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Differential Effects of Monoclonal Antibodies to Tumor Necrosis Factor Alpha and Gamma Interferon on Induction of Hepatic Nitric Oxide Synthase in Experimental Gram-Negative Sepsis

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To investigate the stimuli required for the induction of nitric oxide synthase (NOS) in sepsis, we have analyzed the levels of this enzyme in the livers of mice infected with a 90% lethal dose of *Escherichia coli* in a model of gram-negative sepsis. Hepatic NOS levels are markedly induced in this model, with peak values occurring 12 to 22 h following infection. Treatment with TN3-19.12, a neutralizing monoclonal antibody to tumor necrosis factor alpha (TNF-α), resulted in complete protection from death in this model of sepsis but had no significant effect on the level of induction of hepatic NOS. Treatment with H22, a monoclonal antibody to gamma interferon (IFN-γ), also gave significant protection against death and, in addition, did lead to a decrease in the level of induction of the hepatic NOS. Treatment of mice with pure TNF-α (0.2 μg), IFN-γ (2,000 U), or a combination of the two did not induce the hepatic NOS, but treatment with the combination led to significant mortality (probability of survival at 22 h, 0.32). Thus, the level of induction of NOS within the liver either in sepsis or by the coadministration of TNF-α and IFN-γ does not correlate with death.

Infection with gram-negative organisms results in increased production of a number of cytokines, an effect largely mediated by the endotoxin component of the gram-negative cell wall (for a review, see reference 12). It has become apparent that these cytokines are responsible for many of the deleterious effects seen in sepsis. A central mediator of endotoxin's lethality is tumor necrosis factor alpha (TNF-α), which can reproduce many of the features of septic shock (36). Moreover, levels of circulating TNF-α correlate with poor outcome in sepsis (39), and monoclonal antibodies to this cytokine protect animals in several models of gram-negative infection (30, 37). However, high levels of circulating TNF-α alone do not inevitably lead to death, indicating that TNF-α is necessary but not sufficient for mediating the lethality of endotoxin (29). Other cytokines which participate in the pathogenesis of septic shock include interleukin-1 (IL-1) and gamma interferon (IFN-γ), which act synergistically with TNF-α. A natural antagonist of IL-1 action on its receptor, the IL-1 receptor antagonist (IL-1 ra), and monoclonal antibodies to IFN-γ have both been found protective in animal models of sepsis (13, 23, 31).

The hypotension characteristic of septic shock is now believed to be mediated at least in part by excess production of the vasodilator nitric oxide (NO), which is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (for a review, see reference 21). NOS exists broadly in two main isoforms: a constitutive form found in brain and endothelial cells and an inducible form produced in response to treatment with various combinations of endotoxin, IFN-γ, TNF-α, and IL-1 (21). Originally, the inducible NOS was found in macrophages activated with endotoxin and IFN-γ, in which the NO formed mediates killing of intracellular parasites such as leishmanias and inhibition of the metabolism of tumor cell targets (15, 20). This enzyme has now been found in a variety of other activated cells, such as cytokine-treated endothelial cells, vascular smooth muscle cells, and hepatocytes (5, 7, 8, 17). In addition, NOS is markedly induced in the liver and lungs of animals treated with endotoxin (19). It has been suggested that while the constitutive production of NO is important in maintaining resting vascular tone, the excess production of NO formed by induction of NOS in sepsis produces profound hypotension, refractory to vasoconstrictor therapy. By using an inhibitor of NOS, N^G-monomethyl-L-arginine (L-NMA), it has been shown that the hypotension produced by endotoxin and by TNF-α is indeed due to excess NO production (18, 35). However, under certain conditions, treatment with L-NMA can lead to exacerbation of hypotension and accelerated death rather than protection, suggesting that a background level of NO production is necessary to maintain vascular patency to essential organs (40). Moreover, in hepatocytes, inhibition of NO production increases cellular damage induced by endotoxin (1), so there may be a protective role for NO in this case.

