

German Cockroach Extract Induces Activation of Human Eosinophils to Release Cytotoxic Inflammatory Mediators

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Key Words

Eosinophils · Cockroach extract · Degranulation ·
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protein kinase

Abstract

Background: Eosinophils play an important role in the pathogenesis of allergic diseases. Sensitization and exposure to cockroach allergen have been demonstrated to be one of the major risk factors for the development of bronchial asthma. However, little is known regarding the functional capacity of cockroach extract antigen to activate human eosinophils. **Objective:** We investigated whether German cockroach extract can activate human eosinophils to release cytotoxic inflammatory mediators. **Methods:** Purified eosinophils from the peripheral blood were incubated with various concentrations (0–200 µg/ml) of German cockroach extract antigen. Effector functions of eosinophils were checked by degranulation and superoxide anion production. In addition, we examined surface expression of CD11b and CD69, and intracellular activation of p38 mitogen-activated protein kinase (p38 MAP kinase) in cockroach-stimulated eosino-

phils. **Results:** German cockroach extract induced degranulation and superoxide production from human eosinophils. In addition, incubation of eosinophils for 3 h with the cockroach extract resulted in an increased level of the surface expression of CD11b and CD69. Furthermore, cockroach-induced superoxide production from eosinophils was significantly inhibited by the pretreatment of cells with a p38 MAP kinase inhibitor SB202190. Indeed, a large amount of phosphorylated forms of p38 MAP kinase was detected in cockroach-stimulated eosinophils. **Conclusions:** Our results suggest that German cockroach extract induces activation of human eosinophils to release cytotoxic inflammatory mediators such as superoxide and granular proteins.

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Introduction

Eosinophils play crucial roles in tissue inflammatory reactions associated with allergic diseases, such as bronchial asthma and atopic dermatitis [1, 2]. During such inflammatory reactions, transmigrated eosinophils at the inflamed tissues are activated by stimulation of appropriate stimuli [3–6], thereby the activated eosinophils release proinflammatory cytokines [7], lipid mediators [8], toxic oxygen metabolites [9], and cytotoxic granular

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proteins [3]. These proinflammatory mediators released from eosinophils contribute to the pathogenesis of eosinophil-mediated tissue inflammation. However, the exact mechanisms and pathophysiological factors that are involved in the regulation of eosinophilic inflammation in allergic diseases and bronchial asthma remain largely unknown.

Cockroach allergen has been demonstrated to be one of the major risk factors for the development of asthma [10]. In particular, it has been suggested that sensitization and exposure to cockroach allergen may be closely associated with increased asthma morbidity in inner-city areas [11–13], since cockroaches are ubiquitous and highly allergenic [14]. Cockroach-associated asthmatic children have also been reported in many other regions of the world [15]. In Korea, the German cockroach, which is the most common domiciliary cockroach species, has also been considered as an important aeroallergen in house dust for asthmatic children [16, 17]. Although the precise mechanisms and the causative factor(s) responsible for cockroach-induced allergic inflammation are not well understood, it is clear that cockroach exposure can induce airway hyperreactivity and accumulation of eosinophil-rich inflammatory cells in the blood and tissue in sensitized asthmatic patients and experimental animals [18, 19]. In addition, recent evidence indicates that cockroach extract contains biological molecules showing proinflammatory effects on airway epithelial cells *in vitro*. For example, American cockroach extract induced the production of vascular endothelial growth factor, which is well known to increase microvascular permeability required for airway edema and inflammation, from the bronchial airway epithelial cells [20]. It has also been found that German cockroach extract, but not recombinant Bla g 2, Bla g 4, or Bla g 5, synergistically increased TNF- α -induced IL-8 and IL-6 production in human bronchial epithelial cells, although cockroach extract alone showed little stimulatory effect on epithelial cells [21]. These results led us to speculate that cockroach extract may act on eosinophils at allergic inflammation sites to secrete various inflammatory mediators that could initiate and exacerbate eosinophil-associated tissue hypersensitivity. However, there is no available information on the direct roles of the cockroach extract in the functions of eosinophils. Therefore, we investigated whether German cockroach extract induces activation of human eosinophils to secrete their inflammatory products. We also examined the surface expression of CD11b and CD69, and intracellular signaling pathways involved in superoxide production of eosinophils in response to cockroach extract.

