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Nitric Oxide Regulates Clonal Expansion and Activation-Induced Cell Death Triggered by Staphylococcal Enterotoxin B

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Increased interest has recently been focused on nitric oxide (NO) due to its several biological roles. Apart from being a potential antimicrobial defense and a mediator of autoimmune diseases, NO also appears to be a strong mediator of T-cell responses. In this report, we have characterized the effect of NO on T-cell function. For this purpose, we analyzed in vivo T-cell responses to the bacterial superantigen produced by *Staphylococcus aureus*, staphylococcal enterotoxin B (SEB), in mice treated with an NO donor (isosorbide dinitrate [ISO]). We show that ISO partially prevents SEB-triggered activation-induced cell death of spleen and lymph node CD4Vβ8$^+$ T cells but not of CD8Vβ8$^+$ T cells. SEB-promoted thymic deletion is not abolished by ISO; however, a rapid recovery of thymocyte numbers due to increased double-positive (DP) CD4$^+$ CD8$^+$ thymocyte proliferation was clearly observed in ISO-treated, SEB-injected mice but not in controls (untreated SEB-injected mice). It was also found that ISO inhibits the early SEB-induced cell proliferation (i.e., that found 12 h after SEB injection), accelerating the clonal anergy usually observed 3 days after SEB injection. Inhibition of T-cell proliferation by the NO donor does not appear to be due to inhibition of cytokine production. These results show that NO interferes with apoptosis and facilitates thymic proliferation of DP thymocytes, although it inhibits peripheral T-cell proliferation.

Nitric oxide (NO) is a free radical gas derived from molecular oxygen and guanidino nitrogen of L-arginine, in a reaction catalyzed by NO synthase (NOS) (4). NO is involved in a variety of biological functions: it acts as a neurotransmitter, accounts for the activity of endothelium-derived relaxing factor, prevents platelet aggregation, and is an important effector in the destruction of pathogens and tumor cells (26, 33, 35). In addition, NO participates in inflammatory and autoimmune-mediated tissue destruction (19, 31, 50).

NO is also involved in the regulation of T-lymphocyte proliferation, since T-cell mitogen responses can be inhibited by macrophage-produced NO (27). Th1, but not Th2, cells can be activated by specific antigens or T-cell mitogens to produce large amounts of NO (45). In addition, NO can inhibit interleukin-2 (IL-2) and gamma interferon (IFN-γ) secretion by Th1 cells, although it has no effect on IL-4 produced by Th2 cells (45). Thus, NO exerts a self-regulatory effect on Th1 cells. There are also several reports showing that NO facilitates T-cell proliferation (11, 19), suggesting that it has a dual effect on T-lymphocyte regulation.

Here, we studied the effects of NO on T-cell function, examining the T-cell response to a so-called superantigen. T cells are activated by the recognition of an appropriate peptide presented by the major histocompatibility complex. Bacterial exotoxins, upon binding to a particular T-cell receptor (TCR) Vβ chain, also trigger T-cell activation, regardless of the TCR α-chain partner (39) or TCR specificity. A much larger proportion of T cells reacts with a superantigen than with conventional antigens, and they are therefore used as antigen surrogates in the study of cell activation. Bacterial superantigens such as staphylococcal enterotoxin B (SEB) cause a monophasic depletion of SEB-reactive (Vβ8$^+$) thymocytes (8, 51) and a biphasic change in the specific peripheral T-cell frequency. This consists of an early deletion (minimum of Vβ8$^+$ cells at 12 to 24 h), followed by expansion (36 to 92 h) and a second deletion (>4 days) (22, 28, 47). In addition, superantigens exert dual effects on peripheral T cells. On the one hand, SEB causes transient T-cell activation manifested by acute cytokine release (17, 32) and the proliferation of the SEB-reactive T cells (22, 28). On the other, it causes both specific unresponsiveness of SEB-reactive T cells, as a consequence of the physical elimination (deletion) of a fraction of the Vβ8$^+$ T cells via apoptosis (7, 21) and the induction of functional antigen responsiveness (anergy of the remaining Vβ8$^+$ T cells) (21, 25, 37).

The published reports on the effect of NO on SEB-induced shock appear to have derived contradictory conclusions. Thus, while Sarawar and coworkers (43) found that aminoguanidine, an NOS inhibitor, protects against SEB-induced shock in lymphocytic choriomeningitis virus-infected mice, Florquin et al. (15) showed that endogenous NO produced after SEB administration has a protective effect, since administration of L-NAME (*N*-nitro-L-arginine methyl ester), an NOS inhibitor, dramatically increased the mortality rate in this model.

The data describing the effect of NO in lipopolysaccharide (LPS)-induced cytotoxicity are also controversial. Administration of an N$^{\omega}$-substituted L-arginine analog promotes glomerular thrombosis and liver damage, with an increase in mortality rates in LPS-injected animals, while disease is ameliorated with the administration of the NO donor *S*-nitro-*N*-acetyl-penicillamine (3, 6, 44). In contrast, other authors (23, 38) showed that NO overproduction is implicated in the pathogenesis of LPS-induced arterial hypotension and that NOS inhibitors increase systemic vascular resistance in experimental endotoxemia and in septic shock patients. Finally and most recently,

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mutant mice lacking inducible NOS (iNOS) have been described; there is, however, some controversy regarding their resistance to LPS-induced mortality (24, 29, 49). The discrepancies among these three studies are probably due to genetic background differences in the animals used.

