Aspergillus fumigatus and *Candida albicans* in Cystic Fibrosis: Clinical Significance and Specific Immune Response Involving Serum Immunoglobulins G, A, and M

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OBJECTIVE: The aim of this study was to analyze the clinical significance of *Aspergillus fumigatus* and *Candida albicans* in respiratory secretions from patients with cystic fibrosis and to assess the immune response to these fungi in serum.

PATIENTS AND METHODS: The study included 66 patients with cystic fibrosis (34 men; mean age, 16.2 years). Sera from 15 healthy individuals were used as controls.

RESULTS: The serum concentrations of immunoglobulin (Ig) G, IgA, and IgM against *A fumigatus* and *C albicans* were higher in patients than in the control group. There was no correlation between the presence of *A fumigatus* in respiratory secretions and the immune response to the fungus measured in serum. In contrast, the presence of *C albicans* in respiratory secretions was correlated with the immune response to that fungus. The likelihood of obtaining *A fumigatus* cultures from respiratory secretions increased with age. The presence of these fungi in respiratory samples was not a risk factor for greater respiratory impairment.

CONCLUSIONS: In response to increased colonization of the lower respiratory tract by *A fumigatus* and *C albicans*, patients with cystic fibrosis have elevated serum levels of IgG, IgA, and IgM against those fungi. In patients with cystic fibrosis, culture of sputum and oropharyngeal secretions is adequate for the assessment of lower respiratory tract colonization by *C albicans* but not *A fumigatus*. Fungal colonization of the lower respiratory tract is not a risk factor for greater respiratory impairment in patients with cystic fibrosis.

Key words: Aspergillus fumigatus. Candida albicans. *Cystic fibrosis. Fungal colonization.*

Aspergillus fumigatus y *Candida albicans* en la fibrosis quística: significado clínico e inmunorrespuestas séricas específicas de inmunoglobulinas G, A y M

OBJETIVO: Estudiar el significado clínico de *Aspergillus fumigatus* y *Candida albicans* en las secreciones respiratorias de los pacientes con fibrosis quística (FQ) y las inmunorrespuestas séricas frente a estos hongos.

PACIENTES Y MÉTODOS: Se estudió a 66 pacientes con FQ (34 varones; edad media: 16,2 años). Como grupo control se utilizaron los sueros de 15 individuos sanos.

RESULTADOS: Las concentraciones de inmunoglobulina (Ig) G, IgA e IgM frente a *A. fumigatus* y *C. albicans* estuvieron más elevadas en los pacientes con FQ que en el grupo control. No hubo correlación entre el hallazgo de *A. fumigatus* en las secreciones respiratorias y las inmunorrespuestas séricas frente al hongo. Sí hubo correlación entre la presencia de *C. albicans* en las secreciones respiratorias y las inmunorrespuestas frente a *C. albicans*. A medida que aumentaba la edad de los pacientes, aumentaba la probabilidad de cultivar *A. fumigatus* en las secreciones respiratorias. La presencia de estos hongos en muestras respiratorias no fue un factor de riesgo para un mayor deterioro respiratorio.

CONCLUSIONES: Como respuesta a la elevada colonización del aparato respiratorio inferior por *A. fumigatus* y *C. albicans*, los pacientes con FQ presentan respuestas séricas elevadas de IgG, IgA e IgM frente a estos hongos. En los pacientes con FQ el cultivo de esputos y aspirados orofaríngeos no sirve para evaluar la colonización del aparato respiratorio inferior por *A. fumigatus*, pero sí por *C. albicans*. En estos pacientes, la colonización fúngica del aparato respiratorio inferior no es un factor de riesgo para el deterioro respiratorio.

