

# TOXIC SHOCK SYNDROME TOXIN 1 AS AN INDUCER OF HUMAN TUMOR NECROSIS FACTORS AND $\gamma$ INTERFERON

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The toxic shock syndrome toxin 1 (TSST-1)<sup>1</sup> is an extracellular single chain protein (1, 2) produced by several *Staphylococcus aureus* strains that has been associated with the multisystem illness toxic shock syndrome (TSS) (reviewed in references 3–5). Most of the clinical, laboratory, and pathologic abnormalities observed during the course of toxic shock syndrome are similar in many respects to endotoxin shock (5–7). Moreover, animal studies have shown that, in common with other staphylococcal and streptococcal exotoxins, TSST-1 has the capacity to sensitize the host to lethal injury by small amounts of endotoxin (2, 7–9). It has been hypothesized that this interaction may be important in the pathogenesis of TSS (9, 10). Many features of the disease are as yet unexplained, and a better understanding of the mechanisms involved in the response to TSST-1 would allow us to test the efficacy of therapeutic modalities.

Many studies have been directed at elucidating host mediator activation in endotoxic shock. The role of cachectin was suggested by experiments in which mice passively immunized against the factor were protected from the lethal effect of endotoxin (11). Cachectin and tumor necrosis factor (TNF), both produced by activated macrophages, have then appeared as the same molecule (12). Like endotoxin, TSST-1 is a potent inducer of IL-1 production by monocytes/macrophages (13, 14), and IL-1 may in turn be responsible for the fever and for some other features of TSS. Here we report that TSST-1 could also stimulate human monocytes to produce TNF. Since TSST-1 is mitogenic for T cells (15, 16) and induces IL-2 production (17), we have evaluated its ability to stimulate human peripheral lymphocytes by determining lymphotoxin and IFN- $\gamma$  production, endogenous mediators that could play a role in many pathophysiological processes, either alone or in synergism with other cytokines.

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<sup>1</sup>Abbreviations used in this paper: LAF, lymphocyte-activating factor; LT, lymphotoxin; MTT, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; rHu, recombinant human; rMu, recombinant murine; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1.

## Materials and Methods

**Purified TSST-1.** The toxin was purified to apparent homogeneity from an 18-h culture of *S. aureus* FRI 1169 strain according to Schlievert et al. (7) with some modifications (Anderson, S., and J. E. Alouf, manuscript submitted for publication). The homogeneity of TSST-1 preparation was assessed by SDS-PAGE (silver staining), immunoprecipitation, and immunoblotting by using a specific antiserum kindly provided by Dr. J. Iandolo (Kansas State University, Manhattan, KS). 1 ng of toxin is equivalent to  $10^{-4}$  nmol. The lyophilized TSST-1 was dissolved in pyrogen-free saline, divided into working samples, and stored at  $-20^{\circ}\text{C}$  until used. Purified TSST-1 contained  $<20$  ng LPS/mg protein, as determined by a standard *Limulus* amoebocyte lysate assay (M.A. Bioproducts, Walkersville, MD).

**Reagents.** LPS from *Salmonella enteritidis* was obtained from Difco Laboratories Inc. (Detroit, MI). PHA was purchased from Wellcome Laboratories (Beckenham, England). PMA-, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet, and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO). Nonspecific esterase staining kit was purchased from Technicon Chemicals (Domont, France). Recombinant human TNF (rHuTNF) used to standardize the cytotoxic assay was kindly provided by Biogen (Geneva, Switzerland) and Knoll A.G. (Ludwigshafen, Federal Republic of Germany). Rabbit antisera raised to rHuTNF or to recombinant murine TNF (rMuTNF) were generous gifts from Professor W. Fiers (State University of Ghent, Belgium) and Dr. J. Tavernier (Biogent, Ghent, Belgium). Their respective neutralizing capacity was assessed as  $5 \times 10^6$  U/ml for rHuTNF and  $3.2 \times 10^7$  U/ml for rMuTNF.

