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Staphylococcal Toxic Shock Syndrome Toxin 1-Induced Tumor Necrosis Factor Alpha and Interleukin-1β Secretion by Human Peripheral Blood Monocytes and T Lymphocytes Is Differentially Suppressed by Protein Kinase Inhibitors

RAYMOND H. SEE AND ANTHONY W. CHOW*

Division of Infectious Diseases, Departments of Medicine, Microbiology, and Pathology, and Canadian Bacterial Diseases Network, University of British Columbia and Vancouver General Hospital, Vancouver, British Columbia V5Z 3J5, Canada

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The signal transduction pathways by which staphylococcal toxic shock syndrome toxin 1 (TSST-1) induces tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) secretion were examined with various protein kinase inhibitors. TNF-α secretion by normal human monocytes and T cells in response to TSST-1 was suppressed by inhibitors of protein kinase C (H7) and tyrosine kinases (genistein). In contrast, the secretion of IL-1β was blocked by a cyclic AMP- and cyclic GMP-dependent kinase inhibitor (HA1004) as well as by H7 and genistein. These results suggest that the secretion of TNF-α and IL-1β may be differentially regulated by TSST-1 and that protein kinases play an important role in mediating cytokine responses to the toxin.

Toxic shock syndrome (TSS) is a multisystem disease associated with *Staphylococcus aureus* infection. A 22-kDa exotoxin, TSS toxin 1 (TSST-1), is thought to play a central role in this disease. TSST-1 is a member of the superantigen family with the ability to stimulate a large population of T cells through the V_β sequences of their T cell antigen receptors in a major histocompatibility complex class II-dependent manner (3, 8, 11). Aside from T cell stimulation, TSST-1 is also a potent inducer of the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) from human blood monocytes. These two mediators are thought to play a major role in TSS pathogenesis (5, 15, 16). Recently, we reported that TSST-1 induction of TNF alpha (TNF-α) and IL-1β from normal human blood monocytes required the presence of T lymphocytes (19). Direct cell contact between monocytes and T cells was essential for cytokine production in response to TSST-1 stimulation (19).

In general, the interaction of agonists with their respective membrane receptors results in the generation of a group of cytosolic compounds known as second messengers. These second messengers, which include diacylglycerol, inositol trisphosphate, calcium, cyclic AMP (cAMP), and cyclic GMP (cGMP), serve to transmit and amplify extracellular signals by activating cytosolic protein kinases. These kinases then phosphorylate specific cellular proteins, resulting in their activation for a variety of cell functions, such as cell proliferation, protein secretion and exocytosis, and gene expression (13, 14). The series of reactions that mediate the cellular responses to extracellular stimuli are known as signal transduction pathways.

The signal transduction pathways by which TSST-1 stimulates cytokine release from human peripheral blood mononuclear cells have not yet been defined. TSST-1 appears to activate human monocytes by dephosphorylating several cellular phosphoproteins in a dose- and time-dependent manner (18). Whether dephosphorylation of these proteins is related to cytokine induction has not been determined. In this study, various protein kinase inhibitors were used to explore the signal transduction pathways involved in the secretion of TNF-α and IL-1β by human monocytes and T cells in response to TSST-1 stimulation.

TSST-1 was purified from culture supernatants of *S. aureus* MN8 by preparative isoelectric focusing and chromatofocusing as previously described (9a, 17). The purity of TSST-1 was determined to be >99% by silver staining of sodium dodecyl sulfate-polyacylramide gels and by immunoblotting with rabbit polyclonal antisera against the crude culture filtrate or with pooled normal human serum.

