

## **The Protective Role of Endogenously Synthesized Nitric Oxide in Staphylococcal Enterotoxin B-induced Shock in Mice**

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### **Summary**

Nitric oxide (NO) synthesis during experimental endotoxemia has been shown to have both deleterious and beneficial effects. In the present study, we analyzed the *in vivo* production and the regulatory role of NO in the shock syndrome induced by staphylococcal enterotoxin B (SEB) in mice. First, we found that intraperitoneal administration of 100  $\mu$ g SEB in BALB/c mice induced a massive synthesis of NO as indicated by high serum levels of nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) peaking 16 h after SEB injection. The inhibition of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  release in mice injected with anti-tumor necrosis factor (TNF) and/or anti-interferon  $\gamma$  (IFN- $\gamma$ ) monoclonal antibody (mAb) before SEB challenge revealed that both cytokines were involved in SEB-induced NO overproduction. *In vitro* experiments indicated that NO synthase (NOS) inhibition by *N*-nitro-L-arginine methyl ester (L-NAME) enhanced IFN- $\gamma$  and TNF production by splenocytes in response to SEB. A similar effect was observed *in vivo* as treatment of mice with L-NAME resulted in increased IFN- $\gamma$  and TNF serum levels 24 h after SEB challenge, together with persistent expression of corresponding cytokine mRNA in spleen. The prolonged production of inflammatory cytokines in mice receiving L-NAME and SEB was associated with a 95% mortality rate within 96 h, whereas all mice survived injections of SEB or L-NAME alone. Both TNF and IFN- $\gamma$  were responsible for the lethality induced by SEB in L-NAME-treated mice as shown by the protection provided by simultaneous administration of anti-IFN- $\gamma$  and anti-TNF mAbs. We conclude the SEB induces NO synthesis *in vivo* and that endogenous NO has protective effects in this model of T cell-dependent shock by downregulating IFN- $\gamma$  and TNF production.

Nitric oxide (NO) overproduction is known to be involved in the pathogenesis of LPS (endotoxin)-induced arterial hypotension. Indeed, nitric oxide synthase (NOS) inhibitors were found to increase systemic vascular resistance in experimental endotoxemia (1) and in patients with septic shock (2). However, the therapeutic benefit of NOS inhibitors in severe sepsis is controversial as these inhibitors were also found to promote glomerular thrombosis and liver damage and to increase mortality rates in animals injected with LPS (3–5). These observations suggest that vasodilatation and inhibition of platelet aggregation and adhesion induced by NO might be critical to maintain adequate perfusion of vital organs during endotoxemia (6).

Whereas the toxicity of LPS from gram-negative bacteria is related to macrophage activation (7), staphylococcal enterotoxins exert their pathogenic effects by activating T cells expressing a given  $V\beta$  gene segment on their TCR (8). Thus, injection of staphylococcal enterotoxin B (SEB) in BALB/c

mice induces a shock syndrome due to the release of inflammatory cytokines by  $V\beta 8$ -positive T cells (9). The present study was undertaken to determine whether NO is produced and has a regulatory role in this model of T cell-dependent shock.

### **Materials and Methods**

*Mice.* 10-wk-old BALB/c mice purchased from Bantin and Kingman Ltd. (Grimston Aldbrough Hull, UK) were maintained in our animal facilities on standard laboratory chow.

*mAbs.* The R46A2 rat anti-mouse IFN- $\gamma$  IgG1 mAb (10) and LO-DNP-2, a control rat IgG1 mAb (kindly provided by Dr. H. Bazin, Experimental Immunology Unit, Université Catholique de Louvain, Belgium) were produced in ascites form. Purified TN3 19-12 hamster anti-mouse TNF IgG1 mAb and its isotype control MOPC21 (CB1) were generously provided by Cell Tech (Berkshire, UK).

