

A Deletion Variant of the *Aspergillus fumigatus* Ribotoxin Asp f 1 Induces an Attenuated Airway Inflammatory Response in a Mouse Model of Sensitization

E Álvarez-García,¹ E Batanero,¹ R García-Fernández,² M Villalba,¹ JG Gavilanes,¹ Á Martínez-del-Pozo¹

¹Department of Biochemistry and Molecular Biology I, Faculty of Chemistry, Universidad Complutense, Madrid, Spain

²Department of Veterinary Medicine and Surgery, Faculty of Veterinary Medicine, Universidad Complutense, Madrid, Spain

Short title: An Asp f 1 Mutant Attenuates Airway Inflammation

Abstract

Background: *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen, and the ribotoxin Asp f 1 is one of its major allergens. α -Sarcin is a natural variant of Asp f 1 produced by the nonpathogenic fungus *Aspergillus giganteus*. Both proteins show a sequence identity of 87% and almost identical 3-dimensional structures. α -Sarcin $\Delta(7-22)$ is a deletion mutant that displays reduced immunoglobulin (Ig) E reactivity and is much less cytotoxic than wild-type proteins against human transformed cells.

Objective: A murine model of sensitization to Asp f 1 was established to test the response elicited by this α -sarcin $\Delta(7-22)$ deletion mutant.

Methods: BALB/c mice were treated intraperitoneally with different mixtures of recombinant wild-type Asp f 1 and/or a suspension of a commercially available *A. fumigatus* standard extract. Mice were then intranasally challenged with Asp f 1 or α -sarcin $\Delta(7-22)$. Sera were collected for subsequent measurement of Ig levels and histological analysis of the nostrils and lungs.

Results: Sensitization to Asp f 1 was successful only when the purified protein was first administered together with the *A. fumigatus* suspension. The model was characterized by elevated levels of total IgE in serum and histological lesions in the lungs and nostrils. These symptoms were less severe when the deletion variant was the protein administered, thus confirming in vivo its lower toxic character.

Conclusions: An easily reproducible mouse model of *A. fumigatus* Asp f 1 sensitization was established. This model revealed α -sarcin $\Delta(7-22)$ to be a potential candidate for immunotherapy.

Key words: *Aspergillus*. Sarcin. Ribotoxin. Asp f 1. Allergen. Ribonuclease.

Resumen

Antecedentes: *Aspergillus fumigatus* es el patógeno fúngico aerotransportado más abundante, siendo la ribotoxina Asp f 1 uno de sus principales alérgenos. La α -sarcina es una variante natural de Asp f 1 producida por el hongo no patógeno *Aspergillus giganteus*. Ambas proteínas muestran una identidad de secuencia del 87% y una estructura tridimensional prácticamente idéntica. La α -sarcina $\Delta(7-22)$ es un mutante de delección mucho menos tóxico que la proteína silvestre cuando se ensaya frente a células humanas transformadas y además muestra una reactividad reducida frente a IgE.

Objetivo: El establecimiento de un modelo murino de sensibilización a Asp f 1 y su uso para ensayar la respuesta desencadenada por el mutante de delección α -sarcina $\Delta(7-22)$.

Métodos: Ratones BALB/c fueron tratados intraperitonealmente con diferentes mezclas de Asp f 1 recombinante silvestre y/o una suspensión de un extracto estándar comercial de *A. fumigatus*. Los ratones fueron posteriormente estimulados intranasalmente con Asp f 1 o α -sarcina $\Delta(7-22)$. Posteriormente, se recogió suero, que se utilizó para la cuantificación de los niveles de inmunoglobulinas, y se realizó un análisis histológico de fosas nasales y pulmones.

Resultados: La sensibilización a Asp f 1 sólo tuvo éxito cuando la proteína pura se suministró simultáneamente con la suspensión de *A. fumigatus*. El modelo se caracterizó por sus elevados niveles de IgE en suero así como por lesiones histológicas en pulmones y fosas nasales. Estos síntomas fueron menos graves cuando se administró la variante de delección lo que confirmó *in vivo* su carácter menos citotóxico.

