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Toxic Shock Syndrome-Associated Staphylococcal and Streptococcal Pyrogenic Toxins Are Potent Inducers of Tumor Necrosis Factor Production

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Toxic shock syndrome-associated staphylococcal and streptococcal exotoxins were tested for an ability to induce the production of tumor necrosis factor (TNF). Staphylococcal enterotoxins B and C1, along with streptococcal pyrogenic exotoxin A, all induced TNF production in a dose-dependent manner, with production peaking on the average at 3 days but continuing over the 6 days tested. This time course of exotoxin-induced TNF production contrasts with the 1-day peak–2-day duration observed with endotoxin as the stimulus and may be significant to development of toxic shock syndrome.

Toxic shock syndrome (TSS) is a disease characterized by fever, hypotension, a scarlatiniform rash, and the involvement of three major organ systems (10, 30). In most cases associated with menstruation and in approximately 50% of nonmenstrual cases (i.e., surgical wound infection), TSS is associated with strains of *Staphylococcus aureus* that produce and secrete the exotoxin toxic shock syndrome toxin 1 (TSST-1) (2, 12, 26). It is now evident, however, that TSS and TSS-like disease can also be associated with staphylococci or streptococci producing other exotoxins (15). Among these, staphylococcal enterotoxin B (Ent B) is most common (12, 17, 23), but staphylococcal enterotoxin C1 (Ent C) (12) and streptococcal pyrogenic exotoxin A (SPE A) (11) have also been reported to be associated with TSS-like disease.

These TSS-associated exotoxins are members of a larger group of pyrogenic exotoxins (PT) which are produced by *S. aureus* and *Streptococcus pyogenes*. The group includes TSST-1 (4), the staphylococcal pyrogenic exotoxins A and B (22), the staphylococcal enterotoxins A through E (3), and the streptococcal pyrogenic exotoxins A through C (1). Each PT is serologically distinct when tested by Ouchterlony immunodiffusion, but all share the properties of being pyrogenic, mitogenic for T cells, and inhibitory for immunoglobulin production and of increasing susceptibility to lethal endotoxin shock (8, 21, 22, 25, 28, 29). They differ functionally, however, in that the staphylococcal enterotoxins induce emesis and diarrhea upon ingestion (3), whereas the streptococcal pyrogenic exotoxins induce heart damage and cause scarlet fever (27).

We have previously reported that TSST-1 induces tumor necrosis factor (TNF)-alpha production by human peripheral blood monocytes (MNC) in vitro and that TNF, in turn, has a negative influence on neutrophil chemotactic function (13). We have proposed that these phenomena may in part account for the failure of the host to make a pyogenic response to infections involving TSST-1-producing staphylococci. TSST-1-stimulated production of TNF has also been reported by another laboratory (18). In this report, we provide evidence that three other TSS-associated exotoxins share with TSST-1 the ability to stimulate TNF production by MNC in vitro and demonstrate that the time course of TNF production by all exotoxins tested is considerably longer than that caused by endotoxin.

The exotoxins were purified by methods described previously (1, 26). Briefly, each strain was grown in pyrogen-free beef heart dialysate medium at 37°C. Exotoxins were precipitated by addition of absolute ethanol, dissolved in pyrogen-free distilled water or acetate-buffered saline, and isolated from the cell-free supernatant by preparative thin-layer isoelectric focusing; toxin preparations were homogeneous when tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 μg) and staining with Coomassie brilliant blue R250. Toxin identity was confirmed by Ouchterlony immunodiffusion with specific hyperimmune antisera. All glassware used was baked at 190°C for 3 h to destroy any adherent endotoxin. TSST-1 was isolated from *S. aureus* MN8, a TSS clinical isolate. Ent B was isolated from *S. aureus* MNLO, and Ent C was isolated from strain MNDon, both clinical isolates from patients with nonmenstrual TSS (6, 23). SPE A was isolated from *Streptococcus pyogenes* 594 (24).

PT were tested for contaminating endotoxin by a quantitative chromogenic limulus amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, Md.). At a concentration of 1 mg of PT per ml, net endotoxin levels of ≤33 ng/ml were found in the four PT preparations tested. Therefore, PT at the highest concentration used to stimulate TNF production (10 μg/ml) contained less than 1 ng of endotoxin per ml. That this level of endotoxin does not contribute significantly to PT-stimulated TNF production is shown by failure of addition of 100 μg of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) per ml to reduce TNF production in response to PT.

