

Increased chitinase expression and fungal-specific antibodies in the bronchoalveolar lavage fluid of asthmatic children

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Summary

Background Increasing evidence highlights the contribution of chitinases and fungal infection to the development of asthma.

Objective The purpose of this study was to characterize chitinase expression and serological markers of fungal infection in children with severe asthma.

Methods Bronchoalveolar lavage fluid (BALF) was collected from children undergoing clinically indicated flexible bronchoscopy. A diagnosis of asthma was confirmed by pulmonary function testing. BALF was tested for chitinase activity and YKL-40 (an enzymatically inactive chitinase) concentrations. Specimens were cultured for fungal organisms and tested for cryptococcal antigen by ELISA. IgG and IgA reactivity to whole extract fungal (*Aspergillus fumigatus*, *Alternaria alternata*, *Cryptococcus neoformans* and *Candida albicans*) proteins were determined by immunoblot assay.

Results Among the 37 patients studied, 30 were asthmatic and 7 were non-asthmatic. Asthmatics exhibited elevated serum IgE levels (median: 748 IU/mL, IQR: 219–1765 IU/mL). Chitinase activity was greater in the BALF of asthmatics (mean, 0.85 ± 1.2 U/mL) compared with non-asthmatics (mean: 0.23 ± 0.21 U/mL, $P = 0.012$). Likewise YKL-40 concentrations were higher in the BALF of asthmatics and correlated with chitinase activity. There was a trend towards increased fungal-specific IgG in the BALF of asthmatics compared with non-asthmatics and for *C. albicans* this difference reached statistical significance. IgA reactivity to *C. neoformans* and *A. fumigatus* was greater in the BALF of asthmatics compared with non-asthmatics.

Conclusions and Clinical Relevance Compared with non-asthmatics, asthmatic children exhibited increased chitinase activity and increased YKL-40 levels in BALF. Increased IgG and IgA reactivity to fungal proteins in the BALF of asthmatics may reflect a local response to fungal infection. Our findings are consistent with and suggest a role for chitinases in asthma pathogenesis among Bronx children and provide serological evidence of an association between fungal infection and severe asthma.

Keywords asthma, bronchoalveolar lavage fluid, chitinase, fungal infection, YKL-40

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Introduction

Fungi are well-recognized environmental allergens that are mainly thought to exacerbate asthma in association with recurrent, transient respiratory exposures. Findings from epidemiologic studies highlight the importance of fungi as allergens. Fungal sensitization is more common in children than adults [1, 2]. In addition, fungal

sensitization is associated with severe, life-threatening asthma [3, 4].

Fungi can also elicit asthma symptoms in association with persistent colonization or superficial invasion of host tissue. The best-documented example of asthma related to fungal colonization is allergic bronchopulmonary aspergillosis (ABPA) [5]. Affected patients experience both airway obstruction and damage that is

mediated by an inappropriate inflammatory response (reviewed in [6]). While *Aspergillus* spp. is the most commonly implicated fungus, a role for other fungi has been hypothesized [7, 8]. Fungal sinusitis and dermatophytic infections have also been associated with asthma exacerbations [9, 10]. Recent studies by Denning *et al.* [11] suggest that severe asthmatics, who do not meet criteria for ABPA but have evidence of fungal sensitization, benefit from antifungal therapy, suggesting that unrecognized fungal infection contributes to their asthma symptoms.

Cryptococcus neoformans is an encapsulated fungal pathogen that is well known for its environmental association with pigeon droppings, where densities of 1×10^7 organisms per gram can be reached [12]. *Cryptococcus neoformans* infection is acquired through inhalation and can result in persistent pulmonary infections in immunocompetent individuals [13, 14]. Serologic studies indicate that subclinical cryptococcal infection is common among individuals from New York City, including children from the borough of the Bronx [15–17]. In animal models, experimental *C. neoformans* infection induces allergic inflammation and has been used to study the pathogenesis of allergic bronchomycosis [18–20]. Experimental persistent pulmonary infection in a rat model is associated with enhanced allergic inflammation and airway hyper-reactivity that is ameliorated with fluconazole treatment [21]. Based on these observations, we have hypothesized that subclinical cryptococcosis may contribute to the disproportionately high rates of severely poorly controlled asthma in urban areas [22].