We have studied the effects of NO on T-cell responses to the superantigen SEB, which activates principally Vβ8-expressing T cells (39). We found that the SEB-induced deletion of CD4Vβ8$^+$ T cells was delayed in mice pretreated with the NO donor isosorbide dinitrate (ISO). Clonal expansion of SEB-reactive T cells was also affected in these mice compared to untreated animals. Interestingly, NO donor administration increases and maintains production of SEB-triggered cytokines, mainly IFN-γ and IL-4. These results suggest that NO prevents SEB-induced deletion by preventing T-cell proliferation.

MATERIALS AND METHODS

Animals and in vivo treatments. Specific-pathogen-free BALB/c mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, Mass.) and kept in our specific-pathogen-free animal facilities. When appropriate, they were treated with the NO donor ISO (Sigma), 150 mg/kg of body weight/day, supplied in the drinking water. Mice received intravenous injections of 50 μg of SEB (Toxin Technology, Sarasota, Fla.) diluted in 0.2 ml of phosphate-buffered saline (PBS) or PBS alone as a vehicle control. Mice were killed at various times after injection, and thymus, spleen, and lymph nodes (pooled inguinal and axillary) were removed for analysis. Nitrites in urine were determined by using commercial test strips (Multistix 10SG [Bayer Diagnostics, Leverkusen, Germany]; sensitivity, 13 to 22 μmol/liter). All ISO-treated mice were positive for nitrites in urine. To study whether ISO has a protective effect on SEB-induced septic shock, that is, when it is coadministered with D-galactosamine (GALN), mice received 20 mg of GALN intraperitoneally together with SEB.

Immunofluorescence analysis. Thymocytes or spleen leukocytes enriched by hypotonic erythrocyte lysis, as well as lymph node leukocytes, were stained with monoclonal antibodies (MAbs) against CD4, CD8, and Vβ8, labeled with phycoerythrin (PE), fluorescein isothiocyanate (FITC; Pharmingen, San Diego, Calif.), or biotin (Becton Dickinson, San Jose, Calif.). An unlabeled, purified hamster anti-mouse Fas MAb (Pharmingen) was also used and developed with a FITC-labeled polyclonal goat anti-hamster immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Biotinylated MAbs were developed either with streptavidin-PE (Southern Biotechnology) for double staining or with RED613 (Sigma, St. Louis, Mo.) for three-color staining. The results, expressed as change in mean fluorescence intensity, indicate the ratio of the mean fluorescence intensity of the indicated antibody divided by the mean fluorescence intensity obtained in control staining, i.e., anti-hamster-FITC staining in the absence of anti-mouse Fas MAb.

For cell cycle analysis, freshly purified CD3$^+$ splenocytes were stained with propidium iodide, using a semiautomatic procedure (DNA-Prep; Coulter, Hialeah, Fla.), followed by analysis on an EPICSXL flow cytometer (Coulter).

Proliferation assays. Total splenocytes were cultured in triplicate in 96-well plates in RPMI 1640 (Gibco, Gaithersburg, Md.) supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine, 10 U of penicillin per ml, and 10 μg of streptomycin per ml. Cells were activated with concanavalin A (2.5 μg/ml), SEB (10 μg/ml), or plastic-bound antibodies (10 μg/ml) specific for CD3 (clone 2C11) or Vβ8 (F23.1). After 48 h, cultures were pulsed for 12 h with [^{3}H]thymidine (1 mCi/ml; Amersham International, Aylesbury, United Kingdom) cells were harvested, and radioactivity was measured with a liquid scintillation β counter.

Purification of T cells. Spleen T cells were purified by using Cellect columns (Biotecx, Edmonton, Alberta, Canada) as instructed by the manufacturer. The B-cell contamination level was <3%. Alternatively, spleen T cells were purified by B-cell panning as described previously (21). For cell cycle analysis, purified T cells were cultured for 6 h at 37°C.

Sera and ELISAs. Sera were obtained 90 min, 12 h, and 24 h after SEB injection, and tumor necrosis factor alpha (TNF-α), IL-2, IL-4, IL-10, and IFN-γ levels were assayed by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, Mass.) as recommended by the manufacturer.

Statistical analysis. Statistical comparisons were made by using Student's *t* test.

RESULTS

NO partially prevents the SEB-induced deletion of CD4Vβ8$^+$ but not of CD8Vβ8$^+$ T cells. Injection of the *Staphylococcus aureus* superantigen SEB stimulates T cells which express Vβ8, inducing proliferation, anergy, and deletion by programmed cell death (PCD) (21, 22, 40). Lymph node or spleen Vβ8$^+$ T cells from SEB-injected mice show a first deletion which peaks 12 to 24 h after injection, followed by clonal expansion of these cells and a second deletion beginning 3 to 4 days after SEB injection (Fig. 1).

