

Upregulated levels of human β -defensins in patients with seasonal allergic rhinitis after allergen-specific immunotherapy treatment

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Background: Antimicrobial peptides (AMPs) are important actors in the innate immune system. One class of AMPs is the human β -defensins (HBDs), a group of small peptides with a broad spectrum of antimicrobial activities. Expression of HBDs is downregulated in different manifestations of allergic disease. In this study, we examine whether allergen-specific immunotherapy (ASIT) affects the nasal levels of HBDs in patients with seasonal allergic rhinitis (SAR).

Methods: Nasal biopsies were examined for the occurrence of HBD1-3 with real-time reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. Nasal lavage (NAL) fluids from healthy individuals, untreated SAR patients and SAR patients before and after ASIT were analyzed for levels of HBD1-3 using enzyme-linked immunosorbent assay (ELISA).

Results: Examination of nasal biopsies revealed HBD1-3 expression at gene level as well as at protein level in all samples tested. HBD1 and HBD3 messenger RNA (mRNA)

levels were downregulated in SAR patients compared to healthy individuals. All HBDs were found in NAL fluids. SAR patients having completed 3 years of ASIT displayed higher levels of HBD1 and HBD2 than before treatment, whereas levels of HBD3 were unaffected.

Conclusion: The present study demonstrates an upregulation of HBD1 and HBD2 in SAR patients after completion of ASIT. This may reflect the importance of an intact innate immune response as part of our defense against infections among allergic individuals. © 2013 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; allergy immunotherapy; rhinitis; skin prick test; subcutaneous immunotherapy

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The nasal epithelium provides through tight junctions and mucociliary activity a barrier that prevents microbes from entering. To increase the protection it also releases an array of antimicrobial peptides (AMPs) and cytokines. These mediators in turn initiate and regulate the inflammatory response by inducing recruitment of phagocytes such as neutrophils and monocytes.^{1,2} AMPs are gene-

encoded short (<100 amino acids) amphipathic molecules with hydrophobic and cationic amino acids arranged spatially, which exhibit a broad spectrum of antimicrobial activity. As such, they have a significant role in our innate immune system. Defensins is a class of AMPs that act against bacteria, fungi, and several types of viruses. Based on the site of expression, size, structure, and pattern of disulphide bridges, they are classified into 3 types; α , β , and θ defensin.^{3,4} The human β -defensins (HBD) constitute 4 members (HBD1-4), which in previous studies have been found in various tissues of the upper airway, including the nasal mucosa.⁵ Studies in patients with asthma and atopic dermatitis have reported downregulated levels of AMPs such as cathelicidin (LL-37), HBD2, and HBD3. These patients have T helper type 2 (Th2) cell-driven diseases, suggesting that the Th2 cytokine milieu may be detrimental for the antimicrobial defense provided by the epithelial cells.⁶ For example, in cell culture experiments, Th2 cytokines have been found to reduce the ability of differentiated human bronchial epithelial cells to clear *Pseudomonas aeruginosa* infections.² Because infections in the upper

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airway are known to cause exacerbations of allergic disease,⁷ theories of an altered expression of AMPs have been proposed similar to the findings in atopic dermatitis and asthma.⁸

Allergen-specific immunotherapy (ASIT) is a well-documented treatment that suppresses the number of Th2 cells and increases the levels of immunoglobulin G1 (IgG1) and IgG4 subclasses. This results in clinical and immunological tolerance that persists years after discontinuation. It has also been shown that immunotherapy prevents the progression of allergic rhinoconjunctivitis to asthma,⁹ and the de novo sensitizations to new allergens.¹⁰ In this study, we investigate the occurrence of HBD1-3 in the nose and the possible effect of ASIT on the HBD production among patients with seasonal allergic rhinitis (SAR).

Subjects and methods

Subjects

The study was approved by the local Ethics Committee, and informed consent was obtained from all participants. A detailed description of the subjects included is presented in Table 1. Nasal biopsies were obtained from 20 healthy volunteers and from 10 untreated SAR patients, and investigated for the occurrence of HBD1-3 by real-time reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. Nasal lavage (NAL) fluids were collected from 13 healthy controls, 14 untreated SAR patients, and 9 SAR patients before and 3 years after completion of ASIT. The NAL specimens were analyzed for levels of HBD1-3 using enzyme-linked immunosorbent assay (ELISA). All samples were taken outside pollen season.

