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Sinus aspirates in chronic rhinosinusitis: fungal colonization of paranasal sinuses, evaluation of ICAM-1 and IL-8 and studying of immunological effect of long-term macrolide therapy*

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SUMMARY

Purpose: *In patients with chronic fungal sinusitis, concentrations of interleukin-8 (IL-8), immunoglobulin E (IgE), and soluble intercellular adhesion molecule-1 (sICAM-1) were compared in paranasal sinus aspirates and serum. Furthermore, immunological effects of macrolide treatment of our patients with chronic fungal rhinosinusitis were also studied.*

Materials and methods: *In our cohort study, 108 patients with chronic rhinosinusitis undergoing sinus surgery were selected. Sinus aspirates were collected, and used for immunological assays and cultured to study fungi. All patients were examined for the presence of characteristic allergic mucin of chronic allergic fungal rhinosinusitis and this was confirmed later by measurement of total serum IgE.*

Results: *Our cases were classified into 3 groups: chronic rhinosinusitis with positive fungal culture and negative allergic mucin, chronic rhinosinusitis with positive fungal culture and positive allergic mucin and chronic rhinosinusitis without fungal growth. A control group was included. We found 57.4% of the patient cultures positive for fungus and 36.4% of the control subjects. Aspergillus ssp. were the most prevalent followed by Bipolaris ssp., and Curvularia. IgE levels were increased in group II compared to group I, III and IV. ICAM-1 and IL-8 levels were increased in groups I, II and III compared to the control group. Erythromycin given in group II decreased the levels of IL-8 and ICAM-1.*

Conclusion: *Aspergillus ssp. were the most common. These results confirm the role of ICAM-1 and IL-8 in all types of rhinosinusitis. Erythromycin modulated the immune status of the patients.*

Key words: fungal rhinosinusitis, fungal immunology, IL-8, ICAM-1, macrolides therapy, chronic rhinosinusitis, IgE in fungal rhinosinusitis

INTRODUCTION

Fungi are omnipresent in nature. They are found as normal flora in the oral cavity and presumably on the nasal mucosa [(1)]. Current papers report fungi to be present in 6% to 93% of patients with chronic rhinosinusitis [(2,3)]. The reason for this large difference relates to the problem of detecting fungi and their role in the disease process [(4)].

There are four categories of paranasal sinus fungal disease: chronic indolent fungal rhinosinusitis (invasive), fulminant fungal rhinosinusitis (invasive), fungal ball (noninvasive), and allergic fungal rhinosinusitis (noninvasive). Chronic indolent fungal rhinosinusitis is typically found in immunocompetent patients with unilateral rhinosinusitis and fungal hyphae invading surrounding tissue [(2)]. Fulminant fungal rhinosinusitis is usually found in immunosuppressed patients. The fungus ball is found in healthy, non-atopic patients with unilateral sinus symptoms who developed a tangled mass of fungi with little associated inflammatory reaction [(5)]. Allergic fungal rhinosinusitis is found in atopic immunocompetent patients with chronic rhinosinusitis and nasal polyps, who develop an allergic immune response to extramucosal fungal hyphae depicted histologically as allergic mucin [(2)].

To distinguish allergic fungal rhinosinusitis from chronic bacterial rhinosinusitis and other forms of fungal rhinosinusitis, Bent and Kuhn [(2,6)] elaborated a set of five diagnostic criteria: evidence of type I hypersensitivity, nasal polyps, characteristic computed tomography (CT), eosinophilic mucus and fungi identified on

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stain (without tissue invasion of the surgical specimen). Kuhn and Swan [6] added several minor criteria, which included (asthma history, unilateral predominance, radiographic bone erosion, Charcot-Lyeden crystals, fungal culture, and peripheral eosinophilia). One of the prominent features of chronic rhinosinusitis is persistent purulent discharge containing numerous migrated neutrophils in the paranasal sinuses. Two positive feedback mechanisms explain the chronic neutrophil accumulation in the sinus. First, IL-1β secreted by monocytes, macrophages and fibroblasts upregulates the expression of E-selectin and ICAM-1 in vascular endothelial cells, and thereby induces the extravascular transmigration of neutrophils. The emigrated neutrophils then secrete IL-1β, which amplifies the expression of E-selectin and ICAM-1, resulting in further neutrophil infiltration. Second, chemoattractants including IL-8 in the sinus effusion initiate neutrophil exudation. Emigrated neutrophils then secrete IL-8, which elicits further neutrophil accumulation in the sinus effusion β [7,8].

