α activity was prominent in plasma samples of pups treated with normal rabbit serum (Fig. 3). Anti-TNF-α markedly reduced IL-6 and IFN-γ, but not IL-1α, levels at both 24 and 48 h (Fig. 3). Treatment with anti-TNF-α did not affect the number of CFU in blood in septic rat pups (data not shown).

Rat pups pretreated with anti-TNF-α serum and infected with 1 and 5 LD_{90} of strains H738 and 259 showed enhanced early (48 to 72 h) survival (Fig. 4). However, by 96 h this protection was no longer apparent (Fig. 4).

DISCUSSION

Cytokines are considered major mediators of circulatory septic shock caused by gram-negative organisms. GBS are the main cause of serious neonatal infections in many countries, but little is known about the role of cytokines in the pathophysiology of sepsis caused by these and other gram-positive organisms.

Using a rat model of rapidly fatal neonatal sepsis, we have shown here that GBS consistently induce the production of TNF-α, IL-1α, IL-6, and IFN-γ. Our data support the notion that similar cytokine mediators are produced by host cells upon interaction with gram-positive and gram-negative bacteria and that endotoxin is not required to produce the full sequence of cytokine responses observed during endotoxin or gram-negative shock. Recent reports have indicated that the i.v. injection of killed gram-positive bacteria can produce increases in circulating TNF-α and IL-1β levels (41) and a shock-like state (18, 26, 41). The mean peak levels of TNF-α that we measured (232 U/ml) are very similar to those observed in association with hypotension and tissue damage in rabbits injected i.v. with killed *E. coli* or *S. epidermidis* (41).

In our model, TNF-α appeared before any other cytokine and was at least in part responsible for inducing IL-6 and IFN-γ but not IL-1α. This conclusion was reached by observing marked reductions in IL-6 and IFN-γ levels after neutralization of circulating TNF-α with specific antibodies.

As for the induction of IL-6 by TNF-α, our data are in agreement with similar observations in baboons infected with *E. coli* (16). In addition, a rapid increase in circulating

TABLE 3. Cytokine kinetics in neonatal rats infected with 1 LD_{50} of three different GBS strains

Strain or control[a]	Time after challenge (h)	Mean ± SD (median)[b]				
		CFU/ml (10^6)[c]	TNF-α (U/ml)[d]	IL-1α (U/g)[e]	IL-6 (U/ml)[d]	IFN-γ (U/g)[e]
H738	12	0.33 ± 0.42 (0.04)	12 ± 9 (15)	32 ± 19 (19)	29 ± 15 (20)	37 ± 13 (44)
	24	4.31 ± 7.43 (1.43)	180 ± 171 (148)[f]	479 ± 327 (449)[f]	592 ± 484 (865)[g]	130 ± 69 (129)[f]
	48	37.17 ± 64.10 (3.50)	66 ± 41 (56)[g]	506 ± 398 (385)[f]	313 ± 300 (205)[f]	172 ± 48 (94)[f]
	72	4.51 ± 6.56 (1.60)	47 ± 31 (33)[g]	36 ± 26 (19)	154 ± 97 (158)	69 ± 63 (46)
259	12	0.04 ± 0.01 (0.04)	13 ± 3 (14)	35 ± 15 (45)	36 ± 11 (38)	32 ± 16 (40)
	24	1.47 ± 0.88 (0.98)	165 ± 92 (158)[f]	401 ± 309 (282)	330 ± 195 (328)	131 ± 29 (120)[f]
	48	1.63 ± 0.98 (1.58)	63 ± 43 (52)[f]	526 ± 464 (511)[f]	340 ± 205 (233)[f]	115 ± 44 (101)
	72	0.39 ± 0.31 (0.46)	15 ± 7 (15)	40 ± 21 (40)	101 ± 56 (103)	40 ± 26 (35)
90	12	0.25 ± 0.41 (0.08)	12 ± 12 (8)	34 ± 19 (21)	32 ± 26 (20)	35 ± 31 (16)
	24	4.42 ± 3.67 (3.60)	226 ± 139 (270)[g]	485 ± 405 (482)[f]	437 ± 248 (408)[f]	126 ± 91 (74)[f]
	48	9.02 ± 10.12 (3.90)	61 ± 32 (48)[f]	309 ± 308 (150)	322 ± 174 (309)[f]	138 ± 105 (85)
	72	3.38 ± 4.57 (1.39)	39 ± 28 (36)[f]	39 ± 19 (45)	148 ± 92 (152)	43 ± 13 (44)
PBS	12	ND[h]	4 ± 0 (4)	18 ± 1 (18)	16 ± 3 (15)	29 ± 13 (35)
	24	ND	4 ± 0 (4)	20 ± 2 (19)	17 ± 3 (18)	36 ± 11 (42)
	48	ND	4 ± 0 (4)	19 ± 1 (19)	18 ± 5 (15)	36 ± 10 (40)
	72	ND	4 ± 0 (4)	20 ± 2 (19)	16 ± 3 (17)	31 ± 15 (25)