Conclusiones: Se ha establecido un modelo murino fácilmente reproducible de sensibilización al alérgeno Asp f 1 de *A. fumigatus*. Este modelo es coherente con que la α -sarcina $\Delta(7-22)$ sea un potencial candidato para estudios de inmunoterapia.

Palabras clave: *Aspergillus*. Sarcina. Ribotoxina. Asp f 1. Alérgeno. Ribonucleasa.

Introduction

Aspergillus fumigatus is exceptional in that it is both a primary and opportunistic pathogen as well as an important source of allergens [1]. Its ubiquitous spores are continuously inhaled by humans, with the result that exposure of the respiratory tract is almost constant. Inhalation very rarely causes adverse effects, as the conidia are usually eliminated efficiently by the innate immune system. More widespread use of immunosuppressive therapy is leading to increasingly frequent conditions involving infection by *A. fumigatus*. This microorganism is one of the most prevalent airborne fungal pathogens [2] and the etiological agent in 80% of *Aspergillus*-related diseases, including hypersensitivity, pneumonitis, allergic rhinitis, immunoglobulin (Ig) E-mediated asthma, severe allergic bronchopulmonary aspergillosis (ABPA), and different invasive infections in immunocompromised patients [1,3].

The ribotoxin Asp f 1 is one of the major allergens of *A. fumigatus*, with a prevalence of more than 80% in patients with ABPA [1,4-6]. α -Sarcin, a natural variant of the nonpathogenic *Aspergillus giganteus*, is the best-characterized member of this ribotoxin family, a group of fungal and highly specific secreted ribonucleases [7]. Asp f 1 shows 87% amino acid sequence identity with α -sarcin [4] and an almost identical 3-dimensional structure [6,8,9]. Both proteins are so similar in enzymatic and structural terms that the only factor preventing *A. giganteus* from being infectious is considered to be its inability to grow above 30°C. The toxicity of ribotoxins comes from their ability to reach the cytosol via receptor-independent endocytosis [10]. Once inside the host cell, ribotoxins inhibit protein biosynthesis by inactivating the ribosomes, thus causing cell death [7]. The NH₂-terminal β -hairpin has been shown to play a key role both in the cytotoxic effect of ribotoxins and in IgE-mediated responses [6,11]. In fact, the deletion mutant α -sarcin $\Delta(7-22)$, from which this β -hairpin is eliminated [11], shows much lower cytotoxicity against human rhabdomyosarcoma cells in culture and a 50% reduction in human IgE reactivity. Interestingly, α -sarcin $\Delta(7-22)$ maintains the wild-type 3-dimensional structure [12] and its prevalence in sera from patients with ABPA [6]. These results indicate that α -sarcin $\Delta(7-22)$ is a hypoallergenic variant of Asp f 1.

Immunotherapy against *A fumigatus* allergy is based on allergenic extracts that are difficult to standardize because they contain up to 200 different molecules [13]. In addition, the risk of anaphylactic side effects during treatment cannot be ruled out. The use of recombinant allergens with reduced IgE-binding capacity [14-16] could help resolve these drawbacks, and, in this regard, the deletion variant of the ribotoxins described above is a promising molecule for immunomodulating therapies. In the present study, we attempt to establish an easily reproducible murine model of sensitization to Asp f 1 and use it to compare the response elicited by this deletion variant with that of the wild-type protein.

Materials and methods

Mice

Female, 6- to 8-week-old BALB/c mice (Harlan Interfauna Ibérica SA, Barcelona, Spain) were maintained at the Animal Care Services of the Faculty of Biology (Universidad Complutense, Madrid, Spain) according to the local guidelines for animal care. The study was approved by the Animal Experimentation Ethics Committee of our institution.

Protein Production and Purification

Escherichia coli BL21 (DE3) cells cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding Asp f 1 or the deletion mutant α -sarcin $\Delta(7-22)$ plasmids were used to produce the proteins, as previously described [6,11]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins, protein hydrolysis, amino acid analysis, and circular dichroism spectra were performed according to standard procedures [5,17-20]. Western blots were performed as described elsewhere [6] using rabbit sera raised against wild-type α -sarcin or Asp f 1. Homogeneity of the protein samples was assessed using the procedures described above.

