

Immunoreactivity of antigen extracts of *Aspergillus fumigatus* isolated from different sources

José L. Blanco, Marta-E. García and Viswanath P. Kurup

Allergy-Immunology Division, Department of Medicine, Medical College of Wisconsin, and Zablocki Veterans Administration Medical Center, Milwaukee, Wisconsin, USA.

Summary

Allergic bronchopulmonary aspergillosis (ABPA) is the result of hypersensitivity to *Aspergillus* antigens in patients with long-standing atopic asthma. In the present study mycelial and culture filtrate antigens from *Aspergillus fumigatus* cultures isolated from diverse sources were tested against sera of 10 ABPA patients and 10 control individuals by an ELISA methodology. The results indicate higher antibody reactivity against both antigens in the sera of ABPA patients, while culture filtrate antigens also gave non-specific reactivity with control sera. Mycelial extracts, in general, were useful in the diagnosis of ABPA.

Key words

Aspergillus fumigatus, Antigens, ELISA, ABPA, Immunoreactivity

Inmunorreactividad de extractos antigénicos de *Aspergillus fumigatus* aislados de diferentes orígenes

Resumen

La aspergilosis broncopulmonar alérgica (ABPA) es el resultado de una hipersensibilidad frente a los antígenos de *Aspergillus* en pacientes con asma atópica de larga duración. En el presente estudio se han utilizado antígenos de cepas de *Aspergillus fumigatus* aisladas de diferentes orígenes. Se prepararon soluciones antigénicas tanto de extracto miceliar como de filtrado de cultivos, que fueron utilizados para determinar por ELISA el nivel de anticuerpos específicos tanto en suero de pacientes de ABPA como de individuos control. Los resultados indicaron una mayor reactividad de anticuerpos frente a ambos tipos de antígenos en el suero de pacientes de ABPA, si bien en el caso de los filtrados de cultivo también presentaron reactividad no específica con sueros de individuos control. Por tanto, los extractos miceliar parecen más adecuados para su utilización en el diagnóstico inmunológico de ABPA.

Palabras clave

Aspergillus fumigatus, Antígenos, ELISA, ABPA, Inmunorreactividad

Allergic bronchopulmonary aspergillosis (ABPA) is the result of hypersensitivity to *Aspergillus* antigens in patients with long-standing atopic asthma. Originally considered a rarity, ABPA is currently recognized with much greater frequency. ABPA with varied clinical presentation has been reported to occur in 20% of asthmatic patients admitted to hospitals and in 5% of all rhinitis cases, while the incidence in patients with cystic fibrosis may vary from 10 to 25% [1-3].

Nevertheless, the onset of ABPA can be traced to early childhood or even infancy, and the disease may remain undiagnosed for years or decades [4]. Thus, the actual incidence of ABPA seems to be much higher than is indicated from the reported number of cases [5]. ABPA is specially difficult to diagnose in patients with cystic fibrosis because of the presence of recurrent or chronic infiltrates, wheezing, and frequent colonization with *Aspergillus fumigatus* [6].

Although *A. fumigatus* is the main etiological agent of ABPA, other species such as *Aspergillus terreus*,

Aspergillus flavus, *Aspergillus nidulans*, *Aspergillus oryzae* and *Aspergillus niger* have also been associated with the disease [7-9].

Sera from patients with ABPA consistently demonstrate high levels of specific circulating antibodies [10]. No standardized antigen is available for immunological assay. In addition, diversity in antigenicity among strains of *A. fumigatus* have been reported [11].

In the present study, we have isolated strains from different clinical and environmental sources and studied their antigens in immunological assays of sera from patients and control subjects.

MATERIALS AND METHODS

Strains of *A. fumigatus*. In the present study we used 12 strains of *A. fumigatus* from different origins: three from patients with invasive aspergillosis (AF-102, AF-103, AF-125), two from patients with aspergilloma (AF-118, AF-120), two from environment (AF-101, AF-126), and five from children with cystic fibrosis (CF-001, CF-014, CF-020, CF-039, CF-044). These strains have been maintained in long term storage in our culture collection.

***Aspergillus fumigatus* antigen.** Antigens from the different strains of *A. fumigatus* were prepared as descri-

Dirección para correspondencia:

Dra. Marta-Eulalia García
Dpto. Patología Animal I, Facultad de
Veterinaria, Universidad Complutense, 28040
Madrid, Spain.
Tel. (+34 1) 394 37 17, Fax: (+34 1) 394 3908

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bed [10, 12]. Briefly, the methods are as follows:

Culture filtrate antigens (CF): The organism was grown in synthetic broth (AOAC Broth and Czapek-Dox Broth [1:1, v/v] containing 0.7 % Dextrose) for three weeks at 37°C in stationary culture. The broth was separated from the mycelium after the incubation period, dialyzed extensively against deionized water, filtered through a 0,45 mm membrane and freeze dried.

Mycelial extract antigens (ME): The organism was grown in the same synthetic broth for 96 h in aerated culture. After the incubation period, mycelium was obtained by filtration, washed three times with phosphate buffered saline (PBS) and homogenized in a French Press (SLM Instruments, USA) at 10,000 p.s.i.. Microscopic examination of the preparation revealed less than 80 % breakage of the hyphae. The extract obtained was centrifuged at 10,000 rpm for 30 min, and the supernant collected, dialyzed extensively against deionized water, the retentate filtered through a 0,45 µm filter and freeze dried.

Both antigens were evaluated for their protein content by the Bradford method BCA protein assay (Pierce, USA).

Sera. Twenty sera, ten from patients with ABPA and ten from normal control subjects, were studied using ELISA.

Enzyme linked immunosorbent assay. The method followed was the same as the one described before [11, 12].

