Role of a Carboxy-Terminal Site of Toxic Shock Syndrome Toxin 1 in Eliciting Immune Responses of Human Peripheral Blood Mononuclear Cells

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Staphylococcus aureus toxic shock syndrome toxin 1 (TSST-1) is involved in the pathogenesis of toxic shock syndrome and perhaps other staphylococcal diseases. Recently, the C-terminal part of the TSST-1 toxin has been shown to be responsible for mitogenic activity in animal models. We studied the role of the C-terminal structural unit of TSST-1 with regard to proliferation, cytokine release (tumor necrosis factor alpha [TNF- α], interleukin-6 [IL-6], and IL-8), mRNA expression for IL-6, IL-8, IL-10, TNF- α , and CD40 ligand (CD40L), synthesis of immunoglobulin E (IgE), IgA, IgG, and IgM, CD23 expression, and soluble CD23 (sCD23) release from human peripheral blood mononuclear cells (PBMC). For this purpose, we used the recombinant wild-type TSST-1 (p17) mutant toxin Y115A (tyrosine residue modified to alanine) and toxin H135A (histidine residue modified to alanine). Unmodified toxin p17 and mutant toxin Y115A, at a concentration below 5 ng, to a lesser degree, induced a strong proliferation. Toxin p17 followed by toxin Y115A was the most pronounced inducer for mRNA expression for IL-10 and CD40L and cytokine generation (mRNA and protein) for TNF- α , IL-6, and IL-8. Mutant protein H135A failed to activate human PBMC. Both toxins p17 and, to a lesser degree, Y115A significantly suppressed IL-4- and anti-CD40-induced synthesis of all four Igs as well as IL-4-induced CD23 expression and sCD23 release. Mutant toxin H135A failed to do so. Thus, our data show that a region in the C terminus of TSST-1 is responsible not only for mitogenic activity but also for additional immunomodulating biological activities of TSST-1. More specifically, histidine residue H135A of the 194-amino-acid toxin appears to be critical for the expression of biological activities in a human in vitro model.

Staphylococcus aureus TSST-1 plays a crucial role in TSS, Kawasaki syndrome, and atopic dermatitis (8, 29, 30). In this regard, it seems that the capacity of TSST-1 to induce massive proliferation of T cells and concomitant release of proinflammatory cytokines, particularly TNF- α , is an essential precursor of lethal shock (32).

TSST-1 is a 22-kDa single-chain polypeptide including 194 amino acid residues (5). TSST-1 belongs to the group of superantigens, bacterial exotoxins that strongly and specifically stimulate CD4⁺ and CD8⁺ T lymphocytes by cross-linking the TCR with MHC II molecules on accessory or target cells (16, 17, 31). The three-dimensional structure of TSST-1 has been recently determined. Structural domains responsible for specific biological activities have been studied (1). In this regard, Kokan-Moore and Bergdoll showed that modification of one or two of the nine tyrosine residues leads to an 85% decrease in the mitogenic activity in a murine model (25, 26). Recently, the computer modeling studies of Acharya et al. suggested that both the N and C termini of TSST-1 are involved in binding to MHC class II molecules, while the C terminus is probably involved in binding TCR (1). Mutations at residues 132, 135, and 140 result in partial (35) or total (4, 6) loss of mitogenic activity for murine lymphocytes. Bonventre et al. reported that amino acid exchange at position 135 abolished the lethality of TSST-1, as measured in a rabbit infection model of TSS (6).

Cytokines play an important role in the regulation of many immune responses. It has been shown that activation of PBMC

by TSST-1 results in the release of several cytokines. TNF- α (3, 32, 42) is prominent in sepsis, IL-1 α exhibits pyrogenic activity, and IL-6 is a potent inducer of acute-phase responses (21). Interleukin-8, a recently described protein of 6 to 10 kDa known for its neutrophil chemotactic activity, is another potent mediator of host response during injury and infection (2, 15, 18, 20). IL-10, a Th2-derived cytokine, is also involved in immune response processes (33). High serum levels (>500 ng/ml) of IgE are characteristic of atopic dermatitis (19). IL-4 and the recently described cytokine IL-13 have been described as switch factors for IgE synthesis (39). In addition to elevation of IgE levels, IL-4 has several other effects on PBMC. In this regard, CD23 expression is observed after stimulation with IL-4. CD23 is the low-affinity receptor for IgE (Fc ϵ R2), represents a marker for activated B cells, and is an important differentiation marker (11, 28). Recently, it has been shown that the staphylococcal enterotoxins A and B affect the regulation of IgE synthesis and CD23 expression on human PBMC (36).