Materials and Methods

Materials

Magnetic cell separation system (MACS) microbeads conjugated with mouse anti-human CD16 mAb were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant human IL-5 were purchased from R&D Systems (Minneapolis, Minn., USA). Phycoerythrin (PE)-conjugated mouse anti-human CD11b, CD69 mAb, PE-conjugated mouse IgG1, and FITC-conjugated annexin V were purchased from BD Pharmingen (San Diego, Calif., USA). The selective p38 mitogen-activated protein kinase (p38 MAP kinase) inhibitor (SB202190), serine protease inhibitor AEBSF and protease inhibitor set III were obtained from Calbiochem-Novabiochem (La Jolla, Calif., USA). Anti-phosphorylated p38 MAP kinase Ab, anti-p38 MAP kinase Ab, and goat anti-rabbit Ig conjugated with horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, Mass., USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) unless stated otherwise.

Preparation of Whole Body Extract of German Cockroach

Live or frozen adult German cockroaches (*Blattella germanica*), which had been reared at the Department of Parasitology, Yonsei University College of Medicine, were pulverized in liquid nitrogen. The defatted cockroach sample was extracted at 4°C with phosphate-buffered saline (PBS, pH 7.4) containing 6 mM of 2-mercaptoethanol, 0.1% (v/v) of protease inhibitor set III and 1 mg/ml of 1-phenyl-3-(2-thiazolyl)-2-thiourea for blocking melanization [22]. The extract was then centrifuged at 10,000 g for 30 min at 4°C and the resulting supernatant was dialyzed against distilled water and finally filtered through a 0.22- μ m filter (Millipore, Bedford, Mass., USA). The endotoxin level, which was measured by using the E-toxate kit (Sigma), was found to be free in the cockroach extract sample. The kit was sensitive to 0.05–0.1 endotoxin units/ml. Total protein in the extract was 10.8 mg/ml, as evaluated with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill., USA). The amounts of recombinant Bla g 1 in the cockroach extract were measured by using a sandwich ELISA (Indoor Biotechnologies, Cardiff, UK). German cockroach extract stock used in this study contained 0.63 U/ml Bla g 1.

Measurement of Protease Activity in Cockroach Extract

Cockroach extract protease activity was measured by casein protease assay using QuantiCleave™ protease assay kit (Pierce) according to the manufacturer's instructions. TPCCK trypsin was used as an active protease control. No protease activity was detected in 50 μ g of cockroach extract/ml.

Isolation of Peripheral Blood Eosinophils

Human eosinophils were isolated by a method described previously [23] using anti-human CD16 conjugated with magnetic beads and the MACS (Miltenyi Biotec) from the peripheral blood of normal individuals without allergic symptoms or medication of allergic drugs. Briefly, venous blood (30 ml) anticoagulated with 10 U/ml heparin was diluted with piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer [25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose (pH 7.4)] at a 1:1 ratio. Diluted blood was overlaid on a Histopaque (density, 1.083 g/ml) and centrifuged at 100 g for 4°C for 30 min. After removal of supernatant and mononuclear cells at the interface, erythrocytes in the sediment were lysed by exposure to sterile distilled water. Isolated granulocytes

cytes were incubated with anti-human CD16 mAb conjugated with magnetic particles for 30 min on ice and then cells were loaded onto the separation column positioned in the MACS magnetic field. Cells were eluted with 15 ml PIPES buffer with 1% FCS. The purity of eosinophils counted by Randolph's stain was consistently greater than 95%. Neutrophils are found to be less than 5%, and no mononuclear cells or basophils were present. Purified eosinophils were used immediately for experiments. The viability of eosinophils, as judged by trypan blue exclusion, was always shown to be greater than 99%.

Assay for Superoxide Production and Degranulation from Eosinophils

One hundred microliters of cell suspension (0.5×10^6 cells/ml) in Hanks balanced salt solution (HBSS; pH 7.4) with 10 mM HEPES, 0.03% gelatin and 100 μ M cytochrome c were seeded in the wells of 96-well tissue culture plates. The reactions were initiated by adding 100 μ l of HBSS medium containing various concentrations (0.6, 1.2, 2.5, 5, 10, 20 and 40 μ g) of cockroach extract, 2 ng IL-5 as a positive control, or medium alone. Immediately after the addition of stimuli, the reaction wells were measured for absorbance at 550 nm in a microplate reader (Thermomax; Molecular Device, Sunnyvale, Calif., USA), followed by repeated readings for up to 3 h. Between absorbance measurements, the plate was incubated at 37°C. Each reaction was performed in duplicate and superoxide anion production was calculated with an extinction coefficient of 21.1×10^{-3} M/cm for reduced cytochrome c, and was expressed as nanomoles of superoxide production per 10^6 cells. After 3 h of incubation, cell-free supernatants from 96-well tissue culture plates were collected and stored at -20°C until assayed for eosinophil degranulation. In some experiments, to investigate whether superoxide anion production by eosinophils in response to cockroach extract is transduced by activation of p38 MAP kinase pathway, eosinophils were preincubated with SB202190 (p38 MAP kinase inhibitor, 10 μ M) for 30 min at 37°C before exposure to cockroach extract.