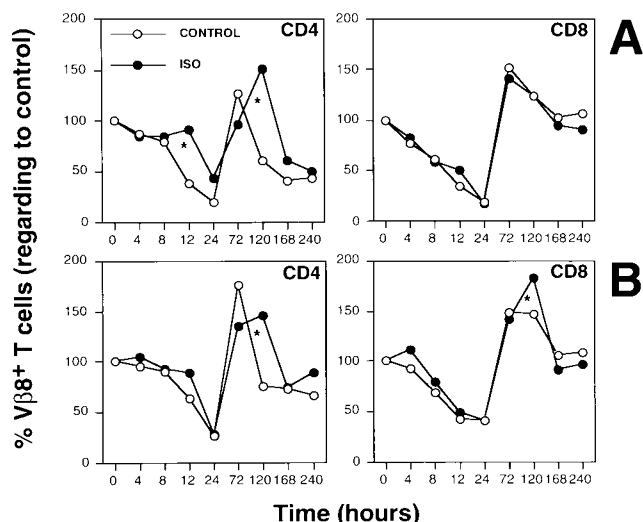

FIG. 1. Effects of NO donors on SEB-induced deletion of Vβ8$^+$ T cells. BALB/c mice pretreated for 1 week with ISO (150 mg/kg/day) or control animals received a single intravenous SEB injection, followed by determination of CD4$^+$Vβ8$^+$ and CD8$^+$Vβ8$^+$ T-cell frequency in lymph node (A) and spleen (B). Each point depicts data for four to six animals at representative time points of a complete kinetic analysis. For simplification, standard deviations are not included. Asterisks mark significant differences between ISO-treated and untreated groups ($P < 0.01$, Student's *t* test).

In ISO-treated, SEB-injected mice, NO delays the first deletion of SEB-induced CD4Vβ8$^+$ T cells. In lymph nodes, more than 60% of CD4Vβ8$^+$ T cells from untreated SEB-injected control mice were deleted 12 h after SEB injection, while in ISO-treated mice, only 10% of the CD4Vβ8$^+$ T cells had been deleted by this time (Fig. 1A). Surprisingly, this effect of ISO could not be detected in CD8Vβ8$^+$ T cells or in CD4Vβ8$^+$ splenocytes (Fig. 1B). The effect of ISO in delaying CD4Vβ8$^+$ lymph node T-cell deletion was transient, however, since no significant difference between the two groups of mice was detected 24 h after SEB injection.

ISO does not prevent SEB-induced clonal expansion; on the contrary, it appears that ISO potentiates clonal expansion (Fig. 1). While CD4Vβ8$^+$ T cells in control mice declined after 5 days to values below those found in untreated mice (time zero), these cells remained at increased levels in ISO-treated animals (Fig. 1). This effect is less pronounced in CD8Vβ8$^+$ T cells, although SEB-induced expansion also appears delayed in ISO-treated animals compared to the normal kinetics observed in SEB-injected control mice. Concomitantly, ISO enhances the SEB-induced splenomegaly detected on day 3 (total number of splenocytes [10^6]: for SEB, 204 ± 16; for ISO plus SEB, 268 ± 14; for PBS, 102 ± 9; for ISO plus PBS, 97 ± 4). Since activated T cells die rapidly in vivo (28, 48), at least part of this increase may be due to the enhanced half-life of activated T cells.

In untreated SEB-injected mice, CD4Vβ8$^+$ T cells decline to values below those found at day 0 (PBS-injected untreated group), while CD8Vβ8$^+$ T cells decrease to values similar to those found on day 0. This is also true for ISO-treated mice

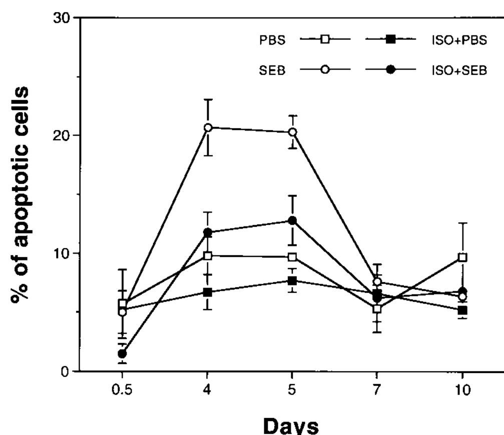

FIG. 2. ISO prevents induction of SEB-triggered PCD in spleen. After 1 week of ISO treatment (150 mg/kg/day), BALB/c mice received a single intravenous injection of SEB. At the indicated time points, freshly isolated $CD3^+$ spleen T cells were cultured in vitro for 6 h at 37°C for detection of PCD-associated changes; cell cycle analysis was performed by with propidium iodide staining. Each point represents data for four to six animals.

(Fig. 1); thus, ISO is not able to prevent the second SEB-induced deletion wave.

Cell cycle analysis shows that ISO also inhibits SEB-induced PCD on days 4 and 5 after SEB injection (Fig. 2). In fact, the percentage of apoptotic spleen T cells found in SEB-injected untreated mice was twofold greater than that found in SEB-injected, ISO-treated mice. We found no difference between days 3 and 4; however, by days 7 and 10, no significant difference in apoptotic cells was detectable between uninjected and SEB-injected mice. This is because PCD occurs for a limited period after SEB priming, contributing to the reduction in $V\beta8^+$ T cells (mainly $CD4^+$).

