Effects of Staphylococcal Enterotoxins on Human Neutrophil Functions and Apoptosis

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Staphylococcal enterotoxins have marked effects on the properties of T cells and monocytes and have recently been reported to affect neutrophil function. In this study, we investigated the abilities of staphylococcal enterotoxins A and B and toxic shock syndrome toxin 1 to affect respiratory burst activity and to delay apoptosis in human neutrophils. When cultures containing approximately 97% neutrophils were tested, the toxins all delayed neutrophil apoptosis in a dose-dependent manner and induced the expression of FcyRI on the neutrophil cell surface. These effects on apoptosis and expression of FcyRI were largely abrogated by the addition of a neutralizing anti-gamma interferon antibody. Similarly, the effects of these toxins on phorbol ester-induced chemiluminescence were decreased after neutralization of gamma interferon. These effects on neutrophil function were mimicked by the addition of conditioned medium from peripheral blood mononuclear cells incubated with the toxins, and again, neutralizing anti-gamma interferon antibodies largely negated the effects. However, when highly purified neutrophils prepared by immunodepletion of T cells and major histocompatibility complex class II-expressing cells were analyzed, the toxins were without effect on apoptosis and FcyRI expression, but granulocyte-macrophage colony-stimulating factor and gamma interferon could still delay apoptosis. These data indicate that these toxins have no direct effect on neutrophil apoptosis but can act indirectly via the production of T-cell-derived and monocyte-derived cytokines. It is noteworthy that such effects are detected in neutrophil suspensions containing only 3% contamination with T cells and other mononuclear cells.

Staphylococcal enterotoxins cause food poisoning and toxic shock and may lead to multiple organ failure resulting from massive activation of the immune system (23). There is also evidence that suggests a critical role for staphylococcal enterotoxins in the pathogenesis of rheumatoid arthritis (27). These toxins are superantigens which activate a subset of T cells (around 20% of T cells have the appropriate Vβ region) by binding to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and cross-linking to the T-cell receptor (16, 24). There is a large production of proinflammatory cytokines from both T cells and monocytes following superantigen activation: T cells produce interleukin-1β (IL-1β), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-2 (17, 20, 32), while monocytes produce both IL-1β and IFN-γ (31, 32, 35).

The effects of superantigens on T cells and monocytes have been well studied, but the effects of superantigens on other cells of the immune system have been relatively neglected. The indirect activation of these other immune cells may be expected in vivo, as cytokine secretion by T cells and monocytes will, in turn, activate other immune cells. There are a few reports of other immune cells being affected apparently without the involvement of T cells or monocytes. For example, CD56-positive natural killer (NK) cells have been shown to be directly stimulated by staphylococcal enterotoxin B (SEB) (4). This action on NK cells is not totally unexpected, as a subset of these cells is known to express MHC class II. There have also been reports of neutrophil responses to toxic shock syndrome toxin 1 (TSST-1), such as expression of heat shock proteins (HSPs) within 30 min of stimulation (15), and changes in surface protein expression and leukotriene B4 synthesis which occur within 10 min of stimulation (14). Such direct effects of TSST-1 on neutrophils are difficult to rationalize, as no surface structures that can act as targets for these superantigens have been identified on freshly isolated neutrophils (14). Recently, neutrophils have been shown to express MHC class II molecules, but only after culture for a day or more with IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-3 (12, 33), but this does not explain the rapid (10 min) effects of TSST-1 on freshly isolated neutrophils.

Neutrophils possess a very short half-life in the circulation because they constitutively undergo apoptosis (10, 25, 34). Cytokines, such as GM-CSF, IFN-γ, IL-1β, and IL-2, which are produced by activated T cells and monocytes, can delay neutrophil apoptosis and extend the functional life span of neutrophils to several days (1, 3, 21, 28). The effects of superantigens on neutrophil apoptosis have not been studied, although it may be predicted that neutrophil apoptosis in vivo may be delayed either directly or indirectly via the production of cytokines by other immune cells. The work of Hensler and colleagues (14, 15) suggests a direct effect of superantigens on neutrophils, including enhanced production of leukotriene B4. This leukotriene is able to delay neutrophil apoptosis (13, 26), and this could provide an indirect mechanism for superantigens to affect neutrophil apoptosis. The aim of this study was to determine if bacterial superantigens have any ability to alter neutrophil survival. We show that these agents have no direct effects on neutrophil apoptosis but can perform this function indirectly, via the production of cytokines by the very low levels of contaminating monocytes and T cells often present in neutrophil preparations.
MATERIALS AND METHODS