Eosinophil-Derived Neurotoxin Release

To quantitate eosinophil degranulation, eosinophil-derived neurotoxin (EDN) concentrations in the culture supernatants were measured by specific radioimmunoassay (RIA), as described previously [23]. The RIA is a double Ab competition assay using radioiodinated EDN, rabbit anti-EDN Ab, and burro anti-rabbit IgG. All assays were done in duplicate.

Flow-Cytometric Analysis for Surface Expression of CD11b and CD69 on Eosinophils

Freshly isolated eosinophils were suspended in RPMI 1640 medium supplemented with 25 mM HEPES, 50 μ g/ml of gentamicin, 0.1% human serum albumin and 10% FCS (Gibco BRL, Grand Island, N.Y., USA). Eosinophils (1×10^5 cells/well) were incubated for 3 h in 96-well flat-bottom tissue culture plates (Costar, Cambridge, Mass., USA) with or without cockroach extract (100 μ g/ml) at 37°C in a 5% CO₂ incubator. Recombinant human IL-5 (1 ng/ml) was used as a positive control. After incubation, the collected eosinophils were washed with cold PBS containing 1% FCS and incubated for 30 min at 4°C with PE-conjugated mouse anti-human CD11b and CD69, respectively. PE-conjugated mouse IgG1 was used as an isotype control Ab. The cells were washed with washing buffer and then fixed with 0.5% paraformaldehyde. Flow-cytometric analysis for fluorescent intensity of CD11b or CD69 expression on eosino-

phils was performed on at least 5,000 cells from each sample with a FACScan (BD Biosciences, San Diego, Calif., USA).

Preparation of Cell Lysates and Western Blot Analysis

Eosinophils (0.5×10^6 cells/group) were stimulated with or without cockroach extract (200 μ g/ml) for various times, and the reaction was stopped by brief centrifugation. The cell pellets were lysed in 60 μ l lysis buffer containing 20 mM Tris-HCl, 60 mM β -glycerophosphate, 10 mM EDTA, 10 mM MgCl₂, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM APMSF, 1% NP-40, and 5 μ g/ml leupeptin. After 40 min of incubation on ice, 20 μ l 4 $\times$ sample loading buffer was added to the cell lysates and boiled for 5 min. The samples were centrifuged at 12,000 g for 3 min to remove nuclear and cellular debris and then stored at -20°C. Samples were subjected to sodium dodecyl sulfate (SDS) 12% polyacrylamide gels under reducing conditions. Proteins from the gels were electrotransferred to polyvinylidene fluoride membrane (Millipore). The membrane was washed with Tris-buffered saline plus Tween 20 (TBS-T), and incubated with 1:1,000 anti-phosphorylated p38 MAP kinase Ab diluted in PBS-T containing 5% skim milk overnight. After incubation, the membranes were washed 3 times for 5 min with TBS-T. Goat anti-rabbit Ig conjugated with horseradish peroxidase diluted 1:2,000 in TBS-T was applied to the membrane for 60 min. The membranes were washed again 3 times with TBS-T and visualized by an enhanced chemiluminescence system (Cell Signaling Technology). In some experiments, to see dose-dependent effects of cockroach extract on phosphorylation of p38 MAP kinase, eosinophils were stimulated with four different concentrations (10, 50, 100 and 200 μ g/ml) of cockroach extract for 15 min at 37°C. Finally, the membranes were reprobbed with anti-p38 MAP kinase Ab, after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS at 50°C for 30 min.

Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM) from three to five independent experiments. Statistical significance between treatment and control groups was assessed by Student's t test. A probability value of less than 0.05 was considered significant.