The liver sequesters the majority of circulating endotoxin (32) and, because of its size, is potentially a major contributor to overall NO synthesis. Recently, we have purified the inducible hepatic NOS from endotoxin-treated rats and found that it differs in biochemical properties from the other isoforms of NOS described to date (10). In the present study, therefore, we wished to determine whether the levels of NOS in liver were increased in mice which had *Escherichia coli* sepsis or which had received injections of pure TNF-α and IFN-γ. In addition, we have studied the effects on hepatic NOS levels of treatment with monoclonal antibodies to TNF-α and IFN-γ, both of which are effective in protecting against death in experimental *E. coli* sepsis, to ascertain whether their protective effects are accompanied by inhibition of the induction of this enzyme.

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MATERIALS AND METHODS

Animals. Six- to eight-week-old male CD1 mice weighing 25 to 30 g (Charles River, Margate, United Kingdom) were used for all experiments. They were fed LabSure PRD (Biosure; Cambridge, United Kingdom) and water ad libitum.

Bacteria. *E. coli* O111:B4 was obtained from B. Appelmelk, Amsterdam, Netherlands. Fresh aliquots stored at −70°C were used for all experiments.

Antibodies. Hamster monoclonal antibodies to recombinant murine TNF-α (TN3-19.12) and recombinant murine IFN-γ (H22) were a gift from Celltech, Berkshire, United Kingdom (26, 28). They were supplied as purified immunoglobulins suspended in isotonic phosphate-buffered saline. TN3-19.12 and H22 were administered at doses of 500 and 350 μg per mouse, respectively. Endotoxin levels of antibodies diluted for injection were determined by a chromogenic *Limulus* amoebocyte assay (6); both antibodies contained less than 50 pg of endotoxin per injected dose.

Cytokines. Recombinant murine TNF-α and IFN-γ were the kind gift of J. Dhillon (Royal Postgraduate Medical School). Both cytokines contained less than 10 pg of endotoxin per injected dose as measured by a chromogenic *Limulus* amoebocyte assay (6).

Biochemical reagents. L-[2,3,4,5-^{3}H]arginine monohydrochloride (35 to 70 Ci mmol^{-1}) was obtained from Amersham International, Amersham, United Kingdom. All other biochemicals were from Sigma, Dorset, United Kingdom.

Animal model of gram-negative sepsis. The animal model of gram-negative sepsis has been described in full elsewhere (30). Briefly, mice were injected intravenously with a 90% lethal dose (LD_{90}) of *E. coli* O111:B4 (2.7×10^8 CFU per animal) and then were treated with gentamicin (David Bull Laboratories, Warwick, United Kingdom) at a dosage of 2 mg kg of body weight^{-1} day^{-1} given as two divided doses starting at 2 h after bacterial challenge. The MIC of gentamicin for this organism is 1.25 mg liter^{-1}. Groups of mice received intravenous injections of 0.3 ml of monoclonal antibodies or pyrogen-free normal saline 1 h before challenge and were then monitored until death or for 48 h, after which no further deaths occurred. Injection of an irrelevant hamster monoclonal antibody in this model has been shown to produce no change in mortality (30). Blood used for measuring TNF levels was taken from the cut tip of the tail 90 min after infection.

Experimental design. We wished to determine hepatic NOS levels at various times after infection as well as the associated mortality in both the control and the monoclonal antibody-treated groups of animals for each experiment. The same bacterial suspension was used to infect a control group and an anticytokine-treated group, so that differences in mortality and hepatic NOS levels depended only on the treatment given. Hepatic NOS levels were determined at various times after infection by sacrificing at random two or three animals from each group and removing their livers for subsequent enzyme assay. The probability of survival in each group of mice was determined by the Kaplan-Meier technique, which treats the results for animals removed from the experiment for NOS assay as censored data, and this is shown on the survival probability curves. The number of mice in each group was such that the effective sample size, which is the size of the total sample minus the number of censored animals, was always greater than six at 48 h after infection.