Several fungal products, including proteases and prostanoids, have been hypothesized to directly promote asthma symptoms [23, 24]. Chitinases are important elements of the innate immune response to fungal infection [25] and have been implicated as important mediators of allergic inflammation [26]. Increased serum YKL-40 levels, a human chitinase, have been found in the serum and lungs of asthmatic patients [27, 28]. Studies in our lab and others suggest that fungal cell wall chitin may promote allergic (TH2) inflammation through the induction of host chitinases [29, 30]. To better understand the relationship between fungal infection, chitinases and asthma in children from the Bronx, we analysed the bronchoalveolar fluid (BALF) from asthmatic children for evidence of chitinase induction and fungal infection.

Materials and methods

Study design

All children (age 2–21 years) with a suspected or confirmed diagnosis of asthma who were referred to the

paediatric pulmonology clinic and required flexible bronchoscopy with lavage between June 2007 and January 2010 were approached for enrollment. Most children in this cohort had severe asthma with confirmed reversible, expiratory airway obstruction as demonstrated by spirometry studies. Children for whom a diagnosis of asthma was initially considered, but ultimately found to have alternative diagnoses were studied as non-asthmatic controls. Informed consent (and child assent, if applicable) was obtained for all subjects.

BALF collection

Lavage was performed in either the right middle lobe or lingula using normal saline (3 mL/kg divided into three equal aliquots, 60 mL maximum). Specimens were sent for cell differential counts and routine bacterial and fungal culture (see below). Surplus fluid was collected and stored in -80°C until analysis. The study was approved by the Committee on Clinical Investigations, Albert Einstein College of Medicine.

Serum immunoglobulin E levels

As part of routine care, serum was sent for IgE level measurements in the majority of patients. For patients with multiple measurements, IgE levels at or near the time of bronchoscopy as well as peak IgE levels were recorded.

Chitinase activity and YKL-measurements

Chitinase activity assay was determined using an established protocol [26]. Briefly, BALF specimens were incubated with 4-methylumbelliferyl-D-N, N'-diacetylchitobioside (Sigma, St. Louis, MO, USA), which is cleaved by bioactive chitinase, thereby releasing a quantifiable fluorogenic substance, 4-methylumbelliferone (excitation 350 nm; emission 450 nm). Bioactive chitinase from *Serratia marcescens* (Sigma) was used for standard measurements. YKL-40 levels in undiluted BALF were measured using a commercially available ELISA (Quidel, San Diego, CA, USA), according to the manufacturer's instructions. The lower limit of detection for this assay is 5.4 ng/mL.

Fungal strains and growth conditions

Cryptococcus neoformans strain 24067 (serotype D), *Candida albicans* (BSMY 212) and *Alternaria alternata* (20084) were obtained from American Type Culture Collection (Manassas, VA, USA). *Aspergillus fumigatus* 293 was a gift of Dr Marta Feldmesser (Albert Einstein College of Medicine). Protein extracts of *C. neoformans*

and *C. albicans* were obtained by glass beading of fungal pellets following growth in Sabouraud's dextrose broth for 3 days as described [31]. *Aspergillus fumigatus* was grown in minimal media [32] for 4–6 days at 30°C. Organisms were collected by filtration and freeze dried, followed by mechanical disruption with a glass pestle in a PBS solution containing protease inhibitors (Boehringer Ingelheim). Protein extracts for *A. alternata* were obtained in a similar manner to *A. fumigatus*, although the organism was grown in Sabouraud's dextrose broth. Protein extracts were quantitated by bicinchoninic acid assay (Pierce, Rockford, IL, USA) and frozen at –80°C prior to use.

Culture and antigen test

All BALF was sent to the microbiology lab at Montefiore Medical Center for routine bacterial and fungal culture. BALF was also tested for cryptococcal antigen using the Premier Cryptococcal Antigen ELISA (Meridian Bioscience Inc., Cincinnati, OH, USA) according to manufacturer's instructions.

Antibody studies

BALF were tested for antibodies against fungal proteins using an immunoblot assay, previously described [33]. Approximately 400 µg of each protein extract was separated by electrophoresis in the Bio-Rad Mini-Protein II system (Bio-Rad, Hercules, CA, USA) with a 10% resolving gel at 100 V. Gels were transferred to nitrocellulose membranes, which were then blocked for 1 h at room temperature or overnight at 4°C in a buffer containing 5% milk in PBS with 0.1% Tween-20. Individual channels on a blotting frame were incubated with human (1 : 1600) sera for 1 h at room temperature or overnight at 4°C. Channels were washed three times with blocking buffer and then incubated with a horseradish peroxidase-conjugated goat antibody to human IgG, or IgA, and then developed with luminol.

