INFECTION AND IMMUNITY, July 1992, p. 2612–2618
0019-9567/92/072612-07$02.00/0

Vol. 60, No. 7

Induction of Tumor Necrosis Factor and Interleukin-1 by Purified Staphylococcal Toxic Shock Syndrome Toxin 1 Requires the Presence of Both Monocytes and T Lymphocytes

RAYMOND H. SEE,[1,2,4] WINNIE W. S. KUM,[1,4] ALEX H. CHANG,[1,4] SWEE HAN GOH,[1,4] AND ANTHONY W. CHOW[1,2,3,4]*

Division of Infectious Diseases, Department of Medicine,[1] and Departments of Microbiology[3] and Pathology,[2] University of British Columbia, Vancouver General Hospital, and the Canadian Bacterial Diseases Network,[4] Vancouver, British Columbia V5Z 3J5, Canada

Received 2 December 1991/Accepted 10 April 1992

Highly purified staphylococcal toxic shock syndrome toxin 1 (TSST-1) was tested for its ability to induce the cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) from fractionated human peripheral blood mononuclear cells prepared from seven healthy donors. Highly purified monocytes alone or T lymphocytes alone did not produce TNF or IL-1 when incubated with TSST-1 at 37°C for up to 72 h. However, the addition of 10 μg of TSST-1 per ml to a 1:1 mixture of monocytes and T cells resulted in significant TNF (predominantly TNF-α) and IL-1β production after 24 h at 37°C. The nature of the monocyte/T-cell interaction did not appear to involve gamma interferon (IFN-γ), since 10 μg of rabbit anti-IFN-γ per ml did not neutralize TNF-α production after TSST-1 induction. Similarly, L243, a monoclonal antibody to HLA-DR which blocks TSST-1 binding to monocytes, did not inhibit TNF-α production following TSST-1 induction. However, direct contact between monocytes and T cells was required, since physical separation of cells in double-chamber culture wells abolished TNF-α secretion after TSST-1 stimulation. Furthermore, paraformaldehyde fixation of either monocytes or T cells prior to addition to viable T cells or monocytes, respectively, also abolished TNF-α secretion, suggesting that aside from cell contact, soluble factors were also involved. Our results suggest that cytokine production involves more than binding of TSST-1 to its receptor on monocytes alone and that cell contact with T cells and the release of a soluble factor(s) other than IFN-γ may be essential for the induction of cytokines by this toxin.

Toxic shock syndrome (TSS) is a multisystem disease associated with *Staphylococcus aureus* infection. A 22-kDa exoprotein, TSS toxin 1 (TSST-1), is thought to be a major etiologic agent in this disease. Other related staphylococcal exoproteins, particularly staphylococcal enterotoxins A (SEA), B (SEB), and C (SEC), have also been implicated (34). TSST-1 and the staphylococcal enterotoxins are members of the superantigen family owing to their ability to stimulate large numbers of T cells via their V_β-specific T-cell receptor in the presence of major histocompatibility complex (MHC) class II molecules on accessory cells (2, 14, 18). TSST-1, SEA, and SEB have all been shown to bind specifically to MHC class II molecules (7, 10, 28, 31). The binding of MHC class II antigens appears to be important in T-cell activation, as the addition of anti-MHC class II antibodies readily neutralizes the mitogenic response (7, 22, 30, 31).

The mechanism by which TSST-1 and the staphylococcal enterotoxins cause TSS remains unclear. TSST-1 and the enterotoxins are potent inducers of the cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) (5, 13, 24, 25). Both cytokines are thought to play an important role in TSS, since injection of either purified IL-1 or TNF in rabbits reproduces many of the features of TSS (13, 23).

Although monocytes are thought to be the main secretors of TNF and IL-1 in response to TSST-1, little is known about whether the presence of other cell types (e.g., T lymphocytes) is also required in cytokine induction. Fast and his associates (5) have reported that a TSST-1 preparation purified by thin-layer isoelectric focusing induced TNF production by human peripheral blood monocytes in vitro. The requirement for T lymphocytes was not commented upon. Trede et al. (33) also reported that a commercial TSST-1 preparation (Toxin Technology Inc., Madison, Wis.) caused transcriptional activation of IL-1β and TNF-α genes in human peripheral blood monocytes and the monocytic cell line THP-1. Fischer et al. (6), however, have found that production of TNF-α and TNF-β by highly purified SEA requires the presence of both monocytes and T lymphocytes. We sought to resolve these discrepancies by investigating the induction of both TNF and IL-1 from fractionated human peripheral blood monocytes and T cells with highly purified TSST-1 prepared in our laboratory.

