
Inhibition of Macrophage-activating Cytokines is Beneficial in the Acute Septic Response

H. PAUL REDMOND, F.R.C.S.I.,* KENNETH D. CHAVIN, M.D.,† JONATHAN S. BROMBERG, M.D., PH.D.,† and JOHN M. DALY, M.D., F.A.C.S.*

Interferon- γ and other cytokines enhance macrophage (M ϕ) antimicrobial function and have been considered for therapeutic use in sepsis. Systemic sequelae of macrophage activation, however, are unclear. This study examined the effects of M ϕ activating cytokines (interferon- γ [IFN- γ] and interleukin-4 [IL-4]) and monoclonal antibodies directed against these cytokines in modulating the acute septic response. CFW/Swiss Webster mice (n = 345) received endotoxin (lipopolysaccharide [LPS]; 60 mg/kg body weight intraperitoneally) and were randomized to five treatment groups: IFN- γ (10^4 units), IL-4 (10^4 units), IgG₁ isotype antibody (TRFK5; 200 μ g), anti-IFN- γ (200 μ g), or anti-IL-4 (200 μ g) monoclonal antibodies (MAbs) given simultaneously or 2 hours after LPS. Animals were divided into two groups and studied for mortality or measurement of peritoneal M ϕ superoxide anion release (O_2^-), tumor necrosis factor (TNF), and IL-6 production 6 hours after administration of LPS \pm experimental regimens. Serum TNF and IL-6 also were assessed at 2 and 4 hours after LPS, respectively. Administration of LPS resulted in a 27% survival compared with 10% in the IFN- γ and 13% in the IL-4 groups. Treatment with anti-IFN- γ offered protection against LPS lethality (93%–100% survival, $p < 0.001$ vs. other groups) when given either simultaneously or 2 hours after LPS. Anti-IFN- γ also significantly decreased PM ϕ O_2^- and TNF release. Thus anti-IFN- γ may have an important role in the modulation of the acute septic response.

BACTERIAL INFECTION REMAINS a common cause of death among critically ill, hospitalized patients despite numerous therapeutic advances.^{1,2} Several studies have reported an increased incidence of gram-negative septicemia with an associated mortality rate of 20% to 35%.^{3,4} In those patients who develop gram-neg-

From the Department of Surgery and the Harrison Department of Surgical Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and the Transplant Program, Department of Surgery, Medical University of South Carolina, Charleston, South Carolina†*

ative septic shock, the mortality rate may be as high as 50% to 80%.⁵ Recent attention has focused on the pathogenesis of gram-negative sepsis and, in particular, the toxic role of endotoxin or lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative and some gram-positive bacteria. Many of the effects of LPS are mediated through the release of cytokines, including tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6). Elevated serum TNF levels occur during experimental endotoxemia in humans, primates, and canines^{6,7} and have been observed in a number of infectious disease states.⁸⁻¹⁰ Furthermore direct intravenous infusion of TNF induces effects mimicking septic shock.¹¹ Moreover in experimental studies passive immunization with anti-TNF monoclonal antibody (MAb) is protective against the development of septic shock and its sequelae.¹²

Although functionally not as well defined, IL-1 appears to act as a potentiator of TNF activity in rabbits¹³ and mice,¹⁴ and high levels of IL-6 have been identified in malaria and meningococcal meningitis.⁹ Monoclonal antibodies directed against IL-6 have recently been shown to be beneficial when given simultaneously with *Escherichia coli* or TNF administration,¹⁵ and an IL-1 receptor (IL-1R) antagonist has proved effective in preventing lethal *E. coli*-induced shock in rabbits and baboons.¹⁶⁻¹⁸

