INFECTION AND IMMUNITY, Sept. 1991, p. 2929–2933
0019-9567/91/092929-05$02.00/0

Vol. 59, No. 9

Toxic Shock Syndrome Toxin 1 Enhances Synthesis of Endotoxin-Induced Tumor Necrosis Factor in Mice

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Received 11 March 1991/Accepted 31 May 1991

Toxic shock syndrome toxin 1 (TSST-1) was tested for its ability to enhance the production of endotoxin-induced tumor necrosis factor (TNF) in C3H/HeN mice. The TNF level in serum was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA). It was found that when mice were injected with 20 μg of TSST-1 12 h before exposure to 1 μg of endotoxin, the serum endotoxin-induced TNF was 20 times as high as that found in mice exposed to endotoxin alone. Although 20 μg of TSST-1 did induce a maximum level of near 1 ng of TNF per ml of serum 1.5 h after exposure, the TNF concentration was greatly diminished after 5 to 6 h and was no longer detectable after 12 h. Pretreatment of mice with 20 μg of TSST-1 or 1 μg of endotoxin did not influence TNF induction by TSST-1 12 h later. Also, pretreatment of mice with 1 μg of endotoxin did not enhance TNF induction by endotoxin 12 h later. Enhancement was achieved only when mice were exposed to TSST-1 more than 4 h and less than 24 h before injection of endotoxin. Despite the relatively high serum TNF levels (30 to 50 ng/ml), no mortality was observed in the mice treated with both TSST and endotoxin.

Endotoxin-induced mortality in animals has been found to be enhanced by streptococcal (11) or staphylococcal (21) toxins. More recently it has been reported that certain microbial products (19) or microorganisms (7, 12) enhance synthesis of endotoxin-induced tumor necrosis factor (TNF). As TNF has been found to be the main mediator in a number of disease conditions (see reference 13 for a review), this study was undertaken to determine whether toxic shock syndrome toxin 1 (TSST-1), produced by *Staphylococcus aureus* and found to enhance endotoxin-induced mortality in rabbits (21), enhanced endotoxin-induced TNF and mortality in an in vivo mouse model. TSST-1 has been reported to enhance slightly (about fourfold) the production of endotoxin-induced TNF in vitro (15).

TSST-1 has been shown to induce interleukin-1, fever (9), and hematologic changes (3) in mice. TSST-1 binds equally well with human and murine major histocompatibility complex class II molecules and activates murine T cells (23), which suggests that the mouse might be a good model for testing cytokine induction by TSST-1.

MATERIALS AND METHODS

Mice. C3H/HeN mice were obtained from Charles River Laboratories, Wilmington, Mass. Female mice weighing 20 ± 1 g were used. Animals were given food and water ad libitum.

Toxins. TSST-1, purified from toxic shock syndrome (TSS)-associated *S. aureus* FRI-1169, was obtained from Toxin Technology, Inc., Madison, Wis., where it was purified by a published method (17). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified toxin, stained with Coomassie blue, revealed only one band of molecular mass 24 kDa. When the purified toxin was subjected to isoelectric focusing, only one band of pI 7.0 to 7.2, characteristic of TSST-1 (1, 17), was observed. TSST-1 was negative (<0.1 ng/ml) for endotoxin by the *Limulus* assay (Entotech kit; ICN Biomedicals, Cleveland, Ohio). Endotoxin from *Salmonella typhimurium* G30/21, purified by a published method (4), was purchased from RIBI ImmunoChem Research Inc., Hamilton, Mont. (Refined Standard Endotoxin ReG30/C21; for additional information, see the manufacturer's data sheet).

Animal inoculations. Toxins (endotoxin or TSST-1) were introduced intraperitoneally (i.p.), with each desired dose prepared in 0.2 ml of sterile nonpyrogenic saline (Abbott Laboratories, Chicago, Ill.). In the synergism studies, endotoxin was introduced either at the same time as TSST-1 or at selected times after TSST-1. Control groups received an equal volume of nonpyrogenic saline in place of one or both toxins. The experiment measuring TSST-induced TNF was repeated three times with 20 μg of polymyxin B (Sigma Chemical Co.) added to the dose of 20 μg of TSST-1 according to a published method (22) to eliminate the possibility that the activity of the TSST protein was due to endotoxin contamination. The addition of polymyxin B did not influence the results.

