Antimicrobial peptides in nasal secretion and mucosa with respect to *S. aureus* colonisation in Wegener’s granulomatosis

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**ABSTRACT**

**Objective.** Nasal *S. aureus* carrier rates are significantly higher in patients with Wegener’s granulomatosis (WG) compared to healthy controls (HC), and nasal colonisation is a risk-factor for relapse. Antimicrobial peptides (AMP) are important defence molecules maintaining an intact barrier function. It is the aim of this study to see if there is a possible link between the nasal AMP pattern and *S. aureus* colonisation, a link which has not been investigated so far.

**Patients and methods.** ELISA was applied to quantify LL-37 and hBD-3 concentrations in nasal secretions (14 WG patients, 13 HC) with and without *S. aureus* colonisation. Immunohistochemistry was used to detect the cellular sources of AMP in the nasal mucosa. Functional analyses of primary nasal epithelial cell cultures (NEC) of these groups stimulated with *S. aureus* were performed.

**Results.** LL-37 was found in significantly higher concentrations in colonised individuals (WG: \(p=0.001\); HC: \(p=0.014\)). Using immunohistochemistry, local cellular sources for AMP could be demonstrated. After stimulation with *S. aureus*, significantly higher concentrations of LL-37 and hBD-3 could be detected in the supernatant of NEC of WG patients (LL-37: \(p=0.001\); hBD-3: \(p=0.001\)) and HC (LL-37: \(p=0.019\); hBD-3: \(p=0.001\)). HBD-3 concentrations were significantly lower in the supernatant of stimulated NEC of WG patients compared to the NEC of HC (\(p=0.032\)), and the dynamic range of the hBD-3 answer was significantly smaller in WG compared to HC (\(p=0.016\)).

**Conclusion.** The dynamic response towards challenges with microbes is dysregulated in WG, and this might be one reason for higher *S. aureus* colonisation rates in WG.

**Introduction**

The etiology of Wegener’s granulomatosis (WG) is unknown. WG is a potentially life- and organ-threatening chronic inflammatory disease. Mortality risk has been reduced by early diagnosis and more effective intervention in the last decades, but negative long term effects of therapy have increased (1). As well as in other autoimmune diseases (e.g. Crohn’s disease) an interaction of genetic susceptibility and environmental factors is discussed (2-5). Airway symptoms with granulomatous inflammation and scar formation occur in almost every WG patient followed over years, and “grumbling disease” within the respiratory tract related to persistent disease activity in WG patients otherwise in clinical remission is frequent (6-8).

The genetic susceptibility is largely unknown, but the environmental *Staphylococcus aureus* colonisation of the upper airways seems to play a potential role in triggering disease activity in WG (9-11). Chronic nasal carriage of *S. aureus* and especially tss-1 superantigen-positive *S. aureus* are associated with WG relapses (12). Even though treatment with trimethoprim/sulfamethoxazole (T/S) is not sufficient to eradicate *S. aureus*, such treatment reduces the incidence of relapses in WG patients in remission (10). The success of such a treatment might be the result of a modified composition of the commensals, which might be disturbed in WG comparable to other diseases associated with barrier dysfunction such as Crohn’s disease (13).

The nasal barrier towards inhaled microorganisms consists of mechanical components (intact epithelium, ciliary function, sneezing, and cough), cellular components (neutrophils, macrophages, dendritic cells) and chemical components (secretory IgA, lysozyme, lactoferrin, antimicrobial peptides (AMP)) (14).