Statistics

Median ages of the two groups were compared by Mann–Whitney test. Comparisons of ELISA absorbance values were done using Student's *t*-test. IgE concentrations and the median numbers of proteins recognized by the sera from different cohorts were compared by Kruskal–Wallis. *Post hoc* analysis was done using the Dunn's test. The proportions of BALF with YKL-40 activity and sera-demonstrating reactivity to fungal proteins were compared by Fisher's exact test. Fisher's exact test was also used to compare proportions of race, gender, residence, allergy and obesity between the two groups. Correlations between chitinase and YKL-40 were done by

Spearman's correlation. All statistics were done with Graph Pad InStat software (San Diego, CA, USA).

Results

Demographics

Forty-five patients were eligible for the study. Eight patients were excluded for one of several reasons including, active pneumonia at bronchoscopy ($n = 1$), insufficient BALF ($n = 3$), insufficient information recorded ($n = 2$), lung nodule, which could be consistent with fungal disease ($n = 1$) or bleeding during bronchoscopy ($n = 1$). Among the remaining patients, 30 were asthmatic and 7 were non-asthmatic. There were no differences in the average age, race or gender distribution between asthmatics and non-asthmatics (Table 1). Final diagnoses for non-asthmatics (e.g. after complete evaluation), included the following: recurrent aspiration, habit cough, recurrent stridor, hyper-trophied lingual tonsil, right middle lobe compression, Type 1A laryngeal cleft with recurrent aspiration. All asthmatics were considered severe since they were failing, at a minimum, Step 4 therapy. Most asthmatics were being treated with a combination of inhaled steroids and a leukotriene inhibitor and/or long-acting beta agonist, whereas some who had previously failed combination therapy were maintained on oral steroids or omalizumab (Table 1). A significantly greater proportion of asthmatics compared with non-asthmatics were noted to have specific food or drug allergies.

Table 1. Epidemiologic and clinical characteristics of asthmatic and non-asthmatic children

	Asthmatics	Non-asthmatics	P-value
Age (years)*	9.1 ± 4.5	8.1 ± 4.6	0.60
Male, <i>n</i> (%)	19 (63)	4 (57)	1.00
Bronx residence (%)	27 (90)	6 (86)	1.00
Race, <i>n</i> (%)			
Multi	9 (30)	3 (43)	0.66
Hispanic	0	0	
Black	9 (30)	0	0.16
White	0	1 (14)	0.19
Not-specified	12 (40)	3 (43)	1.00
Asthma medications			
Inhaled steroid	28 (93)		
Oral corticosteroid†	7 (23)		
LABA	10 (33)		
LI	24 (80)		
Anti-IgE antibody	3 (10)		
Food or drug allergy	14 (47)	0	0.03
Obesity	6 (20)	0	0.57

LABA, long-acting beta agonist; LI, leukotriene inhibitor.

*Age in years ± 1 SD.

†Oral corticosteroid use within 2 weeks of procedure.

Serum immunoglobulin E levels and BALF cell differentials

Twenty-five asthmatics and seven controls had measurements of serum IgE levels. The median peak serum IgE level for asthmatics was 748 IU/mL (IQR: 219–1765 IU/mL) with a mean of 1411 IU/mL (Fig. 1). Eighty percent (20/25) of asthmatics had serum IgE levels >200 IU/mL (laboratory, upper limit of normal). In contrast, the median serum IgE level for non-asthmatics was 8 IU/mL (IQR: 1–293 IU/mL). There were no significant differences in the percentage of eosinophils or neutrophils in the BALF of asthmatics compared with non-asthmatics.

Chitinase activity and YKL-40 levels

The average chitinase activity in BALF was significantly greater for asthmatics compared with non-asthmatics (0.85 ± 1.2 vs. 0.23 ± 0.21 U/mL, $P = 0.012$; Fig. 1). In addition, detectable YKL-40 levels were present in approximately 87% and 29% of BALF specimens from asthmatics and non-asthmatics, respectively ($P = 0.03$). Among BALF samples from asthmatics (with detectable

YKL-40 levels), the average YKL-40 concentration was 14.3 ± 14.3 ng/mL (Fig. 2). There was also a positive correlation between BALF YKL-40 levels and chitinase activity ($R^2 = 0.71$, $P < 0.001$).