*Nitrite/Nitrate ( $\text{NO}_2^-/\text{NO}_3^-$ ) Assay.* Serum samples were as-

sayed for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  (stable end products of NO) after reduction of  $\text{NO}_3^-$  into  $\text{NO}_2^-$  by copper-plated cadmium (11). Briefly, 50  $\mu\text{l}$  of each sample was first deproteinized by incubation with 200  $\mu\text{l}$   $\text{ZnSO}_4$  (75 mM) and 250  $\mu\text{l}$   $\text{NaOH}$  (55 mM) for 10 min at room temperature. After centrifugation at 1,000 g for 10 min, 200  $\mu\text{l}$  of supernatant and 200  $\mu\text{g}$  of activated cadmium were mixed together and stirred at room temperature for 1 h. Activated cadmium was prepared as follows: 5 g cadmium powder (100% mesh; Johnson Matthey, Karlsruhe, Germany) were first plated with copper by stirring in 20 ml of 5 mM  $\text{CuSO}_4$ . Excess metallic Cu was removed by extensive washing with glycine-NaOH buffer (pH 9.7). Copper-plated cadmium was then dried on filter paper and immediately used for the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . Reduced samples were incubated with an equal volume of Griess reagent and absorbance was measured (A450) on a microplate reader (Multiscan MCC/340; Labsystems, Helsinki, Finland).  $\text{NO}_2^-$  concentrations were calculated from a reduced  $\text{NaNO}_3$  standard curve ranging from 5 mM to 0.5  $\mu\text{M}$ . The lower limit of detection of  $\text{NO}_3^-$  in this test was 50  $\mu\text{M}$ .

**Determination of Cytokine Levels by ELISA.** Serum samples were assayed for TNF by two-site ELISA using the TN3 9-12 mAb and rabbit anti-mouse TNF polyclonal Ab kindly provided by Dr. W. Buurman (University of Limburg, Maastricht, The Netherlands) (12).  $\text{IFN-}\gamma$  was also quantitated by two-site ELISA using the  $\text{F}_1$  and Db-1 rat anti-mouse  $\text{IFN-}\gamma$  mAbs, kindly provided by Dr. Billiau (Katholieke Universiteit Leuven, Leuven, Belgium) and P. H. van der Meide (TNO Health Research, Amsterdam, The Netherlands), respectively (13). The lower limits of detection of TNF and  $\text{IFN-}\gamma$  were 20 and 2 U/ml, respectively.

**Analysis of TNF- $\alpha$  and IFN- $\gamma$  mRNA Expression.** Spleens were removed 2 and 24 h after injection of SEB or SEB plus *N*-nitro-L-arginine methyl ester (*L*-NAME; Sigma Chemical Co., St. Louis, MO). Total RNA was extracted using the guanidium thiocyanate method. Preparations of cDNA and PCR for TNF- $\alpha$  and  $\text{IFN-}\gamma$  genes and for hypoxanthine phosphoribosyl transferase (HPRT) housekeeping gene were performed using standard procedures (14). PCR primers used were as follows: TNF- $\alpha$  sense primer 5'-TCTCATCAGTTCTATGGCCC-3' and antisense 5'-GGGAGT-AGACAAGGTACAAC-3';  $\text{IFN-}\gamma$  sense primer 5'-GCTCTGAG-ACAATGAACGCT-3' and antisense 5'-AAAGAGATAATCTGGC-TCTGC-3'; HPRT sense primer 5'-GTTGGATACAGGCCAGAC-TTTTGTG-3' and antisense 5'-GATTCAACTTGCGCTCATC-TTAGGC-3'. Reactions were incubated in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 29 cycles. PCR products were run on a 2% agarose gel and stained with ethidium bromide.

**In Vivo Administration of SEB and NOS Inhibitor.** First, we measured  $\text{NO}_2^-/\text{NO}_3^-$  serum levels at different time points after a single injection of 100  $\mu\text{g}$  i.p. SEB dissolved in 100  $\mu\text{l}$  RPMI (Gibco, Paisley, UK). In experiments designed to analyze the role of NO in vivo, mice received injections of 2 mg i.p. of *L*-NAME, a competitive inhibitor of NOS, 30 min before, simultaneously with, and 2, 4, and 6 h after SEB injection. This protocol of *L*-NAME administration was adapted from that of Shultz and Raji (3). To determine the role of TNF and  $\text{IFN-}\gamma$  in the induction of NO and in the lethality of mice coinjected with SEB and *L*-NAME, mice were pretreated with 500  $\mu\text{g}$  i.p. of either anti- $\text{IFN-}\gamma$  mAb, anti-TNF mAb, or both mAbs, 2 h before SEB challenge. As controls, mice were injected with the isotopic controls of anti- $\text{IFN-}\gamma$  and anti-TNF mAb. Results obtained after injection of the two control mAbs were pooled since they did not differ significantly. The endotoxin levels of SEB, *L*-NAME, and mAb preparations were <15 pg/ml as determined by a *Limulus* amoebocyte lysate assay (LAL-QCL-1000; Whittaker MA, Bioproducts, Walkersville, MD).