One hundred µl of each *A. fumigatus* antigen (1 µg protein/ml of CF and 5 µg/ml of ME) diluted in PBS was added to each well of a microtiter plate (Dynatech Laboratories Inc., USA) and incubated overnight at 4°C. The wells were emptied and washed three times with PBS containing 0.05% Tween 20 (PBS-T). The wells were then blocked by incubation at room temperature for 30 min with 100 µl of blocking solution (PBS-T containing 3% bovine serum albumin). The wells were again washed three times as before with PBS-T.

Next, 100 µl of human serum samples (1:500) were added to each well, and the plates incubated at room temperature for 3 h, and washed again. Subsequent steps included addition of 100 µl of an 1:1,000 dilution of anti-human IgG biotin conjugate (Sigma, USA) and 100 µl of 1:10,000 streptavidin-peroxidase (Sigma) with incubation of 1 h each and washing in between as described above. Finally, 100 µl of the substrate (0.015% H₂O₂ and 0.06% o-phenylenediamine) in citrate buffer (0.1 M pH=4.5) was added to each well. After 15 min the reaction was stopped with 6N H₂SO₄, and the color was read in an automatic enzyme-linked immunosorbent assay reader (Dynatech) with a 490 nm filter. Blanks were subtracted and optical density (OD) values at 490 nm were compared.

The coating concentration of the antigens were determined by a checkerboard titration method. We have used eight to 1,000 ng/ml antigen for coating plates and used four ABPA sera and three normal control sera for arriving at the right coating concentration for each antigen.

RESULTS

The results of ELISA using ABPA and control sera against EM and CF antigen from 12 strains of *A. fumigatus* are shown in Figure 1. Both culture filtrate and mycelial extract detected higher antibodies in the sera of patients with ABPA, while normal controls showed only lower levels of *Aspergillus* specific antibody.

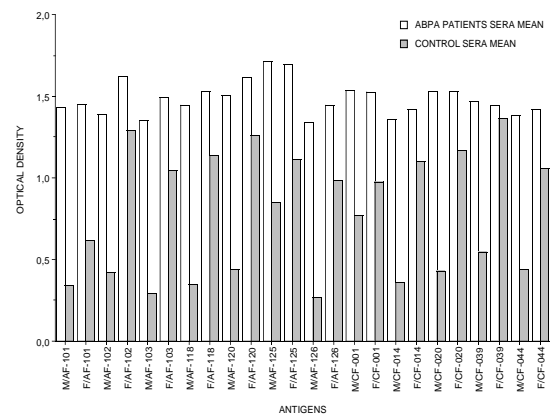


Figure 1. Mean of the antigenic reaction of the mycelial extract and culture filtrate of the 12 strains studied with the groups of control sera and ABPA patients sera.

In Figure 2, the ELISA results of two selected antigens, namely AF-102-CF and AF-102-ME, are shown against individual sera from both ABPA and controls. All patients reacted with both antigens and gave high titers. However, CF antigen showed stronger reactivity with control sera than ME antigen.

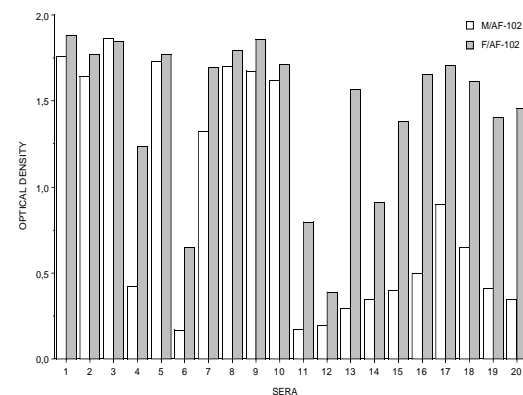


Figure 2. Antigenic reaction of the mycelial extract and culture filtrate of one strain (AF-102) with the 20 human sera studied (sera 1 to 10 from ABPA patients and 11 to 20 from control individuals).

DISCUSSION

Our results indicate that there is considerable difference in the antigenicity among different strains of *A. fumigatus*. This has been recognized in the past by several investigators [5]. However, some strains may show unique antigens specific for few or many patients. In previous studies, mycelial extract has been shown to exert lack of specificity in antibody detection, particularly when agar gel diffusion method was used. In the present study, we have seen high titers of antibody against CF in normal controls compared to ME extract. This may be due to the selection, culture condition, and processing of the antigens.

In some strains, like AF-102, whose results are shown in Figure 2, there was a significant difference between patient and control sera with the use of mycelial extract.

It has been reported that patients with ABPA have quantitatively increased IgE and IgG anti-*A. fumigatus* antibodies as determined by ELISA, which was a helpful adjunctive criteria in the diagnoses of ABPA [6, 11]. The ELISA was more sensitive than immunoblots in quantitating antibodies in patients with cystic fibrosis [6].

Reliability of the results obtained by ELISA is restricted because of the difficulties in standardization of *A. fumigatus* extracts. Knowledge of relevant antigens and allergens is a prerequisite to produce standardized extracts [13]. These authors described in patients with ABPA a stronger immunogenicity of culture filtrate antigens of *A. fumigatus*, the opposite to our results. The content of antigens and allergens of the culture filtrate and the mycelium extract seems to be comparable [13], although controversial results have been reported [14].

In conclusion, our results indicate that there is diversity in the antigenicity of strains of *A. fumigatus* as

well as between mycelial and culture filtrate antigens. The sensitivity of the test and the specificity of the test can be further increased by purifying relevant antigens from different strains of *Aspergillus* and different antigen preparations should be identified and purified for reliable immunodiagnosis of ABPA and related diseases.

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