Fungal studies

Routine fungal cultures of all BALF (asthmatics and non-asthmatics) were negative for fungi. Furthermore, cryptococcal antigen was not detected in BALF by ELISA.

Antibodies to fungi

Immunoglobulin G reactivity. BALF was tested for the presence of IgG to protein extracts by immunoblot using proteins extracted from fungi (Figs 3 and 4). Prior studies have shown that the number of bands can be used as an indicator of prior infection [16, 31, 33]. There was a trend towards greater IgG reactivity to all fungi tested in the BALF of asthmatics compared with non-asthmatics (Fig. 3). For *C. albicans*, this difference was statistically significant. IgG reactivity against *C. neoformans* correlated with reactivity against *C. albicans* (Spearman

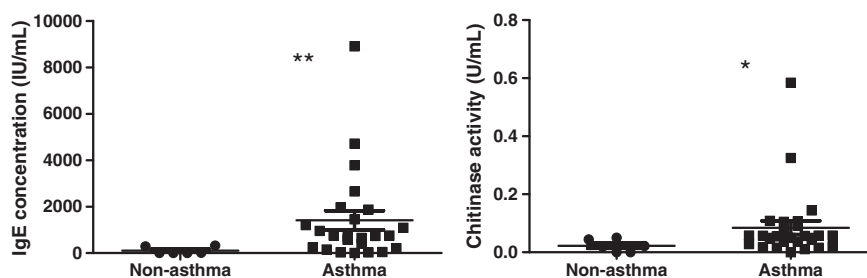


Fig. 1. Serum IgE levels and bronchoalveolar lavage fluid (BALF) chitinase activity in asthma versus non-asthma patients. Graph on left shows peak serum IgE levels for asthmatics and non-asthmatics. Graph on right shows chitinolytic activity of BALF from asthmatic and non-asthmatic samples. Each point represents a value from a single individual. Mean and SEM are shown. $**P < 0.001$, $*P < 0.05$.

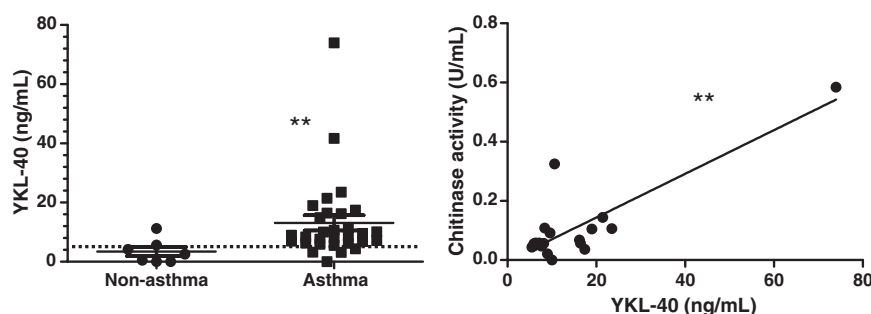


Fig. 2. Bronchoalveolar lavage fluid (BALF) YKL-40 levels in asthmatics. Graph on left shows BALF YKL-40 levels for asthmatics and non-asthmatics as determined by ELISA. Dotted line represents limit of detection of assay. $**P < 0.01$. P -value calculated assuming non-detected values are just below limit of detection for this assay. Mean and SEM are shown. Graph on right shows correlation between YKL-40 levels (horizontal) and chitinase activity (vertical axis) for BALF asthma samples in patients with detectable YKL-40 levels. $R^2 = 0.71$, $**P < 0.01$ for correlation between YKL-40 levels and chitinase activity.