Palabras clave: Aspergillus fumigatus. Candida albicans. *Fibrosis quística. Colonización fúngica.*

Introduction

Cystic fibrosis (CF) is associated with chronic bronchopulmonary bacterial infections that give rise to bronchiectasis. In addition to bacterial colonization, patients with CF are predisposed to fungal colonization due to the

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capacity of some fungi to colonize the lower respiratory tract and to the frequent courses of antibiotics that are required to control the disease.[1,2] The most commonly isolated fungi in respiratory secretions from these patients are *Aspergillus fumigatus* and *Candida albicans*. *A fumigatus* is isolated from respiratory secretions of patients with CF in between 9% and 57% of samples,[3,4] and the rate is somewhat higher in the case of *C albicans*.[4,5] In patients with CF, colonization of the lower respiratory tract by *A fumigatus* results in a high rate of immune responses to the fungus.[6,7] To date, the role of fungi in CF has not been clearly defined, although they are thought not to be pathogenic,[8] except in cases of invasive aspergillosis[9] or allergic bronchopulmonary aspergillosis.[10,11] Although some authors have reported that in certain cases *A fumigatus* can contribute to the pathogenesis of the disease, perhaps due to virulence factors specific to this fungus,[12] others have not found evidence of this.[3]

In this study, we addressed 3 main objectives: *a)* to analyze the serum immune response to *A fumigatus* and *C albicans* involving immunoglobulin (Ig) G, IgA, and IgM in patients with CF; *b)* to determine whether culture of respiratory secretions is a reliable indicator of colonization of the lower respiratory tract by these fungi in patients with CF; and *c)* to analyze the clinical significance of the presence of these fungi in respiratory secretions from patients with CF.

Patients and Methods

Study Population

The study group comprised 66 patients—34 male and 32 female—with a mean (SD) age of 16.2 (8.3) years (range, 3-36 years). In all patients, diagnosis was established based on symptoms indicative of CF and a sweat test with concentrations of chloride greater than 60 mEq/L in 2 determinations.[13] The mean forced vital capacity (FVC) as a percentage of predicted was 79.85% (26.24%) and the forced expiratory volume in 1 second (FEV_1) was 72.09% (31.28%). The study was approved by the ethics committee of the hospital. All patients were informed about the characteristics of the study, but signed consent was not required since patients were cared for according to the standard clinical procedures used in the CF unit.

Study Design and Methods

Patients were assessed prospectively during follow-up appointments in the CF unit (every 3 or 4 months) over a period of 2 to 6 years. At each appointment, in addition to a general physical examination, a selective examination of the mouth was done to determine whether it was colonized by *C albicans*. Patients in whom *C albicans* was found to be present in the mouth were treated with a topical antifungal agent and were temporarily withdrawn from the study until the fungus was eradicated. Additional consultations and tests were carried out if considered necessary by the physicians responsible. An annual follow-up was undertaken to include a chest radiograph (evaluated according to the Brasfield score, always by the same respiratory medicine specialist)[14] and spirometry. All respiratory secretions were cultured for fungi over the 6 years of the study. During the last 4 years of the study, serum samples from each patient were analyzed at least every 8 months. Following the initial phase of the study, which is described elsewhere,[6] the remaining serum was frozen at –20ºC for subsequent processing in the second phase of the study. In this second phase, the humoral immune response to *A fumigatus* and *C albicans* was assessed by analysis of specific IgG, IgA, and IgM in those patients in whom at least 4 samples of respiratory secretions had been analyzed in the last year of the study (n=66). Sera from 15 healthy, nonatopic individuals who were nonsmokers and of a similar age and sex were used as a control group.

Definition of Specific Diagnoses

Various groups were defined according to the microbiologic results: patients with at least 1 positive culture for *A fumigatus* or *C albicans* in respiratory secretions over the course of the study (patients with *A fumigatus* or *C albicans*, respectively) and patients who never had positive cultures of respiratory secretions for *A fumigatus* or *C albicans* (patients without *A fumigatus* or *C albicans*, respectively).

Processing of Respiratory Secretions

Respiratory samples (sputum or oropharyngeal secretions in patients aged less than 2 years or those in whom expectoration was difficult) were collected with varying frequency, usually 1 sample per appointment, with a maximum of 1 sample per month. The samples were treated with N-acetylcysteine for no more than 30 minutes in order to reduce the viscosity and generate a fluid sample. Aliquots were then prepared in duplicate in Sabouraud-chloramphenicol and Sabouraud-chloramphenicol-cycloheximide media and cultured at 30ºC and 37ºC for 4 weeks. The cultures were checked daily and assessed by spectrophotometry every week.

