ORIGINAL ARTICLE

Neutrophil Defensins: Their Possible Role in Allergic Asthma

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Abstract

Background: Neutrophil defensins, originally identified as broad-spectrum antimicrobial peptides, have been implicated in the regulation of inflammatory and immunological processes.
Objectives: To investigate whether the in vitro challenge of neutrophils from patients with bronchial asthma with allergens stimulated the release of α-defensins and whether levels released were dependent on lung infections.
Method: The neutrophils were cultivated with different agonists and the concentration of α-defensin in cell-free supernatant was measured with enzyme-linked immunosorbent assay (ELISA).
Results: Neutrophils from allergic patients released α-defensins via an allergen-dependent mechanism. Our results indicate that the in vitro activation of neutrophils is highly allergen-specific. In this context, allergens other than those which produced clinical symptoms did not elicit α-defensin release, and allergens had no effect on neutrophils from healthy donors. However, neutrophils from both allergic patients and healthy controls were able to release α-defensins upon treatment with PMA. In the allergen-stimulated neutrophils, cells from asthmatic patients stimulated with a sensitizing allergen showed a significantly higher production of α-defensin under respiratory tract infection than cells from the same patients without such an infection
Conclusion: Neutrophils from allergic patients release α-defensins via an allergen-dependent mechanism.

Key words: Neutrophil. IgE. Allergy. Alpha defensins. Allergen challenge. Asthma.

Resumen

Antecedentes: Las defensinas provenientes de los neutrófilos, que originariamente se consideraron sólo como péptidos antimicrobianos, se han implicado en la regulación de procesos inflamatorios e inmunológicos.
Objetivos: Investigar si la provocación *in Vitro* de neutrófilos de pacientes asmáticos liberan α-defensins y si los niveles de esta liberación dependen de procesos infecciosos respiratorios en estos pacientes.
Método: Los neutrófilos se cultivaron con diferentes agonistas y la concentración de α-defensin en el sobrenadante celular se determinó mediante un ELISA.
Resultados: Los neutrófilos de pacientes alérgicos liberaron α-defensinas por un mecanismo dependiente de alérgeno. Nuestros resultados indican que la activación de los neutrófilos *in Vitro* es altamente específica del alérgeno empleado. En este contexto, los alérgenos que no producen síntomas en los pacientes alérgicos no liberan α-defensin. Así mismo, los alérgenos no tienen ningún efecto en las células de sujetos sanos, liberando α-defensina en ambos grupos si estimulamos con PMA.
La liberación de α-defensina dependiente de alérgeno es superior en los pacientes asmáticos con infección respiratoria.
Conclusión: Los neutrófilos de pacientes alérgicos liberan α-defensinas por un mecanismo alérgeno-dependiente

Palabras clave: Neutrófilo. IgE. Alergia. Alfa defensinas. Provocación alergénica. Asma.

Introduction

The integrity of the airway epithelium is an important prerequisite for an efficient host defence system. As has been observed in various inflammatory lung diseases, epithelial injury is followed by a repair process that serves to restore epithelial integrity [1]. During this process, inflammatory cells such as neutrophils are recruited to the site of injury, where they are believed to contribute to host defense, injury, and the repair process itself [2-6].

Defensins are small, arginine-rich cationic peptides that contain 6 highly conserved cysteine residues, forming a compact looped structure. Defensins are divided into α- and ß-defensin families depending on the position of the cysteine residues that participate in disulphide linkages [7]. Defensins released by stimulated neutrophils are members of the α-defensins subfamily and are stored in large amounts in the azurophil granules [8]; these α-defensins are also known as human neutrophil peptides 1-4 [HNP1-4]). Neutrophil defensins, which were originally identified as broad-spectrum antimicrobial peptides, have been implicated in the regulation of inflammatory and immunological processes.

Acute and chronic bronchial inflammation are thought to be central to the pathogenesis of several lung disorders such as asthma. The specific nature of the inflammatory response is determined by the recruitment and activation of immune cells in the lungs. These activated cells produce cytokines, oxidants, and many other mediators which are involved in inflammation. Different stimuli, such as allergens and infections, have been shown to induce bronchial inflammation. The aim of the present study was to investigate whether the in vitro allergen challenge of neutrophils from patients with bronchial asthma stimulated the release of α-defensins and if so, whether the levels released were dependent on lung infections.