SEB can also induce intrathymic tolerance by clonal deletion of thymocytes bearing the appropriate Vβ specificity (7). ISO is not capable of preventing the SEB-induced clonal deletion of $CD3V\beta8^+$ thymocytes (data not shown) or the superantigen-induced PCD of immature double-positive (DP) $CD4^+$ $CD8^+$ thymocytes (Fig. 3 and 4). This is clearly shown by the marked increase in $CD3^{high}$ thymocytes, which is a result of the decrease in the mainly DP $CD3^{low}$ thymocytes (Fig. 4A). However, by day 5 after SEB injection, ISO-treated mice already showed a significant increase in thymocyte number, while untreated mice still showed a very reduced thymocyte count (Fig. 3). This increase in thymocyte number is accompanied by increased DP thymocyte proliferation, as shown by an increment in the percentage of these cells and the decrease in the $CD3^{high}$ population to values near those of control mice (Fig. 4B).

Contrary to data of Fehsel and coworkers, who reported that in vitro donors induce apoptosis in DP mouse thymocytes (13), we found no significant reduction of these cells in NO donor-treated mice. Instead, ISO decreases the effect of SEB on induced cell death of DP thymocytes and enhances rapid recovery by increasing their proliferation.

$V\beta3^+$, $V\beta5^+$, and $V\beta11^+$ T cells are clonally eliminated in the thymuses of BALB/c mice due to their reactivity with self-superantigens encoded by endogenous retroviruses (*mtv* 6, 8, and 9). Mice treated for prolonged periods (2 to 4 months) with ISO showed no increase in the frequency of these cells (data not shown).

Thus, ISO interferes with the PCD of peripheral T cells and facilitates proliferation of DP thymocytes but does not affect PCD of thymocytes which are probably undergoing negative selection.

NO does not regulate Fas expression. Fas (Apo-1, CD95), a cell surface protein belonging to the TNF receptor family, mediates apoptosis after ligation with anti-Fas antibody or the Fas ligand (FasL) (34). Activation-induced T-cell death, which is involved in the downregulation of immune responses, has recently been shown to be mediated through Fas-FasL interactions (1, 5, 9, 20). Recent reports show that SEB-mediated, activation-induced cell death requires Fas-FasL interactions (12, 36, 41, 52). In this study, we analyzed the possible effects of the NO donor on SEB-induced Fas expression.

ISO treatment failed to block the SEB-induced overexpression of Fas which occurs in peripheral $V\beta8^+$ T cells (Table 1). Cell death induced in vitro by anti-Fas antibody on previously SEB-primed T cells was also unaffected by ISO treatment (data not shown).

Upregulation of SEB-induced Fas expression is not restricted to peripheral $V\beta8^+$ T cells; SEB was also observed to increase Fas expression on thymic subpopulations (Table 2). As in the case of the periphery, Fas overexpression in thymus was not prevented by ISO treatment (Table 2). Thus, partial prevention of apoptosis and sustained expansion of peripheral $V\beta8^+$ T cells, as well as the rapid ISO-induced expansion of DP thymocytes, do not appear to be caused by a decrease in Fas expression.

ISO does not prevent death induced by septic shock. The effect of ISO was also assessed in a septic shock model. Coadministration of GALN increases mouse sensitivity to SEB toxicity, with an acutely lethal (<36 h) cytokine release syndrome mediated by T-cell-derived TNF (18, 30, 32, 46). Pretreatment of mice with ISO does not impede the death of animals injected with SEB plus GALN (Table 3).

NO increases cytokine production by in vivo SEB-stimulated T cells. SEB activates both Th1 and Th2 cells (17), as measured by its ability to induce secretion of IFN-γ and IL-2 (Th1 cytokines), as well as IL-4 and IL-10 (Th2 cytokines) (Table 4). IFN-γ and TNF-α are known to induce iNOS to

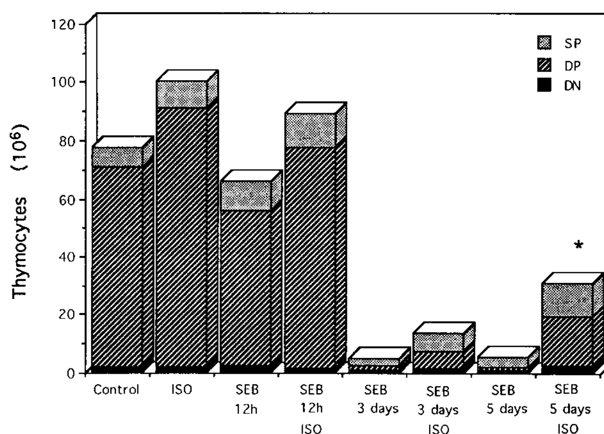

FIG. 3. ISO prevents SEB-induced deletion of DP thymocytes. BALB/c mice were treated with ISO (150 mg/kg/day), followed by a single SEB injection. (A) After 12 h, 3 days, or 5 days, thymocyte suspensions were analyzed by two-color immunofluorescence using FITC- or PE-labeled antibodies specific for CD4 and CD8. The total numbers of the subpopulations indicated, that is, mature single-positive (SP) $CD3^+$ $CD4^+$ $CD8^-$ or $CD3^+$ $CD8^+$ $CD4^-$, DP $CD4^+$ $CD8^+$, and double-negative (DN) $CD4^-$ $CD8^-$ thymocytes, are shown. Asterisks mark significant differences between ISO-treated and untreated groups ($P < 0.01$, Student's t test). The experiment shown is representative of three independent experiments.