Results

Cockroach Extract Induces Degranulation from Eosinophils

To monitor functional roles of cockroach allergen in eosinophil activation, isolated human eosinophils were incubated with various concentrations (3–200 μ g/ml) of cockroach extract and the amount of released EDN from the cells was measured. As shown in figure 1, cockroach extract induced eosinophil degranulation in a dose-dependent fashion. The stimulatory effect of cockroach extract on eosinophil degranulation was observed from the concentrations higher than 10 μ g/ml. At 50, 100 and 200 μ g/ml, cockroach extract induced 86.4 ± 17.0 , 102.4 ± 21.1 and 108.0 ± 25.1 ng EDN release per 10^6 cells (mean $\pm$

SEM, $n = 5$), respectively. In contrast, EDN release induced by medium alone was 47.2 ± 5.4 ng/ 10^6 cells. In addition, to know whether eosinophil degranulation induced by cockroach extract was due to a cytotoxic effect of cockroach extract, we measured the percentage of cells stained with annexin V in eosinophils incubated for 3 h with various concentrations (10, 50, 100 and 200 μ g/ml) of cockroach extract.

There was no difference in the percentages of annexin V-positive cells between cells incubated with medium alone and high concentrations of the cockroach extract. About 6% of eosinophils were found to be annexin V-positive when cells were incubated for 3 h with medium alone or cockroach extract (data not shown). This result suggests that EDN release induced by cockroach extract is likely due to exocytosis, not cytolysis.

Cockroach Extract Induces Superoxide Anion Production from Eosinophils

Next, to investigate whether cockroach extract induces other effector functions of human eosinophils, we checked superoxide anion production by eosinophils. Figure 2 shows that cockroach extract strongly induced superoxide anion production and the amount of released superoxide was dependent upon the concentration of

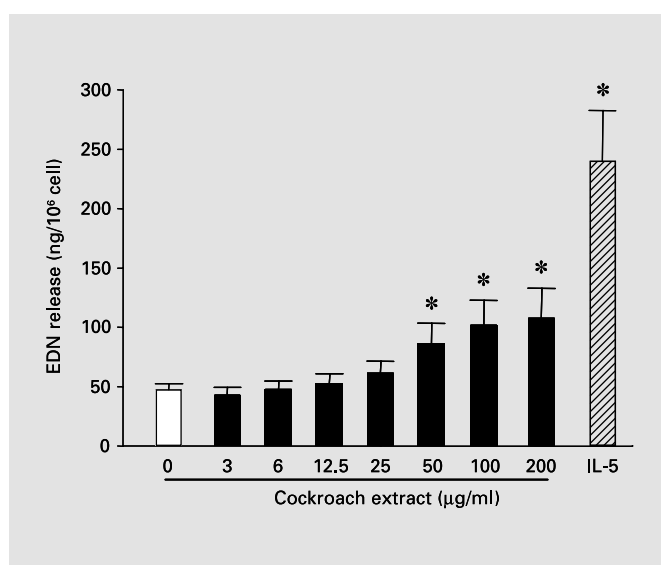
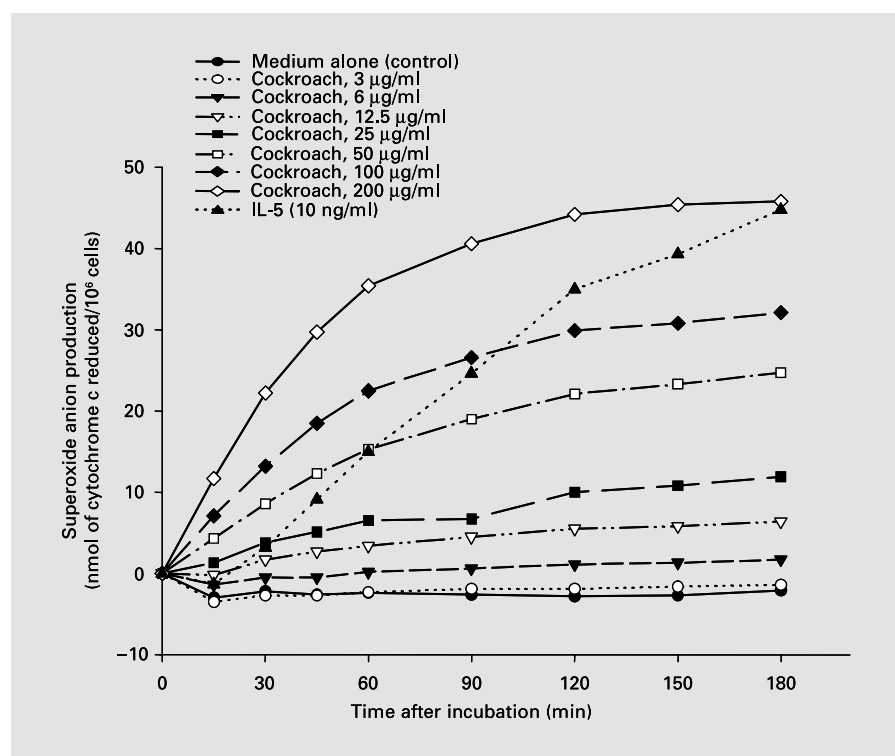


