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Regulation of Staphylococcal Enterotoxin B-Elicited Nitric Oxide Production by Endothelial Cells

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The effect of staphylococcal enterotoxin B (SEB)-elicited inducible nitric oxide synthase (iNOS) in mouse endothelial cells was investigated. Results showed that SEB stimulated the same level of NO production in gamma interferon (IFN-γ)-primed cells as did trichloroacetic acid-extracted lipopolysaccharide. The kinetics of induced NO production and expression of mRNA for iNOS differed markedly in endothelial and macrophage cells. Induced endothelial nitrite production was transient and was 15 to 20% of that generated by macrophage cells; mRNA levels peaked by 2 h and then steadily declined, whereas macrophage message levels continually increased. The ability of endothelial cells to produce SEB-induced NO depended on priming with IFN-γ, although detectable mRNA could be elicited by SEB alone. Induction of endothelial iNOS mRNA was inhibited by cycloheximide, which indicated a requirement for de novo protein synthesis. Niacinamide and interleukin-10 significantly reduced SEB-induced endothelial NO production. Both are reported to affect IFN-γ-induced class II major histocompatibility complex (MHC) expression on antigen-presenting cells. Niacinamide reduced iNOS mRNA levels and markedly reduced IFN-γ induction of endothelial class II MHC surface antigen. Interleukin-10 did not consistently reduce iNOS mRNA expression and had no effect on IFN-γ induction of endothelial class II MHC surface antigen. These results suggest that SEB interacts with IFN-γ-primed endothelial cells to elicit induced NO and that this induction can be effectively modulated at the receptor or transcriptional level.

Staphylococcal enterotoxins (SE) can induce extensive pathophysiological changes in humans and animals, which can lead to incapacitation, shock, and death. Although the mechanisms of intoxication are not completely defined and may differ from one SE to another, there are a number of common features. First, class II major histocompatibility complex (MHC) molecules act as specific receptors for SE (33) through which biological effects, such as transcriptional activation of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), are elicited (48). Upon binding to MHC class II molecules, these exoproteins are potent stimulators of T-cell proliferation by interacting with specific T-cell receptor Vβ segments on both $CD4^+$ and $CD8^+$ T cells (45). Finally, induced cytokines appear to play a critical role in the pathogenesis of the SE (32).

At least two distinct types of nitric oxide synthase (NOS) have been identified (13). Constitutive enzyme synthesis of NO occurs within seconds and depends on cell surface receptor-ligand signaling. This enzyme is dependent on calcium or calmodulin, and NO is produced only at very low levels. The second type of NOS is induced within 4 to 18 h by discrete immunostimulators, and NO is produced at relatively high levels. While low-output, constitutive isoforms are found in endothelial cells (4), these cells can also express the high-output, induced isoform (17). Produced in small amounts by the constitutive enzyme, NO plays a central role in control of vascular tone, platelet function, and synaptic neuronal transmission (14). The induced NOS (iNOS) produces NO in large quantities, which inhibits DNA (37) and protein (8) syntheses, and inhibits oxidative phosphorylation (51).

The extensive distribution of vascular endothelium and its capacity to produce iNOS suggest that it may be a primary source of this mediator (21). Vascular permeability and leakage associated with the hypotension observed in septic and toxic shock may relate to pathological overproduction of NO. Analogs of L-arginine can inhibit TNF-induced hypotension (22), prevent increased vascular permeability (19), and reverse endotoxin-mediated shock (23). Activated vascular endothelial cells also play an important role in antigen presentation to T cells through MHC class II molecules (41), a major biological receptor for the SE. Selective suppression of the endothelial immune response, such as regulation of MHC class II surface antigen, may provide a therapeutic target.

In this study, we characterized induced NO production by microvascular endothelial cells exposed to SEB (SEB) and compared the responses with those of a previously characterized macrophage cell line, RAW 264.7 (29). We further examined the direct effects of niacinamide and IL-10, both reported to affect the expression of class II MHC surface antigen, on SEB-elicited iNOS. Both cell lines responded in a similar dose-dependent manner to either SEB or trichloroacetic acid-extracted lipopolysaccharide (LPS). Transcriptional regulation of endothelial cell iNOS by SE and nitrite production differed markedly from that of macrophages. Our results suggest that SEB interacts with IFN-γ-primed endothelial cells to elicit inducible NO and that this induction can be effectively modulated at the receptor or transcriptional level.

MATERIALS AND METHODS

Reagents. Purified SEB was reconstituted (5 mg/ml) in RPMI 1640 (GIBCO, Grand Island, N.Y.) and passed through an affinity filtration device designed to remove LPS (END-X5; Associates of Cape Cod, Inc., Woods Hole, Mass.).

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Preparations of SEB migrated as a single band on sodium dodecyl sulfate-polyacrylamide gels and were negative for endotoxin as determined by a chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.) at a sensitivity level of 0.1 endotoxin unit (EU)/ml. LPS extracted by trichloroacetic acid was obtained from Difco Laboratories (Detroit, Mich.) (*Escherichia coli* O111:B4). Aliquots of SEB and LPS were stored at −70°C for future use. Murine recombinant gamma interferon (IFN-γ) and IL-10 were obtained from Genzyme Corporation (Cambridge, Mass.), diluted to a specific activity of 10^4 U/ml in RPMI 1640 plus 10% fetal bovine serum (FBS), and stored at −70°C. Trifluoperazine (TFP), niacinamide, cycloheximide, and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, Mo.). N^G-L-amino arginine (L-NAA) was purchased from Peninsula Laboratories, Inc. (Belmont, Calif.).