Methods

Materials

The allergens used were commercially available antigen extracts (*Dermatophagoides pteronyssinus, Dactylis glomerata, Olea europae*, and *Artemisia vulgaris*) purchased from Bial-Aristegui, Bilbao, Spain. Other biochemicals were obtained from Sigma (Madrid, Spain) and Merck (Barcelona, Spain). All the cultured reagents had endotoxin levels of ≤0.01 ng/mL, as tested by the limulus lysate assay (Coatest; Chromogenix, Mölndal, Sweden).

Patients and Controls

The study group included 20 adult atopic patients with intermittent [9] bronchial asthma and 10 healthy adult nonatopic volunteer controls. They were all lifelong nonsmokers. Asthma was diagnosed on the basis of criteria previously described in detail [10]. The patients had positive skin prick tests (Bial-Arístegui) and specific immunoglobulin (Ig) E (HYTEC 288; Hycor Biomedical Inc.-IZASA, Barcelona, Spain) to at least 1 common allergen (house dust mites and pollens). They were not allowed to take bronchodilators in the 8 hours before the in vitro challenge and none had received treatment or specific hyposensitization. Oral bronchodilators were withheld for 96 hours, and none of the patients took corticosteroids, cromolyn sodium, or nedocromil sodium in the previous week. The controls had no history of allergy or bronchial symptoms; they had negative skin prick tests (Bial-Arístegui) and negative specific IgE (HYTEC 288) to a battery of inhalant allergens (house dust mites, pollens, molds, and animal dander). None of the participants had had a respiratory tract infection in the 6 weeks prior to the challenge. The patients (outpatients) and controls were followed until they developed a respiratory tract infection, diagnosed by the following clinical symptoms: nasal blockage, sneezing, fever, cough and both purulent rhinorrhea and expectoration. No medication was allowed after blood sampling. The hospital ethics committee approved the study, and all the participants gave their informed consent.

Preparation of Polymorphonuclear Leukocytes

Human neutrophils were purified from freshly drawn heparinized (10 U/mL) venous blood [11]. They were then washed twice with phosphate buffer solution containing 2% newborn calf serum. For further purification, the neutrophil preparations were incubated with mouse antihuman CD9 antibody (Immunotech-IZASA, Barcelona, Spain) and goat anti-mouse IgG micromagnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) [12]. The purity of the neutrophils was on average >99% (<0.1 eosinophil contamination). The purified cells were used immediately. Trypan blue exclusion showed greater than 96% viability.

Agonists included PMA (10 µg/mL) as a positive control and allergens at a final concentration of 10 µg/mL [11].

Neutrophil Culture

Neutrophils were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained at 37ºC in an atmosphere of 5% CO_2 and 95% O_2. For stimulation treatments, the cells were incubated with allergens or agonists at 37ºC for 15 minutes. None of the agents affected the viability of the cells at the concentrations used, as confirmed by the trypan blue dye exclusion test.

Measurement of α-defensin by ELISA

The concentration of α-defensin (HNP 1-3) in cell-free supernatant was measured with the HNP 1-3 enzyme-linked immunosorbent assay kit (ELISA) (Cell Sciences, Norwood, Massachusetts, USA) according to the manufacturer´s instructions. This assay measures the 3 main α-defensins, HNP 1-3, which are unique to neutrophils and account for over 99% of the total defensin content of these cells.

Statistical Analysis

Data are expressed as means±SEM. Comparisons between groups were made using 1-way analysis of variance. A *P* value of <.05 was considered significant.

Results

Neutrophil defensins have been implicated in the regulation of inflammatory processes and therefore, like other proinflammatory stimuli, might have the potential to exacerbate the allergic immune response. To test whether neutrophils might be a source of α-defensins during the allergic inflammatory process, we analysed α-defensin levels in the culture supernatant of allergen-stimulated neutrophils from allergic asthmatic patients using ELISA.