The SAR patients had a minimum 2-year history of pollen-induced rhinoconjunctivitis and had experienced severe symptoms during previous pollen seasons (itchy nose and eyes, sneezing, rhinorrhea, and rhinitis). Their allergy was confirmed by a positive specific IgE test and a positive skin prick test (SPT) to birch and/or timothy pollen (wheal reaction diameter >3 mm). ASIT was administered subcutaneously by a standardized, aluminum hydroxide-adsorbed, depot pollen vaccine (Alutard; ALK Abelló, Hørsholm, Denmark). The patients received incremental

doses of allergen, reaching the maintenance dose 100,000 standardized quality units (SQ-U). Thereafter, maintenance injections were given regularly every 6 to 8 weeks for 3 years. Control subjects were all symptom-free, with no history of SAR and a negative SPT to the standard panel of allergens. Subjects with a history or signs of persistent allergic rhinoconjunctivitis or chronic rhinosinusitis including nasal polyposis were excluded along with patients with hypertrophy of turbinates, severe septum deviation, or a history of airway infection within 2 weeks before the first visit.

Sample collection

Nasal biopsies, approximately 2 × 2 × 2 mm in size, were taken from the inferior turbinate after topical application of local anesthesia containing lidocaine hydrochloride:nafazoline (34 mg/mL:0.17 mg/mL) for 20 minutes. NAL fluid was acquired as described.¹¹ Briefly, after clearing excess mucus by exsufflation, a sterile saline solution was aerosolized into the nostrils and nasal fluids were passively collected in a test tube until 7 mL was recovered. NAL fluids were centrifuged at 615 × g at 4°C for 10 minutes to remove the cell content and the supernatants were stored at –80°C until use.

RNA extraction and real-time RT-PCR

Nasal biopsies were homogenized in RLT buffer (Qiagen, Hilden, Germany) with 1% 2-mercaptoethanol. Total RNA was extracted using RNeasy Mini Kit (Qiagen), and the quantity and quality of the RNA were measured by spectrophotometry using the wavelength absorption ratio (260/280 nm). Reverse transcription of total RNA into complementary DNA (cDNA) was performed using the Omniscript™ reverse transcriptase kit (Qiagen) with oligo(dT)16 (DNA Technology, Aarhus, Denmark) in a Mastercycler personal PCR machine (Eppendorf, Hamburg, Germany). The RNA samples were denatured (65° for 5 minutes). The reaction was carried out at 37°C for 1 hour.

Real-time PCR was performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA) using Brilliant® II QPCR Master Mix (Agilent Technologies) in a final volume of 20 µL. Intron over-spanning oligonucleotide primers for detection of HBD1-3 and β-actin were designed to generate PCR products between 100 and 150 bp using Primer Express® 2.0 software (Applied Biosystems, Foster City, CA) and synthesized by DNA Technology A/S (Aarhus, Denmark). The following primers were used for HBD1-3 and β-actin:

HBD1 fwd 5'-ACCTTCTGCTGTTTACTCTCTGCTTAC-3'

HBD1 rev 5'-TCCACTGCTGACGCAATTGTA-3'

HBD2 fwd 5'-TCCTCTTCTCGTTCCTCTTCATATTC-3'

HBD2 rev 5'-GACTGGATGACATATGGCTCCAC-3'

HBD3 fwd 5'-CCATTATCTTCTGTTTGCTTTGCTC-3'

TABLE 1. Patient characteristics

	n	Sample	Sex	Age, years, median (range)
Healthy subjects	20	Nasal biopsies	12 M, 8 F	27 (23–31)
Untreated SAR patients	10	Nasal biopsies	4 M, 6 F	30 (24–43)
Healthy subjects	13	NAL fluid	4 M, 9 F	28 (22–47)
Untreated SAR patients	14	NAL fluid	5 M, 9 F	31 (19–47)
ASIT treated SAR patients	9	NAL fluid	7 M, 2 F	29 (15–40)

ASIT = allergen-specific immunotherapy; F = females; M = males; NAL = nasal lavage; SAR = seasonal allergic rhinitis.