Materials. Neutrophil isolation medium (NIM) was purchased from Cardinal Associates (Santa Ana, Calif.). RPMI 1640 medium was from ICN Biomedicals (Thame, Oxford, United Kingdom), and fetal calf serum (FCS) and l-glutamine were from Gibco-BRL (Paisley, United Kingdom). Neutrophhics, SEB, and TSST-1 were purchased from Sigma. IFN-γ was from Boehringer Mannheim (Lewes, United Kingdom). Monoclonal antibody 32.2 (anti-FcRI) was purchased from Medarex Inc., and monoclonal antibody Leu11a (anti-FcRII), a fluorescein isothiocyanate-conjugated second-layer antibody, and immunoglob- 

Neutrophil isolation and purification. Neutrophils were isolated from hepa-rinized venous blood from healthy volunteers by centrifugation in NIM as de- 

Neutrophil prepara-

Neutrophil prepara-

PBMC isolation. Heparinized venous blood from healthy volunteers used for the preparation of neutrophils was centrifuged through NIM as described above with removal of peripheral blood mononuclear cells (PBMC) at the first step of neutrophil isolation (8). PBMC were then washed with RPMI medium, counted, and resuspended in RPMI 1640 medium supplemented with 5% (vol/vol) FCS and 2-mmol/liter l-glutamine.

PBMC-conditioned medium. PBMC were isolated as described above and resuspended at 2 × 106/ml in RPMI 1640 medium supplemented with 5% (vol/vol) FCS and 2-mmol/liter l-glutamine. PBMC-conditioned medium was added at a volume of 1 liter per ml of neutrophil suspension. The concentration of carryover superantigen in these neutrophil suspensions was therefore less than 1.25 ng/ml for SEA and SEB and less than 0.25 ng/ml for TSST-1.

Neutrophil culture. Neutrophils were suspended at 5 × 106 cells/ml in RPMI 1640 medium supplemented with 5% (vol/vol) FCS. Medium with no further additions served as a control, while SEA, SEB, and TSST-1 were added at 0.01, 0.05, 0.1, 0.5, or 5.0 μg/ml; GM-CSF was added at 50 U/ml; and IFN-γ was added at 100 U/ml. PBMC-conditioned medium was added at 12.5 μl/ml. IFN-γ-neutralizing antisera was added to incubations at a 1:1,000 dilution.

NADPH oxidase activity. Chemiluminescence was assayed after supplement-

Statistical analysis. All experimental data are expressed as means ± the standard deviations (SD). Where appropriate, samples were analyzed by using the Student’s t test, and statistical significance was defined as P ≤ 0.05 or P ≤ 0.01.

RESULTS

Effects of superantigens on neutrophil suspensions. Delayed neutrophil apoptosis is associated with the preservation of functions such as the ability to produce reactive oxygen species in response to PMA or IMLP (10, 25, 34). The chemo- 

Heat shock and de novo protein synthesis. Neutrophils at 107/ml were preincubated with 60 μg/ml I2S]methionine for 15 min at 37°C. Cells were then cultured for either 1 h at 37°C, 30 min at 42°C, 1 h at 42°C, or 1 h at 37°C with TSST-1 added at 1.0 and 5.0 μg/ml. Proteins (from 106 cells) were then solubi-

FIG. 1. Effects of bacterial superantigens on neutrophil respiratory burst activity. Following isolation, neutrophils were cultured in medium alone or supplemented with SEA, SEB, or TSST-1 at the concentrations (conc) shown. After 20 h of culture, aliquots of equal numbers of neutrophils were processed for assessment of oxidative-burst activity in response to stimulation with PMA (0.1 ng/ml). The results shown are means ± SE of three separate experiments. Significant difference from control incubations is indicated by the symbol † (P ≤ 0.05) or ✠ (P ≤ 0.01). A, effects of SEA; B, effects of SEB; C, effects of TSST-1.
above 0.1 µg/ml, with maximal effectiveness at 5.0 µg/ml. The toxins were therefore added to neutrophil suspensions at 0.1 µg/ml for SEA and SEB and at 1.0 and 5.0 µg/ml for TSST-1 in further experiments.

