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ORIGINAL RESEARCH PAPER

The effects of various doses of bacterial lipopolysaccharide on the expression of CD63 and the release of histamine by basophils of atopic and non-atopic patients

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Abstract
Objective We tested the effect of various doses of bacterial lipopolysaccharide (LPS, endotoxin) on the expression of CD63 and the in vitro release of histamine by basophils stimulated with ragweed allergen in patients with or without ragweed and mite allergies.
Methods The peripheral blood of 11 patients with ragweed allergy, 10 patients with mite allergy and 14 control patients was incubated with ragweed allergen extract following pretreatment with varying doses of LPS. The expression of CD63 in basophils was measured by flow cytometry, and the release of histamine was determined by ELISA.
Results In the samples of patients with ragweed allergy that were exposed to specific allergen, only high doses of LPS significantly elevated the expression of CD63 (200 ng/ml; 1,000 EU/ml) and the release of histamine (2,000 ng/ml; 10,000 EU/ml). There was no effect of LPS in any other cases.
Conclusions Bacterial LPS (endotoxin) concentrations higher than 200 ng/ml (1,000 EU/ml), which rarely occurs in nature, could only activate the basophils from atopic patients whilst in the presence of the specific allergen. Thus, the restoration of the urban, "microbe-poor" milieu with endotoxin (as LPS) can be a promising and harmless approach for allergy prevention.

Keywords Bacterial lipopolysaccharide (endotoxin) · CD63 · Histamine · Atopic allergy

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Introduction

Bacterial lipopolysaccharide (LPS, "endotoxin"), a cell wall component of Gram-negative bacteria, represents a prominent pathogen-associated molecular pattern that is recognised by the innate immune system through members of the highly conserved family of Toll-like receptors [1], and it is an ubiquitous and natural component of the external and internal milieu. In the last decade, the biological and ecological significance of LPS has increased from studies showing its ability to protect from and prevent allergy [2–6]. Conversely, it is also known that acute inhalation of high concentrations of endotoxin can cause wheezing and coughing in the first year of life [7], and can aggravate already existing inflammation in asthmatics [8]. It has also been observed that bacterial endotoxin could potentiate the release of histamine from basophils in the presence of a specific allergen [9] and anti-IgE, in addition to potentiating the non-immunological histamine release (HR) [10]. However, the amount of bacterial LPS that can be tolerated in the environment or in the blood of atopic and non-atopic patients without causing harmful activation of circulating basophils remains uncertain. For example, in 229 inner-city school dust samples and in 118 bedroom dust samples tested in Boston, the median endotoxin concentration was 13.4 EU/mg (range 0.7–360.7 EU/mg) [11].

In industrialised nations and high-income regions of the world, the decline of infectious diseases is paralleled by an

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increase in allergic, autoimmune and chronic inflammatory diseases. The changes in lifestyle that individually and collectively impact the intestinal microbiota may, at least in part, account for the pandemic character of these diseases [13]. Therefore, the concept of artificial restoration of the urban milieu with endotoxin or other microbiological derivatives has become prominent [14].

Beyond the classical HR assay, new flow cytometric methods are emerging to assess the activation of basophils [15, 16]. Amongst several other markers, CD63 and CD203c have most commonly been applied in basophil activation assays [17]. The CD63 antigen, lysosome-associated membrane protein (LAMP-3), is not readily detectable on resting basophils but is upregulated rapidly on the outer cell membrane following activation with specific stimuli [18]. We chose to measure CD63 expression in this study, as this antigen was expressed with higher density on IgE-activated basophils than the transiently expressing CD203c antigen [17]. Furthermore, CD63 expression more closely reflects the anaphylactic degranulation pathway that is associated with the release of preformed mediators, compared to piecemeal degranulation, which can be measured by other groups of activation markers such as CD203c [19].

In the current study, we aimed to compare the in vitro effects of various doses of bacterial LPS on the expression of CD63 and the release of histamine in basophils, which were derived from healthy subjects or atopic patients who were sensitised either to ragweed or to mite allergen. The assays were performed in the presence and absence of soluble ragweed allergen.