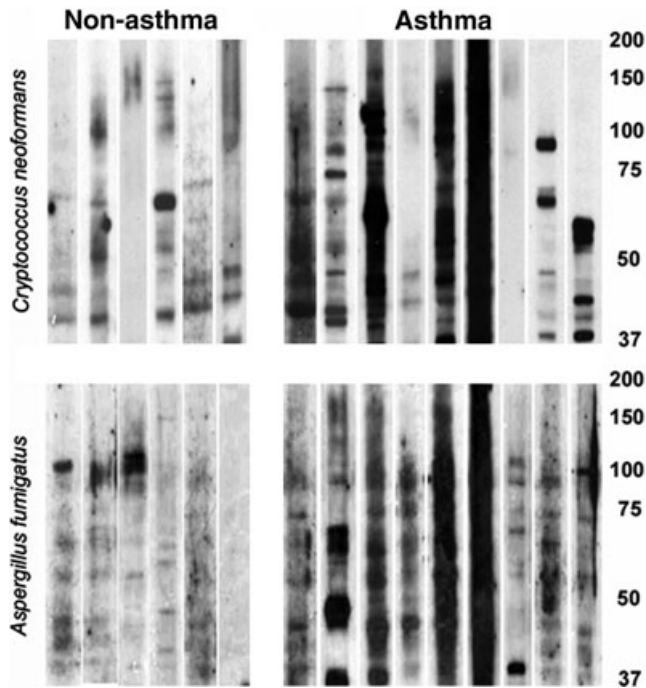


Fig. 3. Fungal-specific IgG in bronchoalveolar lavage fluid (BALF). Representative IgG immunoblots for BALF from asthmatics and non-asthmatics when reacted against *Cryptococcus neoformans* (top) and *Aspergillus fumigatus* (bottom) protein extracts are shown. Each lane represents BALF from a different individual. Top and bottom lanes are done with BALF from the same individual and show correlation between reactivity against *C. neoformans* and *A. fumigatus* proteins. Corresponding IgA blots are shown in Fig. 5. Molecular weight markers in kDa are shown on right.

$r = 0.77$, $P < 0.001$), *A. alternata* (Spearman $r = 0.59$, $P = 0.008$) and *A. fumigatus* (Spearman $r = 0.75$, $P < 0.001$).

Immunoglobulin A reactivity. Limited BALF was available for IgA studies and was only tested for reactivity against *C. neoformans* and *A. fumigatus* extracts (Figs 5 and 6). IgA reactivity to cryptococcal proteins was significantly more extensive in the BALF of asthmatics ($n = 21$) compared with non-asthmatics ($n = 7$) with a median number of bands of 7 and 1 respectively ($P = 0.002$). Likewise, IgA reactivity to *A. fumigatus* was more extensive in the BALF of asthmatics compared with controls ($P = 0.031$).

Discussion

Asthma is increasingly viewed as a heterogeneous disease that encompasses numerous etiologies, all of which result in airway inflammation and obstruction. In this context, phenotype-specific treatment of poorly controlled asthma, which includes both allergic and non-allergic diseases has been proposed [34, 35]. Findings from our study highlight the allergic nature of asthma in children from the Bronx, where 15% of school age children and 6.2% of adults may be affected (nyc.gov/html/doh/downloads/pdf/asthma/facts.pdf).

Human chitinases consist of a family of glycosylhydrolases, which can be further classified into enzymatically active (chitotriosidase and AMCase) and

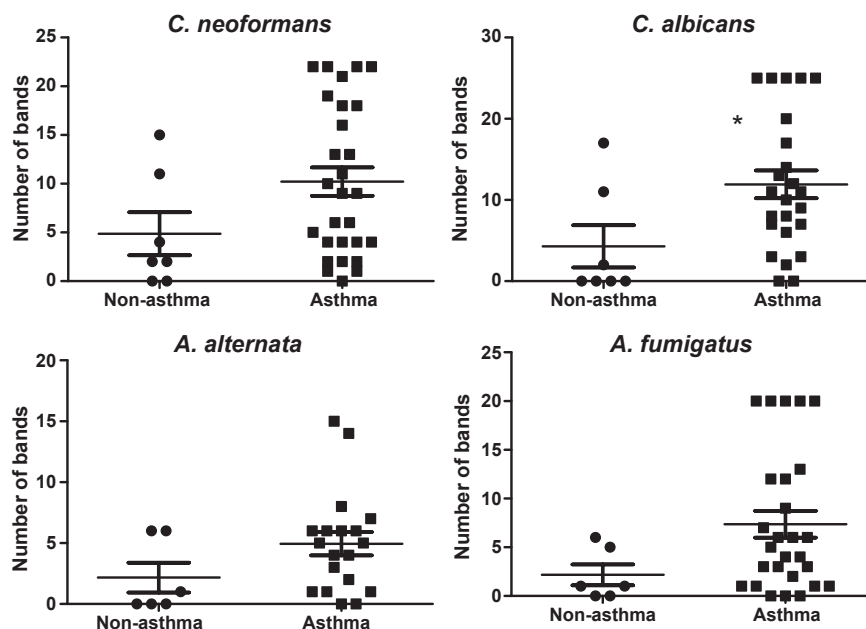


Fig. 4. Analysis of bronchoalveolar lavage fluid (BALF) IgG immunoblots. The number of fungal proteins recognized by BALF IgG for non-asthmatics and asthmatics are shown. Each value represents reactivity of an individual BALF. Reactivity against the various fungi are shown. * $P < 0.05$ for comparison between non-asthmatic and asthmatics.