Cells and media. RPMI 1640 or Dulbecco's minimum essential medium (DMEM) was prepared from a powdered mixture (GIBCO/BRL) and supplemented with (final concentrations given) 3.7 mg of sodium bicarbonate per ml, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (all from ICN/Flow, McLean, Va.), 100 U of penicillin G per ml and 100 μg of streptomycin (both from Pfizer, Atlanta, Ga.) per ml, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma Chemical Co.), and sodium pyruvate and nonessential amino acids (both from ICN/Flow; diluted 1:100 from 100× stocks). FBS (mycoplasma free) was obtained from BioWhittaker. All reagents and culture media were negative for endotoxin, as determined by a chromogenic *Limulus* amebocyte lysate assay (BioWhittaker) at a sensitivity level of 0.1 EU/ml. Hybridomas producing rat monoclonal antibodies (MAb) specific for the mouse IFN-γ receptor were cultured as previously described (27) in DMEM plus 20% FBS. A previously described mouse microvascular endothelial cell line, SVEC, derived from the C3H/HeJ mouse strain (39) was kindly provided by Michael Edidin (Johns Hopkins University, Baltimore, Md.). The RAW 264.7 mouse (BALB/c) monocyte-macrophage cell line was obtained from the American Type Culture Collection (Rockville, Md.). Both cell lines were maintained in RPMI 1640 plus 10% FBS.

Antibodies. MAb were produced in terminal stationary-phase cultures as previously described (27). MAb of the immunoglobulin G2a (IgG2a) subclass were affinity purified with protein G Sepharose 4 Fast Flow (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), according to the manufacturer's instructions. Protein concentrations of purified antibodies were determined by the BCA protein assay (Pierce Chemical Company, Rockford, Ill.). Mouse anti-Ia^k IgG2b was prepared from culture supernatants derived from the hybridoma 10-2.16 (TIB 93; American Type Culture Collection). Biotin-conjugated mouse anti-mouse Ia^k MAb was obtained from Pharmingen (San Diego, Calif.). An irrelevant mouse IgG (Vector Laboratories, Burlingame, Calif.) was used as a nonspecific control antibody. Affinity-purified, monospecific polyclonal chicken, anti-SEB antibody was kindly provided by Paul Lemley (Toxinology Division, U.S. Army Medical Research Institute of Infectious Diseases).

Nitrite assay. Cells were plated in triplicate in 96-well microtiter plates at 10^5 cells per well and allowed to adhere for 3 to 4 h before use. Murine IFN-γ at a concentration of 10 U/ml was used to prime the cells. Either SEB or LPS was added simultaneously at concentrations noted. Determinations of nitrite concentrations in culture supernatants were based on the method of Green et al. (16). Briefly, cell-free culture supernatants (100 μl) or sodium nitrite standards were mixed with 100 μl of Griess reagent (0.1% naphthyl-ethylene diamine dihydrochloride, 1% sulfanilamide, 3% H_3PO_4) and incubated for 10 min at room temperature. A_{540} was measured with a MR5000 microtiter plate reader (Dynatech, Chantilly, Va.). Determinations of nitrite concentrations were based on a standard curve obtained with known concentrations of nitrite.

Cell viability assay. Assessment of cell viability was based on the metabolic activity of cells with a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (10, 35). MTT (Sigma Chemical Co.) was dissolved at 5 mg/ml in RPMI 1640, and aliquots were frozen at −20°C. Reduction of MTT to insoluble formazan crystals by metabolically active cells was measured by solubilizing the crystals in acidified (0.04 N HCl) isopropanol, and the A_{570} was read with a 630-nm reference wavelength by a Dynatech MR5000 plate reader.

RNA isolation and reverse transcription. Endothelial cells and macrophages were cultured in 12-well culture plates at a density of 2×10^6 cells per well. The cells were washed once with cold phosphate-buffered saline and then solubilized by adding 0.5 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) to each well. RNA was extracted by the method of Chomczynski and Sacchi (7), then briefly dried under vacuum (Speed Vac Concentrator; Savant, Farmingdale, N.Y.), and resuspended in 20 μl of nuclease-free water. A reverse transcription system (Promega, Madison, Wis.) was used to prepare cDNA from RNA. The manufacturer's procedure was modified to include RNA denaturation as follows. RNase-free water, 1 μg of total RNA, and 1 μl of oligo(dT) were mixed for a final volume of 10 μl and heated at 65°C for 5 min and then quenched on ice for 5 min. Heated sample tubes were centrifuged for 5 min at 12,000 × *g* (4°C) to bring down any condensation. In a fresh 1.5-ml tube, a master mixture was then prepared that consisted of the following: 0.75 μl of H_2O; 2 μl of 10× reverse transcription buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 [pH 8.8]); 4 μl of 25 mM $MgCl_2$; 2 μl of a deoxynucleoside triphosphate mixture (10 mM); 5) 0.5 μl of RNasin inhibitor; and 0.75 μl of avian myeloblastosis virus reverse transcriptase (25,000 U/ml). A separate mixture was prepared with 0.75 μl of RNase-free water in place of the 0.75 μl of reverse transcriptase as a control. An aliquot of 10 μl from the master mixture was then added to the oligo(dT)-template RNA mixture and incubated at 37°C for 1 h in a water bath. The reaction mixture was then heated at 90°C for 5 min and cooled on ice. Samples were centrifuged again for 5 min at 12,000 × *g* (4°C).

