



Original Article

The inflammatory response of eosinophil-related fungal rhinosinusitis varies with inciting fungi

Pratibha Kale¹, Shivaprakash M. Rudramurthy², Naresh K. Panda³,
Ashim Das⁴ and Arunaloake Chakrabarti^{5,*}

¹Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, at present All India Institute of Medical Sciences, New Delhi, India, ²Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, ³Department of Otolaryngology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, ⁴Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India and ⁵Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

*To whom correspondence should be addressed. Prof. Arunaloake Chakrabarti, MD, Head, Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India. Tel: +91 172 2755155; Fax: +91 172 2744401; E-mail: arunaloake@hotmail.com

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Abstract

Earlier studies demonstrated immunological response to *Alternaria alternata* in patients with eosinophil-related fungal rhinosinusitis (FRS). However, *Aspergillus flavus* rather than *A. alternata* is predominantly isolated from such patients in Asia. We compared immunological response to *A. flavus* and *A. alternata* in our patients with eosinophil related FRS. Total immunoglobulin E, absolute eosinophil count (AEC), cytokine response, and *in vitro* eosinophil degranulation in the presence of *A. flavus*/*A. alternata* were compared among patients with eosinophil-related FRS, non-eosinophilic rhinosinusitis (NECRS), and healthy individuals. Eosinophil-related FRS patients were subgrouped into: Group A – presence of mucin with fungus in tissues and positive immediate hypersensitivity; group B – presence of mucin with fungus in biopsies and no immediate hypersensitivity; and group C – presence of mucin without fungi and hypersensitivity. *A. flavus* was the predominant (89%) isolate. Significantly higher major basic protein (MBP) was induced by *A. flavus* in Group A (279.15 ± 32.29 ng/ 2.5×10^5 cells) compared to Group B (254.9 ± 17.14 ng), Group C (238.33 ± 17.56 ng), NECRS (56.96 ± 10.97 ng), and normal subjects (28.73 ± 7.04 ng). *A. alternata* – eosinophil interaction failed to induce detectable MBP. AEC and serum cytokines, interleukin (IL)- 2, IL-4, IL-5, IL-10, tumor necrosis factor α , and interferon- γ were significantly higher ($P < 0.001$) in eosinophil-related FRS compared to NECRS and control. Thus a mixed Th1 and Th2 cytokine response was observed in eosinophil-related FRS. In conclusion, immune response in eosinophil-related

FRS depends on locally inciting fungi rather than *A. alternata* in all instances, and the categorization of this group appears to be arbitrary.

Key words: allergy, *Aspergillus flavus*, *Alternaria alternata*, cytokines, eosinophils, fungal rhinosinusitis.

Introduction

Chronic rhinosinusitis (CRS) is a common disorder characterized by hypertrophic nasal and paranasal mucosa containing lymphocytes, plasma cells with or without polyps, and/or eosinophils and presents with nasal obstruction, discharge, sometimes pain/tenderness, and facial and/or orbital swelling for more than 12 weeks [1]. Several putative factors like bacteria, viruses, anatomical obstruction, and fungi are associated with the development of CRS. However, major controversy persists surrounding the etiopathogenic role of fungi in this infection [2]. Noninvasive group of fungal rhinosinusitis (FRS) may be categorized into localized colonization, fungal ball, and eosinophil-related infection [3]. There are controversies regarding the classification of eosinophil-related FRS, in that one school of thought suggests that an allergy to fungi as the central mechanism of FRS and describes the condition as allergic fungal rhinosinusitis (AFRS). The latter is defined by the presence of characteristic CT findings, nasal polyposis, presence of allergic (eosinophilic) mucin with evidence of fungi, and type-I hypersensitivity to fungal antigens [4,5]. However, Ponikau et al. did not find type-I hypersensitivity in the majority of eosinophil-related FRS and based on this indicated that eosinophilic inflammation and not type-I hypersensitivity, is the chief factor in the disease process and have accordingly introduced the term eosinophilic fungal rhinosinusitis (EFRS) for this pathology [6]. Ferguson et al. recognized a subgroup of EFRS in older patients in which the fungi were absent in the eosinophilic mucin, but the patients had asthma with higher risk of aspirin sensitivity. They introduced the term eosinophilic mucinous rhinosinusitis (EMRS) for such patients [7].

