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The Effect of Topical Amphotericin B on Inflammatory Markers in Patients With Chronic Rhinosinusitis: A Multicenter Randomized Controlled Study

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Background: It has been suggested that an exaggerated immune response to fungi is crucial in the pathogenesis of chronic rhinosinusitis (CRS). Based on this rationale, the use of topical antifungals (amphotericin B) has been advocated. Studies on its clinical effectiveness are, however, contradictory.

Objectives: To examine the effect of nasal antifungal treatment on secreted mediators in samples of nasal lavage fluid from patients with CRS with or without nasal polyps (NP).

Methods: Part two of a prospective double-blind, placebo-controlled multicenter clinical trial investigating the effect of 13 weeks of treatment with amphotericin B or placebo on the levels of pro-inflammatory cytokines, chemokines and growth factors (i.e., IL-1β, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin, RANTES, MCP-1, MCP-2, MCP-3, VEGF, EGF, FGF-basic, HGF, Gro-α) and albumin via a fluorescent enzyme immunoassay in nasal lavage specimens of CRS patients with or without NP.

Results: Topical amphotericin B had no significant effect on the level of any of the tested pro-inflammatory cytokines, chemokines, and growth factors in CRS nasal lavage samples. Treatment with placebo, however, increased the level of MIP-1α and MIP-1β, which are mediators involved in wound healing.

Conclusions: Topical amphotericin B has no significant effect on activation markers of nasal inflammatory cells in chronic rhinosinusitis with or without nasal polyps.

Key Words: Randomized controlled trial, prospective study, double-blind method, human, amphotericin B, intranasal administration, rhinosinusitis, nasal polyps, chronic disease, fungi, cytokines, chemokines, intracellular signalling peptides and proteins, nasal lavage fluid, chemokine CCL3, chemokine CCL4.

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INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by distinctive symptoms (e.g., nasal blockage, nasal discharge, facial pain, and/or reduced sense of smell) and either endoscopic signs or computed tomography (CT) changes characteristic of the disease.[1] The etiology of CRS with or without nasal polyps (NP) is debated, and its pathophysiology remains controversial. Recently, a fungal etiology has been proposed.[2] Various studies have shown that under optimal conditions, fungi can be identified within the nose and paranasal sinuses of nearly every individual.[3,4] While the presence of these

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TABLE I.
Baseline Demographic and Clinical Characteristics.

	Placebo (n = 19)	Amphotericin B (n = 20)	*P* value
Age (years), mean (SD)	41.7 (15.3)	46.8 (8.11)	.21
Male gender, n (%)	11 (58%)	16 (80%)	.14
ASA intolerance, n (%)	4 (21.1%)	7 (35.0%)	.33
Asthma, n (%)	11 (58%)	9 (45%)	.42
Allergy (general), n (%)	12 (63%)	8 (40%)	.30
Fungal allergy, n (%)	1 (7%)	3 (16%)	.61
Smoking habits, n (%)			
Current smoker	3 (15.8%)	3 (15.0%)	.95
Ex-smoker	9 (47.4%)	9 (45.0%)	.88
Nonsmoker	7 (36.8%)	8 (40.0%)	.84
Mean CT score, mean (SD)	18.1 (3.40)	18.1 (4.25)	1.00
Presence of nasal polyps, n (%)	17 (89.5%)	18 (90%)	1.00
Number of surgical interventions, median (IQ range)	3 (1.0–5.0)	2.50 (2.0–4.75)	.93
Use of local steroids, n (%)	12 (63%)	14 (72%)	.56

SD = standard deviation; ASA = acetyl salicylic acid; CT = computed tomography.

fungi may not cause the disease CRS in itself, it has been suggested that fungi may trigger a cascade of events, ultimately resulting in the accumulation and degranulation of eosinophils in susceptible individuals.[2] Based on this rationale, the use of topical antifungals (amphotericin B) has been advocated.[5] Studies on its clinical effectiveness are, however, contradictory.[5–9]