Materials and methods

Subjects

Atopic donors suffering from moderate allergic rhinitis with ragweed (w1)-, house dust mite *Dermatophagoides pteronyssinus* (d1)- or *D. farinae* (d2)-specific IgE took part in the study. Eleven of the patients (six males, five females; mean age 13.0 ± 7.8 years, range 6–34) were monosensitised to ragweed. They were investigated before the pollen season, and they did not take any anti-histamine drugs. Ten of the patients (six males, four females; mean age 10.2 ± 3.61 years, range 5–17) were monosensitised to house dust mite d1 or d2. Their anti-histamine treatment was stopped at least 72 h before the experiments. None of the atopic patients were treated with corticosteroids during the study. Patients were excluded if they had specific IgE to any inhalant allergens other than w1, d1 or d2. Fourteen non-atopic healthy individuals (six males, eight females; mean age 11.38 ± 7.9 years, range 6–33) were the controls. All patients, or their parents in the case of children, gave written informed consent.

Treatment with LPS

Heparinised whole blood (1 ml) was preincubated for 15 min at 37 °C with various amounts of *Escherichia coli* bacterial LPS (Serotype 0111:B4, Sigma-Aldrich, Steinheim, Germany) that was diluted in sterile HEPES buffer to final concentrations of 20, 200 or 2,000 ng/ml. LPS diluted to 0.2 ng/ml was equivalent to an endotoxin value of 1 EU/ml. Ambrosie mix allergen extract (Lopharma, Milan, Italy) was used as ragweed allergen. 100 µl of the extract was diluted 1:50 with sterile HEPES buffer and added to the blood at 37 °C for 45 min. The reaction was stopped by cooling the tubes on ice. Following centrifugation, the supernatants were collected and stored for histamine determination at −70 °C. The pelleted cells were resuspended in cold sterile HEPES buffer containing 5 mM EDTA, and they were used for the CD63 expression assay of basophils.

Analysis of CD63 expression in basophils by flow cytometry

The pelleted cells were labelled with anti-human IgE-FITC (goat) and monoclonal anti-human CD63-PE antibodies (Caltag, Burlingame, CA) at 4 °C for 60 min. Basophils presenting highly fluorescent IgE-FITC were gated (100 % reference). Amongst these cells, the percentage of CD63 positive basophils was determined by the Becton–Dickinson FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany) [12]. The CD63 percentage in ragweed and/or LPS activated samples was corrected with the percentage obtained with buffer-treated samples. At least 500–1,000 basophils were counted.

The enhancement of ragweed-induced basophil CD63 expression in the presence of LPS was calculated using the following equation:

$$\text{Enhancement}\ (\%) = \frac{\text{Mean\% CD63}_{\text{ragweed+LPS}} - \text{Mean\% CD63}_{\text{ragweed}}}{\text{Mean\% CD63}_{\text{ragweed}}} \times 100$$

Measurement of histamine release

Histamine levels in the culture supernatants were determined by ELISA (Immunotech, Marseille, France). After lysing the cells with distilled water and repeated freezing and thawing, the total histamine contents were measured. The results were expressed as the percent of the total histamine content. The enhancement of ragweed-induced HR in the presence of LPS was calculated:

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$$\text{Enhancement}\ (\%) = \frac{\text{Mean}\%\ \text{HR}_{\text{ragweed+LPS}} - \text{Mean}\%\ \text{HR}_{\text{ragweed}}}{\text{Mean}\%\ \text{HR}_{\text{ragweed}}} \times 100$$

Testing for IgE

Patients were tested for total serum IgE and ragweed- and house-dust-mite-specific IgE (*D. pteronyssinus, D. farinae*) by ELISA (Adaltis, Casalecchio di Reno, Italy). Some other potential inhalative allergens were also tested, and samples showing positive results for allergens other than ragweed- or mite-specific IgE were excluded from the study.

