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Staphylococcal Enterotoxin A-Induced Injury of Human Lung Endothelial Cells and IL-8 Accumulation Are Mediated by TNF-α

Nobumitsu Fujisawa,* Shinichiro Hayashi,† Anna Kurdowska,* James M. Noble,* Keiko Naitoh,† and Edmund J. Miller*2

Staphylococcal enterotoxin A (SEA), a superantigen produced by some strains of Staphylococcus aureus, causes a variety of clinical manifestations ranging from food poisoning to shock. S. aureus can also be associated with the development of acute respiratory distress syndrome, and SEA has been shown to cause an inflammatory reaction in the lung. Therefore, we examined possible interactions between SEA, PBMCs, polymorphonuclear cells (PMNs), and normal human lung microvascular endothelial cells (HMVEC-L), as well as the role of these interactions on the secretion of IL-8. Injury to HMVEC-L, as measured by the release of 51 Cr, increased significantly when HMVEC-L were incubated with SEA and PBMCs. IL-8 was secreted by both PBMCs and HMVEC-L. The accumulation of IL-8 in the culture medium of HMVEC-L was increased by SEA in a dose-dependent manner and was directly related to the number of PBMCs present. Although neither anti-human IL-8 nor IL-1 mAb inhibited HMVEC-L. The accumulation of IL-8 in the culture medium of HMVEC-L was increased by SEA in a dose-dependent manner and was directly related to the number of PBMCs present. Although neither anti-human IL-8 nor IL-1 mAb inhibited HMVEC-L cytotoxicity, anti-human TNF-α mAb inhibited both the cytotoxicity and IL-8 accumulation completely. When HMVEC-L were incubated with supernatants from SEA-treated PBMCs, HMVEC-L cytotoxicity was comparable with HMVEC-L incubated with SEA and PBMCs at the same time. Although high concentrations of purified PMNs induced HMVEC-L lysis in a dose-dependent manner, the effect of PMNs was not changed in the presence of SEA. These findings suggest that TNF-α secreted by SEA-stimulated PBMCs plays a leading role in HMVEC-L injury. The Journal of Immunology, 1998, 161: 5627–5632.

Acute respiratory distress syndrome (ARDS) is an acute deterioration of lung function that occurs in association with severe clinical disorders such as sepsis (1). Although the pathogenesis of ARDS remains unclear, the interaction between polymorphonuclear cells (PMNs), their toxic products such as myeloperoxidase, and cytokines derived from PBMCs and pulmonary endothelium are thought to increase microvascular permeability to plasma proteins. The resultant lung edema is considered a major component of ARDS (2–4). Sepsis is one of the clinical disorders that is most often associated with ARDS (5, 6), and many studies have examined the possible role of endotoxin from Gram-negative bacteria, and in particular the cell wall component LPS, in the development of ARDS (7–9). However, there is a significant group of patients who develop ARDS associated with Gram-positive bacteria such as Staphylococcus aureus which do not produce LPS (10). Staphylococcal enterotoxin A (SEA) is a 28-kDa exoprotein produced by several strains of S. aureus (11). SEA is known to induce TNF-α and IL-1β in human monocytic cells (12–15). Proinflammatory cytokines such as TNF-α and IL-1β can stimulate the production of IL-8 (15), and there are reports that describe TNF-α increasing the permeability of the endothelium (16–18) and being associated with the acute lung injury (19). IL-8, a potent PMN chemotactic and activating factor, has also been implicated in the pathogenesis of ARDS (20). Furthermore, we have found that IL-8 concentrations are higher in the lungs of patients with ARDS associated with sepsis than in nonseptic ARDS patients (21), and that i.v. SEA increases the IL-8 concentration of plasma and epithelial lining fluid in rabbits (22). In these studies, we examined the mechanism of lung endothelial cell injury associated with SEA.

Materials and Methods

Human subjects

All work involving human subjects was approved by the Institutional Human Subjects Committee at the University of Texas Health Center.