TNF assay. TNF was quantified by a sandwich-type enzyme-linked immunosorbent assay (ELISA) as previously described (18). Recombinant murine TNF-α (rMuTNF-α), rabbit preimmune serum, rabbit polyclonal anti-TNF-α serum (polyclonal anti-rMuTNF-α), and hamster monoclonal anti-recombinant murine TNF-α (monoclonal anti-rMuTNF-α) were purchased from Genzyme Corp., Boston, Mass. The specific activity of rMuTNF-α was 4×10^7 U/mg assayed on L-929 cells in the presence of actinomycin D (20). Rabbit polyclonal anti-rMuTNF-α had a neutralizing activity of 10^6 neutralizing units of mouse TNF bioactivity per ml in the L-929 assay. The monoclonal anti-rMuTNF-α, obtained by in vivo immunization of hamsters with purified rMuTNF-α followed by fusion of hamster spleen cells with a hypoxanthine-aminopterin-thymidine (HAT)-sensitive mouse myeloma cell line, had an antibody concentration of 2 mg/ml with 25,000 neutralizing units of mouse TNF bioactivity per ml in the L-929 assay. The specificity of polyclonal anti-rMuTNF-α, monoclonal anti-rMuTNF-α, and the MuTNF ELISA has been previously documented (18). The ELISA was run to detect TNF concentrations as low as 30 pg/ml. Blood was obtained from mice by cardiac puncture at

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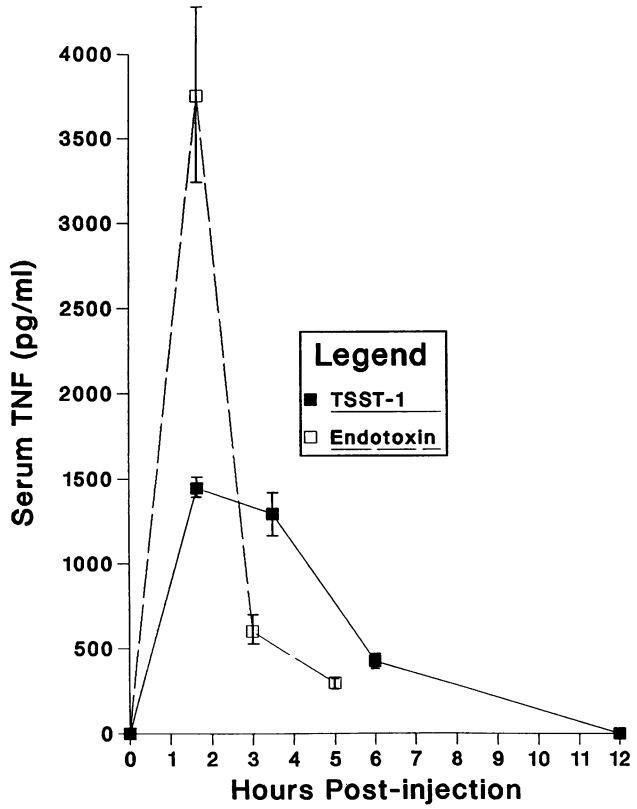

FIG. 1. Time appearance of TNF in serum following i.p. injection of 20 μg of TSST-1 or 1 μg of endotoxin. Each point represents the average for four to six mice with ± 1 SE indicated.

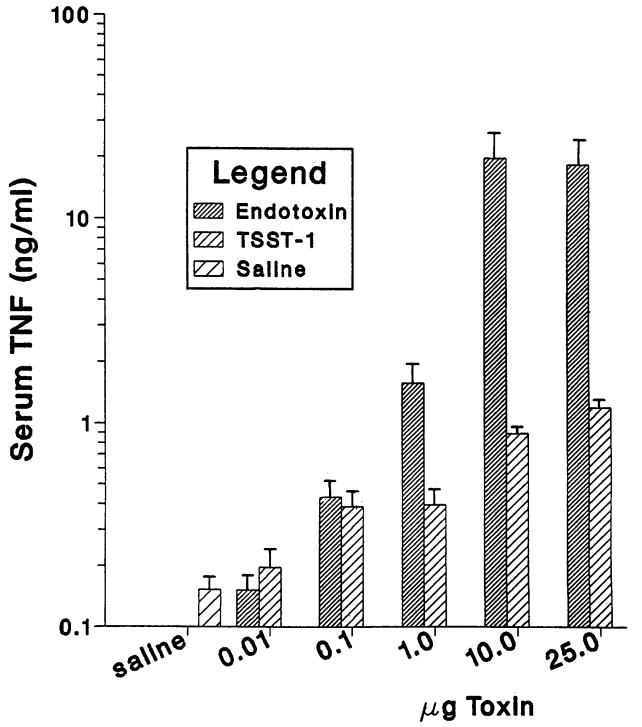

FIG. 2. Serum TNF response 1.5 h after i.p. injection of selected doses of TSST-1 or endotoxin. Bars represent the average for four to six mice with ±1 SE indicated.

selected times postinoculation with serum removed after centrifugation.