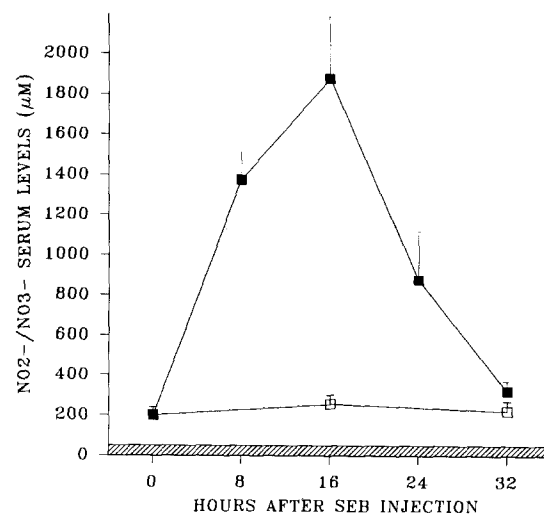
**In Vitro Studies.** After lysis of red cells,  $5 \times 10^6$  spleen cells from normal BALB/c mice were cultured in duplicates in 0.5 ml complete medium consisting of RPMI 1640 supplemented with 2% Ultrosor (Gibco), 1% sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids, penicillin, streptomycin, and  $5 \times 10^{-5}$  M 2-M. SEB at a concentration of 10  $\mu\text{g}/\text{ml}$  was added in experimental wells together with increasing concentrations (0.25–2 mg/ml) of *L*-NAME. After 4 d of incubation in 6%  $\text{CO}_2$  in humidified air, supernatants were collected and assayed for TNF and  $\text{IFN-}\gamma$  production.

**Statistical Analysis.** Statistical comparisons were made using the unpaired two-tailed Mann-Whitney test except for lethality data which were analyzed by the two-tailed Log-Rank test.

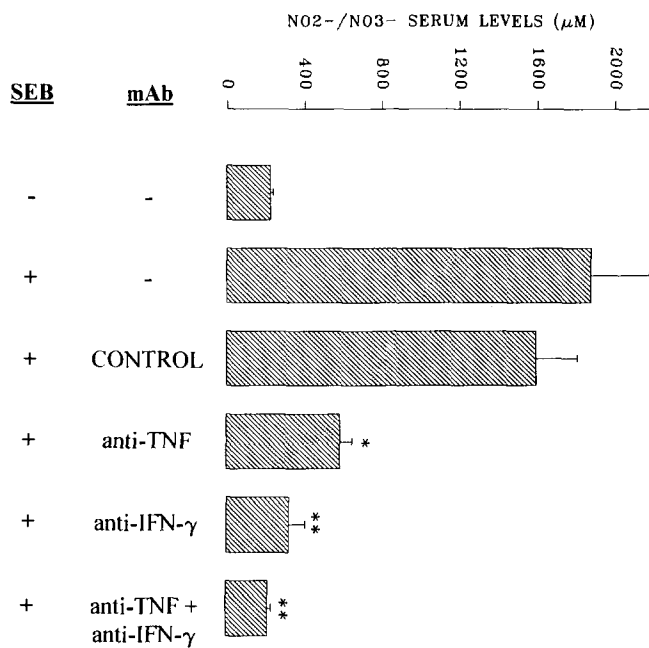
## Results and Discussion

**Systemic Release of NO after SEB Injection in BALB/c Mice.** We first established that injection of 100  $\mu\text{g}$  i.p. SEB in BALB/c mice induces a massive production of NO metabolites. Indeed,  $\text{NO}_2^-/\text{NO}_3^-$  serum levels rose from  $200 \pm 40 \mu\text{M}$  (mean  $\pm$  SEM) before SEB injection to  $1,876 \pm 305 \mu\text{M}$  16 h later while they were not influenced by injection of medium alone (Fig. 1).