We found that neutrophils from allergic asthmatic patients stimulated with sensitizing allergens showed significantly higher α-defensin release (173±7.7) than those from either healthy donors (34.9±1.9) or asthmatic patients incubated with nonsensitizing allergens (35.6±1.7) (*P*<.001) (Figure 1). No statistical differences were observed between the levels of α-defensin released by neutrophils from allergic patients cultured without an allergen (33.3±1.8) and those released by neutrophils from either healthy donors or allergic patients incubated with a nonsensitizing allergen.

When we stimulated neutrophils with PMA, there was a significantly higher production of α-defensin in asthmatic patients (407.2±10.5) and healthy donors (398.4±9.8) with a respiratory tract infection than in asthmatic patients (228±6.3) and healthy donors

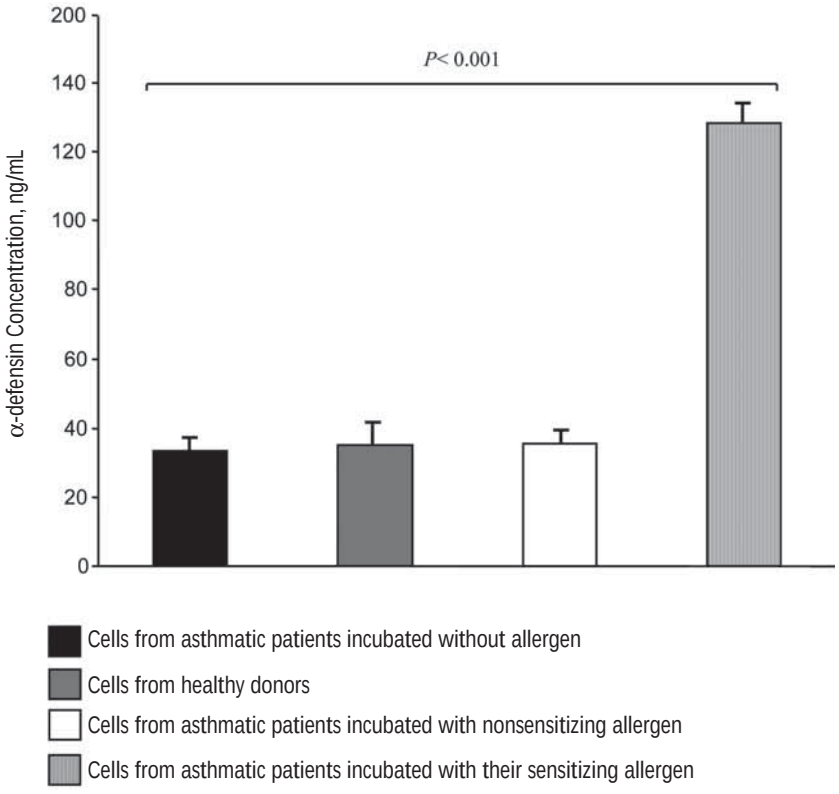

Figure 1. In the case of allergen-stimulated neutrophils, cells from the group of asthmatic patients stimulated with a sensitizing allergen showed a significantly higher production of α-defensin than cells from either healthy donors or asthmatic patients incubated with an allergen to which they were not sensitized (*P*<.001). No statistical differences were observed between the levels of α-defensin released by unstimulated cells and those released by either cells from healthy donors or cells stimulated with a nonsensitizing allergen from allergic patients.

