INFECTION AND IMMUNITY, Dec. 1993, p. 5333–5338
0019-9567/93/125333-06$02.00/0

Vol. 61, No. 12

Toxicity of Staphylococcal Enterotoxins Potentiated by Lipopolysaccharide: Major Histocompatibility Complex Class II Molecule Dependency and Cytokine Release

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Received 23 June 1993/Returned for modification 13 August 1993/Accepted 21 September 1993

The biological effects of staphylococcal enterotoxins (SE), potentiated by bacterial lipopolysaccharide (LPS), were studied with mice. Control animals survived the maximum dose of either SE or LPS, while mice receiving both agents died. SEA was 43-fold more potent than SEB and 20-fold more potent than SEC_1. The mechanism of toxicity was further examined with transgenic mice deficient in major histocompatibility complex class I or II expression. Class II-deficient mice were resistant to SEA or SEB. However, class I-deficient animals were less susceptible to SEA (30% lethality) than wild-type mice (93% lethality). In vitro stimulation of T cells from the three mouse phenotypes by SEA correlated well with toxicity. T cells from transgenic or wild-type mice were similarly responsive to SEA when presented by irradiated, wild-type mononuclear cells. These data confirmed that the toxicity of SE was mainly exerted through a mechanism dependent on the expression of major histocompatibility complex class II molecules. Toxicity was also linked to stimulated cytokine release. Levels in serum of tumor necrosis factor alpha, interleukin-6, and gamma interferon peaked 2 to 4 h after the potentiating dose of LPS but returned to normal within 10 h. Concentrations of interleukin-1α were also maximal after 2 h but remained above the background for up to 22 h. Relative to the levels in mice given only SEA or LPS, the levels in serum of tumor necrosis factor alpha, interleukin-6, and gamma interferon increased 5-, 10-, and 15-fold, respectively, after injections of SEA plus LPS. There was only an additive effect of SEA and LPS on interleukin-1α concentrations.

Staphylococcal enterotoxins (SE) are 26- to 30-kDa proteins that are produced by *Staphylococcus aureus* and are responsible for most common cases of human food poisoning (1, 12). Different toxin serotypes, such as SEA, SEB, and SEC_1, share considerable amino acid sequence homology (24). Molecular structure data from X-ray crystallography of SEB (28) and circular dichroism spectroscopy of SEA, SEB, and SEC_1 (18) suggest that SE are conformationally similar. SE were originally defined by type-specific antibodies; however, common epitopes are shared (24, 25).

SE bind to major histocompatibility complex (MHC) class II molecules, and the toxin-class II complex subsequently stimulates T cells (5). The release of cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), and the ensuing sequelae are associated with the toxic effects of SE (11, 19). SE, certain viral proteins, and other bacterial toxins, such as *S. aureus* toxic shock syndrome toxin 1 (TSST-1), *Streptococcus pyogenes* pyrogenic exotoxins A to C, and *Clostridium perfringens* type A enterotoxin, are commonly called superantigens because they engage T-cell antigen receptors at exceptionally low concentrations (3, 17). Each superantigen stimulates T cells bearing a distinct V_β subset of antigen receptors (17). Collectively, these proteins share very little amino acid sequence homology with SE or each other.

SE affinities for MHC class II receptors are considerably lower in nonprimates (1, 30a). Nonetheless, mice have been used in toxicity assays, such as those measuring weight loss (16), or in other studies, such as those of mortality after a potentiating dose of D-galactosamine (19). The biological effects of SE, TSST-1, and streptococcal pyrogenic toxins are also enhanced by lipopolysaccharide (LPS) (22, 26, 27). LPS stimulates the production and/or release of various cytokines, including potentially lethal amounts of TNF-α, gamma interferon (IFN-γ), and IL-1 (2, 9, 20).