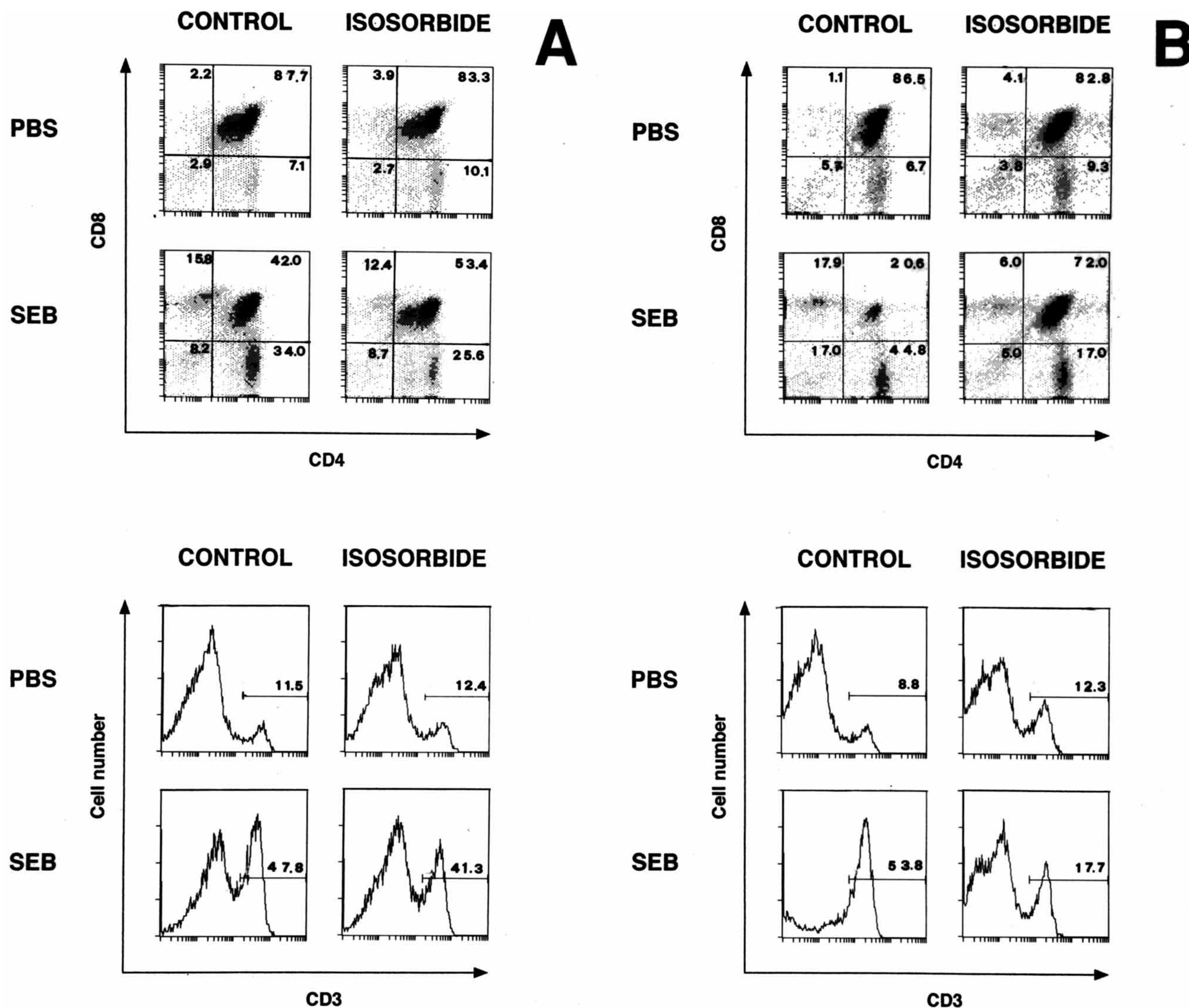

FIG. 4. Effect of ISO on SEB-induced deletion of DP thymocytes. BALB/c mice were treated with ISO (150 mg/kg/day) and then given a single injection of SEB. Frequencies of $CD4^-$ $CD8^-$, $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, and $CD3^{high}$ thymic subpopulations 3 (A) and 5 (B) days after SEB injection are shown as percentages. The experiment shown is representative of three independent experiments.

produce NO, which in turn inhibits cytokine secretion by Th1 cells, although it has no effect on cytokines secreted by Th2 cells (45). As shown in Table 4, ISO-treated SEB-injected mice also show an indiscriminate stimulation of Th1 and Th2 cells, although production of cytokines such as IFN-γ, IL-4, and TNF-α is potentiated by ISO very shortly after SEB injection (90 min). ISO per se increases TNF-α production by T cells and/or macrophages compared to control PBS-injected mice. This effect might explain why ISO does not prevent the septic shock induced by the combination of SEB and GALN (Table 3), as TNF-α is known to have an important role in in vivo apoptosis induction by SEB (18, 30, 32, 46).