PCR. Primers for PCR of iNOS were obtained from Clontech (Palo Alto, Calif.). Glyceraldehyde-3-phosphate dehydrogenase (G3PHD) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) primers were used for reverse transcription PCR amplification for each sample to compare equal quantities of RNA and control for cDNA synthesis efficiency. Sequence information pertaining to housekeeping gene mRNA primers for HPRT was obtained from a published source (46) or for G3PDH (10a). For labelling of amplified DNA fragments, a digoxigenin-coupled dUTP (Boehringer Mannheim, Indianapolis, Ind.) was incorporated directly into the PCR. Reaction components (Perkin Elmer, Norwalk, Conn.) were combined in the following order: 77 μl of nuclease-free water; 10 μl of 10× reaction buffer; 2 μl (each) of 10 mM deoxynucleoside triphosphates except 1.95 μl of dTTP; 0.5 μl of digoxigenin-coupled dUTP (1 mM); 1 μl of sense primers and 1 μl of antisense primers (100 μM); and 0.5 μl of AmpliTaq DNA polymerase (5 U/μl). The reaction mixture was split between two tubes, each containing 49 μl to which 1 μl of the reverse transcriptase mixture was added. The PCR was carried out at different cycle numbers to obtain results greater than the level of detection but below the point of PCR plateau.

PCR product detection and quantitation. After it was established that a single PCR product was present by agarose gel electrophoresis, PCR products were applied directly to a nylon membrane in a 96-well filtration manifold (Schleicher & Schuell, Keene, N.H.) and then washed with Tris-borate-EDTA. DNA was then UV cross-linked to the nylon membrane with a Stratalinker 1800 (Stratagene, La Jolla, Calif.). Digoxigenin-dUTP-labelled PCR fragments were detected on the nylon membrane by a chemiluminescent antidigoxigenin alkaline phosphatase-conjugated antibody detection system as provided by Boehringer Mannheim. PCR products were detected by making multiple X-ray film exposures (XAR-2; Kodak, Rochester, N.Y.) ranging from 1 min to overnight at room temperature. Exposed films were scanned by an IBAS imaging system (Roche Image Analysis Systems Inc., Elon College, N.C.). Numerical data corresponding to the total number of pixels in any given band were obtained and referred to as optical density. To obtain relative comparisons of steady-state levels of mRNA, values obtained were normalized against the corresponding levels of housekeeping gene expression so that equivalent amounts of RNA were being assayed. Measurements for specific mRNA expression were adjusted to equal the highest concentration of RNA, as determined by G3PDH expression. HPRT levels for each sample were also measured to verify the G3PDH measure of RNA.

Cytofluorometry. The levels of auto- and background fluorescence of cell populations were determined by omitting either primary and secondary antibodies or primary antibody, respectively. Fluorescence labelling of cells for flow cytometric analysis of IFN-γ-induced endothelial class II MHC surface expression was accomplished as previously described (27) with mouse anti-Ia^k IgG2b as the primary antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse $F(ab)_2$ for detection. An irrelevant mouse IgG (Vector Laboratories) was used as a irrelevant control primary antibody. Stained cells were analyzed by a Becton-Dickinson FACScan (San Jose, Calif.).

Statistics. Values are expressed as the means ± standard errors of the means (SEMs), unless otherwise noted. When it was necessary to determine whether or not differences for more than one group were statistically significant, homogeneity of variances was tested. A one-way analysis of variance followed by a Tukey post hoc comparison of means was used with homogeneous data sets. For heterogeneous data sets, a Kruskal-Wallis nonparametric one-way analysis of variance was used. *P* values of <0.05 were considered statistically significant.

RESULTS

Dose response of macrophage and endothelial cells to SEB and LPS. Purified SEB was negative for endotoxin as described in Materials and Methods at a sensitivity level of 0.25 ng of LPS per mg of protein. The LPS used in these studies was extracted by trichloroacetic acid (34) and previously shown to induce the production of NO (47) in cells of C3H/HeJ origin. Endothelial nitrite production elicited by SEB did not differ significantly from that elicited by LPS (Fig. 1A) with respect to the level produced or the shape of the dose-response curve. We obtained similar results with the endotoxin-responsive macrophage cell line RAW 264.7 (Fig. 1B), although the levels were approximately fivefold higher. Cell viability was unaffected by either SEB or LPS within the dose ranges shown.

Antagonism of nitrite production. Endothelial cells express both constitutive NOS (31) and iNOS (17). TFP, a calmodulin antagonist that inhibits the constitutive (17, 43), but not the inducible enzyme (12, 36), had no detectable effect on nitrite