Hepatic NOS levels. Mice were randomly selected from each treatment group at various times after infection for the assay of hepatic NOS. Animals were killed by cervical dislocation, and the livers were removed and frozen immediately in liquid nitrogen. All subsequent procedures were performed at 4°C. The livers were thawed and homogenized in 5 volumes of ice-cold buffer A (0.32 M sucrose, 50 mM Tris-Cl [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol, 10 μg of soybean trypsin inhibitor per ml, 1 μg of antipain per ml, 1 μg of pepstatin per ml, 1 μg of aprotonin per ml, and 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 100,000 × *g* for 60 min, the lipoprotein layer was discarded, and the supernatants were removed for the NOS assay, which was performed within 1 h of preparation. NOS activity was assayed by the conversion of radiolabelled arginine to citrulline as described elsewhere (4). Citrulline is produced with equimolar amounts of nitric oxide. Reaction buffer contained 50 mM Tris-Cl [pH 7.4], 1 mM NADPH, 1 mM dithiothreitol, and 50 μM tetrahydrobiopterin. An assay mix was composed of 100 μl of reaction buffer, 25 μl of 100 nM radiolabelled arginine, and 10 μl of the enzyme fraction to be tested. In addition, endogenous arginase and other urea cycle enzymes were inhibited by including 50 mM valine, 1 mM ornithine, and 1 mM citrulline in the reaction buffer as described elsewhere (19). Reactions were for 15 min at 37°C; reaction rates were linear with respect to time and the amount of added enzyme for the conditions specified. All reaction rates are for the conditions described here. The specificity of this assay for hepatic NOS has been demonstrated before (19). We have independently repeated the following measures of specificity. (i) After removal of endogenous NADPH by ion-exchange chromatography, the reaction is entirely dependent on added NADPH, as expected for NOS (21). (ii) The reaction is completely inhibited by 0.4 mM L-NMA but not by D-NMA, confirming the expected stereospecificity of NOS.

TNF-α assay. TNF-α levels in blood were examined by enzyme-linked immunosorbent assay (ELISA) as described previously (30). This assay has a lower limit of sensitivity (0.05 ng ml^{-1}). We have shown previously that the mean concordance of samples from the serum of experimental animals examined by ELISA and bioassay with L929 cells was 92% (30).

Statistical analysis. Survival data were calculated by using the Kaplan-Meier technique and compared by using the log rank test. The differences in NOS levels over time in control and treated animals were compared by a two-way analysis of variance with interaction by using the Minitab computer program. The data were tested for fit to the normal distribution by the Shapiro-Francia test (27).

RESULTS

Hepatic NOS is induced in gram-negative sepsis. In order to determine whether hepatic NOS is induced in gram-negative sepsis, we infected a group of mice ($n = 20$) with an LD_{90} of *E. coli*. In this model of sepsis, death occurs 12 to 24 h after infection, with very few animals dying after 24 h. At various times after inoculation, groups of animals were sacrificed, their livers were removed, and the hepatic NOS levels were measured. The results of a typical experiment are shown in Fig. 1A. In uninfected animals, the level of hepatic NOS is barely detectable, with the lower limit of detection of this assay being approximately 2.5 amol of citrulline formed min^{-1} μg^{-1}. After infection, the levels rise, reaching a maximum 12 to 22 h after infection before falling towards the unstimulated levels by 28 h. In a separate experiment with