Low-dose, long-term administration of a 14-membered lactone ring macrolide has been reported to be very effective for patients with chronic upper respiratory tract infections. Macrolide therapy was first reported by Kudoh et al., [9] who demonstrated a significant effect of erythromycin in patients with diffuse panbronchiolitis. Subsequently, the efficacy of macrolide therapy for patients with sinobronchial syndrome, chronic rhinosinusitis, and otitis media with effusion has also been reported [10].

In this work, we aimed to evaluate the prevalence and type of fungal species in chronic rhinosinusitis. Also, we wanted to evaluate IL-8 and ICAM-1, which have a striking effect on cellular infiltration, especially neutrophil chemotaxis in all types of rhinosinusitis. We selected erythromycin to study its effect on IL-8 and ICAM-1 in rhinosinusitis as its effect may be immunologically and not due to its bactericidal effect.

SUBJECTS AND METHODS

Ethical considerations

Approval for this paper was obtained from the Research Ethics committee at our department as a long study assessment for chronic rhinosinusitis (CRS) patients (Cohort Strategy).

Patients

One hundred and eight consecutive patients with the clinical diagnosis of chronic rhinosinusitis were selected from patients attending the Otorhinolaryngology Department of the Mansoura University Hospital.

Clinical diagnosis of chronic rhinoinusitis criteria

Rhinosinusitis (including nasal polyps) is defined as: inflammation of the nose and the paranasal sinuses characterized by two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip), ± facial pain/pressure, ± reduction or loss of smell, and either endoscopic signs of: polyps and/or mucopurulent discharge primarily from middle meatus and/or oedema/mucosal obstruction primarily in middle meatus and/or CT changes, especially mucosal changes within the ostiomeatal complex and/or sinuses [11].

Sinus aspirates were collected by wide pore spinal needle, 1 ml sterile 0.9 % saline was introduced through the middle meatus, left in place for 2 minutes and then aspirated in sterile containers. A part of the aspirate was stored at -30°C for the immunological assay; the other part was cultured for mycologic growth. All patients were examined for the presence of characteristic allergic mucin (AM) of chronic allergic fungal rhinosinusitis and smears were stained by haematoxylin and eosin to elucidate eosinophils, which was confirmed later by measurement of total serum IgE.

After fungal culture (F), we have classified our cases into 3 groups:

Group I: chronic rhinosinusitis with positive fungal culture and negative allergic mucin (n = 48), CRS F+/AM-. The mean age was (35 ± 3) years, with 32 males and 16 females.

Group II: chronic rhinosinusitis with positive fungal culture and positive allergic mucin (n = 14), CRS F+/AM+. The mean age was (43 ± 5.2) years, with 8 males and 6 females. The mean age was (48.3 ± 5.4) years, with 18 males and 28 females.

Group III: chronic rhinosinusitis without fungal growth (n = 46), CRS F-/AM-

Finally, *Group IV* was included: a control group (n = 11), in which no history of nasal or paranasal sinus disease, with no symptoms of inhalant allergy and with normal-appearing mucosa confirmed by nasal endoscopy, undergoing sinus aspirate for other surgical conditions. The mean age was (35 ± 2.4) years, with 7 males and 4 females.