HBD3 rev 5'-CCGCCTCTGACTCTGCAATAATA-3'
 β -actin fwd 5'-GCCAACCGCGAGAAGATG-3'
 β -actin rev 5'-ACGGCCAGAGGCGTACAG-3'

The thermal cycler was set to perform 95°C for 15 minutes, followed by 46 cycles of 94°C for 30 seconds and 55°C for 60 seconds (initially 65°C, followed by a 2°C decrease for the first 6 cycles). Melting curve analysis was performed to ensure specificity of the amplified PCR products. The mRNA expression was assessed using the comparative cycle threshold (Ct) method in which the relative amounts of mRNA for HBD1-3 were determined by subtracting the Ct value for these genes with the Ct value for β -actin (Ct). The amount of mRNA is expressed as $2^{-Ct} \times 10^5$ (Refs. 12 and 13).

Immunohistochemistry

The morphological localization of HBD1-3 proteins in nasal mucosa was assessed using immunohistochemistry. Immunostaining was performed according to the Envision⁺ System-horseradish peroxidase (HRP) kit (Dako, Copenhagen, Denmark) as described in detail.^{12,13} Briefly, the sections were incubated overnight in 4°C with a mouse anti-human monoclonal antibody (mAb) to HBD1 (Abcam, Cambridge, UK), a rabbit anti-human pAb to HBD2 (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit anti-human pAb to HBD3 (Chemicon International, Temecula, CA). The antibodies were diluted 1:100 in antibody diluent from Dako. Thereafter, the sections were incubated with HRP-labeled goat anti-rabbit or goat anti-mouse polymer for 30 minutes, followed by 3,3'-diaminobenzidine substrate-chromogen for 5 minutes. Counterstaining was performed with hematoxylin. Finally, the slides were mounted in Faramount Aqueous Mounting Medium (Dako). As negative controls, N-series universal negative control reagents against mouse and rabbit (both from Dako) were used. Tris-buffered saline (pH 7.6) supplemented with 0.05% Tween 20 was used for all washing steps.

ELISA

NAL fluids were analyzed for levels of HBD1, HBD2, and HBD3 using ELISA plates from Alpha Diagnostics (San Antonio, TX).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All data are expressed as median and range, and n equals the number of subjects. Statistical differences were analyzed using Mann-Whitney *t* test (unpaired data) or Wilcoxon matched pairs test (paired observations). A *p* value ≤ 0.05 was considered statistically significant.

Results

Expression and localization of HBD1, 2, and 3 in nasal mucosa

The expression of HBD1-3 was investigated by real-time RT-PCR in healthy subjects and patients with untreated SAR. mRNAs for HBD1, HBD2, and HBD3 were detected in all samples investigated. A downregulated expression of HBD1 and HBD3 was observed in the SAR group (Fig. 1A-C).

To support the molecular data and provide evidence for AMP synthesis in the nose, immunohistochemistry was performed. In line with the mRNA data, a clear immunopositivity was seen for HBD1 and HBD2, both in the surface epithelium and in the submucosa, whereas the staining was less intense for HBD3. When the primary specific antibodies were omitted, a complete loss of staining was seen (Fig. 2A-D).

ASIT increases the HBD release in SAR patients

The release of HBD1-3 in NAL fluid from healthy subjects, untreated SAR patients and SAR patients before and after completion of ASIT was determined by ELISA. Overall, stable levels of HBD1 and HBD2 were found, whereas the level of HBD3 was very low or even absent. No differences were seen between healthy and allergic patients, neither untreated SAR patients nor SAR patients before ASIT. Three years of ASIT treatment markedly induced the production

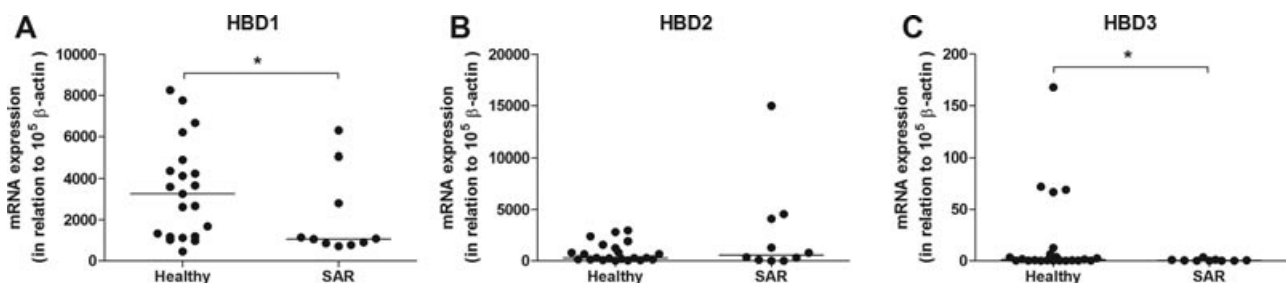


FIGURE 1. Expression of HBD1-3 in nasal mucosa from healthy and allergic subjects. (A-C) Levels of HBD1-3 in nasal mucosa from healthy subjects ($n = 20$) and from untreated SAR patients ($n = 10$) were determined by real-time RT-PCR. Data are presented in relation to β -actin as $2^{-\Delta Ct} \times 10^5$ and depicted as median and range. * $p \leq 0.05$ (Mann-Whitney *t* test). HBD = human β -defensin; RT-PCR = reverse-transcription polymerase chain reaction; SAR = seasonal allergic rhinitis.