[a] Rat pups (five per group) were inoculated i.p. with 1 LD_{50} of the indicated GBS strains or PBS and sacrificed at different times after challenge.
[b] Calculated from five observations. Each observation was made for a different animal.
[c] Measured in whole blood.
[d] Measured in plasma.
[e] Measured in spleen homogenates.
[f] $P < 0.05$ versus the corresponding PBS control, as determined by one-factor ANOVA for factorial models with four classes (H738, 259, 90, and PBS).
[g] $P < 0.01$ versus the corresponding PBS control, as determined by one-factor ANOVA for factorial models with four classes (H738, 259, 90, and PBS).
[h] ND, not determined.

IL-6 was measured in patients infused with recombinant TNF-α (9). Stimuli other than TNF-α are also able to induce the release of IL-6 (22). Since IL-6 levels were not totally abrogated by anti-TNF-α, it will be of interest to ascertain whether GBS and their components are able to directly induce the production of IL-6 in vivo and in vitro.

Our results are also in agreement with the notion that TNF-α is an important stimulus for the release of IFN-γ, as shown in *Listeria monocytogenes*-infected *scid* mice (5). In that model, the simultaneous presence of bacterial products and TNF-α stimulates natural killer cells to produce IFN-γ (43).

Endotoxin is capable of inducing IFN-γ in mice (7, 23). However, this cytokine is often undetectable in sera of patients with septic shock (10, 39), and serum IFN-γ elevations measured in a baboon *E. coli* sepsis model were not statistically significant (25). Accordingly, we found only variable elevations of circulating IFN-γ in GBS-infected rat

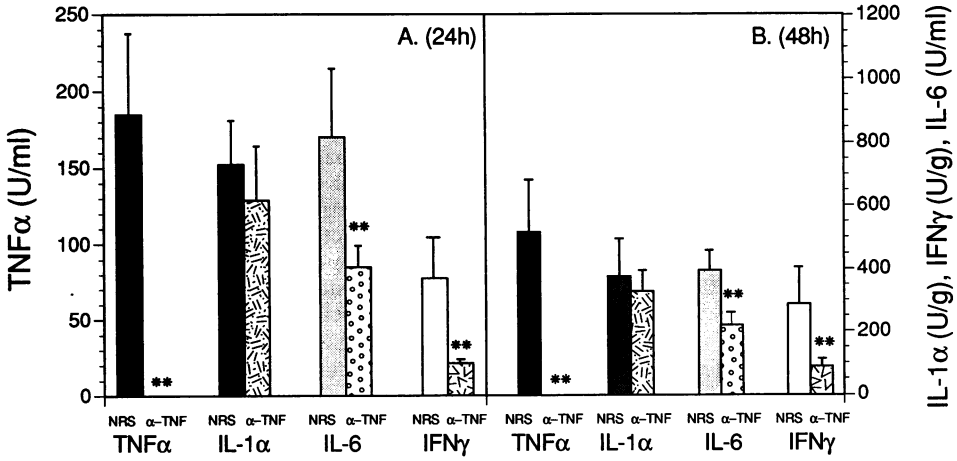

FIG. 3. Effect of anti-TNF-α antibodies on cytokine levels of GBS-infected rat pups. Rat pups (five per group) received two intracardiac injections of anti-TNF-α (α-TNF) or normal rabbit serum (NRS) 2 h before and 26 h after i.p. challenge with 3×10^3 CFU of strain H738. The animals were sacrificed at 24 (A) and 48 (B) h after infection. TNF-α and IL-6 levels were measured in plasma, while IL-1α and IFN-γ levels were measured in spleen homogenates. Columns and bars, means ± standard deviations of five observations, each conducted on a different animal; **, $P < 0.01$ versus normal rabbit serum, as determined by Fisher's PLSD test by using one-factor ANOVA for factorial models with two classes.