Spirometry

Spirometry was performed in 60 patients aged over 5 years who were able to perform the maneuvers. FVC and FEV_1 were measured without bronchodilation using a VMax 20 spirometer (SensorMedics Corporation, Yorba Linda, California, USA) according to the recommendations of the Spanish Society of Pulmonology and Thoracic Surgery.[15] The results were expressed as a percentage of the reference values of the European Community for Steel and Coal according to age, sex, weight, height, and ethnicity.[16] The mean values for each patient over the course of the study were used for statistical analysis.

Brasfield Score

The Brasfield score considers 5 radiologic variables: air trapping, linear markings, nodular cystic lesions, segmental or lobular consolidations, and impression of overall severity. In the case of air trapping, linear markings, and nodular cystic lesions, a score of 0 indicates absence and scores of 1 to 4 are applied for increasing severity. In the large lesions (segmental or lobar consolidation), a score of 0 indicates absent and scores of 3 to 5 are applied according to severity. For overall impression of severity, a score of 0 indicates normal appearance and scores of 1 to 5 are applied according to increasing severity. The total score is obtained by subtracting the sum from 25. Thus, a higher score reflects a better radiographic classification, with 25 indicating a chest radiograph without alterations. The mean values for each patient over the course of the study were used for statistical analysis.

Analysis of Specific Serum IgG, IgA, and IgM Against A fumigatus *and* C albicans

Enzyme-linked immunosorbent assay was used to analyze specific IgG, IgA, and IgM to *A fumigatus* and *C albicans*.

For *A fumigatus*, each well of the microtiter plate (Costar 3590, Costar Corp, Cambridge, USA) was coated with 0.1 mL of *A fumigatus* antigen diluted 1:2000 in phosphate buffered saline (PBS), pH 7.2. Following incubation for 18 hours at 4ºC the plate was washed with PBS containing Tween 20 (PBST; Sigma, St Louis, Missouri, USA) and then 0.1 mL of PBS containing 2% bovine serum albumin (Sigma) was added to each well and incubated at 37ºC for 1 hour. The plate was then washed again with PBST and 0.1 mL of patient serum was added at a dilution of 1:1000 in PBST and incubated for 1 hour at ambient temperature. The plates were then washed again with PBST and the following antibodies (Biosource International, Camarillo, California, USA) added prior to incubation for 1 hour at ambient temperature: peroxidase-conjugated anti-human IgG diluted 1:8000 in PBST containing 25% fetal bovine serum for detection of IgG; anti-human IgA at a dilution of 1:2000 for detection of IgA; and anti-human IgM at a dilution of 1:2000 for detection of IgM. The wells were incubated in PBST and 0.1 mL of substrate (2 mg/mL *o*-phenylenediamine in distilled water and 0.03% hydrogen peroxide) was added at ambient temperature. After 15 minutes, the color reaction was stopped by addition of 0.1 mL of 4N sulfuric acid. The results were expressed as optical density (OD) measured at 492 nm in a Titertek-Multiscan spectrophotometer (Flow Laboratories, Irvine, UK). All tests were performed in duplicate. For analysis of specific IgG, IgA, and IgM against *C albicans*, the same method was used with anti-human immunoglobulin antibodies from Sanofi Diagnostic Pasteur (Laboratorio Arístegui, Bilbao, Spain). Sera from 15 healthy nonatopic nonsmokers were used as controls. The test was considered positive for a given immunoglobulin when a value greater than the mean plus 2 SDs of the control value for that immunoglobulin was obtained. The following values were considered positive for specific immunoglobulins against *A fumigatus*: IgG, OD >0.67; IgA, OD >0.21; IgM, OD >0.94. The following values were considered positive for immunoglobulins against *C albicans*: IgG, OD >0.56; IgA, OD >0.41; IgM, OD >0.90.