Preparation of Aspergillus fumigatus Suspension

A standard commercially available mixture of spores and mycelia inactivated by gamma radiation (Allergon AB, Ängelholm, Sweden) was resuspended in phosphate-buffered saline (PBS) at 1 mg/mL (w/v) and shaken for 2 hours at 250 rpm and 4°C. This suspension is referred to as the Af suspension throughout the text.

Sensitization and Challenge

Different approaches were tried in order to establish a murine model of sensitization to recombinant Asp f 1. The success of each protocol was evaluated by measuring total and specific antibody levels, including IgE, IgG1 and IgG2a (see below), as well as by assessing clinical manifestations such as histopathological alterations, pilar erecti, and weight loss. Finally, the successful procedure was as follows: Mice (n=5) were sensitized by 2 intraperitoneal injections with 1 µg of Asp f 1 and 10 µL of the Af suspension adsorbed to 2 mg of Al(OH)₃ in 150 µL of PBS at 7 day-intervals. After 7 days mice were challenged by intranasal administration of different amounts of Asp f 1 or the mutant α-sarcin Δ(7-22) in 50 µL of PBS on 3 consecutive days under mild anesthesia with ketomine/xylazine (Figure 1). With the mouse supine, the antigen solution was injected into the nostrils using a micropipette. The animal was restrained in this position for a minimum of 10 minutes until it awoke from the anesthesia. Six different groups were studied. Group A were not treated, and were used as controls, whereas group B mice received the intraperitoneal mixture but were intranasally challenged with PBS only. Groups C, D, and E were intranasally challenged with 1, 5, and 10 µg of purified Asp f 1, respectively. Group F were challenged with 1 µg of α-sarcin Δ(7-22). Seven days after the last intranasal challenge, blood samples were collected from the retro-orbital plexus (terminal bleeding) and tissues were removed for histological examination.

Determination of Specific IgG1, IgG2a, and Total IgE in Serum

Antibody (Ab) binding was measured by an enzyme-linked immunosorbent assay (ELISA) as previously described [21]. In order to quantitate Asp f 1 specific antibodies, serum samples were diluted 1:25 000 for IgG1, 1:400 for IgG2a, and 1:5 for IgE. Antibody levels were expressed as optical density (OD) values read at 492 nm. Total IgE (diluted 1:40) levels in serum were measured by sandwich ELISA using the OptEIA mouse IgE set (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions.

Histopathology

Nostril and lung samples were fixed in 10% buffered formalin, routinely processed, and paraffin-embedded. Sections (3 μ m) were stained with hematoxylin-eosin to examine general morphology and cellular infiltration, and the periodic acid-Schiff (PAS) technique was applied to observe mucus production. Sections were examined under a light microscope by a pathologist who was blinded to the protocol design. The intensity and severity of lung and nostril involvement were assessed on a semiquantitative score ranging from 0 to 4, with the respective values corresponding to negative, faint, weak, moderate, or severe lesions.

Results

Sensitization Protocol

Intraperitoneal immunization with purified Asp f 1 only was insufficient to induce a response, even when administered in amounts as high as 10 µg. Given that allergy to *A fumigatus* usually occurs alongside fungal infection, a second approach was tried using the Af suspension as the only protein source to achieve sensitization. Once again, antibody levels remained unaffected and toxic effects were not observed. This result was consistent with the absence of Asp f 1 in the Af suspension as revealed by immunoblotting. Consequently, in the third approach, purified Asp f 1 and the Af suspension were combined in the hope that the latter would contain some component that would lead to an allergic response, as described elsewhere [22].

Thus, the final successful strategy was based on the intraperitoneal administration of a mixture containing both the Af suspension (10 µl) and the recombinant protein (1 µg) in Al(OH)₃, followed by three intranasal challenges with different doses of recombinant Asp f 1 only. Mice challenged with 10 µg of Asp f 1 systematically died after the third intranasal challenge (Figure 1). Therefore, group E was not further studied. The groups treated with 1 or 5 µg did experience adverse effects, as evidenced by weight loss and piloerection, but fully recovered once the Asp f 1 challenge was interrupted. These results suggest the existence of a dose-dependent and more complex toxic effect.