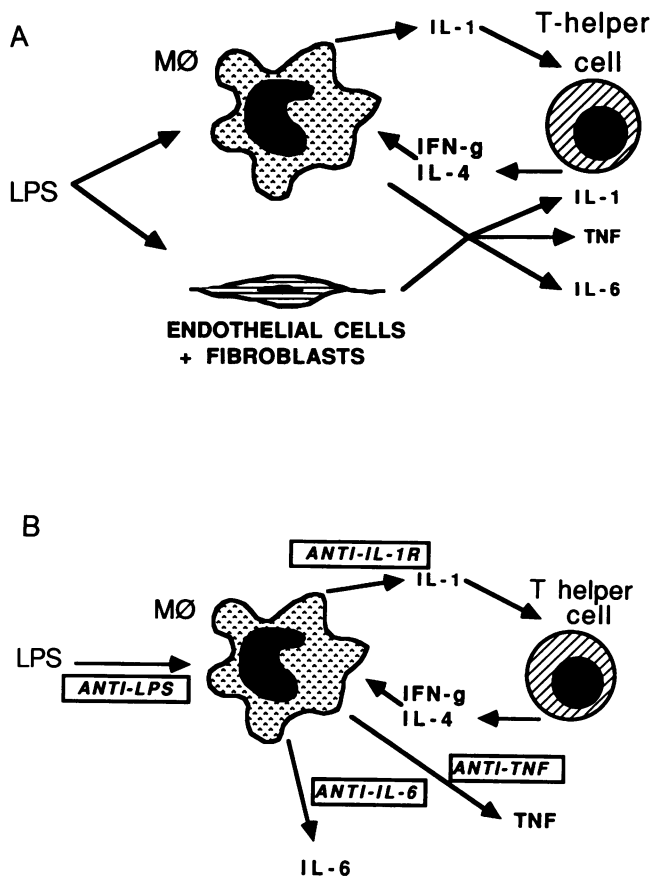
Central to the production and release of these cytokines is the mononuclear phagocyte, which includes both peripheral blood monocytes and fixed tissue macrophages (M ϕ). Macrophage activation results in increased release of these cytokines as well as reactive oxygen intermediates. As shown in Figure 1, recent studies have used MAbs against these cytokines to alter the acute septic response.

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Address reprint requests to Dr. John M. Daly, M.D., 4th Floor, Silverstein Pavilion, Hospital of the University of Pennsylvania, Philadelphia, PA 19104.

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FIGS. 1A and 1B. (A) Monoclonal antibodies have been developed and used against endotoxin (LPS), the macrophage proinflammatory cytokines TNF and IL-6, and IL-1R. (B) Proposed steps in the activation of macrophages by endotoxin. Macrophage–endotoxin interaction may lead to IL-1 release, which in turn, stimulates T-helper cell subsets, with subsequent release of the macrophage-activating factors, interferon- γ and IL-4. These factors, in turn, activate macrophages for release of TNF and possibly IL-6. Endothelial cells, epithelial cells, and fibroblasts may also secrete proinflammatory cytokines when appropriately stimulated.

Macrophage activation is induced *in vivo* and *in vitro* by interferon- γ (IFN- γ)¹⁹ and *in vitro* by interleukin-4 (IL-4)^{20,21} (Fig. 1A). Elevation in serum IFN- γ levels occurs during the course of septic shock.⁵ It is reasonable to postulate that IFN- γ , as well as other MØ-activating cytokines such as IL-4, may have a role in modulating the acute septic response because of their potent macrophage-activating capacity.

These studies were designed to assess the role of macrophage activation during the acute septic response and, in particular, the potential benefit of monoclonal antibodies directed against the macrophage-activating factors IFN- γ and IL-4 (Fig. 1B).

Methods

Mice

Three hundred forty-five virus-free, female Swiss-Webster (CFW) mice (Charles River Laboratories, Wil-

ington, MA), 6 to 8 weeks old, were studied. After a 7-day period of acclimation on a regular diet, animals were weighed and randomized for either *in vivo* mortality analyses or *in vitro* studies (Fig. 2).

Reagents

Endotoxin (LPS) from *E. coli* serotype 055:B5, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), phorbol myristate acetate, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO).

Cytokines

Murine rIFN- γ and rIL-6 were purchased from Genzyme Corp. (Cambridge, MA), specific activity: 4.5 to 9 $\times 10^6$ $\mu\text{m}/\text{mg}$. Murine rIL-4 was provided by Immunex (Seattle, WA), specific activity: 10.5 $\times 10^6$ $\mu\text{m}/\text{mg}$. Murine rTNF α was kindly donated by Dr. L. Moldover (Cornell University, New York, NY).