It is generally accepted that the presence of eosinophilic inflammation with predominant Th2 cytokine (interleukin [IL]-4, IL-5, IL-13) production is characteristic of eosinophil-related FRS. The disease is considered as an enhanced immune response to ubiquitous airborne fungi, particularly *Alternaria alternata*, inducing eosinophilic degradation and triggering inflammation [8]. However, a significant geographical dichotomy has been observed among the fungi linked with this disease. Melanized fungi including *Alternaria* species, *Bipolaris* species, and *Curvularia* species have been isolated from patients with eosinophil-related FRS in the western world [8–11]. In contrast, *Aspergillus flavus* has been noted as the predominant

etiologic agent across South Asia, the Middle East, and Sudan [12–14]. As eosinophil-related FRS is the common FRS presentation in our patients, it would be important to study whether *A. flavus* produces enhanced immune response in our patients. However, a study of the eosinophil-*Aspergillus* interaction, failed to induce any eosinophil degradation [15]. In another investigation, *Aspergillus* failed to induce cytokines from peripheral blood mononuclear cells (PBMCs) in such patients, whereas *A. alternata* extract produced non-immunoglobulin E (IgE) mediated eosinophilic degradation thereby attracting Th2 cytokines when exposed to PBMCs [8]. We therefore undertook this study to compare *A. flavus* and *A. alternata* interactions with eosinophils, and the cytokine and IgE production in different groups of CRS patients at our center.

Methods

Patients

Patients with chronic rhinosinusitis were enrolled for the study after providing informed consent. Chronic rhinosinusitis (CRS) was defined as a clinical condition marked by nasal blockage, discharge and inflammation of the nose and paranasal sinuses (detected by endoscopy and/or computed tomography [CT] images), persisting for more than 12 weeks without complete resolution at any time. Only CRS patients who met the definition and were within the age group of 12–60 years, without diabetes, immunosuppression, or any malignancy were included. All patients underwent CT imaging, aspergillin skin test for immediate hypersensitivity, total IgE, and absolute eosinophil counts in blood. Endoscopic biopsy of nasal tissue was performed for histopathology to categorize the group of noninvasive CRS. These patients were further differentiated into two groups of 26 patients each, in other words, eosinophilic and noneosinophilic CRS by demonstration of eosinophilic mucin on histopathology.

The eosinophilic CRS group was further divided into three groups, in other words, A, B and C. Patients in group A (possible AFRS patients) were defined as CRS patients having a) characteristic radiographic findings (bony erosion and heterogeneous opacity with sinus expansion on CT scan), b) presence of eosinophilic mucin, c) evidence of fungi without invasion across mucosa on histopathology and/or culture, d) immediate hypersensitivity, positive

on skin test using aspergillin antigen (Hollister–Steir, USA). The skin testing was done using *A. fumigatus* antigen (100 PNU/ml, Hollister–Steir, USA) injected intradermally on anterior aspect of left forearm and 0.85% saline injected on the right forearm. The test was read after 15 minutes and following up to 1 hour and then at 6–8 hours. The test was considered positive with the development of wheal and flare reaction of more than 8 mm in the test arm as compared to control arm, maximal after 10–20 minutes and resolving within 1 to 1 and one-half hours. Group B (possible EFRS) was defined as CRS patients having first three characteristics of group A but negative for immediate hypersensitivity. Group C (possible EMRS) was defined as CRS patients having only the first two characteristics of group A. This group had no fungus in tissue even after examining multiple tissue sections. Twenty-six healthy individuals residing in same environment/region without any symptom of nasal congestion, polyposis, and allergy were enrolled as controls. Our Institute Review Board approved the study and informed consent was taken from each patient enrolled.