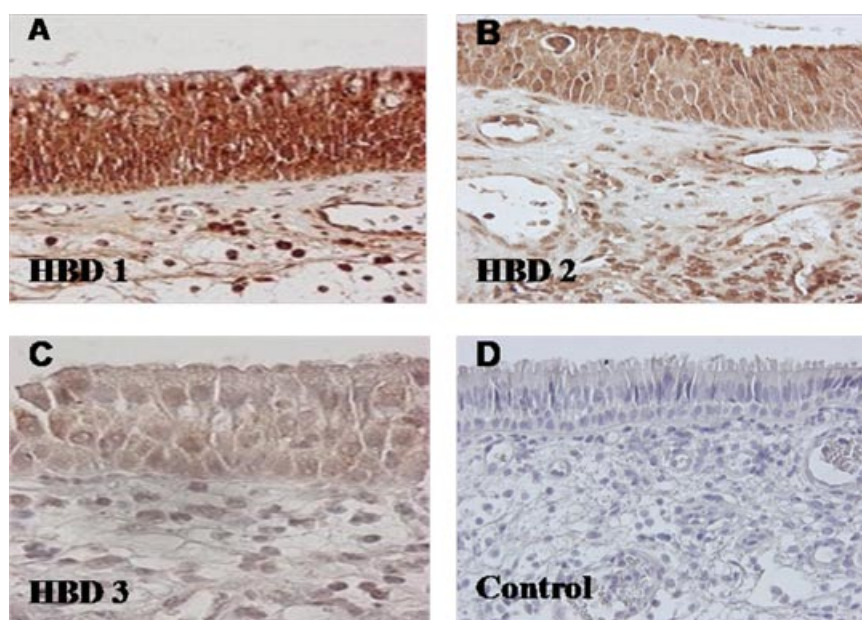


FIGURE 2. Immunohistochemical staining of nasal mucosa. (A-C) Nasal biopsies were incubated with antibodies against HBD1-3 and visualized by 3,3'-diaminobenzidine (brown). All sections were counterstained with hematoxylin. (D) In control slides, N-series universal negative control reagent was used. Magnification $\times 40$ to $\times 100$. HBD = human β -defensin.