Monoclonal Antibodies

The rat IgG1 antimurine IFN- γ (R4-6A2) was purchased from ATCC (Rockville, MD), grown in culture, and purified over a protein G column (Pharmacia, Piscataway, NJ). The 11B11 (anti-IL-4) also was purchased from the ATCC, grown in culture, and purified over a protein G column. The TRFK5 (anti-IL-5) hybridoma, used as a control antibody, was a gift of Dr. R. L. Coffman (DNAX, Palo Alto, CA). Monoclonal antibodies and cytokines were tested for the presence of LPS using the Limulus Assay (Sigma Chemical Co, St. Louis, MO) and found to contain less than 0.4 ng/mL LPS per milliliter of antibody. Monoclonal antibodies and cytokines were administered intravenously in LPS-free phosphate-buffered saline (PBS: 200 $\mu\text{L}/\text{mouse}$)

In Vivo Mortality Studies

Mice ($n = 15/\text{group}$) were randomized to receive LPS (60 mg/kg body weight [BW] intraperitoneally [I.P.]) immediately followed by a treatment regimen as outlined in Figure 1. Survival was assessed daily up to 5 days, after which time no mortality was noted in these studies.

In a second experiment, mice ($n = 15/\text{group}$) received LPS (60 mg/kg BW I.P.) Two hours later, animals were randomized to a treatment group as outlined in Figure 1. Survival was assessed daily up to 5 days.

Isolation and Assays of Peritoneal Macrophages

Peritoneal macrophages (PMØ) were harvested by peritoneal lavage with sterile PBS, centrifuged, washed with PBS, and resuspended in Krebs Ringer's phosphate dextrose (KRPD).²² Cells were plated at a concentration

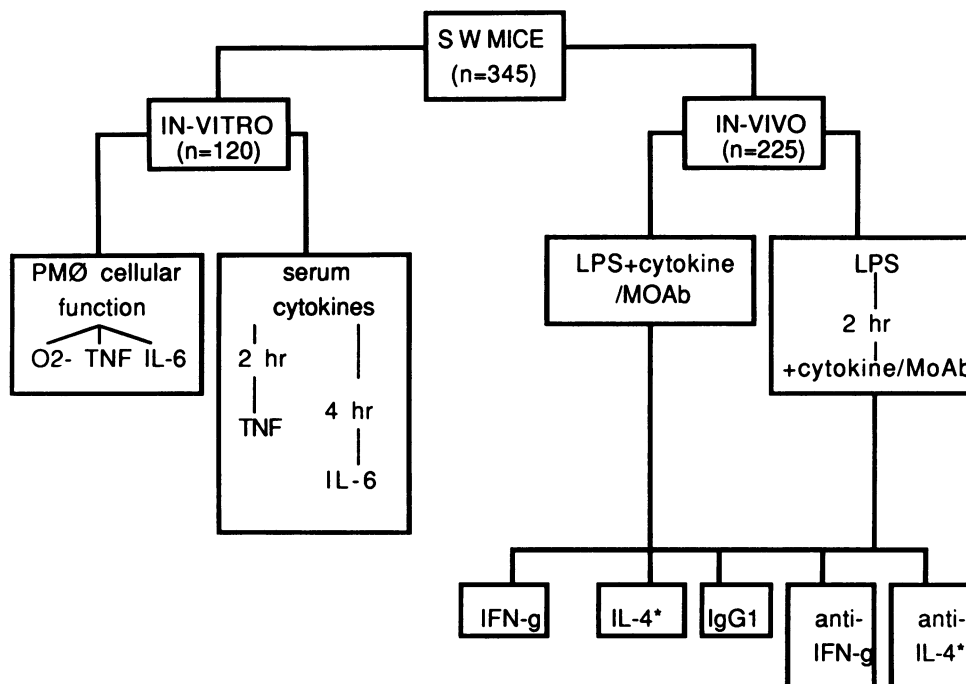


FIG. 2. This flow diagram highlights the different *in vitro* and *in vivo* groups used in this study. *Not studied in delayed (2-hr) treatment group.

of 1×10^6 cells/mL, allowed to adhere for 2 hours at 37 C, washed twice in PBS to remove nonadherent cells, and studied for superoxide anion production, TNF, and IL-6 release.