Assessment of protection from apoptosis was determined by measuring CD16 surface expression in neutrophils cultured for 20 h as shown in Fig. 2. Previous work has shown that surface levels of this receptor correlate well with other indicators of neutrophil apoptosis (5, 10). Each toxin gave significant protection from apoptosis as measured by this assay, with the protection from apoptosis comparable to that found with GM-CSF treatments. TSST-1 gave the best protection from apoptosis, with the higher TSST-1 concentration being the most potent in delaying apoptosis. These results were also confirmed by morphological assessment of apoptosis (data not shown), with the same pattern of results obtained by both measurements.

Role of PBMC-derived cytokines in superantigen-mediated effects. The isolation of neutrophils routinely gave >97% polymorphonuclear cells or <3% contamination with PBMC. However, the activation of PBMC by superantigen yields vast quantities of cytokines, such that contamination of neutrophil preparations with less than 3% PBMC could produce sufficient cytokines to delay neutrophil apoptosis. Figure 3 shows the results of experiments done to determine if the proportion of PBMC in a neutrophil preparation correlated with the delayed apoptosis observed following superantigen treatment. SEB added to a normal neutrophil preparation delayed apoptosis, as assessed by both morphology and preservation of the ability to produce reactive oxygen species in response to PMA. Addition of extra PBMC at a ratio of 50:1 (neutrophils to PBMC) gave no further delay in apoptosis in SEB-treated neutrophils, but as more PBMC were added to the culture, there was clear enhancement of the ability of SEB to delay the apoptosis of neutrophils. The effect was greatest at neutrophil-to-PBMC ratios between 5:1 and 1:1, which is similar to the ratio of neutrophils to PBMC in the circulation. The addition of extra PBMC to neutrophil suspensions at the highest ratio tested (one neutrophil to two PBMC) showed a delay in neutrophil apoptosis without SEB treatment, but this effect was much less than that found when SEB was included. It therefore seemed likely that cytokine production from PBMC activated by superantigen in normal neutrophil preparations could be responsible for the delayed apoptosis observed.

Of the cytokines produced by superantigen-activated PBMC, IFN-γ is perhaps the simplest to assay for, as it is
known to induce surface expression of FcγRI on neutrophils (2, 29). Overnight culture with each of the superantigens (SEA or SEB at 0.1 μg/ml or TSST-1 at 5 μg/ml) clearly induced FcγRI expression determined by flow cytometry, as shown in Fig. 4A. At these concentrations, the toxins were almost as effective as IFN-γ used at 100 U/ml. The preparation of conditioned medium from PBMC incubated in the absence (control) or presence of each superantigen for 20 h provided confirmation that FcγRI expression could be induced by a PBMC-derived product. Untreated (control) conditioned medium from PBMC gave no induction of FcγRI on neutrophils, but conditioned medium from PBMC treated with any of the superantigens gave a marked induction of FcγRI. The addition of IFN-γ-neutralizing antisera prevented induction of FcγRI by IFN-γ (mean channel fluorescence: IFN-γ-treated cells, 40.7 ± 12.6 [n = 3]; cells treated with IFN-γ plus neutralizing antibody, 20.8 ± 7.5 [n = 3]). Neutralization of IFN-γ with this antibody also decreased FcγRI expression stimulated by superantigen-activated PBMC-conditioned medium, indicating that the induction of FcγRI on neutrophils was almost entirely due to IFN-γ secreted from the contaminating PBMC found in neutrophil preparations.