Cytokines, chemokines, and growth factors are potent biologic factors involved in the regulation of inflammation, immune defense, and wound healing. Although we have not yet achieved a full understanding of the precise mechanisms underlying the pathogenesis of CRS, a variety of these mediators is suggested to be involved.[1] Interleukin (IL)-5, eotaxin, and transforming growth factor-beta (TGF-β) seem to be crucial players in the regulation of eosinophilic inflammation and extracellular matrix breakdown and/or deposition in CRS patients with concurrent NP. In addition, a variety of other inflammatory mediators including IL-1, IL-3, IL-4, IL-6, IL-8, IL-13, tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), regulated upon activation normal T-cell expressed and secreted (RANTES), and growth-related oncogene-α (Gro-α) may be increased in CRS tissue specimens with or without NP.[1,10] As is the case for at least some of these mediators, cytokine levels have been shown to correlate with clinical signs and symptoms of CRS, and effective anti-inflammatory treatment (a.o. macrolides) has been shown to significantly reduce cytokine levels in some individuals.[11] In this study, we aimed to examine the effect of topical amphotericin B on various pro-inflammatory mediators. Using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA), we assessed nasal lavage specimens of 39 CRS patients for a variety of inflammatory markers before and following 13 weeks of nasal lavage with topical amphotericin B. This study was performed as part of a double-blind randomized controlled multicenter clinical study in which we observed no effect on clinical outcome of topical amphotericin B treatment in patients suffering from CRS with or without NP.[9]

MATERIALS AND METHODS

Participants

This study was based on a double-blind, placebo-controlled, multicenter trial assessing the effectiveness of intranasal amphotericin B (100 mg/mL) when used for 3 months in adult patients with CRS with or without NP.[9] It included patients presenting to the Otorhinolaryngology Department of the Academic Medical Center (Amsterdam, The Netherlands), Erasmus Medical Center (Rotterdam, The Netherlands), Royal National Hospital (London, United Kingdom), University Hospital Ghent (Ghent, Belgium), University Hospital Leuven (Leuven, Belgium), or Hospital Clinic de Barcelona (Barcelona, Spain) between February 2002 and December 2004. All adult patients with clinical symptoms of CRS, endoscopic signs of CRS with or without NP, and sinus CT scan score of at least 5 according to the Lund and Mackay scoring system[12] who had undergone previous endoscopic sinus surgery were eligible to enroll. To guarantee adequate access to sinonasal mucosa on irrigation with intranasal amphotericin B and to improve the representativeness of the lavage material, previous endoscopic sinus surgery (ESS) was obligatory for inclusion. Exclusion criteria included immunodeficiency (AIDS, chronic systemic steroid use, immunosuppressive treatment, immunoglobulin deficiency, complement deficiency), inability to provide consent or concerns regarding compliance, actual or suspected pregnancy, use of oral antifungals, use of topical decongestants or antihistamines, Mycobacterium infection, osteoporosis, and chronic renal or liver failure.

Intervention

In this study, patients applied 25 mL of a 100 mg/mL amphotericin B solution or placebo to each nostril twice daily using an Emcur (also named Rhinicur) nasal douching device (Emcur GmbH, Bad Ems, Germany). Amphotericin B is active against most moulds frequently identified within the paranasal sinuses while fractions involuntarily ingested are not