MNC were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma) from heparinized venous blood drawn from healthy control volunteers according to methods previously described (7) and were then washed three times with Hanks balanced salt solution (Biologos, Naperville, Ill.). Monocytes were enumerated by microscopic examination of Cytospin slides stained for myeloperoxidase activity (19). To prepare supernatant fluids, MNC providing 5×10^4 monocytes per well were cultured in 96-well microtiter plates in Earle minimal essential medium (MEM) (Biologos) containing 15% pooled human serum and three different concentrations of each of the four PT. The supernatant fluids were

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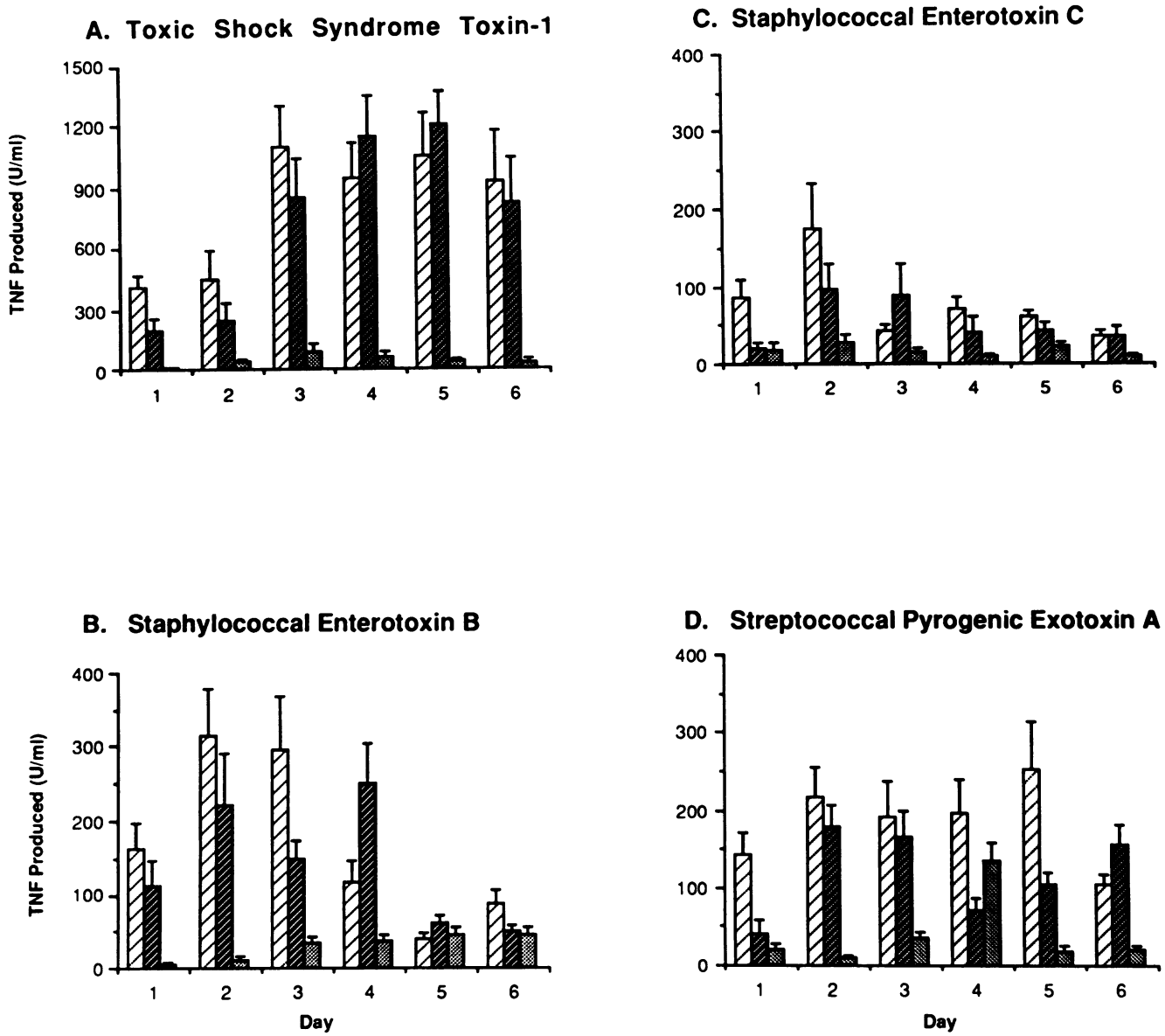