MATERIALS AND METHODS

Purification of TSST-1. TSST-1 was purified from *S. aureus* MN8 culture supernatants by preparative isoelectric focusing and chromatofocusing, as described elsewhere (14a). The purified TSST-1 migrated as a single band, as demonstrated on silver-stained gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoblotting with polyclonal rabbit antiserum against the crude culture supernatant or with pooled normal human serum. The final preparations were endotoxin-free, as measured by the *Limulus* amoebocyte lysate assay (sensitivity limit, 10 pg/ml) (16). A commercial preparation of TSST-1

* Corresponding author.

was purchased from Toxin Technology Inc. (Madison, Wis.).

Preparation of donor cells. Fresh human peripheral blood mononuclear cells were obtained from seven healthy donors by centrifugation of plateletpheresis buffy coats over Histopaque 1.077 (Sigma Chemical Co., St. Louis, Mo.). Mononuclear cells at the interface were washed five times with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution and additionally separated into T- and non-T-cell populations by rosetting with sheep erythrocytes as described before (17). Monocytes were then separated from B lymphocytes by density centrifugation over Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) as outlined by de Boer et al. (3). Briefly, the non-T-cell fraction was suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, Utah), at a concentration of 5×10^7 cells per ml. The cells were then mixed with Percoll to give a final specific gravity of 1.062 g/ml. Then 1 ml of RPMI 1640 with 10% FBS was gently layered on top of each suspension. The gradient was then centrifuged at $850 \times g$ without braking for 15 min. The monocyte-containing interface was removed, washed three times, and suspended in RPMI 1640 containing 10% FBS. The monocyte preparation was ≥90% pure, as assessed by nonspecific esterase staining of cytospin preparations (35), with the contaminating cells being mainly natural killer cells. The trypan blue dye exclusion assay showed ≥97% cell viability.

For isolation of purified human T lymphocytes, E-rosetted cells were treated with ammonium chloride to remove sheep erythrocytes, washed three times, and subjected to antibody-directed complement lysis with an antibody to the HLA-DR antigen (L243) and to the monocyte-specific CD11b antigen (OKM1) and pooled rabbit complement, as described before (1). Monoclonal antibodies L243 and OKM1 were purified by using the protein G MAb Trap Kit (Pharmacia) from ascites fluid obtained by injecting hybridomas (American Type Culture Collection, Rockville, Md.) into pristane-primed BALB/c mice. Purified human T cells were >98% $CD2^+$ and <2% $HLA\text{-}DR^+$ as determined by flow cytometric analysis.

Paraformaldehyde fixation of cells. Purified cells were incubated in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min at room temperature. Fixed cells were washed three times with Hanks balanced salt solution and suspended in RPMI 1640 with 10% FBS.

TSST-1 stimulation of monocytes and T lymphocytes. Purified monocytes or T cells, alone or mixed 1:1, were cultured in 24-well plates in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 10 μg of polymyxin B sulfate per ml. Cells were stimulated with 10 μg of TSST-1 per ml for 24 h at 37°C. For antibody studies, cells were preincubated with the indicated concentrations of rabbit anti-human gamma interferon (anti-IFN-γ; Endogen, Boston, Mass.) or purified anti-HLA-DR monoclonal antibody L243.

For chamber experiments, monocytes were cultured in the upper chambers of 12-mm Transwells (0.45-μm pore size; Millipore, Mississauga, Ontario, Canada), while T cells were added to the lower chambers. Supernatants were collected, microcentrifuged at $800 \times g$ for 5 min, and frozen at −70°C until analysis.