Fresh human peripheral blood mononuclear cells from normal healthy donors were obtained by centrifugation of platelet pheresis buffy coats over Histopaque 1.077 (Sigma Chemical Co., St. Louis, Mo.). Cells at the interface were washed five times with Hanks' balanced salt solution and additionally separated into T and non-T cell populations by rosette formation with sheep erythrocytes as described previously (10). Monocytes were then separated from B lymphocytes by density centrifugation with Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) as outlined by de Boer et al. (4). In brief, the non-T cell fraction was suspended in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). The cells were then mixed with Percoll to yield a final specific gravity of 1.062 g/ml. One milliliter of RPMI 1640 with 10% FBS was gently layered on top of each suspension. The gradient was then centrifuged at 850 × *g* for 15 min. The monocyte-containing interface was removed, washed three times, and suspended in RPMI 1640 containing 10% FBS, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, and 10 μg of polymyxin B sulfate per ml. The purity of the monocytes was determined to be >90% by nonspecific esterase staining of centrifuged preparations. For isolation of purified T lymphocytes, erythrocyte-rosetted cells were treated with ammonium chloride to remove sheep erythrocytes, washed three times, and subjected to antibody-directed complement-mediated lysis with monoclonal antibodies directed against the HLA-DR antigen, L243, and against the monocyte

* Corresponding author.

antigen, OKM1, and pooled rabbit complement as described previously (2). T lymphocytes were suspended in the above-described medium at a concentration of 2×10^6 cells per ml. The composition of the T cells was confirmed by flow cytometric analysis to be >98% $CD2^+$ and <2% $HLA\text{-}DR^+$.

A 1:1 ratio (10^6 cells) of monocytes and T cells in the above-described medium was cultured with various concentrations of TSST-1 for 24 h at 37°C in 24-well culture plates in the presence or absence of inhibitors of second messenger pathways. The inhibitors included H7 (1-[5-isoquinolinylsulfonyl]-2-methylpiperazine) and HA1004 (*N*-[2-guanidinoethyl]-5-isoquinolinesulfonamide), both from Sigma, and genistein, from ICN, Mississauga, Ontario, Canada. Cells were pretreated with the kinase inhibitors for 15 min prior to stimulation with TSST-1. Culture supernatants were centrifuged at 800 × *g* to pellet cells and frozen at −70°C for cytokine analysis.

Levels of TNF-α and IL-1β in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISAs) developed in our laboratory. Goat anti-human TNF-α or goat anti-human IL-1β (R & D Systems, Minneapolis, Minn.) at 2 μg/ml in 0.05 M bicarbonate-carbonate buffer (pH 9.6) was used to coat flat-bottomed 96-well microtiter plates (Immulon I; 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. Human recombinant TNF-α or IL-1β standards (R & D Systems) serially diluted from 8,000 to 62.5 pg/ml in PBS with 3% bovine serum albumin (BSA) were added in triplicate 100-μl volumes to respective wells, and the plates were incubated at 37°C for 1.5 h. After three washes with PBS–0.1% Tween 20, 100 μl of biotinylated goat anti-human TNF-α or biotinylated goat anti-human IL-1β (diluted 1/4,000 in PBS with 3% BSA) was added to respective wells, and the plates were incubated for 1.5 h. The plates were 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. In brief, 50 μl of the BRL substrate was pipetted into each well, and the plates were incubated at room temperature for 15 min in the dark. Without removal of the substrate solution, 50 μl of the BRL amplifier reagent was added, and the plates were incubated for 15 min at room temperature. The reaction was stopped by the addition of 50 μl of 0.3 M H_2SO_4, and the optical density at 495 nm was measured in a Titertek Multiskan spectrophotometer (Flow Laboratories, Mississauga, Ontario, Canada). For the TNF-α ELISA, no cross-reactivity was observed with IL-1α, IL-1β, or gamma interferon. For the IL-1β ELISA, no cross-reactivity was observed with IL-1α, TNF-α, or gamma interferon. Statistical analyses of cytokine levels in culture supernatants in the presence or absence of kinase inhibitors were performed with the paired Student *t* test.