Since it has been reported that *Staphylococcus aureus* toxins are involved in allergic and inflammatory disease processes, we analyzed the influence of the recombinant wild-type p17 as well as recombinant mutant toxins Y115A and H135A on human PBMC with regard to proliferation, Ig synthesis, CD23 expression, sCD23 release, and cytokine expression and release.

MATERIALS AND METHODS

Abbreviations. aCD40, anti-CD40 monoclonal antibody; BSA, bovine serum albumin; CD23, low-affinity IgE receptor (Fc ϵ R2); CD40L, CD40 ligand; EA-

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SIA, enzyme-amplified sensitivity immunoassay; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MAb, monoclonal antibody; MHC, major histocompatibility complex; NSB, nonspecific binding; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; rIL, recombinant human interleukin; RIA, radioimmunoassay; RT, reverse transcription; sCD23, soluble CD23; SD, standard deviation; SSC, saline-sodium citrate buffer; TCR, T-cell antigen receptor; TNF, tumor necrosis factor; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1.

Isolation of lymphocytes. Lymphocytes were obtained from healthy volunteers. Isolation was performed by centrifugation on a Ficoll-sodium metrizoate (Sigma Chemical Co., Munich, Germany) gradient by the method of Böyum (9). Briefly, EDTA-treated blood (200 ml) was layered over Ficoll-sodium metrizoate (density, 1.075 g/ml) and centrifuged at $375 \times g$ for 45 min. Cells at the interphase above the Ficoll-metrizoate were removed and washed three times with RPMI 1640. These cells are referred to as PBMC. The composition of the PBMC fraction was determined by FACS analysis as follows: $84.6\% \pm 4.6\%$ lymphocytes, $14.4\% \pm 4.1\%$ monocytes, and $1.2\% \pm 0.5\%$ basophilic granulocytes.

Cell culture conditions. The basic culture medium was RPMI 1640 supplemented with 2 mM glutamine, streptomycin (100 µg/ml), penicillin (100 IU/ml), 10 mM HEPES, and 20 mM sodium hydrogen carbonate. Medium containing 10% FCS is referred to as RPMI 1640–10% FCS. Cell suspensions containing 10^6 viable cells per ml in RPMI 1640–10% FCS were dispensed into each well of 6-well (CD23 detection and RT-PCR analysis), 24-well (Ig synthesis), and 96-well (proliferation) plates. Stimuli were added, and the cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for the indicated time periods.

Stimuli. The recombinant TSST toxins p17, Y115A, and H135A were purified and characterized as described before (6). rIL-4 was obtained from W. Sebald (Theodor-Boveri-Institut für Biowissenschaften, Universität Würzburg). aCD40 (G28-5) was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Wash.

Determination of mitogenicity by [³H]thymidine incorporation. After 3 days in culture (96-well plates [Falcon]; 10^6 cells per ml; 200 µl per well), DNA synthesis was assessed by adding 7.4 kBq of [³H]thymidine (Amersham Buchler, Braunschweig, Germany) per well for the final 4 h of culture. Triplicate cultures were harvested and fixed onto glass fiber filters, and incorporation of [³H]thymidine was measured by liquid scintillation.

Determination of IgE. The determination of human IgE was performed by an ELISA sandwich assay. Plates (96-well; Nunc) were coated with MAb 4-15 and MAb 7-12 (kind gift from A. Saxon, University of California, Los Angeles) in bicarbonate buffer (0.1 M, pH 9.6) overnight at room temperature. The IgE standard was obtained from Behring-Werke (Marburg, Germany; highest standard, 200 ng/ml; 1:2 diluted in PBS–0.05% Tween; 12 standards, 1 NSB). Cell culture supernatants were added undiluted (100 µl to each well) and incubated likewise overnight at room temperature. After washing, the secondary antibody (Kirkegaard & Perry, Gaithersburg, Md.) goat anti-human IgE, phosphatase labeled, diluted 1:3,000 in PBS–0.05% Tween, was added, and the plates were incubated for 6 h at 37°C. The amount of IgE was determined according to the IL-8 protocol.