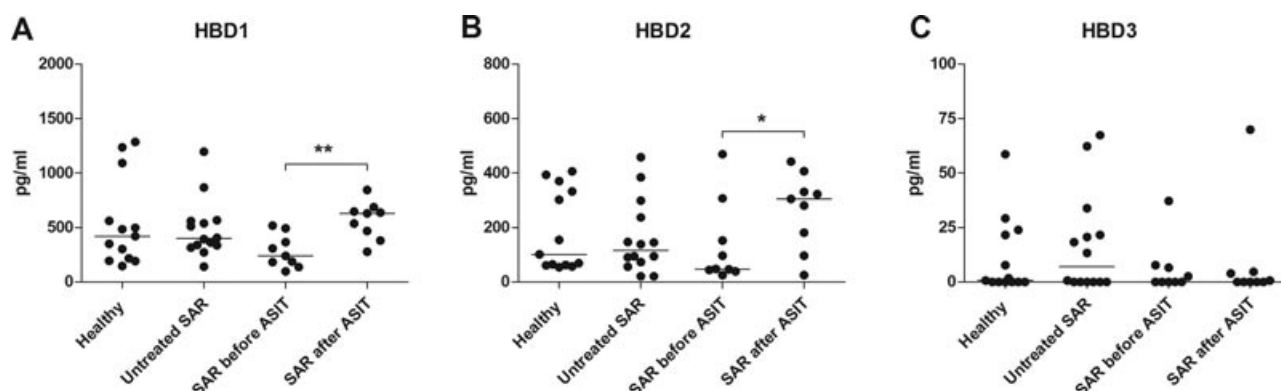


FIGURE 3. Levels of HBD1-3 in NAL fluid from healthy and allergic subjects. NAL fluid was collected from healthy controls ($n = 13$), untreated patients with SAR ($n = 14$) and SAR patients before and 3 years after completion of ASIT treatment ($n = 9$). NAL fluids were analyzed for levels of HBD1-3 using ELISA. (A-C) Healthy subjects were compared with untreated SAR patients or SAR patients before ASIT (Mann Whitney t test). SAR patients were compared before and after ASIT (Wilcoxon matched pairs test). Data are presented as median and range. $*p \leq 0.05$; $**p \leq 0.01$. ASIT = allergen-specific immunotherapy; ELISA = enzyme-linked immunosorbent assay; HBD = human β -defensin; NAL = nasal lavage; SAR = seasonal allergic rhinitis.

of HBD1 and HBD2, whereas no difference was seen for HBD3 (Fig. 3A-C).

Discussion

Airway infections are known to cause exacerbations of allergy and asthma. The present study describes HBD1-3 in the nose and their regulation in SAR patients in response to ASIT. Expression and release of HBD1-3 are shown in nasal biopsies and NAL fluid, respectively. SAR patients generally have lower mRNA levels of HBDs than healthy controls. SAR patients having completed ASIT acquire up-regulated levels of HBD1 and HBD2 in NAL compared to before treatment.

The presence of HBDs in nasal mucosa and secretions has been described.^{5,14-16} Their expression can be induced after administration of bacterial lysates or upon infection with rhinovirus and *Pseudomonas aeruginosa*.^{14,17,18} Further, a deficient HBD expression in the nasal epithelium has a tendency to make individuals more prone to persistent nasal colonization with *Staphylococcus aureus*.¹⁹ Other AMPs, such as S100A7 (psoriasin), have shown reduced levels in upper airway tissues during infection,⁷ chronic rhinosinusitis, and SAR.^{20,21} In line with our finding of reduced mRNA levels of HBD1-3 in nasal biopsies from patients with SAR, lower levels of HBD1-4 have been documented in allergic compared to nonallergic mucosa.²² Moreover, recent results from our laboratory show a diminished tonsillar expression and secretion of HBD1-3 in

SAR.²³ A similar pattern with reduced levels of AMPs has also been seen in other Th2-driven diseases, such as asthma and atopic dermatitis.^{6,24} Strong evidence points to a role of Th2 cytokines in this process. We have recently shown that interleukin 4 (IL-4), IL-5, and histamine downregulate the epithelial release of HBD1-3 and S100A7.^{21,23} Likewise, exposure of airway epithelial cells to IL-4 and IL-13 has been found to decrease the antimicrobial activity; eg, by suppressing mRNA levels of HBD2.²⁵ An overexpression of IL-4, IL-10, and IL-13 in the skin of patients with atopic dermatitis has been reported to cause a reduction of HBD2 and HBD3 levels, something that has been related to the increased numbers of skin infections seen among these patients.^{24,26} Moreover, IL-4 and IL-13 have been found to inhibit the expression of HBD3 by keratinocytes in response to inflammatory stimuli.²⁷

The beneficial effects of ASIT are partly related to a suppressed infiltration of Th2 cells and a shift from a Th2-polarized toward a Th1-polarized cytokine profile.^{9,28} It therefore seems likely that the increased levels of HBDs seen among our SAR patients after ASIT are a result of such cytokine shift. This finding is supported by a recent study reporting lower levels of S100A7 in NAL from SAR patients than from healthy controls, and the restoration of these levels to normal 3 years after completion of ASIT.²¹

Based on our findings, future studies are planned to further examine the expression and function of other AMPs

in the upper airway, such as LL-37, RNase7, and LEAP-2. These AMPs have not been fully studied, especially not in relation to SAR. A weakness in our study is the lack of comparison data on untreated SAR patients after 3 years. Therefore, a possible effect of natural variation in expression of HBDs cannot be completely ruled out.

Conclusion

The present study demonstrates expression, localization, and secretion of HBD1-3 in the nose and that ASIT increases the nasal release of HBD1 and HBD2 in patients with SAR. This clear link between allergic status and AMP levels indicates that SAR patients have an impaired antimicrobial defense that render them more susceptible to microbes. Microbes could also play a part in worsening the allergy. The elevated levels of HBDs after ASIT suggest that these patients are more resistant to respiratory tract infections. 

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