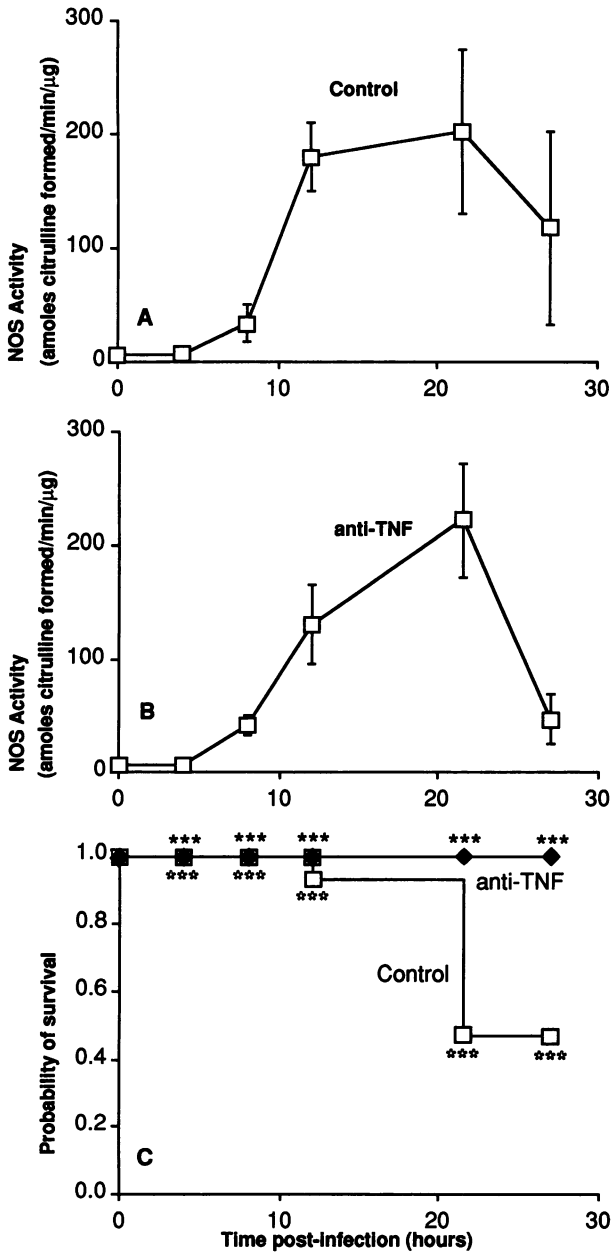

FIG. 1. Hepatic NOS activity and associated mortality in mice infected with *E. coli* treated with saline (control) or anti-TNF-α monoclonal antibody TN3-19.12. Hepatic NOS levels over time after infection are shown for control animals (n = 20) (A) and for animals treated with TN3-19.12 (n = 20) (B). Each point represents the mean of values from the livers of three separate animals. Bars indicate 1 standard error of the mean. (C) Kaplan-Meier survival probabilities for both groups. The data contain censored observations, as the animals sacrificed for the enzyme assay have unknown mortality outcomes. ☆ and ★, results for censored animals in the control and the anti-TNF-α-treated groups, respectively.

another group of infected animals which were not sampled at other time points, enough animals survived to 48 h after infection to allow their hepatic NOS levels to be measured. This examination showed that the enzyme levels had returned to the levels seen in animals prior to infection (data not shown). This time course of induction of hepatic NOS was extremely consistent and has been repeated in five separate experiments with the same results. The associated survival curve for the group of animals whose NOS levels are presented in Fig. 1A is shown in Fig. 1C. The probability of survival at 28 h after infection was 0.42.

Hepatic NOS levels in septic animals treated with anti-TNF-α. We wished to determine whether the known protective effect of anti-TNF-α antibody treatment in gram-negative sepsis correlated with an inhibition of induction of the hepatic NOS. In the experiment whose results are shown in Fig. 1A, a separate group of animals (n = 20) were treated 1 h before infection with a neutralizing monoclonal antibody to TNF-α (TN3-19.12). Groups of animals were sacrificed at various times after inoculation as before, and their hepatic NOS levels were determined. The results of this experiment are shown in Fig. 1B. As can be seen, the enzyme levels in this treated group of mice were indistinguishable from those of the control untreated group (no significant difference between the treatment groups as determined by a two-way analysis of variance with interaction). In contrast, the protective effect of this antibody was clearly demonstrated (Fig. 1C). Five animals remained in the anti-TNF-α-treated group after 28 h; no further deaths occurred in this group up to 48 h after infection, at which time the level of hepatic NOS was found to be the same as in the uninfected animals (data not shown).