Cell culture

Human lung microvascular endothelial cells (HMVEC-L) (Clonetics, San Diego, CA) were maintained in EGM medium containing human epidermal growth factor (10 ng/ml), bovine brain extract (12 mg/ml), gentamicin sulfate (50 mg/ml), amphotericin-B (50 ng/ml) (Clonetics), and 10% FCS (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere containing 5% CO2. HMVEC-L were grown as monolayers in tissue culture flasks. Cells were passaged when they reached 70–80% confluence using trypsin (0.025%/EDTA (0.01%) in HBSS (Clonetics), centrifuged at low speed (220 × g for 5 min), and resuspended in fresh medium. HMVEC-L were maintained for no longer than 3 wk.

Preparation of human PMNs and PBMCs

Human blood from healthy donors was anticoagulated with heparin (Elkins-Sinn, Cherry Hill, NJ). PMNs were isolated by dextran (Pharmacia, Piscataway, NJ) sedimentation and E lysis using the method of Boyum (23) as modified in our earlier studies (24, 25) and were further purified in gradients of Ficoll-Hypaque (density 1.14; ICN Biomedicals, Costa Mesa, CA) (26) for cytotoxic assays. The isolated PMNs were ≥99% pure and viable. PBMCs were also isolated in gradients of Ficoll-Hypaque. The isolated PBMCs were ≥98% pure and 99% viable.
and/or PMNs (8.5 3 10^5 cells/ml) and/or PMNs (8.5 3 10^6 cells/ml) in the presence or absence of 10 ng/ml of SEA for 21 h. Next, percent lysis was calculated as described in Materials and Methods.

51Cr release cytotoxicity assay

The assay was performed as described previously (27). In brief, HMVEC-L monolayers in 96-well plates were incubated with 2 Ci/well of Na251CrO4 (DuPont-New England Nuclear, Wilmington, DE) alone or with indicated concentrations of SEA (Toxin Technology, Sarasota, FL). Following the incubation, the wells were washed three times and incubated in culture medium for an additional 30 min at 37°C to allow spontaneous lysis of marginally viable cells. After washing twice, a 100-μl aliquot of SEA, purified mouse IgG1 anti-IL-8 mAb (R&D Systems, Minneapolis, MN), anti-TNF-α mAb, anti-IL-1β mAb (Biosource International, Camarillo, CA), freshly isolated PBMCs, and/or PMNs were added to each well. The cells were cultured for 21 h, and the radioactivity in the supernatants was counted using a gamma radiation spectrometer. Each well received culture medium alone or 2% SDS (EM Industries, Cherry Hill, NJ) to determine spontaneous and maximum release, respectively. Percent lysis was calculated using the following formula: % Lysis = ([experimental cpm - spontaneous cpm]/[maximum cpm - spontaneous cpm]) 3 100.

Quantitation of IL-8

IL-8 accumulation in the supernatants was quantitated using an ELISA as described previously (28, 29). The assay employed an anti-IL-8 mAb (IgG1) (purified from ascites that had been developed using HB9467 hybridoma cells (American Type Culture Collection, Manassas, VA, with permission from Dr. E. J. Leonard, National Cancer Institute, Frederick, MD)) (28) and rabbit polyclonal anti-human IL-8 polyclonal antiserum (Upstate Biotechnology, Lake Plasid, NY) followed by swine anti-rabbit Igs conjugated with horseradish peroxidase (Dako, Carpinteria, CA). The immunoassay was specific for IL-8 and did not cross-react with other members of the α-chemokine family (29).

Flow cytometric analysis

Flow cytometric analysis was performed as described previously with some modifications (30). Briefly, PMNs that had been freshly isolated using dextran and Ficoll-Hypaque were incubated with FITC-labeled anti-CD16 mAbs (Exalpha, Boston, MA) for 30 min at 4°C to identify the PMN population. Cells were then washed three times with cold PBS and incubated with phycoerythrin-labeled mAbs for Mac-1 (CD11b; Monosan, Uden, Netherlands) or L-selectin (CD62L; Exalpha) for an additional 30 min at 4°C. Cells were washed again three times and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Control leukocytes were prepared by hypotonic lysis of freshly isolated blood and incubated with mAbs as described above.

Mean fluorescence intensity (MFI) was calculated, and the percent stimulation of expression was calculated using the following formula: % Stimulation = ([MFI of purified PMNs − MFI of control leukocytes]/MFI of control leukocytes) 3 100.