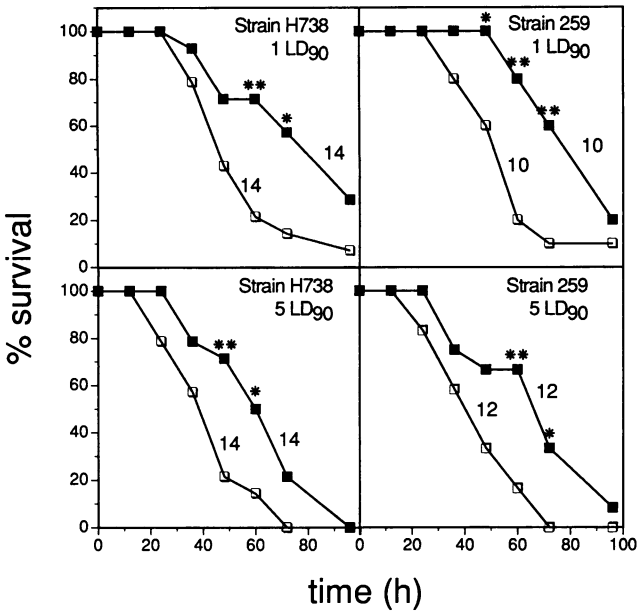

FIG. 4. Effect of anti-TNF-α antibodies on survival of GBS-infected rat pups. Rat pups received two intracardiac injections of anti-TNF-α (■) or normal rabbit serum (□) 2 h before and 26 h after i.p. challenge with the indicated strains and doses of GBS. Numbers near the lines indicate the number of rat pups per group. *, $P < 0.05$; **, $P < 0.01$ versus normal rabbit serum, as determined by χ^2 analysis.

pups. However, reproducible increases in IFN-γ levels were measured in spleen homogenates. Studies are under way to identify the spleen cell types involved in IFN-γ production.

Although circulating IL-1β can be induced by TNF-α (16), in our model spleen IL-1α levels were not affected by anti-TNF-α antibodies. Studies are under way to ascertain whether IL-1 production is regulated by different factors in the spleen and in the blood after challenge with gram-positive and gram-negative bacteria.

A unique feature of the cytokine kinetics reported here is that TNF-α persisted at elevated levels for 1 to 2 days until the death of septic rat pups, after reaching maximal values earlier during infection. In contrast, TNF-α elevations are typically transient and of short duration in other gram-positive (41) and gram-negative shock models (17, 25, 32, 38) involving i.v. injections of bacteria or endotoxin. We are not able to completely explain these differences. It is unlikely that the persistence of high circulating TNF-α levels we observed is in some way related to GBS per se. In a previous study, using the same GBS strain in adult mice, we have detected only transient TNF-α and IL-6 elevations (35).

Our working hypothesis is that the persistence of high circulating cytokine levels is in some way related to the continuing presence of very high (~10^8 CFU/ml) numbers of bacteria in the blood. This is not a feature of the adult mouse model, because of the low virulence of GBS for the adult host. Persistence of elevated TNF-α levels in the present study (Table 3) was observed only with two highly virulent strains (H738 and 90), not with strain 259, which is about 100- to 1,000-fold less virulent. Finally, our observation that, in septic rat pups, the logs of bacterial concentrations are significantly correlated with cytokine levels is also in agreement with this hypothesis. The possibility that the prolonged elevations in circulating TNF-α levels are in part due to the presence of a persistent focus in the peritoneal cavity should also be considered. Although quantitative data are not available, cytokine levels in rat pups rose concomitantly with the development of macroscopically visible peritonitis.

It is interesting that persistence of high TNF-α levels can be observed in adult patients with gram-negative shock. For example, in 79% of patients with gram-negative sepsis, detectable levels of TNF-α were present 10 h after the development of shock (10). After this time, two different evolutions of serum TNF-α levels were observed. In survivors, TNF-α had decreased to undetectable levels 10 days after the beginning of shock, whereas in 11 of 13 nonsurvivors TNF-α levels remained elevated. Persistence of elevated TNF-α activity in patients who die could be related to failure in eliminating infection by gram-negative bacteria despite appropriate antimicrobial therapy (10).