Hepatic NOS levels in septic animals treated with anti-IFN-γ. IFN-γ is known to induce NOS in a number of different cells, and antibodies directed against this cytokine are protective against death in experimental gram-negative sepsis (31). Groups of mice were treated with saline (n = 27) or anti-IFN-γ (n = 21) 1 h prior to an intravenous injection of an LD_{90} of *E. coli*. The levels of their hepatic NOS following infection are shown in Fig. 2A. As before, the saline-treated animals showed a marked rise in enzyme levels, which peaked 12 to 22 h after infection. In the animals treated with anti-IFN-γ, however, levels of hepatic NOS were attenuated at all time points after 6 h. There was a significant difference between the two treatment groups over time as determined by a two-way analysis of variance with interaction (P < 0.01).

The associated survival curves for the two groups of animals in this experiment are shown in Fig. 2B. The anti-IFN-γ is not as effective as the anti-TNF-α at protecting against death, as has been observed in other experiments (31). However, the difference in the probability of survival between the two groups was highly significant (P < 0.001) as determined by the log rank test. Six animals remained in the anti-IFN-γ-treated group after 28 h; no further deaths occurred in this group at up to 48 h after infection, at which time their hepatic NOS levels were the same as those of uninfected animals (data not shown).

Effects of TNF-α and IFN-γ on hepatic NOS levels. In order to explore further the possible role of TNF-α and IFN-γ in the induction of hepatic NOS in the whole animal, we injected the purified recombinant cytokines both separately and together into groups of mice. At various times following this injection, animals were sacrificed and their hepatic NOS levels were determined. The results of this experiment are shown in Fig. 3A. As can be seen, neither TNF-α (0.2 μg per mouse) nor IFN-γ (2,000 U per mouse) alone produced any significant stimulation of NOS levels up to 24 h after injection. No deaths occurred in either of these two groups of cytokine-treated animals, as shown in Fig. 3B. When the same doses of TNF-α and IFN-γ were administered together, there was a striking synergistic effect; the mice

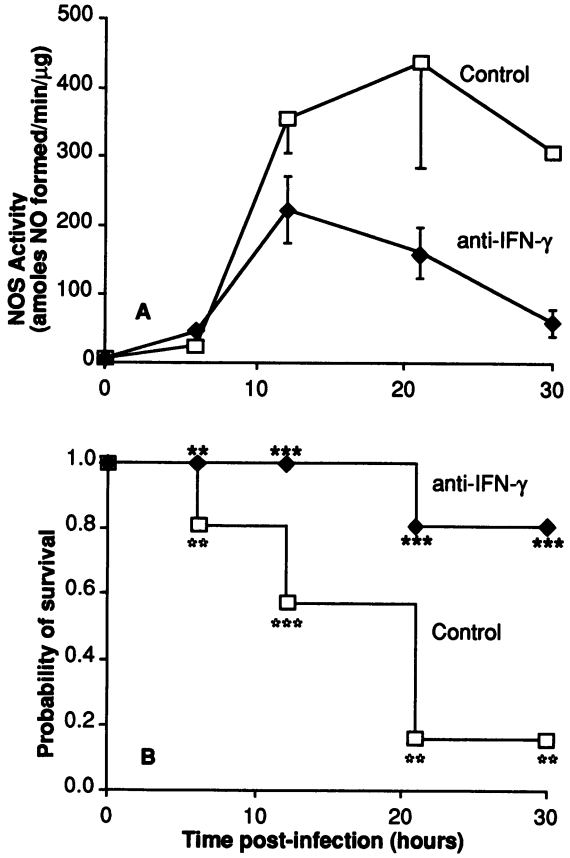

FIG. 2. Hepatic NOS activity and associated mortality in mice infected with *E. coli* treated with saline (control; $n = 27$) or anti-IFN-γ monoclonal antibody H22 ($n = 21$). (A) Hepatic NOS levels over time after infection for control animals and animals receiving H22. Each point represents the mean of values obtained for two or three animals. Bars indicate 1 standard error of the mean. (B) Kaplan-Meier survival curves for both groups. Censored data as described in the legend to Fig. 1 are shown for the control (☆) and anti-IFN-γ-treated (★) groups.