RIA for IgA, IgG, and IgM. The IgA, IgG, and IgM contents of culture supernatants were determined by a solid-phase RIA on day 10. Cell viability was assessed by trypan blue exclusion analysis. Briefly, purified goat anti-human IgA (100 µg), IgG (100 µg), and IgM (100 µg) antibodies (1 mg/ml; Dianova, Hamburg, Germany) were labeled with 37 MBq of Na¹²⁵I (specific activity, 520.2 MBq/µg of iodine; Amersham Buchler, Braunschweig, Germany) as described by Klinman and Taylor (24).

Removawell U-bottomed wells (Dynatech, Denkendorf, Germany) were coated with anti-IgA, -IgG, or -IgM (each at 100 µl; diluted 1:1,000 in PBS–0.05% Tween 20) for 4 h. Then 100 µl of supernatant (1:20 dilution) was added and incubated overnight at room temperature. [¹²⁵I]anti-IgA, [¹²⁵I]anti-IgG, or [¹²⁵I]anti-IgM (3.7 kBq), respectively, was added for an additional 4 h at 37°C. In parallel, standard curves for IgA, IgG, and IgM (0.3 to 300 ng/ml) were determined. All determinations were made at least in triplicate.

Determination of CD23. The detection of CD23 on PBMC was performed as previously described on day 3 of cell culture (10). MAb 135 was a kind gift from G. Delespesse (University of Montreal, Montreal, Canada). Briefly, 10^6 cells were incubated with ¹²⁵I-labeled MAb 135 (50 ng/200 µl; 14.8 kBq) for 1 h at room temperature. Separation of unbound and bound antibodies was carried out by centrifugation through a 500-µl FCS cushion (500 × g, 10 min, 4°C) in minitubes (Greiner, Nürtingen, Germany). After removal of unbound radioactivity and washing with PBS–0.05% Tween 20, the cell-bound radioactivity was measured with a gamma counter (Packard Cobra; Packard-Canberra, Frankfurt, Germany). All determinations were made at least in triplicate. The results were indicated as the percentage of the applied ¹²⁵I-labeled MAb bound per 10^6 cells.

Determination of sCD23. The quantification of sCD23 was carried out by a sandwich RIA as described in detail elsewhere (10). Briefly, microtiter plates with removable round-bottomed wells (Dynatech, Denkendorf, Germany) were coated with MAb 176 (1 µg/ml). The unoccupied protein-binding capacity of the plastic surface was saturated with 0.05% Tween 20 (Serva, Heidelberg, Ger-

many) and with 1% BSA (Boehringer, Mannheim, Germany) in PBS; incubation with the culture supernatants of the stimulated cells was then performed, and the bound sCD23 was detected with ¹²⁵I-labeled MAb 135 (12.5 ng/100 µl; 3.7 kBq). Results are indicated as percent of binding of the total applied ¹²⁵I-labelled MAb 135.

Determination of TNF-α. TNF-α was measured with a commercial EASIA assay obtained from Medgenix.

Determination of IL-8. IL-8 release was determined by a sandwich ELISA as detailed by M. Ceska (Boehringer, Vienna, Austria). Each well of a 96-well plate (Nunc Maxisorb) was precoated overnight at 4°C with 100 µl of bicarbonate buffer (0.1 M, pH 9.6) containing anti-IL-8 antibodies at a concentration of 5 µg/ml. The plates were washed three times with PBS–0.05% Tween 20, the appropriate sample or IL-8 standard was added, and incubation proceeded for 2 h at 37°C. Thereafter, alkaline phosphatase-linked anti-IL-8 antibody was added. After addition of *p*-nitrophenylphosphate (15 mg/ml) for quantification, an ELISA reader and, for calculation, Mikrotek software (SLT-Labinstruments, Crailsheim, Germany) were used.