Antibody Response

Intraperitoneal injection of the Asp f 1–Af suspension combination was sufficient to induce a 7-fold increase in total IgE serum levels when compared to controls (Figure 2). This response was further strengthened after intranasal challenge with 5 µg of Asp f 1 (Figure 2). No significant differences were observed when mice were challenged with lower amounts of protein. Asp f 1–specific IgE, IgG1, or IgG2 antibodies were not detected in the sera of any of the immunized mice within the limits of ELISA and Western blot analysis.

Lung and Nostril Histology

Lung tissue was obtained from each experimental group, except for group E, 7 days after the last intranasal challenge to assess the effect of the sensitization protocol on airway inflammation. Histologic analysis revealed that Asp f 1 challenge promoted severe perivascular and peribronchial infiltrations of inflammatory cells—lymphocytes, neutrophils and occasional eosinophils and macrophages—in all lobes of the lungs. These effects were even more evident in mice challenged intranasally with 5 µg of protein, in which marked re-epithelialization and fibrosis, characterized by enrichment of collagen fibers, were also observed throughout the parenchyma (Figure 3).

Examination of nostril tissue revealed that it was also affected by Asp f 1 sensitization (Figure 4). A mixed infiltrate of cells, especially lymphocytes and neutrophils, was observed in the nostrils of the experimental animals. Again, the most severe effects were obtained for the higher Asp f 1 dose. Mucus hypersecretion in the nostrils was a notable histopathologic feature of the Asp f 1–challenged mice.

Effect of a Deletion Mutant With Diminished IgE Response

The $\Delta(7-22)$ deletion mutant of α -sarcin was also studied to evaluate its effect on the onset of sensitization. Thus, group F mice were intranasally challenged with 1 µg of $\Delta(7-22)$ α -sarcin, that is, the amount of recombinant Asp f 1 used for group C mice. No significant effects on total IgE levels were observed in the mutant-challenged mice compared with group C mice (Figure 2). Nevertheless, intranasal administration of the mutant resulted in diminished perivascular and peribronchial cellular infiltration and inhibited epithelial cell hyperplasia in the lungs (Figure 3). With respect to the nostrils, the use of the mutant induced a similar cellular inflammatory response, although mucus release was notably diminished (Figure 4).

Discussion

One of the most promising alternatives to conventional immunotherapy for allergy is the use of hypoallergenic derivatives as vaccines [16,23]. However, before clinical application, in vitro and in vivo evaluation of these presumed hypoallergens is required to identify the best candidates. We established a murine model of sensitization to Asp f 1 in order to test the safety and in vivo suitability of a variant of Asp f 1, the α -sarcin $\Delta(7-22)$ mutant, as an immunotherapeutic agent for allergy.

Most animal models of *A fumigatus* sensitization have been achieved in mice by intranasal administration of crude extracts of the fungus or of intact organisms, particularly the conidia [22,24,25]. We were not able to sensitize mice to the commercially available extract used in this study. This could be due to nondetectable levels of Asp f 1, the heterogeneity and lack of reproducibility of the extracts used by other authors, or both. In fact, intraperitoneal administration of purified Asp f 1 alone also rendered healthy animals with undetectable levels of total IgE. However, when BALB/c mice were sensitized by intraperitoneal administration of a combination of Asp f 1 and Af suspension in $Al(OH)_3$ followed by intranasal challenge, onset of an allergic-like state was observed in the high levels of total IgE in serum and histological lesions in the lung and nostrils. However, Asp f 1-specific IgE, IgG1, IgG2a antibodies were not detected, in agreement with previous studies of other authors [26] who observed that immunization of animals with an *A fumigatus* extract induced an increased level of total serum IgE, but not specific IgE or IgG antibodies.

Therefore, the advantage of this model over others described elsewhere [25-29] is its reproducibility, which is based on the use of a widely available extract and a purified and well-characterized recombinant protein. The other procedures presented above were based on poorly described homemade extracts or allergen-enriched mixtures.