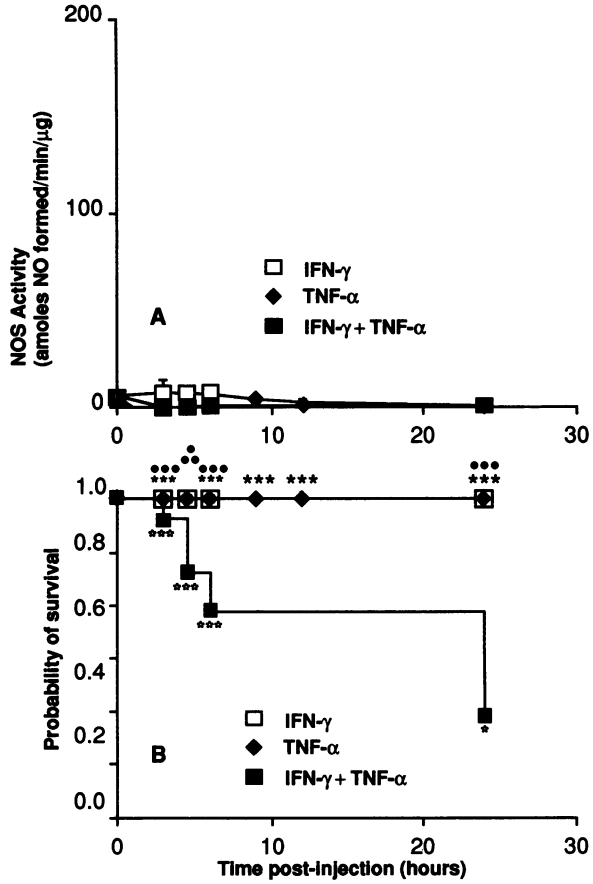

FIG. 3. Hepatic NOS levels in mice injected with TNF-α, IFN-γ, or a combination of both cytokines. (A) NOS levels with respect to time after the injection of 0.2 μg of TNF-α alone ($n = 15$), 2,000 U of IFN-γ ($n = 15$), or a combination of both cytokines ($n = 15$). (B) Kaplan-Meier survival curves for these treated animals, with censored data shown as for Fig. 1 for the TNF-α-treated group (★), the IFN-γ-treated group (●), and the combination-treated group (☆).

became ill within 1 h, showing marked lethargy and ruffled fur, and then deaths occurred, commencing at 3 h after injection (Fig. 3B). At 24 h after injection, the probability of survival in the group treated with the combination of TNF-α and IFN-γ was only 0.32. Despite this marked lethal effect, there was no detectable rise in hepatic NOS levels in these mice (Fig. 3A). In order to be sure that we had not missed an early rise in the hepatic NOS, we repeated this experiment with exactly the same doses of TNF-α and IFN-γ and took three livers for the assay of NOS 1 h after injection. This assay showed that there still was no increase in the hepatic NOS above the level seen in untreated animals at this time after injection (data not shown).

TNF-α levels in septic and cytokine-treated animals. We wished to compare the levels of TNF-α produced by the injection of the recombinant cytokine with those produced by infection with *E. coli* in the model of gram-negative sepsis used in these experiments. The time course of TNF-α release in this model has already been determined, with peak levels being observed at 90 min after injection of the bacteria (30). Levels at this time after infection are shown in Fig. 4 in comparison with the levels seen 30 min after injection of 0.2 μg of recombinant TNF-α. As can be seen, there is no significant difference in the peak levels of TNF-α in these two groups of mice. Also shown in Fig. 4 are the peak levels of TNF-α produced in the animals treated with the monoclonal anti-TNF-α antibody (TN3-19.12). As expected, this antibody reduces the immunoreactive TNF-α levels significantly ($P < 0.01$ as determined by Student's t test).