Preparation of Serum Samples

Blood samples for determination of serum cytokines were obtained by cardiac puncture of mice after CO₂ asphyxiation. Mice ($n = 3$ /group), were randomized to receive a specific treatment regimen, followed immediately by LPS administration (60 mg/kg BW I.P.), and were killed at either 2 or 4 hours for TNF and IL-6 estimation, respectively. Serum samples were obtained from pooled blood and stored at -70 C until assays were performed.

Assay for Superoxide Anion Generation

O₂⁻ generation was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome C in response to a stimulus of phorbol myristate acetate (PMA 1 μ g/mL). Results are expressed as nmol O₂⁻/mg protein/90 minutes. Protein content of adherent cells was determined by Lowry's method.²³

Tumor Necrosis Factor Bioassay

The tumor necrosis factor assay used L929 mouse fibroblasts, which were grown to confluency in a 75-cm² flask. L929 cells were plated at 5×10^5 cells/well for samples and standard curve analysis. After 24 hours all wells were aspirated and replaced with 80 μ L actinomycin D and complete media. Samples and standards then were

added in a 20- μ L volume, and incubated for 18 hours. For cellular TNF release, MØ were stimulated with LPS (1 μ g/mL) for 2 hours and samples were stored at -70 C until used. 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT: 10 μ L/well) was then added to each well and incubation was continued for 4 hours. All wells then were aspirated and 100 μ L isopropanol and 0.04 N HCl were added. The plate then was incubated for a further 10 minutes and 100 μ L distilled water was added per well. Absorbance was read with an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular devices, Menlo Park, CA) at 570 nm with 690 nm subtraction.

Interleukin-6 Bioassay

Interleukin-6 was assayed according to the technique of Aarden et al.²⁴ Briefly $5 \times 10^3 - 1 \times 10^4$ B9 cells (subclone of B13.29 kindly provided by Dr L. Moldover) were cultured in 96-well flat-bottomed microtiter plates in 200 μ L complete RPMI. For cellular IL-6 release, MØ were stimulated with LPS (100 ng/ml) for 12 hours and samples were stored at -70 C until used. Freshly thawed samples were assayed in duplicate and added to the cells at 1:5, 1:25, 1:125, 1:625 dilutions. Sixty hours later 10 μ L Dulbecco's modified Eagle's medium containing 6 mg/mL MTT was added to each well and incubated for 4 hours. The assay was completed as described for the TNF assay. One unit of IL-6 was defined as the concentration required for half-maximal MTT incorporation. Murine rIL-6 (Genzyme Corp Cambridge MA) was used as the

standard. Neither rIL-1-alpha nor rTNF induce B9 growth under these experimental conditions.

Statistical Analysis

Superoxide anion generation, cellular TNF, and IL-6 were performed on pooled MØ, using at least five mice per assay. Each experiment was performed three to five times. Serum cytokine analysis was performed on pooled serum, using three to five mice per assay. *In vivo* mortality studies used at least 15 mice per group. Statistical analysis was performed using the unpaired Student's t test for comparison of two groups and ANOVA for multiple comparison of means of several groups. The chi square analysis was used to assess survival in LPS studies. The level of significance was determined at $p < 0.05$. Data are presented as the mean \pm standard deviation.

Results

Superoxide Anion Generation

As shown in Figure 3, endotoxin administration primed PMØ *in vivo* for enhanced O_2^- release. Interferon- γ synergized with LPS to induce the highest state of macrophage activation as reflected in mean O_2^- production compared with all other groups ($p < 0.05$). Both anti-IFN- γ and anti-IL-4 inhibited LPS-induced priming for O_2^- release, indicating their potential to inhibit macrophage activation.

Peritoneal Macrophage TNF Release

As shown in Figure 4, IFN- γ and IL-4 were associated with significantly elevated mean cellular TNF release compared with the control group ($p < 0.05$) (Fig. 4). Anti-IFN- γ significantly inhibited mean cellular TNF release from PMØ compared with the isotype control ($p < 0.01$). Anti-IL-4 failed to downregulate *in vivo* LPS-induced mean cellular TNF release.