**TNF and  $\text{IFN-}\gamma$  Mediate SEB-induced NO Overproduction.** TNF and  $\text{IFN-}\gamma$  are released after SEB injection (9, 15) and both cytokines are known to induce NOS (16, 17). We therefore studied the respective roles of TNF and  $\text{IFN-}\gamma$  in the in vivo production of  $\text{NO}_2^-/\text{NO}_3^-$  in SEB-challenged mice. For this purpose, mice were pretreated with either anti-TNF, anti- $\text{IFN-}\gamma$ , or both mAbs 2 h before injection of 100  $\mu\text{g}$  of SEB, and peak serum levels of  $\text{NO}_2^-/\text{NO}_3^-$  were determined 16 h later. In preliminary experiments, we ascertained that the injected amounts of anti- $\text{IFN-}\gamma$  and anti-TNF mAb efficiently neutralized corresponding cytokines in the circulation of SEB-injected mice (data not shown). As shown in Fig. 2, anti-TNF mAb pretreatment



**Figure 1.** Serum levels of  $\text{NO}_2^-/\text{NO}_3^-$  after a single injection of 100  $\mu\text{g}$  i.p. SEB in BALB/c mice (■). Controls (□) received RPMI medium alone. Results are represented as mean  $\pm$  SEM of at least five mice for each time point. (Hatched area) Detection limit of  $\text{NO}_2^-/\text{NO}_3^-$ .



**Figure 2.** Involvement of TNF and IFN- $\gamma$  in SEB-induced NO overproduction. Mice were treated before SEB challenge (100  $\mu$ g i.p.) with either anti-TNF mAb, anti-IFN- $\gamma$  mAb, or anti-TNF plus anti-IFN- $\gamma$  mAbs, as described in Materials and Methods. The control mAb group included five mice injected with LO-DNP-2 rat mAb and five mice injected with CB1 hamster mAb. NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> peak serum levels were measured 16 h after SEB injection and are represented as mean  $\pm$  SEM of at least five mice in each group. (\*)  $p < 0.02$ ; (\*\*)  $p < 0.01$ , as compared with mice injected with control mAb and SEB.

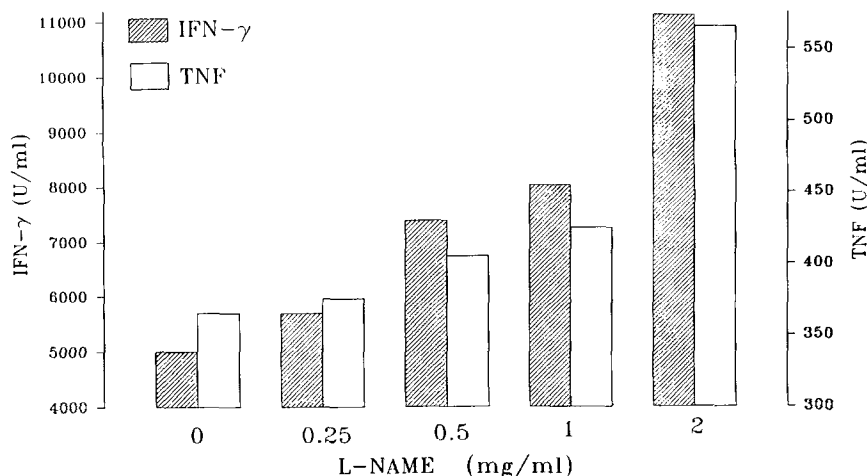
reduced by 63% peak serum levels of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> (585  $\pm$  64 vs. 1,594  $\pm$  213  $\mu$ M in mice pretreated with control mAb,  $p < 0.02$ ). Anti-IFN- $\gamma$  mAb pretreatment was even more efficient since it reduced peak NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels to 321  $\pm$  83  $\mu$ M ( $p < 0.01$  as compared with mice pretreated with control mAb). Coinjection of anti-IFN- $\gamma$  and anti-TNF mAbs before SEB completely prevented the increase in

NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels. These in vivo data are in keeping with the in vitro observations indicating that IFN- $\gamma$  and TNF interact synergistically to induce NO synthesis by macrophages and hepatocytes (18, 19).

*Inhibition of NO Synthesis Enhances IFN- $\gamma$  and TNF Synthesis and Induces Lethality in SEB-injected Mice.* After the demonstration that IFN- $\gamma$  and TNF were responsible for NO overproduction, we aimed to determine whether NO would in turn control the synthesis of those cytokines. This question was first addressed in vitro by analyzing the effect of L-NAME, a NOS inhibitor, on the secretion of cytokines by spleen cells stimulated with SEB. As shown in Fig. 3, the addition of L-NAME enhanced in a dose-dependent manner the secretion of TNF and IFN- $\gamma$  triggered by SEB. As both cytokines are known to be produced by T cells in this setting (9, 15, 20), these data confirm and extend recent observations made in experimental parasitic diseases demonstrating that NOS inhibition enhanced IFN- $\gamma$  production by T cells in vitro (21, 22).