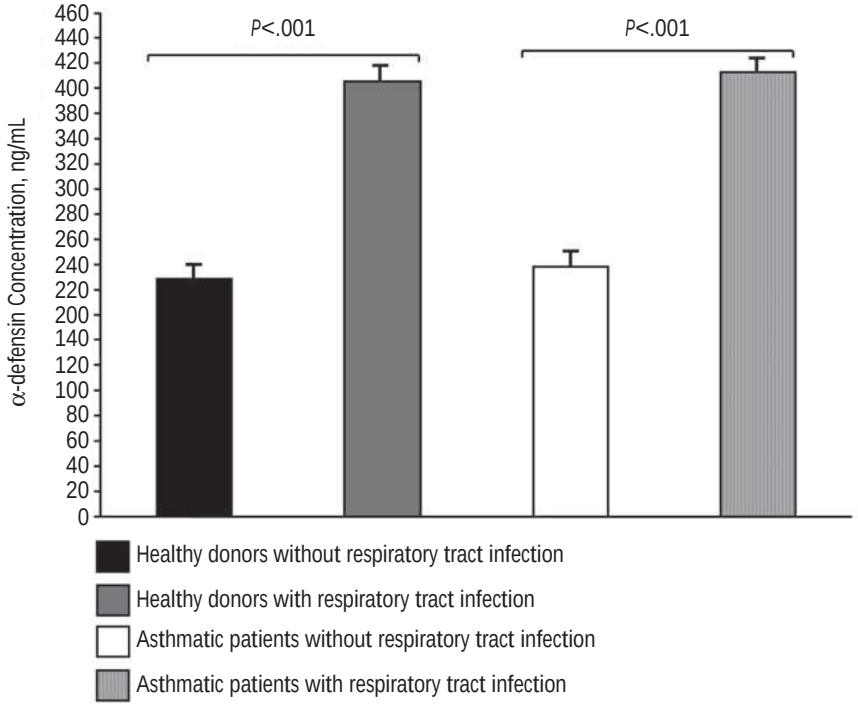

Figure 2. In the case of PMA-stimulated neutrophils, there was a significantly higher production of α-defensin in both asthmatic patients and healthy donors with a respiratory tract infection than in healthy donors without a tract infection (*P*<.001). No statistical differences were observed between α-defensin levels released by cells from healthy donors and by cells from allergic patients with a respiratory tract infection.

(216.9±6.1) without an infection (*P*<.001). No statistical differences were observed in α-defensin levels between the 2 groups with a respiratory tract infection (Figure 2).

The postchallenge production of α-defensin was significantly higher in asthmatic patients with a respiratory infection (269±14) than in those without (128.5±7.7) (*P*<.001) (Figure 3). No statistical differences were observed between the levels of α-defensin released by unstimulated neutrophils and those released by neutrophils from either healthy donors or allergic patients incubated with a nonsensitizing allergen, regardless of the presence or not of a respiratory tract infection (data not shown).

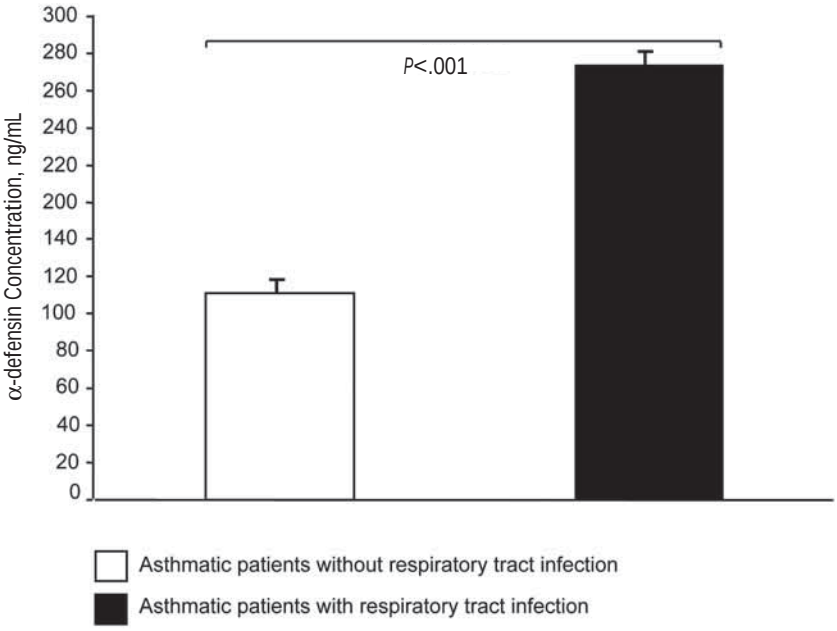

Figure 3. In the case of allergen-stimulated neutrophils, cells from asthmatic patients stimulated with a sensitizing allergen produced significantly higher levels of α-defensin when the patients had a respiratory tract infection (*P*<.001). No statistical differences were observed between the levels of α-defensin released by unstimulated cells and those released by cells from healthy donors or by cells from allergic patients stimulated with a nonsensitizing particular allergen, regardless of whether or not they had a respiratory tract infection (data not shown).