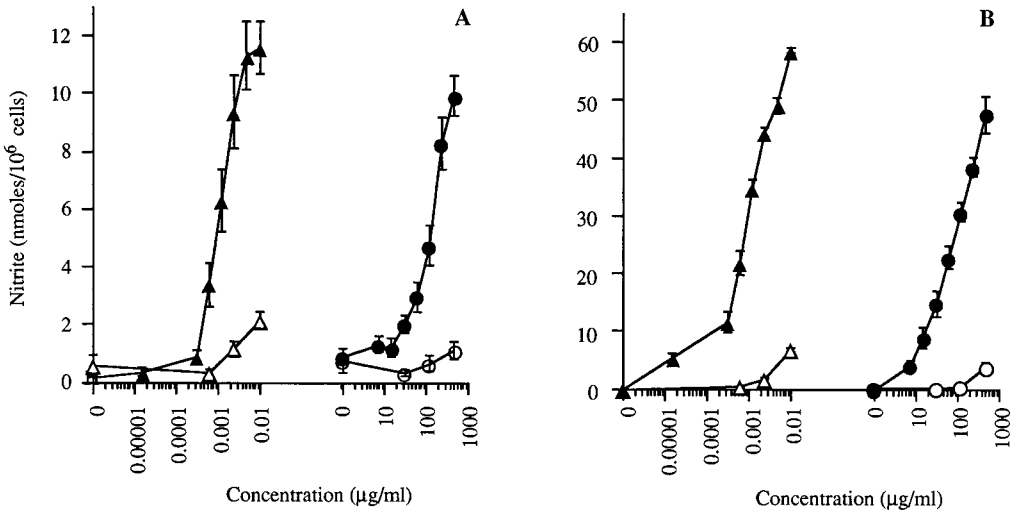

FIG. 1. Dose-response curves for SEB- and LPS-elicited nitrite production by endothelial and macrophage cells. Adherent cells were incubated with the indicated concentrations of SEB (○) or LPS (△) alone or with 10 U of IFN-γ per ml (SEB [●]; LPS [▲]) for 15 h. The datum points shown represent the means ± SEMs of four separate experiments done in triplicate. The ordinate values for endothelial cells (A) are one-fifth of those of the macrophage cells (B), since endothelial nitrite production was typically lower. Nitrite production levels stabilized at concentrations of LPS of >10 ng/ml or SEB of >500 μg/ml. The 50% effective doses ± SEMs derived from log dose-response data from four experiments for SEB- and LPS-induced nitrite production in IFN-γ-primed endothelial cells were 81 ± 1 μg/ml and 1 ± 0.9 ng/ml, respectively. For the IFN-γ-primed macrophage cells, these values were 73 ± 1 μg/ml and 1 ± 0.9 ng/ml, respectively. The correlation coefficients were >0.90.

production (Fig. 2E and F). The L-arginine analog L-NAA significantly reduced nitrite production by both endothelial and macrophages (Fig. 2C and D) but had no effect on induced TNF-α production by macrophage cells (data not shown). Monospecific chicken anti-SEB antibody significantly reduced SEB-induced nitrite production in a dose-dependent manner in endothelial and macrophage cells (Fig. 2A and B) but did not significantly alter the response to LPS, which indicated the response was specific for SEB. The rat MAb GR-20 recognizes an epitope in the ligand-binding domain of the mouse IFN-γ receptor and blocks binding and biological function of this cytokine (1, 27). We used this antibody to abrogate the effects of IFN-γ. A rat IgG2a MAb, GR-3, that recognizes an antigen on the mouse IFN-γ receptor but does not interfere with ligand binding or biological function was purified in a similar manner and used as a control. Priming cells with IFN-γ was necessary, because the MAb GR-20 effectively blocked induced endothelial nitrite production (Table 1).

Time course characteristics of iNOS. As shown in Fig. 1, the nitrite levels in activated endothelial cell culture supernatants after 15 h were approximately five times lower than those seen in activated macrophages. Because of this, we examined differences in nitrite production over time. Although both endothelial and macrophage cells express iNOS, temporal responses differed markedly. Both cell types exhibited a lag phase of approximately 4 h, followed by increased output of NO (Fig. 3A and B). While macrophage nitrite production continued to increase, endothelial nitrite concentrations typically began to decrease between 14 and 24 h. On the basis of these observations, we evaluated the temporal response of mRNA for iNOS. As shown in Fig. 4, the kinetic profile varied significantly between macrophage and endothelial cells. Message levels in the macrophages were detectable at 2 h and continued to increase over 24 h. In contrast, endothelial message levels were maximal by 2 h and then began to decline to almost undetectable levels by 24 h. A linear relationship between serially diluted template endothelial and macrophage mRNAs and PCR products isolated at 16 h was demonstrated (data not shown).