In our model, lung lesions typically consisted of perivascular and peribronchial infiltrates of inflammatory cells, emigration of some eosinophils into the lumen, and, in severe cases, epithelial hyperplasia. Accumulation of collagen was frequent in the parenchyma, and this effect of fibrosis was much

more severe in group D mice. In addition, the strong inflammation in the lungs induced by *A fumigatus* antigens persisted over 7 days after the last intranasal challenge. Similar results have been reported by other groups [27-29]. In the present analysis, the histological study was further extended to the nostrils and the results obtained were also compatible with the induction of an allergic response to *A fumigatus* antigens.

Thus, this model of *A fumigatus* sensitization is not only easy to reproduce but also suitable for the in vivo testing of its wild-type and mutant allergens. Overall, however, the results suggest that the allergic-like phenotype observed in the present model is due not only to the typical IgE-dependent response, but also to the result of a much more complex interaction between the host immune system and *A fumigatus* antigens, including ribotoxins. The results suggest that the allergic state induced by the combination of Asp f 1 and Af suspension could result from the cumulative toxic effects of several toxins, esterases, and proteases present in the extract. These could also act as adjuvants, perhaps by inducing epithelial damage and allowing normal antigens to bypass the mucosal barrier [22,30,31]. Thus, the specific roles of the individual allergens have yet to be determined. In addition, it has been shown that proteases preferentially induce T_H2 responses, suggesting that they could be skewing the response to *A fumigatus* antigens to a more allergic phenotype [32,33].

Challenge with the α -sarcin $\Delta(7-22)$ mutant resulted in a substantial decrease in lung and nostril lesions when compared to those induced by wild-type Asp f 1, although total IgE levels remained unaffected. These data confirm the low toxic character suggested previously for the deletion mutant in mice [6]. Toxicity at higher doses cannot be ruled out and warrants further investigation.

In summary, we established an easily reproducible mouse model of *A fumigatus* sensitization. Despite its limitations, this in vivo model is a suitable system for preclinical testing of recombinant allergens and their derivatives. The decreased toxicity induced by α -sarcin $\Delta(7-22)$, in terms of a reduced inflammatory response in the airway and lower human IgE binding activity, confirms this molecule as a suitable candidate for immunotherapy.

Acknowledgements

This work was supported by grant BFU2006-04404 from the Ministerio de Educación y Ciencia (Spain). EA-G. holds a fellowship from the Ministerio de Educación y Ciencia (Spain). The useful discussion and suggestions made by Dr. Rosalía Rodríguez are greatly appreciated.

References

1. Greenberger PA. Allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol.* 2002;10:685-92.
2. Latgè JP. The pathobiology of *Aspergillus fumigatus*. *Trends Microbiol.* 2001;9:382-9.
3. Wark PA, Gibson PG. Allergic bronchopulmonary aspergillosis: new concepts of pathogenesis and treatment. *Respirology.* 2001;6:1-7.
4. Arruda LK, Platts-Mills TA, Fox JW, Chapman MD. *Aspergillus fumigatus* allergen I, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med.* 1990;172:1529-32.
5. Kao R, Martínez-Ruiz A, Martínez-del-Pozo A, Cramer R, Davies J. Mitogillin and related fungal ribotoxins. *Methods Enzymol.* 2001;341:324-35.
6. García-Ortega L, Lacadena J, Villalba M, Rodríguez R, Crespo JF, Rodríguez J, Pascual C, Olmo N, Oñaderra M, Martínez-del-Pozo A, Gavilanes JG. Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Asp f 1 displaying reduced IgE binding. *FEBS J.* 2005;272:2536-44.
7. Lacadena J, Álvarez-García E, Carreras-Sangrà N, Herrero-Galán E, Alegre-Cebollada J, García-Ortega L, Oñaderra M, Gavilanes JG, Martínez-del-Pozo A. Fungal ribotoxins: molecular dissection of a family of natural killers. *FEMS Microbiol Rev.* 2007;31:212-37.
8. Yang X, Moffat K. Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of the highly specific *Aspergillus* ribotoxin, restrictocin. *Structure.* 1996;4:837-52.
9. Pérez-Cañadillas JM, Santoro J, Campos-Olivas R, Lacadena J, Martínez-del-Pozo A, Gavilanes JG, Rico M, Bruix M. The highly refined solution structure of the cytotoxic ribonuclease α -sarcin reveals the structural requirements for substrate recognition and ribonucleolytic activity. *J Mol Biol.* 2000;299:1061-73.
10. Olmo N, Turnay J, González de Buitrago G, López de Silanes I, Gavilanes JG, Lizarbe MA. Cytotoxic mechanism of the ribotoxin α -