RNA preparation and PCR analysis. The preparation of total cellular RNA was performed by the protocol of Chomczynski and Sacchi (12). The reverse transcription of total RNA from 5×10^6 cells to cDNA was performed by using cloned Moloney murine leukemia virus reverse transcriptase (2.5 U/µl) and oligo(dT)₁₆ (2.5 µM) according to the method described by Mullis and Fallona (34). The cDNA solution (10 µl) was amplified in reaction mixes (100 µl) containing 50 pmol of each appropriate primer pair (Table 1), 200 µmol of each nucleotide (dATP, dCTP, dGTP, and dTTP; Boehringer, Mannheim, Germany), and 2 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus) in 25 mM Tris-HCl (pH 8.3)–1.5 mM MgCl₂–50 mM NaCl–0.01% (wt/vol) gelatin. The samples were overlaid with mineral oil (Sigma, Munich, Germany) to prevent evaporation. The amplification was carried out in a DNA thermal cycler (Perkin Elmer Cetus) with the following temperature conditions: 5 min at 94°C to denature the DNA and then 25 to 35 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Aliquots (20 µl) of each reaction mix were applied to a 2% (wt/vol) agarose (Serva, Heidelberg, Germany) gel containing ethidium bromide (0.5 µg/ml) and electrophoresed in Tris-acetate-EDTA buffer. The 123-bp ladder (Bethesda Research Laboratories) was run in parallel as molecular size markers. The specificity of the amplified bands was validated by their predicted size. In all PCR experiments, β-actin was coamplified to evaluate similar amounts of mRNA. To evaluate that toxin treatment did not result in altered amounts of total RNA, the RNA was analyzed on a formaldehyde-agarose gel.

Slot blot analysis. Total RNA from 5×10^6 human lymphocytes diluted in 100 µl of H₂O–20× SSC–formaldehyde (5:3:2) was sequentially diluted (1:2) and applied to a convertible filtration manifold system obtained from Gibco BRL (Eggenstein, Germany). For the processing of the slot blot, a Digoxigenine Hybridization and Luminescent Detection Kit from Boehringer, Mannheim, Germany, was used according to the manufacturer's instructions.

Analysis of data. If not stated otherwise, all data show the mean values $\pm$ SD of at least three individual experiments with cells from different donors. The significance was evaluated with Student's *t* test for independent means. *P* < 0.05 was considered significant.

RESULTS

Mitogenicity. The mitogenicity of TSST-1 and recombinant wild-type p17 as well as mutant toxins Y115A and H135A for human PBMC was analyzed in a thymidine proliferation assay. As far as is known, TSST-1 and the mutant proteins activate only T cells. The superantigen toxins do not act as B-cell mitogens (6, 17). TSST-1 and recombinant wild-type p17 showed similar mitogenic activities over the whole concentration range tested (0 to 500 ng/ml) (data not shown). The superantigen toxins are active at picogram levels. As presented in Fig. 1, the mitogenicity of toxin p17 is significantly greater than that of toxin Y115A when tested at physiologically realistic conditions (up to 1 ng). In this regard, the mitogenicity of mutant Y115A is 30 to 50% less than that of p17. Toxin H135A showed no mitogenic activity.

Ig synthesis (IgE, IgA, IgG, and IgM). IL-4 alone as well as in combination with aCD40 represents a switch factor for IgE synthesis and an inducer for the humoral immune response (IgA, IgG, and IgM) (22, 38, 39). Therefore, we studied the effects of recombinant TSST toxins p17, Y115A, and H135A (10 ng/ml) by themselves as well as in combination with 1 nM rIL-4 and aCD40 (100 ng/ml) on Ig synthesis. IL-4 plus aCD40 induced a significant increase in Ig synthesis (6.42 ± 0.53) compared with that in unstimulated cells (0.98 ± 0.21). As is apparent from Fig. 2, toxins p17 and Y115A downregu-

FIG. 1. Mitogenicity assays. The mitogenic activity of fast protein liquid chromatography (FPLC)-purified recombinant wild-type p17 and mutant toxins Y115A and H135A from transformed *S. aureus* in a thymidine proliferation assay with human PBMC was determined. The toxins p17, Y115A, and H135A were used at concentrations of between 0 and 500 ng/ml.

lated the IL-4/aCD40-induced IgE, IgA, IgG, and IgM synthesis. In this regard, toxin p17 diminished IL-4/aCD40-induced IgE/A/G/M synthesis (6.42 ± 0.53 , $1,384 \pm 172$, $1,563 \pm 156$, and 630 ± 83 ng/ml, respectively) to nearly baseline levels (0.848 ± 0.18 , 398 ± 112 , 357 ± 97 , and 275 ± 59 ng/ml, respectively). Toxin Y115A downregulated IL-4/aCD40-induced Ig synthesis to baseline levels. Similar results were obtained when rhIL-4 (1 nM) alone was used as the stimulus for Ig synthesis (data not shown). The toxins by themselves did not induce Ig synthesis in a concentration range from 10 ng/ml down to 10 fg/ml (data not shown).