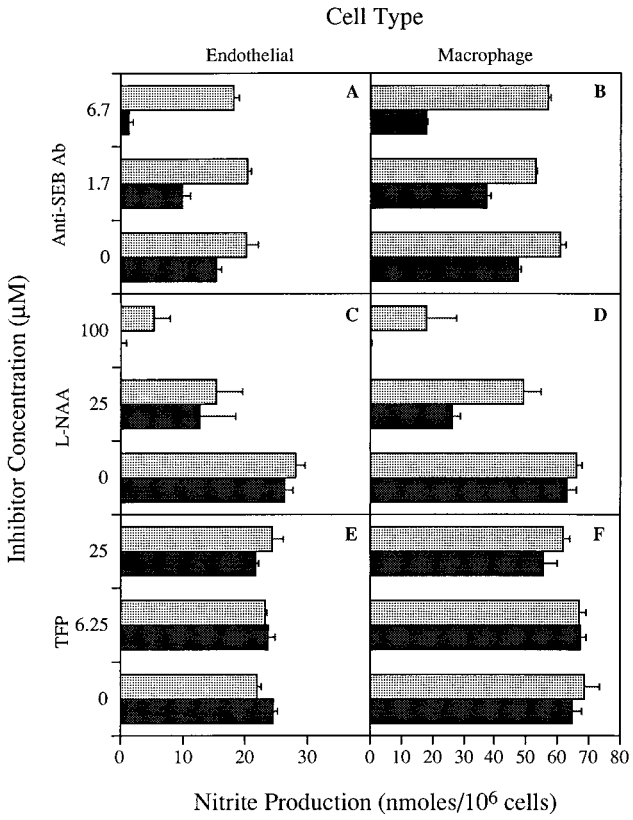

FIG. 2. Effect of anti-SEB antibody, arginine analog antagonism (L-NAA), or calmodulin inhibition (TFP) on nitrite production in endothelial and macrophage cells. Adherent cells were incubated for 15 h with SEB (100 μg/ml) (■) or LPS (2 ng/ml) (▨) and IFN-γ (10 U/ml) in the presence of various concentrations of either monospecific anti-SEB antibody (Ab) (A and B), L-NAA (C and D), or TFP (E and F) as indicated. Supernatants were assayed for nitrite. Results shown for endothelial cells (A, C, and E) and macrophage cells (B, D, and F) are the means ± SEMs from a representative experiment done in triplicate.

TABLE 1. Effects of anti-mouse IFN-γ receptor antibodies on SEB-elicited endothelial NO production[a]

Antibody (62.5 nM)	Nitrite concn (mean ± SEM) (nmol/10^6 cells) elicited by:	
	SEB	LPS
None	7.1 ± 0.49	5.6 ± 0.34
GR-20	0.9 ± 0.14[b]	0.2 ± 0.16[b]
GR-3	5.6 ± 0.31	5.1 ± 1.10

[a] Cells were primed with 10 U of IFN-γ per ml as described in Materials and Methods. The concentrations of SEB and LPS used for activation were 100 μg/ml and 1 ng/ml, respectively.

[b] Differences in nitrite production from control (no antibody, medium alone) were significant at $P < 0.05$.

Regulation of endothelial SEB-induced NOS expression. Protein synthesis is reported to be required for transcriptional activation of iNOS by macrophages (29) and hepatocytes (15). The requirement for endothelial cell protein synthesis for transcriptional activation of iNOS was evaluated and found to be similar. Adding the protein synthesis inhibitor cycloheximide decreases endothelial iNOS mRNA, as does the transcriptional inhibitor actinomycin D (Fig. 5). Reductions in mRNA levels corresponded with subsequent reduction in nitrite production. Neither SEB nor LPS was capable of triggering nitrite production over the dose ranges used (Fig. 1) or time period evaluated (Fig. 3). Neither IFN-γ nor SEB alone induced significant endothelial nitrite production, although SEB alone significantly ($P < 0.05$) increased iNOS mRNA levels (Fig. 5 and Table 2) while IFN-γ did not (Table 2).

Niacinamide and IL-10, two compounds previously reported to modulate the effects of IFN-γ-induced class II MHC expression (9, 40), were further evaluated with regards to their effect on SEB-elicited endothelial iNOS. As can be seen in Fig. 5, both IL-10 and niacinamide reduced endothelial SEB-elicited nitrite production. Niacinamide significantly reduced SEB-elicited iNOS mRNA levels while IL-10 did not (Table 2). A dose response was consistently noted with niacinamide but not with IL-10. Also, niacinamide diminished endothelial IFN-γ-induced class II MHC expression (Fig. 6), while IL-10 had no effect at concentrations up to 40 U/ml.

DISCUSSION

Endothelial interaction with the SE through the MHC class II surface antigen can trigger the production of mediators such as NO, important in disorders caused by the SE, and can allow for toxin interaction with the T-cell receptor. In this study, we first characterized the ability of SEB to activate microvascular endothelial cells and induce the production of NO. We then examined the time course, regulation, and modulation of this response. Results described here show that the SEB-elicited response in IFN-γ-primed endothelial cells is comparable to that previously described for LPS (42), but the response and transcriptional regulation of iNOS mRNA differed markedly from those previously described for the macrophage cell line (29) or hepatocytes (15).

Endothelial cells express constitutive and inducible forms of NOS (13). In contrast to the constitutive enzyme, iNOS is not regulated by calcium or calmodulin. TFP, a calmodulin inhibitor previously shown to block enzymatic activity of constitutive NOS (5), had no effect on SEB induction of endothelial NOS. Formation of NO by either isoform of NOS requires L-arginine as a substrate. The L-arginine analog L-NAA inhibited SEB-induced nitrite production in a dose-dependent manner in both endothelial and macrophage cells. The induction of NOS enzymatic activity by SEB, but not LPS, could also be blocked with the polyclonal anti-SEB antibody. These results suggest that the production of NO by IFN-γ-primed endothelial cells in response to SEB was not due to increased activity of the constitutive enzyme, depended on the presence of L-arginine, and could be neutralized by SEB-specific antibodies.

Activation of endothelial cells and subsequent iNOS activity induced by SEB depended on IFN-γ priming. If IFN-γ was absent or if the biological effects of IFN-γ with neutralizing antibody against its receptor were blocked, SEB-induced NO production was negligible. Priming cells with IFN-γ alone induced only very low levels of iNOS mRNA and negligible enzymatic activity in endothelial cells. In contrast, SEB alone induced transcriptional activation of endothelial iNOS mRNA, as has been described for other monokines (48). Although detectable message was induced, SEB alone did not elicit detectable nitrite production. Cotreatment with IFN-γ and SEB augmented message levels produced by SEB and synergisti-