Eosinophil isolation

Total eosinophil count was done by flow cytometry (FACS CANTO, BD Biosciences, New Jersey, USA). Blood (10 ml) was collected from all subjects from the three groups (eosinophilic, noneosinophilic CRS, and healthy controls). Human eosinophils were isolated by Percoll density gradient centrifugation using granulocyte separation medium (Granulosep, Himedia, Mumbai, India) and magnetic eosinophil isolation kit (Big Easy Sep Magnet, Stem Cell Technology). The purity of eosinophils was maintained above 98%.

Culture Filtrate Antigen

Isolates

Culture filtrate antigens were prepared from *A. flavus* and *A. alternata* isolated from patients with AFRS and maintained in the NCCPF (National Culture Collection of Pathogenic Fungi, Postgraduate Institute of Medical Education and Research, Chandigarh, India). A single clinical isolate of *A. flavus* NCCPF 70947 and *A. alternata* NCCPF 650022 were used for the study.

Procedure

Isolates were cultured on Sabouraud dextrose agar (SDA) slants, incubated at 25°C for 2 days. The fungi were then subcultured in 200 ml glucose asparagine broth in a 500 ml flask by inoculating 0.5 cm² of the fungal colony block. The broth was incubated at 25°C for 5 weeks in an incubator shaker. After 5 weeks, formalin was added to a final

concentration of 0.5% in the broth. Before adding formalin, the broth was subcultured onto Robertson's cooked meat medium and sheep blood agar for purity check. Direct microscopy was also performed to check for contamination. The formalized broth culture was filtered using Whatman filter paper no. 40. The filtrate was chilled at 4°C, and two volumes of cold acetone were added drop by drop. The culture filtrate was maintained cold and in constant agitation with a magnetic stirrer during this period and for 15 minutes thereafter. The precipitated filtrate was transferred to a large centrifuge vessel and centrifuged at 3500 g for 30 minutes in cold. The supernatant was discarded, and the open centrifuge bottles were placed in a dessicator containing calcium chloride. The precipitate was allowed to dry overnight at 25°C. The pellet was resuspended in sterile distilled water at a volume half the original culture filtrate. It was agitated for a few minutes and centrifuged at 3500 g for 30 minutes to remove undissolved precipitate. The supernatant was recovered and its carbohydrate content measured using phenol-sulfuric acid method. The antigen was adjusted to contain 1000–1500 µg carbohydrate per ml by diluting it in sterile distilled water. Merthiolate was added to a final concentration of 1:10000, and the antigen was stored at 4°C.

Eosinophil degranulation assay

To compare the interaction of eosinophils with *A. flavus* and *A. alternata*, degranulation of eosinophils was measured by quantifying eosinophil granule major basic protein (MBP). The eosinophils collected from each patient and control were incubated separately with 100 µl fungal extracts of *A. flavus*/*A. alternata* in the media containing HBSS with 25 mM HEPES and 0.01% gelatin at 2.5×10^5 cells/ml in 96-well tissue culture plates for 6 hours at 37°C and 5% CO₂. The cell-free supernatants were stored at –20°C. The concentration of MBP (ng/ 2.5×10^5 cells) in the supernatants was determined by enzyme-linked immunosorbent assay (ELISA; MBP ELISA Kit, GenxBio Health Sciences Pvt. Ltd. New Delhi, India. Catalog No. CSB-E11240r). The detection limit of the kit was 12.5–800 ng/ml.

Cytokine analysis of serum assay

The sera from patients and controls were used to investigate the difference in cytokine profile of the patient and control groups. The serum samples were processed using human Th1/Th2 cytokine kit (Cytometric Bead Array, BD Biosciences, New Jersey, USA. Cat. No. 550749). The levels of interleukin (IL)-2, IL-4, IL-5, IL-10, tumor necrosis factor α (TNFα) and interferon-γ (INFγ) in the

samples were measured by flow cytometry (FACS CANTO, BD Biosciences, New Jersey, USA). The results were analyzed by FCAP Array Software. The detection limit was 200–5000 pg/ml.