TNF cytotoxicity assay. A standard L929 cytotoxicity assay was used to detect TNF activity in supernatants of TSST-1-stimulated cell cultures (9). Briefly, the murine fibroblast L929 cell line (American Type Culture Collection) was cultured in flat-bottomed 96-well microtiter plates at a concentration of 5×10^4 cells per well in RPMI 1640 medium containing 10% FBS and 4 μg of dactinomycin (Sigma) per ml. Serial dilutions of recombinant human TNF-α standards (R & D Systems, Minneapolis, Minn.) or test samples, preincubated for 2 h at 37°C with or without added goat anti-human TNF-α or TNF-β (R & D Systems), were placed in 50-μl volumes into appropriate wells. After incubation for 20 h at 37°C, the culture medium was removed and 0.5 mg of MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma) per ml in phenol red-free RPMI 1640 was added, and the culture plate was incubated for a further 3 h at 37°C. Excess dye was removed, and 100 μl of dimethyl sulfoxide was added to the wells to dissolve the dye precipitate. The purple color was quantitated at an optical density of 540 nm (OD_{540}) with a Titertek multiscan spectrophotometer (Flow Laboratories, Inc., McLean, Va.). Cytotoxicity was calculated as follows: percent cytotoxicity = [1 − (OD_{540} of sample/OD_{540} of control)] × 100. The sensitivity of the assay was 20 pg/ml.

ELISA for assay of TNF-α and IL-1β. An enzyme-linked immunosorbent assay (ELISA) was developed for the measurement of TNF-α in all culture supernatants. Goat anti-human TNF-α (R & D Systems), 2 μg/ml in 0.05 M bicarbonate–carbonate buffer, pH 9.6, was used to coat flat-bottomed 96-well microtiter plates (Immulon 1; Dynatech Laboratories, Inc., Alexandria, Va.) for 20 h at 20°C (100 μl per well). Unbound antibodies were removed by three 2-min washes with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T). Human recombinant TNF-α standards (R & D Systems), serially diluted from 8,000 to 62 pg/ml in PBS with 3% bovine serum albumin (BSA), were added in triplicate 100-μl volumes to the wells and incubated at 37°C for 1.5 h. After the wells were washed with PBS-T, 100 μl of biotinylated goat anti-human TNF-α (diluted 1:4,000 in PBS with 3% BSA) was added, and the plates were incubated at 37°C for 1.5 h. The plates were then washed and incubated with streptavidin-alkaline phosphatase (diluted 1:2,000 in PBS with 3% BSA) for 20 min at 37°C. The wells were then washed five times with 200 μl of 50 mM Tris-buffered saline, pH 7.5. An ELISA amplification system (GIBCO/BRL, Burlington, Ontario, Canada) was then used to increase the sensitivity of the assay. Briefly, 50 μl of the BRL substrate was pipetted into each well and incubated at room temperature for 15 min in the dark. Without removal of the substrate solution, 50 μl of BRL amplifier reagent was added, and the plate was incubated for a further 15 min at room temperature. The reaction was stopped by the addition of 50 μl of 0.3 M H_2SO_4, and the OD_{495} was measured in a Titertek multiscan spectrophotometer (Flow Laboratories). The sensitivity limit of the TNF-α ELISA was 62 pg/ml. No cross-reactivity was observed with human TNF-β, IL-1α, IL-1β, and IFN-γ. In parallel experiments, the TNF-α ELISA results correlated strongly with the cytotoxicity assay results (correlation coefficient, 0.84; $n = 13$; $P < 0.001$).

Human IL-1β was quantitated with an ELISA kit (sensitivity limit, 10 pg/ml) purchased from R & D Systems.

Biotinylation of antibodies. Antibodies were biotinylated by using freshly prepared biotinyl-Σ-amino caproic acid *N*-hydroxysuccinimide ester (biotin-X-NHS; Calbiochem, La Jolla, Calif.) at a biotin-X-NHS-to-antibody ratio of 100:1, as described previously (11). After 4 h at room temperature, the reaction was stopped by the addition of 1 M Tris-HCl, pH 8.0. Biotinylated antibody was separated from