Fig. 1. Degranulation of eosinophils stimulated with German cockroach extract. Eosinophils were incubated for 3 h with or without various concentrations (3–200 μ g/ml) of cockroach extract. After incubation, released EDN into the culture supernatants by degranulation was measured by RIA as described in Materials and Methods. IL-5 (10 ng/ml) was used as a positive control. Data are presented as mean $\pm$ SEM obtained from five independent experiments performed in duplicate. Significant differences from the control are as follows: * $p < 0.05$.

Fig. 2. Superoxide anion production induced by German cockroach extract. Eosinophils were stimulated with cockroach extract (3–200 μ g/ml), IL-5 (10 ng/ml), or medium alone (control) for up to 3 h at 37°C. Superoxide production from eosinophils was measured by superoxide dismutase-inhibitable reduction of cytochrome c, as described in Materials and Methods. The results represent mean values from five independent experiments performed in duplicate.



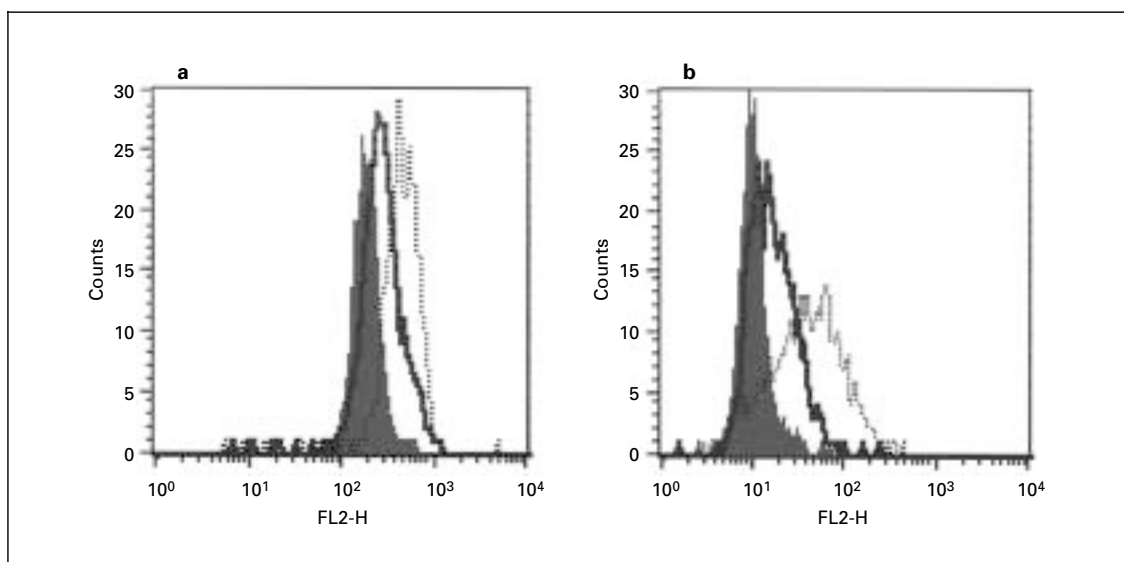


Fig. 3. Increased surface expression of CD11b (**a**) or CD69 (**b**) on eosinophils stimulated with German cockroach extract. Eosinophils were incubated for 3 h with medium alone (shaded area), 100 µg/ml cockroach extract (bold line), and 1 ng/ml IL-5 (dotted line) as a positive control. After incubation, eosinophils were collected and stained with PE-labeled mouse anti-human CD11b and CD69 mAb, respectively. PE-labeled mouse IgG1 was used as an isotype control Ab. Changes in fluorescent intensity of CD11b or CD69 expression were then analyzed by flow cytometry. The results are representative of three independent experiments showing similar results.

cockroach extract. For example, after 3 h of incubation, when eosinophils were stimulated with 50, 100 and 200 µg/ml cockroach extract, 24.7, 32.0 and 45.8 nmol of superoxide per 10^6 cells were produced. The amount of released superoxide induced by the highest concentration of cockroach extract was comparable to that induced by eosinophil-active cytokine IL-5. Little or no production of superoxide was shown when eosinophils were stimulated with lower concentrations (3–12.5 µg/ml) of the cockroach extract or medium alone.

