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Inhalation of *Aspergillus fumigatus* spores induces airway inflammation in mice in a similar manner as observed in Recurrent Airway Obstruction in horses

Inhalación de esporas de *Aspergillus fumigatus* induce en ratones una inflamación de las vías aéreas similar a la observada en equinos con Obstrucción Recurrente de las Vías Aéreas

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RESUMEN

El objetivo de este estudio fue determinar si ratones expuestos a esporas de *Aspergillus fumigatus* desarrollan una inflamación alérgica de las vías respiratorias similar a la observada en equinos con Obstrucción Recurrente de las Vías Aéreas (ORVA). Para esto se utilizaron ratones Rockefeller (RK) de 2, 5, 6 y 8 meses respectivamente, los cuales se mantuvieron por 16 días en jaulas que contenían heno contaminado con *A. fumigatus*. Posterior a la exposición, los animales fueron sangrados y sacrificados para la obtención de suero sanguíneo, lavado broncoalveolar (LBA) y muestras de tejido pulmonar. Además, se midieron los niveles de anticuerpos específicos para *A. fumigatus* a través de un ELISA indirecto. Los resultados mostraron que la inhalación de esporas de *A. fumigatus* en ratones induce una respuesta alérgica inflamatoria en pulmones similar a lo observado en ORVA equino, con un aumento del porcentaje de neutrófilos en el LBA y cambios histológicos pulmonares característicos de una hipersensibilidad bronquial. Asimismo, se observó un aumento de los niveles de inmunoglobulinas IgE, IgG1 e IgG2a específicas para este hongo en fluidos bronquiales. Además, los animales remitieron el estrés respiratorio cuando fueron expuestos en un ambiente de remisión. Se puede concluir que la simple exposición de esporas de *A. fumigatus* produce una condición alérgica similar al ORVA en equinos. Por lo tanto, este modelo experimental puede ser de utilidad para el estudio de los mecanismos inmunológicos, y probar nuevas opciones terapéuticas para esta enfermedad respiratoria de los caballos.

Key words: *Aspergillus fumigatus*, RAO, airway inflammation, mice, horses.

Palabras clave: *Aspergillus fumigatus*, ORVA, inflamación de las vías aéreas, ratones, equinos.

INTRODUCTION

Recurrent Airway Obstruction (RAO) in horses, also known as heaves, is a complex, severe and common respiratory disorder that affects stabled horses (Dixon *et al* 1995, Jackson *et al* 2000, Robinson 2001). It is caused by an aberrant immune response to inhaled antigens and is characterized by coughing, respiratory distress, reversible airway obstruction and reduced physical performance (Jackson *et al* 2000, Robinson 2001).

Aspergillus fumigatus is a saprophyte fungus, which survives and grows over a large variety of organic remains and whose most common ecological niche is on the ground. It is one of the most ubiquitous fungi, due to the ease of dispersion of its conidia (Rementeria *et al* 2005). This fungus is commonly found in the horse environment and is considered one of the inciting agents in equine heaves (McPherson *et al* 1979, Derksen *et al* 1988). Studies suggest that RAO-affected horses have higher titers of antibodies to *A. fumigatus* extracts in bronchoalveolar lavage fluid (BALF) compared to healthy

control horses (Halliwell *et al* 1993, Schmallenbach *et al* 1998, Eder *et al* 2000). Moran *et al* (2009) showed that healthy and RAO-affected animals have specific circulating antibodies against the crude mold extract from this fungus. In addition, using an *in vitro* bioassay, Moran *et al* (2010) demonstrated that 30% of thirty RAO-affected horses tested positive for *A. fumigatus*, which indicates a wide distribution for this allergen.

Airway inflammation is a component of a RAO affected horse's response to aeroallergens and is considered to be one of the primary characteristics of this disease. In general, airway inflammation in RAO-affected horses involves the activation of pathogen specific inflammatory cells, modulation of gene transcription factors and the release of inflammatory mediators (Bureau *et al* 2000^{a,b}). Type I hypersensitivity, which is IgE-mediated (Halliwell *et al* 1993, Schmallenbach *et al* 1998, Eder *et al* 2000, Eder *et al* 2001, Curik *et al* 2003, Künzle *et al* 2007, Tahon *et al* 2009, Moran *et al* 2010^{a,b}), and type III hypersensitivity reactions have been suggested to play a role in airway inflammation in RAO-affected horses (Franchini *et al* 2000, Lavoie *et al* 2001).

On the other hand, in humans, *A. fumigatus* represents a real threat for immunocompromised individuals, such as patients suffering from leukemia, individuals infected with

HIV, and those who have undergone organ transplants. In normal individuals, cases of allergic disease and mycotoxicosis have also been reported (Rementeria *et al* 2005). Several experimental mouse models have been proposed for the study of allergic bronchopulmonary aspergillosis (ABPA) in humans. The majority of these models require parenteral immunization, followed by nasal instillation of the antigen. This model results in a Th2-oriented immune response, the production of *A. fumigatus*-specific IgE, and eosinophilic pulmonary inflammation (Kurup *et al* 2001, Randolph *et al* 1999, Huang *et al* 2001).

It is commonly accepted that RAO disease is a bronchial hypersensitivity reaction induced by inhaled molds and organic dust (Derksen *et al* 1988, McGorum *et al* 1993). However, although the immunological aspects of RAO have been extensively studied, the precise sequence of events is not fully understood (Leguillette 2003, Wagner 2009). In addition, these investigations have been hampered by problems with the use of antibodies for the detection of equine IgE (Tahon *et al* 2009) and the lack of suitable immunological tools. A rodent model with controlled environmental conditions would be useful and may provide additional information about RAO in horses. In this study, we show that mice naturally exposed to *A. fumigatus* develop a disease with characteristics that are similar to RAO in horses. These characteristics include remission of signs after antigen removal, recurrence of disease upon antigen re-exposure, intense neutrophilic infiltration in the airways, and histopathological lung changes.