**Human Peripheral Blood Leukocytes (PBL) Populations.** Buffy coats from adult healthy volunteers were obtained from the Centre de Transfusion Sanguine (Hôpital Pasteur, Paris, France) through the courtesy of Dr. Dighiero. Mononuclear cells were isolated by density centrifugation (Lymphoprep; Nyegaard, Oslo, Norway). They were seeded in 24-well culture plates (F 3047; Falcon Labware, Oxnard, CA) at  $2 \times 10^6$ /ml nonspecific esterase-positive cells in complete serum-free medium consisting of RPMI 1640 (Seromed, Berlin, FRG), antibiotics, 2 mM L-glutamine, and 0.01 M HEPES (Seromed). After adherence for 2 h, nonadherent lymphocytes were removed by washing the wells twice with medium. Adherent mononuclear cells were then cultured in fresh serum-free complete medium overnight at  $37^{\circ}\text{C}$  (5%  $\text{CO}_2$  in air) in the presence of various concentrations of stimulants. The supernatant fluids were collected, centrifuged, and stored at  $-20^{\circ}\text{C}$  until use. Nonadherent mononuclear cells were obtained after adherence for 2 h to plastic flasks (F 3024, Falcon Labware) of total mononuclear cells ( $10^7$ /ml). The nonadherent cell preparation contained  $<5\%$  nonspecific esterase-positive cells. They were cultured overnight in 24-well culture plates (F 3072, Falcon Labware) at  $6 \times 10^6$ /ml/well in medium supplemented with 5% FCS (Irvine Scientific, Santa Ana, CA) and in the presence of the various stimulants. The cell-free supernatant fluids were then stored at  $-20^{\circ}\text{C}$ .

**Cytotoxicity Assay.** Cytotoxic activity in supernatant fluids was measured on mouse L929 $\alpha$  cells treated with actinomycin D, essentially as described by Ruff and Gifford (18). Cells were seeded into flat-bottomed 96-well microtiter plates at  $1.25 \times 10^4$  per well in RPMI with 5% FCS. The next day, threefold serial dilutions of the test samples were applied in triplicate to the cells with a final concentration of 1  $\mu\text{g}$ /ml actinomycin D. They were further incubated at  $37^{\circ}\text{C}$  for 24 h. The culture medium was removed by inverting and flicking the plate. The remaining adherent cells were stained for 15 min with a solution of 15% methanol with 0.5% crystal violet. The plates were then washed and the remaining dye was solubilized with SDS (1%) for 15 min. Absorbance was read at 550 nm with a microplate spectrophotometer (Titertek Multiscan; Flow Laboratories, Helsinki, Finland). The cytotoxic activity (in units per milliliter) was defined as the reciprocal of the dilution resulting in 50% cytotoxicity determined by plotting the regression line of log of the dilution against absorbance.

**Neutralization of TNF.** Antibody neutralization was performed by incubation for 1 h at  $37^{\circ}\text{C}$  of 1:2,500 dilution of rabbit serum with pretested human supernatant fluids that had been adjusted to  $\sim 40$  U/ml of cytotoxic activity. Lytic activity of the samples was

then determined in the microplate assay using twofold dilutions. Three rabbit sera were comparatively used: a preimmune serum, and two immune sera, one specific for rHuTNF and the other for rMuTNF.

*IL-1 Assay.* IL-1 production was quantitated in a lymphocyte activation (LAF) assay using thymocytes from 8-wk-old C3H/HeJ mice cultured in the presence of PHA (19).

*IL-2 Assay.* The IL-2 content in supernatant fluids was determined by incubating an IL-2-dependent T cell line (CTLL2) at  $10^4$  cells/well with twofold dilutions of the test sample for 24 h at 37°C. At the end of the assay the viability of the cells was evaluated using the MTT assay (20). The potency of IL-2 is expressed in terms of reference units of bioactivity based on the National Institutes of Health reference reagent supplied by the Biological Response Modifiers Program of the National Cancer Institute (21).

*IFN- $\gamma$  Assay.* Interferon was quantified as the reciprocal of the dilution that produced a 50% reduction in vesicular stomatitis virus-induced lysis of human amniotic WISH cells (22) as compared with a standard preparation of rIFN- $\gamma$  of  $2 \times 10^7$  U/mg (RU 42369; Roussel-Uclaf, Romainville, France). Its species specificity was characterized by substituting rat fibroblasts for human WISH cells in the assay and by determining its inhibition by mAbs to IFN- $\gamma$  (Centocor, Malvern, PA).