Statistical analysis. Data were analyzed by the Student unpaired *t* test or analysis of variance (Dunnett's test) (24). All experiments were carried out more than once, with reproducible results.

RESULTS

TNF elevation following injections of TSST-1 or endotoxin. To determine the time of peak TNF induction, groups of six C3H/HeN mice were injected with endotoxin (1 μg) or TSST-1 (20 μg) and the TNF level in serum was measured at selected times. Figure 1 shows that both toxins induced peak levels of TNF at 1.5 h postinjection, with TNF activity greatly diminished by 5 to 6 h postinjection.

In a dose-response study, TSST-1 was compared with endotoxin for its ability to induce TNF. It was found that as little as 0.1 μg of TSST-1 or endotoxin resulted in a significant ($P < 0.02$ by Dunn's test) increase in TNF levels in serum. Endotoxin induced maximum TNF levels in serum (nearly 30 ng/ml), whereas TSST-1 induced about 1/20 as much (Fig. 2).

Augmented synthesis of endotoxin-induced serum TNF after injection of TSST-1. In Fig. 3 the endotoxin-induced TNF concentrations in serum samples collected at progressive times after i.p. injection of 20 μg of TSST-1 are presented. After exposure to 20 μg of TSST-1, mice were injected at selected times with 1 μg of endotoxin. The mice were bled 1.5 h after the endotoxin injection, and the TNF level in serum was determined by the ELISA for each mouse. Figure 3 shows that when endotoxin was injected 12 h after TSST-1 exposure, the endotoxin-induced TNF (average, 25 ng/ml) was 20 times greater than that present (1.3 ng/ml) when endotoxin was injected into mice without any previous exposure to TSST-1. No synergism was seen when the two toxins were injected simultaneously or when TSST-1 was injected 24 h before endotoxin. Serum samples from mice exposed to TSST-1 alone had no detectable TNF levels 12 h after exposure.

In a separate experiment to determine the duration of peak TNF production after endotoxin exposure in TSST-1-treated groups of mice, animals were injected with 20 μg of TSST-1 and exposed to 1 μg of endotoxin 12 h later. One group of mice was bled 1.5 h after the endotoxin injection, and another group was bled 3 h after endotoxin exposure. It was found that the TNF level at 3 h after endotoxin exposure was 10% of the level 1.5 h after endotoxin exposure (30.0 ± 1.5 ng and 3.1 ± 1.0 ng at the 1.5- and 3-h postendotoxin sampling times, respectively; $n = 4$ to 6 with data represented as mean ± one standard error [SE]).

An experiment was conducted to determine whether a 12-h pretreatment with TSST-1 or endotoxin influenced TSST-1-induced TNF or whether a 12-h pretreatment with endotoxin influenced endotoxin-induced TNF. Table 1 shows that pretreatment with TSST-1 or endotoxin did not enhance the production of TNF induced by TSST-1 injected 12 h later. Pretreatment with endotoxin inhibited TNF induction by endotoxin 12 h later.

To determine the dose of TSST-1 necessary to obtain the synergism with endotoxin on the TNF response, groups of mice were injected with selected doses of TSST-1 12 h before endotoxin administration and the TNF level was