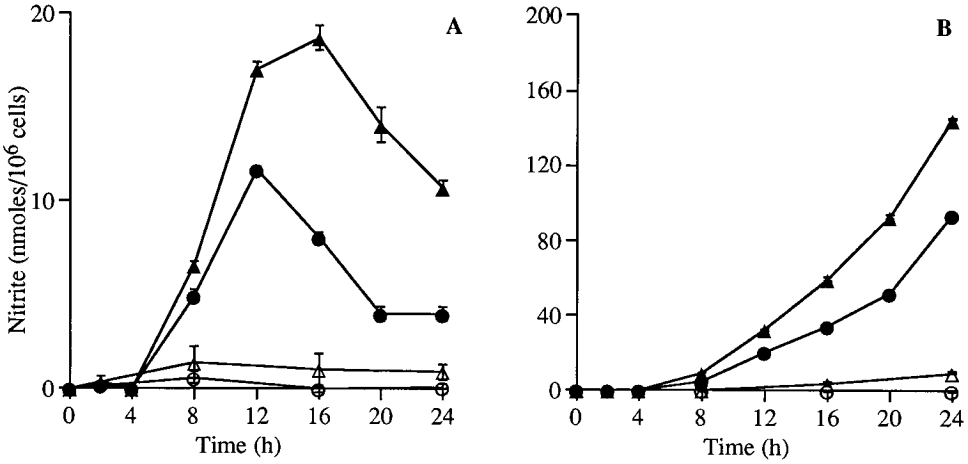

FIG. 3. Time course of SEB- or LPS-elicited nitrite production in endothelial (A) and macrophage cells (B). Cells were incubated with SEB (100 μg/ml) (○) or LPS (2 ng/ml) (△) alone or with 10 U of IFN-γ per ml (SEB [●]; LPS [▲]) for the duration indicated for each point up to 24 h. Points represent the means ± SEMs of nitrite concentrations from a representative experiment done in triplicate. Toxin and IFN-γ were present throughout the time indicated for each point. Culture supernatants were assayed for nitrite content, as described in Materials and Methods. Ordinate values for endothelial cells are one-tenth of those of the macrophage cells, since endothelial nitrite production was proportionally lower.

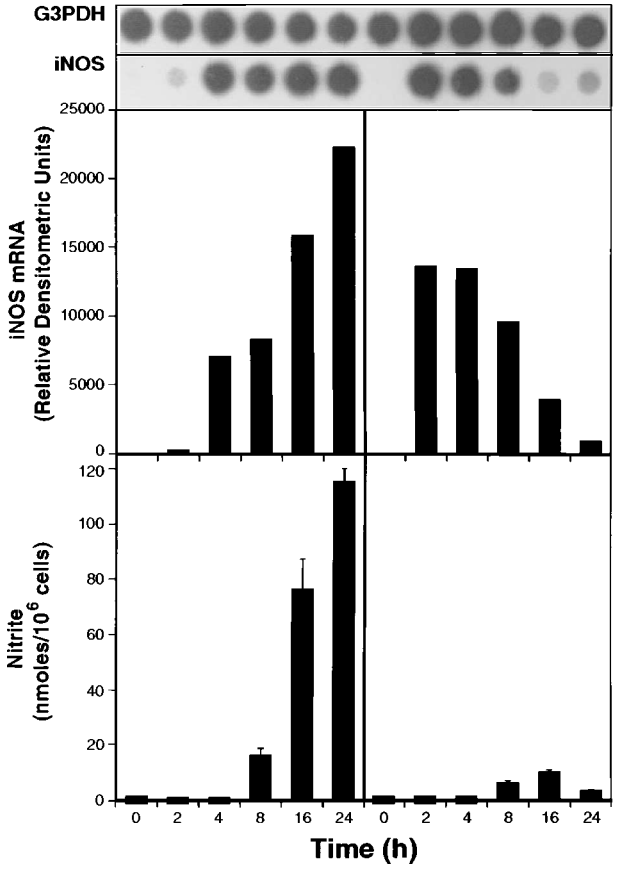

FIG. 4. Time course of SEB-elicited mRNA for iNOS by endothelial (right) and macrophage cells (left). Total RNA was isolated from stimulated cells, reverse transcribed, subjected to 20 PCR cycles, and detected as described in Materials and Methods. The relative levels of iNOS mRNA were determined after normalization to the respective G3PDH (housekeeping gene) signal to account for variability in the amounts of mRNA analyzed by PCR. A representative autofluorogram from a dot blot is shown in the upper panel. Results from densitometric analysis of the means from two experiments are presented as histograms in the center panel. The corresponding nitrite production levels are shown in the lower panels. As shown in Fig. 3, SEB (100 μg/ml) alone did not stimulate significant nitrite production over 24 h.