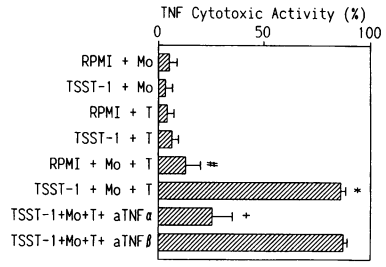

FIG. 1. Production of TNF by human monocytes and T cells in response to TSST-1. Human monocytes (Mo) (10^6 cells per ml) or T cells (T) (10^6 cells per ml), alone or cocultured 1:1, were incubated with 10 μg of toxin per ml for 24 h at 37°C. Supernatants were collected and assayed for TNF by the L929 fibroblast cytotoxicity assay. A 100% cytotoxic activity denotes complete L929 cell lysis. A recombinant human TNF-α standard produced a cytotoxic activity of 54.5% ± 2.7% (mean ± standard error for five assays). For neutralization experiments, supernatants were incubated with 10 μg of goat anti-human TNF-α (aTNFα) or anti-human TNF-β (aTNFβ) per ml for 2 h prior to assay for TNF activity. The results represent the means ± standard errors (SE) for four separate donors. TNF production induced by TSST-1 in the presence of monocytes and T cells (*) is of borderline significance compared with RPMI controls (#) ($P = 0.06$, one-tailed Wilcoxon's signed rank test). Neutralization of activity by anti-TNF-α (+) is of borderline significance compared with TSST-1 positive control levels (*) ($P = 0.06$, one-tailed Wilcoxon's signed rank test).

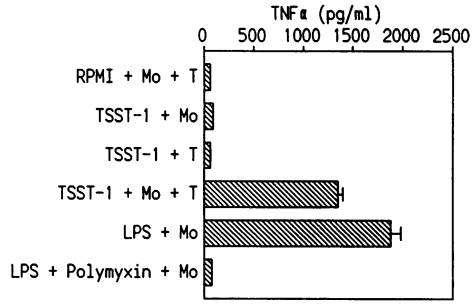

FIG. 2. Comparison of TSST-1- and LPS-induced TNF-α secretion by human monocytes and T cells. Monocytes (10^6 cells per ml) or T cells (10^6 cells per ml), alone or cocultured 1:1, were incubated with 10 μg of TSST-1 per ml for 24 h at 37°C. Monocytes alone were also incubated with 1 μg of *E. coli* LPS per ml in the absence or presence of 10 μg of polymyxin B sulfate per ml. TNF-α levels in culture supernatants were measured by an ELISA as described in Materials and Methods. Results (mean ± SE) are from one representative donor studied in triplicate experiments. See Fig. 1 legend for abbreviations.

free biotin by gel filtration with a PD-10 gel filtration column (Pharmacia Fine Chemicals, Dorval, Quebec, Canada).

RESULTS

Monocytes and T cells, alone or in combination, were stimulated with TSST-1 purified by preparative isoelectric focusing and chromatofocusing from culture supernatants of *S. aureus* MN8. With the L929 cytotoxicity assay, the data show that treatment of monocytes or T cells alone with TSST-1 (10 μg/ml) resulted in little TNF cytotoxic activity (Fig. 1). No TNF activity could be detected in monocyte or T-cell cultures incubated for up to 72 h with 10 μg of TSST-1 per ml (data not shown). When TSST-1 (10 μg/ml) was added to a 1:1 mixture of monocytes and T cells, a large increase (up to sixfold) in cytotoxic activity was found at 24 h compared with the medium control (Fig. 1). TSST-1 itself had no cytotoxic effect on L929 cells (data not shown). The cytotoxic activity of culture supernatants was neutralizable with antibodies directed against TNF-α but not against TNF-β (Fig. 1).

Since the TNF activity was predominantly the alpha form, an ELISA for TNF-α (sensitivity limit, 62 pg/ml) was developed to quantitate the levels of this cytokine in culture supernatants. The TNF-α ELISA was less labor-intensive to perform than the L929 bioassay. The TNF-α-specific ELISA also confirmed the requirement for T cells for cytokine induction in human monocytes by TSST-1. As shown in Fig. 2, TSST-1 did not induce TNF-α secretion unless monocytes and T cells were both present. In contrast, 1 μg of *Escherichia coli* lipopolysaccharide (LPS) (O26:B6; Sigma) per ml stimulated TNF-α secretion from human monocytes in the absence of T cells (Fig. 2). This effect of LPS on human monocytes was abolished in the presence of polymyxin B sulfate (10 μg/ml) (Fig. 2).

To determine whether the induction of another cytokine, IL-1β, also involved both cell types, human T cells and monocytes, alone or mixed 1:1, were incubated with TSST-1 for 24 h at 37°C. The results indicate that IL-1β induction by TSST-1 also occurred only in the presence of both cell types and that monocytes stimulated with TSST-1 in the absence of T cells could not produce this cytokine (Fig. 3).