FIG. 1. TNF produced in response to four PT: TSST-1, Ent B, Ent C, and SPE A. MNC were incubated with three concentrations of PT (▨, 10 μg/ml; ▨, 100 ng/ml; ▩, 1 ng/ml) for 1 to 6 days. Cell-free supernatant fluids were harvested every day and tested for the presence of TNF as measured by cytotoxicity toward the TNF-sensitive murine fibroblast cell line L-929. Data are from four experiments and are expressed as mean ± standard error of produced TNF. Supernatants from untreated control cells contained no TNF activity on any day (data not shown). The exotoxins had no direct effect on the L-929 cells.

collected at 24-h intervals over 6-day period, centrifuged to remove cellular debris, and stored at −70°C until used.

TNF in the stimulated supernatants was assayed by a modified L-929 cytotoxicity assay that is based on reduction of a tetrazolium dye (14, 20). Murine TNF-sensitive L-929 cells (American Type Culture Collection, Rockville, Md.) were cultured in 96-well microtiter plates as a concentration of 2.5×10^4 per well in MEM–5% fetal calf serum (Biologos) supplemented with MEM nonessential amino acids solution (Biologos) and incubated for 3 h at 37°C. A 50-μl volume of neat supernatant or supernatant diluted in MEM–5% fetal calf serum was then added to the L-929 cell cultures. After incubation for 18 h at 37°C, the supernatants were discarded, and the wells were washed three times with phosphate-buffered saline. The wells were refilled with 200 μl of serum-free MEM. An additional 20 μl of phosphate-buffered saline containing 5 mg of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] per ml was then added to the wells, and the plates were incubated for 4 h at 37°C. After incubation, the medium in the wells was discarded and 100 μl of acidified isopropanol–1% sodium dodecyl sulfate was added to dissolve the MTT-formazan precipitate. The optical density of the dissolved precipitate was read at 550 and 630 nm on a Titertek Multiscan MCC 340 automatic plate reader. TNF activity in the supernatant fluids was estimated from a standard curve produced by using known amounts of recombinant human TNF (Genzyme, Boston, Mass.).

All four PT induced the production of considerable amounts of TNF (Fig. 1). At a concentration of 10 μg/ml,

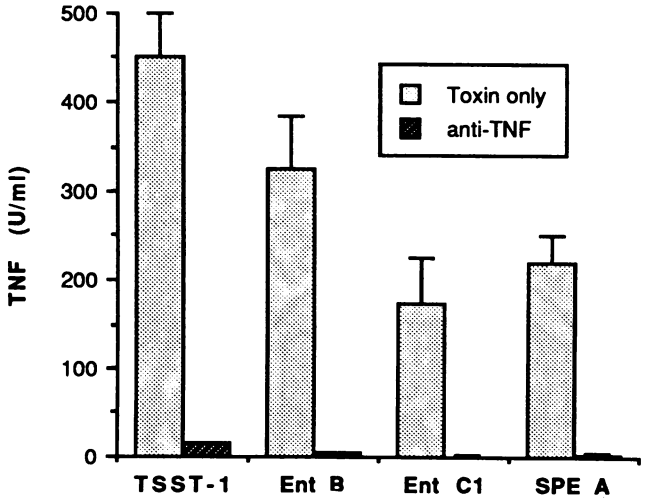

FIG. 2. Anti-TNF antiserum neutralization of exotoxin-stimulated supernatant fluids. Cell-free supernatant fluids from MNC stimulated with 10 μg of the four exotoxins per ml were preincubated for 30 min at 22°C with anti-TNF-alpha antiserum before addition to the target cells in the TNF cytotoxicity assay. Data are from three experiments and are expressed as mean ± standard error of TNF produced. The antiserum itself had no effect on the viability of the L-929 cells (data not shown).