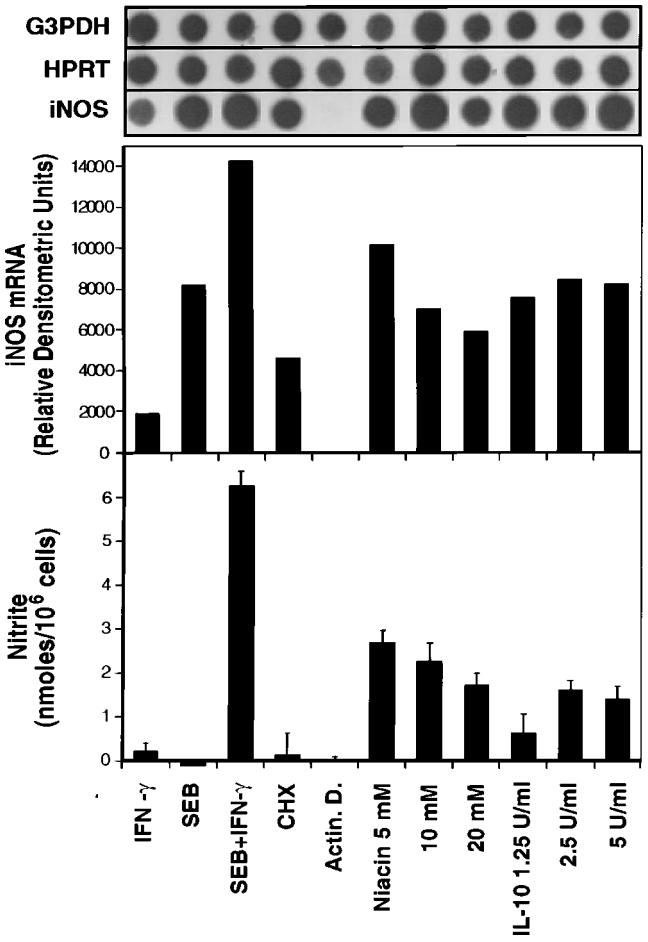

FIG. 5. Effects of cycloheximide, actinomycin D, niacinamide, and IL-10 on SEB-induced endothelial nitrite production and iNOS mRNA. To assess the effect of cycloheximide (CHX), actinomycin D (Actin. D.), niacinamide, or IL-10 on endothelial nitrite production, cells were incubated with SEB (100 μg/ml) and IFN-γ (10 U/ml) with or without the various concentrations of the test reagent shown. The upper histogram represents the corresponding effect of these agents on endothelial iNOS mRNA as analyzed by reverse transcription PCR as described in the legend to Fig. 4. Values shown are relative levels of iNOS mRNA determined after normalization to the respective G3PDH (housekeeping gene) signal to account for variability in the amounts of mRNA analyzed. The corresponding supernatant nitrite concentrations were determined (lower histogram). Values shown are the means ± SEMs of nitrite concentrations of a representative experiment done in triplicate.

cally increased the levels of nitrite produced. The need for endothelial cell priming with IFN-γ to induce production of NO has been described for TNF, IL-1, and endotoxin (21, 26). Further, IFN-γ has been shown to be the time-dependent trigger for endotoxin-induced NO synthesis by endothelial cells (52). Two regulator upstream regions of the macrophage iNOS gene have recently been described (30), one of which mediates IFN-γ regulation. One might expect similar positive and negative regulatory elements in the 5′-flanking region of the endothelial iNOS gene.

The kinetics of endothelial SEB-elicited iNOS activity differed considerably from that of the macrophage. In contrast to the macrophage response, endothelial iNOS mRNA levels peaked within 2 h and then steadily declined to baseline levels over 24 h. For both cell lines, nitrite levels were not detectable until 4 h after stimulation and 2 to 4 h after mRNA detection. Although endothelial iNOS mRNA levels after 2 h were comparable to that of the macrophage at 16 h, enzymatic activity was modest compared with that of the macrophage. The expression of endothelial iNOS must be tightly regulated to prevent damage to surrounding tissues. Recently, it has been shown that endothelial cell constitutive NO production is suppressed under the inflammatory induction of iNOS stimulated by LPS and IFN-γ (52). The likely coregulatory effect of constitutive NO synthesis on induced NOS needs to be further evaluated. The strong induction of iNOS mRNA within only a few hours suggests that the regulation of expression is primarily transcriptional. Inhibition of iNOS mRNA and nitrite production by actinomycin D supports this concept. Induction of iNOS by SEB in IFN-γ-primed endothelial cells depended on de novo protein synthesis on the basis of the finding that cycloheximide significantly suppressed iNOS mRNA levels and nitrite production in activated cells. This result is consistent with previous reports for macrophage (29) and hepatocyte (15) origin iNOS. Radomski et al. (42) demonstrated that the protein synthesis inhibitor, cycloheximide, inhibits the expression of the endothelial induced but not constitutive NOS.

Interferon-γ acts on the endothelium in a unique manner in that it regulates the expression of MHC class II surface anti-

TABLE 2. Effects of niacinamide and IL-10 on SEB-elicited endothelial inducible NOS mRNA levels

Treatment	% Maximal NOS mRNA response (mean ± SEM) normalized to[a]:	
	G3PDH	HPRT
Medium alone	7.0 ± 4.7	12.8 ± 9.1
IFN-γ alone (10 U/ml)	12.0 ± 6.0	19.1 ± 9.5
SEB alone (100 μg/ml)	56.7 ± 2.0	77.1 ± 6.5
Niacin (20 mM)	23.8 ± 12.0	37.0 ± 17.6
IL-10 (5 U/ml)	61.8 ± 23.4[b]	72.2 ± 18.3

[a] The values shown represent averages of results obtained from three independent experiments. Relative levels of iNOS mRNA were determined after normalization to either G3PDH or HPRT signal to account for variability in the amounts of mRNA analyzed by PCR as described in Materials and Methods. In each experiment, maximal mRNA levels were elicited by 10 U of IFN-γ per ml and 100 μg of SEB per ml. This value was used as the maximal (100%) response within each experiment. Paired-sample *t* tests on G3PDH versus HPRT-normalized values showed no significant difference ($P < 0.05$) within treatment groups. The doses of niacinamide or IL-10 used did not significantly alter cell viability or proliferative response.

[b] Levels of iNOS mRNA did not differ significantly ($P < 0.05$) from that induced by 10 U of IFN-γ per ml and 100 μg of SEB per ml, as determined by a Kruskal-Wallis one-way analysis of variance. All other treatment effects on iNOS mRNA shown were significantly reduced.