Statistical analysis

Statistical analysis was carried out using SPSS v19.0. The results from patients and controls were compared using ANOVA. Normalcy of the two groups was determined by Kolmogorov–Smirnov test. The correlation between absolute eosinophil count and serum immunoglobulin E (IgE) and their association with disease was measured by Pearson's correlation. The nonparametric Mann–Whitney U test was used to compare cytokine levels, serum IgE, and absolute eosinophil count. A *P* value <.05 was considered significant.

RESULTS

Twenty-six consecutive patients each from eosinophil-related and non-eosinophil related CRS were included in the

study. A total of 26 healthy unrelated controls were also included from the same geographical location after informed consent. The patients with eosinophil related chronic rhinosinusitis were further classified into group A (*n* = 13), group B (*n* = 10), and group C (*n* = 3).

Demographic characteristics

The demographic characteristics of the patients and controls are presented in Table 1. This was an age and sex-matched study. Mean ages of patients in group A, B, and C was 23.8, 27.8, and 32.33 years, respectively (Table 1). The groups were homogenous in composition (*P* = .081). Male preponderance was observed in all study groups (Table 1).

Clinical characteristics

The clinical characteristics of the study groups are summarized in Table 1. Similar family history was predominantly noted in group A patients as compared to other groups (*P* = .021). A history of previous surgery, indicating recurrence of disease was also observed in group A (*P* = .006).

Table 1. Demographic and clinical characteristics of different study groups and controls

Patient characteristics	Eosinophil-related fungal rhinosinusitis			Non-eosinophil related CRS N (%)	Healthy hosts N (%)	Level of significance. <i>P</i> value
	Group A N (%)	Group B N (%)	Group C N (%)			
Total no. of subjects	13	10	3	26	26	
Mean age in years	23.8	27.8	32.33	34.31	32.65	0.189
Male (%)	69.2	60	66.7	80.8	53.8	0.344
Female (%)	30.8	40	33.3	19.2	46.2	
Allergic symptoms	13 (100)	10 (100)	3 (100)	2 (7.7)	0 (0)	0.490
Salicylate sensitivity	5 (38.5)	0 (0)	0 (0)	0 (0)	0 (0)	<0.001
Nasal obstruction	13 (100)	10 (100)	3 (100)	22 (84.6)	0 (0)	<0.001*
Nasal discharge	13 (100)	10 (100)	3 (100)	26 (100)	0 (0)	<0.001*
Postnasal drip	11 (84.6)	9 (90)	3 (100)	23 (88.5)	0 (0)	<0.001*
Headache	12 (92.3)	7 (70)	3 (100)	26 (100)	0 (0)	<0.001*
Epistaxis	4 (30.8)	2 (20)	0(0)	6 (23.1)	0 (0)	0.062
Hyposmia	10 (76.9)	6 (60)	3 (100)	16 (61.5)	0 (0)	<0.001*
Family history of similar disease	5 (38.5)	0 (0)	0 (0)	1 (3.8)	4 (15.4)	0.021
Previous surgery for same disease	5 (38.5)	1 (10)	1 (33.3)	2 (7.7)	0 (0)	0.006*
Nasal/oral steroids	6 (46.2)	3 (30)	1 (33.3)	2 (7.7)	0 (0)	0.001*
Telecanthus	6 (46.2)	2 (20)	1 (33.3)	10 (38.5)	0 (0)	0.005*
Proptosis	7 (53.8)	2 (20)	2 (66.7)	8 (30.8)	0 (0)	0.001*
Polyps	11 (84.6)	9 (90)	3 (100)	11 (42.3)	0 (0)	<0.001*
Immediate hypersensitivity on <i>Aspergillus</i> skin test	13 (100)	0 (0)	0 (0)	4 (15.4)	0(0)	<0.001*
Bilateral disease	6 (46.2)	7 (70)	3 (100)	14 (53.8)	0 (0)	<0.001*
Intraorbital extension	3 (23.1)	1 (10)	1 (33.3)	9 (34.6)	0 (0)	0.019
Intracranial extension	0 (0)	1 (10)	1 (33.3)	6 (23.1)	0 (0)	0.028

N = Number; **P* < .05 considered as significant.