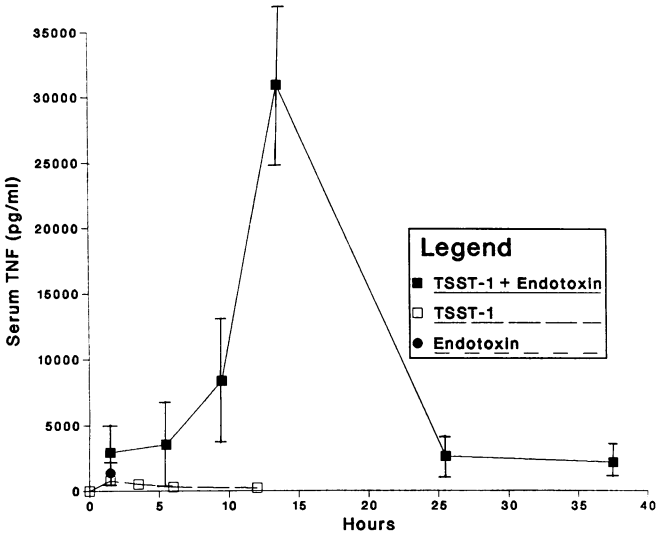

FIG. 3. TNF level in serum in mice at progressive times after i.p. injection of 20 μg of TSST-1 in combination with 1 μg of endotoxin, where TSST-1 was injected at time zero and endotoxin was introduced 1.5 h before each sampling time. The figure also shows the TNF concentrations in serum at progressive times following injection of 20 μg of TSST-1 alone. The level of TNF in serum 1.5 h after injection of 1 μg of endotoxin in mice not exposed to TSST-1 is also shown. Each point represents the average for four to six mice with ±1 SE indicated.

measured 1.5 h later. Figure 4 shows that reduction of the TSST-1 dose also reduced the mean TNF response in the synergism with 1 μg of endotoxin. More variability was seen within groups receiving the lower TSST-1 doses.

To determine the dose of endotoxin necessary to obtain the TSST-enhanced synergism of endotoxin-induced TNF, groups of mice were exposed ip to selected doses of endotoxin 12 h after injection with 20 μg of TSST-1. The level of TNF in serum was measured 1.5 h later. Figure 5 shows that the TSST-1 pretreatment enhanced (by about 20-fold) the ability of the smaller endotoxin doses (0.01, 0.1, and 1 μg) to induce TNF. The TNF-binding ability of the larger (10-μg) endotoxin dose was not significantly affected by the TSST-1 pretreatment.

TABLE 1. Enhancement of endotoxin-induced serum TNF in mice by pretreatment with TSST-1.

Animal group (n = 4 to 6)	Pretreatment[a]	Treatment	TNF concn in serum (pg/ml)[b]
1	TSST-1	Endotoxin	39,450 ± 5,510
2	Saline	Endotoxin	4,750 ± 1,000[c]
3	Endotoxin	Endotoxin	1,150 ± 513
4	Saline	TSST-1	671 ± 78
5	TSST-1	TSST-1	675 ± 55
6	Endotoxin	TSST-1	917 ± 192
7	TSST	Saline	ND[d]
8	Endotoxin	Saline	ND
9	Saline	Saline	ND

[a] Pretreatment was 12 h before treatment; the TSST-1 dose was 20 μg per mouse, and the endotoxin dose was 1 μg per mouse.
[b] TNF was measured 90 min after treatment.
[c] Significantly different from group 1 by $P < 0.001$ and group 3 by $P < 0.01$ (least significant difference).
[d] ND, not detectable.

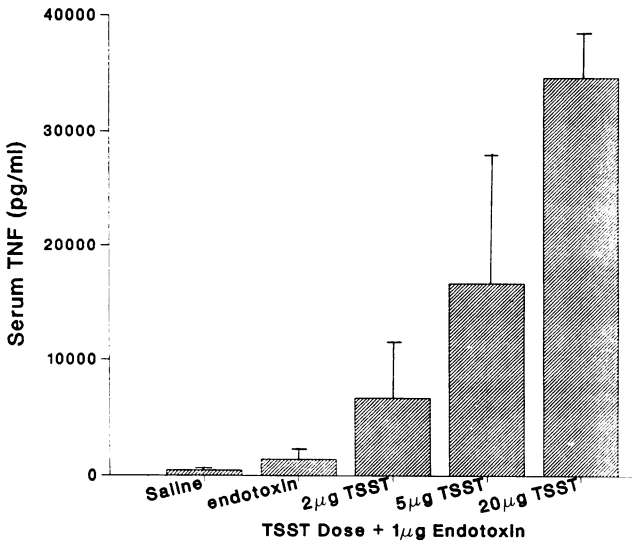

FIG. 4. Serum TNF response when selected doses of TSST-1 were injected i.p. 12 h before 1 μg of endotoxin. TNF was measured 1.5 h later. Bars represent the average for four to six mice with ±1 SE indicated.

Morbidity in TSST-1-primed mice. After endotoxin exposure, mice were observed over a 2-week period for mortality; no mortality was observed in animals exposed to TSST-1 (20 μg) alone, endotoxin (1 μg) alone, or TSST-1 (20 μg) followed by endotoxin (1 μg) 12 h later (n = 5 for all groups).