## Results

*Stimulation of Human Monocytes with TSST-1.* Human peripheral blood monocytes isolated under LPS-free conditions were cultured for 20 h with varying concentrations of TSST-1 in a serum-free medium. Unstimulated monocytes from any of the donors did not produce detectable levels of TNF activity, as assayed on actinomycin D-treated L929 $\alpha$  cells. Treatment of monocyte cultures with TSST-1 resulted in the production of significant titers of TNF activity, even for as low as 10 or 100 pg/ml. By itself, the toxin had no direct toxic effect on the target cells. In a typical assay presented in Fig. 1A, the level of cytotoxicity increased in a dose-dependent manner. Cells from 10 different donors were exposed to several concentrations of TSST-1 in comparable experiments. In all assays, a significant titer of TNF activity was induced by TSST-1, but some of the donor cells (2 of 10) were more responsive to the toxin. Moreover, the peak of the response varied between 1 ng/ml to 1  $\mu$ g/ml of TSST-1, depending on the donor. With 10 ng/ml of TSST-1 (Fig. 1B), the mean level of cytotoxic activity found in cell cultures from the 10 donors (67.3 U/ml) corresponds to a concentration of 3.6 ng/ml of rHuTNF used as control in the assay.

A higher dispersion in the cytotoxic activity level was still observed when monocytes from the same donors were stimulated with two to four different concentrations of LPS used as control. In terms of TNF activity, two types of responses were determined; thus with 100 ng/ml LPS in cell cultures from six of the donors the mean titer was  $48 \pm 22.6$  U/ml, whereas it ranged from 250 to 2,150 U/ml in cell cultures from the four other donors (data not shown). It is worth noting that high responders to LPS were not those producing higher levels of TNF activity in response to TSST-1.

IL-1 activity was estimated in some of the supernatant fluids by the LAF assay. As expected, TSST-1 induced the release of IL-1 from human monocytes (13, 14). Due to the high degree of proliferation caused by TSST-1 alone added to the mouse thymocytes, its direct effect should be subtracted. Under these conditions, the addition of supernatant fluids from monocytes cultured for 20 h in the presence of TSST-1 dramatically enhanced proliferation. A high level of IL-1 activity could be demonstrated with low dosage level of TSST-1, i.e., at 1

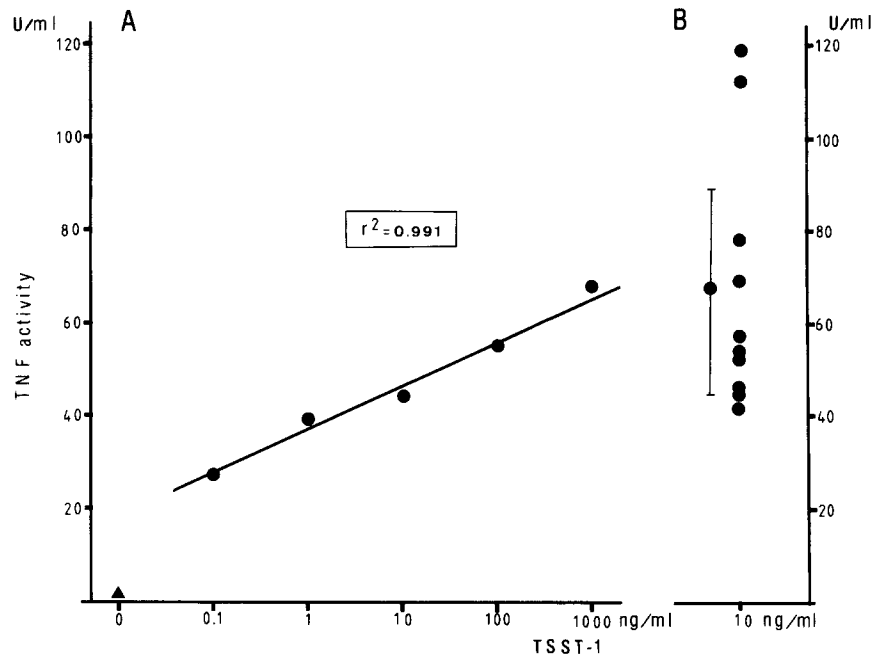


FIGURE 1. Cytotoxic activity (U/ml) on actinomycin D-treated L929 $\alpha$  cells in supernatant fluids from  $2 \times 10^6$ /ml human monocytes incubated for 20 h with TSST-1 in a serum-free medium. (A) Dose response to TSST-1 in cell culture from one donor; (B) variations in the response to 10 ng/ml TSST-1 in cell cultures from 10 different individuals.