ISO does not inhibit SEB-driven acute activation, since serum IL-2 and IL-4 levels were significantly elevated in both SEB-injected untreated and ISO-treated animals (Table 4). In fact, serum IL-4 levels in the latter group were threefold higher than in the former. The increased IL-4 levels may reflect an attempt at regulation of NO production either by iNOS inhibition, since IL-4 is known to inhibit this enzyme (27, 42), or by regulation of Th1 cells (45), which produce cytokines that potentiate NO production, such as IFN-γ and TNF-α (10, 16).

Twenty-four hours after SEB injection, serum IL-2 and IL-4 levels had already decreased to values near those of noninjected control mice, while IFN-γ and TNF-α levels remain elevated in ISO-treated mice. Moreover, the increase in IFN-γ levels was markedly enhanced and sustained by ISO in SEB-injected mice. Florquin et al. (15) reported that TNF-α and IFN-γ secretion by SEB-stimulated cells was also enhanced by an iNOS inhibitor causing SEB-triggered lethality in mice, suggesting that NO produced endogenously in the presence of SEB protects against septic shock. Here, we show that ISO, an NO donor, also potentiates production of these cytokines. However, when we used these authors' protocol (that is, 2 mg of ISO intraperitoneally 30 min prior to, simultaneous with, and 2, 4, and 6 h after injection of 100 μg of SEB), no lethality occurred in SEB- and ISO-injected mice (SEB, 0% mortality [0

TABLE 1. Expression of Fas on peripheral Vβ8$^+$ T cells[a]

Treatment	Change in mean fluorescence intensity			
	CD4Vβ8$^+$ cells		CD8Vβ8$^+$ cells	
	Spleen	Lymph nodes	Spleen	Lymph nodes
PBS	2.50	2.78	2.13	2.58
ISO + PBS	2.43	2.28	2.02	2.29
SEB	4.78	4.27	4.09	4.05
ISO + SEB	4.98	3.82	4.62	3.78

[a] BALB/c mice pretreated for 1 week with ISO (150 mg/kg/day) and control animals were injected with SEB or PBS as a vehicle control. Twenty-four hours after injection, cell suspensions from spleen and lymph nodes were triple stained with anti-CD4 or anti-CD8, anti-Vβ8, and anti-Fas antibodies. CD4Vβ8$^+$ and CD8Vβ8$^+$ gated populations were analyzed for Fas expression, and results are expressed in terms of change in the mean fluorescence intensity (see Materials and Methods).

TABLE 2. Expression of Fas on thymic populations[a]

Treatment	Change in mean fluorescence intensity		
	CD4$^+$ CD8$^+$	CD8$^+$ CD4$^-$	CD4$^+$ CD8$^-$
PBS	4.66	3.14	3.62
ISO + PBS	4.34	2.53	3.57
SEB	6.33	3.67	4.30
ISO + SEB	6.38	3.75	4.15

[a] Expression of Fas in thymocytes was performed, 24 h after injection of SEB or of PBS as a vehicle control, in BALB/c mice that had been previously treated for 1 week with ISO (150 mg/kg/day) and in control animals. Thymocyte suspensions were subjected to three-color immunofluorescence analysis by antibodies specific for CD4, CD8, and Fas. Expression of Fas antigen was analyzed on the gated cell population for CD4$^+$ CD8$^+$ thymocytes, CD4$^+$ CD8$^-$ thymocytes, and CD4$^-$ CD8$^+$ thymocytes. Results are expressed in terms of change in the mean fluorescence intensity (see Materials and Methods).

deaths/10 mice]; ISO, 0% [0/12]; ISO plus SEB, 0% [0/20]). Therefore, although TNF-α and IFN-γ levels are greatly increased in ISO-treated SEB-injected mice, other factors must be involved in SEB-induced mortality.

A protective role for IL-10 against SEB-induced septic shock has also been described (14). Using a commercial ELISA kit, we detected no appreciable amounts of IL-10 90 min after SEB injection, either in untreated or in ISO-treated mice (Table 4).

NO does not prevent anergy induction by SEB in T cells. Clonal expansion and cell death can occur as sequential stages in an immune response to SEB. After exposure to this toxin and following proliferation, peripheral Vβ8$^+$ T cells progress into a state of anergy, unable to respond to SEB in vitro. This anergy is accompanied by PCD of the Vβ8$^+$ T-cell subpopulation (21, 22, 40, 51). Since SEB-induced acute T-cell activation as regards cytokine production was unaffected by ISO, we evaluated the capacity of ISO to prevent SEB-induced clonal anergy.