DISCUSSION

Mice have not been used extensively in the study of TSS because of their resistance to death from TSST-1 (for a review of animal models, see reference 16). We have shown that TSST-1 does induce TNF in mice, although peak levels are lower than those of endotoxin-induced TNF. Still,

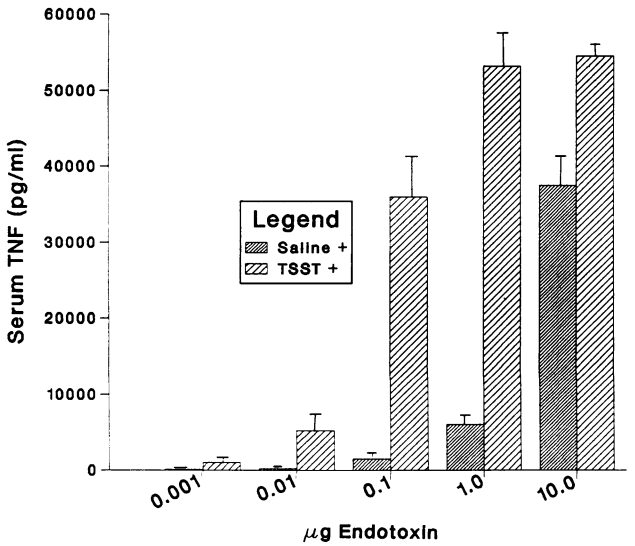

FIG. 5. Serum TNF response when 20 μg of TSST-1 was injected i.p. 12 h before selected doses of endotoxin. Bars represent the average for four to six mice with ±1 SE indicated.

TSST-1 acted similarly to endotoxin (25), inducing peak levels of TNF at 1.5 h after exposure, with TNF activity greatly diminished by 5 to 6 h and no longer detectable by 12 h. TSST-1 has been found to induce TNF in rabbits (8) and in human monocytes (10, 15). Unlike our finding in the in vivo mouse model, in the in vitro human monocyte model TSST-1 has been found to induce higher levels of TNF than does endotoxin, and TSST-1-induced-TNF was produced for a much longer period than was endotoxin-induced TNF (5).

In this study we have found that pretreatment of mice with TSST-1 12 h before exposure to endotoxin resulted in a 20-fold enhancement of endotoxin-induced serum TNF. In addition, an endotoxin dose of 0.001 or 0.01 μg, too low to induce detectable TNF, did induce significant levels of TNF when mice were pretreated with TSST-1. The enhancement of the TNF-inducing ability of these low levels of endotoxin may be important in the animals immune defense because TNF has been shown to potentiate the antimicrobial activity of neutrophils (6). Temporal production of the TSST-1-enhanced endotoxin-induced TNF appeared to resemble the unenhanced endotoxin TNF induction (25), with high levels of serum TNF found 1 to 2 h after endotoxin exposure and with TNF concentrations in serum greatly diminished by 4 h after injection. TSST-1 has been reported to enhance slightly (about fourfold) endotoxin-induced TNF levels in human monocytes (15). In the monocyte study, variability between repeated experiments and lack of dose response suggested the sequential exposure to TSST-1 and endotoxin resulted in monocyte damage and loss of viability (15).

It is interesting that although levels of 35 to 50 ng of TNF in serum were achieved by the TSST-1–endotoxin synergism, no deaths or changes in clinical chemistry were observed in mice exposed to the dual TSST-1–endotoxin dose. The relationship between circulating TNF and toxic effects is unclear. Although a number of studies indicate that TNF is an important mediator of endotoxic shock (for a review, see reference 13), other studies have failed to find an association between toxicity and levels of circulating endogenous TNF in mice (14) and recombinant human TNF in baboons (2). A synergism between TNF and bacteria or bacterial products has been reported to be necessary in the establishment of TNF-mediated toxicity (14, 19).

The resemblance of the kinetics of the development of an increased ability of endotoxin to induce TNF production in mice to the effect of the synergism between these two toxins on rabbit mortality (21) is striking. In both cases, in order for the synergism to take place, animals had to be treated with TSST-1 at least 4 h but less than 24 h before being treated with endotoxin, and a minimum TSST-1 dose of 0.1 to 0.5 μg/kg was necessary. It would be interesting to determine which, if any, cytokines were synergistically induced in the rabbits treated with TSST-1 and endotoxin and the role of these cytokines in the observed mortality.

ACKNOWLEDGMENTS

This work was supported by the American Heart Association of Michigan.

L. Riipi is acknowledged for her expert assistance with the ELISA. C. Wingerson is gratefully acknowledged for assistance in manuscript preparation.

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