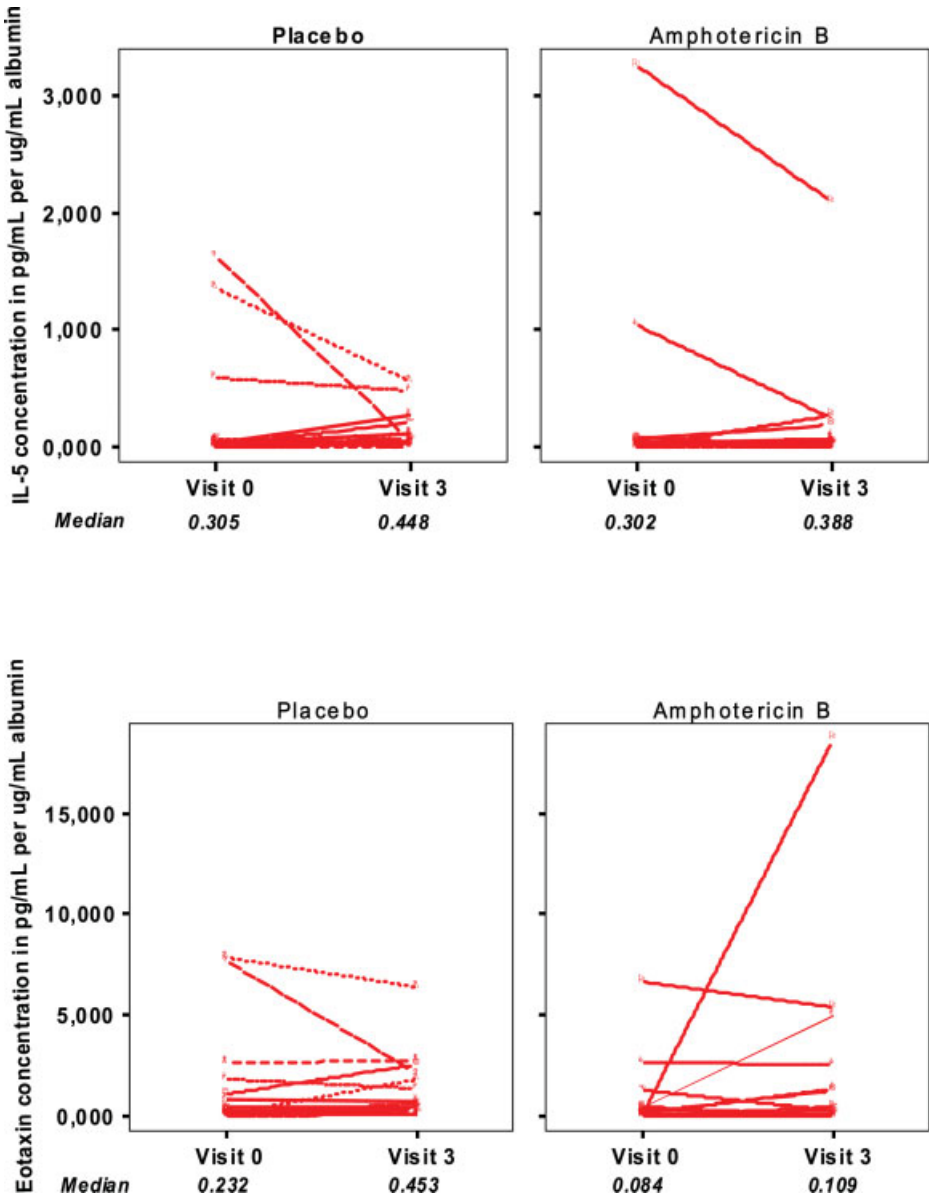

Fig. 1. Change in albumin-adjusted IL-5, IL-8, eotaxin, and GRO-a concentrations between both groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

systemically absorbed. The applied concentration (100 mg/mL) is approximately 30 to 100 times higher than the minimum inhibitory concentration for all relevant fungi.[13] The study protocol was approved by the medical ethical committee of each participating center, and all participating patients read and signed an informed consent form before enrollment.

