Effect of Nasal Antifungal Therapy on Nasal Cell Activation Markers in Chronic Rhinosinusitis

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Objective: To examine the effect of nasal antifungal treatment on eosinophil cationic protein (ECP) and tryptase levels in samples of nasal lavage fluid from patients with chronic rhinosinusitis and nasal polyps.

Design: Prospective double-blind placebo-controlled clinical trial.

Setting: Tertiary surgical center.

Patients: Subjects with severe chronic rhinosinusitis and nasal polyps. Of 120 screened patients, 76 were eligible. Six patients withdrew because of minor adverse events, and 10 dropped out for other reasons. In total, 60 patients completed the study according to the study protocol.

Interventions: Nasal treatment with amphotericin B or saline control for 8 weeks.

Main Outcome Measures: Nasal lavages were performed before and after treatment. Fungal elements were assessed by culture and with different polymerase chain reaction assays. Levels of ECP and tryptase were determined by fluorescent enzyme immunoassay.

Results: No correlation between cell activation markers and fungus detection was observed before treatment (all P > 0.20). Nasal amphotericin B treatment had no effect on levels of ECP (P = 0.17) or tryptase (P = 0.09) in nasal lavage samples. Moreover, successful fungus eradication, defined as fungus detection before but not after treatment, did not influence nasal ECP or tryptase levels (all P > 0.40).

Conclusion: Neither topical amphotericin B therapy nor fungal state before and after treatment had any significant influence on activation markers of nasal inflammatory cells in chronic rhinosinusitis.
The active drug contained amphotericin B (3 mg/mL), and the control contained 0.02% tartrazine (wt/vol) (Synopharm GmbH, Barsbüttel, Germany), 0.013% chinin sulfate (wt/vol) (Caesar & Loretz GmbH, Bonn, Germany), 0.02% 1-(4-sulfoo-1-phenylazo)-2-naphthol-6-sulfo acid (wt/vol) (Synopharm GmbH), and 1.0% (3-sn phosphatidyl)choline (vol/vol) (Lipostabil N; Nattermann & Cie, Cologne, Germany) in 5% glucose solution. Both sprays were indistinguishable regarding taste, color, and sensation. The study medication (200 µL per nostril) was administered 4 times daily. Exclusion criteria included the recent initiation of antifungal therapy (<3 weeks before study entry), corticosteroid therapy (topic or systemic), antihistamine therapy, specific immunotherapy, and acetysalicylic acid desensitization.

STUDY DESIGN

Following an initial nasal lavage, the patients were randomly allocated either to the amphotericin B arm or to the control arm (Figure 1). Both patients and physicians were unaware of their treatment allocation. After 8 weeks, a second nasal lavage was performed. Nasal lavage samples were examined for fungal elements and cell activation markers (ECP and tryptase).

DETECTION OF ECP AND TRYPTASE IN NASAL LVAGE

Nasal lavages were performed after mucosal decongestion with 0.1% xylometazoline using 5 mL of sterile isotonic saline per nostril and kept at 37°C for 2 hours to allow the cells to degranulate. The nasal lavage samples were then centrifuged at 1000g for 10 minutes at room temperature. The supernatant was examined for ECP and tryptase using a fluorescent enzyme immunoassay (UniCAP 100; Pharmacia, Freiburg im Breisgau, Germany) according to the manufacturer's recommendations.

FUNGAL CULTURE

Microbiological examinations were performed as described previously. In brief, lavage fluids were treated with dithiothreitol (0.3 mg/mL) to dissolve viscous mucus. Then, 2 Sabouraud glucose (4%) agar plates (Becton-Dickinson, Heidelberg, Germany) containing chloramphenicol (0.4 g/L) and gentamicin sulfate (0.04 g/L) were each inoculated with 0.5 mL of the samples. The plates were incubated for 30 days at 37°C and 30°C, respectively. The remainder of the fluid was centrifuged, and 0.5 mL of the resuspended sediment was cultured on Sabouraud agar and in Sabouraud bouillon, respectively, for 30 days at 30°C. One droplet was stained with calcofluor white and examined by fluorescent microscopy for the presence of fungal elements. All fungal isolates were identified morphologically and biochemically by standard methods.

