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Negative impact of *Aspergillus* galactomannan and DNA detection in the diagnosis of fungal rhinosinusitis

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A proportion of patients with chronic rhinosinusitis, especially if nasal polyps are present, have a diagnosis of fungal rhinosinusitis. The diagnosis is difficult to establish because the symptoms and clinical and radiological signs are non-specific. Also current diagnostic methods, i.e. histology, fungal staining and culture, are insensitive. The performance of the *Aspergillus* galactomannan (GM) ELISA and real-time PCR for *Aspergillus fumigatus* mitochondrial DNA was evaluated for the detection of *Aspergillus* in sinus mucus samples from 25 patients with chronic rhinosinusitis with nasal polyposis. The results were compared with those from nasal lavage fluid from 19 healthy volunteers. Seven patients (28 %) were diagnosed as having fungal rhinosinusitis according to the presence of filaments in histology or direct microscopy using Calcofluor white. All fungal rhinosinusitis patients were negative in the GM ELISA. GM ELISA was positive in five patients whose samples were negative using conventional methods and *A. fumigatus* PCR. Two out of seven patients with fungal rhinosinusitis were positive by *A. fumigatus* PCR: one also had a positive *A. fumigatus* culture, and one had hyphae consistent with *Aspergillus* in histology. One additional patient had a weak positive PCR result, but other fungal tests were negative. In control subjects, the GM ELISA was positive in 21 %, whereas direct microscopy, culture and *A. fumigatus* PCR were negative in all samples. Direct microscopy and culture together with histology remain pivotal in defining fungal rhinosinusitis diagnosis. *A. fumigatus* PCR may have additional value in allowing the diagnosis to be made sooner, whereas the GM ELISA is not reliable in diagnosing *Aspergillus* infection of the paranasal sinuses.

INTRODUCTION

Chronic rhinosinusitis (CRS), defined as an inflammatory condition of the mucosa of the nose and the paranasal sinuses that persists longer than 12 weeks, is one of the most common chronic respiratory tract diseases, affecting approximately 5 % of the adult population (Fokkens *et al.*, 2005). In recent years, there has been an ongoing debate about the possible fungal aetiology of CRS. A positive fungal culture has been found in over 90 % of nasal lavage samples from CRS patients as well as from healthy volunteers (Braun *et al.*, 2003; Buzina *et al.*, 2003; Ponikau *et al.*, 1999). In these studies, there was no marked difference between the fungal species grown from the patient and the control samples, reflecting more likely the fungal colonization of the nose than the actual infectious cause of the disease. However, it is known that a number of fungi are capable of invading the paranasal sinuses, including *Aspergillus* species. It is estimated that approximately 5–10 % of CRS patients have a diagnosis of fungal rhinosinusitis (deShazo, 1998; Granville *et al.*,

Abbreviations: CRS, chronic rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyposis; C_T, threshold cycle; GM, galactomannan; NAL, nasal lavage.

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2004). Fungal rhinosinusitis presents as five distinct clinicopathological forms: three invasive forms, i.e. acute fulminant, chronic and granulomatous invasive fungal rhinosinusitis; and two non-invasive variants, i.e. sinus mycetoma and allergic fungal rhinosinusitis (deShazo, 1998). The non-invasive forms are more common, accounting for over 90 % of cases (Granville *et al.*, 2004). The diagnosis is based on clinical appearance, radiological imaging and histopathological evidence of fungal hyphae, but the diagnosis is difficult to establish because of the non-specific nature of symptoms and clinical and radiological signs as well as the insensitivity of current laboratory diagnostic methods (deShazo, 1998).

The prognosis of patients with invasive fungal rhinosinusitis depends upon early diagnosis. In recent years, rapid, non-culture-based methods to detect fungi have been developed, such as ELISA for *Aspergillus* galactomannan antigen (GM ELISA) and fungal PCR. The clinical utility of GM ELISA has been demonstrated in several studies, mainly in patients with haematological malignancies, and the test has been shown to cross-react with a number of hyaline moulds (Ascioglu *et al.*, 2002; Mennink-Kersten *et al.*, 2004). The GM ELISA has been evaluated in one study of 23 rhinosinusitis patients (Kauffmann-Lacroix *et al.*, 2001). Here the histology compared favourably with antigen detection while direct microscopic examination and culture were less sensitive. Also, PCR has proven to be more sensitive compared to fungal cultures, but results obtained by pan-fungal PCR have differed from those obtained by genus-specific primers, and genus-specific PCR results have been different from fungal culture results (Kim *et al.*, 2005; Polzehl *et al.*, 2005). Moreover, pan-fungal PCR has been reported to detect fungal DNA in comparable levels in nasal samples from CRS patients and controls (Catten *et al.*, 2001; Kim *et al.*, 2005). Similar to fungal culture results, the presence of fungal DNA in the nasal/sinus specimen is not enough to explain the role of fungi in the disease pathogenesis. However, PCR may have value as an adjunctive tool in diagnosing fungal rhinosinusitis and identifying the organism present in the microbiological/histological specimen (Buzina *et al.*, 2003; Willinger *et al.*, 2003).