A similar effect of NOS inhibition was observed in vivo by measuring cytokine serum levels in L-NAME-treated mice. First, we verified that NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> serum levels remained at basal values after coinjection of SEB and L-NAME (mean  $\pm$  SEM 16 h after SEB injection: 265  $\pm$  55  $\mu$ M). As shown in Table 1, in vivo inhibition of NOS did not modify the peak serum levels of TNF and IFN- $\gamma$ , but prolonged the period during which these cytokines persist in the circulation. In parallel, we found that IFN- $\gamma$  and TNF- $\alpha$  mRNA expression in spleen 24 h after SEB injection was increased in L-NAME-treated mice (Fig. 4). Moreover, L-NAME did not modify the disappearance rate of radiolabeled TNF, indicating that the effect of NOS inhibition on cytokine serum levels was not related to impaired cytokine clearance (data not shown). Taken together, these results demonstrate the existence of a regulatory loop by which NO inhibits the production of TNF and IFN- $\gamma$  which induce its own synthesis.

The sustained release of TNF and IFN- $\gamma$  caused by NOS inhibition was associated with an increased toxicity of SEB.

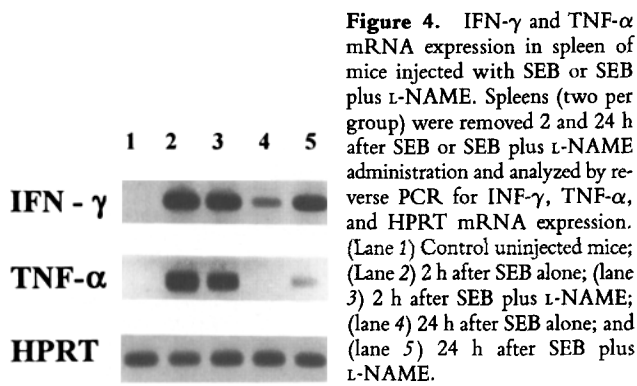


**Figure 3.** TNF and IFN- $\gamma$  secretion by spleen cells stimulated with SEB in the presence or absence of the NOS inhibitor L-NAME.  $5 \times 10^6$  spleen cells were incubated with 10  $\mu$ g/ml of SEB together with increasing concentrations of L-NAME (0–2 mg/ml). After 4 d incubation, TNF and IFN- $\gamma$  production was assayed by ELISA. Hatched (IFN- $\gamma$ ) and open (TNF) columns represent means of duplicate samples in one representative experiment out of two. TNF and IFN- $\gamma$  levels in the absence of SEB (200 and 20 U/ml), respectively) were not modified by L-NAME addition.

**Table 1.** TNF and IFN- $\gamma$  Serum Levels after SEB Injection in L-NAME-treated Mice

Mice injected with*	TNF (U/ml) <sup>†</sup>		IFN- $\gamma$ (U/ml) <sup>‡</sup>	
	90 min	24 h	4 h	24 h
SEB	110 $\pm$ 4	<20	300 $\pm$ 30	<2
SEB plus L-NAME	120 $\pm$ 13	90 $\pm$ 7 <sup>§</sup>	270 $\pm$ 60	42 $\pm$ 4 <sup>§</sup>
L-NAME	<20	<20	<2	<2

\* Mice were injected with 100  $\mu$ g SEB alone, 10 mg L-NAME alone, or 100  $\mu$ g SEB and 10 mg L-NAME, as described in Materials and Methods.  
<sup>†</sup> TNF and IFN- $\gamma$  serum levels were measured at their peak (90 min for TNF, 4 h for IFN- $\gamma$ ) and 24 h after SEB injection. Results were expressed as mean  $\pm$  SEM of at least five mice in each group.  
<sup>§</sup>  $p < 0.01$  as compared with mice injected with SEB alone.