To investigate further the nature of the monocyte/T-cell interaction for cytokine production, several antibodies, including those directed against the T-cell lymphokine IFN-γ and against the TSST-1 receptor (HLA-DR), were added to the cell cultures before TSST-1 stimulation. Rabbit anti-IFN-γ immunoglobulin G (10 μg/ml; Endogen, Boston, Mass.) did not inhibit TSST-1-induced TNF-α production in the presence of monocytes and T cells (Fig. 4A). A monoclonal antibody, L243, known to block binding of TSST-1 to the HLA-DR receptor on human monocytes as well as to inhibit TSST-1-triggered accessory cell-dependent T-lymphocyte proliferation (29, 31) also did not neutralize TSST-1-induced TNF-α secretion in the presence of both cell types (Fig. 4B).

Experiments were then performed to determine whether there was a requirement for cell contact between monocytes and T cells for TNF-α induction by TSST-1. A Transwell

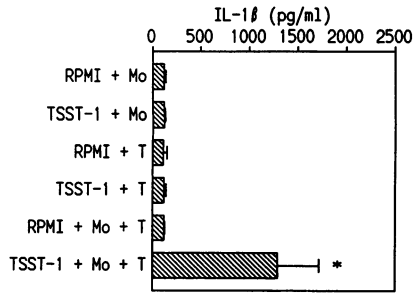

FIG. 3. Induction of IL-1β in human monocytes by TSST-1 in the presence and absence of T cells. Monocytes (10^6 cells per ml), cocultured with T cells in a 1:1 ratio, were stimulated with 10 μg of TSST-1 per ml for 24 h at 37°C. Supernatants were assayed for IL-1β with a commercial ELISA kit. *, significant difference compared with RPMI 1640 medium control ($P = 0.008$, one-tailed Wilcoxon's signed rank test). Results represent the means ± SE for seven separate donors. See Fig. 1 legend for abbreviations.

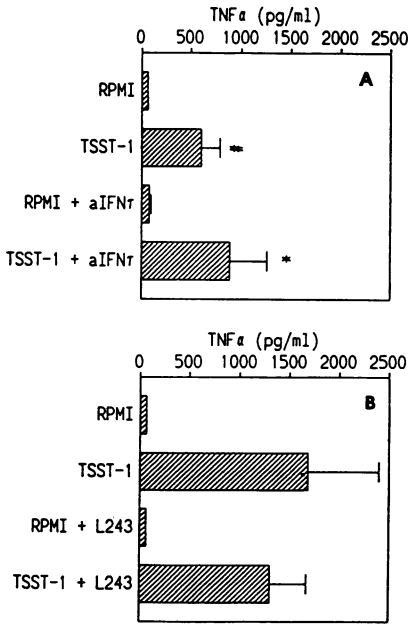

FIG. 4. Effects of different antibodies on TNF-α induction in human monocytes and T cells by TSST-1 as measured by ELISA. Monocytes cocultured 1:1 with T cells were treated with antibody for at least 1 h prior to the addition of TSST-1 (10 μg/ml) for 24 h at 37°C. (A) Cells treated with rabbit anti-human IFN-γ (10 μg/ml). Each bar represents the mean ± SE for five separate donors. *, borderline significant increase in TNF-α levels ($P = 0.06$, one-tailed Wilcoxon's signed rank test) compared with TSST-1 controls (#). (B) Cells treated with anti-HLA-DR monoclonal antibody L243 (100 μg/ml). Each bar represents the mean ± SE for four separate donors. No significant difference ($P = 0.3$, one-tailed Wilcoxon's signed rank test) was found for TSST-1-treated monocytes plus T cells in the presence or absence of L243. See Fig. 1 legend for abbreviations.

chamber insert was used to physically separate monocytes from T cells but still allow the diffusion of soluble cytokines through its 0.45-μm pores. The results show that TSST-1 did not induce TNF-α when monocytes were separated from T cells, suggesting that contact between these cell types was

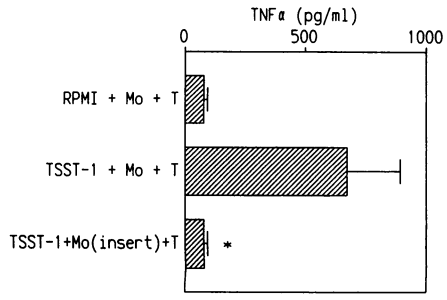

FIG. 5. Requirement for monocyte/T-cell contact for the induction of TNF-α by TSST-1. Equal numbers of monocytes and T cells were cultured in the upper (insert) and lower chamber, respectively, in Transwell plates and stimulated with TSST-1 (10 μg/ml) for 24 h at 37°C. Supernatants were assayed for TNF-α by ELISA. Each bar represents the mean ± SE for four separate donors. *, borderline significant difference ($P = 0.06$, one-tailed Wilcoxon's signed rank test) compared with supernatants of unseparated monocytes plus T cells stimulated with TSST-1. See Fig. 1 legend for abbreviations.