To determine the second messenger pathways involved in cytokine induction by TSST-1, we used various protein kinase inhibitors. Human monocytes cocultured 1:1 with T cells were stimulated with various concentrations of TSST-1 in the presence or absence of the following inhibitors of protein kinases: H7 (protein kinase C), HA1004 (cAMP- and cGMP-dependent kinases), and genistein (tyrosine kinases). As shown in Fig. 1A, both H7 and genistein suppressed TNF-α secretion in a dose-dependent manner. Strong inhibition of TNF-α secretion was observed with 25 μM H7 and

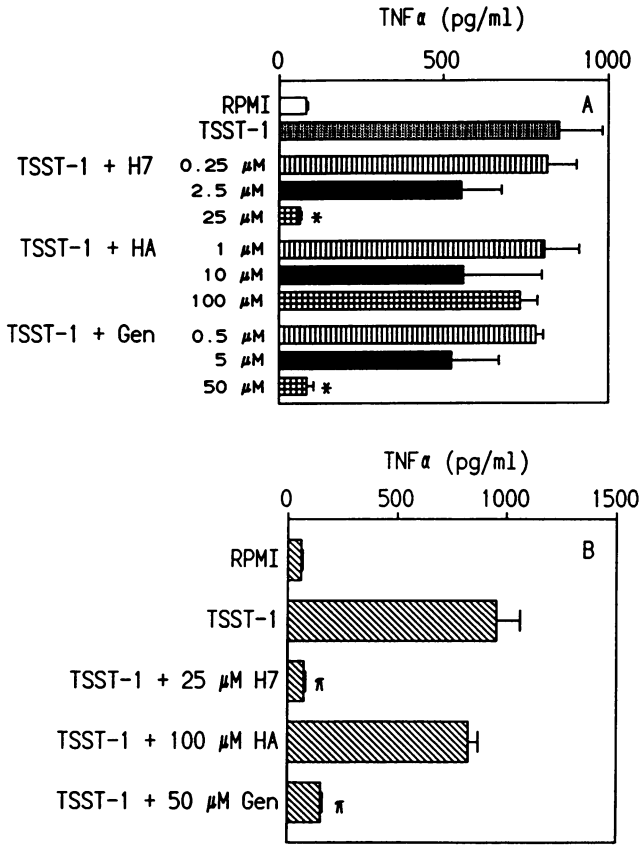

FIG. 1. Effect of kinase inhibitors on TSST-1-induced TNF-α secretion by human monocytes (10^6 cells) cocultured 1:1 with T lymphocytes in RPMI 1640 medium. Cells were pretreated with H7, HA1004 (HA), or genistein (Gen) for 15 min prior to the addition of TSST-1 for 24 h at 37°C. TNF-α levels in culture supernatants were determined by an ELISA. (A) Dose-dependent inhibition of TNF-α secretion by kinase inhibitors after stimulation of cells with 1 μg of TSST-1 per ml. The results represent the mean ± standard error of the mean (SEM) for three donors. *, significantly different ($P < 0.05$; paired *t* test) from the TSST-1 control. (B) TNF-α levels in culture supernatants of cells stimulated with 10 μg of TSST-1 per ml in the presence or absence of 25 μM H7, 100 μM HA1004, or 50 μM genistein. The results represent the mean ± SEM for 12 donors. π, significantly different ($P < 0.001$) from the TSST-1 control.

50 μM genistein (92 and 83% inhibition, respectively; Fig. 1B). The inhibitory effect was not attributed to an alteration in cell viability, as judged by trypan blue staining. HA1004, an inhibitor of cAMP- and cGMP-dependent kinases but not of protein kinase C, did not affect TSST-1-induced secretion of TNF-α, as determined by an ELISA. Furthermore, the concentrations of inhibitors used in these experiments are well within the range used in a number of other studies (1, 7, 12, 20).