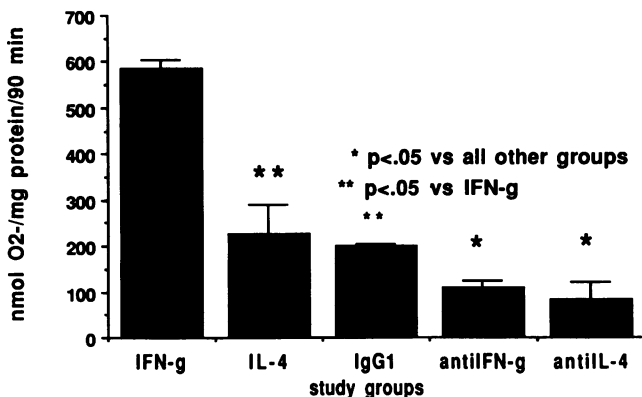


FIG. 3. Bar graph of superoxide anion production (O_2^-) by peritoneal macrophages from each of the study groups ($n = 3-5$ /group) after *in vivo* priming with LPS (60 mg/kg) for 6 hours and *in vitro* stimulation with PMA (1 μ g/ml) for 90 min, after 2 hours of macrophage adherence.

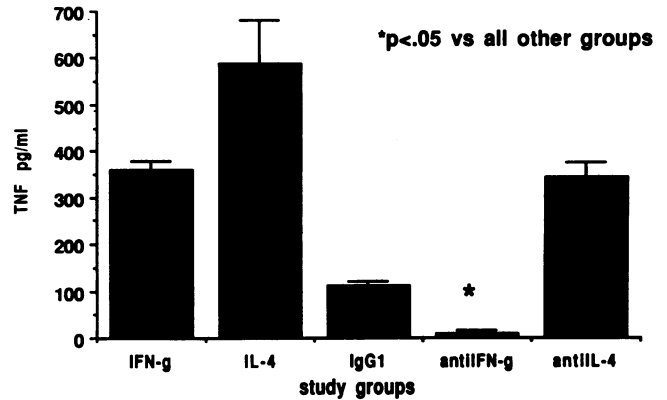


FIG. 4. Bar graph of TNF release by peritoneal macrophages (mean \pm SD) from each of the study groups ($n = 3-5$ mice/group) after *in vivo* LPS challenge (60 mg/kg) for 6 hours, and *in vitro* LPS stimulation (1 μ g/ml) for 2 hours, after 2 hours of macrophage adherence.

Serum TNF Levels

As shown in Figure 5, IFN- γ administered simultaneously with LPS was associated with significantly higher mean serum TNF levels compared with all other groups ($p < 0.05$), indicating an apparent synergy with LPS (Fig. 5). Lowest levels of mean serum TNF were found in mice treated with the isotype antibody.

Peritoneal Macrophage IL-6 Release and Serum IL-6 Levels

Mean LPS-induced macrophage IL-6 release was not significantly different among study groups (Fig. 6). The highest mean serum IL-6 levels, however, were found in IFN- γ and IL-4 treatment groups (Fig. 7). Treatment with anti-IL-4 was associated with significantly lower mean serum IL-6 levels compared with all other groups ($p < 0.01$).

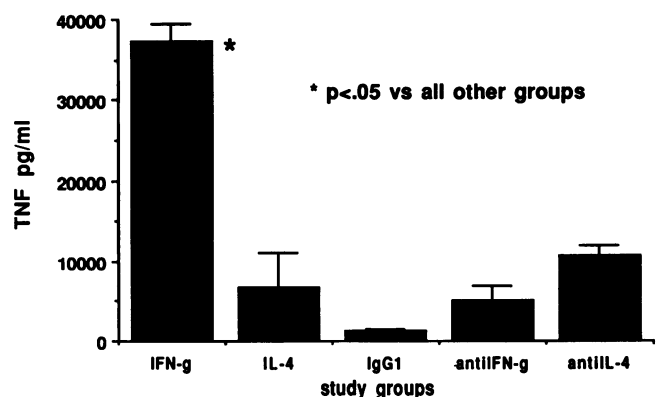


FIG. 5. Graph showing serum TNF levels (mean \pm SD) for each study group. Serum was harvested ($n = 3$ mice/group) 2 hours after simultaneous LPS challenge (60 mg/kg) and cytokine/monoclonal antibody administration.