Study Design

Randomized allocation to one of two treatment groups took place on visit 0. Before randomization, each patient was required to participate in a 2-week run-in period on saline in order to get acquainted with the Emcur nasal douching device. Follow-up visits were scheduled at 2, 6, and 13 weeks after randomization. On randomization, the hospital pharmacist provided each participating patient with trial medication. Amphotericin B nasal lavage solution was prepared by dissolving amphotericin B for injection (Bristol-Myers-Squibb, New York, NY), resulting in a clear yellow solution. Placebo nasal lavage solution was prepared by dissolving 3.4 mL/L Cernevit (Baxter, Deerfield, IL) in sterile water containing 2.5% (w/v) glucose, resulting in a solution identical in color and smell to the amphotericin B solution. At visit 0 and visit 3 (13 weeks of treatment), mucus samples were collected from all patients by flushing each nostril with 20 mL of sterile saline using a sterile syringe with a blunt, curved needle. At the start of this procedure, patients were asked to take a deep inspiratory breath and hold it until one of the investigators injected sterile saline into one of the nostrils. Upon injection, patients were asked to forcefully exhale through the nose during flushing. The return was collected in a sterile pan and stored at −80°C until analysis.

Detection of Pro-inflammatory Mediators in Nasal Lavage Specimens

Upon analysis, a random sample of nasal lavages obtained from 39 of 116 patients (19 treated with placebo and 20 treated with amphotericin B) was defrosted to room temperature,