**Competing interests:** none declared.
It has been recently demonstrated that the mechanical barrier in WG is disturbed by the severely impaired ciliar beat frequency in the upper airways in WG, and by the obviously defective epithelium detected by endoscopy which could facilitate S. aureus colonisation and invasiveness possibly triggering chronic inflammation (15, 16). The two main classes of AMP found in humans are cathelicidins and defensins (17). They exhibit a broad spectrum of efficient antimicrobial activity against both Gram-positive and Gram-negative bacteria, yeasts and enveloped viruses (18). Human beta-defensin-3 (hBD-3) and LL-37, the only cathelicidin found in humans, develop especially antimicrobial activity directed against S. aureus (19-21). Being effector molecules, the expression of AMP were found to be up-regulated by inflammatory stimuli, such as cytokines (IL-6, TNF-α, IFN-γ), as well as in direct response to bacteria, including S. aureus (21).

In addition, the diverse immunomodulatory properties of these peptides play a profound role in the complicated network of innate and acquired immunity. These properties include chemotactic for monocytes, CCR-6 expressing cell-like dendritic, memory T cells, influence on proliferation, release of cytokines, homeostasis, wound healing and the balance of proteases and protease inhibitors (14, 22, 23). Immunohistochemical and RT-PCR analyses revealed epithelial cells, inflammatory cells in subepithelial layers and submucosal glands as the source for LL-37 and hBD-3 in the nasal mucosa (21; 24-30).

Nothing is known about these AMP in correlation were investigated. In addition, immunohistochemistry was used to locate the expression of these AMP in corresponding nasal mucosa biopsies. To determine the ability of nasal epithelial cells to secrete AMP in response to S. aureus stimulation, an ex vivo stimulation assay was established. The aim was to demonstrate a barrier dysfunction of the nasal mucosa in respect to LL-37 and hBD-3 in WG patients, and to explore the effect of S. aureus colonisation to the expression of these AMP.

Patients and methods

Patients

From April 2008 to February 2009, 14 patients with Wegener’s Granulomatosis (6 women, 8 men, mean age 56 years) and 13 healthy controls (HC) (4 women, 9 men, mean age 41 years) were included in this study. The study was approved by the ethics committee of the University of Kiel, Germany, and participants gave written informed consent. Exclusion criteria included pregnancy and underage. WG was diagnosed in accordance to the ACR classification criteria and the Chapel Hill definitions for WG as recommended by EULAR (31). In 86% of cases (12 out of 14 patients), WG was proven by biopsy. Disease activity was measured using the Birmingham Vasculitis Activity Index (BVAS), organ damage as a consequence of granulomatous inflammation and vasculitis were diagnosed using the Vasculitis Damage Index (VDI) (32), and organ involvement was assessed by ELK-classification (33). WG subgroups (localised, early systemic and generalised WG), relapse and remission were diagnosed and defined according to the European Vasculitis Study Group (EUVAS) definitions and the recent recommendations by the European League Against Rheumatism (EULAR) (31). To assess WG activity, all patients were subjected to a standardised interdisciplinary evaluation as described earlier, and examined endoscopically by an ENT surgeon as proposed by Paulsen and Rudert (7; 34).

Control individuals had no anamnestic, visual or serological signs of acute or chronic rhinosinusitis, and biopsies were taken while performing airway passage improving surgery on the turbinates.

The status of systemic inflammation was assessed by analysis of the serum levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and white blood cell count (WBC).

Nasal secretions

Both anterior nostrils were swabbed following a standard operational procedure. The cotton swab was transferred into a sterile tube and incubated for 45 minutes at room temperature under rotation in a 10mM sodium phosphate buffer containing 0.1% BSA. The solution was stored at -80°C until it was used for ELISA.

Bacterial culture

One hundred micro litres of the swab supernatant described above were plated onto the Chapman agar for quantification of S. aureus colonisation. Furthermore, the swab tip was incubated in tryptic soy broth overnight at 37°C, the resulting bacterial growth being isolated on sheep blood agar and Chapman agar. Isolates were identified as S. aureus by the typical appearance of the colonies, haemolysis, the expression of the clumping factor, protein A, the ability to coagulate citrate plasma, and by acid production from mannitol fermentation.