The possible role of TNF-α in the pathogenesis of GBS disease deserves some consideration. Theoretically, TNF-α could have both protective and detrimental activities for the host. TNF-α and IFN-γ are potent activators of phagocytosis and killing by polymorphonuclear and mononuclear phagocytes (31). Since complement- and antibody-dependent opsonic phagocytosis is the main defense mechanism of the host against GBS (4), TNF-α and TNF-α-induced IFN-γ may play beneficial roles. Conversely, as discussed above, overproduction of these cytokines may lead to shock and death. This is best illustrated by i.v. endotoxin and *E. coli* sepsis models in which prophylactic administration of anti-TNF-α antibodies completely prevents mortality (6, 31, 38).

Much less dramatic effects of anti-TNF-α antibodies were observed here. In fact, abrogation of circulating TNF-α by specific antibodies did not affect GBS-induced mortality at 96 h, although survival time was moderately increased. In our previous study (35), rabbit anti-TNF-α serum had no effect on survival or survival time of GBS-infected adult mice. The moderate increase in survival time observed here for similarly treated rat pups may be explained by the fact that elevations in plasma TNF-α levels were more prolonged in the rat model than in the mouse model. Alternatively, the different response to anti-TNF-α prophylaxis may be related to differences in inflammatory mechanisms of neonatal versus adult individuals. Limited data are available on cytokine production in neonates. Production of TNF-α, IL-1 (42), and IL-6 (44) was found to be reduced in preterm, but not full-term, neonates.

Our data provide further evidence that anti-TNF-α antibodies have different effects in different septic shock models. For example, anti-TNF-α prevented endotoxin lethality in D-galactosamine-sensitized mice but not carrageenan-sensitized mice (17). Similarly, anti-TNF-α monoclonal antibodies prevented *E. coli*-induced lethality in gentamicin-treated mice but had only a limited effect in mice injected with *Pseudomonas aeruginosa* (32). In the same study, no effect of anti-TNF-α was noted for neutropenic mice challenged with *Klebsiella pneumoniae*. Anti-TNF-α increased mortality in an experimental peritonitis-sepsis model, while administration of recombinant TNF-α had a protective effect (14).

The moderate increase in survival time produced by anti-TNF-α prophylaxis in septic rat pups suggests that anticytokine agents deserve some attention as possible adjunctive measures in the treatment of GBS disease. In addition, preliminary data from our laboratory indicate that anti-TNF-α antibodies are highly protective in neonatal rats injected with a lethal dose of heat-killed GBS. Clearly, more studies involving therapeutic as well as prophylactic regimens are needed. It will be of interest to test several agents

aimed at blocking the production or activity of different cytokines and inflammatory mediators.