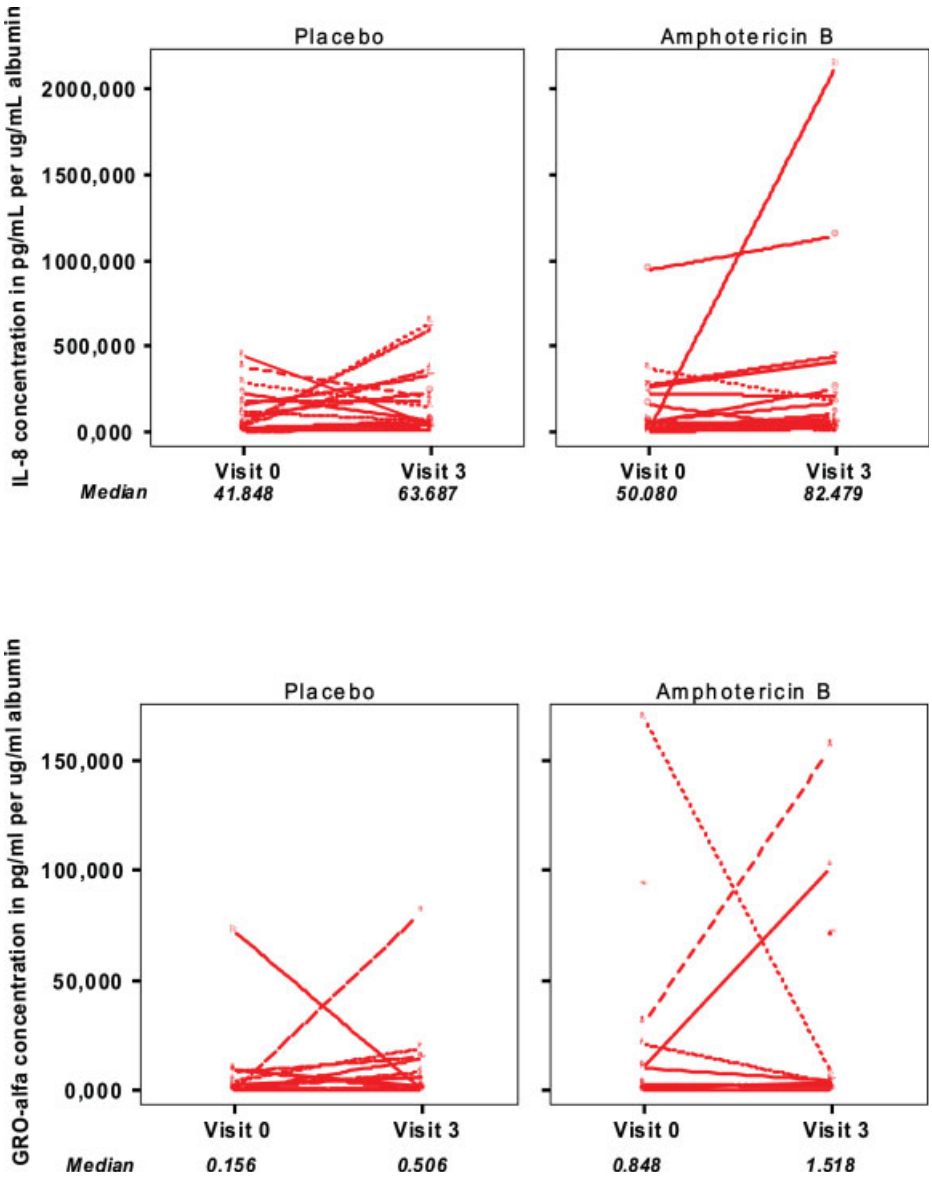

Fig. 1. (Continued).

vortexed, and centrifuged at 1,400 rpm. Supernatants were collected for subsequent analysis. All study personnel were blinded to the treatment allocation for the duration of the investigations. Randomization codes were revealed to the researchers once data collection was complete.

Determination of IL-5 and eotaxin concentrations was performed using a sandwich immunoassay containing 30 analytes (i.e., IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-2 receptor (IL-2R), IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, interferon α (IFN-α), IFN-γ, granulocyte colony-stimulating factor (G-CSF), GM-CSF, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, interferon-inducible protein of 10 kDa (IP-10), monokine induced by IFN-γ (MIG), eotaxin, RANTES, monocyte chemoattractant protein 1 (MCP-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF-basic), and hepatocyte growth factor (HGF; Biosource^{TM}, Invitrogen, Breda, The Netherlands). Gro-α concentrations were determined using a custom-made sandwich immunoassay containing three analytes (i.e., MCP-2, MCP-3, and GRO-α; Biosource^{TM}, Invitrogen). IL-3 and albumin concentrations were determined by performing separate sandwich immunoassays (Biosource^{TM}, Invitrogen, and Bethyl Laboratories Inc., Montgomery, TX, respectively). All assays were performed as described by the manufacturer (Biosource^{TM}, Invitrogen [30-plex, 3-plex, and IL-3 assay] and Bethyl Laboratories [albumin assay]). Plates were analyzed using a Luminex 100^{TM} instrument (Luminex BV, Oosterhout, The Netherlands; 30-plex, 3-plex, and IL-3 assay) or using a Versamax microtiter plate reader (Versamax, Molecular Devices Ltd., Sunnyvale, CA; albumin) at a wavelength of 450 nm.

Statistical Analysis

Statistical analysis was carried out using SPSS 14.0 (Chicago, IL). All variables were tested for normality both graphically and by using the Kolmogorov-Smirnov test. Cytokine, chemokine, and growth-factor values reported as below threshold were recoded to zero. Adjusted values were computed by dividing the observed mediator concentration by the corresponding albumin concentration of the same lavage specimen.