PANFUNGAL AND ASPERGILLUS-SPECIFIC PCR ASSAY

Standard precautions to prevent polymerase chain reaction (PCR) assay contamination were followed. After DNA extraction and purification, PCR assays were performed. Briefly, for panfungal PCR, a 194-base pair segment of the 18S ribosomal RNA gene of a wide variety of fungal species was amplified. For Aspergillus-specific nested PCR, a 236-base pair segment of the 18S ribosomal RNA that is specific for Aspergillus species was detected.

The PCR runs were performed in a thermocycler (Tersonal; Biometra, Goettingen, Germany) as described previously.3 For the Aspergillus-specific nested PCR, 5 µL of the amplified DNA of panfungal PCR was hybridized with the biotinylated oligonucleotide probes V2AS (10 ng/mL, specific for Aspergillus and Penicillium) and V2CA (50 ng/mL, specific for Candida) at 48°C.

Hybridization products were detected with a DNA enzyme immunoassay (Gen-Eti-K DEIA; BykDiaSorin, Düsseldorf, Germany) according to the instructions of the manufacturer. The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and detected under UV light. Sensitivity of the panfungal PCR assay had been determined to 1 pg Aspergillus fumigatus and 1 pg Candida albicans DNA or 40 A fumigatus conidia per reaction after hybridization with the corresponding probes. The sensitivity level of the Aspergillus-specific nested PCR assay was 100 fg A fumigatus DNA or 10 A fumigatus conidia per reaction.3

STATISTICAL EVALUATION

Cell activation markers before treatment in fungus-positive and fungus-negative patients with CRS were compared with the Mann-Whitney U test. Cell mediator levels before and after treatment were compared with the Wilcoxon signed rank test. The differences in scores after and before treatment in the 2 treatment arms (amphotericin B vs control, fungus persistent vs fungus eliminated) were compared with the Mann-Whitney U test. The type I error was set to 0.05 (2-sided). All results are expressed as median ± interquartile range.

Figure 1. Consort diagram of the pretest-posttest double-blind control group study, with random allocation of patients to the 2 treatment arms. ECP indicates eosinophil cationic protein.

STUDY MEDICATION AND CONCOMITANT THERAPY

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Of 120 screened patients with CRS, 50 men and 26 women (mean age, 49 years; range, 25-77 years) were eligible according to the study protocol. Of these, 16 patients dropped out because of intolerance of the study medication (5 amphotericin B, 1 control), failure to show up for final examination (3 amphotericin B, 4 control), discontinuation of study medication owing to an acute exacerbation of CRS (1 amphotericin B), and lack of material for serologic examinations (2 amphotericin B). Finally, 60 patients were evaluable per protocol (Figure 1). Clinical characteristics and concomitant treatment are listed in the Table.

FUNGUS DETECTION AND CELL ACTIVATION MARKERS BEFORE TREATMENT INITIATION

In the nasal lavage samples from 45 of 76 patients with CRS, fungal elements were detected by at least 1 of the 3 detection methods used (calcofluor, culture, or PCR). These patients were classified as fungus positive. The nasal lavage samples from 16 patients showed fungal growth in culture. In 3 specimens, more than 10 colony-forming units were recorded. The most commonly detected fungi were *Aspergillus* species followed by *Penicillium* and *Alternaria* species. The detection of fungal elements before and after treatment allowed us to identify a subgroup of patients with detectable fungal elements before, but not after, treatment (fungus eliminated, n=17 [8 control, 9 amphotericin B]). This subgroup was compared with the patients in whom fungal elements were detectable before and after treatment (persistent fungus, n=20 [14 control, 6 amphotericin B]).