Examination of sinus aspirate for fungal growth

The collected sinus aspirate was placed in centrifuge tubes and sent directly to the Mycology Laboratory in the Medical Microbiology and Immunology Department. Samples were processed in a laminar flow hood. One vial (10 ml) of sterile dithiothretol was used to liquefy the mucus. The mixture was centrifuged in 20 ml tubes for 10 minutes at 3000 rpm/min. The supernatant was discarded and the sediment was vortexed for 30 seconds. Half a milliliter was inoculated onto an inhibitory mold agar plate containing chloramphenicol (125 µg/ml), ciprofloxacin (5 µg/ml) (for isolation of cyclohexamide sensitive fungi), brain-heart infusion agar containing 5% sheep blood, gentamicin (5 µg/ml) and chloramphenicol (15 µg/ml) and cycloheximide (5 mg/ml) (for isolation of fastidious pathogenic fungi). The plates were incubated at 30°C and organisms were allowed to grow for 4 weeks. The plates were examined at 4 days intervals. All cultures were identified by colony morphology, microscopically, and selective media growth.

Examination of sinus aspirate for ICAM-1

Sinus aspirate samples were stored at -30°C until assay time. Concentrations of sICAM-1 were determined (in duplicate) by commercial ELISA kits (Biosource-International, Inc., CA, USA)

according to the manufacturer's descriptions using a 100 µl sample.

Examination of sinus aspirate for IL-8

IL-8 concentrations in sinus aspirates were determined using the R&D International (Minneapolis, MN, USA) ELISA kit according to the manufacturer's instructions.

Assay of total serum IgE

Total serum IgE was measured by ELISA kit (Elitech Diagnostics, Salon-de-Provence, France). All ELISA's were measured using a Spectra III reader .

Macrolide (Erythromycin) treatment of chronic fungal sinusitis patients

Group II CRS F+/AM+ were subjected to an erythromycin course consisting of a daily 1500 mg oral dose for a period of 2 months (without corticosteroids or other medications). The sinus aspirates were taken at the end of the treatment and examined for ICAM-1 and IL-8.

Statistical analysis

Mean values, standard deviations, and p-values for ICAM-1, IL-8, and IgE were determined for all tested groups using cross tabulation and Chi square analysis.

RESULTS

Mycological study

Fungal cultures resulted in cultures positive for fungus in 62 of 108 (57.4%) patients with chronic rhinosinusitis. A total of 89 positive cultures grew from the patients, with an average of 1.48 organisms per patient and a maximum of 3 different organisms per patient. Group I CRS F+/AM- was diagnosed in 48 cases out of 108 patients (44.4%) with *Aspergillus fumigatus* (36.2 %) being most prevalent followed by *Bipolaris ssp and Aspergillus niger*. Group II CRS F+/AM+ was diagnosed in 14 cases out of 108 patients (12.9%). The most commonly isolated fungus from chronic rhinosinusitis patients was *Aspergillus fumigatus* (50%) followed by *Aspergillus niger*, *Bipolaris*, and *Curvularia* (15% for the 3 strains) (Table 1).

A total of 7 different genera of fungi were isolated. The control group was 36.4% culture positive for fungi with a maximum of 1 organism per subject. Only three genera were isolated from the control group; the number of organisms grown from the controls was markedly different from those isolated from the patients.

Eosinophil test

Eosinophilic mucin was only found in (all) patients of group II. The allergic fungal mucin was thick, tenacious and highly viscous in consistency; its colour varied from light tan to brown or dark green. The number of eosinophils varied from 10-15 / HPF.

Immunological assay

As shown in (Table 2), the mean values of sICAM-1 were 112.8 ± 20.7 ng/ml in group I, 111.6 ± 15.0 ng/ml in group II, 115.3 ± 17.8 ng/ml in group III and 20.8 ± 5.4 ng/ml in the control group. The levels of sICAM-1 were statistically significantly increased in group I, group II and group III compared to the control group IV. There were no statistically significant differences between the first 3 groups compared to each other.

For IL-8, the mean values were 176.7 ± 20.4 ng/ml in group I, 152.3 ± 23.6 ng/ml in group II, 173.1 ± 25.3 ng/ml in group III and 8.7 ± 2.3 ng/ml in control group. IL-8 levels were statistically significantly increased in group I, group II and group III compared to the control group IV. There were no statistically significant differences between the first 3 groups compared to each other.