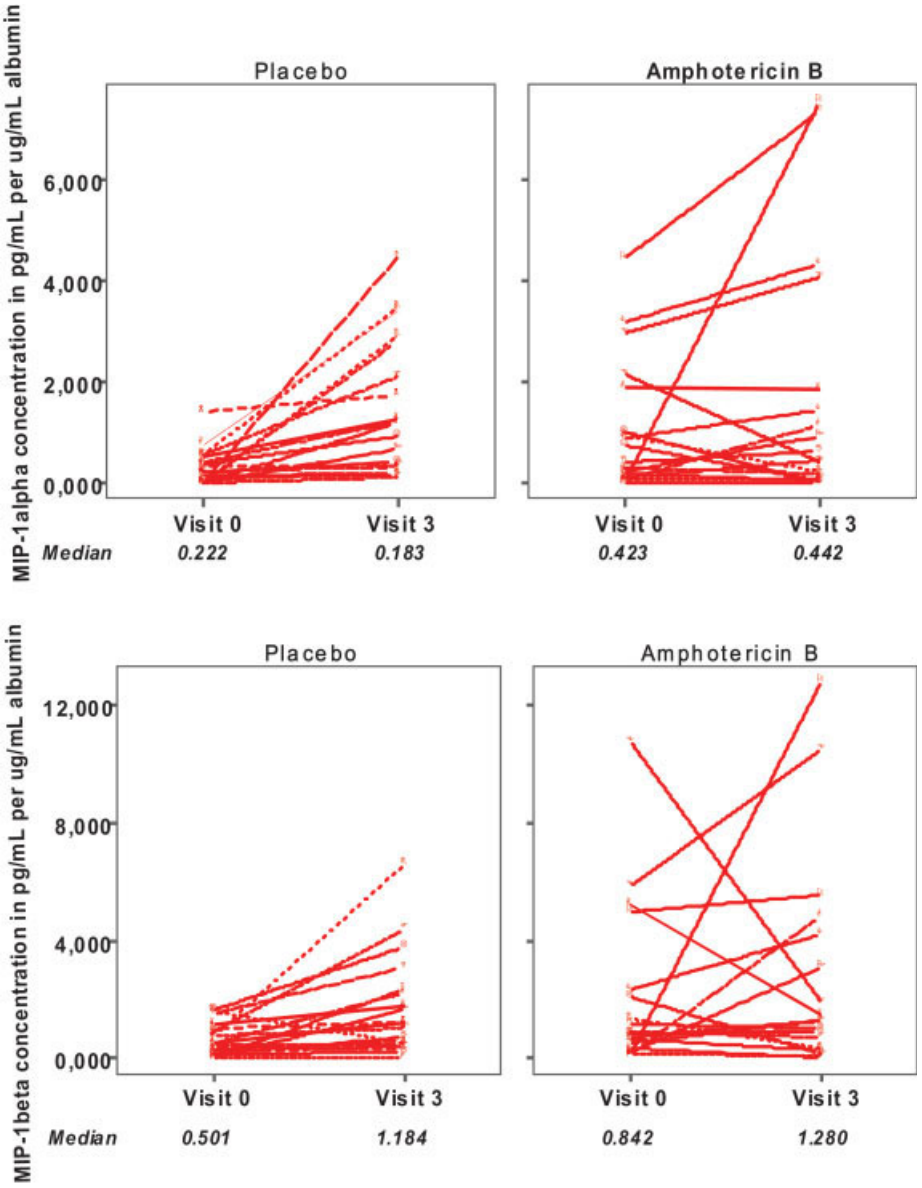

Fig. 2. Change in albumin-adjusted MIP-1α and MIP-1β concentrations between both groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Both unadjusted and adjusted values were analyzed. Change values (3 months minus baseline) were computed to evaluate treatment effect. Demographic and clinical characteristics in both groups at baseline were compared using χ^2 and Fisher exact tests (proportions) and two-sided t tests and Mann-Whitney U tests (continuous variables) as required. Changes in mediator concentrations *within* each group were assessed using Wilcoxon signed-rank tests. Differences in mediator changes *between* both groups were assessed using Mann-Whitney U tests. Using the Bonferroni adjustment for multiple comparisons, the level of statistical significance was set to .0015 (two-sided).

RESULTS

Participants

Of the 116 patients included in this double-blind, placebo-controlled multicenter trial, 99 patients completed the study per protocol. From these 99 patients who completed the trial, paired nasal lavage samples of 39 patients (20 from the amphotericin B-treated group and 19 from the placebo-treated group) were randomly selected. No significant differences in demographic and clinical characteristics were observed at baseline between both groups (Table I).

Albumin and Cytokine Concentrations

Baseline. The median albumin concentration in nasal lavage fluid at baseline was 109.6 mg/mL in the amphotericin B group and 96.8 mg/mL in the placebo group ($P = .29$). No significant differences were observed for all cytokine, chemokine, and growth-factor concentrations, either before or after adjustment for the observed albumin concentration, between both groups. Although the albumin-adjusted G-GSF concentration was slightly

higher in the placebo group ($P = .03$), this difference was statistically not significant after Bonferroni adjustment for multiple comparisons.