The ECP levels in nasal secretions decreased in both the amphotericin B group and the control group. In the amphotericin B group, the median ECP concentration in nasal lavage was 13.9 μg/L (range, 2.9-70.3 μg/L) before treatment and 8.0 μg/L (range, 2.1-102.0 μg/L; P=.45) after treatment. In the control group, the median ECP level was 12.7 μg/L (range, 2.5-54.5 μg/L) before treatment and 5.8 μg/L (range, 1.5-53.2 μg/L; P=.04) after treatment. The tryptase level was 0.3 μg/L (range, 0.1-1.1 μg/L) before treatment and 0.8 μg/L (range, 0.6-1.5 μg/L; P=.03) after treatment. In the control group, the tryptase level was 0.7 μg/L (range, 0.1-1.3 μg/L) before treatment and 0.6 μg/L (range, 0.5-0.9 μg/L; P=.65) after treatment.

To compare the effects of treatment, the differences in ECP levels after and before treatment were calculated for the 2 treatment arms. The median difference in the ECP level was −0.08 μg/L (range, −7.9 to 1.0 μg/L) in the amphotericin B group and −3.0 μg/L (range, −43.9 to 0.1 μg/L) in the control group (P=.17) (Figure 3). The median difference in the tryptase level was 0.5 μg/L (range, −0.1 to 1.0 μg/L) in the amphotericin B group and 0.2 μg/L (range, −0.5 μg/L to 0.5 μg/L; P=.09) in the control group.

FUNGAL ELIMINATION AND CELL ACTIVATION MARKERS

The detection of fungal elements before and after treatment allowed us to identify a subgroup of patients with chronic rhinosinusitis with and without fungus in nasal lavage samples (median line in box; interquartile range, box; and highest and lowest values, whiskers).
Fungi are well known pathogenic factors in certain types of CRS, such as allergic fungal rhinosinusitis or invasive fungal rhinosinusitis. Irrespective of the type of CRS, fungal elements may be isolated from the nasal cavity and paranasal sinuses with varying frequencies.\textsuperscript{2,3} Since airborne fungal spores are regularly inhaled and deposited within the nasal cavity, it is unclear whether fungal elements detected within the nose are innocent bystanders or represent a frequent cause of CRS in general.\textsuperscript{12} In allergic and nonallergic CRS, eosinophils and mast cells are regularly detected. These cells may be involved in the immune response to fungi as effector cells.\textsuperscript{1,4} Their secretory products—ECP and tryptase—are valuable indicators of their inflammatory activation and have been used to monitor therapy of CRS.\textsuperscript{6,7}

In this study, we aimed to examine the effect of fungal elements on the inflammatory activation of characteristic effector cells in patients with CRS. Therefore, we compared the ECP and tryptase levels in nasal lavage fluid samples from patients with severe CRS with and without detectable fungal elements. Moreover, we studied the effect of nasal antifungal treatment and the effect of successful elimination of initially detectable fungus on these inflammatory cell activation markers.

At study entry, the presence or absence of fungal elements had no relevant influence on cell activation markers in patients with CRS and nasal polyps. Moreover, neither nasal amphotericin B treatment nor the successful elimination of fungal elements had any significant influence on the inflammatory cell activation markers assessed in the samples of nasal lavage fluid. It appears to us that these results favor an innocent bystander role for fungal elements in CRS with nasal polyps.

Some remarks on the study design may aid in the interpretation of the results. For this study, patients with CRS and extensive nasal polyps were recruited to allow recognition of medication-related improvement. Moreover, patients with known contributing factors for CRS, such as evident fungal disease, were excluded. Thus, the conclusions may be invalid for patients with less severe CRS, for patients with conditions that predispose them to CRS, and for patients with sinusitis of well-established fungal origin.\textsuperscript{13} Nasal lavage has been regarded as the optimal diagnostic procedure to obtain fungus-containing mucus, because it covers a large mucosal surface and reaches relevant (maxillary and ethmoid) sinuses.\textsuperscript{14} Lavage specimens were pretreated with dithiothreitol to further enhance fungal detection.\textsuperscript{12} However, investigating mucus collected from within the sinuses might have resulted in a higher yield.\textsuperscript{15}

Two highly sensitive techniques were applied for fungus detection: standard culture methods and fungus-specific PCR technique. The results with sterile laboratory controls that paralleled all preparative steps remained negative. Therefore, we regard the applied detection system as highly sensitive and specific.