Statistical analysis

A repeated measures ANOVA was used to analyse the differences in the various experimental groups. Multiple comparisons were performed using the post hoc Tukey test. The results are presented as the mean ± standard error. The Mann–Whitney test was performed to compare the enhancement of basophil CD63 expression and the HR at a given LPS concentration. *P*-values <0.05 were considered significant.

Results

The effect of LPS on CD63 expression of basophils

LPS administered in the range of 20–2,000 ng/ml dose-dependently increased the expression of CD63 in basophils from ragweed-sensitised patients in the presence of ragweed allergen (repeated measures ANOVA, $P < 0.001$). The lower dose of LPS (20 ng/ml, 100 EU/ml) caused a slight, but insignificant, increase in CD63 expression compared to the LPS-free milieu. Conversely, there were significant enhancements in CD63 expression following the addition of 200 ng/ml (1,000 EU/ml) and 2,000 ng/ml (10,000 EU/ml) of LPS compared to 20 ng/ml (100 EU/ml) and LPS-free systems (post hoc Tukey's test, $P < 0.05$ for both). In the patients with mite allergy and in the non-atopic healthy individuals, the ragweed allergen did not cause an increase in basophil CD63 expression following preincubation with the three LPS concentrations. Furthermore, LPS alone (without a specific allergen or IgE) did not enhance the expression of CD63 in any patient group at any concentration (Fig. 1).

Influence of LPS on the release of histamine from basophils

The ability of various doses of LPS to enhance the release of histamine in whole blood from ragweed-sensitised patients in the presence of ragweed allergen was investigated and found to result in a significant change (repeated measures ANOVA, $P < 0.002$). The analysis revealed that only the 2000 ng/ml (10,000 EU/ml) dose of LPS resulted in a significant elevation of CD63 expression compared either to untreated cells or to the group treated with 20 ng/ml (100 EU/ml) of LPS, but only in those cells which were stimulated by the specific ragweed allergen (post hoc Tukey's test, $P < 0.05$). In the patients with mite allergy and in the non-atopic healthy individuals, the ragweed allergen did not induce HR from the basophils either with or without LPS (Fig. 2).

Comparison of the activating capacity of LPS on the expression of CD63 and the release of histamine in basophils activated by specific allergen

As shown in Fig. 3, much smaller doses of LPS were required to increase the expression of CD63 than the release of histamine in the basophils of atopic patients

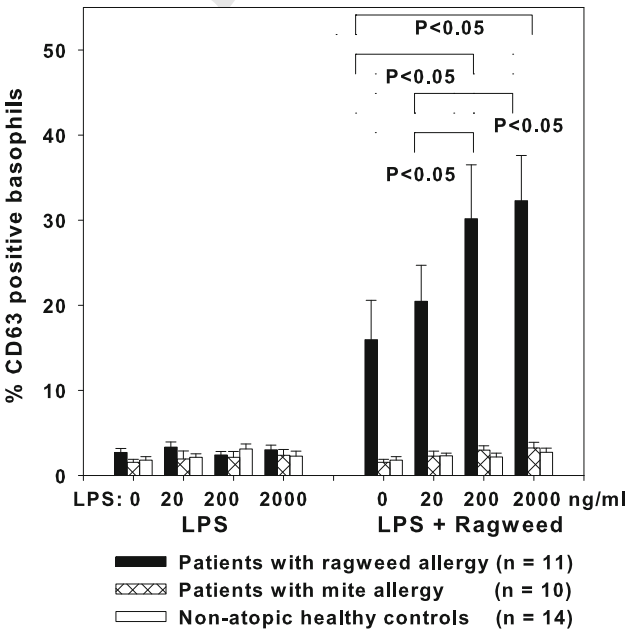

Fig. 1 The effect of LPS on the basophil expression of CD63 in the absence and presence of ragweed allergen in ragweed and mite allergy patients and in non-atopic healthy controls. Heparinised whole blood samples obtained from ragweed- or mite-sensitised atopic donors and non-atopic healthy controls were preincubated with various amounts of LPS for 15 min followed by incubation with ragweed allergen for 45 min at 37 °C. After staining the cells with anti-IgE-FITC and CD63-PE, basophil cells presenting highly fluorescent IgE-FITC were gated (100 % reference). Amongst these cells, the percent of CD63 positive basophils was determined by flow cytometry. A repeated measures ANOVA test was carried out in all groups that were treated with ragweed allergen, giving a result of $P < 0.001$. All pairwise multiple comparisons were performed by the post hoc Tukey's test, and the significant *P* values are shown. The results are expressed as the group mean ± SEM

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stimulated by their specific allergen in vitro. These findings show that the increase in the CD63 expression is a more sensitive marker of the activation of basophils than the release of histamine. However, the release of histamine indicates a functional activation of the basophils.