DISCUSSION

This study has demonstrated for the first time the induction of an NOS in animals challenged with live gram-negative bacteria. We have found that treatment with a monoclonal antibody to TNF-α does not result in any diminution of this induction, but that in contrast, a monoclonal antibody to IFN-γ attenuates the rise in hepatic NOS after infection; yet both antibody treatments were effective in significantly reducing the mortality seen in this model of sepsis.

The kinetics of NOS production differ for animals treated with endotoxin and those described here: the induction of NOS in the rat liver by endotoxin is maximal 6 h after injection, after which the levels decline rapidly to near unstimulated levels after 19 h, a more rapid and shorter-lived induction than seen in our experiments using live *E. coli* (19). The liver cell homogenates which we used to measure the

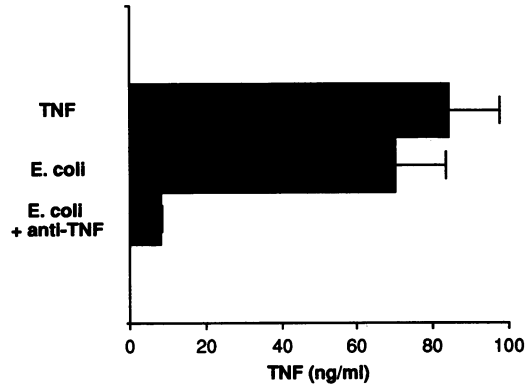

FIG. 4. TNF-α levels in septic and cytokine-treated animals as determined by ELISA. Results for animals injected with 0.2 μg of pure TNF-α (n = 5) 30 min after injection (TNF), animals with *E. coli* sepsis (n = 7) 90 min after infection (E. coli), and animals with *E. coli* sepsis treated with TN3-19.12 (n = 5) 90 min after infection (E. coli + anti-TNF) are shown. Bars indicate 1 standard error of the mean.

NOS activity are derived mainly from the hepatic parenchymal cells, which contribute about 90% of the total protein (38). However, there is a contribution from the nonparenchymal cell population, mostly from Kupffer cells, the fixed hepatic macrophages, which are also known to possess an inducible NOS (3). One group has found that the NOS activity 6 h after endotoxin challenge was localized predominantly in hepatocytes (19), although another group has found that endotoxin alone caused a minor increase in NOS activity in the nonparenchymal cell population (2). The exact requirements for induction of NOS have been found to differ, depending upon the cells studied. Primary hepatocytes in culture require a combination of TNF-α, IFN-γ, IL-1β, and endotoxin for maximal stimulation of NOS production (8). Vascular smooth muscle cells produce the inducible form of NOS on stimulation with IL-1β alone (5). Endothelial cells, however, require a combination of at least two stimuli, such as IFN-γ and TNF-α, for induction of NOS, as do macrophages (9, 17). Additional cytokines such as IL-1β and endotoxin itself can further increase the level of induction of NOS in most of these cell types, although macrophages are notable for the lack of effect of IL-1β in producing any further induction of NOS (9). A previous report has demonstrated that live *Mycobacterium bovis* BCG can induce NO production in peritoneal macrophages of infected mice (33).

The central role of TNF-α in the pathogenesis of septic shock is well established (12). It is known that in vitro, TNF-α contributes to the induction of NOS in a number of different cells and that the hypotension seen on infusion of this cytokine is due to excess production of NO (18). We found that the effects of a monoclonal antibody to TNF-α were full protection of mice from death due to *E. coli* sepsis but no effect on the production of hepatic NOS (Fig. 1). Not all circulating TNF-α is neutralized by this antibody, although clearly, at this dose the antibody is able to reduce the lethal effects of TNF-α. We do not know what effect the anti-TNF-α treatment has upon the induction of NOS in other tissues, in particular the vasculature, in which the local production of NO could produce vasodilatation and contribute to the hypotension of septic shock. However, certainly in vitro, TNF synergizes with other factors to produce increased production of NOS in hepatocytes, vascular smooth muscle, and endothelial cells (5, 17). It is possible that in sepsis, increased levels of a number of cytokines can effectively substitute for TNF-α, so that neutralization of its action does not inhibit the induction of NOS within the liver. A lower inoculum of bacteria would induce less TNF-α and might allow an effect of the anti-TNF-α on hepatic NOS levels to be seen. However, in this model, reducing the dose of bacteria lowers the level of induction of the hepatic NOS and sharply reduces mortality, making any intervention difficult to interpret (10a).