In this study, we examined the mechanism of toxicity when SE were coadministered with LPS. MHC class II-deficient mice were resistant to a lethal dose of SE and LPS, while MHC class I-deficient or wild-type animals were susceptible, confirming that the biological activity of SE was dependent on MHC class II molecules. It was of interest that SEA and LPS greatly increased levels in serum of TNF-α, IL-6, and IFN-γ relative to those in mice injected with only SEA or LPS. Taken together, these results indicate that the toxicity of SE can be potentiated by LPS and directly reflects the biological activity of bacterial superantigens.

MATERIALS AND METHODS

SE and LPS. Purified SEA, SEB, and SEC_1 were obtained from Toxin Technology (Sarasota, Fla.) and stored at −50°C. SE preparations contained <1% (wt/wt) endotoxin, as determined by a *Limulus* lysate assay (Sigma Chemical Co., St. Louis, Mo.). LPS B from *Escherichia coli* (O55:B5) contained 6.4% lipid A (Difco Laboratories, Detroit, Mich.) and was reconstituted with sterile phosphate-buffered sa-

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line, pH 7.4 (PBS), to a 5-mg/ml concentration and kept at 4°C.

LPS potentiation of SE toxicity in mice. C57BL/6 mice weighing 18 to 20 g (Harlan Sprague-Dawley, Inc., Frederick Cancer Research and Development Center, Frederick, Md.) were each injected intraperitoneally with 200 μl of PBS containing various amounts of SEA, SEB, or SEC_1 and then injected 4 h later with 150 μg of LPS (200-μl intraperitoneal injection). Controls were each injected with either SE (30 μg) or LPS (150 μg). Animals were observed for 72 h after the LPS injection. Calculations of 50% lethal doses were done by probit analysis with 95% fiducial limits (SAS Institute Inc., Cary, N.C.).

The biological effects of SEA and SEB were also tested with transgenic C57BL/6 mice (GenPharm International, Mountain View, Calif.) deficient in MHC class I or II expression (8, 32) as described above with a single dose of toxin (30 μg per mouse). Genetic homozygosity was confirmed by Southern analysis of parental tail DNA with β_2-microglobulin and MHC class IIβ DNA probes.

Detection of cytokines in serum. Mice (18 per group) were injected with SEA (10 μg), LPS (150 μg), or SEA plus LPS. Sera were collected and pooled from three mice per group at each time point (2, 4, 6, 8, 10, and 22 h) after LPS injection. Sera were collected at various times following SEA injection (−4 h for data tabulation). Collection of LPS control sera began at the time of injection (0 h).

Levels in serum of TNF-α and IL-1α were detected by an enzyme-linked immunosorbent assay (ELISA). TNF-α was first captured by use of a monoclonal antibody against mouse TNF-α (GIBCO-BRL, Grand Island, N.Y.) and then incubated with rabbit anti-mouse TNF-α antibody (Genzyme, Boston, Mass.). The ELISA plate was washed, and a peroxidase conjugate of anti-rabbit antibody (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to the wells. After the plate was washed and the substrate (Kirkegaard and Perry, Gaithersburg, Md.) was added, TNF-α concentrations were measured by use of the mean A_{450} reading for duplicate samples and a standard curve generated from recombinant mouse TNF-α (GIBCO-BRL). Levels in serum of IL-1α were determined from the mean reading of duplicate samples with an ELISA kit that specifically detects murine IL-1α (Genzyme). The standard error of the mean (SEM) for TNF-α and IL-1α readings was ±5%.

IL-6 and IFN-γ levels were measured by bioassays (13). An IL-6-dependent cell line, 7TD1, was used in a proliferation assay with serial twofold dilutions of serum samples assayed in triplicate. The proliferation of 7TD1 cells in a microtiter plate was measured on the basis of uptake of [^{3}H]thymidine (1 μCi per well; Amersham, Arlington Heights, Ill.), and the activity of IL-6 in serum was compared with that of recombinant mouse IL-6 standard (R and D Systems, Minneapolis, Minn.) as previously described (13). The SEM for triplicate samples was ±10%.