Discussion

Our present study provides experimental in vitro evidence that, in the presence of a specific allergen and IgE, only extremely high concentrations of bacterial LPS (>200 ng/ml, 1,000 EU/ml) can cause significant increases in the expression of CD63 in the basophils of atopic patients. To induce a statistically significant release of histamine in the same system, however, a much higher (2,000 ng/ml, 10,000 EU/ml) dose of LPS was required. This difference in the LPS doses shows that the elevated expression of CD63 expression on the surface of basophils could be a more sensitive, and potentially earlier, marker of basophil activation than the release of histamine. It is the release of histamine, however, that may have more important clinical importance. In agreement with our results, other studies have shown that HR does not strictly correlate with basophil CD63 expression [20]. In the patients with mite allergy and in the healthy controls, neither ragweed allergen nor any dose of LPS (20–2,000 ng/ml, 100–10,000 EU/ml) could induce basophil activation. These results show that only unusually high (1,000, 2,000 EU/ml) amounts of LPS may increase the severity of an acute allergic reaction based on basophil activation (CD63 expression and HR). LPS alone is not effective in the cases where the allergen and its specific IgE are not present.

Notably, Lopharma informed us that the ragweed allergen extract used in this study was not completely free of endotoxin contamination. The amount of contamination was far below the 20 ng/ml used as the smallest LPS dose in the experiments and therefore was not activating the basophils.

A great number of studies confirm that endotoxin is a protective and preventive factor for asthma in older children, whereas it is a risk factor for wheezing in younger children. [21]. Although histamine may have a pathogenic role in non-specific coughing and wheezing [22, 23], our results indicate that the naturally existing concentrations of endotoxin in the environment (367 mg EU/mg, 100 U/m^3,

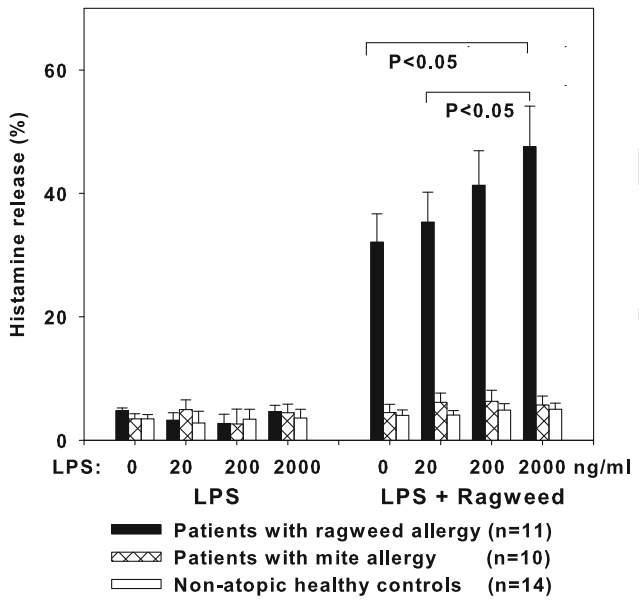

Fig. 2 The effect of LPS on the release of histamine from basophils treated with ragweed allergen in patients with ragweed and mite allergy and in non-atopic healthy controls. Heparinised whole blood samples obtained from ragweed or mite sensitised atopic donors and non-atopic healthy controls were preincubated with various amounts of LPS for 15 min followed by incubation with ragweed allergen for 45 min at 37 °C. The cells were pelleted, and the histamine content in the supernatants was determined by ELISA. The histamine release was calculated as the percent of the total histamine content. A repeated measures ANOVA test was carried out in all groups treated with ragweed allergen, giving a result of $P = 0.002$. Pairwise multiple comparisons were performed by the post hoc Tukey's test, and the significant P values are shown. The results are expressed as the group mean ± SEM