Figure 4B shows the results of experiments done to determine if the effects of superantigen treatment on the preservation of the ability of neutrophils to generate a respiratory burst was also mediated by IFN-γ. Treatment of neutrophil cultures with 100-U/ml IFN-γ gave preservation of the oxidative burst (and protection from apoptosis) similar to that provided by treatment with all of the superantigens or superantigen-activated PBMC-conditioned medium. The inclusion of IFN-γ-neutralizing antisera in superantigen-treated neutrophil suspensions prevented most of the effects of these agents on neutrophil function, but some effects remained at higher toxin concentrations. This may be due to inefficient neutralization of IFN-γ after time in culture. Therefore, the neutralizing antibody was added to conditioned medium 20 min before the addition of conditioned medium to neutrophils. This pretreatment completely abolished the effects of 100-U/ml IFN-γ. The superantigen-treated conditioned medium was, however, still slightly effective after neutralization of IFN-γ. The effects of superantigen were therefore mediated largely by IFN-γ. The remaining effect may be mediated by other cytokines produced by the PBMC or may be due to a direct effect of the toxins on neutrophils themselves. Similarly, the effects of superantigens on delayed neutrophil apoptosis (as assessed by morphology or surface CD16 expression) were not seen after neutralization of IFN-γ (data not shown).

Short-term effects of superantigens on neutrophils. Experiments using fluo-3-loaded neutrophils (11, 37) indicated that addition of superantigens did not result in detectable increases in intracellular Ca²⁺ levels, thus excluding the possibility that these toxins stimulate receptor-mediated pathways leading to Ca²⁺ mobilization (data not shown). Also, we could not detect any changes in the levels of phosphorylation of neutrophil proteins on tyrosine residues (22, 36) after treatment with superantigens (data not shown). Likewise, the superantigens could neither prime nor activate the respiratory burst themselves (data not shown).

Delayed apoptosis in superantigen-treated neutrophil suspensions depleted of T cells. The above-described experiments indicate that most, if not all, of the effects of superantigens on neutrophil responses are due to the effects of cytokines (especially IFN-γ) generated by PBMC present in the neutrophil suspensions. The production of cytokines by PBMC is reported to require the presence of both T cells and antigen-presenting
cells (32), so depletion of contaminating T cells from neutrophil preparations should prevent this cytokine production. T-cell-depleted neutrophils were rescued from apoptosis by GM-CSF, as assessed by PMA-induced chemiluminescence after 20 h of culture (Fig. 6A). Treatment with superantigens at both 0.1 and 1.0 μg/ml (which effectively delayed apoptosis in normal neutrophil suspensions) had no effect on these T-cell-depleted suspensions. However, at the highest concentration of superantigen used (5.0 μg/ml), SEA, SEB, and TSST-1 were still effective at preserving neutrophil function after 20 h of culture.

Figure 6B shows apoptosis of neutrophils following 20 h of culture as assessed by the percentage of cells retaining high levels of surface CD16. It is clear that the depletion of T cells had no adverse effect on the ability of exogenously added GM-CSF or IFN-γ to delay apoptosis (Fig. 6B). Superantigen treatment of T-cell-depleted neutrophils at 0.1 μg/ml showed a normal rate of apoptosis. However, at higher concentrations of superantigen, the entry into apoptosis was still delayed by a slight but significant extent at 1.0 μg/ml and more markedly at 5.0 μg/ml. The protection from apoptosis afforded by the higher concentrations of superantigen was not as great in T-cell-depleted neutrophils as it was in normal neutrophil preparations, suggesting that part of the protection from apoptosis is T cell dependent.

Apoptosis in superantigen-treated neutrophil suspensions depleted of both T cells and MHC class II-expressing cells. The protection from apoptosis provided by high concentrations of superantigens in T-cell-depleted neutrophil suspensions suggested either (i) a direct effect of these superantigen treatments on neutrophil apoptosis or (ii) an effect mediated by cytokines from other, contaminating cells. For example, MHC class II-expressing cells may be able to produce cytokines when stimulated with high concentrations of superantigen, even in the absence of T cells. It was therefore necessary to deplete neutrophil suspensions of both T cells and MHC class II-expressing cells to confirm if the superantigens had a direct effect on neutrophil apoptosis.

Figure 7A and B shows PMA-stimulated chemiluminescence and apoptosis results of neutrophil preparations that had been depleted of both T cells and MHC class II-expressing cells and then cultured for 20 h. Both GM-CSF and IFN-γ were effective at preserving the ability of neutrophils to generate a respiratory burst (Fig. 7A) and protected against apoptosis in these highly purified preparations. However, none of the superantigens (used at 5 μg/ml) could preserve function or protect against apoptosis in these purified neutrophils.