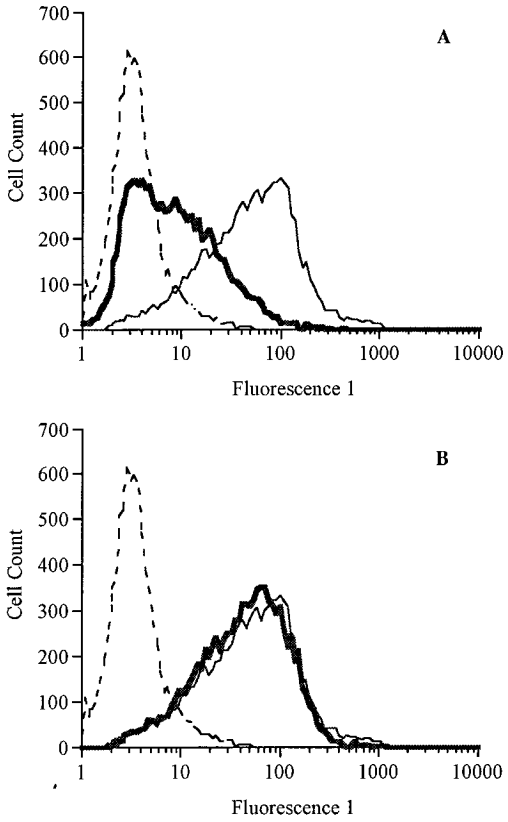

FIG. 6. Measurement of niacinamide and IL-10 effects on IFN-γ-induced endothelial cell surface expression of MHC class II antigen. Endothelial cells were treated for 72 h with 100 U of IFN-γ per ml either alone (——) or with 20 mM niacinamide (A, ▬▬) or 40 U of IL-10 per ml (B, ▬▬). The dashed line represents incubation with medium alone. Cells were analyzed for MHC class II surface antigen expression flow cytometrically as described in Materials and Methods. Fluorescence levels detected with the control IgG as the primary antibody in IFN-γ-stimulated cells were not significantly different from medium-treated cells stained with the anti-Ia^k primary antibody or when the secondary fluorescein isothiocyanate-tagged anti-mouse IgG was omitted.

gen, a receptor protein for the SE (49, 50). Additionally, upon binding to MHC class II antigens, the SE induce the production of a number of monokines (18, 48) and, as reported here, NO. IL-10 has been reported to down-regulate class II MHC expression in monocytes (9), suppress macrophage NO and TNF-α production (3), reduce the in vivo release of TNF-α, and prevent lethality in experimental endotoxemia. Niacinamide has immunomodulating activity in experimental autoimmune models and reduces IFN-γ-induced class II MHC expression in endothelial cells (40). Also, IL-10 suppresses macrophage iNOS activity and protects mice from SEB-induced lethal shock (2). Additionally, it has been shown to augment macrophage IFN-γ- and LPS-induced iNOS mRNA (6) levels. In this study, we found that niacinamide significantly repressed endothelial SEB-induced iNOS mRNA at the transcriptional level and subsequent nitrite production. IFN-γ-induced MHC class II expression on endothelial cells was also reduced in the presence of niacinamide. The reduction in iNOS activity was probably not due entirely to decreased SEB-MHC class II interaction, since significant levels of iNOS transcription were detectable within hours and niacinamide also repressed LPS elicited iNOS (data not shown). In contrast, IL-10 did not consistently reduce SEB-elicited iNOS mRNA but did reduce its enzymatic product in endothelial cells. The effect of IL-10 had a narrow range of dose dependency, with a maximal response between 1 and 5 U/ml (approximately). IFN-γ-induced expression of endothelial MHC class II antigen was not significantly altered by IL-10, even at levels 40 times that where biological activity was noted. Reports on the effect of IL-10 on macrophage MHC class II induction differ (9, 11). It appears that IL-10 may affect accessory cell function in a manner independent of MHC class II–T-cell receptor interactions.

The role of endothelial cell-derived NO as a mediator in SE intoxication has yet to be clearly defined (38). Immunologically generated NO from various cell types alters leukocyte and platelet adhesion (25) and function (20), modulates microvascular permeability (24), and is cytotoxic at high levels (28). These factors may contribute to the vasculitis and perivascular edema associated with septic shock. Specific inhibition of iNOS by L-arginine analogs such as L-NAA is protective in cytokine- and endotoxin-mediated shock (22, 23). The results of this study suggest that SEB directly interacts with IFN-γ-primed endothelial cells to elicit the production of NO. Transcriptional activation of endothelial iNOS occurred several hours earlier than that previously reported for other cell types. Although detectable nitrite levels in culture did not occur until approximately 4 h, levels of NO physiologically active at the vascular level are far less than the sensitivity of the assay used (ca. 0.5 μM). Presumably, levels of SEB-induced NO found at the vascular level were produced within 2 h. Further, endothelial responses were regulated more tightly at the transcriptional level than that of the macrophage. Levels of endothelial iNOS mRNA peaked within 2 h and steadily declined thereafter, even in the presence of sustained stimulation. This decline may reflect a functional difference between the two cell types of NO as an inflammatory mediator. In view of the important physiological role of NO in the vascular system, modulation cellular activation by microbial superantigens like the SE and subsequent NO production might prove to have clinical importance. Substrate analogs and immunomodulators described in this report and elsewhere (44) may prove beneficial.

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