Statistics

Data are expressed as mean values ± SD. Significant differences between the means of two groups were assessed using the Student t test. Data were considered statistically significant if p values were ≤0.05. The experiments were performed at least twice with at least four replicate cultures per experiment.

Results

Cytotoxic effect toward HMVEC-L incubated with SEA, PBMCs, and/or PMNs

Endothelial cell injury was estimated as the release of chromium from prelabeled HMVEC-L. When HMVEC-L were incubated with PBMCs and SEA, HMVEC-L cytotoxicity increased significantly compared with the incubation with PBMCs alone (p < 0.0001) (Fig. 1). Alternatively, when HMVEC-L were incubated with PMNs in the presence of SEA, there was no increase in endothelial cell lysis as compared with the incubation with PMNs alone. There was also no additional increase in cytotoxicity when PBMCs and SEA were incubated with PMNs. However, when HMVEC-L and SEA were incubated with PBMCs, the percentage of cell lysis was increased in a dose-dependent manner. There were significant differences from control cultures (incubated without PBMCs) at concentrations ≥7.5 3 10^5 cells/ml of PBMCs (p < 0.007) (Fig. 2A). When HMVEC-L and PBMCs were incubated with SEA, the percentage of cell lysis also increased in a dose-dependent manner. There were significant differences from control cultures (incubated without SEA) at concentrations ≥1.5 3 10^5 cells/ml of PBMCs (p < 0.007) (Fig. 2B).

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Cytotoxic effect of SEA, PBMCs, and/or PMNs toward HMVEC-L. HMVEC-L were incubated with PBMCs (1.5 3 10^5 cells/ml) and/or PMNs (8.5 3 10^6 cells/ml) in the presence or absence of 10 ng/ml of SEA for 21 h. Next, percent lysis was calculated as described in Materials and Methods.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Cytotoxic effect of different concentrations of PBMCs and SEA toward HMVEC-L. A, HMVEC-L were incubated with 10 ng/ml of SEA and indicated concentrations of PBMCs for 21 h. B, HMVEC-L were incubated with 1.5 3 10^5 cells/ml of PBMCs and indicated concentrations of SEA for 21 h.
cultures (incubated without SEA) at concentrations ≥0.1 ng/ml of SEA (p < 0.01) (Fig. 2B).

**IL-8 accumulation in supernatants of HMVEC-L incubated with SEA, PBMCs, and/or PMNs**

SEA contributed to IL-8 production and secretion when it was incubated with PBMCs (Fig. 3). When HMVEC-L and PBMCs were incubated with SEA, IL-8 accumulation was increased significantly compared with incubation in the absence of SEA (p = 0.0003). The addition of PMNs did not contribute to the accumulation of IL-8 regardless of the presence of SEA or PBMCs. Interestingly, HMVEC-L produced and secreted IL-8 in the absence of any additional stimuli. When HMVEC-L and SEA were incubated with PBMCs, IL-8 accumulation was increased and was related to the number of PBMCs added; there were significant differences from control cultures (incubated without PBMCs) at concentrations ≥1.5 × 10⁵ cells/ml of PBMCs (p < 0.0001) (Fig. 4A). When HMVEC-L and PBMCs were coincubated in the presence of SEA, IL-8 accumulation also increased in a dose-dependent manner, and there were significant differences from control cultures (incubated without SEA) at concentrations ≥0.001 ng/ml of SEA (p < 0.0001) (Fig. 4B).

**Effect of supernatants of PBMCs incubated with or without SEA**

To determine whether the cytotoxic factor and/or the stimulus for IL-8 production and secretion was present in the medium, or whether cell contact was required, PBMCs were incubated for 21 h in the presence or absence of SEA. Next, supernatants were collected and incubated with HMVEC-L. The cytotoxic effects induced by the coincubation of HMVEC-L, PBMCs, and SEA were due to soluble factors released into the medium. When HMVEC-L were incubated with SEA alone (no PBMCs), there was no significant increase in cytotoxicity compared with the incubation with medium alone (Fig. 5A). When HMVEC-L were incubated with supernatants that had been incubated with PBMCs in the presence or absence of SEA, the percentage of cell lysis was increased significantly compared with the incubation with medium alone (p < 0.0001 and p = 0.0261, respectively). Furthermore, supernatants from SEA-stimulated PBMCs were equally cytotoxic to HMVEC-L compared with when SEA, PBMCs and HMVEC-L were present at the same time (p = 0.61). IL-8 accumulation in the supernatants before and after incubation with HMVEC-L is shown in Fig. 5B. Both PBMCs and HMVEC-L produced and secreted IL-8, and SEA increased the accumulation only when PBMCs had been incubated previously in the medium.