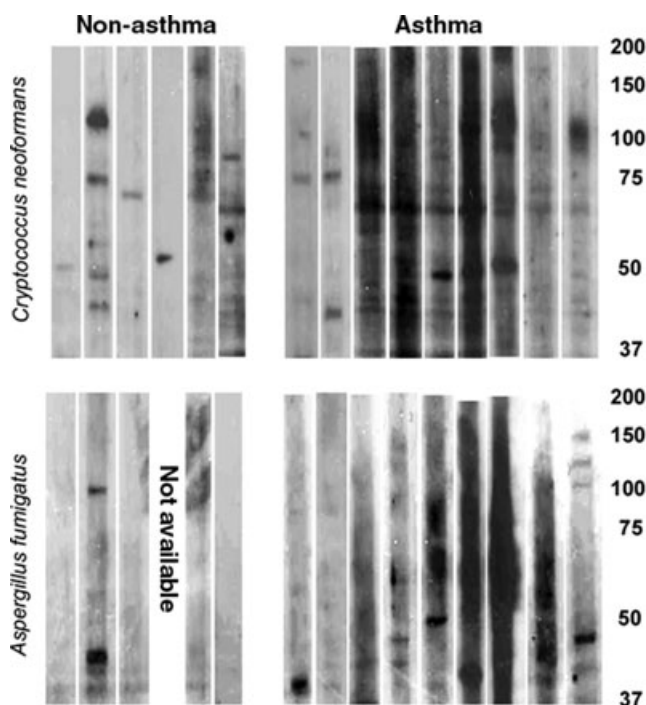


Fig. 5. Fungal-specific IgA in bronchoalveolar lavage fluid (BALF). Representative IgA immunoblots for BALF from asthmatics and non-asthmatics when reacted against *Cryptococcus neoformans* (top) and *Aspergillus fumigatus* (bottom) protein extracts are shown. Each lane represents BALF from a different individual. Top and bottom lanes are done with BALF from the same individual. Corresponding IgG blots are shown in Fig. 3. Molecular weight markers in kDa are shown on right.

inactive forms (YKL-40). Our results show that the chitinase pathway is activated in the respiratory tract of Bronx children with severe asthma and can be identified in BALF. They also suggest a coordinated up-regulation of both enzymatically active and inactive forms. Early studies in mice demonstrated that Ym1, YM2 (murine specific chitinases) and AMCase are induced in an experimental model of asthma [26, 36]. AMCase, in this model, is elicited by IL-13 and is an essential down-stream mediator of IL-13 activity,

including the induction of eosinophilia and airway hyper-reactivity [26]. Studies in humans have shown that YKL-40 levels are elevated in the serum of asthmatics and correlate with asthma severity and exacerbations [27, 28]. Elevated YKL-40 levels in serum and BALF have also been reported in response to allergen challenge [37].

Increased BALF YKL-40 levels in asthmatics suggest local chitinase production and are consistent with animal experimentation [26] and immunohistochemistry studies in humans that localized expression to respiratory epithelial cells and associated inflammatory cells [27]. Nonetheless, levels in this study were low when compared with reported serum levels and near the level of detection for this assay [27, 28]. This may reflect a dilutional effect of lavage and suggest that serum levels may serve as a better parameter for diagnosis and monitoring YKL-40 levels. We did not find a correlation between IgE levels and chitinase or YKL-40 expression as observed in previous studies [28], but suspect that this reflects relatively small number of patients in this study, which may have limited our ability to detect this relationship. In addition, patients in this study were on a variety of therapies, which could have introduced additional heterogeneity that may have differentially affected these parameters and reduced the likelihood of observing such a correlation.