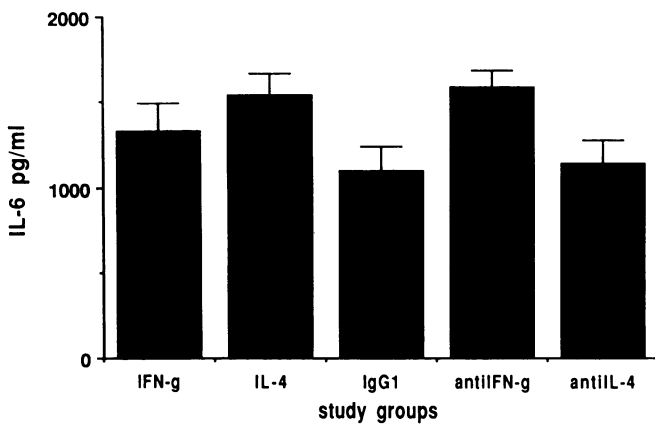


FIG. 6. Bar graph of IL-6 release by peritoneal macrophages (mean \pm SD) from each of the study groups ($n = 3-5$ mice/group) after *in vivo* LPS challenge (60 mg/kg) for 6 hours, and *in vitro* LPS stimulation (100 ng/ml) for 12 hours, after 2 hours of macrophage adherence.

Simultaneous LPS Administration and Cytokine/MAb Administration

Endotoxin administration was associated with only a 27% survival in the control MAb study group (Fig. 8). Simultaneous administration of either IFN- γ or IL-4 with LPS resulted in less survival (10% and 13%) compared with the control group. Anti-IFN- γ treatment significantly enhanced survival to 93% compared with results in all other groups ($p < 0.001$). Anti-IL-4 conferred only a slight survival advantage (27% vs. 47%) compared with the control isotype antibody.

LPS and Delayed (2-hour) IFN- γ /MAb Administration

Administration of the IgG₁ isotype control antibody 2 hours after LPS challenge was associated with a survival rate of 33%, which was similar to that obtained when this antibody was administered simultaneously with LPS (Fig.

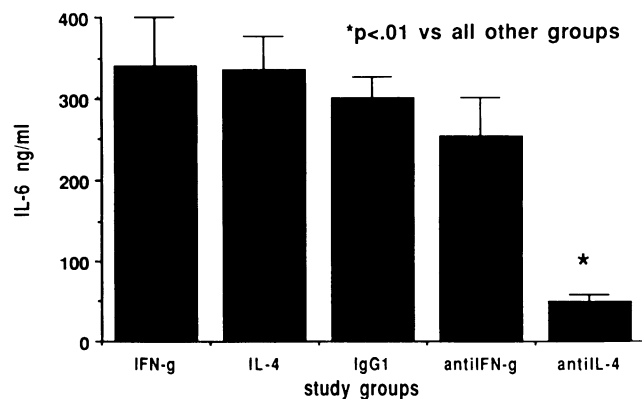


FIG. 7. Graph showing serum IL-6 levels (mean \pm SD) for each study group. Serum was harvested ($n = 3$ mice/group) 4 hours after simultaneous LPS challenge (60 mg/kg) and cytokine/monoclonal antibody administration.

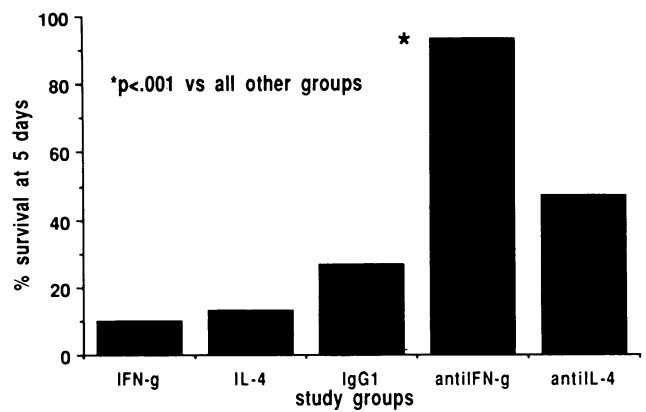


FIG. 8. Bar graph of survival after 5 days in each of the study groups after simultaneous LPS challenge (60 mg/kg) and cytokine/monoclonal antibody administration.