- sarcin. Induction of cell death via apoptosis. *Eur J Biochem.* 2001;268:2113-23.
11. García-Ortega L, Masip M, Mancheño JM, Oñaderra M, Lizarbe MA, García-Mayoral MF, Bruix M, Martínez-del-Pozo A, Gavilanes JG. Deletion of the NH₂-terminal β -hairpin of the ribotoxin α -sarcin produces a nontoxic but active ribonuclease. *J Biol Chem.* 2002;277:18632-9.
 12. García-Mayoral MF, García-Ortega L, Lillo MP, Santoro J, Martínez-del-Pozo A, Gavilanes JG, Rico M, Bruix M. NMR structure of the noncytotoxic α -sarcin mutant $\Delta(7-22)$: The importance of the native conformation of peripheral loops for activity. *Protein Sci.* 2004;13:1000-11.
 13. Kurup VP, Kumar A. Immunodiagnosis of aspergillosis. *Clin Microbiol Rev.* 1991;4:439-56.
 14. Cramer R, Hemmann S, Ismail C, Menz G, Blaser K. Disease-specific recombinant allergens for the diagnosis of allergic bronchopulmonary aspergillosis. *Int Immunol.* 1998;10:1211-6.
 15. Kurup VP, Knutsen AP, Moss RB, Bansal NK. Specific antibodies to recombinant allergens of *Aspergillus fumigatus* in cystic fibrosis patients with ABPA. *Clin Mol Allergy* 2006;4:11.
 16. Valenta R, Niederberger V. Recombinant allergens for immunotherapy. *J Allergy Clin Immunol.* 2007;119:826-30.
 17. Lacadena J, Martínez-del-Pozo A, Barbero JL, Mancheño JM, Gasset M, Oñaderra M, López-Otín C, Ortega S, García J, Gavilanes JG. Overproduction and purification of biologically active native fungal α -sarcin in *Escherichia coli*. *Gene.* 1994;142:147-51.
 18. Lacadena J, Martínez-del-Pozo A, Martínez-Ruiz A, Pérez-Cañadillas JM, Bruix M, Mancheño JM, Oñaderra M, Gavilanes JG. Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α -sarcin. *Proteins.* 1999;37:474-84.
 19. García-Ortega L, Lacadena J, Lacadena V, Masip M, De Antonio C, Martínez-Ruiz A, Martínez-del-Pozo A. The solubility of the ribotoxin α -sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. *Lett Appl Microbiol.* 2000;30:298-302.

20. Martínez-Ruiz A, García-Ortega L, Kao R, Lacadena J, Oñaderra M, Mancheño JM, Davies J, Martínez-del-Pozo A, Gavilanes JG. RNase U2 and α -sarcin: A study of relationships. *Methods Enzymol.* 2001;341:335-51.
21. Batanero E, Barral P, Villalba M, Rodríguez R. Sensitization of mice with olive pollen allergen Ole e 1 induces a Th2 response. *Int Arch Allergy Immunol.* 2002;127:269-75.
22. Kurup VP, Grunig G. Animal models of allergic bronchopulmonary aspergillosis. *Mycopathologia.* 2001;153:165-77.
23. Vrtala S. From allergen genes to new forms of allergy diagnosis and treatment. *Allergy.* 2008;63:299-309.
24. Kurup VP, Kumar A, Kenealy WR, Greenberger PA. *Aspergillus* ribotoxins react with IgE and IgG antibodies of patients with allergic bronchopulmonary aspergillosis. *J Lab Clin Med.* 1994;123:749-56.
25. Kurup VP, Xia JQ, Cramer R, Rickaby DA, Choi HY, Flückiger S, Blaser K, Dawson CA, Kelly KJ. Purified recombinant *A. fumigatus* allergens induce different responses in mice. *Clin Immunol.* 2001;98:327-36.
26. Svirshchevskaya EV, Viskova N, Shevchenko M, Alekseeva L, Marchenko A, Benevolensky S, Kurup VP. High-affinity IgG to a major *A. fumigatus* allergen, Asp f 2, retards allergic response. *Med Sci Monit.* 2004;10:371-80.
27. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, Lukacs NW. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by *Aspergillus fumigatus*. *Am J Pathol.* 2000;156:723-32.
28. Kurup VP, Choi H, Resnick A, Kalbfleisch J, Fink JN. Immunopathological response of C57BL/6 and C3H/He mice to *Aspergillus fumigatus* antigens. *Int Arch Allergy Appl Immunol.* 1990;91:145-54.
29. Kurup VP, Mauze S, Choi HY, Seymour BWP, Coffman RL. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J Immunol.* 1992;148:3783-8.
30. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. *J Allergy Clin Immunol.* 2004;114:997-1008.