We also carefully considered which drug and application mode should be used. Amphotericin B is effective

\textbf{COMMENT}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Difference in nasal lavage eosinophil cationic protein (ECP) concentrations after and before treatment with nasal amphotericin B spray or control (line in box; median; box, interquartile range; and whiskers, highest and lowest values, except outside values [asterisks] and far-outside values [circle]).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Difference in eosinophil cationic protein (ECP) concentrations in nasal lavage samples from patients with positive fungus detection before, but not after, treatment and positive fungus detection before and after treatment (median, line in box; interquartile range, box; and whiskers, highest and lowest values, except outside values [asterisks]).}
\end{figure}
against a broad spectrum of common nasal fungal organisms, such as Aspergillus and Penicillium species. The applied amphotericin B concentration of 3 mg/mL is well above the minimum inhibitory concentrations for common nasal fungi, and resistance to this drug is rare.18 Amphotericin B was used as a nasal spray for several reasons. First, various studies using nasal corticosteroids have shown that nasal sprays may effectively alter the course of CRS and decrease the levels of cell activation markers in nasal secretions.6,17 Second, nasal lavage is an active treatment method of CRS itself; therefore, its application might interfere with the effects of the antifungal agent.18 Finally, it is much more convenient to apply nasal spray than to perform nasal lavage, and patients’ compliance was considered more reliable and easier to audit with nasal sprays. Although nasal sprays distribute well within the nasal cavity, their penetration into the paranasal sinuses of healthy persons or in patients after sinus surgery is inferior to nasal lavage.19 Also, because our patients had advanced polyps, it was assumed that their sinus ostia were blocked and that fluid would not be able to enter the sinus lumen. Thus, the advantages of nasal spray application appeared to outweigh the possible benefits of nasal saline irrigation. Moreover, the efficacy of nasal spray treatment to eliminate fungal elements has been proved by a posttest microbiological examination. An application period of 8 weeks was chosen for 3 reasons: (1) there were ethical considerations because all study participants had severe CRS, requesting urgent surgical treatment to relieve their symptoms; (2) topical anti-inflammatory treatment has been shown to reduce cell activation markers in CRS as soon as 4 to 6 weeks after initiation17; and (3) because of the expected topical intolerance of study medication, the shortest possible period of time had to be chosen to reduce the dropout rate. Therefore, a study period of 8 weeks seemed to be appropriate.

Demographic and clinical characteristics, other than sex, were similarly distributed in both groups (amphotericin B and control). To our knowledge, there has been no reported influence of sex on the effectiveness of sinusitis therapy or antifungal treatment. Thus, the uneven distribution should not bias the treatment results.

In a recent controlled study, Ponikau et al20 found a significant decrease of eosinophil-derived neurotoxin in nasal lavage samples from patients with CRS who were treated with nasal amphotericin B. This positive effect may be explained by the longer application of nasal antifungal agents (6 months in their study vs 8 weeks in our study), the nonexclusion of patients with allergic fungal rhinosinusitis, and the additional use of nasal irrigation in patients after sinus surgery. In the patients with enlarged sinus ostia, the lavage fluid might actually have entered the sinus lumen. Although the sample size in this pilot trial was comparatively small, it may help to identify a subgroup of patients with CRS who could benefit from nasal antifungal treatment.

In conclusion, in our study, neither 8 weeks of topical treatment with amphotericin B nor the fungal state before and after treatment had any significant effect on nasal cell activation markers in CRS with nasal polyps.

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