Expression of the low-affinity IgE receptor CD23 and sCD23 (Fc ϵ RII). CD23 is expressed on human PBMC upon stimulation with IL-4. We analyzed the influence of the various toxins on rhIL-4 (1 nM)-induced CD23 expression and sCD23 release from human PBMC (Fig. 3). As is apparent from Fig. 3, p17 and, to a lesser extent, mutant toxin Y115A inhibited the rhIL-4-induced CD23 expression (Fig. 3A) and sCD23 release (Fig. 3B) from human PBMC. In this regard, toxin p17 at a concentration of 10 ng/ml downregulated CD23 expression and sCD23 release by approximately $30\% \pm 6.5\%$ and $46\% \pm 9.2\%$. The toxin Y115A induced downregulation of IL-4-induced CD23 expression, and sCD23 release was significantly less pronounced (CD23, $18\% \pm 5.2\%$; sCD23, $29.5\% \pm 7.3\%$). Toxin H135A showed no effects on rhIL-4-induced CD23 expression and sCD23 release. At lower concentrations of p17 and Y115A, the observed effects were less pronounced (data not shown). Toxin p17 and Y115A by themselves led to a slight upregulation of CD23 expression compared with the unstimulated control. Toxin H135A showed no effects.

Cytokine release and mRNA expression for TNF- α , IL-6, and IL-8. TSST-1 is known to induce proinflammatory cytokine release. Therefore, PBMC were stimulated with the various toxins (20 ng/ml) for 24, 48, and 72 h and analyzed for TNF- α , IL-6, and IL-8 release as well as cytokine mRNA expression. Our data show that all three toxins followed similar kinetics. The results for TNF- α release are presented in Fig. 4. Toxin p17 induced strong TNF- α release from human PBMC after 24 h (4,200 ng/ml), followed by a decline at later time points; mutant toxin Y115A showed a less pronounced upregulation. Mutant toxin H135A showed no significant effect on TNF- α release at all three time points compared with unstimulated cells. Identical results were obtained for IL-6 release

TABLE 1. List of primers used for cDNA amplification

Type	5' Primer	3' Primer
IL-6 (human)	5'-ATG AAC TCC TTC ACA AGC GC-3'	5'-GAA GAG CCC TCA GGC TGG ACT G-3'
IL-8 (human)	5'-CTT AGA TGT CAG TGC ATA AAG ACA TAC TCC-3'	5'-CTC AGC CCT CTT CAA AAA CTT CTC CAC A-3'
IL-10 (human)	5'-GCA CAG CTC AGC ACT GCT CTG TTG-3'	5'-TCA GTT TCG TAT CTT CAT TGT CAT GTA-3'
TNF- α (human)	5'-CAG AGG GAA GAG TTC CCC AG-3'	5'-CCT TGG TCT GGT AGG AGA CG-3'
CD40L (human)	5'-GCC GAT ATC CCA CCA TGA TCG AAA CAT ACA ACC AA-3'	5'-GGC GCT AGC TCA TCA GAG TTT GAG TAA GCC AAA-3'
β -actin (human)	5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'	5'-CTA GAA GCA TTG CGG TGG TGG ACG ATG GAG GG-3'

FIG. 2. Ig synthesis. Human PBMC were stimulated with the various toxins (p17, Y115A, and H135A at 10 ng/ml) in the absence or in the presence of rhIL-4 (1 nM), aCD40 (100 ng/ml), rhIL-4 plus aCD40, or rhIL-13 (10 ng/ml) for 10 days. Ig amounts were measured by ELISA for IgE (A) or by RIA for IgA (B), IgG (C), and IgM (D). *, $P < 0.05$ compared with IL-4/aCD40.

(data not shown). In contrast to TNF- α and IL-6, the release of the cytokine IL-8 was not affected by all three toxins up to an incubation time of 72 h (data not shown). Consistent with the results obtained for cytokine release, toxin p17 (lane 2) was the most pronounced inducer for TNF- α (Fig. 5A) and IL-6 (Fig. 5B) mRNA expression, followed by toxin Y115A (lane 3), compared with the medium control (lane 1). Again, toxin H135A (lane 4) was ineffective in mRNA expression. With regard to IL-8, all three toxins failed to induce mRNA expression (data not shown).

