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Staphylococcus aureus enterotoxins A and B inhibit human and mice eosinophil chemotaxis and adhesion in vitro

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ABSTRACT

Staphylococcus aureus aggravates the allergic eosinophilic inflammation. We hypothesized that Staphylococcus aureus-derived enterotoxins directly affect eosinophil functions. Therefore, this study investigated the effects of Staphylococcal enterotoxins A and B (SEA and SEB) on human and mice eosinophil chemotaxis and adhesion in vitro, focusing on p38 MAPK phosphorylation and intracellular Ca^{2+} mobilization. Eosinophil chemotaxis was evaluated using a microchemotaxis chamber, whereas adhesion was performed in VCAM-1 and ICAM-1-coated plates. Measurement of p38 MAPK phosphorylation and intracellular Ca^{2+} levels were monitored by flow cytometry and fluorogenic calcium-binding dye, respectively. Prior incubation (30 to 240 min) of human blood eosinophils with SEA (0.5 to 3 ng/ml) significantly reduced eotaxin-, PAF- and RANTES-induced chemotaxis ($P < 0.05$). Likewise, SEB (1 ng/ml, 30 min) significantly reduced eotaxin-induced human eosinophil chemotaxis ($P < 0.05$). The reduction of eotaxin-induced human eosinophil chemotaxis by SEA and SEB was prevented by anti-MHC monoclonal antibody (1 μg/ml). In addition, SEA and SEB nearly suppressed the eotaxin-induced human eosinophil adhesion in ICAM-1- and VCAM-1-coated plates. SEA and SEB prevented the increases of p38 MAPK phosphorylation and Ca^{2+} levels in eotaxin-activated human eosinophils. In separate protocols, we evaluated the effects of SEA on chemotaxis and adhesion of eosinophils obtained from mice bone marrow. SEA (10 ng/ml) significantly reduced the eotaxin-induced chemotaxis along with cell adhesion to both ICAM-1 and VCAM-1-coated plates ($P < 0.05$). In conclusion, the inhibition by SEA and SEB of eosinophil functions (chemotaxis and adhesion) are associated with reductions of p38 MAPK phosphorylation and intracellular Ca^{2+} mobilization.

1. Introduction

The Gram-positive pathogen *Staphylococcus aureus* is the leading cause of serious and fatal infectious diseases that can progress to bacteremia, sepsis, pneumonia and multiorgan dysfunction [1]. These bacteria are capable of secreting a family of 25–30 kDa exotoxins that are classified into distinct immunological types, namely toxic shock syndrome toxin-1 (TSST-1) and Staphylococcal enterotoxin serotypes A–E and G–Q. Staphylococcal exotoxins are a prototypic group of microbial superantigens that induce extensive proliferation of T cells mediated by cross-linking of the variable region of the β chain of the T-cell receptor (TCR) with MHC class II (MHC II) molecules on antigen-presenting cells (APC) [2].

Staphylococcal enterotoxins trigger a variety of immune-modulatory and inflammatory effects due to their ability to activate macrophages, mast cells and granulocytes in different experimental models and animal species [3]. Airways exposure to Staphylococcal enterotoxin A (SEA) promotes marked neutrophil influx through activation of different pathways, resulting in increased expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as enhanced production of nitric oxide (NO), eicosanoids, TNF-α and IL-6 [4]. The neuropeptide substance P also takes part in the inflammatory responses induced by Staphylococcal enterotoxin B (SEB) in mice [5]. In humans, *S. aureus* and its enterotoxins worsen the allergic inflammation in upper airway diseases [6], allergic rhinitis [7], atopic dermatitis [8] and rhinosinusitis [9], and have also been involved with food allergy [10]. In murine models of allergic diseases, SEA or SEB enhances allergic skin inflammation [11], pulmonary eosinophilic inflammation [12–14] and nasal polypoid lesions [15]. A previous study showed that SEA and SEB inhibit human eosinophil apoptosis [16]. However, no studies exist examining the direct effects of Staphylococcal superantigens on eosinophil effector functions such as chemotaxis and adhesion, and

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its signaling pathway. Therefore, in the present study we designed experiments to investigate the effects of SEA and SEB on human and mice eosinophil chemotaxis and adhesion in vitro, focusing on p38 MAPK phosphorylation and intracellular Ca^{2+} mobilization. Our findings show that SEA and SEB down-regulates the downstream signaling of CCR3 in eosinophils leading to reduced chemotaxis and adhesion.