TABLE I  
Human Lymphocyte Responses Induced with TSST-1 Toxin

Exp.	Assay	Cytokine activity in cultures incubated with:								
		Nothing	PHA (5 $\mu$ g)	PMA (20 ng)	LPS (10 ng)	TSST-1 toxin (ng)				
						0.1	1	10	100	1,000
			U/ml			U/ml				
A	Cytotoxicity L929 cells	<3	150	786	—	56	109	77	63	39
	IFN- $\gamma$	<2	2,990	570	—	NT	1,375	1,470	2,125	3,250
	IL-2	0	89	3	—	82	118	120	123	113
B	Cytotoxicity L929 cells	<3	40	256	21	74	171	103	85	29
	IFN- $\gamma$	<2	1,750	595	80	1,220	1,430	1,930	2,000	2,750
	IL-2	3	20	1	0	75	176	176	136	127

Cytokine content was determined in supernatant fluids of  $6 \times 10^6$ /ml lymphocytes from two donors incubated with different inducers (dose/ml) for 24 h.

and 10 ng/ml (data not shown). There was no IFN activity detectable in these monocyte cultures.

*Stimulation of Human Lymphocytes with TSST-1.* Enriched preparations of freshly isolated lymphocytes (nonadherent cells containing <5% nonspecific esterase-positive cells) were cultured for 20 h with the various inducers. In the same cell-free supernatant fluids, the cytotoxic activity on L929 $\alpha$  cells, IL-2 and IFN- $\gamma$  levels were determined. Two representative experiments are reported in Table I, but several partial assays have shown that the results are reproducible.

Cytotoxic activity for L929 $\alpha$  cells was produced by nonadherent mononuclear cells incubated with the toxin and the titer peaked for a low dose of TSST-1 (1 ng/ml). IFN- $\gamma$  titers obtained after exposing nonadherent cells to TSST-1 were comparable to those induced by PHA, the highest dose of toxin used in the assay (1  $\mu$ g/ml) being more potent than 5  $\mu$ g/ml PHA. Under these conditions, the production of IFN- $\gamma$  was lower in lymphocyte cultures stimulated with the phorbol ester at 20 ng/ml (Table I). An increased production of IL-2 was also found as already reported by other investigators (17). No clear dose-dependent response was observed with the doses of toxin used in the assay. A single dose of PHA, PMA, or LPS was used in control cultures. The level of responses to these inducers did not show any correlation with the titers obtained in cultures stimulated with TSST-1.

Costimulation with PMA of mitogen-activated human lymphocytes synergistically enhances cytokine production (23, 24). Such a response was obtained in our assays depicted in Fig. 2. The cytotoxic activity, IFN- $\gamma$  and IL-2 levels were strongly potentiated in cell cultures stimulated with PHA plus PMA. Although to a lesser extent, responses to TSST-1 were also markedly increased by the addition of PMA. Thus, costimulation with PMA and TSST-1 (100 ng/ml) resulted in a greater than threefold increase in both cytotoxic activity or IFN- $\gamma$  level over those observed after treatment with each inducer separately, and a greater than eightfold increase in IL-2 level (Fig. 2).