IFN-γ was measured on the basis of the reduction of vesicular stomatitis virus (New Jersey strain) cytopathic effects on L929 cells as previously described (30). In brief, serial twofold dilutions of serum were made in duplicate and added to microtiter wells containing L929 cells (5×10^4 per well). After incubation for 24 h, virus (5×10^5 PFU per well) was added, and the cytopathic effects were measured at 48 h by taking A_{570} readings of reduced 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma). The activity in each serum sample was determined with recombinant mouse IFN-γ as a standard (Biosource, Camarillo, Calif.). The SEM for duplicate samples was ±5%.

TABLE 1. Titration of SEA, SEB, and SEC_1 in the C57BL/6 mouse lethality assay

Stimulus[a]	% Lethality (no. of mice tested) with the following dose of SE, in micrograms/mouse[b]:			
	30	10	1	0.1
SEA + LPS	93 (15)[b]	85 (20)	80 (15)	20 (10)
SEB + LPS	80 (15)	27 (15)	0 (15)	0 (15)
SEC_1 + LPS	80 (10)	60 (10)	10 (10)	0 (10)

[a] LPS was injected into each mouse (150 μg) 4 h after the SE injection. Control mice injected with 150 μg of LPS (n = 20) or 30 μg of SEA, SEB, or SEC_1 (n = 10) survived.

[b] Results are from a combination of separate experiments with five mice per experiment.

T-cell stimulation assays. Untreated mice were euthanized, and spleens were removed aseptically. Single-cell suspensions were prepared with RPMI 1640 tissue culture medium, and splenocytes were washed three times with RPMI 1640. The cell pellet was suspended in 6 ml of medium, layered onto 3 ml of Histopaque 1119 (Sigma), and centrifuged at 400 × g for 40 min. Mononuclear cells were harvested from the medium-Histopaque interface and washed three times by centrifugation. The cells were resuspended in medium containing 10% heat-inactivated fetal bovine serum and counted, and viability was determined (>95%) by Trypan blue dye exclusion. Mononuclear cells were added to 96-well plates (10^6 cells per well). For some experiments, additional antigen-presenting mononuclear cells (irradiated with 1,500 rads) were obtained from naive, wild-type C57BL/6 mice. An equal number of responding mononuclear cells was cocultured in a 96-well plate with irradiated antigen-presenting mononuclear cells (10^6 total cells per well). SEA-stimulated cultures were incubated for 72 h at 37°C (5% CO_2, 95% humidity) and then labeled with 1 μCi of [^{3}H]thymidine per well for an additional 10 h. Cells were harvested onto glass fiber filters, and incorporated [^{3}H]thymidine was measured by liquid scintillation.

RESULTS

Biological activity of SE potentiated by LPS. In comparison with primates, mice are not very susceptible to the toxic effects of SE, and we therefore sought to increase sensitivity with a potentiating dose of LPS. There was no apparent effect in control animals injected with any of the SE (up to 30 μg per mouse) or LPS (150 μg per mouse) alone (Table 1). Incremental injections of LPS were also not lethal when given in amounts of up to 250 μg per mouse (data not shown). However, mice died between 24 and 48 h after SE and LPS were given to the same animal (Table 1). SEA was much more toxic than either SEB or SEC_1, and the calculated 50% lethal doses of SEA, SEB, and SEC_1 (95% fiducial limits) were 18.5 (6.5 to 38.5), 789.0 (582.5 to 1,044.5), and 369.0 (197.5 to 676.0) μg of toxin per kg, respectively.