Group A – presence of mucin and hyphae in nasal biopsies and immediate hypersensitivity positive; Group B – presence of mucin and hyphae in nasal biopsies without hypersensitivity; Group C – presence of mucin without fungus or hypersensitivity; CRS = Chronic rhinosinusitis.

Occurrence of nasal polyps was significantly higher in group A (84.6%) group B (90%), and group C (100%) as compared to NECRS group (42.3%) ($P < .001$).

Humoral IgE and eosinophil response

Total IgE levels were significantly elevated in the Group A and B patients compared to NECRS patients or normal controls ($P < .001$). Mean absolute eosinophil count was 684 ± 142 cells/mm³ in group A, 577 ± 86 cells/mm³ in group B, 503 ± 79 cells/mm³ in group C, 189 ± 57 cells/mm³ in NECRS, and 157 ± 54 cells/mm³ in normal subjects (Table 2), the difference being statistically significant ($P < .001$). All the patients of the three study groups had eosinophil counts more than 350/mm³, while none of controls had significant eosinophilia.

There was a positive correlation between serum IgE levels and absolute eosinophil count in patients of group A (0.599), group C (1.00), and NECRS (0.934).

Eosinophil degradation response

The eosinophils isolated from the study subjects were exposed to culture filtrate antigens of *A. flavus* and *A. alternata*, and the eosinophil degradation products were quantified in terms of major basic protein (ng/ 2.5×10^5 cells). The MBP response was significantly higher for *A. flavus* in group A (mean MBP: 279.15 ± 32.3 ng/ 2.5×10^5 cells) compared to group B (mean MBP: 254.9 ± 17.14 ng/ 2.5×10^5 cells) ($P = .05$), Group C (mean MBP: 238.33 ± 17.56 ng/ 2.5×10^5 cells) ($P < .026$), NECRS (mean MBP: 56.96 ± 10.97 ng/ 2.5×10^5 cells) ($P < .001$) and normal subjects (mean MBP: 28.73 ± 7.04 ng/ 2.5×10^5 cells) ($P < .001$). MBP response to *A. alternata* was significantly lower in group A (mean MBP: 48.77 ± 12.95 ng/ 2.5×10^5 cells), NECRS (mean MBP: 40.83 ± 6.92 ng/ 2.5×10^5 cells), and normal subjects (mean MBP: 21.81 ± 4.72 ng/ 2.5×10^5 cells). Thus, there was significant eosinophil degranulation in response to *A. flavus* but not to *A. alternata* in patients with eosinophil-related fungal rhinosinusitis.

There was significant correlation between absolute eosinophil count, serum IgE levels (correlation coefficient: 0.607) and major basic protein released by the eosinophils in response to *A. flavus* in patients with group A (0.092), group B (0.991), and group C (1.00). However, no significant correlation was observed with NECRS patients and control group.

Cytokine analysis

Serum samples were tested for IL-2, IL-4, IL-5, IL-10, TNF α , and INF γ cytokines. Results showed that all these

cytokines were significantly elevated in patients with group A, B, and C as compared to NECRS and normal subjects ($P < .001$) (Table 2).