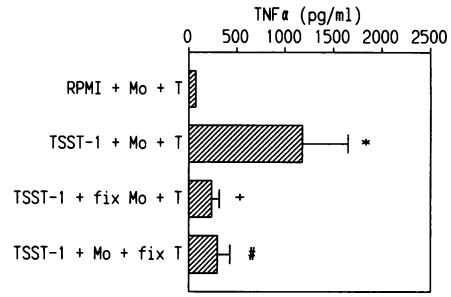

FIG. 6. Effect of fixation of human monocytes or T cells on the induction of TNF-α by TSST-1. Monocytes or T cells were metabolically inactivated by fixation with 4% paraformaldehyde and added in a 1:1 ratio (10^6 cells of each per ml) to viable T cells or monocytes, respectively. Cells were stimulated with TSST-1 (10 μg/ml) for 24 h at 37°C, and the supernatants were assayed for TNF-α by ELISA. Results represent the means ± SE for seven donors. Significant differences in TNF-α levels were found for TSST-1 plus fixed monocytes (fix Mo) plus T cells (+) versus positive controls (*) ($P = 0.008$, one-tailed Wilcoxon's signed rank test) and for TSST-1 plus monocytes plus fixed T cells (fix T) (#) versus positive controls (*) ($P = 0.015$, one-tailed Wilcoxon's signed rank test).

required for cytokine secretion (Fig. 5). Although the double-chamber culture experiments indicated that cognate interactions between monocytes and T cells were important, there remained the possibility that soluble factors were still involved (i.e., cell contact plus soluble factors were required for the induction of TNF-α secretion by monocytes or T cells). To address this question, either monocytes or T cells were paraformaldehyde fixed and reconstituted with viable counterpart cell types. The results show that fixing either monocytes or T cells almost completely abolished TNF-α secretion by the viable cells (Fig. 6), suggesting that in addition to cell contact, a soluble factor(s) secreted by monocytes or T cells was important in cytokine production.

It has been reported that TSST-1 is able to induce TNF-α and IL-1β production in monocytes in the absence of T cells (33). The discrepancy between our results and those of other workers may lie in the purity of the fractionated monocytes (e.g., contamination with T cells) or in the purity of the TSST-1 preparations. To investigate the latter possibility, a commercial preparation of TSST-1 (Toxin Technology) was compared with our own in-house toxin for induction of TNF-α secretion by human monocytes. In contrast to our TSST-1 preparations, commercial TSST-1 induced TNF-α from monocytes alone, in the absence of T lymphocytes (Table 1). However, further evaluation of the commercial TSST-1 by silver staining and immunoblotting with pooled normal human serum after SDS-PAGE revealed multiple protein impurities (14a). When commercial TSST-1 was further purified to homogeneity by a chromatofocusing procedure and then incubated with monocytes alone, no TNF-α activity was found. Like our TSST-1 preparation, the further-purified commercial TSST-1 induced TNF-α activity only when T cells were added to the monocyte cultures (Table 1). Additionally, a protein impurity fraction eluted from the chromatofocusing column was able to stimulate TNF-α secretion from monocytes alone (data not shown). These data also excluded the possibility that the commercial TSST-1 preparation was contaminated by LPS, since all studies with TSST-1 preparations were performed in the presence of polymyxin B sulfate (10 μg/ml).