Statistical Analysis

The χ^2 test was used to compare qualitative variables. The Brasfield score was considered a semiquantitative variable and nonparametric tests were used for analysis of associations with other variables (Kruskal-Wallis test, Spearman rank correlation coefficient). If one of the variables was considered quantitative and the other qualitative, the difference between the means was analyzed and the homogeneity of the variances confirmed with the Bartlett test. If both variables were quantitative, correlations were analyzed with the Pearson correlation coefficient. Multivariate analysis was performed to study the possible association of the immune responses to *A fumigatus* and *C albicans* with age, sex, spirometric variables, and the Brasfield score. The sample mean was used as an estimate of the population mean. To estimate the variability of the population (variance, SD), the *t* test was used with n–1 degrees of freedom. All analyses were performed with the statistical programs DBase-IV, EpiInfo version 6.04-b (Borland International, Scotts Valley, California, USA), and SPSS for Windows version 8.0 (SPSS Inc, Chicago, Illinois, USA).

Results

A total of 1239 samples of respiratory secretions were cultured from 66 patients (mean number of cultures per patient, 18.77; range, 4-48). *A fumigatus* was isolated in 256 cultures (20.7%) and *C albicans* in 588 (47.5%). Forty patients had at least 1 positive culture for *A fumigatus* and 58 had at least 1 positive culture for *C albicans*, as reported previously.[6]

Over the course of the study, a positive immune response to *A fumigatus* and *C albicans* was observed on at least 1 occasion in 92.1% and 91% of the patients, respectively. The serum titers of IgG, IgA, and IgM to *A fumigatus* and *C albicans* were significantly higher in patients with CF than in the control group ($P<.001$, Table 1).

Patient Characteristics According to the Presence or Absence of A fumigatus *and* C albicans *in Respiratory Secretions*

The characteristics of the patients according to the presence or absence of *A fumigatus* and *C albicans* are shown in Table 2. Patients with positive cultures for *A fumigatus* were older than those in whom no positive cultures for that fungus were obtained, whereas there were no differences in age between patients with and without positive cultures for *C albicans*. In addition, age was associated with an increased probability of obtaining a positive culture for *A fumigatus* in respiratory secretions ($r=0.28$, $P<.05$) but not with the probability of a positive culture for *C albicans* ($r=0.10$, $P=.41$). No significant differences were found in the titers of specific immunoglobulins against *A fumigatus* in patients with or without positive culture for *A fumigatus* and there was no significant correlation between levels of specific immunoglobulins and the probability of obtaining positive cultures for *A fumigatus* in respiratory secretions.

The probability of culturing *C albicans* in respiratory secretions was associated with greater respiratory impairment, although statistical significance was only achieved for the Brasfield score ($\rho=-0.24$, $P<.05$). In contrast to the situation with *A fumigatus*, the levels of specific immune responses to *C albicans* were significantly higher in patients with positive cultures for *C albicans* than in those in whom no positive culture was obtained. In addition, there was a significant correlation between the probability of obtaining a positive culture for *C albicans* in respiratory samples and the levels of specific immunoglobulins ($r=0.63$, $P<.001$ for specific IgG against *C albicans*; $r=0.61$, $P<.001$ for specific IgA; and $r=0.40$, $P<.05$ for specific IgM).

TABLE 1
Comparison of the Specific Immune Responses to *Aspergillus fumigatus* and *Candida albicans* in Patients With Cystic Fibrosis and Healthy Control Subjects[a]

Immune Response	Patients With CF (n=66)	Control Subjects (n=15)	*P*
IgG – *A fumigatus*	0.94 (0.49)	0.43 (0.12)	<.001
IgA – *A fumigatus*	0.32 (0.23)	0.13 (0.04)	<.001
IgM – *A fumigatus*	0.99 (0.51)	0.50 (0.22)	<.001
IgG – *C albicans*	1.23 (0.81)	0.36 (0.10)	<.001
IgA – *C albicans*	0.74 (0.67)	0.19 (0.11)	<.001
IgM – *C albicans*	0.85 (0.47)	0.48 (0.21)	<.001

Abbreviations: CF, cystic fibrosis; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
[a]Data are shown as means (SD) of the optical density at 492 nm.