Increased Surface Expression of CD11b and CD69 on Eosinophils Stimulated with Cockroach Extract

Next, we investigated the surface expression of CD11b and CD69, which has been known to be increased in activated eosinophils [24–26], on eosinophils stimulated with cockroach extract. As shown in figure 3, when eosinophils were incubated for 3 h in the presence of cockroach extract (100 µg/ml), fluorescent intensity of CD11b or CD69 expression was remarkably increased compared with results for cells incubated with medium alone. IL-5 (1 ng/ml) also strongly augmented fluorescent intensity of CD11b or CD69 expression on eosinophils.

Intracellular p38 MAP Kinase Signaling Pathways Are Involved in Superoxide Production of Eosinophils Induced by Cockroach Extract

To examine the functional relevance of p38 MAP kinase activation in effector functions of eosinophils in response to cockroach extract, we investigated whether cockroach-induced superoxide production of eosinophils is mediated through activation of the p38 MAP kinase signaling pathway, which is known to participate in various proinflammatory responses. As shown in figure 4, preincubation of eosinophils with a p38 MAP kinase inhibitor SB202190 (10 µM) significantly, but not completely, reduced the cockroach-induced superoxide production by 45%. We also observed that SB202190 more potently inhibited superoxide production from eosinophils induced by IL-5 than cockroach extract.

Finally, to verify whether cockroach extract can induce activation of intracellular p38 MAP kinase signaling pathway, we examined the phosphorylation of p38 MAP kinase in cockroach-stimulated eosinophils. Figure 5 shows that cockroach extract induces phosphorylation of p38 MAP kinase in eosinophils. A low level of phosphorylated p38 was observed in freshly isolated or nonstimulated eosinophils. In contrast, incubation of eosinophils

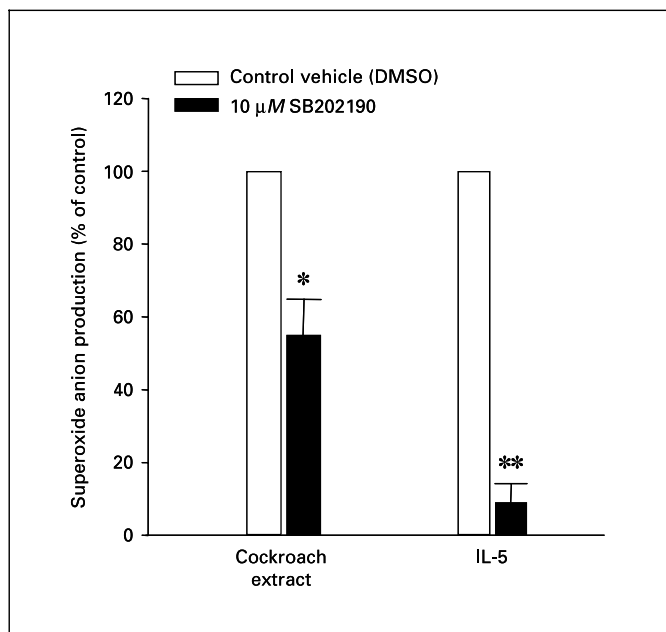


Fig. 4. Effects of p38 MAP kinase inhibitor SB202190 on cockroach-induced eosinophil superoxide anion production. Eosinophils were preincubated with 10 μ M SB202190 and 0.1% DMSO (control vehicle) for 30 min at 37°C and stimulated with cockroach extract (50 μ g/ml) or IL-5 (10 ng/ml) for 3 h at 37°C in a CO₂ incubator. The amounts of superoxide anion released from eosinophils were measured as described in the Materials and Methods. Data are normalized to mean superoxide production induced by cockroach extract or IL-5 without the inhibitors, taken as 100% (control value: 16 $\pm$ 1.0 nmol for cockroach extract or 38 $\pm$ 2.7 nmol for IL-5). Data are presented as mean $\pm$ SEM from 3 separate experiments using different eosinophil donors. * p < 0.05; ** p < 0.01 between cells with and without a MAP kinase inhibitor. No cytotoxic effect of SB202190 alone at the concentration tested in this study was observed.

for 15 min with 200 μ g/ml cockroach extract resulted in a marked induction of the phosphorylated form of p38 MAP kinase when compared to cells incubated with medium alone (fig. 5a). This stimulatory effect of the cockroach extract on phosphorylation of p38 was similar to that of IL-5, and persisted after incubation of 60 min. In addition, the cockroach extract-induced phosphorylation of p38 MAP kinase was concentration-dependent (fig. 5b). Reprobing the membrane with anti-p38 MAP kinase Ab were used as a measurement for equal protein loading on the gel.