Primary nasal epithelial cell (NEC) culture

Turbinate specimens were taken from a subset of 5 WG patients and 5HC. The NECs were obtained by a standard protocol using the dispase method (Invitrogen, Karlsruhe, Germany) and the Airway Epithelial Cell growth medium (Promocell, Heidelberg, Germany). Experiments were performed with preconfluent monolayers of NEC in 96 well plates keeping cell amounts equal.

Bacterial stimulation of NEC

A nasal S. aureus isolate (T190-2, kindly provided by B.M. Bröker, University of Greifswald, Germany), which has been described as predominant in Western Europe, (35) was used as a standard S. aureus strain in stimulation experiments. The strain was grown in tryptic soy broth (TSB) overnight at 37°C until a concentration of about 5x10⁸ per ml was reached. After centrifugation (15min, 4000xg), the bacterial supernatant was removed and sterile filtered. Prior to stimulation, the epithelial morphology of the NEC was verified by the inverted-phase contrast microscopy. Subsequently, the medium of the NEC
was removed and a fresh medium was added containing the bacterial supernatant in a final dilution of 1:5. NEC incubated in fresh culture medium without bacterial supernatant served as the control. After stimulation for 16 hours, cell culture supernatants were collected and stored at -80°C. In preliminary investigations, dose and time dependent stimulations were tested (data not shown). Cell viability was controlled by trypan blue dye exclusion, and cell morphology was observed microscopically. Furthermore, absence of bacterial contamination was proven by overnight incubation of 10μL of cell culture supernatant on sheep blood agar at 37°C.

ELISA
Nasal secretions and supernatants of stimulated NEC were analysed by sandwich ELISA for LL-37 and hBD-3. Briefly, ninety-six well immunoplates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with polyclonal rabbit anti-LL-37 (0.5μg/ml, Innovagen, Lund, Sweden) and incubated overnight at room temperature. Biotinylated polyclonal rabbit anti-LL-37 (0.25μg/ml, Innovagen, Lund, Sweden) served as quantification standard. Human recombinant LL-37 (0.5μg/ml, Acris, Herford, Germany) served as a positive control as described (21, 36). Negative controls were processed in the same way without the primary antibody followed by counterstaining with haematoxylin. Tonsil biopsy served as a positive control as described (21, 36). Negative controls were processed in the same way without the primary antibody and as a specificity control by isotype control staining with negative control serum and as a specificity control by isotype control staining with negative control serum (Vector, Burlingame, USA) instead of the primary antibody. Immunohistochemistry was performed on 4μm-thick formalin-fixed, paraffin embedded biopsies. Biopsies were taken for scientific research only at sides macroscopically unaffected by inflammation or damage (e.g. granuloma, edema, bloody patches, purulent secretion, crusts or septal perforation). After deparaffinisation and rehydration, the slides were microwave treated in 0.01M citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide/methanol for 10 minutes. The slides were incubated with 3% normal swine serum (Vector, Burlingame, USA) to avoid non-specific binding and with polyclonal rabbit anti-LL-37 antibody (6μg/ml, Innovagen, Lund, Sweden) or with polyclonal rabbit anti-hBD-3 (5μg/ml, Acris, Herford, Germany) affinity-purified antibody for 1 hour at room temperature. After the slides were rinsed with Tris-buffered saline (TBS), they were incubated with biotinylated polyclonal swine anti-rabbit immunoglobulin (4.4μg/ml, DAKO, Glostrup, Denmark) for 30 minutes at room temperature. After washing with TBS, the slides were incubated with streptavidine-peroxidase reagent "Vector ABC kit" (Vector, Burlingame, USA) for 30 minutes. The activity of peroxidase was detected with the "Vector NovaRed substrate kit" (Vector, Burlingame, USA), followed by counterstaining with haematoxylin. Tonsil biopsy served as a positive control as described (21, 36). Negative controls were processed in the same way without the primary antibody and as a specificity control by isotype control staining with negative control rabbit immunoglobulin fraction (Dako, Glostrup, Denmark) instead of the primary antibody (Fig. 2c).