A role for unrecognized fungal infection in the pathogenesis of asthma is supported by studies demonstrating the utility of anti-fungal therapy in the treatment of patients with severe asthma and fungal sensitization [11]. Recently, we described six children with severe asthma and fungal sensitization three of whom were treated with Itraconazole [38]. These children were found to be sensitized to *Aspergillus*, *Penicillium* or a combination of fungi. In a recent study of adult asthmatics, detection of *A. fumigatus* in the sputum was associated with a poor response to bronchodilator therapy [39]. In our current study, we did not observe direct evidence of fungal infection by routine culture or

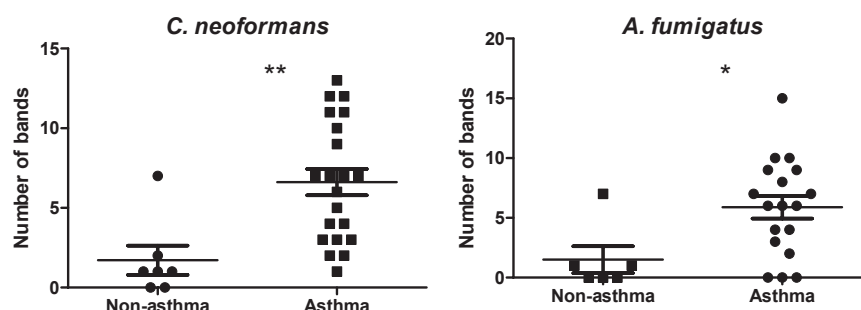


Fig. 6. Analysis of bronchoalveolar lavage fluid (BALF) IgA immunoblots. The number of fungal proteins recognized by BALF IgA for non-asthmatics and asthmatics are shown. Each value represents reactivity of an individual BALF. Reactivity against the various fungi are shown. * $P < 0.05$, ** $P < 0.001$ for comparison between non-asthmatics and asthmatics.

cryptococcal antigen testing. Since these assays may be relatively insensitive to infection, especially those characterized by localized disease with a low fungal burden, we examined BALF for antibody responses to fungal proteins. A trend towards increased fungal-specific IgG in the BALF of the asthma group compared with the non-asthmatic group is consistent with a higher prevalence of fungal infection and/or disproportionately stronger antibody response among asthmatics. Furthermore, the presence of fungal-specific IgA reactivity in BALF suggests local antibody production to pulmonary infection, though we did not have serum samples for comparative analysis. Our findings are consistent with earlier studies that demonstrated local production of *Aspergillus*-specific IgE and IgA in the BALF of patients with ABPA [40].

Fungal infection can elicit chitinase activity [29, 30] and Ym1 expression in animal models [41, 42]. Furthermore, chitinases have *in vitro* and *in vivo* fungicidal activity and have been implicated in the host response to fungal infection [25, 43]. In our study, there was no correlation between serologic responses to fungal antigens and either chitinase activity or YKL-40 levels. The relationships between fungal exposures, chitinase and asthma are likely to be complex and influenced by genetic predisposition, given that AMCase, YKL-40 and chitotriosidase polymorphisms are each associated with asthma [44–48]. Recently, severe asthma exacerbations have been associated with fungal exposures in children with certain chitinase polymorphisms [31]. We hypothesize that chitinase polymorphisms could increase the risk for certain individuals risk for fungal infection, which in turn acts to enhance asthma possibly through

the induction of YKL-40 levels. In our small series of children with severe asthma and fungal sensitization, we found that all six were heterozygous for a 24-bp duplication in the CHIT1 gene, which has been associated with decreased levels of circulating chitotriosidase [38].

Several caveats must be taken in the interpretation of our antibody studies. First, fungal-specific antibodies in the BALF are not necessarily indicative of active infection. Secondly, the presence of infection does not infer causality to asthma as infection may be secondary to the immunologic derangements associated with allergic asthma. Finally, our IgG studies suggest that there is significant cross-reactivity between BALF antibodies to the various fungi, making it difficult to discern the infecting fungus, with the methodology used in this study.

In summary, we describe a cohort of Bronx children with severe asthma and evidence of activation of chitinase pathway within the respiratory tract. These children also demonstrate serologic evidence of fungal infection. Although causality between fungal infection and asthma cannot and should not be inferred from these data, the results are nevertheless consistent with a large body of animal and human evidence suggesting that certain fungal infections can contribute to the pathogenesis of asthma. Additional study is needed to understand the contribution of fungal infection to asthma, its relationship to the induction of chitinases and the potential therapeutic role of anti-fungals.

Acknowledgements

The authors have no conflict of interest to declare.

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