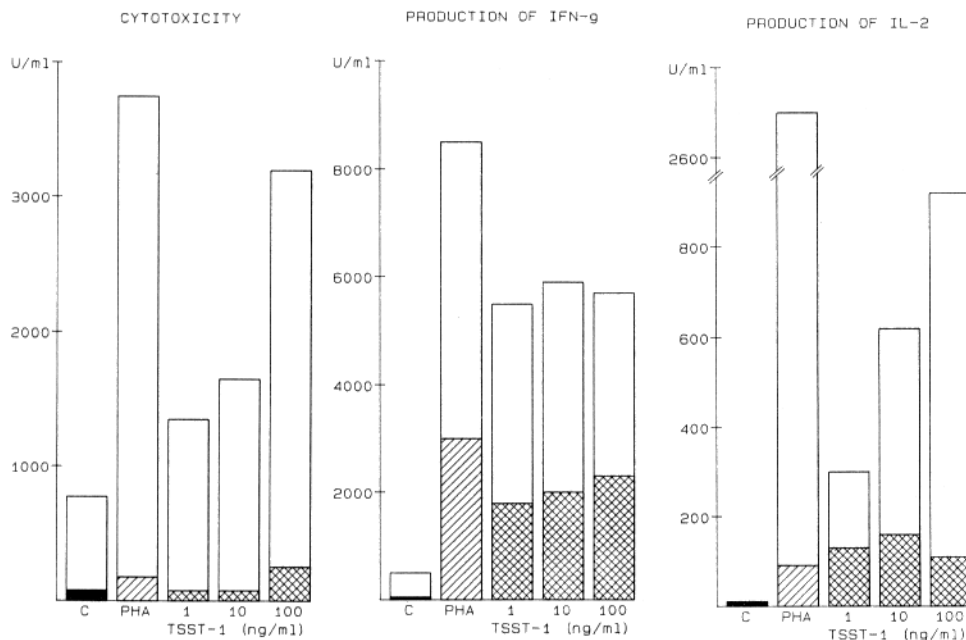


FIGURE 2. Cytokine production by nonadherent human PBL ( $6 \times 10^6$ /ml) cultured for 20 h in the presence of PMA plus TSST-1 or PHA. Cell-free supernatants were collected and tested for cytotoxic activity on actinomycin D-treated L929 $\alpha$  cells, and for IFN- $\gamma$  and IL-2 content. The open bars represent values obtained in the presence of PMA, while the shaded parts correspond to control cultures with the respective stimulant (■, medium; ▨, PHA; ▩, TSST) in the absence of PMA. The results depicted in the figure are representative of three identical experiments.

TABLE II  
*Cytotoxic Activity on L929 Cells in Supernatant Fluids from Human PBL after Treatment with Rabbit anti-rHuTNF-specific Antiserum*

Cytotoxic sample source	Inducer in PBL cultures (dose/ml)	Cytotoxicity (U/ml) after treatment			
		Nothing	Anti-rHuTNF	Pre-immune	Anti-rMuTNF
Adherent cells	Medium	0	0	0	0
	TSST-1 (1 ng)	35	0	38	36
	TSST-1 (10 ng)	39	0	43	37
	LPS (10 ng)	28	0	27	30
Nonadherent cells	Medium	0	0	0	0
	TSST-1 (10 ng)	28	21	31	29
	TSST-1 (100 ng)	34	23	33	35
	TSST-1 (10 ng) + PMA	45	13	43	40
	TSST-1 (100 ng) + PMA	43	12	43	45
	PHA (5 $\mu$ g) + PMA	28	9	28	29
rHuTNF solution	—	44	0	47	37
rMuTNF solution	—	38	37	42	0

Supernatant fluids from PBL collected as described were pretested for cytotoxic activity and adjusted to 20–50 U/ml before being incubated for 1 h at 37°C with the indicated rabbit serum at 1:2,500 dilution. The data are representative of three separate assays.

*Effect of Neutralizing Antibody to rHuTNF on TSST-1-induced Cytotoxic Activity.* The cytotoxic activity on L929 $\alpha$  cells found in both monocyte and lymphocyte supernatant fluids was further characterized in neutralization experiments using a polyclonal antiserum directed against rHuTNF that did not react with lymphotoxin. When these antibodies were added to supernatant from monocyte cultures stimulated either with TSST-1 or LPS, a complete inactivation of the cytotoxic effect was obtained, whereas control normal or immune sera were ineffective (Table II). When nonadherent cells were cultured in the presence of TSST-1, most of the cytotoxic activity found in the supernatant fluids was not affected by treatment with antibody directed to rHuTNF, suggesting that lymphotoxin (LT or TNF- $\beta$ ) was the major cytotoxin produced. However, co-stimulation with PMA, which potentiated the production of cytotoxic activity by the two mitogens, TSST-1 or PHA, modified the relative level of the two cytotoxins secreted in lymphocyte preparations since a greater inhibition of cytotoxic activity was found after addition of rHuTNF antibodies to the supernatant fluids (Table II). Mitogenic lectins, alone or synergistically with phorbol ester, have already been reported as efficient inducers of TNF production by highly purified human lymphocytes (25).

### Discussion

In addition to LPS or Gram-negative bacteria, other microorganisms or their components were found to induce the production of cytotoxic factor(s) by monocytes/macrophages (23, 26–28). In this study, staphylococcal TSST-1 was demonstrated as a potent inducer of TNF production by human blood mono-

cytes. It has already been reported that TSST-1 could stimulate human monocytes to release large quantities of IL-1 (13, 14). A significant production of both IL-1 and TNF was achieved by stimulating the cells with very low amounts of TSST-1 (0.1 or 1 ng/ml), showing that the toxin was an inducer still more efficient than LPS. The TNF activity produced by adherent mononuclear cells was completely neutralized by a polyclonal antibody prepared against rHuTNF. Our results do not support unpublished data mentioned by Beutler and Cerami (29) indicating that TSST-1 was incapable of eliciting cachectin/TNF biosynthesis. Different experimental conditions could perhaps explain the discrepancy. Since the monocyte population used in this study was not pure, it remains that some cells other than monocytes may be required for TNF production. Additional experiments have shown that a human promyelocytic cell line could be stimulated with TSST-1 to produce TNF activity (De Azavedo, J. C., unpublished results), showing that TNF induction by TSST-1 could be established without the influence of other cell types.