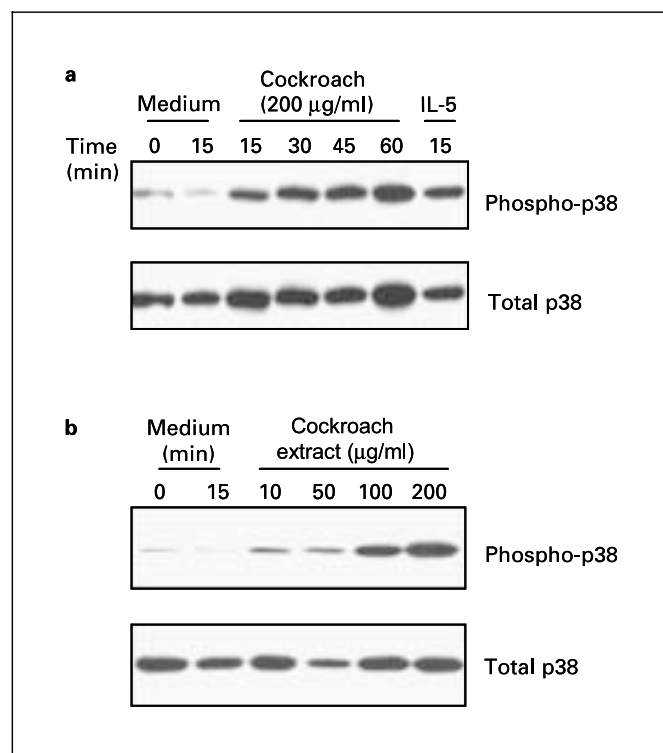


Fig. 5. Phosphorylation of p38 MAP kinase in cockroach-stimulated eosinophils. Time (a)- and dose (b)-dependent effects of cockroach extract on phosphorylation of p38 MAP kinase in eosinophils. Purified eosinophils were incubated with or without 200 μ g/ml cockroach extract for indicated times. In addition, eosinophils were incubated with or without various doses (10, 50, 100 and 200 μ g/ml) of cockroach extract for 15 min at 37°C. After reaction with the cockroach extract, eosinophils were lysed, and the cell lysates were mixed with sample buffer and loaded on 12% SDS-PAGE followed by immunoblotting with anti-phosphor-specific p38 Ab and anti-p38 Ab, respectively, as described in Materials and Methods. Eosinophils stimulated with IL-5 (10 ng/ml) for 15 min were used as a positive control of phosphorylation of p38 MAP kinase. The results are representative of three different experiments showing similar results.

Discussion

In the present study, we have demonstrated that German cockroach extract induces activation of human eosinophils to release superoxide and cytotoxic granular proteins. Incubation of eosinophils with cockroach extract resulted in significant degranulation and superoxide production from eosinophils. The fluorescent intensity of CD11b or CD69 expression, which is regarded as an activation marker for eosinophils [24–26], was remarkably augmented when cells were incubated for 3 h with the cockroach extract. In addition, the cockroach extract di-

rectly stimulates induction of phosphorylation of p38 MAP kinase in eosinophils. This intracellular activation of p38 MAP kinase induced by cockroach extract was associated with superoxide production from eosinophils in response to the cockroach extract, because pretreatment of eosinophils with p38 MAP kinase inhibitor SB202190 caused significant attenuation of cockroach-induced superoxide production from eosinophils. Taken together, our results suggest that exposure of German cockroach extract antigen might contribute to eosinophil-mediated airway inflammation in cockroach-induced asthma.