The effect of the same kinase inhibitors on IL-1β secretion by cocultures of human monocytes and T cells was evaluated with an ELISA specific for IL-1β. Figure 2A shows the dose-dependent inhibition by H7, HA1004, and genistein of TSST-1-induced IL-1β secretion. IL-1β levels were strongly reduced in the presence of 25 μM H7, 100 μM HA1004, and 50 μM genistein (82, 75, and 87% inhibition, respectively) (Fig. 2B). All kinase inhibitors in medium alone did not induce TNF-α or IL-1β release (data not shown). H7 and

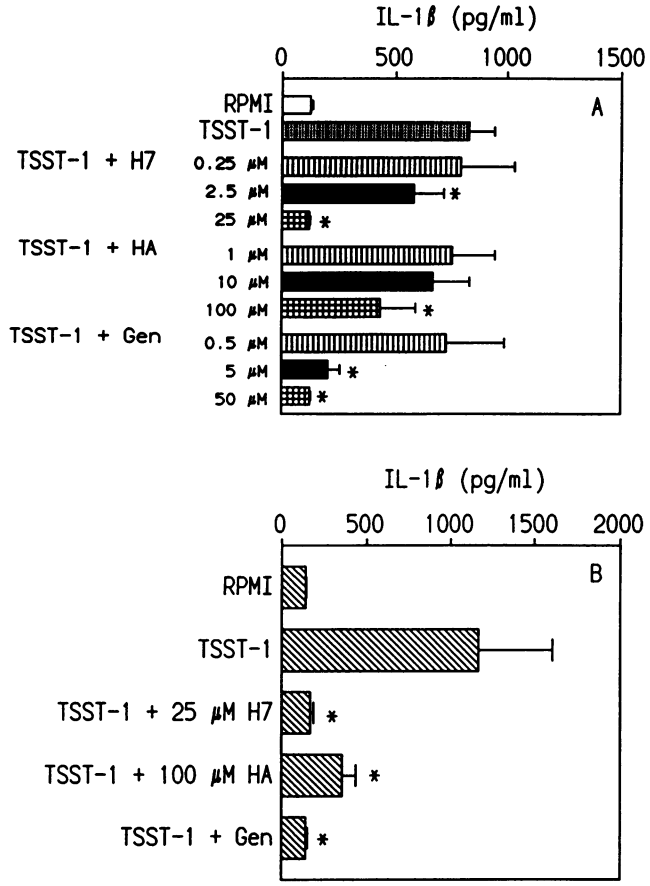

FIG. 2. Effect of kinase inhibitors on TSST-1-induced IL-1β secretion by human monocytes (10^6 cells) cocultured 1:1 with T lymphocytes in RPMI 1640 medium. Cells were pretreated with H7, HA1004 (HA), or genistein (Gen) for 15 min prior to the addition of TSST-1 for 24 h at 37°C. Culture supernatants were assayed for IL-1β by an ELISA. (A) Dose-dependent inhibition of IL-1β secretion by kinase inhibitors after stimulation of cells with 1 μg of TSST-1 per ml. The results represent the mean ± standard error of the mean (SEM) for three donors. *, significantly different ($P < 0.05$; paired t test) from the TSST-1 control. (B) IL-1β levels in culture supernatants of cells stimulated with 10 μg of TSST-1 per ml in the presence or absence of 25 μM H7, 100 μM HA1004, or 50 μM genistein. The results represent the mean ± SEM for 11 donors. *, significantly different ($P < 0.05$) from the TSST-1 control.

genistein also strongly inhibited (>80%) TNF-α and IL-1β secretion from lipopolysaccharide (LPS)-stimulated human monocytes, whereas HA1004 partially blocked IL-1β but not TNF-α secretion from these LPS-treated cells (data not shown).