DISCUSSION

The present study shows exaggerated IgE levels, eosinophilia, combined Th1 and Th2 cytokines, and eosinophilic inflammatory response to *A. flavus* in Group A patients with eosinophil-related FRS (possibly classified as AFRS). Ever since the first description by Millar et al. [16] and Katzenstein et al. [17] of *Aspergillus* fungal sinusitis, many studies have attempted to understand the role of fungi in pathogenesis of chronic rhinosinusitis [2,3,6–23]. The equivocal and often contradictory results from these studies have failed to delineate the pathogenesis of this disease. Though type I hypersensitivity and presence of fungi in allergic mucin are considered as the hall mark of AFRS, Ponikau et al. showed that fungi in mucin, which they described it as eosinophilic mucin rather than allergic mucin, may be present in many cases of CRS without type I hypersensitivity and coined the term EFRS to describe most cases of CRS [6]. Their hypothesis was further supported by subsequent studies [24,25]. The eosinophilic inflammatory response is believed to be due to Th2 cytokine production (IL-4, IL-5, IL-13), independent of any hypersensitivity reaction. In their study, Shin et al. found a strong correlation between IL-5 levels and fungus specific immunoglobulin G (IgG) but no correlation with IgE [8]. However, incongruous findings have also been reported by subsequent studies [21,26]. The eosinophilic mucin contains sloughed epithelial cells, eosinophils, charcot-leyden crystals, and other inflammatory cells arranged in a laminar pattern. Presence of fungi is rare and scattered in the eosinophilic mucin. The sensitivity of its detection can be improved by enzymatic digestion technique utilizing antibody and PCR techniques [25,27–29]. However, eosinophilic mucin without any fungal hyphae has been noted in a group of patients with CRS. Ferguson classified this group as EMRS [7]. A study employing DNA microarray could not find any significant difference between the expression profiles of AFRS and EMRS, rendering the clinico-pathological distinction obscure [30].

Despite the prevailing controversies, all experts in the field agree on the presence of eosinophilic mucin in these patients and contend that eosinophil degranulation induces the inflammatory reaction seen in this disease. This raises the obvious query of what triggers the eosinophil degradation. Unlike mast cells and basophils, the eosinophils have minimal surface expression of FC ϵ RI, so the IgE rise in these patients cannot explain eosinophil degranulation [31]. In vitro eosinophil degranulation has been

Table 2. Laboratory findings of the study groups & controls

	Group A mean $\pm$ 2 SD	Group B mean $\pm$ 2 SD	Group C mean $\pm$ 2 SD	Non-eosinophil related CRS mean $\pm$ 2 SD	Healthy host; mean $\pm$ 2 SD	Level of significance, <i>P</i> value
Serum IgE IU/ml.	162.88 $\pm$ 34.18	139.7 $\pm$ 26.32	118.67 $\pm$ 28.15	104.12 $\pm$ 16.99	117.2 $\pm$ 15.46	<0.001* Group A vs. B -0.047
Absolute eosinophil count Cells/ μ l	684 $\pm$ 142	577 $\pm$ 86	503 $\pm$ 79	189 $\pm$ 57	157 $\pm$ 54	
Major basic protein ng/ 2.5×10^5 cells in response to <i>A. flavus</i> extract	279.15 $\pm$ 32.29	254.90 $\pm$ 17.14	238.33 $\pm$ 17.56	56.96 $\pm$ 10.97	28.73 $\pm$ 7.04	Group A vs. C -0.026* Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.05
Major basic protein ng/ 2.5×10^5 cells in response to <i>A. alternata</i> extract	48.77 $\pm$ 12.95	44 $\pm$ 12.26	21.17 $\pm$ 12.91	40.83 $\pm$ 6.93	21.81 $\pm$ 4.72	Group A vs. C -0.026 Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.264
IL-2 pg/ml	389.12 $\pm$ 437.02	322.69 $\pm$ 538.92	116.95 $\pm$ 131.97	1.97 $\pm$ 2.88	0.99 $\pm$ 2.06	Group A vs. C -0.018* Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.93 Group A vs. C -0.31
IL-4 pg/ml	342.38 $\pm$ 233.39	319.03 $\pm$ 390.85	159.92 $\pm$ 165.09	3.18 $\pm$ 4.36	2.04 $\pm$ 4.16	Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.05 Group A vs. C -0.026
IL-5 pg/ml	709.19 $\pm$ 530.51	643.46 $\pm$ 816.07	289.12 $\pm$ 249.87	0.98 $\pm$ 1.12	0.57 $\pm$ 0.92	Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.53 Group A vs. C -0.15
IL-10 pg/ml	599.17 $\pm$ 460.66	517.45 $\pm$ 675.49	244.99 $\pm$ 215.65	5.59 $\pm$ 8.61	1.49 $\pm$ 2.42	Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001* Group A vs. B -0.53 Group A vs. C -0.15 Group A vs. NECRS - <0.001* Group A vs. healthy - <0.001*