Additionaly, double staining of AMP and cytokeratin 18 (clone DC10, Dako, dilution 1:50) using the EnVision™ G2 Doublestain System (Dako) following the manufacturer’s instructions was performed. Negative controls were performed using isotype controls (IR600 and IR750, Dako).

Statistical analysis
Results were analysed using SPSS statistical software for Windows (version 15.0, SPSS Inc., Chicago, USA). Data were described by mean (± standard deviation (SD)). Although we found no evidence against normal distribution assumption in our data (Kolmogorov-Smirnov test) the Mann-Whitney test was used to compare concentrations of LL-37 in nasal secretions between WG and healthy controls (with / without S. aureus). Because of small sample sizes (n=5) and very homogenous results we used t-test statistics for the evaluation of stimulation data in LL-37 and hBD-3. As a level of significance we used p≤0.05 for all statistical tests.

Results
Patient characteristics
The mean time from the first manifestation of WG until study entry was 5 years (range 26–0 years), and the mean time from first diagnosis until study entry was 4 years (range 18–0 years). Signs for systemic inflammation (CRP, ESR, WBC) were heterogeneous (Table I).

Systemic medication was heterogeneous with one HC and 13 WG patients receiving steroids, three WG patients receiving systemic antibiotics, one local antibiotic ointment, four oral cyclophosphamide, four methotrexate, three azathioprine, one leflunomide and one mycophenolate mofetil or rituximab respectively in different combinations and dosages. The cumulative cyclophosphamide dosage of the 15 patients who ever received this treatment was 19g (SD 14.8). The mean cytoplasmic ANCA (c-ANCA) level for WG patients was 1:308 (SD 674).

One WG patient was classified as early systemic, 13 as generalised (two of which were refractory) according to the subgroup classification by EULAR.

The mean BVAS-1 (indicating new/worse disease activity) was 3.6 (SD 4.5, max. 13, min. 0), and the mean BVAS-2 (indicating persistent disease activity) was 0.57 (SD 1.22, max. 4, min. 0). The mean VDI was 1.8 (SD 1.2, max. 4, min. 0). Seven (50%) WG patients showed involvement of the upper respiratory tract by vasculitis according to the ELK classification. No endonasal activity was detected endoscopically in 7 patients (50%), mild activity was detected in 6 patients (43%), and one WG patient showed moderate activity.
The *S. aureus* yield of swabbing the anterior nostrils was the following: 8 patients had a value of more than 500, and respectively one patient 10, 16, 73, 398, 466 and 480 CFU/swab.

**LL-37 and hBD-3 in nasal secretions**

No statistical difference in the concentrations of LL-37 in nasal secretion of WG patients and HC could be detected (28.2 (SD 29.3, max. 89.9, min. 0) and 17.7 (SD 19.04, max. 61.9, min. 0) ng/ml, respectively). There was also no statistical difference in the concentration of hBD-3 in nasal secretion of these groups with 2.7 ng/ml (SD 3.2, max. 9, min. 0) and 2.7 ng/ml (SD 5.4, max. 19.77, min. 0), respectively, for WG patients versus HC.

Based on correlation analyses, only weak dependency of endonasal activity determined by endoscopic evaluation and concentration of AMP in nasal secretion with $r^2$ of 0.078 for LL-37 and 0.184 for hBD-3 could be detected.

Eight (57%) of the WG patients and 6 (46%) healthy controls were colonised endonasally by *S. aureus*. The colonised WG patients and HC had significantly higher levels of LL-37 in the nasal secretion than non-colonised individuals ($p=0.001$ and $p=0.014$, respectively, Fig. 1).

In contrast to LL-37, no effect of *S. aureus* colonisation on the expression of hBD-3 in nasal secretion was detected.