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REFERENCES

1. **Anthony, B. F., N. F. Concepcion, S. A. McGeary, J. I. Ward, D. C. Heiner, P. Shapshak, and R. A. Insel.** 1982. Immunospecificity and quantitation of an enzyme-linked immunosorbent assay for group B streptococcal antibody. J. Clin. Microbiol. **16:**350–354.
2. **Arditi, M., K. R. Manogue, M. Caplan, and R. Yogev.** 1990. Cerebrospinal fluid cachectin/tumor necrosis factor-α and platelet activating factor concentrations and severity of bacterial meningitis in children. J. Infect. Dis. **162:**139–147.
3. **Bagby, G. J., K. J. Plessala, L. A. Wilson, J. J. Thompson, and S. Nelson.** 1991. Divergent efficacy of antibody to tumor necrosis factor-α in intravascular and peritonitis models of sepsis. J. Infect. Dis. **163:**83–88.
4. **Baker, C. J.** 1986. Group B streptococcal infections in newborns. N. Engl. J. Med. **314:**1702–1708.
5. **Bancroft, J. G., K. C. F. Sheehan, R. D. Schreiber, and E. R. Unanue.** 1989. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in *scid* mice. J. Immunol. **143:**127–130.
6. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
7. **Blanchard, K., J. Y. Djeu, T. W. Klein, H. Friedman, and W. E. Stewart II.** 1986. Interferon γ induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. J. Immunol. **136:**963–970.
8. **Brakenhoff, J. B., J. E. R. Degroot, R. F. Evers, H. Pannekock, and L. A. Aarden.** 1987. Molecular cloning and expression of hybridoma growth factor in *E. coli*. J. Immunol. **139:**41–46.
9. **Brouckaert, P., D. R. Spriggs, G. Demetri, D. W. Kufe, and W. Fiers.** 1989. Circulating interleukin 6 during a continuous infusion of tumor necrosis factor and interferon-γ. J. Exp. Med. **169:**2257–2262.
10. **Calandra, T., J. Baumgartner, G. E. Grau, M. Wu, P. Lambert, J. Schellekens, J. Verhoef, M. P. Glauser, and Swiss-Dutch J5 Immunoglobulin Study Group.** 1990. Prognostic values of tumor necrosis factor/cachectin, interleukin-1, interferon-α, and interferon-γ in the serum of patients with septic shock. J. Infect. Dis. **161:**982–987.
11. **Carey, R. B., T. K. Eisenstein, G. D. Shockman, T. F. Greber, and R. M. Swenson.** 1980. Soluble group- and type-specific antigens from type III group B streptococcus. Infect. Immun. **28:**195–203.
12. **Dinarello, C. A.** 1988. Biology of interleukin 1. FASEB J. **2:**108–115.
13. **Dinarello, C. A.** 1991. The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome. J. Infect. Dis. **163:**1177–1184.
14. **Echtenacher, B., W. Falk, D. M. Mannel, and P. H. Krammer.** 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. J. Immunol. **145:**3762–3766.
15. **Fong, Y., L. L. Moldawer, M. Marano, H. Wei, S. B. Tatter, R. H. Clarick, U. Santhanam, D. Sherris, L. T. May, P. B. Sehgal, and S. F. Lowry.** 1989. Endotoxemia elicits increased circulating β_2 interferon/interleukin-6 in man. J. Immunol. **142:** 2321–2324.
16. **Fong, Y., K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. R. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, and A. Cerami.** 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. **170:**1627–1633.
17. **Franks, A. K., K. I. Kujawa, and L. J. Yaffe.** 1991. Experimental elimination of tumor necrosis factor in low-dose endotoxin models has variable effects on survival. Infect. Immun. **59:**2609–2614.
18. **Freudenberg, M. A., and C. Galanos.** 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. Infect. Immun. **59:**2110–2115.
19. **Gibson, R. L., G. J. Redding, W. R. Henderson, and W. E. Truog.** 1991. Group B streptococcus induces tumor necrosis factor in neonatal piglets. Am. Rev. Respir. Dis. **143:**598–604.
20. **Gunther, C., M. Rollinghoff, and H. U. Beusher.** 1989. Proteolysis of the native murine IL-1β precursor is required to generate IL-1β bioactivity. Immunobiology **178:**436–448.
21. **Hakansson, S., and S. Holm.** 1986. Influence of polysaccharide capsule and ionic strength on buoyant density of group B streptococci. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **94:**139–143.
22. **Havell, E. A., and P. B. Sehgal.** 1991. Tumor necrosis factor-independent IL-6 production during murine listeriosis. J. Immunol. **146:**756–761.
23. **Heinzel, F. P.** 1990. The role of IFN-γ in the pathology of experimental endotoxemia. J. Immunol. **145:**2920–2924.
24. **Helfgott, D. C., S. B. Tatter, U. Santhanam, R. H. Clarick, N. Bardwaj, L. T. May, and P. B. Sehgal.** 1989. Multiple forms of IFNβ_2/IL-6 in serum and body fluids during acute bacterial infection. J. Immunol. **142:**948–953.
25. **Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino, Jr., A. Cerami, G. T. Shires, and S. F. Lowry.** 1988. Cytokine appearance in human endotoxemia and primate bacteremia. Surg. Gynecol. Obstet. **166:**147–153.
26. **Nathanson, C., R. L. Danner, R. J. Elin, J. M. Hosseini, K. W. Peart, S. M. Banks, T. J. McVittie, R. I. Walker, and J. E. Parrillo.** 1989. Role of endotoxemia in cardiovascular dysfunction and mortality. *Escherichia coli* and *Staphylococcus aureus* challenges in a canine model of human septic shock. J. Clin. Invest. **83:**243–251.
27. **Nathanson, C., P. W. Eichenholz, L. R. Danner, P. Q. Eichacker, W. D. Hoffman, G. C. Kuo, S. M. Banks, T. J. McVittie, and J. E. Parrillo.** 1989. Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. J. Exp. Med. **169:**823–832.
28. **Nelson, S., G. J. Bagby, B. G. Bainton, L. A. Wilson, J. J. Thompson, and W. R. Summer.** 1989. Compartmentalization of intraalveolar and systemic lipopolysaccharide-induced tumor necrosis factor and the pulmonary inflammatory response. J. Infect. Dis. **159:**189–194.
29. **Okusawa, S., J. A. Gelfand, T. Ikejima, R. J. Connolly, and C. A. Dinarello.** 1988. Interleukin-1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J. Clin. Invest. **81:**1162–1172.
30. **Orencole, S. F., B. D. Clark, C. A. Dinarello, and P. Ghezzi.** 1991. Measurement of in vivo and in vitro interleukin-1α levels in mouse stimulated with endotoxin using a radioimmunoassay for murine IL-1α. Abstr. Third Int. Workshop Cytokines, abstr. no. 257. Cytokine **3:**492.
31. **Shalaby, M. R., B. B. Aggarwal, E. Rinderchneht, L. P. Sverdesky, B. S. Finkle, and M. A. Palladino.** 1985. Activation of human polymorphonuclear leucocyte functions by interferon-γ and tumor necrosis factors. J. Immunol. **135:**2069–2072.
32. **Silva, A. T., K. F. Bayston, and J. Cohen.** 1990. Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-α in experimental gram negative shock. J. Infect. Dis. **162:**421–427.
33. **Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J.-C. Zou, and J. S. Abrams.** 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. J. Immunol. **145:**4185–4191.
34. **Teti, G., M. Calapai, G. Calogero, F. Tomasello, G. Mancuso, A. Galli, and G. Riggio.** 1992. Specificity and protective activity of