The staphylococcal toxin could also stimulate other cell population(s) among human blood mononuclear cells. TSST-1 is known to induce proliferative response of T cells functioning as a nonspecific mitogen (16, 17). The present study shows that after exposure to TSST-1, nonadherent cells could produce several cytokine activities, namely cytotoxic factors, IL-2, and IFN- $\gamma$ . The cytotoxic activity found in lymphocyte preparations stimulated with TSST-1 could be partly related to TNF production as indicated by a slight inhibition with anti-rHuTNF. Whether or not it is related to the presence of contaminant monocytes remains to be determined, but low levels of TNF can be produced by T cells upon mitogen stimulation (23, 24). Nevertheless, the remaining cytotoxic activity observed after addition of anti-rHuTNF antibodies indicates that the predominant source of cytotoxin is probably lymphotoxin. Higher levels of TNF were still produced when lymphocyte preparations were stimulated in the presence of PMA as has been demonstrated with other mitogens (23, 25). A possible role of IFN- $\gamma$  in the cytotoxic activity of both TNF and LT contained in nonadherent cell cultures cannot be excluded in this report. Regarding the release of IL-2 and IFN- $\gamma$ , TSST-1 was found to be more efficient than PHA or PMA used as positive controls in the assay. However, in combination with PMA, the staphylococcal toxin appeared less potent than PHA, although it displayed a synergistic effect.

The significance of the cytokine production in the pathophysiological mechanisms of TSS has still to be determined in a suitable animal model. TSS is a syndrome with some clinical features that are not generally seen in the shock caused by endotoxin-producing bacteria. The production of TNF has been suspected to be an important mediator of endotoxicity (29), and the pathophysiological effects elicited by intravenous injection of purified rTNF into mice or rats could not be distinguished from those produced by endotoxin (30, 31). Moreover, treatment that increased the mouse sensitivity to the lethal effect of LPS also enhanced the toxicity of TNF, e.g., in galactosamine-treated mice (32) or in adrenalectomized mice (Parant, M., unpublished results). TSST-1, like LPS, is capable of activating human monocytes to produce TNF and IL-1. However, unlike LPS, the toxin also has an effect on cytokine production by

lymphocytes. It is possible that lymphotoxin, a molecule that is similar in several aspects to TNF, plays a comparable role in inflammatory disease states (30, 31, 33). In addition, there are many examples of synergism between TNF or LT and IFN- $\gamma$ , even if toxicity of a combined treatment has not been evaluated yet. Thus, IFN- $\gamma$  was shown to permit the LPS-induced production of TNF/cachectin by C3H/HeJ (LPS-resistant) macrophages (34). Moreover, mutual stimulatory interactions that occur with these different cytokines may be responsible for pathophysiological mechanisms distinct from shock caused by LPS. Differences in *in vitro* responses to TSST-1 in various animal models could provide pertinent information in relation to great variations in animal susceptibility to TSS.

### Summary

We present evidence that toxic shock syndrome toxin 1 (TSST-1) induces the production of high levels of TNF by human blood monocytes. Enriched lymphocyte preparations incubated with the staphylococcal toxin produced significant levels of TNF-like activity that is not neutralized by anti-rHuTNF antibodies and is likely to be lymphotoxin (LT or TNF- $\beta$ ). We demonstrate also that TSST-1 is a potent inducer of IFN- $\gamma$ . When lymphocyte preparations were costimulated with PMA, the TSST-1 effect was strongly potentiated and the levels of cytotoxic factors, IFN- $\gamma$ , and IL-2 present in supernatant fluids were comparable to those observed after treatment with PMA and PHA. Thus, TSST-1, which is also known as an inducer of IL-1 and IL-2, stimulates the production of endogenous mediators that could play a role in the physiopathological processes of toxic shock syndrome (TSS). The described results suggest that the discrepancies in the clinical features between TSS and endotoxin shock may be related to qualitative differences in cytokine production.

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