**Effect of cytokine neutralizing Abs on SEA-induced cytotoxicity and IL-8 accumulation**

51Cr-labeled HMVEC-L were incubated with SEA and PBMCs in the presence of neutralizing Abs. When anti-IL-8 mAb was used, the percentage of cell lysis did not change significantly (Fig. 6A). However, when anti-TNF-α mAb was incubated with HMVEC-L, SEA, and PBMCs, the percentage of cell lysis was decreased in a dose-dependent manner; in addition, there were significant differences from control cultures (incubated without anti-TNF-α mAb) at concentrations ≥5 µg/ml of anti-TNF-α mAb (p < 0.02) (Fig. 6B). The SEA-induced cytotoxicity was completely inhibited when anti-TNF-α mAb was coincubated at concentrations of ≥20 µg/ml (p = 0.267). Anti-IL-β mAb, another proinflammatory cytokine neutralizing Ab, was also tested (Fig. 6C). In this case, there was no significant change in cytotoxicity from control cultures grown in the absence of anti-IL-β mAb.

IL-8 accumulation in the culture supernatants from the coincubation of HMVEC-L, SEA, and PBMCs in the presence of anti-TNF-α mAb was also quantitated (Fig. 7). The accumulation of IL-8 was inhibited by anti-TNF-α mAb in a dose-dependent manner and was completely inhibited at concentrations ≥1 µg/ml.
Effect of PMNs on HMVEC-L

HMVEC-L were incubated with various concentrations of PMNs (Fig. 8). In the absence of any added stimulants, the percentage of cell lysis increased with the number of PMNs added; there were significant differences from control cultures (incubated without PMNs) at concentrations $\geq 1 \times 10^6$ cells/ml of PMNs ($p < 0.002$).

Expression of Mac-1 and L-selectin on the surface of PMNs

Flow cytometry was performed to determine whether PMNs were activated by the purification procedure (Table I). The expression of Mac-1 (CD11b) was increased and the expression of L-selectin (CD62L) was decreased in CD16$^+$ cells.

Discussion

The loss of endothelial integrity by cytolysis is a common mechanism causing increased vascular permeability, and vascular damage plays an important role in the pathogenesis of vasculitis associated with ARDS (31). Infectious diseases are most commonly associated with ARDS, and Seidenfeld et al. showed that 36% of infection-induced ARDS were due to Gram-positive cocci (1).

In this study, we describe a mechanism for SEA-induced endothelial damage. TNF-α, which was produced and secreted by SEA-stimulated PBMCs, is an essential component of endothelial injury. To our knowledge, this is the first report that demonstrates a mechanism of SEA-induced endothelial damage.

Many previous reports have described PMN-dependent endothelial damage and increase in endothelial permeability (9, 16, 32). However, ARDS has been reported in patients who are neutropenic (33–36), suggesting that PMNs are not essential for its development. We found that the SEA-induced cytotoxic effect occurred when the toxin was incubated with PBMCs alone and was independent of the presence of PMNs. However, the cytotoxicity was dependent upon the concentrations of both SEA and PBMCs. Furthermore, supernatants from PBMCs incubated with SEA also induced the same level of cytotoxicity in HMVEC-L. These data support the hypothesis that the ability of SEA to cause endothelial cell lysis is not related to the presence of PMNs, suggesting a possible mechanism of endothelial injury in neutropenic patients.
IL-8 has been identified as a potent PMN chemotactic and activating factor (37, 38). Our previous study showed that the concentration of IL-8 in the airspaces is elevated in patients with ARDS. Additionally, the IL-8 in the lungs of patients with ARDS associated with sepsis reached greater concentrations than in nonseptic ARDS patients (20, 21). In this study, we found that IL-8 was produced and secreted by both PBMCs and HMVEC-L without any added stimulants. Although the accumulation of IL-8 was increased in parallel with the cytotoxicity when HMVEC-L were incubated with PBMCs and SEA, neutralizing Ab for IL-8 did not reduce the cytotoxicity. These data suggest that IL-8 is not directly responsible for HMVEC-L injury.