9). Despite a 2-hour treatment delay, anti-IFN- γ conferred a significant survival advantage (100%), compared with either IFN- γ (47%) or the control IgG₁ antibody (33%). Administration of IFN- γ 2 hours after LPS was less lethal than simultaneous injection (47% vs. 10%)

Discussion

The pathophysiology of the septic response to endotoxemia and bacterial infection is based in part on the inflammatory mediators released by M ϕ in various states of activation. Tumor necrosis factor, IL-1, IL-6, platelet-activating factor, complement, eicosanoids, and reactive oxygen intermediates all have been implicated in inducing the deleterious effects that are associated with the host's acute septic response. Aggressive use of antimicrobial agents may combat bacteremia, but often falls short of ameliorating adverse responses of the host during sepsis. Use of monoclonal antibodies specifically directed against key inflammatory mediators, including LPS, TNF, IL-6, and IL-1R has proven to be a major development in therapy aimed at modulating the acute septic response.^{12,15,16,25} This study provides evidence that endotoxemia is associated with M ϕ activation, and that administration of cytokines with *in vitro* M ϕ activating properties (IFN- γ and IL-4), in association with LPS results in even higher states of macrophage activation, as evidenced by significantly higher peritoneal macrophage mean O₂⁻ generation, mean TNF release, and mean serum TNF levels (IFN- γ only). Administration of monoclonal antibodies aimed at specifically blocking each of these M ϕ activating factors was beneficial in inhibiting release of one or more of these cytokines, indicating the importance of IFN- γ and IL-4 as M ϕ activating cytokines during endotoxemia. Furthermore, anti-IFN- γ significantly enhanced survival from lethal endotoxin challenge through modulation of M ϕ activation state.

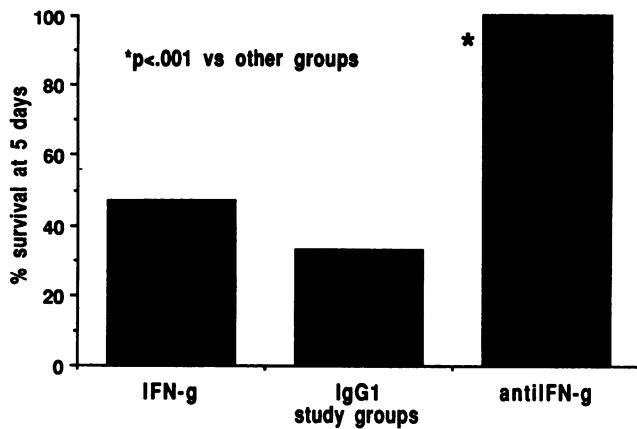


FIG. 9. Bar graph of survival 5 days after LPS challenge (60 mg/kg) with delayed treatment (2 hours) administration, consisting of either interferon- γ , IgG1 isotype control, or anti-interferon- γ study groups.

Interferon- γ is a T cell lymphokine, released during M ϕ -T-cell interaction. It has a broad range of functions and is primarily recognized for its potent capacity to activate M ϕ for enhanced microbicidal activity, including oxygen free radical production, phagocytosis, and killing of intracellular pathogens.²⁶ A number of studies have examined the potential benefit of IFN- γ administration in various disease states. In a rodent model of hemorrhagic shock, Livingston and Melangoni²⁷ demonstrated a reduction in abscess size and bacterial count using a combination of IFN- γ and cefamandole, indicating the ability of IFN- γ to restore host immune function. Hershmann et al.²⁸ showed that IFN- γ was beneficial in a model simulating bacterial infection after trauma. Subsequent studies from this laboratory, however, demonstrated a detrimental effect when IFN- γ treatment was instigated concomitant with cecal ligation and puncture.²⁹ Heremans et al.³⁰ have analyzed the endogenous role of IFN- γ in the generalized Schwartzman reaction. A good correlation was found between levels of interferon and mortality. Treatment with IFN- γ rendered mice more susceptible to the reaction, and anti-IFN- γ administration was protective. Heinzel³¹ has demonstrated a deleterious role for IFN- γ in the pathogenesis of experimental endotoxemia.