Table 2. continued

	Group A mean $\pm$ 2 SD	Group B mean $\pm$ 2 SD	Group C mean $\pm$ 2 SD	Non-eosinophil related CRS mean $\pm$ 2 SD	Healthy host; mean $\pm$ 2 SD	Level of significance, P value
TNF- α pg/ml	1003.73 $\pm$ 894.55	743.47 $\pm$ 1098.41	300.64 $\pm$ 192.95	3.08 $\pm$ 3.51	1.05 $\pm$ 2.63	Group A vs. B – 0.31 Group A vs. C – 0.15 Group A vs. NECRS – <0.001* Group A vs. healthy – <0.001*
INF- γ pg/ml	369.44 $\pm$ 356.99	302.25 $\pm$ 502.51	92.63 $\pm$ 77.88	8.04 $\pm$ 10.42	4.51 $\pm$ 7.71	Group A vs. B – 0.38 Group A vs. C – 0.10 Group A vs. NECRS – <0.001* Group A vs. healthy – <0.001*

*P < .05 considered as significant.

Group A – presence of mucin and hyphae in nasal biopsies and immediate hypersensitivity positive; Group B – presence of mucin and hyphae in nasal biopsies without hypersensitivity; Group C – presence of mucin without fungus or hypersensitivity; CRS = Chronic rhinosinusitis.

demonstrated in direct fungi or fungal products engagement or after stimulation by soluble inflammatory mediators like IL-5, GM-CSF, RANTES, eotaxin, IFN- γ , platelet activating factors (PAF), C5a, and plasma-activated zymosan [15,32]. In a detailed study on fungi-eosinophil direct interaction, it was observed that *Alternaria* species and *Penicillium* species could release eosinophil-derived neurotoxin (EDN); but isolates under *Aspergillus*, *Bipolaris*, *Candida*, *Cladosporium*, and *Curvularia* species failed to induce EDN release [15].

Airborne fungi commonly found in patients with AFRS and EFRS include *Alternaria*, *Aspergillus*, *Penicillium*, *Bipolaris*, *Curvularia*, and *Cladosporium* species [8–11]. Manning and Holman from the United States reported 87% of their AFRS cases were due to dematiaceous fungi and 13% due to *Aspergillus* species [33]. Schubert claimed that in most of their AFRS cases, allergic mucin was culture positive for *Bipolaris spicifera* or *Curvularia lunata* [11]. In contrast *A. flavus* was isolated from 50 to 80% of AFRS cases in India [12,13,34,35] and 50% of AFRS cases from the Middle East [14]. Conforming to our earlier studies *A. flavus* was isolated from 70% patients with AFRS and EFRS in the present study. This raises the question of how *Aspergillus* species trigger eosinophil degranulation in AFRS patients and induce the subsequent inflammatory cascade. The first possibility is that the fungus can induce cytokines like IL-5 and IFN- γ , which may lead to eosinophil degranulation. The second possibility is that the eosinophils from different patient groups and geographical locations behave differently and contrary to earlier reports [15], *Aspergillus* species may also be capable of degranulating eosinophils by direct interaction.