murine monoclonal antibodies directed against the capsular polysaccharide of type III group B streptococci. Hybridoma **11:**13–22.

35. **Teti, G., G. Mancuso, F. Tomasello, and M. S. Chiofalo.** 1992. Production of TNFα and IL-6 in mice infected with group B streptococci. Circ. Shock **38:**138–144.
36. **Teti, G., F. Tomasello, M. S. Chiofalo, G. Orefici, and P. Mastroeni.** 1987. Adherence of group B streptococci to adult and neonatal epithelial cells mediated by lipoteichoic acid. Infect. Immun. **55:**3057–3064.
37. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science (Washington, D.C.) **234:**470–474.
38. **Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. C. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature (London) **330:**662–665.
39. **Waage, A., P. Brandtzaeg, A. Halstelsen, P. Kierulf, and T. Espevik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. J. Exp. Med. **169:** 333–338.
40. **Wakabayashi, G., J. A. Gelfand, J. F. Burke, R. C. Thompson, and C. A. Dinarello.** 1991. A specific receptor antagonist for interleukin-1 prevents *E. coli* induced shock. FASEB J. **5:**338–343.
41. **Wakabayashi, G., J. A. Gelfand, W. K. Jung, R. J. Connolly, J. F. Burke, and C. A. Dinarello.** 1991. *Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. Comparison to *Escherichia coli*. J. Clin. Invest. **87:**1925–1935.
42. **Weatherstone, K. B., and E. A. Rich.** 1989. Tumor necrosis factor/cachectin and interleukin 1 secretion by cord blood monocytes from premature and term neonates. Pediatr. Res. **25:**342–346.
43. **Wherry, J. C., R. D. Schreiber, and E. R. Unanue.** 1991. Regulation of gamma interferon production by natural killer cells in *scid* mice: roles of tumor necrosis factor and bacterial stimuli. Infect. Immun. **59:**1709–1715.
44. **Yachie, A., N. Takano, K. Ohta, T. Uehara, S.-I. Fujita, T. Miyawaki, and N. Taniguchi.** 1992. Defective production of interleukin-6 in very small premature infants in response to bacterial pathogens. Infect. Immun. **60:**749–753.