Discussion

Host defense against infection involves a multitude of factors and cells that together form the elements of innate and acquired immunity. A substantial number of studies have focused on defensins, a family of low-molecular weight, multifunctional cationic peptides that interact and disrupt microbial membranes. They are antimicrobial for gram-negative and gram-positive bacteria, fungi, and enveloped viruses [13], and have been identified in plants, animals, and humans.

Besides their involvement in the host's defense against infection, defensins are also thought to play a role in inflammation, wound repair, and specific immune responses. They also induce interleukin 8 (IL-8) synthesis in airway epithelial cells [14], suggesting that they may contribute to the perpetuation of the inflammatory response by stimulation of local chemokine release. Furthermore, defensins have been shown to stimulate the production of the neutrophil chemoattractants leukotriene B_4 and IL-8 by alveolar macrophages [15]. Other studies have shown that neutrophil defensins display chemotatic activity for monocytes and T cells [16,17]. Defensins have also been shown to induce the release of cytokines such as interferon-γ, IL-6, and IL-10 by T cells [18]. In addition, neutrophil defensins induce histamine secretion from mast cells [19] and mucin gene expression in lung epithelial cells [6]. Therefore, the excessive stimulation of mucin expression by defensins may lead to mucus hypersecretion, a feature of bronchial asthma.

An increase in epithelial permeability and epithelial injury is a frequent finding in both asthma and a variety of other inflammatory lung disorders [20,21]. Although this phenomenon has been attributed to the effect of proteinases, defensins may also contribute to airway epithelial cell damage. This is supported by in vitro studies showing that defensins, in concentrations that are likely to be relevant to the in vivo situation, cause lysis of airway epithelial cells [22]. Defensins may also promote epithelial cell damage by binding to members of the serine proteinase inhibitor (serpin) family, such as the α1-proteinase inhibitor. This binding results in an inability of α1-proteinase inhibitor to bind to and inactivate the injurious neutrophil elastase [23].

It has been recognized that asthmatic patients are more likely to develop infections. Allergic inflammation of the airways inhibits antibacterial host defense, and Th2-type inflammation in the lung results in suppressed antibacterial host defense [24]. This effect may be induced by ADP-ribosylation of α-defensins. ADP-ribosylation alters the biological properties of these defensins, decreasing antimicrobial activity and maintaining proinflammatory properties. ADP-ribosylated α-defensins have been found in the bronchoalveolar lavage fluid of asthmatic patients [25,26].

Human ß-defensin (HBD)-1 polymorphisms have been associated with asthma diagnosis [27]. The *HBD1* gene has also been linked to asthma, atopy, and plasma total IgE concentrations in asthmatic children [28]. Whatever the reason for the maintenance of a balanced variant, it is interesting to note that variation in *HBD1* might fit a previously proposed hypothesis [29] whereby alleles that conferred resistance to pathogens in ancient settings are now associated with susceptibility to atopic disorders; *HBD1* haplotypes associated with protection against infections seem to predispose to asthma and atopy. A similar link between past selection and present disease predisposition has been suggested [30] in the case of polymorphic variants in the IL-4 receptor alpha gene and might help to explain the high prevalence of atopic conditions in modern societies.

The experiments described here provide the first evidence

that neutrophils from allergic patients release α-defensins through an allergen-dependent mechanism. Our results indicate that in vitro activation of neutrophils is highly allergen-specific. In this context, allergens other than those which produced clinical symptoms did not elicit α-defensin release, and allergens had no effect on neutrophils from healthy donors. By contrast, PMA-stimulated neutrophils from both allergic patients and healthy controls released α-defensins.

It has been demonstrated that infections may cause exacerbation of chronic airway diseases such as asthma [31,32]. Studies involving patients with allergic rhinitis have demonstrated increased responsiveness to allergens in individuals with common cold symptoms [33-34]. These exacerbations may be in part due to the release of α-defensins by neutrophils both nonspecifically (in our case demonstrated by the stimulation of neutrophils with PMA) and specifically, with increased responsiveness to allergens. In both cases, α-defensins may contribute to the inflammatory processes in asthma.

Acknowledgments

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