Splenocytes from SEB-injected control or ISO-treated mice reexposed in vitro to SEB or to immobilized anti-Vβ8 3 days after SEB injection exhibited low proliferative responses (Fig. 5B), indicating that ISO did not abolish SEB-induced clonal anergy. This loss of reactivity to SEB was maintained until at least day 10 (Fig. 5C). In contrast, when splenocytes from SEB-injected control mice were reexposed in vitro to SEB 12 h after SEB injection, they showed much higher proliferative responses than splenocytes from PBS-injected control mice. This proliferation was less pronounced in SEB-injected ISO-treated mice (Fig. 5A). ISO therefore does not prevent SEB-induced anergy but rather is able to decrease the earlier in vitro proliferation in response to SEB. Although NO has been described to inhibit T-cell proliferation by blocking IL-2 production (26), this is not the case in our experimental model, as we detected no difference in IL-2 serum levels between the SEB-injected untreated and the ISO-treated mice (Table 4). In conclusion, ISO accelerates clonal anergy when T cells are reexposed in vitro to SEB, as seen by the decrease of earlier proliferation response to SEB, although there is no in vivo inhibition of cytokine production.

DISCUSSION

As shown here, NO is involved in the regulation of the cellularity of lymphoid organs, mainly the thymus, as well as in PCD of peripheral T cells and specific superantigen responses. NO is the end product of the oxidation of one guanido nitrogen of L-arginine by NOS (4). Several NOS isoforms, representing the products of three separate genes on distinct chromosomes, have been identified. Two different enzymes are expressed spontaneously in neurons and endothelial cells, respectively, while the third, usually absent in resting cells, can be induced by a variety of stimuli such as LPS, IFN-γ, or pathogens (4). NO can act as an intracellular signalling molecule regulating intracellular enzyme function or as a mediator, affecting cell communication and immune function. When present at a high concentration or when produced chronically, NO leads to cell and tissue toxicity. Thus, its overproduction has been shown to play an important part in various tissue injuries associated with chronic inflammatory processes such as asthma (2), rheumatoid arthritis (31), and nephritis (50). The NO produced during an adaptive response induces growth inhibition of microbial pathogens and behaves as a potent agent in nonspecific defense mechanisms by upregulating the release of inflammatory mediators (26). As we show here, NO might also be important in the fine-tuning of superantigen-triggered T-cell responses. Thus, the SEB-triggered cytokine release pattern, known to activate both Th1 and Th2 cells, is notably affected in the presence of NO donors, heightening IL-4 production and, therefore, preferential Th2 T-cell stimulation. Since cloned, antigen- or mitogen-activated murine Th1 cells express NOS and produce substantial amounts of NO, this preferential production of IL-4 will establish feedback control to prevent continuous Th1 stimulation.

Shortly after SEB injection (8 h), the number of Vβ8$^+$ T cells begins to decline, reaching an early minimum at 12 to 24 h. This phenomenon involves PCD-mediated deletion and is

TABLE 3. Failure of ISO to inhibit SEB-induced lethal septic shock[a]

Treatment	Sensitizing agent	% Mortality (deaths/total)
None	20 mg of GALN	0 (0/5)
150 mg of ISO	None	0 (0/43)
	20 mg of GALN	0 (0/5)
50 μg of SEB	None	0 (0/40)
	20 mg of GALN	100 (6/6)
150 mg ISO + 50 μg of SEB	None	0 (0/46)
	20 mg of GALN	100 (8/8)

[a] BALB/c mice previously pretreated for 1 week with ISO (150 mg/kg/day) dissolved in drinking water, and control animals received simultaneous injections of SEB (intravenously through the tail vein) and GALN (intraperitoneally) suspended in 200 μl of PBS. Death rates were monitored during the next 7 days. In all cases, animals died with an interval of 12 to 36 h after injection.

TABLE 4. Cytokine concentrations detected in serum[a]

Treatment	IL-2 (pg/ml)		IFN-γ (pg/ml)		IL-4 (pg/ml)		TNF-α (pg/ml)		IL-10 (pg/ml)	
	90 min	24 h	90 min	24 h	90 min	24 h	90 min	24 h	90 min	24 h
PBS	18.7 ± 5.4	29.6 ± 4.3	139 ± 22.2	131 ± 28.1	14.5 ± 8.6	5.9 ± 1.5	46.6 ± 15.6	24.7 ± 2.5	0.14 ± 0.02	ND
ISO + PBS	ND	ND	47.0 ± 12.9	53.2 ± 15.3	7.1 ± 1.0	5.2 ± 2.6	104 ± 44.2	131 ± 8.7	0.14 ± 0.04	ND
SEB	5,900 ± 800	99.8 ± 27.2	8,218 ± 1,574	10,550 ± 1,458	599 ± 171	46.4 ± 15.8	1,889 ± 114	419 ± 66.7	0.37 ± 0.12	ND
ISO + SEB	5,733 ± 694	53.0 ± 15.4	10,725 ± 816	17,430 ± 2,857	1,755 ± 437	70.2 ± 10.6	2,890 ± 248	321 ± 66.5	0.37 ± 0.05	ND