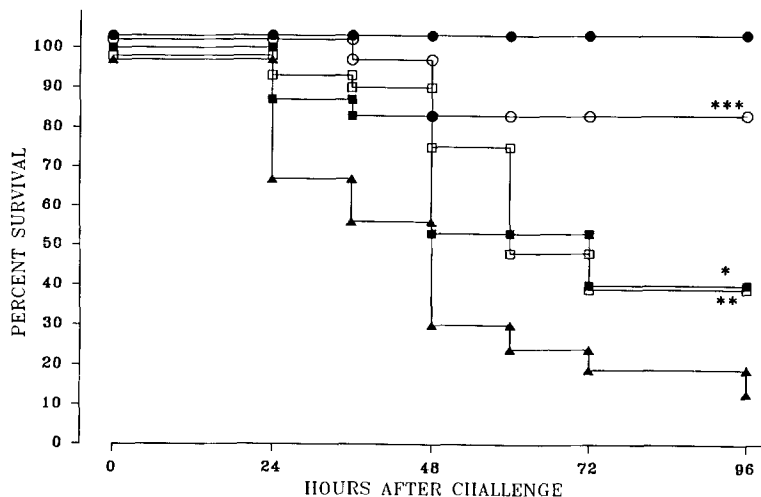


**Figure 4.** IFN- $\gamma$  and TNF- $\alpha$  mRNA expression in spleen of mice injected with SEB or SEB plus L-NAME. Spleens (two per group) were removed 2 and 24 h after SEB or SEB plus L-NAME administration and analyzed by reverse PCR for INF- $\gamma$ , TNF- $\alpha$ , and HPRT mRNA expression. (Lane 1) Control uninjected mice; (Lane 2) 2 h after SEB alone; (lane 3) 2 h after SEB plus L-NAME; (lane 4) 24 h after SEB alone; and (lane 5) 24 h after SEB plus L-NAME.

Indeed, 95% of mice (15 of 16) coinjected with L-NAME and SEB died within 96 h after SEB challenge (Fig. 5) whereas no lethality occurred in mice injected with SEB alone ( $n = 20$ ) or L-NAME alone ( $n = 20$ ). To study the involvement of IFN- $\gamma$  and TNF in the mortality induced by the combination of L-NAME plus SEB, groups of animals were pretreated

with anti-IFN- $\gamma$  and/or anti-TNF mAbs. As shown in Fig. 5, simultaneous neutralization of IFN- $\gamma$  and TNF dramatically reduced the mortality induced by SEB in L-NAME-injected mice whereas pretreatment with either anti-TNF mAb alone or anti-IFN- $\gamma$  mAb alone merely delayed animal death (Fig. 5). The role of TNF in SEB-induced shock has previously been demonstrated in D-galactosamine-sensitized mice (9) and we recently observed that IFN- $\gamma$  is involved in the lethality induced by SEB in mice treated with anti-IL-10 mAb (20). The data presented herein indicate that IFN- $\gamma$  and TNF might act synergistically in mediating SEB toxicity as they do in other models of inflammation (23).

NO in SEB-induced shock not only downregulates the production of inflammatory cytokines as shown in this paper but might also reduce their pathogenic effects. As a matter of fact, the vasoactive properties of NO as well as its ability to inhibit platelet aggregation and adhesion could be important in counteracting the prothrombotic properties of TNF and IFN- $\gamma$  (24, 25). Indeed, we observed lesions of coagulative necrosis in the liver of mice coinjected with SEB and



**Figure 5.** Involvement of TNF and IFN- $\gamma$  in the lethality induced by SEB in L-NAME-treated mice. Mice were injected with L-NAME (total dose, 10 mg) and SEB (100  $\mu$ g) after pretreatment with either control mAb ( $\blacktriangle$ ,  $n = 54$ , including 30 mice injected with LO-DNP-2 mAb and 24 mice injected with CB1 mAb), anti-TNF mAb ( $\blacksquare$ ,  $n = 30$ ), anti-IFN- $\gamma$  mAb ( $\square$ ,  $n = 33$ ), or anti-TNF plus anti-IFN- $\gamma$  mAbs ( $\circ$ ,  $n = 30$ ). Survival was 100% at 96 h in mice receiving SEB alone or L-NAME alone ( $\bullet$ ,  $n = 20$  in each group). (\*)  $p < 0.05$ , (\*\*)  $p < 0.005$ , and (\*\*\*)  $p < 0.0005$ , as compared with mice pretreated with control mAb.

L-NAME (data not shown) similar to those described in animals receiving LPS and L-NAME (4).

We conclude that NO overproduction is a major protective mechanism in the T cell-dependent shock induced by

SEB and that NOS inhibition might have detrimental consequences in T cell-mediated inflammatory disorders by enhancing both the production and the toxicity of inflammatory cytokines.

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