Although it remains possible that other intracellular signaling pathways may be linked to eosinophil activation induced by the cockroach extract, the results in our report are the first to show evidence that activation of p38 MAP kinase pathway is closely involved in cockroach-induced eosinophil activation, which might have important relevance in the pathogenesis of eosinophil-mediated airway tissue inflammation in cockroach-induced asthma. It has been known that MAP kinases such as ERK, p38 kinase, and JNK, are important mediators of signal transduction processes that serve to coordinate the cellular processes in immune responses to a variety of extracellular stimuli [27]. There is *in vivo* and *in vitro* evidence for an involvement of MAP kinase in allergic inflammation. For example, treatment with p38 MAP kinase inhibitor resulted in the reduction of antigen-induced accumulation of eosinophils in the airways in experimental animals [28]. In particular, the close involvement of p38 MAP kinase and ERK in specific functions of eosinophils has been described. Eotaxin induced phosphorylation of p38 kinase and ERK in eosinophils, and both SB202190 and PD98059 caused a significant reduction of eosinophil degranulation and chemotaxis in response to eotaxin [29]. In addition, it has been reported that both p38 kinase and ERK play an important role in LTB₄-induced NADPH oxidase activation, which is responsible for the generation of superoxide, in eosinophils [30]. IL-5-stimulated eosinophil degranulation and C5a-induced macrophage inflammatory protein-1 α production by eosinophils was inhibited by pretreatment with PD98059 or SB202190 [31]. Furthermore, activation of p38 MAP kinase plays an essential role in TNF- α -induced eotaxin production of human eosinophils [32]. Therefore, it is likely that cockroach-induced activation of p38 MAP kinase in eosinophils may contribute to the secretion of various proinflammatory mediators such as cytokines and chemokines that can trigger and amplify allergic inflammatory responses in the affected tissues in bronchial asthma.

Cockroach allergens are found throughout the house, including beds, furniture, and carpets [10]. Although there has been no reports on the airway levels of cockroach antigen in patients with positive skin test responses, cockroach allergen Bla g 1 was detectable in bedding and bedroom floor samples of 85.3% of homes of inner-city children with asthma, and levels of Bla g 1 of 8 U/g of dust or greater, which are considered high and proposed as a disease induction threshold, were found in 50.2% of those bedrooms [33]. It has also been reported that children exposed to greater than 2 U/g Bla g 1 or Bla g 2 in the family room or kitchen were associated with a higher risk of developing incident asthma and recurrent asthmatic wheezing [10]. In contrast to animal allergens, which are carried on small airborne particles, cockroach aeroallergens are associated with particles larger than 10 μ m in diameter [34]. Once in the airways, cockroach proteins elute from these particles and may reach high local concentrations in a restricted space. It is, therefore, imaginable that cockroach-carrying particles could have sufficient allergen to induce chronic inflammation on inhalation. However, it is unclear whether the protein amounts of cockroach extract (50–200 μ g/ml) or Bla g 1 (0.63 U/ml) present in the cockroach extract used in this study could be encountered by children and adults in cockroach-infested houses.

It has been shown that many allergens share sequence homology with known enzymes or contain functional enzymatic activity that directly influences inflammatory or immune cells. It becomes evident that several mite allergens are proteolytic enzymes (serine and cysteine proteases), and that proteolytic activity of the mite proteins directly renders the host sensitized or allergic conditions. For example, Der p 1 of house dust mite, *Dermatophagoides pteronyssinus*, cleaves surface expression of CD23 on B cells [35] and α -subunit of CD25 on T cells [36], and the cleavage effect of the allergen was dependent on its cysteine proteolytic activity. In addition, Der p 1 and other mite allergens showing proteolytic activity cause disruption of intercellular tight junctions, detachment of airway epithelial cells, and profound secretion of proinflammatory cytokines and chemokines *in vitro* [37–40]. There is no clear evidence for similar enzymatic effects with cockroach allergens, although it has been reported that the German cockroach extract containing a high dose of Bla g 2, which shares sequence homology with aspartic protease, contains active serine proteases but not active aspartic proteases [41]. In this study, however, we found that detectable levels of protease activity, as measured by using the QuantiCleaveTM protease assay kit, were not

observed even in the high concentration of the German cockroach extract. In addition, we found superoxide production induced by 50 µg/ml cockroach extract was not affected by pretreatment of cockroach extract with 2 mM AEBSF, a potent serine protease inhibitor (data not shown). Our results suggest that other factor(s) rather than serine proteases may be involved in the cockroach-induced responses of eosinophils.

In summary, we report that the cockroach extract of *B. germanica* induces activation of human eosinophils to release cytotoxic inflammatory mediators. Our findings may have clinical implications suggesting that exposure of cockroach antigens plays an important role in the regulation of eosinophilic inflammation of the airway tissues. Detailed studies on how and what factors are involved in

eosinophil activation induced by cockroach extract will be required for unraveling the immunobiological role of eosinophils and for seeking a potential therapeutic drug for bronchial asthma associated with cockroach allergens.

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