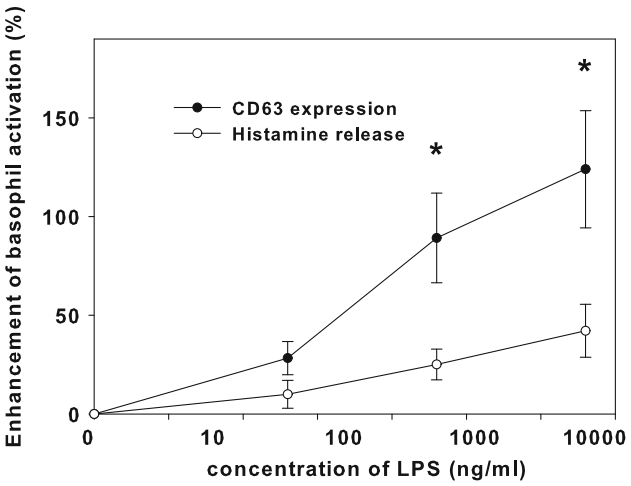

Fig. 3 Comparison of the activating capacity of LPS on the expression of CD63 and the release of histamine in basophils activated by ragweed allergen in patients with ragweed allergy. The enhancement of ragweed-induced basophil activation in the presence of LPS was defined as the following: $\text{Enhancement } (\%) = \frac{\text{Mean\% activation}_{\text{ragweed+LPS}} - \text{Mean\% activation}_{\text{ragweed}}}{\text{Mean\% activation}_{\text{ragweed}}} \times 100$B-asophil activation was measured as basophil CD63 expression or histamine release from basophils. The *solid line* and *filled circle* represent the enhancement in CD63 expression, and the *dotted line* and *open circle* represent the enhancement in histamine release. The data are presented as the means, and *the bars* represent the SEM ($n = 11$). The *asterisks* indicates a significant ($P < 0.05$) enhancement of CD63 expression versus the enhancement of histamine release at a given LPS concentration (Mann–Whitney test). *LPS* lipopolysaccharide

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1,550 EU/m^3 [24–28] are insufficient to induce enough HR to provoke clinical symptoms. However, naturally occurring endotoxin concentrations can trigger the release of leukotrienes [29] and activate the complement system, resulting in the production of bronchoconstrictor C3a and C5a [30] in some patients. For example, the importance of leukotrienes was recognised in the use of leukotriene D4 in the bronchial provocation test [31]. We hypothesise that the release of leukotrienes and activation of complement by high doses of LPS could be a potential cause of non-specific wheezing and coughing.

The restoration of the urban environment by using a "tolerable" dose and form of bacterial LPS can be a plausible new method of allergy prevention in the future [14]. LPS taken from the environment may influence the development and expansion of $CD4^+Foxp3^+$ regulatory (immunosuppressive) T cells [32]. The airway responsiveness of a patient on endotoxin depends on the existence of individual Toll-like receptor 4 variants [29]. According to our present data, a naturally occurring high level of endotoxin in the dust of a swine facility (1,550 EU/m^3) is approximately 1,000-fold lower than the lowest concentration of LPS required to activate basophils in atopic patients (1,000 EU/ml). Thus, the artificial enrichment of the environment with special types of endotoxin (having Th1 stimulating effects without causing non-specific wheezing and coughing) can be a realistic and harmless new approach to allergy prevention in infants.

In conclusion, our results show that only unusually high doses of bacterial LPS in the presence of specific allergen can trigger the expression of CD63 and the release of histamine in the basophils of atopic patients. Other activation processes of the blood may be involved in the pathomechanism of non-specific coughing or wheezing induced by high doses of LPS.

These data support the idea that restoration of the urban environment with a tolerable dose and form of bacterial LPS can serve to prevent allergy.

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