MHC class II dependency. The role of MHC class I and class II molecules in SE toxicity, potentiated by LPS, was addressed by use of transgenic MHC-deficient mice (Table 2). Class II-deficient animals were unaffected by a dose of SE (30 μg) plus LPS (150 μg) that was lethal for 93% of wild-type and 30% of class I-deficient mice. Mononuclear cells from class II-deficient animals were not able to present SEA, as measured by proliferative responses. MHC class I-deficient cells were functional in supporting T-cell proliferation but at levels <30% of the proliferative response

TABLE 2. Lethality of SEA and SEB in C57BL/6 mice lacking MHC class I or class II

Stimulus[a]	% Lethality (no. of mice tested) in mice with the following MHC class phenotype		
	$I^- II^+$	$I^+ II^-$	$I^+ II^+$
SEA + LPS	30 (10)	0 (5)	93 (15)
SEB + LPS	ND[b]	0 (5)	80 (15)
SEA only	0 (2)	0 (2)	0 (2)
SEB only	ND[b]	0 (2)	0 (2)
LPS only	0 (5)	0 (5)	0 (5)

[a] Mice were injected with 30 μg of SEA or SEB and, 4 h later, with 150 μg of LPS, as indicated. Control mice were injected with only SEA, SEB, or LPS.
[b] ND, not determined.

supported by wild-type MHC-presenting cells (Fig. 1A). Cell surface expression levels were normal, when compared with those in nontransgenic C57BL/6 mice, for A^b in class I-deficient mice and K^b/D^b in class II-deficient mice (21a). However, thymic selection of $CD4^+$ and $CD8^+$ lymphocytes was potentially affected by MHC deficiencies (15, 32). Therefore, it was necessary to test the T cells from each phenotype to determine whether they retained the ability to respond normally when SEA was presented by wild-type cells. The T-cell response of MHC class I- or class II-deficient mice was essentially equivalent to that of the wild type when SEA was presented by mononuclear cells expressing both class I and class II molecules (Fig. 1B).

Serum cytokine levels. The levels in serum of TNF-α, IL-1α, IL-6, and IFN-γ in mice injected with SEA, LPS, or SEA plus LPS were measured at various times following injection (Fig. 2). Compared with those in mice injected with either SEA or LPS alone, the levels in serum of TNF-α, IL-6, and IFN-γ increased 5-, 10-, and 15-fold, respectively, in animals given SEA plus LPS. SEA alone did not elicit any detectable increase in TNF-α, IL-6, or IFN-γ levels above the background. In contrast to those of the other cytokines, IL-1α levels in mice injected with SEA plus LPS showed a simple additive effect.

Levels in serum of TNF-α, IL-6, and IFN-γ were maximal 2 to 4 h after the LPS injection but returned to normal by 10 h. The concentration of IL-1α in mice given SEA plus LPS also peaked 2 h after the LPS injection but stayed above the background for the remaining determinations. Levels of IL-1α in mice given only LPS or SEA peaked at 4 and 6 h, respectively. Unlike profiles for other cytokines, the highest level of IL-1α in mice injected with SEA and LPS corresponded to the peak stimulated by SEA but not LPS.