The mean values total serum IgE were 60.3 ± 16.4 IU/ml in group I, 576.1 ± 181.8 IU/ml in group II, 58.8 ± 16.2 IU/ml in group III and 68.7 ± 21.5 IU/ml in control group. There was a statistically significant increase in IgE in group II compared to group I, group III and the control group IV. There were no statistically significant differences between these latter 3 groups.

Table 1. Distribution of fungi isolated by culture in different groups.

Fungus Ssp.	**GroupI** (F+/AM-)		**Group II** (F+/AM+)		**Group IV** control		**Total**
	n	**%**	**n**	**%**	**n**	**%**	
Asper. fumigatus	25	36.2	10	50	2	18.2	37
Asper. niger	11	15.9	3	15	1	9.1	15
Asper. flavus	3	4.3	0	0	0	0	3
Bipolaris	13	18.8	3	15	0	0	16
Curvularia	10	14.4	3	15	0	0	13
Alternaria	4	5.7	1	5	0	0	5
Mucor	3	4.3	0	0	1	9.1	4
Total	69		20		4		93
p-value	0.001(x31)*		0.01(x24)**				

* - Group I x Group IV
** - Group II x Group IV

Table 2. Assay of ICAM-1 and IL-8 in sinus aspirate and serum IgE.

	Group 1 F+/AM-	Group11 F+/AM+	Group111 F-/AM-	Group1V control
ICAM 1				
Mean (ng/ml)	112.8	116.6	115.3	20.8
SD	20.7	15.0	17.8	5.4
IL-8				
Mean (ng/ml)	176.7	152.3	173.1	8.7
SD	20.4	23.6	25.3	2.3
IgE				
Mean (IU/m)	60.3	567.1	58.8	68.7
SD	16.4	181.8	16.2	21.5

p-value < 0.05 (= significant)
ICAM 1: p-value of gp 1 versus gpII, III, IV is 0.781, 0.882 and 0.001 ; p-value of gp II versus gp III and IV is 0.779 and 0.01; p-value of gpIII versus gpIV is 0.001; **IL8:** p-value of gp 1 versus gpII, III, IV is 0.891, 0.572, 0.02; p-value of gp 1 versus gpII, III, IV is 0.671, 0.01; p-value of gpIII versus gpIV is 0.001; **IgE:** p-value of gp 1 versus gpII, III, IV is 0.001, 0.696, 789; p-value of gp II versus gp III and IV is 0.01, 0.01; p - value of gp III versus gp IV is 0.11.

Table 3. Study of the effect of Macrolide (Erythromycin) on ICAM-1 and IL-8 levels in chronic fungal sinusitis patients.

	ICAM-1			IL-8		
	Mean	**SD**	**p-value**	**Mean**	**SD**	**p-value**
Group II (F+/AM+) (Before treatment)	112.8	20.7	0.01	176.7	20.4	0.001
Group II (F+/AM+) (After treatment)	57.4	10.8		75.1	12.1	

p-value < 0.05 (= significant)

Macrolide (erythromycin) treatment of group II CRS F+/AM+

As indicated in Table 3, after erythromycin treatment, the mean value of sICAM-1 expression decreased from 112.8 ± 20.7 ng/ml to 57.4 ± 10.8 ng/ml with a statistically significant value ($p < 0.01$). Also, after finishing the course of erythromycin, the mean value of IL-8 decreased from 176.7 ± 20.4 ng/ml to 75.1 ± 12.1 ng/ml with a statistically highly significant difference ($p < 0.001$).

DISCUSSION

In our study, the collection and culturing method resulted in positive fungal cultures in 57.4% of the patients with chronic rhinosinusitis and *Aspergillus fumigatus* being the most prevalent. A total of 7 different species were isolated.