In this study administration of IFN- γ was not protective during endotoxemia whether given simultaneously or 2 hours after the administration of LPS. Moreover IFN- γ was associated with a higher mortality when given at the same time as LPS, compared with the control group (10% vs. 27%). Interestingly this deleterious effect was not seen when IFN- γ treatment was delayed for 2 hours after LPS administration. Administration of anti-IFN- γ was highly protective after lethal endotoxin challenge, whether given at the same time as LPS or as a delayed treatment regimen. These findings indicate that although IFN- γ may be beneficial as an immunostimulant in immunosuppressive disease states, during endotoxemia IFN- γ is a pathogenic

mediator. Thus IFN- γ may have a therapeutic role when *in vivo* M ϕ antimicrobial function is impaired, but it may be harmful when given during the acute septic response when M ϕ s appear to be in an activated state.

Interleukin-4 (IL-4) is a T-cell-derived glycoprotein that was originally characterized a B cell stimulatory factor-1.³² Functions of IL-4 on human monocytes and mouse macrophages also have been reported. Interleukin-4 has been shown to increase antigen-presenting ability, surface 1a expression, and tumoricidal activity in mouse macrophages.^{33,34} Preliminary studies from our laboratory indicate that IL-4 can prime M ϕ for enhanced release of reactive oxygen species.²¹ Little is known regarding the *in vivo* antimicrobial potential of this cytokine. McBride et al.³⁵ failed to demonstrate a protective effect when IL-4 was administered before a lethal dose of either LPS or IL-2. We also have failed to identify any beneficial effect when this cytokine was administered concurrent with LPS. Administration of anti-IL-4, however, was associated with a slightly greater survival after LPS, compared with controls (27% vs. 47%) and was associated with significantly greater survival compared with the IL-4 group (13%).

The role of M ϕ -derived antimicrobial mediators during the acute septic response has been well documented. Previous studies have attempted to correlate serum cytokine levels with survival after sepsis. In studies comparing levels of serum cytokines, TNF appears to most closely correlate with ultimate outcome.^{5,36} Serum cytokine levels do not appear to be as predictive as other physiologic parameters, however.⁵ Our study attempted to correlate cellular production and serum levels of these mediators with survival. In animals treated with IFN- γ , the highest macrophage mean O₂⁻ production and mean serum TNF levels were identified and were associated with greatest mortality. Treatment with IL-4 also was associated with elevated mean O₂⁻ and significantly elevated cellular TNF release. Anti-IFN- γ significantly downregulated mean O₂⁻ and cellular TNF release, but not mean serum TNF levels. In contrast to TNF, mean cellular IL-6 release was similar between groups; however anti-IL-4 significantly downregulated mean serum IL-6 levels, a potential mechanism by which this monoclonal antibody may be beneficial. Overall peritoneal macrophage production of O₂⁻ most closely correlated with eventual outcome. These findings add further evidence to the role of the activated M ϕ in mediating the deleterious effects of LPS.

The consequences of septic shock remain a serious therapeutic dilemma. Treatment of the septic source, antimicrobial agents, and supportive care are vital but do not offer complete protection against the often lethal consequences of cellular immune responses to infection. Use of monoclonal antibodies aimed at modulating the degree of macrophage activation, possibly combined with antimicrobial agents, offer a novel treatment strategy in the septic host.

Acknowledgment

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DISCUSSION

DR. JOHN MANNICK (Boston, Massachusetts): I think the key question is, what is the take-home message for the treatment of seriously injured patients from this study? Unfortunately I am uncertain about that.

The problem, it seems to me, is that the kind of critically injured patient that one has to deal with in the intensive care unit probably has not got the profile of cytokine production that is seen in the model that

Dr. Daly has described to you, which is a model produced by a lethal or near-lethal injection of endotoxin as a bolus.

What we generally see in the critically injured patient is a situation in which there is a massive and continual hypersecretion of the pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF), presumably by monocytes and macrophages, although possibly also by polymorphonuclear leukocytes. But what one sees on the other