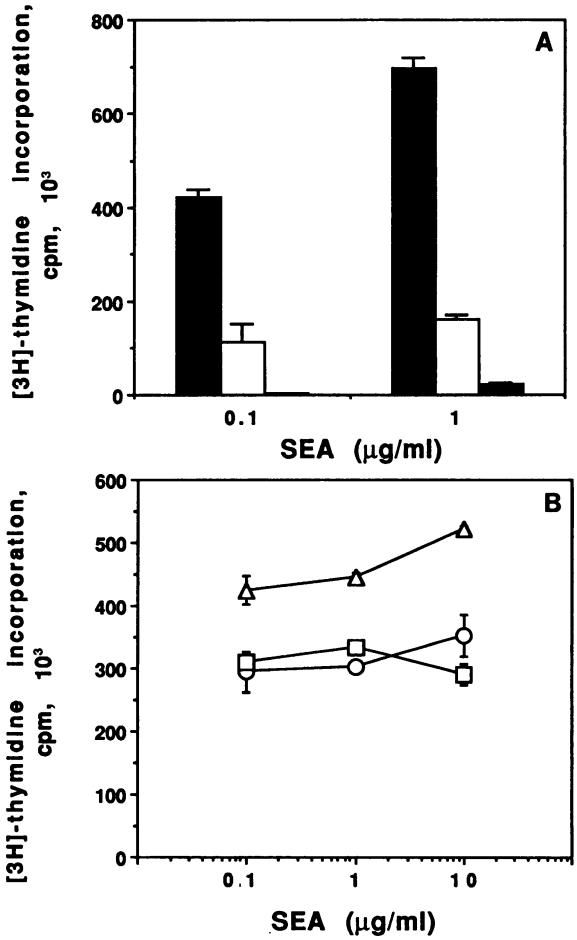

FIG. 1. T-cell responses of MHC-deficient mice to SEA. (A) Autologous antigen-presenting cells of MHC-deficient mice are defective in the T-cell response to SEA. Symbols: ■, wild type; □, class I deficient; ▩, class II deficient. (B) T cells from MHC-deficient mice respond normally to SEA presented by wild-type mononuclear cells (irradiated). Symbols: ○, wild type; △, class I deficient; □, class II deficient. Data represent the mean of triplicate determinations ± the SEM. The incorporation of [^{3}H]thymidine by irradiated wild-type mononuclear cells with SEA added to the cultures was 68 cpm.

DISCUSSION

The mouse strain used in this study was of the $H\text{-}2^b$ haplotype and was selected on the basis of previous data showing that this class II allelic product was an efficient presenter of SE (29). It is likely that the biological effects of SE in mice are dependent on both the MHC class II isotype and allele(s) expressed. In agreement with previous studies indicating a higher affinity of SEA than of SEB or SEC_1 for MHC class II molecules (6, 7), we found that SEA was also much more toxic in mice. For optimal T-cell stimulation, the binding of SEA to antigen-presenting cells reportedly favors *I-A* expression (31). Both isotypes of mouse MHC class II bind SEA or SEB, as shown by previous inhibition studies with antibody against class II (29, 31).

There are previous reports suggesting weak binding of SE to class I molecules in vitro (6, 10). We have observed that transgenic class I-deficient mice exhibit reduced sensitivity to SE, relative to that of wild-type animals, thus suggesting that MHC class I or other receptors may play at least a minor role in mediating toxicity. In addition, SEC_1 but not SEA, SEB, or TSST-1 bound specifically to a receptor on class II-negative L cells at a level 5 to 10% of that for cells transfected with the HLA-DR gene (30a). Cantor and colleagues (4) found a short amino acid sequence which is common to SEC_1, SEC_2, and SEC_3, but not present in other SE, and which shares homology with the sequence of VCAM-1, a vascular adhesion molecule found on the surface of endothelial cells (21). Only cells expressing VLA-4, the receptor for VCAM-1, were lysed by cytotoxic T cells when incubated with SEC_2 (4), suggesting that this molecule may also be a receptor for SECs. The identity of the SEC_1