With this background, we wished to evaluate the clinical usefulness of the *Aspergillus* GM ELISA and *Aspergillus fumigatus* PCR in detecting *Aspergillus* in chronic rhinosinusitis with nasal polyposis (CRSwNP). CRSwNP was chosen because it is a disease that is most often related to fungal infection of the paranasal sinuses (deShazo, 1998). Because fungal colonization in the nose is common, we also evaluated these assays in healthy volunteers.

METHODS

Patients and controls. Twenty-five patients (11 males, 14 females, aged 28–68 years) undergoing a paranasal sinus operation due to CRSwNP were enrolled. All patients had a history of CRS lasting longer than 12 weeks and findings of mucosal swelling and retention in some of the paranasal sinuses in preoperative CT scan. The presence of polyps was assessed by anterior rhinoscopy and further confirmed during the operation. Eleven patients (44 %) had self-reported allergic rhinitis, 14 patients (56 %) had asthma and 4 of them (29 %) had a history of aspirin intolerance. Fifteen patients (60 %) had earlier had one or more sinus operations. Nineteen healthy volunteers (8 males, 11 females, aged 29–60 years) from the Department of Otorhinolaryngology without any history of nasal or sinus operations served as volunteer controls.

The study was approved by the Ethical Committee of Helsinki University Central Hospital. Written consent was obtained from each patient.

Specimen collection. Patients' specimens were collected during the operation. Mucus from the paranasal sinus was placed into an empty sample tube for fungal examination. A specimen for fungal staining was taken with a sterile cotton swab and spread directly onto a microscope slide. The slide was then air-dried. The biopsy for histological analysis was obtained from the same paranasal sinus as the mucus.

The nasal lavage (NAL) method earlier described by Hirvonen *et al.* (1999) was used with some modifications for collecting samples from the controls. In brief, each nostril was flushed through with 5 ml PBS using a 5 ml sterile syringe and a sterile butterfly cannula of about 2 cm in length. The patient closed the nostrils by pinching them firmly together and leaned forward. PBS was pushed back and forth twice and finally collected into the syringe. Any residual PBS remaining in the nostril was collected in a pan placed underneath the nose and collected into the syringe.

Fungal staining and culture. The standard methods used in the diagnostic laboratories of Helsinki University Central Hospital to culture and identify fungi were used. The specimen was first vortexed to mechanically disperse the mucus. Calcofluor white fluorescence staining was carried out using the processed NAL sample or the sample placed directly onto a microscope slide and the preparation was examined using a fluorescence microscope with 40-fold magnification. The remaining sample was plated out on Sabouraud dextrose agar containing 6 mg penicillin l^{-1} and 26 mg streptomycin l^{-1}. The cultures were incubated at 28 and 37 °C and examined at 7 and 10 days.

All histological samples were stained with haematoxylin and eosin and with periodic acid–Schiff. If the periodic acid–Schiff staining was negative for fungi, the Gomori methenamine silver staining method was done.

GM ELISA. The *Aspergillus* GM antigen was detected in sinus mucus/NAL fluid by a direct double-sandwich ELISA (Platelia) according to the manufacturer's instructions. Positive and negative controls were included in each assay. Results were interpreted from the relationship of the absorbance of the sample to that of the threshold control (GM index=absorbance sample/absorbance threshold control).

A. fumigatus* PCR.** The samples were concentrated for DNA purification by centrifugation for 5 min at 3000 ***g, the supernatant was discarded, and 200 µl of the pellet was processed further. The release of fungal DNA was enhanced by lyticase treatment (7 U) at 30 °C for 1 h and homogenization with 0.3 g of zirconia–silica beads and a Mini-Beadbeater. The DNA purification was done with a High Pure PCR template preparation kit (Roche Diagnostic) following the instructions of the kit.