Ponikau et al. [3] isolated up to 4 different fungal species belonging to the common environmental genera in the nasal lavage fluid of both volunteers and patients with chronic rhinosinusitis. Thus, these fungi may well exist in the sinus without causing inflammation or a fungus ball. This is consistent with the findings of Vennewald et al., [12] who reported on 132 tissue samples obtained by endoscopic operation from the paranasal sinuses of 117 patients, which were examined for mycotic infections. Fungi were isolated 34 times from 132 surgical specimens (25.7%). The following fungal species were isolated: *Aspergillus fumigatus* [17], other *Aspergillus ssp* [6], *Alternaria alternata* [2], *Penicillium rugulosum* [1], and moulds without differentiation [5]. These findings represented a commensal colonization of the paranasal sinuses, but not a mycosis [12].

Aspergillus ssp. is the most commonly reported cause of fungal rhinosinusitis and fungus balls followed by Dematiaceous fungi [13]. In the paranasal cavities of patients with a suspicion of chronic rhinosinusitis, by standard mycological as well as serological examinations, *Aspergillus* was found in 9 of 37 patients [14]. These results were similar to our results since *Apergillus ssp*. were the most prevalent in our patients causing chronic rhinosinusitis. Buzina et al. [15], studied mucus samples by flushing the noses of chronic rhinosinusitis patients with saline or by endoscopic sinus surgery. Fungi from mucus were cultivated on agar plates. They indicated that the most prevalent isolates belonged to the genera *Penicillium, Aspergillus, Cladosporium, Alternaria and Aureobasidium*. Whereas *Aspergillus* and *Penicillium ssp*. occurred in more or less the same numbers throughout the year, *Cladosporium ssp.*, *Alternaria ssp.* and *Aureobasidium pullulans* showed a significantly higher occurrence during late summer and early autumn. In our study, we did not detect the effect of seasonal variation on the prevalence of fungi causing chronic rhinosinusitis.

In our study, there were increased levels of ICAM-1 in the three rhinosinusitis groups compared to the control. There were no differences between the three disease groups. These results confirmed the role of this adhesion molecule in all types of rhinosinusitis and are in agreement with many studies concerning this molecule. Soluble ICAM-1 levels did not increase in healthy controls, but patients with chronic rhinosinusitis had higher sICAM-1 levels that also increased in patients with allergic rhinitis after a provocation test with the specific allergen. These results show that ICAM-1 is involved in the pathogenesis of chronic rhinosinusitis and allergic rhinitis [16].

In another study [17], there were significantly elevated concentrations of sICAM-1 in nasal secretion detected only in patients with chronic non-allergic rhinosinusitis (79.4 ± 45.6 ng/ml). The elevated sICAM-1 nasal secretion values in this group correlated significantly to the serum values. Elevated concentrations of sICAM-1 in patients with chronic non-allergic rhinosinusitis pointed to its key role in the recruitment of neutrophils into the inflamed tissue, whereas an important role in eosinophil recruitment was ruled out [17]. Our results about sICAM-1were in agreement with these results since we found a significant difference in Group I CRS F+/AM- rhinosinusitis in comparison to healthy control group.

Another study investigated maxillary sinus mucosal specimens from patients with chronic rhinosinusitis and from normal subjects were immunostained with specific antibodies directed against several cytokines (IL-1alpha, IL-1beta, IL-6, IL-8 and TNF-alpha) and two adhesion molecules (ICAM-1 and VCAM-1). The number of immunoreactive cells for IL-1alpha, IL-1beta, IL-6, IL-8, and TNF-alpha was increased significantly in patients with chronic rhinosinusitis compared with normal controls. Immunoreactivity for ICAM-1 was also increased significantly in patients with chronic rhinosinusitis compared with normal controls, whereas VCAM-1 is only minimally expressed or is absent in both groups.[18]

We found increased levels of IL-8 in the three rhinosinusitis groups compared to the control group. These results indicate the importance of IL-8 regarding its chemotactic activity for neutrophils in all types of rhinosinusitis and this has previously be explained by many authors. The IL-8 level in nasal discharge was significantly higher in the chronic rhinosinusitis group than in the allergic rhinitis group suggesting that chemotactic factors in sinus effusion, including IL-8 derived from nasal gland duct cells and epithelial cells, attract neutrophils out of the mucosa. The neutrophils that have migrated into the sinus effusion secrete IL-8. This induces further neutrophil accumulation in the sinus effusion of patients with chronic rhinosinusitis [19]. Whereas IL-8 mRNA was expressed in the maxillary mucosa, IL-8 transcripts were not detected in the inferior turbinate by Northern blot analysis. IL-8 transcripts were detected in 45% of chronic rhinosinusitis RNAs and in 50% of allergic rhinitis RNAs by RT-PCR, suggesting that

IL-8 may contribute to neutrophil involvement in chronic rhinosinusitis [20].