In the present study, we have subgrouped the patients with eosinophil-related CRS into three groups (A, B, C) based on their histopathological and cultural characteristics. When the eosinophils from these groups of study subjects and controls groups were interacted with *A. flavus* and *A. alternata* extracts, the eosinophil degradation product MBP, increased much higher in response to *A. flavus* compared to *A. alternata*. Rather *A. alternata* extracts induced minimal response in all our patient groups and controls. MBP level was significantly higher in interaction of *A. flavus* extract with eosinophils from patients in group A (279.15 $\pm$ 32.29 ng/2.5 $\times$ 10⁵ eosinophils), group B (254.9 $\pm$ 17.14 ng/2.5 $\times$ 10⁵ eosinophils), and group C (238.3 $\pm$ 17.56 ng/2.5 $\times$ 10⁵ eosinophils) compared to NECRS (56.96 $\pm$ 10.97 ng/2.5 $\times$ 10⁵ eosinophils) and healthy controls (28.73 $\pm$ 7.04 ng/2.5 $\times$ 10⁵ eosinophils). This result confirms our second hypothesis that eosinophils from patients of different ethnic origins may behave differently and responds to the prevalent fungal species of that particular region. There is a possibility that the β -D glucan

present in the *Aspergillus* cell wall and secreted products may also induce eosinophil degranulation [36]. However, the *in vitro* experiment may not entirely explain the *in vivo* pathogenic mechanisms where several cellular, cytokine, and chemokine interactions take place.

It is believed that cytokines liberated by immune cells induce eosinophil recruitment to the nasal mucosa. A decline in Th1 cell response parallels increase in Th2 response and triggers Th2 cell dependent fungal antigen mediated immune response. These events are believed to orchestrate the central mechanism of allergic response in these patients. Although attention has been focused on Th2 cells only, human mast cells also store and secrete IL-4 and TNF- α [37]. Studies have demonstrated increased IL-4 and IL-10 levels in sinus washings of patients with allergic sinusitis as compared to elevated IL-1 β and IL-1ra in bacterial sinusitis [38]. However, many studies have shown high levels of IFN- γ , mediator of Th1 response, in asthma and allergy [8,15,39]. Shin et al. had observed increased IFN- γ and exaggerated Th1 and Th2 humoral and cellular responses in their CRS patients [8]. While their study was limited to local cytokine production in sinus tissues, we demonstrated systemic cytokine response, which corroborated their findings. In the present study, both Th1 and Th2 cytokines were significantly raised in group A, B, and C as compared to NECRS ($P < .001$). Among the raised cytokines, levels of IL-4 and IL-10 were highest. Our results also revealed a significant positive correlation between elevated absolute eosinophil count, MBP release and Th1/Th2 cytokine levels (correlation coefficient 0.994). Therefore, the possible central mechanism of eosinophil-related fungal rhinosinusitis is exposure of susceptible patients to *A. flavus* leading to eosinophilia, systemic Th1 and Th2 response and cytokine release, recruitment of eosinophils in the mucosa of nose and paranasal sinuses. Both fungi and cytokines may thus play a role in eosinophil degranulation and release of eosinophil granule proteins, leading to subsequent immunological (cytokines) and inflammatory processes (eosinophil granule protein) which initiate destruction of mucosal barrier function.

The distinction between the three groups appears more arbitrary than real, as absolute eosinophil count was significantly higher in all three groups, total IgE significantly raised in both group A and B and Th1/Th2 cytokines raised in all three groups. Moreover, clinical features, radiology, immunological parameters, and histopathology are similar in these three groups. The distinction of the third group, C lies in the absence of demonstrable fungi [7], which also depends on the technique used for detection. To differentiate between group A and B, we assessed immediate hypersensitivity to aspergillin. Our group B patients might

be hypersensitive to other allergens or fungal extracts as well, although those were not examined. The distinction of NECRS also requires further study, as IgE encoding transcripts have been detected in the sinus mucosa of such patients [40]. A large multi-centric evaluation considering all these parameters may resolve these issues.

In conclusion, the study demonstrated considerable overlap among the categories of eosinophil-related FRS. The patients especially manifest exaggerated humoral IgE, cellular eosinophilic and elevated Th1/Th2 cytokine response to noninvasive fungi or their extract. The response is to the commonly isolated fungus *A. flavus* in this region rather than *A. alternata*.

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Declaration of conflict of interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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