In addition to PCR, these results were confirmed by slot blot analysis instead of PCR. The results are presented in Fig. 6 for IL-6 mRNA expression of human PBMC treated with toxins p17 and H135A.

Detection of mRNA for IL-10 and CD40L. The CD40/CD40L system and IL-10 are important parameters for the regulation of IgE synthesis (22). We analyzed the effects of TSST toxins p17, Y115A, and H135A on mRNA expression for CD40L and IL-10 in PBMC after 24, 48, and 72 h. Figure 5C and D clearly show for an incubation time of 24 h that p17 (lane 2), followed by toxin Y115A (lane 3), led to an increase in expression of IL-10 (Fig. 5C) and CD40L (Fig. 5D) mRNA compared with unstimulated cells (lane 1). Mutant toxin H135A (lane 4) failed to induce CD40L as well as IL-10

mRNA expression. Similar results were obtained at later time points (48 and 72 h) (data not shown). In all experiments, β -actin was coamplified to evaluate similar amounts of mRNA (Fig. 5E). The toxin treatment of human PBMC did not alter the total amount of RNA, as is shown in Fig. 5F by comparing 28S and 18S rRNAs on a formaldehyde-agarose gel.

DISCUSSION

Staphylococcal TSST-1 toxin as well as staphylococcal enterotoxins have been implicated in the pathogenesis of TSS, the Kawasaki syndrome, sepsis, and atopic dermatitis. The induction of proinflammatory cytokines and T-cell proliferation are believed to play an important role in the diverse diseases. However, the pathophysiological mechanism of toxin action is not yet clear. We report for the first time that the region shown to be important in mitogenesis is also important for cytokine release and for the modulatory effects on Ig synthesis, CD23 expression, and sCD23 release. In this regard, the C-terminal region of TSST-1, especially the amino acid histidine at position 135, plays a central role in the induction of the described cellular responses.

Because of the ability of superantigens to stimulate strong T-cell responses in vivo and in vitro, many attempts have been

FIG. 3. Expression of the low-affinity IgE receptor CD23 (A) and the soluble low-affinity IgE receptor sCD23 (B). Human PBMC were stimulated with the various toxins (p17, Y115A, or H135A at 10 ng/ml) in the absence or in the presence of rIL-4 (1 nM). The cultures were harvested on day 5 of culture. Data are expressed as percent binding of MAb 135.

undertaken to analyze the molecular interaction involved in T-cell activation with superantigens. The X-ray crystallographic structure of TSST-1 has recently been reported (1). The authors suggest from the structure that the C-terminal region of TSST-1 (residues 88 to 194) is implicated in mitogenic activity. Regions of TSST-1 which are important in mitogenesis may be class II binding sites but are considered more likely to be TCR binding sites (1). Indeed, Murray et al. found that amino acid residues 132 to 140 of TSST-1 are important for mitogenic activity in a rabbit model (35). Recently, Bonventre et al. (6) studied the effect of recombinant TSST-1 (p17), mutant toxin Y115A (tyrosine residue modified to alanine), and toxin H135A (histidine residue modified to alanine) in a murine system for mitogenic activity. They found that mutant toxin H135A was nearly devoid of mitogenic activity for murine splenocytes, whereas toxin Y115A showed a reduction of mitogenic activity of ~50% compared with p17. We support and extend these findings in a human in vitro system. Mutant toxin H135A was inactive in a thymidine proliferation assay, whereas mutant Y115A was less active than p17 at low toxin concentrations (0.5 to 0.05 ng/ml).

B cells can be stimulated to proliferate in response to cell contact-dependent signals provided by activated but not resting T cells (11). In this regard, the CD40/CD40L system plays

FIG. 4. TNF- α release. Human PBMC were stimulated with the various toxins (p17, Y115A, and H135A) at a concentration of 20 ng/ml for 24, 48, or 72 h. The amounts of TNF- α in the cell culture supernatants were measured by EASIA.