In our work, we considered total IgE levels as one of the parameters to assure the diagnosis of allergic fungal rhinosinusitis and its differentiation from other types of rhinosinusitis. We have found increased IgE levels in group II compared to group I, group III and the control group. Therefore, total IgE levels has been proposed as a useful indicator of allergic fungal rhinosinusitis clinical activity. Total IgE values are generally elevated in allergic fungal rhinosinusitis, often to more than 1000 U/ml [21]. Allergic fungal rhinosinusitis patients showed significantly more IgE in sinus mucosa tissue specimens [22]. The local tissue specific IgE is more specific than the systemic serum IgE profile in determining the allergic status of allergic fungal rhinosinusitis patients [23]. Mabry and Manninig [24] compared 16 patients with histologically confirmed allergic fungal rhinosinusitis with a control group with chronic rhinosinusitis. Levels of fungal-specific IgE were uniformly elevated in all patients with allergic fungal rhinosinusitis and corresponded with the results of fungal cultures. In contrast, levels of fungal-specific IgE were not elevated within the control group.

Long-term low-dose macrolide therapy was first introduced for the treatment of diffuse panbronchiolitis in the 1980's. In the 1990's, it was also shown to be an effective treatment for chronic rhinosinusitis. The inhibitory effect of macrolides on neutrophil infiltration in inflammatory sites has been well documented in these diseases. Several lines of evidence indicate that macrolides do not function simply as a bactericide, but they inhibit the production of IL-8 and IL-1beta as well as the expression of ICAM-1, suggesting that macrolides block the dual positive feedback system of neutrophil recruitment and thereby exert their clinical efficacy in the treatment of chronic rhinosinusitis. The inhibitory effects of macrolides on multiple steps in the process of neutrophil recruitment are presumably mediated by the inhibition of transcription factors such as nuclear factor-kB and activator protein-1 [8].

We selected erythromycin as a model for the macrolides to study its effect in the GroupII CRS F+/AM+ and after finishing treatment, the mean value of ICAM-1 had decreased. Also, the mean value of IL-8 decreased after treatment, indicating the efficacy of this drug to modulate the immunological aspect of this disease. These results concur that of many authors who demonstrated the effect of macrolides in chronic rhinosinusitis.

To clarify the basis of macrolide therapy for improvement of chronic rhinosinusitis, Iino et al. [25] investigated the effect of macrolides on the expression of HLA-DR and co-stimulatory molecules such as CD54 and CD80 on macrophages in nasal polyps. Nasal polyps taken from 54 patients who had or had not been treated with macrolides were immunohistochemically studied. The percentages of CD68-positive macrophages expressing HLA-DR or CD54 were not significantly different between patients treated with macrolides or not. However, among patients exhibiting no atopic predisposition, the number of CD80-positive macrophages was higher in patients treated with macrolides than in those not treated. In addition, the percentage of CD80-positive macrophages was negatively correlated with the percentage of infiltrating eosinophils in the polyps. These results demonstrated that macrophages act as antigen-presenting cells, expressing both major histocompatibility complex II and costimulators, and that the expression of CD80 may play a key role in the immune responses occurring in a nasal polyp. Macrolides may modulate the mucosal immune responses through CD80 expression.

CONCLUSION

Aspergillus fumigatus was the most prevalent in chronic rhinosinusitis with positive fungal culture with or without allergic mucin. ICAM-1 and IL-8 have a role in all types of rhinosinusitis. ICAM-1 and IL-8 expression decreased significantly after macrolides therapy indicating the efficacy of this drug to affect the immunological part of the disease.

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