The PCR primers and probes used have been described by Rantakokko-Jalava *et al.* (2003). Briefly, the PCR amplified a 134 bp fragment from the *A. fumigatus* mtDNA encoding mitochondrial tRNA gene with primers 5′-GAA AGC TCA GGT GTT CGA

GTC A-3′ and 5′-CTT GGT TGC GGG TTT AGG GAT T. The *Aspergillus* tRNA gene was detected by fluorescence resonance energy transfer using the pair of probes tRNA FL (5′-TTC TTA TTT ATA TGC GGG TTG ATG TAA TAG TAA CA-3′) and tRNA LC (5′-AGA TGG CTC ATG ACC ATA ATA TTT AGG TGC p).

The DNA amplification was done using the LightCycler instrument as described by Rantakokko-Jalava *et al.* (2003). Forty-five cycles were performed, and the fluorescence was measured at the end of the annealing phase of each cycle. All runs contained distilled water as negative isolations and reaction controls and *A. fumigatus* DNA in four concentrations (20 ng, 2 ng, 200 pg and 20 pg per reaction mixture) as amplification standards. Samples with detectable exponential amplification on the fluorescence versus cycle plot were regarded as positive. The threshold cycle (C_T), i.e. the cycle at which the exponential amplification becomes detectable over the background fluorescence, has an inverse linear relation to the amount of target DNA in the original sample. All samples were tested for PCR inhibition by amplification of the human growth hormone gene as described previously (Rantakokko-Jalava *et al.*, 2000).

RESULTS AND DISCUSSION

A. fumigatus is a ubiquitous fungus capable of causing life-threatening infections usually in immunocompromised patients. *Aspergillus* species are the most common reported cause of fungal rhinosinusitis, accounting for up to 80 % of fungal pathogens (deShazo *et al.*, 1997). According to our clinical experience, *Aspergillus* is also the most common fungal genus found in the paranasal sinuses in the Finnish otorhinolaryngology patient population. Both ELISA for GM antigen and *Aspergillus* PCR have recently been developed for the rapid, more sensitive, and thus earlier diagnosis of *Aspergillus* infection. The clinical studies applying these assays have ascertained that both assays have significantly better sensitivity and specificity in patients with high likelihood of disease when compared to patients with merely a possibility of fungal infection (Jones & McLintock, 2003). This makes it problematic in the case of CRS, in which the prevalence of fungal infection may be as low as 5 % (Granville *et al.*, 2004). Moreover, at least 40 different fungal genera are described in CRS patients, although the causal association has not always been apparent, limiting the clinical usefulness of these assays in CRS (Braun *et al.*, 2003; Buzina *et al.*, 2003; Ponikau *et al.*, 1999). The inevitable colonization of the nose by *Aspergillus* species, which has been described in up to 60 % of nasal samples from healthy volunteers, complicates further the interpretation of positive results (Braun *et al.*, 2003; Buzina *et al.*, 2003; Ponikau *et al.*, 1999). However, taking into consideration the major role of *Aspergillus* in fungal rhinosinusitis and the low sensitivity of conventional methods to detect fungi, adjunctive tests to improve the accuracy and accelerate the diagnosis of fungal rhinosinusitis are needed.

The results for GM ELISA, *A. fumigatus* PCR, fungal staining and culture of the sinus mucus and fungal staining of tissue specimens in CRSwNP patients together with the clinical diagnosis are summarized in Table 1. A total of seven patients (28 %) were diagnosed as having fungal rhinosinusitis according to the presence of filaments in histological specimens or direct microscopic examination, whereas in the control group no sample was positive for fungi by either fungal staining or culture. The clinical diagnosis was mycetoma in two patients (patient nos 2 and 9). They had fungal hyphae morphologically consistent with *Aspergillus* visible in both sinus mucus and tissue specimens. The remaining five patients were considered to have allergic fungal rhinosinusitis-like syndrome. No invasive fungal infection was discovered. The occurrence of fungal rhinosinusitis in the present study (28 %) exceeds the estimated prevalence of fungal infection among rhinosinusitis patients in the literature (Granville *et al.*, 2004). Vennewald *et al.* (1999) reported an equal prevalence of fungal positive findings in 117 CRSwNP patients who also had granulomatous material within the sinus by endoscopy. The authors interpreted these results to represent fungal colonization of the paranasal sinuses instead of a fungal rhinosinusitis. However, these results may indicate that when the typical findings of fungal rhinosinusitis are present, there is a strong possibility of fungal infection (deShazo, 1998).