TABLE 2
Characteristics of Patients With Cystic Fibrosis (n=66) With and Without *Aspergillus fumigatus* and *Candida albicans* in Respiratory Secretions[a]

Characteristics	*A fumigatus*		*P*	*C albicans*		*P*
	Yes (n=40)	No (n=26)		Yes (n=58)	No (n=8)	
Age, y	17.17 (8.20)	12.23 (7.81)	<.05	15.15 (8.64)	15.75 (6.32)	.85
Sex, male/female	19/21	15/11	.58	29/29	5/3	.71
Brasfield score	17.47 (4.30)	18.92 (5.42)	.23	17.86 (4.67)	19.37 (5.73)	.41
FEV_1, % predicted	70.40 (26.72)	74.35 (40.33)	.53	71.72 (31.23)	72.09 (38.20)	.97
FVC, % predicted	78.44 (25.32)	81.61 (29.45)	.67	77.60 (31.08)	79.80 (26.27)	.84
IgG – *A fumigatus*, OD_{492}	0.95 (0.34)	0.83 (0.48)	.22	NA	NA	NA
IgA – *A fumigatus*, OD_{492}	0.30 (0.20)	0.30 (0.26)	1.00	NA	NA	NA
IgM – *A fumigatus*, OD_{492}	1.04 (0.43)	0.84 (0.33)	.14	NA	NA	NA
IgG – *C albicans*, OD_{492}	NA	NA	NA	1.36 (0.74)	0.65 (0.47)	<.05
IgA – *C albicans*, OD_{492}	NA	NA	NA	0.83 (0.63)	0.18 (0.08)	<.001
IgM – *C albicans*, OD_{492}	NA	NA	NA	0.93 (0.48)	0.63 (0.21)	<.05

Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; NA, not assessed; OD_{492}, optical density at 492 nm.
[a]Data are shown as the mean (SD) except for sex, which is shown as the ratio of men to women.

TABLE 3
Correlation Between Specific Serum Immunoglobulin Titers Against *Aspergillus fumigatus* and *Candida albicans* and Age and Respiratory Condition in Patients With Cystic Fibrosis (n=66)[a]

Characteristics	IgG – *A fumigatus*	IgA – *A fumigatus*	IgM – *A fumigatus*	IgG – *C albicans*	IgM – *C albicans*	IgM – *C albicans*
Age, y	0.30b	0.27[b]	0.07	0.20	0.11	0.11
Brasfield score	–0.43[c]	–0.53[c]	–0.28[b]	–0.47[c]	–0.35[b]	–0.35[b]
FEV_1, % predicted	–0.46[c]	–0.50[c]	–0.18	–0.37[b]	–0.15	–0.15
FVC, % predicted	–0.35[b]	–0.36[b]	–0.18	–0.25	–0.19	–0.19

Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
[a]Results are expressed as the Pearson correlation coefficient (*r*), except for the Bradfield score, which is expressed as the Spearman rank correlation coefficient (ρ).
[b]*P*<.05. [c] *P*<.001.

Correlation Between Specific Immune Responses and Age, Sex, and Respiratory Function in Patients With Cystic Fibrosis

Table 3 shows the correlations between the serum titers of specific IgG, IgA, and IgM against *A fumigatus* and *C albicans* and the characteristics of the patients. There was no statistically significant correlation between sex and concentrations of IgG, IgA, and IgM against *A fumigatus*. In contrast, women were found to have higher concentrations of specific IgG, IgA, and IgM against *C albicans* than did men, and this observation was statistically significant for IgG and IgM (*P*<.05).

To eliminate possible confounding factors, multiple linear regression analysis was undertaken. In that analysis, using FEV_1 as the dependent variable and IgG, IgA, and IgM against *A fumigatus*, presence of *A fumigatus* in respiratory secretions, age, and sex as independent variables, age was the variable that showed the greatest contribution to a lower FEV_1 ($\beta = -0.31$, SE=0.32, *P*<.05). Similar results were obtained when FVC was used as the dependent variable ($\beta = -0.18$, SE=0.42, *P*<.05). Following logistic regression analysis, age was the main variable associated with a lower Brasfield score (β=–0.14, SE=0.12, *P*<.05), defined for this analysis as a Brasfield score of less than 18.

When FEV_1 was used as the dependent variable and immune responses to *C albicans*, presence of *C albicans* in respiratory secretions, age, and sex as independent variables, age was the variable that showed the greatest contribution to a reduced FEV_1 (β=–0.22, SE=0.34, *P*<.05). Similar results were obtained when FVC was used as the dependent variable. In the logistic regression analysis, age was also the only variable associated with a lower Brasfield score.