Anti-IFN-γ monoclonal antibodies did lead to a significant reduction in the level of hepatic NOS (Fig. 2). This reduction is specific to this antibody, as the injections of similar amounts of anti-TNF-α were without effect. Circulating IFN-γ levels are raised in a number of models of septic shock, typically rather later than the peak levels of TNF-α (14, 16). Antibodies to IFN-γ are protective against death in endotoxin-induced shock and also in models of live sepsis (13, 31). IFN-γ on its own does not produce the features of septic shock but synergizes very strongly with other cytokines, particularly with TNF-α (34). We have again demonstrated this strong synergy in producing death in mice injected with combinations of pure TNF-α and IFN-γ (Fig. 3). However, despite the production of a significant mortality, there was no demonstrable induction of hepatic NOS. Again, we do not know what effect this combination of cytokines would have on NOS in other tissues, but as discussed above, both of these cytokines stimulate the induction of NOS in a variety of different cell types. The levels of TNF-α achieved by injection of the pure cytokine were very similar to those seen in the model of gram-negative sepsis used in these experiments (Fig. 4), suggesting that we did inject sufficient TNF-α to reproduce the level seen in sepsis. In fact, the levels achieved are higher than expected from the dose of TNF-α injected and its half-life; we feel that this reflects the different source of TNF-α used to standardize the immunological assay. That animals can have these high levels of TNF-α and not die is further demonstration that while TNF-α is necessary for mediating death in sepsis, it is not sufficient by itself to cause death. The clear effect of anti-IFN-γ in reducing the induction of the hepatic NOS after infection contrasts with the lack of effect of this cytokine administered alone or in combination with TNF-α. One explanation may be that in sepsis, the induction of other cytokines, such as IL-1, as well as the presence of endotoxin provides other stimuli which may combine synergistically with IFN-γ to induce hepatic NOS; under these conditions, neutralization of IFN-γ with a monoclonal antibody would then counter this synergy, leading to inhibition of NOS induction.

What is the role of the hepatic NOS? In this study, we have demonstrated that the high levels induced within the liver in sepsis do not seem to correlate with death, as animals fully protected from death by anti-TNF-α have hepatic NOS levels that are as high as those of control animals. In vitro, the production of NO within hepatocytes leads to inhibition of protein synthesis (3). Thus, it might be expected that inhibition of NO production should lead to less hepatic damage. However, in mice sensitized to the effects of lipopolysaccharide by injection of killed *Corynebacterium parvum*, the inhibition of NO production by L-NMA, in fact, led to increased hepatic injury (1). This suggests that the NO formed within the liver may have some protective role in this case. Certainly, hepatic injury is a feature of the sepsis syndrome, contributing to the mortality of this condition

(25). Although not the prime aim of this study, the effects of the different anticytokines on hepatic function in sepsis are of interest. However, it is difficult to demonstrate a specific effect of antibody treatment on hepatic function, as the anti-TNF-α reduces the levels of ornithine carbamoyltransferase (a marker of hepatocellular damage) in serum compared with those in control animals, despite the two groups having the same levels of hepatic NOS (31a). Clearly, there are additional factors other than local NO production which contribute to hepatic damage.

The work described here has begun to define the important stimuli necessary for the induction of the hepatic NOS in vivo, as well as the effects of therapeutic intervention with monoclonal antibodies to TNF-α and IFN-γ which are being developed for possible clinical use. Nevertheless, it is apparent that the relationship between endotoxin-induced cytokines and stimulation of NOS is complex, and care will be needed in considering the clinical consequences of modifying cytokine release (11) or the inhibition of NOS (22, 24).

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