Five specimens (20 %) in the patient group and four (21 %) in the control group were positive by GM ELISA when the index cut-off value for a positive result was 1.0. It is noteworthy that the specimens from controls were collected using NAL, whereas sinus mucus samples collected during the operation were used in the patient group. Thus the positive results in the control samples may represent the colonizing presence of aspergilli in the nostrils, although every sample was negative for fungi when tested using conventional methods and *A. fumigatus* PCR. This discrepancy may be explained by the low sensitivity of conventional methods for detecting fungi and the specificity of the PCR method used here for *A. fumigatus*, not any other *Aspergillus* spp. (Rantakokko-Jalava *et al.*, 2003). However, in the patient group also every GM ELISA positive sample tested negative for fungi by both conventional methods and PCR. It is difficult to estimate how many of the positive results in both groups are true positive. It is well established that this assay gives false-positive results, especially in children, in whom a false-positive rate of as much as 83 % has been described (Mennink-Kersten *et al.*, 2004). The false-positive reactivity in serum may be due to the translocation of GM present in food across the intestinal mucosa or intravenous administration of the antibiotic piperacillin-tazobactam (Mennink-Kersten *et al.*, 2004). However, it is unlikely that the GM load of the digestive tract could explain its presence inside the paranasal sinuses. None of the patients had intravenous or topical antibiotic treatment before the operation. It is known that there is some degree of cross-reactivity between GM of various filamentous fungi (Giacchino *et al.*, 2006). Therefore, environmental exposure to other fungal species or other material rich in protein, like pollen, could also have caused the positive reactivity (Ansorg *et al.*, 1997; Giacchino *et al.*, 2006; Murashige *et al.*, 2005).

Table 1. Results for fungal examination and clinical diagnosis in patients operated on for chronic rhinosinusitis with nasal polyposis

GM index >1.0; AFRS-like sdr, allergic fungal rhinosinusitis-like syndrome; CRSwNP, chronic rhinosinusitis with nasal polyposis; C_T, threshold cycle.

Patient no.	Patient sex and age (years)	GM ELISA	*Aspergillus* PCR (C_T)	Fungal staining of sinus mucus	Fungal hyphae in tissue specimen	Culture	Clinical diagnosis
1	Male 36	−	−	Yeast cells	−	−	AFRS-like sdr
2	Female 49	−	+ (33.71)	Fungal hyphae	+	−	Mycetoma
3	Male 36	−	−	−	+	−	AFRS-like sdr
4	Female 47	Not taken	−	−	−	−	CRSwNP
5	Female 29	−	−	−	−	−	CRSwNP
6	Female 53	−	−	−	−	−	CRSwNP
7	Male 44	−	−	−	−	−	CRSwNP
8	Male 68	Not taken	Inhibitory	−	−	−	CRSwNP
9	Male 58	−	Not taken	Fungal hyphae	+	−	Mycetoma
10	Female 56	Not taken	+ (35.63)	Fungal hyphae	−	*A. fumigatus*	AFRS-like sdr
11	Female 56	+	−	−	−	−	CRSwNP
12	Female 38	−	−	−	−	−	CRSwNP
13	Female 28	−	−	−	−	−	CRSwNP
14	Female 52	+	−	−	−	−	CRSwNP
15	Female 66	−	−	−	−	−	CRSwNP
16	Female 56	−	Not taken	−	+	−	AFRS-like sdr
17	Female 42	−	+ (37.70)	−	−	−	CRSwNP
18	Male 36	+	−	−	−	−	CRSwNP
19	Male 62	−	−	−	−	−	CRSwNP
20	Female 54	−	−	−	−	−	CRSwNP
21	Male 48	−	−	−	−	−	CRSwNP
22	Male 53	−	−	Fungal hyphae	+	*A. niger*	AFRS-like sdr
23	Male 45	+	−	−	−	−	CRSwNP
24	Male 41	−	−	−	−	−	CRSwNP
25	Female 51	+	−	−	−	−	CRSwNP