**Immunohistochemistry**

for LL-37 and hBD-3

Positive staining for LL-37 and hBD-3 was found in all nasal biopsy specimens both in WG patients and in HC. LL-37 was located in the epithelial cell layer, in the submucosal glands and in some cells of the connective tissue (Fig. 2a). LL-37 positive cells in the connective tissue appear morphologically as leukocytes (Fig. 2d). HBD-3 showed positive staining in the epithelium and submucosal glands (Fig. 2b).

Additionally, double staining of AMP and cytokeratin 18 confirmed our results for epithelial cells as a major source for AMP (Fig. 3). Biopsies showed no histological abnormalities, especially no WG-specific abnormalities.

**Stimulation of NEC**

Patients for NEC were selected randomly, and when comparing HC and WG patients, no differences regarding *S. aureus* colonisation could be detected. Endonasal activity of the NEC group was comparable to the complete WG group with no activity in 2 patients and mild activity in 3 patients.

The NEC of WG patients secreted statistically significant higher amounts of LL-37 (mean 46.9, SD 5.4, max. 56.1, min. 34.4 ng/ml, $p=0.001$) and hBD-3 (mean 92.1, SD 4, max. 98.9, min. 88.8 ng/ml, $p=0.001$) after stimulation with *S. aureus* culture supernatants...
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than in non-stimulated NEC (LL-37 mean 0.4, SD 0.6, max. 1.5, min. 0ng/ml; hBD-3 mean 2.3, SD 0.6, max. 3, min. 1.3ng/ml).

Likewise, the NEC of HC secreted statistically significant higher amounts of LL-37 (with stimulation mean 39.5, SD 22.9, max. 52.2, min. 0, without stimulation mean 0.7, SD 0.9, max. 2, min. 0ng/ml, $p=0.019$) and hBD-3 (with stimulation mean 100.7, SD 4.6, max. 107.4, min. 95ng/ml; without stimulation mean 1.4, SD 0.2, max. 1.6, min. 1.2ng/ml, $p=0.001$) after stimulation. Remarkably, in WG the increase in hBD-3 response was significantly smaller than those in HC ($p=0.032$) and also the dynamic range (hBD-3 stimulated minus hBD-3 unstimulated) was significantly more restricted in WG ($p=0.016$, Fig. 4).

**Discussion**

**AMP in nasal secretion and cellular sources**

In living organisms, over two billion years ago, mechanisms of the innate immunity evolved to defend themselves against invading microorganisms. AMPs are detected on the barriers of vertebrates such as the skin, the urogenital tract, the intestine and the respiratory tract (17).

In this study, the inducible AMP LL-37 and hBD-3 were demonstrated in nasal secretions in HC and WG patients and quantified by ELISA. For healthy volunteers, results are in line with previously reports of Lysenko et al. (37) who reported that LL-37 was present in nasopharyngeal surface fluids and nasal secretions. However, the concentration appears to vary widely from specimen to specimen, and Tjabringa et al. (38) detected very low concentrations of LL-37 in nasal secretion by Western-blot.

HBD-3, one of the four known human beta defensins, can be predominantly detected in the trachea, tonsil, tongue and skin (21). The expression of hBD-3 mRNA was found to be below detection level by RT-PCR in nasal mucosa in patients with chronic sinusitis, nasal polyps, and in HC (39). The study presented here is the first investigation to demonstrate hBD-3 in nasal secretion by Western-blot.

Cellular sources of AMP in nasal secretion might be cells of the mucosa (epithelium, glands, connective tissue), or infiltrating cells like granulocytes (38). By immunohistochemistry, LL-37 was detected in epithelial cells, submucosal glands and cells of the connective tissue as previously reported in HC and chronic rhinosinusitis patients (25, 26, 30). At this time, there are no reports about the cellular sources for local se-
cretion of hBD-3 of the nasal mucosa. In this study, epithelial cells and submucosal glands could be demonstrated by immunohistochemistry as sources of AMP secretion.