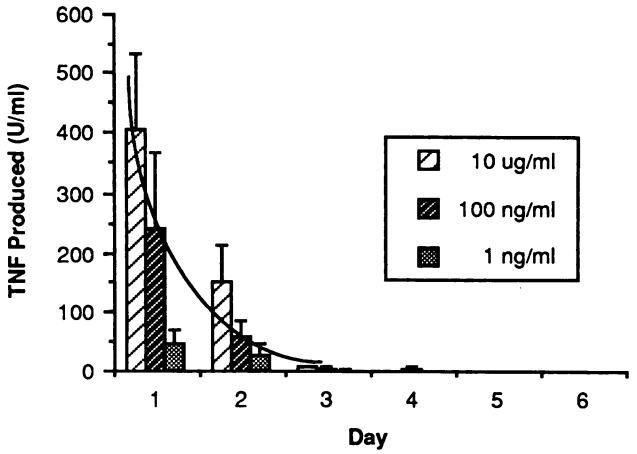

FIG. 3. TNF produced in response to endotoxin from *E. coli* O111:B4. MNC were incubated with three concentrations of endotoxin for 1 to 6 days. Cell-free supernatant fluids were harvested every day and tested for the presence of TNF as measured by cytotoxicity for L-929 cells. Data are from four experiments and are expressed as mean ± standard error of TNF produced. The curve represents cytotoxic activity from recombinant human TNF incubated under cell-free conditions for 1 to 6 days. Supernatants from untreated control cells contained no TNF activity on any day (data not shown). Endotoxin was not directly cytotoxic effect for the L-929 cells (data not shown).

TNF production stimulated by all PT was maximum (175 to 1,200 U/ml) after 2 to 3 days of culture, but the pattern of decline in TNF production varied with each PT. For TSST-1, maximum TNF production continued through the 6 days of culture; for SPE A, TNF production appeared to begin to decline after 5 days of culture; for Ent B, TNF production began to decline after 3 days of culture; and for Ent C, TNF production began to decline after 2 days of culture. At the two lower concentrations of PT, 100 and 1 ng/ml, the level of TNF production reached, the time to maximum TNF production, and the pattern of decline of production were more variable among the PT. The presence of TNF was confirmed by the ability of anti-TNF-alpha antiserum (Genzyme) to neutralize the cytotoxic activity of supernatants from MNC stimulated with PT for 48 h (Fig. 2). We have previously shown that anti-TNF-alpha antiserum neutralizes the chemotactic inhibitory activity found in 48-h supernatants from MNC stimulated with TSST-1 (13). These observations indicate that multiple staphylococcal and streptococcal exotoxins associated with TSS share with endotoxin (5) an ability to stimulate TNF production. The extended period over which the PT stimulate TNF production differs, however, from the time course of endotoxin-stimulated TNF production.

The duration of endotoxin-stimulated interleukin 1 production by human monocyte-derived macrophages has been noted to be less than 2 days (9, 16). TNF production in response to endotoxin also decreases in cultured monocytes but can be augmented by the addition of interferon-gamma (9). Our experiments to describe the temporal characteristics of endotoxin-stimulated TNF production confirm the short duration of this endotoxin effect. TNF production in response to each of the doses of endotoxin (1 ng/ml to 10 μg/ml) (lipopolysaccharide W *Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.) was maximum within the first 24-h period but negligible after 3 days of culture (Fig. 3). This phenomenon has been associated with desensitization of the target cell to the endotoxin stimulus, but we have considered that the rate of decline in TNF production may also reflect in part the time over which TNF remains biologically active under the culture conditions.

To test the lifetime of TNF in culture, we incubated 500 U of recombinant human TNF per ml in our culture medium (without cells) and monitored biological activity after 1 to 6 days in culture. TNF activity declined with a temporal pattern matching that observed for endotoxin-stimulated TNF production (Fig. 2). This observation suggests that endotoxin-stimulated TNF production must occur for less than 24 h; it also suggests that the activity of TNF present in the 1- to 6-day culture supernatants of exotoxin-stimulated cells is the result of continued production of the monokine, not the result of accumulation of the product.

In summary, we have demonstrated that PT associated with TSS share an ability to induce the production of TNF by monocytes in vitro and that the production of TNF stimulated by PT is maintained over a prolonged period. The former but not the latter characteristic of PT is shared with endotoxin, another potent inducer of TNF production. We propose that a comparably extended time over which PT might stimulate monokine production in vivo implicates a role of TNF in mediating shock symptoms of TSS (31) and the failure of the infected host to respond pyogenically to PT-producing staphylococci and streptococci.

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