Three definite false-negative GM ELISA results (GM index cut-off value 1.0) were obtained in the present study: two patients with morphologically suspected *Aspergillus* mycetoma and the patient with *Aspergillus* grown in culture. One explanation may be that the environment inside the paranasal sinuses is lacking in essential nutrients, especially glucose and oxygen, leading to a reduced amount of antigen released (Mennink-Kersten *et al.*, 2004). Antifungal drug therapy is known to reduce mycelial growth, resulting in suppression of GM expression, but none of the patients received antifungal drugs (Mennink-Kersten *et al.*, 2004). Another factor influencing the assay performance is the optimal index cut-off value to define positivity of GM ELISA. When the Platelia ELISA kit was first introduced a decade ago, the recommended index cut-off value for serum samples was 1.5. Since then, the cut-off value 1.0 has been applied in several studies. The US Food and Drug Administration have now approved the ELISA kit with a cut-off index of 0.5 when applied to serum. Musher *et al.* (2004) achieved the optimal performance of GM ELISA performed on bronchoalveolar lavage fluid by also using an index cut-off of 0.5. If the cut-off value of the GM index was lowered to 0.5 in the present study, three more patient samples and two more control samples were positive for GM. Only one of these three patients had positive fungal staining of sinus mucus, and the patient had yeast cells, not hyphae typical of *Aspergillus* species. Thus lowering the cut-off value did not improve the assay performance in this study.

The poor performance of GM ELISA in this study differs considerably from the results obtained by Kauffmann-Lacroix *et al.* (2001). In their study of 23 rhinosinusitis patients, out of which 16 had fungal rhinosinusitis, 20 patients had concordant GM ELISA and mycological and/or histological results in sinus mucus samples obtained during endoscopic operation. The sensitivity and specificity of Platelia *Aspergillus* ELISA were 87 % and 88 %, respectively. Perhaps the lower prevalence of fungal rhinosinusitis among the patients explains partly the much poorer performance of GM ELISA seen in the present study. However, recent studies have shown significant variation in the performance of the GM assay with the overall sensitivity being as low as 29.4 % (Herbrecht *et al.*, 2002; Weisser *et al.*, 2005). The importance of serial screening has been highlighted because of false-positive results, whereas we tested a single sample only. However, in the recent study even the serial screening of GM in serum

from patients at risk for invasive aspergillosis did not improve the performance of the test, as the positive GM ELISA result, determined as GM index of $\geqslant 0.5$ in two consecutive measurements, did not precede detection of major lesions by pulmonary CT (Weisser *et al.*, 2005).

The *A. fumigatus* PCR identified the patient with fungal rhinosinusitis caused by *A. fumigatus* (patient no. 10). Also the mycetoma patient (patient no. 2 in Table 1) most likely had a true-positive PCR result, because the hyphae present in histology were morphologically consistent with *Aspergillus*, although culture remained negative. The third patient (no. 17) with a positive *A. fumigatus* PCR had otherwise negative fungal results and did not meet the criteria of fungal rhinosinusitis. The C_T of this sample was >37.70, indicating that the amount of *A. fumigatus* DNA in the sample was low and may be derived from fungal colonization of the nose. Unfortunately we were not able to test the other mycetoma patient (no. 9) with probable *Aspergillus* infection by *A. fumigatus* PCR because of the limited volume of the sample. The PCR assay used in the present study is designed to be specific for *A. fumigatus* (Rantakokko-Jalava *et al.*, 2003). In fact, the one patient whose fungal culture grew *Aspergillus niger* had a negative PCR result. Rantakokko-Jalava *et al.* (2003) in their study of patients with invasive aspergillosis found the sensitivity and specificity of the same *A. fumigatus* PCR method applied to bronchoalveolar lavage and tissue biopsy specimens to be 72 % and 93 %, respectively. All the patients with positive PCR and $C_T < 35$ had a proven or probable invasive pulmonary aspergillosis, whereas samples with higher C_T values were obtained both from patients with invasive disease and from those who were at risk, but had no other evidence of invasive disease. The respiratory tracts of these patients were probably colonized by *A. fumigatus*. In the present study, every control sample tested negative by *A. fumigatus* PCR, although the GM ELISA was positive in 21 %, casting more doubt that fungal colonization would explain the positive GM ELISA results in controls. Detection of GM by ELISA has been suggested to be more sensitive than amplification of *Aspergillus* mitochondrial DNA from serum mainly due to the higher concentration of GM (Costa *et al.*, 2002). However, the lack of any concordance between GM ELISA results and results obtained by other methods used in the patient group makes it impossible to compare these assays in the control group either.

In conclusion, fungal staining and culture of sinus mucus together with histology using specific fungal stains remain pivotal in defining diagnosis and differentiating invasive from non-invasive forms of fungal rhinosinusitis. *A. fumigatus* PCR did not increase the accuracy of fungal rhinosinusitis diagnosis, but it may accelerate the detection of fungi in nasal/sinus samples and thus it may have additional value in diagnosing *Aspergillus* rhinosinusitis. Conversely, the GM ELISA, whether the result is either positive or negative, is not reliable in the diagnosis of *Aspergillus* infection of the paranasal sinuses.

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