31. Shen HD, Tam MF, Tang RB, Chou H. *Aspergillus* and *Penicillium* allergens: focus on proteases. *Curr Allergy Asthma Rep* 2007;7:351-6.
32. Tomee JF, Wierenga AT, Hiemstra PS, Kauffman HK. Proteases from *Aspergillus fumigatus* induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines. *J Infect Dis.* 1997;176:300-3.
33. Lamhamedi-Cherradi SE, Martin RE, Ito T, Kheradmand F, Corry DB, Liu YJ, Moyle M. Fungal proteases induce Th2 polarization through limited dendritic cell maturation and reduced production of IL-12. *J Immunol.* 2008;180:6000-9.

Manuscript received February 10, 2009; accepted for publication April 9, 2009.

Álvaro Martínez del Pozo

Departamento de Bioquímica y Biología Molecular I

Facultad de Ciencias Químicas

Universidad Complutense

28040 Madrid, Spain

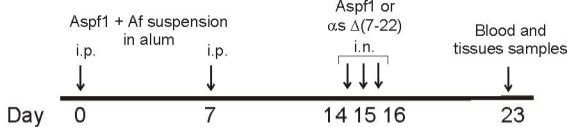
E-mail: alvaro@bbm1.ucm.es

Figure 1. A, Experimental protocol. Mice were sensitized by intraperitoneal injection of a mixture containing Asp f 1 (1 µg) and the Af suspension (10 µL) on Al(OH)₃, followed by intranasal challenge with Asp f 1 (1, 5, or 10 µg; groups C, D, and E, respectively) or α-sarcin Δ(7-22) (1 µg; group F) on 3 consecutive days. After 1 week, blood and tissue samples were collected. B, Survival of mice treated according to these protocols expressed as the percentage of mice surviving as a function of time of treatment. Group A comprised nontreated control mice. Group B mice underwent the intraperitoneal challenge but received intranasal phosphate-buffered saline only. IN indicates intranasal; IP, intraperitoneal.

Figure 2. Total serum immunoglobulin E levels in the different groups of sensitized mice determined by enzyme-linked immunosorbent assay. Bars represent group mean (SEM) of 5 mice per group.

Figure 3. A-F, Representative photomicrograph (hematoxylin-eosin) of lung section from mice in groups A, B, C, D, and F. Histologic analysis revealed numerous zones of inflammation (white arrows) and epithelial hyperplasia (black arrows) in lungs from group C and D animals compared with control mice (groups A and B). Fibrosis was especially noteworthy in group D (see the inset photomicrograph in panel D). G, Histological score for lung inflammation. Data are expressed as the mean (SEM) of 5 mice per group. B indicates bronchium; V, blood vessel.

Figure 4. A-F, Representative photomicrograph (hematoxylin-eosin) of nostrils section from mice in groups A, B, C, D, and F showing cellular infiltration. The insets are photomicrograph periodic acid-Schiff stains of a wider field where the mucus plugs are marked with asterisks. G, Histological score of inflammation and mucus secretion. Data are expressed as the mean (SEM) of 5 mice per group.

A**B****% Survival**