Three Months of Treatment. Albumin concentrations after 3 months of treatment were essentially unchanged (median 88.8 mg/mL in the amphotericin B-treated group and 36.3 mg/ml in the placebo-treated group; $P = .76$ and $P = .1$, respectively). The levels of most mediators at baseline correlated closely with levels at 3 months from baseline. Although the adjusted concentration of 26 of 34 mediators increased in the amphotericin group, none of these changes were statistically significant. More importantly, in the placebo group the adjusted concentration of most tested mediators (20 of 34) increased as well (Fig. 1). Although this increase in the placebo group was more pronounced for IL-15 and HGF ($P = .025$ and $P = .015$, respectively), it was not significant after Bonferonni adjustment for multiple comparisons. The observed increase in MIP-1α and MIP-1β in the placebo group, however, was statistically significant ($P < .0001$ and $P = .001$, respectively) (Fig. 2). When comparing change values for the adjusted mediator levels between both groups, no significant differences were observed (Table II). Although repeat analysis for most unadjusted cytokine concentrations revealed similar results as described above for the adjusted cytokine concentrations (Table III), now we no longer observed a significant change from baseline in the concentration of both MIP-1α and MIP-1β within each group.

TABLE III.
Median Change from Baseline in Raw Mediator Concentrations.

Concentration in pg/mL per μg/mL albumin	Placebo (n = 19)	Amphotericin B (n = 20)	*P* value
IL-1b	0	0	.28
IL-3	75.34	−63.04	.27
IL-4	0	0	.89
IL-5	0	−0.48	.73
IL-6	−3.40	−0.27	.46
IL-8	358.58	3870.19	.18
IL-13	0	0	.96
TNF-α	0.12	1.33	.46
Eotaxin	6.72	1.90	.82
IFN-γ	1.50	1.68	.33
MIP-1α	8.06	−12.50	.19
MIP-1β	9.64	−7.01	.46
GM-CSF	0	0	.11
GRO-α	0	0	.40
RANTES	0	0	.91

IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; MIP = macrophage inflammatory protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; GRO = growth-related oncogene; RANTES = regulated upon activation normal T-cell expressed and secreted.

DISCUSSION

If the inflammation observed in CRS patients is the result of an immune reaction to fungi, reducing the presence of this inflammatory trigger may improve the course of the disease.[2] Ideally, treatment should eliminate the fungus without causing harm to the host. Topical treatment, thus, seems most attractive. Although the injectable formulation of amphotericin B carries U.S. Food and Drug Administration–approved labelling solely for intravenous administration, several alternative routes of administration that use the injectable formulation have been reported, including the administration of amphotericin B into the pleural cavity[14] and bladder.[15] Recently, amphotericin B nasal lavages have been advocated in the treatment of CRS. Studies on its clinical effectiveness, however, have yielded conflicting results.[5–9]

In recent years, several hypotheses have been put forward regarding topical amphotericin's mechanism of action. Besides possessing an antifungal effect, it has been suggested that amphotericin B may reduce inflammation via a direct cytotoxic effect on nasal polyp epithelial cells[16,17] or that it may have anti-inflammatory properties.[18,19] In this study, we aimed to examine the effect of amphotericin B nasal lavages on several pro-inflammatory mediators that are known to be involved in leukocyte migration to sites of inflammation and other stages in the inflammatory cascade.

We used nasal lavages to collect mucus, a technique that has been used frequently by others to collect and subsequently study the presence or absence of pro-inflammatory mediators. This method has been shown to have relatively low within-subject variability.[20] However, since unknown fractions of lavage fluid may be swallowed or absorbed, this technique can be associated with potentially unpredictable dilutions of nasal secretions. This could pose a problem when interpreting the observed cytokine, chemokine, and growth-factor

TABLE II.
Median Change from Baseline in Albumin-Adjusted Mediator Concentrations.