2. Material and methods

2.1. Eosinophil isolation from human blood

Blood was collected from healthy volunteers who were not under medication. Informed consent and approval from the local ethics committee were obtained before the study (Protocol Nº 472/2008) and conforms to the provisions of the Declaration of Helsinki. Eosinophils were isolated from peripheral blood as previously described [17] with minor modifications. Briefly, 60 to 120 mL of blood collected in 3.13% (w/v) sodium citrate was diluted 1:1 with phosphate buffered saline (PBS), and 35 mL of diluted blood was overlaid onto a 15 mL Percoll gradient (1088 g/mL, pH 7.4, 340 mosmol/Kg H_2O). Gradients were centrifuged at 1000 × *g* for 20 min, 4 °C (Sorvall, Thermo Fisher Scientific, USA) and the pellet containing red cells and granulocytes was collected. Red cells were lysed with lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$; 0.1 mM EDTA), and the remaining granulocytes were washed and then incubated with anti-CD16 immunomagnetic microbeads for 30 min before passing on a steel-matrix column in a magnetic field and CD16-negative eosinophils were collected (Miltenyi Biotec Inc., Auburn, CA, USA). Eosinophils were resuspended in RPMI-1640 medium (without phenol red), pH 7.2 (>92% eosinophils, contaminating cells were mononuclear cells).

2.2. Experimental protocols with human eosinophils

After purification, human eosinophils were incubated with SEA (0.5 to 3 ng/ml), SEB (1-30 ng/ml) or minimum essential media (MEM, 10 μl) at 37º C (5% de CO_2) for time-periods of 30 min to 4 h. Next, eosinophils were activated with eotaxin (300 ng/mL), PAF (500 ng/ml), RANTES (100 ng/mL) or MEM. The following assays were then carried out: Cell viability (MTT assay), chemotaxis, adhesion to ICAM-1 and VCAM-1-coated plates, flow cytometry analysis for phosphorylated p38 MAPK and intracellular Ca^{2+} mobilization. In separate assays, anti-MHC monoclonal blocking antibody (anti-HLA-DR; 1 μg/ml, Novus Biologicals, USA) was incubated with the eosinophils for 30 min at 37 °C before addition of either SEA (3 ng/ml) or SEB (1 ng/ml), and migration in response to eotaxin (300 ng/ml).

2.3. Eosinophil viability

Cell viability was estimated using the tetrazolium salt reduction test (MTT assay) by eosinophils after exposure to SEA or SEB, as previously described [18]. Isolated eosinophils were resuspended to a concentration of 2 x 10^6 cells/ml in MEM and then exposed or not to SEA (3 ng /ml) and SEB (1 ng/ml) for 30 min at 37 °C in humidified atmosphere. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, 10 μl/well, 5 mg/ml in PBS) were added in triplicate to a 96-well plates. Cells were allowed to incubate for 3 h at 37 °C and 5% CO_2. After incubation, 100 μl of 10% SDS in 0.01 M HCl was added to each well. Cell samples were then incubated for 18 h at 37 °C, 5% CO_2 and absorbance measured at 540 nm in a microplate reader (Multiscan MS, Labsystems, USA). Results are expressed as MTT reduction (% control).