The present study shows that inhibitors of second messenger pathways effectively block TSST-1-induced cytokine release from cocultures of monocytes and T cells. Furthermore, our studies suggest that the induction of TNF-α and IL-1β secretion by TSST-1 is differentially regulated. Both TNF-α secretion and IL-1β secretion were strongly inhibited by H7, suggesting a role for protein kinase C in cytokine production or secretion after toxin stimulation. The role of protein kinase C has also been implicated in gene expression and protein production by murine macrophages in response to LPS stimulation. Kovacs et al. (9) showed that in murine peritoneal macrophages, IL-1α, IL-1β, and TNF-α mRNA expression was inhibited in a dose-dependent manner by H7 during stimulation with LPS. Furthermore, they showed that the secretion of these cytokines by macrophages was inhibited when H7 was added after LPS stimulation, suggesting that posttranslational events were also influenced by protein kinase C. Taniguchi et al. (20) also reported that the secretion of IL-1 by LPS-stimulated human monocytes was significantly blocked by H7. Recently, in contrast to Kovacs et al. (9), Bakouche et al. (1) showed that protein kinase C played a major role in IL-1 secretion but not production. Using LPS-treated human monocytes, they showed that the protein kinase C inhibitors H7 and staurosporine did not block the production of intracellular IL-1α or IL-1β protein. However, these inhibitors were strongly effective in suppressing the extracellular release of IL-1, suggesting that the regulation of IL-1 production was independent of IL-1 secretion. In our studies, no conclusions could be made as to whether protein kinase C acted at the level of protein production or secretion.

Cytokine secretion induced by TSST-1 appears to involve more than one second messenger pathway. An inhibitor of tyrosine kinases was also effective in reducing both TNF-α and IL-1β levels. To our knowledge, the inhibition of IL-1β and TNF-α by the tyrosine kinase inhibitor genistein, in response to either TSST-1 or LPS has not yet been reported. We recently observed that TSST-1 and the staphylococcal enterotoxins induce the tyrosine phosphorylation of several monocyte cellular phosphoproteins (18). Whether the tyrosine phosphorylation of these monocyte cellular proteins in response to TSST-1 stimulation represents the initial signals that eventually result in cytokine production is not immediately clear. Further characterization of the tyrosine kinases and their substrate proteins are required to address this question.

Our data also suggest that the induction of TNF-α and IL-1β secretion by TSST-1 is differentially regulated. The cAMP- and cGMP-dependent protein kinase inhibitor HA1004 strongly suppressed IL-1β but not TNF-α release in human monocyte-T cell cultures stimulated with TSST-1. Differential inhibition of IL-1 and TNF gene expression and protein production has also been reported for LPS-stimulated murine macrophages. Kovacs et al. (9) showed that while protein kinase C- and calmodulin-dependent pathways were involved in the induction of IL-1 gene expression and production by LPS, only a protein kinase C-dependent pathway was involved in TNF-α expression and secretion. Additionally, Bakouche et al. (1) demonstrated that LPS presented to human monocytes in multilamellar vesicles resulted in the production and secretion of IL-1α but not IL-1β. These findings imply that LPS and possibly TSST-1 can activate the production of one cytokine without activating the concomitant production of the other (1, 9).

One general concern about the use of these inhibitors is that their specificities can vary depending on the concentrations used. For example, H7 can also inhibit both cAMP- and cGMP-dependent kinases but has the greatest activity for protein kinase C (K_i, 6.0 μM) (6). However, the use of several kinase inhibitors with overlapping specificities may allow one to draw some general conclusions. For all the inhibitors used in our experiments, the ranges of concentrations were selected on the basis of the specificities of the inhibitors for the particular kinases at their reported K_is and were similar to those used by others with human monocytes (1, 7, 12, 20). Furthermore, a dose-dependent effect of these inhibitors was observed. Moreover, HA1004 inhibited IL-1β

but not TNF-α secretion, further demonstrating its specific action on cAMP- and cGMP-dependent kinases.

In summary, our results indicate that TSST-1-induced cytokine secretion by human peripheral blood mononuclear cells is controlled by various second messengers. TNF-α secretion is suppressed by inhibitors of both protein kinase C and tyrosine kinases. In contrast, IL-1β release is blocked by inhibitors of cAMP- and cGMP-dependent protein kinases as well as by inhibitors of protein kinase C and tyrosine kinases, suggesting that the two cytokines are differentially regulated in response to TSST-1 induction.

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