Taken together, LL-37 and hBD-3 are detectable in nasal secretions of patients with WG, and in HC, and local tissue seems to be at least partly the source of these AMP.

**AMP dependence on S. aureus**

Bacterial overgrowth in barrier organs, e.g. *S. aureus* skin infection in atopic dermatitis patients, is partly explained by reduced AMP concentrations secondary to disturbed cytokine pattern (23). Similarly, bacterial infections associated with cystic fibrosis are facilitated by reduced AMP activity (40). In WG, higher nasal carrier rates of *S. aureus* are known and associated with relapse (9, 15).

LL-37 is inducible by yeasts and Gram-positive and -negative bacterial components such as lipopolysaccharide and lipoteichoic acid (22, 27-29). Vitamin D, and hypoxia response pathways (HIF1α) are involved in these not fully understood mechanisms controlling LL-37 production (22). In line with these reports, the data of the present study suggest higher levels of LL-37 expression in nasal secretions of patients colonised with *S. aureus*. Strikingly, such an effect was not detectable for hBD-3, even though hBD-3 is also inducible via bacterial infection, cytokines and calcium (23). One reason for this finding might be that different *S. aureus* strains elicit different mRNA expression levels of hBD-3 in epithelial cells as demonstrated by Quinn et al. for *S. aureus* strains of carriers and non-carriers (41).

Under optimal conditions, the antimicrobial activity of AMP is found at concentrations as low as 1–10µg/ml (17, 42). Microorganisms play an active part in preventing antimicrobial effects of AMP. They sense AMP and developed various options to overcome threatening host reactions such as efflux pumps, secreted proteases (aureolysin, V8 proteases), alterations of bacterial cell surface charge (teichoic acid alanylation, phospholipid lysinylation), down regulation of peptide expression or even the inhibition of NFκB-dependent AMP transcription in the host cells (22, 40, 43, 44).

The concentration for LL-37 and hBD-3 detected in nasal secretions was 0.07 to 0.7µg/ml and therefore surprisingly higher than levels detected in the washing fluids of healthy skin (45). Furthermore, concentrations of AMP on mucosal surfaces which are responsible for antimicrobial effects are not directly measurable, and concentration gradients from the side of requested effects like mucosal surface and intercellular space to nasal secretion lead to underestimation of AMP concentrations (38). Furthermore, no studies have been done on nasal secretion concerning synergistic and additive AMP effects which possibly lead to antimicrobial effects even in lower concentrations. (40, 46).

To investigate the contribution of epithelial cells to the AMP response towards *S. aureus* stimulation, the *in vitro*
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primary nasal epithelial cell culture system described in this paper was established. Our results confirm that epithelial cells are one of the main sources for LL-37 and hBD-3 in the nasal mucosa. AMP response of WG patients to the S. aureus threat was significantly altered. Intriguingly, the NEC of WG patients displayed a reduced hBD-3 response to S. aureus compared to healthy controls. In contrast, LL-37 responses of NEC to S. aureus were statistically significantly elicited in WG patients and HC without detectable differences. Thus, the antimicrobial response of NEC in WG is not generally impaired, but differs for individual AMP. A diminished expression of hBD-3 could favour S. aureus colonisation in WG patients as it is described for skin diseases (47). Moreover, this finding could have potential therapeutic implications for relapse prevention in WG by local AMP application (47).

**Conclusions**

By means of ELISA, LL-37 and hBD-3 could be demonstrated in nasal secretions in HC and WG. Nasal S. aureus colonisation leads to significantly higher concentrations of LL-37 in nasal secretion. This could not be demonstrated for hBD-3. Local cellular AMP sources are cells of the nasal barrier (epithelial cells, submucosal glands) and, for LL-37, also cells in the connective tissue. The epithelial cells secreted significantly higher amounts of hBD-3 and LL-37 after stimulation with S. aureus, but the dynamic is disturbed in WG at least for hBD-3, possibly leading to higher S. aureus colonisation rates with known clinical impact.

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