TNF-α and IL-1β, which are known IL-8 inducers, are produced and released by monocytic cells in response to SEA (12–15). Also, both TNF-α and IL-1β have been reported to be associated with ARDS (19). In particular, TNF-α increases pulmonary vascular permeability independent of neutrophils (39). Although the addition of anti-IL-1β mAb did not affect HMVEC-L cytotoxicity when anti-TNF-α mAb was coincubated with HMVEC-L, SEA, and PBMCs, the cytotoxicity decreased in a dose-dependent manner and was inhibited completely by adding 20 μg/ml of the Ab. The accumulation of IL-8 was also inhibited completely by adding 1 μg/ml of anti-TNF-α mAb. These data suggest that TNF-α is essential for the HMVEC-L cytotoxic reaction and the increase in the accumulation of IL-8. Since TNF-α also induces gene expression and the secretion of monocyte chemoattractant protein-1 by human endothelial cells (40), it is also possible that the migration of monocytes to a focus of inflammation accelerates the production and secretion of TNF-α, which could worsen the endothelial injury.

The interaction between toxic products from PMNs such as myeloperoxidase and pulmonary endothelium is thought to increase microvascular permeability to plasma proteins, and the resultant lung edema is considered to be a major component of ARDS (41, 42). Furthermore, it has been shown previously that i.v. administration of IL-8 to rabbits induced changes in the lung histology that were consistent with ARDS (43), and that IL-8 also plays a significant role in PMNs adherence to and transmigration through vascular endothelium (44). As shown in Fig. 8, high concentrations of purified PMNs induced HMVEC-L lysis in a dose-dependent manner. Our PMN-purification protocol, which is a standard method for in vitro study, caused an increase of Mac-1 as described previously (45), as well as a decrease of L-selectin expression on the cell surface (Table I). These changes in adhesion molecules were also noted on IL-8-activated PMNs (46). Therefore, it is possible that the activation of PMNs by the purification procedure participates in the PMN-induced cytotoxic effect. However, as shown in Fig. 1, despite any purification-induced activation, PMNs did not enhance the cytotoxic effect of PBMCs for endothelial cells. These data indicate that PBMCs play an important role in the pulmonary endothelial cytotoxicity induced by SEA.

In conclusion, we have demonstrated a mechanism of SEA-induced human lung endothelium injury. TNF-α, which is secreted by SEA-induced PBMCs, injures HMVEC-L and stimulates the production and secretion of IL-8 from PBMCs and HMVEC-L. Because PMNs can be cytotoxic to the endothelium, it is suggested that the accumulation of PMNs due to IL-8 may accelerate the cytotoxicity of HMVEC-L. From the findings we have reported here, it is expected that antagonists of TNF-α may have an important role in the treatment of SEA-induced pulmonary injury.

### Table I. Effect of density gradient centrifugation on PMNs

<table>
<thead>
<tr>
<th>Expression</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1 (CD11b)</td>
<td>64.8 ± 8.1</td>
</tr>
<tr>
<td>L-selectin (CD62L)</td>
<td>−23.8 ± 8.8</td>
</tr>
</tbody>
</table>

*Cell staining using mAbs and analysis by FACScan were performed as described in Materials and Methods.*

### References


### Figures

**FIGURE 7.** Effect of density gradient centrifugation on PMNs. PMNs were incubated with indicated concentrations of PMNs without any stimulants for 21 h; the percentage of cell lysis was calculated as described in Materials and Methods.

**FIGURE 8.** Effect of PMNs on HMVEC-L cytotoxicity. HMVEC-L were incubated with indicated concentrations of PMNs without any stimulants for 21 h; the percentage of cell lysis was calculated as described in Materials and Methods.