Discussion

Our results show that most patients exhibited an immune response to *A fumigatus* and *C albicans* at some point during the course of the study, confirming the high rate of exposure of patients with CF to these fungi.[7] Comparison of the immune responses of CF patients with those of control subjects extended the findings of other authors[17] and showed that, compared to control subjects, patients with CF have significantly higher serum immunoglobulin titers for specific IgG, IgA, and IgM against *A fumigatus* and *C albicans* as a response to fungal colonization of the lower respiratory tract.[9] This ease with which the lower airways are colonized is due to the damage to the first line of defense against fungi (mucosal epithelial barrier and alveolar macrophages) as a result of bronchiectasis. This

fungal colonization stimulates a T helper 1-type response, with extensive production of IgG, IgA, and IgM against the fungi, and in some patients, a T helper 2-type response also occurs, with production of specific IgE.[6,18]

Consistent with the results of previous studies,[17] we found no relationship between the presence or absence of *A fumigatus* in respiratory secretions (sputum and oropharyngeal secretions) and the titers of IgG, IgA, and IgM against that fungus. In contrast, the titers of immunoglobulins against *C albicans* were higher in patients who had the fungus in respiratory secretions than in those who did not. Since the serum immune responses are indicative of the degree of exposure to the fungi,[19-21] it may be suggested that the culture of these respiratory samples does not reflect the extent of exposure of the lung tissue to *A fumigatus* but does reflect the exposure to *C albicans*. This may be explained by the presence of the fungus in respiratory secretions being dependent on the size of its spores, as observed by Mullins and Seaton.[22] Thus, *A fumigatus*, which has small spores, remains trapped in the distal airways and can be found in the lung more often than would be expected based on culture of sputum samples or oropharyngeal secretion. Another hypothesis to explain this discrepancy between the isolation of *A fumigatus* in respiratory samples and the specific immune responses would be that perhaps *A fumigatus* can trigger a specific immune response that would persist in the organism after the fungus had been eliminated by local phagocytes.[23] Therefore, we believe that, in patients with CF, culture of sputum and oropharyngeal secretions is not a valid method with which to assess colonization of the lower airway by *A fumigatus*, since it underestimates colonization by that fungus, but is valid for assessment of *C albicans* colonization. Our results show that specific humoral immune responses to *A fumigatus* and *C albicans* can function as good markers of respiratory tract colonization by these 2 fungi.[20]

However, our study has certain methodological limitations, since contamination by *C albicans* was only assessed by physical examination of the oral cavity and other possible reservoirs, such as the digestive tract or vagina, were not ruled out. Thus, the finding that women with CF had significantly higher titers of IgG and IgM antibodies against *C albicans* could be explained by the ease with which *C albicans* can colonize the female genitourinary tract, especially in individuals who receive frequent courses of antibiotic treatment, and the associated possibility of generating specific immune responses to that fungus.[24]

In our study, we also sought to determine whether *A fumigatus*, *C albicans*, or the immune responses to those fungi could be risk factors for greater respiratory impairment. Since many of the variables studied are related to others (for instance, respiratory function declines with increasing age), multivariate analysis was done in order to eliminate confounding factors. Once these were eliminated, we demonstrated that the presence in respiratory secretions of neither *A fumigatus* nor *C albicans*, nor the presence of specific serum IgG, IgA, or IgM to those fungi were independent risk factors for greater respiratory deterioration, as has been reported by some authors for *A fumigatus*.[3]

In summary, patients with CF have elevated serum titers for specific IgG, IgA, and IgM against *A fumigatus* and *C albicans* in response to colonization of the lower airway by those fungi. Sputum samples and oropharyngeal aspirates are not valid for the assessment of the extent of colonization of the lower respiratory tract of CF patients by *A fumigatus*; instead, serum immune responses involving specific IgG, IgA, and IgM against *A fumigatus* should be employed. Increasing age of the patients was the main risk factor associated with the probability of obtaining a positive culture for *A fumigatus* in respiratory samples. Finally, we would like to highlight our observation that the presence of *A fumigatus* or *C albicans* in respiratory secretions from patients with CF is not an independent risk factor for increased respiratory impairment and, therefore, that specific treatment of fungal colonization should not be employed.

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