**DISCUSSION**

In this report, we have shown that neutrophil apoptosis is delayed by superantigen treatment but that this delay in apoptosis is dependent upon the presence of a small number of contaminating PBMC in the suspensions. The levels of cyto-
Ptosis was determined by morphology (B). The values shown are means ± SD of three separate experiments, and a value significantly different from the control is indicated by the symbol † (P < 0.05) or * (P < 0.01).

FIG. 7. Effects of bacterial superantigens on apoptosis of highly purified neutrophil suspensions. Neutrophil suspensions depleted of both T cells and MHC class II-expressing cells were incubated for 20 h with the agents indicated. Cells were analyzed for the ability to generate a respiratory burst (A), or apoptosis determined by morphology (B). The values shown are means ± SD of three separate experiments, and a value significantly different from the control is indicated by the symbol † (P < 0.05) or * (P < 0.01).

Kines produced by contaminating PBMC in normal neutrophil preparations are sufficiently high to delay neutrophil apoptosis. IFN-γ accounts for almost all of the delayed apoptosis observed, as neutralization of IFN-γ in superantigen-treated neutrophil suspensions abolished around 80% of the action of superantigen on neutrophil apoptosis (Fig. 4).

From previously published reports, 3% contamination of PBMC could result in the production of cytokines to the following levels: IFN-γ, 65 to 80 U/ml (17, 32); IL-2, 3 to 250 U/ml (17, 32); IL-1β, ~4 U/ml (20, 31); TNF-α, 1 to 2.5 U/ml (17, 20). These cytokine levels are sufficient to delay neutrophil apoptosis, as IFN-γ will delay apoptosis at concentrations between 50 and 500 U/ml (3). IL-1β is able to delay neutrophil apoptosis at concentrations as low as 2 U/ml but is more effective at concentrations between 20 and 200 U/ml (3). IL-2 can delay neutrophil apoptosis, but the concentrations produced by 3% contamination with PBMC is unlikely to be sufficient to delay apoptosis, as concentrations of around 1,000 U/ml are required (28). Neutralization of IFN-γ still leaves the other cytokines present, which could explain the slight effects that remain after IFN-γ neutralization (Fig. 4). IL-1β, which was present at a concentration sufficient to delay neutrophil apoptosis slightly, could be the cytokine present after IFN-γ neutralization that resulted in delayed apoptosis.

The depletion of T cells from the neutrophil suspensions did abolish the delayed apoptosis observed with superantigen concentrations below 5.0 μg/ml, but there was still a delay in apoptosis when they were used at this higher concentration. This may be explained by the observation of Jupin et al. (17), who reported that increasing concentrations of superantigen gave a corresponding increase in IFN-γ production. Therefore, the depletion of T cells, if not complete, would still allow the production of IFN-γ at levels sufficient to delay neutrophil apoptosis. Alternatively, the work of Jupin et al. (17) suggests that monocytes are able to produce cytokines (TNF-α and IL-1β) without the presence of T cells. Therefore, the removal of T cells alone may not prevent all cytokine production, with cytokines derived from the remaining PBMC being responsible for the delayed neutrophil apoptosis.

The depletion of both T cells and MHC class II-expressing cells from neutrophil preparations allowed the action of superantigens on neutrophil apoptosis to be assessed without any contribution from other superantigen-stimulated cells. This clearly demonstrated that superantigens had no direct action on neutrophils that we could measure in our experiments (Fig. 7). Apart from an inability to delay apoptosis or preserve function, the superantigens did not affect levels of phosphorylation of neutrophil proteins on tyrosine residues, did not stimulate intracellular Ca²⁺ transients, and neither activated nor primed the respiratory burst (data not shown).

The possibility remains that superantigens do have other direct actions on neutrophils, such as the ability of TSST-1 to modulate FMLP receptor expression (14), and induce HSP expression (15). We have examined the induction of HSP expression by TSST-1 and found no induction under conditions similar to those used by Hensler et al. (15). This difference in results may be explained by the purity of the neutrophils used. Hensler et al. (15) reported neutrophil purity of 95%, which indicates approximately twofold higher contamination with PBMC than in the preparations used in this report. Therefore, cytokine production would be considerably higher in Hensler’s cultures, and cytokines such as IL-1 and TNF-α are able to induce HSP expression rapidly in neutrophils (18).

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REFERENCES


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