an important role. In the human system, antibodies specific for the surface antigen CD40 as well as the soluble CD40L may substitute for the B- and T-cell contact. Our data show that mRNA for CD40L was strongly induced following activation with TSST-1 (p17) and to a lesser degree with mutant toxin Y115A. Toxin H135A, in contrast, did not elicit mRNA expression for CD40L compared with the unstimulated control. Thus, the capacity of the various toxins to induce proliferation goes in parallel with the capacity to induce CD40L mRNA expression. In addition to its role in proliferation, the CD40/CD40L system is, in combination with IL-4 or IL-13, involved in IgE synthesis and CD23 expression as well (22, 39). Although toxin p17 induced IL-4 mRNA expression (data not shown), toxin p17 downregulated IL-4 as well as IL-4/aCD40-induced Ig synthesis and IL-4-induced CD23 expression and sCD23 release; mutant toxin Y115A was less active. Mutant toxin H135A did not modulate Ig synthesis, CD23 expression, or sCD23 release. Thus, we may conclude that additional factors released from toxin (p17, Y115A, or H135A)-treated PBMC influence Ig synthesis, CD23 expression, and sCD23 release. IFN- γ and IL-12 are known to counteract IL-4 effects (13, 23, 38). Indeed, we have shown that staphylococcal superantigens, e.g., enterotoxins A and B as well as TSST-1, induce significant amounts of IFN- γ and IL-12 (mRNA expression and protein secretion) (27). Recently, it has been reported that IL-10 prevents IL-4-induced IgE synthesis by inhibiting accessory cell functions of monocytes (40). Our data clearly show that toxin p17 and, to a lesser degree, toxin Y115A, but not toxin H135A, induced mRNA expression for IL-10. Thus, we may conclude that Ig synthesis, CD23 expression, and sCD23 release are influenced by CD40L and T helper cell (Th1/Th2) subset-specific cytokines.

The monokine TNF- α is a prominent factor in the onset of TSS and septic shock (41, 42). In this regard, the injection of purified TNF- α either alone or with IL-1 into rabbits reproduced many of the features of TSS (6). Like Parsonnet and Gillis (37), we observed secretion and mRNA expression for TNF- α and IL-6 in human PBMC challenged with TSST-1. Mutation of amino acid 115 diminished and mutation of amino acid 135 abolished the capacity of TSST-1 to induce TNF- α and IL-6 expression (mRNA and protein). TNF- α , IL-6, and IL-10 are produced by lymphocytes and monocytes as well. Since a PBMC cell suspension, a mixture of T and B lympho-

FIG. 5. mRNA analysis for TNF- α (A), IL-6 (B), IL-10 (C), CD40L (D), and β -actin (E). Human PBMC were stimulated with the various toxins (p17, Y115A, and H135A) at a concentration of 10 ng/ml. For detection of mRNA, the cells were incubated for 48 h. Total RNA from 5×10^6 cells was reverse transcribed in a total volume of 40 μ l, and an aliquot of 10 μ l was used for amplification. Lane M, markers; lane 1, medium control; lane 2, p17; lane 3, Y115A; lane 4, H135A. The desired amplification products are indicated with arrows. (F) Total RNA from 5×10^6 human PBMC treated with the various toxins at 10 ng/ml for 24 h on a formaldehyde-agarose gel. Lane 1, medium control; lane 2, p17; lane 3, Y115A; lane 4, H135A.

cytes and monocytes, was used in our experiments, we cannot distinguish the source of expressed mRNA. However, See et al. stated that the induction of TNF- α and IL-1 β by purified staphylococcal TSST-1 requires the presence of both monocytes and T lymphocytes (42).

Recombinant TSST-1 (p17) and mutant toxins Y115A and H135A bind equally well to MHC class II-expressing antigen-presenting cells (murine B cells and human lymphomas) (7, 14). The binding to T cells can be inferred only from the functional assays, e.g., T-cell proliferation. Since residues 132 to 140 are located within the second alpha helix and are exposed and therefore solvent accessible, Acharya et al. speculate that these residues interact with the TCR (1, 33). Since toxin H135A is the only mutant which demonstrates total absence of T-cell activation potential, histidine 135 would appear to be crucial for effective interaction between TSST-1 and the TCR. The structural change in the mutant, however, is minor, since H135A and p17 are antigenically similar and H135A elicits TSST-1 neutralizing antibodies (2, 33).

Obviously, our data contribute to the understanding of toxin-cell interaction. Knowledge about superantigens is a prerequisite for developing strategies against superantigen-induced diseases.

FIG. 6. Slot blot analysis of IL-6 mRNA. Human PBMC were stimulated with the various toxins at a concentration of 10 ng/ml. The total extracted RNA from 5×10^6 cells was diluted 1:2 in H₂O–20 $\times$ SSC–formaldehyde (5:3:2). Lane 1, medium control; lane 2, p17; lane 3, H135A.

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