TABLE 1. TNF production by human monocytes and T cells stimulated with various TSST-1 preparations

Stimulus[a] and cells	TNF-α[b] (pg/ml)	
	Donor 1	Donor 2
RPMI + monocytes	<62	<62
RPMI + monocytes + T cells	<62	<62
TSST-1 (AWC) + monocytes	<62	<62
TSST-1 (AWC) + T cells	<62	<62
TSST-1 (AWC) + monocytes + T cells	1,500	3,700
TSST-1 (TT) + monocytes	1,400	1,550
TSST-1 (TT) + monocytes + T cells	1,500	ND[c]
Further-purified TSST-1 (TT) + monocytes	<62	<62
Further-purified TSST-1 (TT) + monocytes + T cells	2,600	3,500

[a] TSST-1 (AWC), in-house-produced TSST-1; TSST-1 (TT), commercial TSST-1 purchased from Toxin Technology Inc. TSST-1 (TT) was further purified by a chromatofocusing procedure.
[b] TNF-α was quantitated by ELISA (lower sensitivity limit, 62 pg/ml). Values represent the means of duplicate determinations.
[c] ND, not done.

DISCUSSION

The results of this study show that induction of the cytokines TNF-α and IL-1β by TSST-1 requires the presence of both monocytes and T lymphocytes. Obtained with the use of highly purified TSST-1 and fractionated human peripheral blood mononuclear cells, our data show that cultures of human monocytes or T cells alone, stimulated with TSST-1 for up to 72 h, were unable to produce TNF-α or IL-1β. However, when both cell types were added together, significant cytokine production was observed 24 h after TSST-1 stimulation. Both monocytes and T cells were involved in TNF-α production, as evidenced by experiments in which paraformaldehyde fixation of one cell type and reconstitution with the other viable cell type prior to TSST-1 induction were performed. Our findings are in agreement with those observed with another superantigen, SEA. Fischer et al. (6) reported that TNF-α and TNF-β production by SEA requires the presence of both cell types. Using an intracytoplasmic staining technique for TNF-producing cells, they showed that both monocytes and T lymphocytes were actively producing TNF-α in response to SEA stimulation. T lymphocytes were further shown to produce TNF-β in the presence of autologous monocytes, although with later kinetics than observed with TNF-α (6).

The nature of the monocyte/T-cell interaction for cytokine production was investigated in this study. It is well known that TSST-1 and other superantigens bind to nonpolymorphic regions of MHC class II molecules, and together, these proteins are recognized by T cells bearing the appropriate V_β T-cell receptors (2, 8, 14). This would suggest that cytokine production occurs because of monocyte/T-cell contact or as a result of lymphokines released by T cells, which in turn upregulates TNF or IL-1 production by monocytes. Fischer et al. (6) have previously shown that several cytokines, such as IL-2, IL-4, IL-6, IL-7, and IFN-γ, alone or in combination, could not replace the T-cell requirement for TNF-α production by human monocytes stimulated with SEA. These authors also demonstrated that one particular T-cell subset, $CD4^+$ $45R^-$, supported TNF-α production by monocytes more efficiently than any other subset. In our study, consistent with the observations of Fischer et al. (6), rabbit anti-human IFN-γ antibodies did not abolish TNF-α production by monocytes plus T cells after TSST-1 induction, while an increase in TNF-α was observed (Fig. 4A), possibly due to uptake of immune complexes of IFN-γ and its antibody by monocytes. Furthermore, the priming of human monocytes overnight with 250 U of IFN-γ per ml prior to the addition of TSST-1 did not induce TNF-α production in the absence of T cells (unpublished data). These results strongly indicate that the signals transduced by T cells to monocytes do not consist entirely of IFN-γ and that physical contact or other soluble factors, or both, may be required for TSST-1-induced TNF-α production. This hypothesis is supported by our experiments in which Transwell culture chambers were used to physically separate monocytes from T cells but still allow the diffusion of soluble molecules through a filter. The abolition of TNF-α production by human monocytes or T cells in these chambers strongly indicates a requirement for monocyte/T-cell contact. However, it also appears that soluble factors are involved, since the addition of paraformaldehyde-fixed monocytes or T cells did not result in cytokine production by the counterpart viable cell. These results are analogous to those of Mourad et al. (21), who showed that TSST-1-triggered B-cell proliferative responses and immunoglobulin production require physical interaction between T and B cells. Recently, Lands and his associates (15) have shown that induction of IL-1 by anti-CD3 requires two signals, direct contact between monocytes and activated T cells and a soluble T-cell lymphokine. Our results suggest that induction of cytokine secretion by TSST-1 may also occur as a two-signal process involving direct monocyte/T-cell contact as well as lymphokine or monokine production. This is in contrast to LPS induction of cytokine production in human monocytes, for which T cells are not required. Whereas LPS can induce cytokine secretion by interacting directly with human monocytes, TSST-1-stimulated monocytes are unable to do so unless additional signals (cell contact and soluble mediators) from T cells are provided. Thus, the activation of human monocytes by TSST-1 and LPS may involve different signal transduction pathways. This hypothesis is further supported by our recent finding that TSST-1 and LPS induce distinct patterns of protein phosphorylation in human monocytes alone (32).