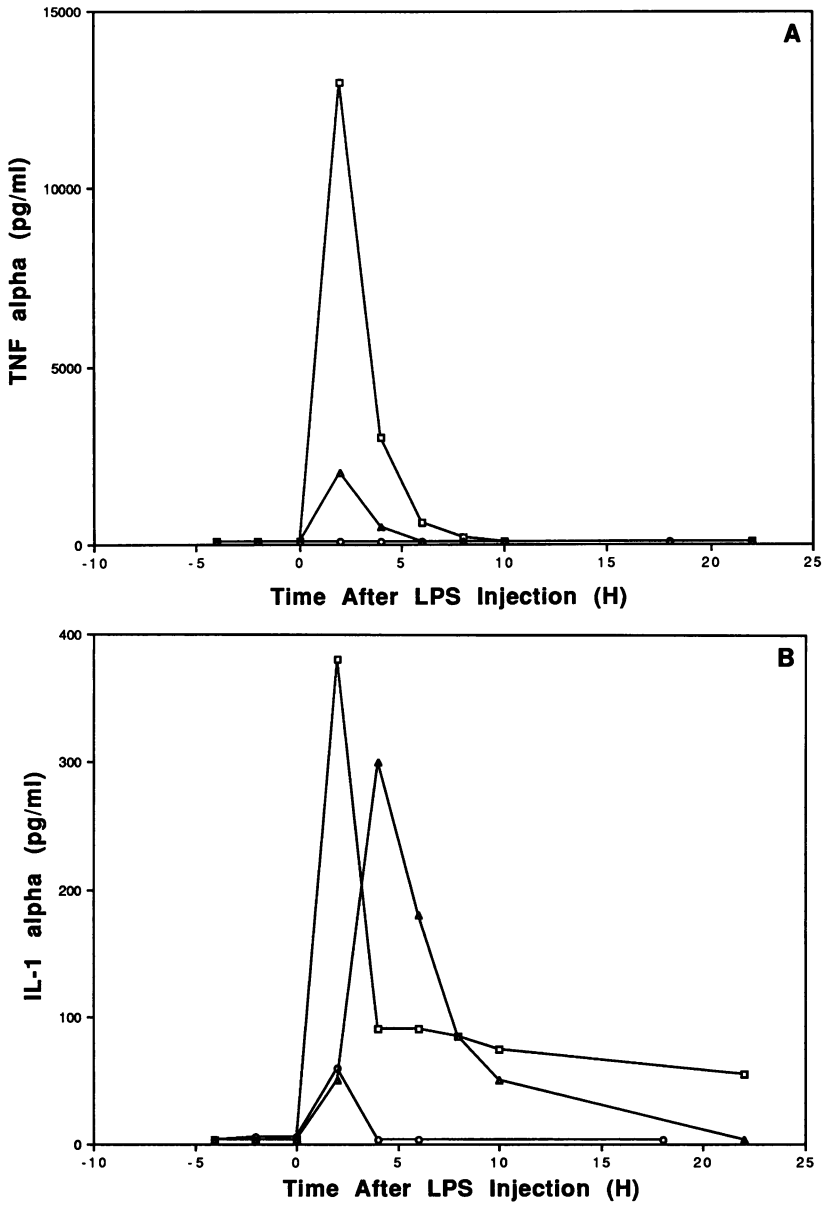

FIG. 2. Detection of TNF-α (A), IL-1α (B), IL-6 (C), and IFN-γ (D) in the serum of mice injected with SEA (○), LPS (△), or SEA plus LPS (□). Values for TNF-α and IL-1α represent the mean of duplicate samples, with an SEM of ±5%. IFN-γ and IL-6 values represent the mean of duplicate and triplicate samples, respectively. The SEMs for IFN-γ and IL-6 readings were ±5% and ±10%, respectively.

receptor on L cells is not clear, since this cell line does not express the VLA-4 molecule.

In this study, mice injected with SEA plus LPS had greatly elevated concentrations of TNF-α, IL-1α, IL-6, and IFN-γ in serum. We found that maximal levels of TNF-α, IL-1α, and IFN-γ in serum were detected 2 h after LPS potentiation, while IL-6 levels peaked at 4 h. SE sensitization may lower the concentration of LPS required to stimulate cytokine synthesis and/or trigger the release of stored cytokines. The appearance of IL-6 in serum correlated with previous studies suggesting that it is induced by TNF-α and IL-1 (23). LPS stimulates the production and release of elevated levels of cytokines such as TNF-α, IFN-γ, and IL-1 (2, 9, 20). In addition, injection of TNF-α or IL-1 produces a concomitant rise in serum IL-6 levels (23) and may ultimately result in lethal shock.