2.4. Chemotaxis assays

The eosinophil migration assay was performed using a 48-well microchemotaxis Boyden chamber, as previously described [18]. The bottom wells of the chamber were filled with the chemoattractant agents eotaxin (300 ng/mL), PAF (500 ng/ml) and RANTES (100 ng/mL) in 27 μl MEM, whereas the upper wells were filled with eosinophils (50 μl) that had been treated or not with SEA (0.5 to 3 ng/ml) or SEB (1 ng/ml) for 30 min, 2 h or 4 h. The bottom and upper cells were separated by a polycarbonate filter (PVP-free; 25 × 80 mm; average pore size, 5 μm; Poretics Products, Osmonics, CA). The chamber was then incubated for 60 min at 371C with 5% CO2 atmosphere. Each incubation was carried out in triplicate and migration was determined by counting eosinophils that had migrated completely through the filter in five random high-power fields (HPF, ×1000) per well.

2.5. Eosinophil adhesion assay

96-well plates were prepared by coating individual wells with 50 μl of ICAM-1 and VCAM-1 solution (100 ng/ml in PBS) overnight at 4 °C. Wells were then washed twice with PBS before blocking non-coated sites with 0.1% (w/v) BSA for 60 min at 37 °C. Wells were washed twice again with PBS before allowing plates to dry. Eosinophils (50 μL of 7 × 10^4 cells/ml) were incubated (0.5 to 4 h) with SEA (3 ng/ml) or MEM (50 μl). Cells were then activated with eotaxin (0.3 μg/ml) for 30 min, after which eosinophils were added to the coated wells. Cells were allowed to adhere to wells for 30 min at 37 °C, 5% CO_2. After incubation, non-adhered cells were removed and the remaining cells were washed twice with PBS. Fifty μl of MEM were added to each well, and varying concentrations of the original cell suspension were added to empty wells to form a standard curve. Eosinophil adhesion was calculated by measuring residual eosinophil peroxidase (EPO) activity of adherent cells [19]. Fifty μl of EPO substrate (1 mM H_2O_2, 1 mM o-phenylenediamine and 0.1% Triton X-100 in Tris buffer, pH 8.0) were added to each well. After 30 min at room temperature, 25 μl of H_2SO_4 (4 M) were added to each well to stop the reaction and absorbance was measured at 490 nm in a microplate reader. Adherence was calculated by comparing absorbance of unknowns to that of the standard curve.

2.6. Flow cytometry

Eosinophils (100 μL of 3 × 10^6 cells/ml) were pre-incubated with SEA (3 ng/ml), SEB (1 ng/ml) or MEM (10 μl) for 30 min. Next, cells were activated with eotaxin (300 ng/ml) for another 30 min. Expression of p38 MAPK of eosinophils was detected using flow cytometry. After 30 min of incubation, eosinophils were washed and incubated for 30 min with perm buffer III (BD Biosciences, San Jose, CA, USA). Cells were washed and incubated for 20 min at 4 °C with 5 μl of RPE-conjugated mouse IgG1, k monoclonal immunoglobulin isotype control, and PE p38 MAPK (phospho flow). Cells were analyzed on a Becton-Dickinson FACScalibur (San Jose, USA). The mean fluorescence intensity was compared to that of isotype control reacting cells.

2.7. Intracellular Ca^{2+} measurements

Isolated eosinophils (2 × 10^6 cells/ml) incubated with SEA (3 ng/ml) and SEB (1 ng/ml) were suspended in MEM in the presence of Fluoforte (3 μM) for 45 min at room temperature protected from light. Thereafter, the eosinophil suspension was centrifuged at 400 *g* for 10 min. The eosinophil pellets were resuspended in MEM. Aliquots of eosinophils (1 ml) were dispensed into cuvettes (Hitachi-F 2000 — Japan) equipped with a stirring device. To obtain the total intracellular calcium levels, the external Ca^{2+} concentration was adjusted to 1 mM with $CaCl_2$, following equilibration for at least 30 s. Next, eotaxin (300 ng/ml) was added to induce eosinophil activation. Fluoforte fluorescence was