It is surprising that for four separate donors, the addition of HLA-DR monoclonal antibody L243 did not abolish TNF-α production by monocyte/T-cell cultures stimulated with TSST-1. Previous work in our laboratory as well as in others has demonstrated that the L243 monoclonal antibody strongly blocks binding of TSST-1 to the HLA-DR receptor in human monocytes (22, 28–31). In addition, this monoclonal antibody also abrogates monocyte-dependent T-cell activation and B-cell production of immunoglobulins induced by TSST-1 and other staphylococcal exotoxins (19, 21, 29). It is quite possible that binding of TSST-1 to HLA-DR on monocytes and subsequent activation of T-cell proliferation are unrelated to the production of TNF-α. Support for this contention comes from the findings of Grossman et al. (12), who showed that monocyte-stimulatory activity (e.g., TNF-α secretion) in response to SEA and SEB could be dissociated from that of T-cell proliferation. Alternatively, induction of TNF-α by TSST-1 may involve other class II MHC molecules, such as HLA-DQ and HLA-DP, with which L243 does not cross-react. Recently, it has been shown that in the presence of both the I-E and I-A haplotypes on antigen-presenting cells of recombinant congenic mice, presentation of SEB to a murine T-cell receptor $V_{\beta 8+}$

T-cell clone is dominated by I-E (26). However, in the absence of I-E, some antigen-presenting cells bearing I-A alleles can present SEB as effectively as those expressing both I-A and I-E. Therefore, in our study, it is possible that when HLA-DR receptors on monocytes are blocked by the L243 monoclonal antibody, HLA-DQ and HLA-DP play a more prominent role in TNF-α induction by TSST-1. This hypothesis can be easily tested in future studies by examining the relative effectiveness of anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP antibodies, alone and in combination, on cytokine production. Finally, TSST-1 induction of TNF-α in human monocytes and T cells may be mediated by other receptors, such as the adhesion molecules CD2 and LFA-1 (4, 20).

TSST-1 has been reported to stimulate plastic-adherence-purified monocytes alone to produce TNF and IL-1 (5, 13, 24, 25). The difference between our results and those of others may lie in the purity of the monocyte cultures used. A few contaminating T cells in the monocyte preparations may be sufficient for triggering TNF-α and IL-1β production in response to the toxin. Alternatively, protein impurities in the TSST-1 preparations may produce misleading results. We have consistently found, by silver staining or by immunoblotting (with either pooled normal human serum or rabbit antiserum against *S. aureus* MN8 culture supernatant) following SDS-PAGE, that several lots of commercially available TSST-1 contained protein impurities (14a). Further-purified commercial TSST-1 induced TNF-α only when monocytes were mixed with T cells (Table 1). Recently, we have isolated a protein impurity fraction by chromatofocusing from the commercial TSST-1 preparation; this impurity fraction alone was capable of inducing TNF-α production from monocytes in the absence of T cells (unpublished data). These results emphasize that for cytokine studies, more-stringent methods of assessing the purity of TSST-1 preparations must be used (27).

In conclusion, unlike LPS, the induction of the cytokines TNF-α and IL-1β by TSST-1 was found to require the presence of both human monocytes and T lymphocytes. The induction of these cytokines was not abrogated by antibodies directed against the lymphokine IFN-γ or HLA-DR, the receptor for TSST-1 in human monocytes. Direct physical contact between both cell types and the release of a soluble factor(s) other than IFN-γ appear to be essential for the induction of cytokines by TSST-1.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the Medical Research Council of Canada (MT-7630), the British Columbia Health Care Research Foundation, and the Canadian Bacterial Diseases Network Centers of Excellence.

We thank Donna Hogge and the nursing staff at the Cell Separator Unit, Vancouver General Hospital, for the provision of plateletpheresis packs, Niamh Kelly for provision of the L929 murine fibroblast cell line, and Annie Lam for preparation of the manuscript.

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