[a] Serum samples were obtained from BALB/c mice untreated or pretreated for 1 week with ISO (150 mg/kg/day) and injected with SEB or PBS for 90 ± 5 min and 24 h. IL-10 levels at 24 h were not determined. ND, not detectable.

delayed by NO donors. NO seems more effective in impairing the deletion of CD4Vβ8$^+$ T cells than in impairing that of CD8Vβ8$^+$ T cells. The reason for this differential effect of NO is unknown, although it may involve distinct signaling requirements for CD4$^+$ and CD8$^+$ T cells. In addition, the effect of delaying clonal deletion of CD4Vβ8$^+$ T cells occurred only in lymph nodes, not in spleen cells. The difference observed between lymph nodes and spleen cells, and between CD4Vβ8$^+$ and CD8Vβ8$^+$ T cells, is not new. Using mice made genetically deficient for ICAM-1, we thus have shown that SEB-induced proliferation of CD8$^+$ T cells from lymph node (but not from spleen) is independent of the interaction mediated by ICAM-1 (17b). The distinct features of these populations might correlate either with different costimulatory requirements, e.g., due to their activation status, or with different antigen-presenting cell types in lymph node or in spleen.

The delay in SEB-triggered PCD may be associated with sustained cytokine production in the presence of NO donors. If this is indeed the case, other cell types may also be involved in this process, and the outcome would be a steady state between the normal proliferation rate and delayed induction of apoptosis. Alternatively, NO might also act on endothelial cells, affecting the expression of molecules implicated in cell trafficking; this effect could contribute to the final phenotype observed here.

NO donors also partially prevent the SEB-triggered acute depletion of DP thymocytes. More relevant, the presence of NO donors assists significantly in thymocyte replenishment. Interestingly, the role of NO in preventing SEB-triggered PCD is not mediated by Fas level regulation; it therefore probably acts on the signal transduction pathway triggered by Fas ligation.

In summary, the in vivo effects mediated by superantigens acting on Vβ8$^+$ T cells are regulated by the presence of NO

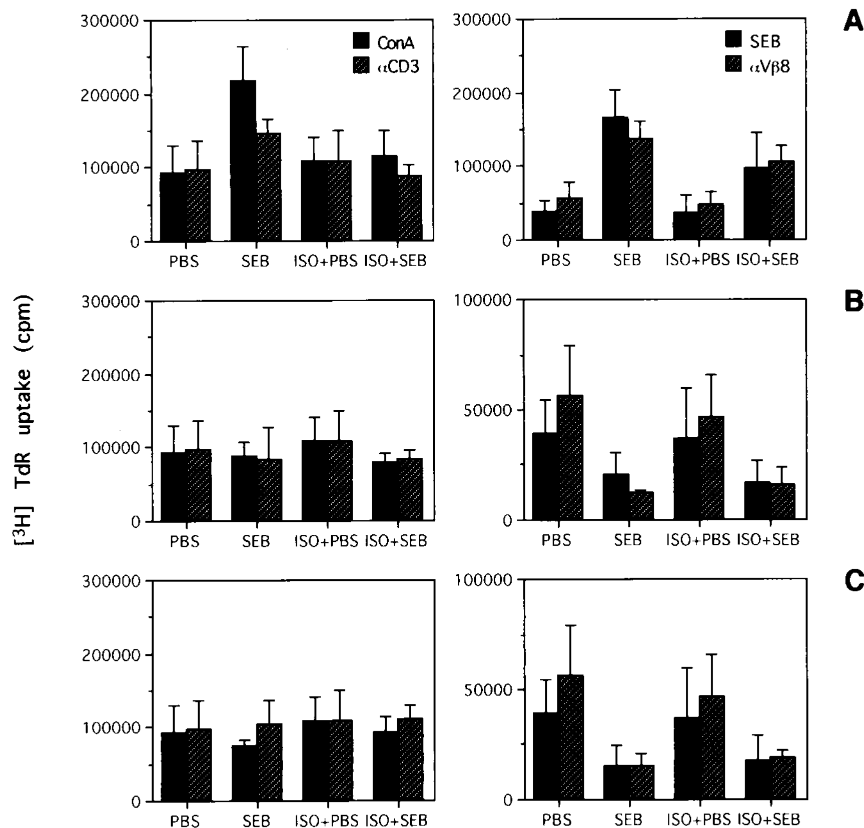

FIG. 5. ISO inhibits SEB-induced in vitro T-cell proliferation. Splenocytes from BALB/c mice treated for 1 week with ISO (150 mg/kg/day) were recovered 12 h (A), 3 days (B), and 10 days (C) after SEB injection and cultured in the presence of optimal doses of soluble mitogens (concanavalin A [ConA] and SEB) or plastic-immobilized stimulators (anti-CD3 [αCD3] and anti-Vβ8 [αVβ8]) as described in Materials and Methods. Note the different scale of the proliferative response of cells obtained 12 h after in vivo stimulation (A, right panel).

donors. The effect of NO appears to be dual: it delays T-cell deletion and promotes an in vivo increase in cytokine production early after stimulation; however, when the same cell population is stimulated in vitro, it displays a lower proliferative response.

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