Other murine models have been described for studying the biological effects of SE. A high dose of D-galactosamine (20 mg per mouse) has been shown to enhance SEB toxicity (19), possibly by impairing RNA synthesis and increasing sensitivity to TNF-α (14). Another study measured weight loss (3 to 11%), perhaps a consequence of increased cytokine levels, after an injection of 10 to 100 μg of SEB per mouse (16). We have found both weight loss and D-galactosamine treatment to be less sensitive and consistent than the technique described in this report (unpublished data). It is apparent that all of the hallmark indications of SE toxicity are maintained by increasing the susceptibility of mice with

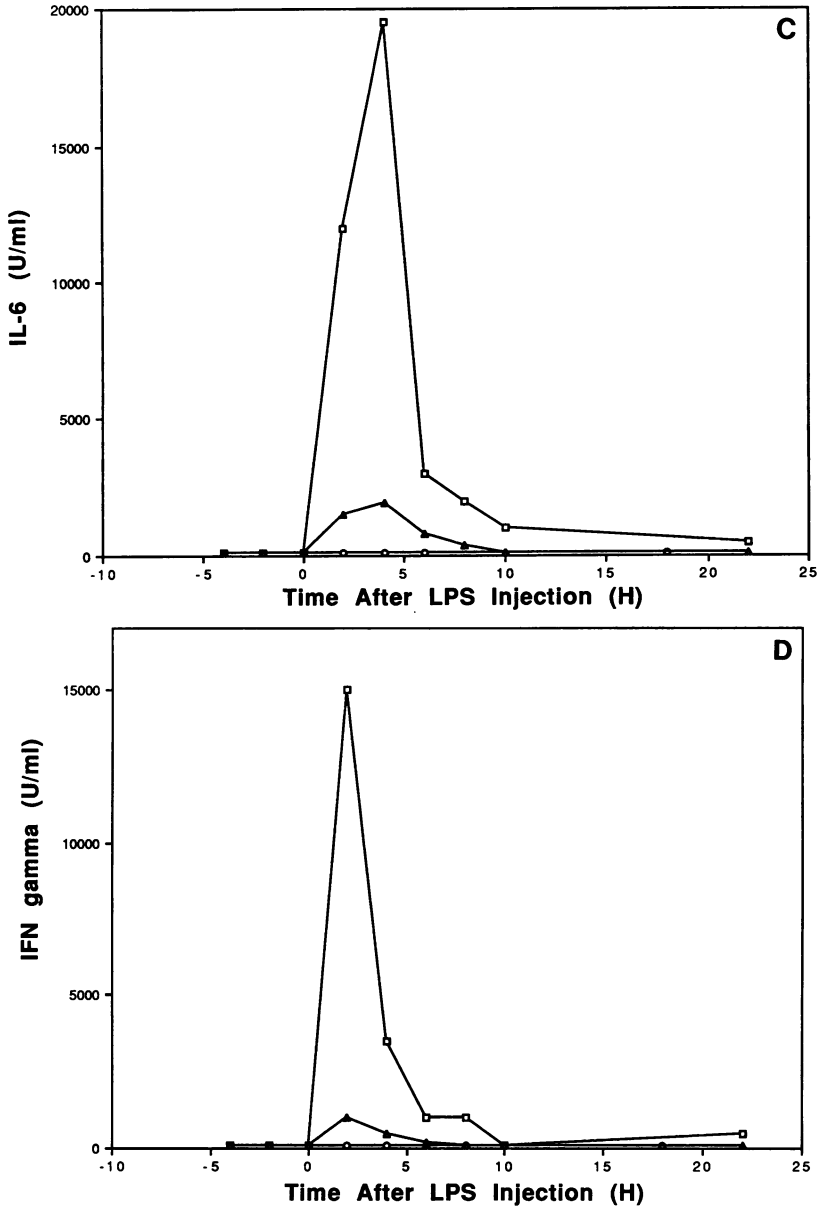

FIG. 2—*Continued.*

LPS. This very reproducible and sensitive method will make it possible to study the complex effects of superantigens on the immune system.

ACKNOWLEDGMENTS

The expert technical contributions of Francis Sexton and Dathane Turner are greatly appreciated, as is the probit analysis by Gene Nelson.

Sina Bavari is supported by a National Research Council postdoctoral fellowship.

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