Concentration in pg/mL per μg/mL albumin	Placebo (n = 19)	Amphotericin B (n = 20)	*P* value
IL-1β	0	0	.41
IL-3	1.33	−0.64	.52
IL-4	0.008	0	.85
IL-5	0.01	0.004	.80
IL-6	0.033	0.186	.60
IL-8	10.53	36.29	.33
IL-13	0	0.01	.79
TNF-α	0.015	0.01	.71
Eotaxin	0.088	0.0059	.78
IFN-γ	0.023	0.0087	.56
MIP-1α	0.696	0	.06
MIP-1β	0.617	0.04	.17
GM-CSF	0	0	.11
GRO-α	0.09	0	.60
RANTES	0	−0.02	.97

IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; MIP = macrophage inflammatory protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; GRO = growth-related oncogene; RANTES = regulated upon activation normal T-cell expressed and secreted.

concentrations. It has been shown that the use of albumin as a marker of dilution improves the accuracy of quantifying endogenous substances in nasal secretions.[21] Although we did not observe major differences in outcome when comparing the unadjusted and adjusted mediator concentrations, in our opinion adjusted mediator concentrations provide a more accurate estimate of true mediator concentrations.

If effective, one would expect that any clinical effect of topical amphotericin B is associated with concurrent demonstrable effects on CRS mediators. However, in agreement with our clinical data,[9] we demonstrate that 13 weeks of treatment with topical amphotericin B does not result in a statistically significant reduction in the level of any of the tested cytokines, chemokines, or growth factors. Our results are in line with recent data by Shin et al., who showed that topical amphotericin B treatment does not result in a significant reduction in the level of IL-5, IL-8, IFN-γ, and RANTES (all pro-inflammatory cytokines),[18] and Weschta et al., who showed that topical amphotericin B treatment does not result in a significant reduction in eosinophilic cationic protein (ECP) and tryptase levels.[19] Thus, any direct or indirect anti-inflammatory effect of topical amphotericin B in patients with CRS appears highly unlikely. However, although Weschta et al. observed that neither topical amphotericin B treatment nor fungal state before and after treatment significantly influenced the level of any of the tested inflammatory activation markers,[19] we cannot exclude the possibility that treatment with topical amphotericin B may have an effect on the inflammatory markers in those patients who are fungus positive at inclusion. However, since fungi have been shown to be omnipresent,[3,4] it seems highly unlikely that only fungus-negative patients were included in our study.

Although no significant differences were observed when comparing both treatment groups, within the placebo group a clear increase in both MIP-1α and MIP-1β (adjusted concentrations) is observed. Although these results could be a chance occurrence, MIP-1α and MIP-1β belong to the CC family of chemokines and are crucial for T-cell chemotaxis to inflamed tissue. They both play an important role in the regulation of transendothelial migration of monocytes, dendritic cells, and NK cells. Thus, it is not surprising that MIP-1α and MIP-1β are key players in the pathogenesis of many inflammatory conditions including asthma, granuloma formation, arthritis, multiple sclerosis, pneumonia, and psoriasis.[22] Moreover, MIP-1α is also critical for macrophage chemotaxis to sites of cutaneous wound repair and may promote healing by inducing inflammatory responses against various pathogens such as viruses[23] and parasites.[24,25] Since most patients who received placebo performed better on all clinical outcome measures when compared with those who were treated with amphotericin B,[9] we suggest that the increase in MIP-α and MIP-1β may reflect enhanced tissue repair in those patients treated with placebo, a result that is in accordance with clinical data demonstrating a positive effect of saline douching in patients suffering from CRS.[26–28] However, further studies that could characterize the role of MIP-1α and MIP-1β in the pathogenesis of CRS are needed. In the meanwhile, our study adds to the body of evidence that suggests a limited (if any) role for topical amphotericin B in the treatment of patients suffering from CRS.

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