MATERIAL AND METHODS

ANIMALS

For all experiments, we used 2- to 8-month-old, sex- and age-matched Rockefeller (RK) mice. The animals were obtained from, and maintained at the Animal Facility of the Universidad Austral de Chile. During exposure to *A. fumigatus*, the animals were placed in an isolation room with the appropriate ventilation and filtering systems. This study was approved by the Bioethics Committee for the Use of Animals in Biomedical Research of the Universidad Austral de Chile.

ANTIGENIC PREPARATIONS OF *A. FUMIGATUS*

The culturing of *A. fumigatus* was carried out in two steps. The fungus was first cultured in peptone agar for 7 days at 37 °C and then in malt broth for an additional 7 days. The resulting fungi were used for soluble protein extraction and for the contamination of mouse bedding. For protein extraction, fungi were blended and subsequently sonicated (Ultrasonic Homogenizer, series 4710 Cole Parmer, USA). The resulting suspension was centrifuged at 12,500 x g for 10 min and the supernatant, which contained the soluble proteins, was stored at -20 °C for later

use. To expose mice to *A. fumigatus*, under conditions that are similar to those confronted by horses affected with this pathogen, good quality hay was sterilized and sprinkled with water containing fungus spores. The contaminated hay was then placed into plastic bags and incubated for 48 hours or more until mold contamination became visible. At this point, the contaminated hay was used as bedding in the mouse cages. During the experiment, animals were maintained under these conditions according to the design of each experiment.

EXPOSURE OF MICE TO *A. FUMIGATUS* SPORES

Different groups of 2-, 5-, 6- and 8-month-old mice (eight mice per group) were housed in cages with sterile or *A. fumigatus* contaminated hay bedding and were kept in an isolation room for 16 days. Each day, after being exposed to the fungus, the animals were carefully observed twice a day for clinical signs of respiratory distress. After 16 days of mold exposure, the mice were bled and sacrificed to obtain bronchoalveolar lavage fluid (BALF) or lung tissues for histological analysis.

In addition, different groups of 2-, 5-, 6- and 8-month-old mice (eight mice per group) were used to evaluate the recurrence of respiratory distress. For this purpose, the animals that were housed in the contaminated environment and demonstrated clear signs of respiratory distress (such as tachypnea and audible sibilance) were placed in a remission environment, allowing us to observe the disappearance of signs associated with respiratory distress.

BRONCHOALVEOLAR LAVAGE

Different groups of mice were used for bronchoalveolar lavage (BAL) and analysis of lung tissues to prevent any possible alterations in the histological appearance of the lungs that were due to the lavage. The animals were sacrificed using an overdose of a sodium barbital anesthetic (Serve, USA). For BAL, the trachea was surgically exposed and cannulated with an intravenous catheter (22 G x 1/in), and 500 µL of saline solution was slowly administrated into the trachea and immediately recovered. The obtained BALF was centrifuged at 690 x g, and the supernatant was stored at -20 °C for later use. The supernatant was used for the analysis of antibody titers, and the cell pellet was used for cytological analyses.

ANALYSIS OF ANTIBODY PRODUCTION

The levels of *Aspergillus*-specific immunoglobulins present in BALF and serum were determined by ELISA. Briefly, 96-well polystyrene microplates (Sarstedt, USA) were coated with 10 µg/ml of *A. fumigatus* total protein that was dissolved in carbonate buffer (pH 9) and incubated overnight. The plates were blocked with a solution of 5% non-fat milk, followed by the addition of 100 µl of

the appropriate dilution of BALF (1:1) or serum (1:100) into each well. The plates were incubated at 37 °C for 2 hours, washed three times and incubated with peroxidase-conjugated anti-mouse IgE, IgG1 or IgG2a antibodies (Santa Cruz, Inc, USA) for an additional 2 hours at 37 °C. After incubation, the plates were thoroughly washed and the reaction was revealed with o-phenylenediamine (OPD; Sigma, USA). The colorimetric reaction was evaluated at 450 nm using a microplate reader (ELx800, Bio-Tek Instruments, USA).

CYTOLOGY OF BALF AND HISTOLOGICAL ANALYSIS

Cells recovered from BALF were resuspended in 100 µL of saline solution. A 10 µL drop of this cell suspension was dried on a glass slide and stained with May-Grünwald-Giemsa. For histological sections, control or experimental lung tissues were fixed in 4% formaldehyde, embedded in Paraplast, cut into 5-µm sections, and stained with hematoxylin-eosin (Ortloff *et al* 2010)

STATISTICAL ANALYSIS

The results of the percentage of neutrophils in the BALF and the analysis of the antibody levels were assessed by ANOVA. When significant, the means were compared using a Tukey's Multiple Comparison Test. The results are reported as the mean ± SD. A value of $P < 0.05$ was considered significant.

RESULTS

ANALYSIS OF THE CELLULAR COMPOSITION OF THE BALF

The differential neutrophil counts in the BALF from *A. fumigatus*-exposed and non-exposed mice, which were different ages, are shown in figure 1. The cells recovered from the BALF of the exposed mice exhibited a significant increase in the proportion of polymorphonuclear neutrophils. There were significantly greater proportions of neutrophils in 5-, 6- or 8-month-old mice that were exposed to *A. fumigatus* compared to the non-exposed mice. Neutrophils were present in the BALF from 2-month-old mice that were exposed to *A. fumigatus*, but there was no statistically significant difference in this group compared to the non-exposed controls.

HISTOPATHOLOGICAL EVALUATION

Figures 2 and 3 demonstrate the histopathological changes that occurred in the airways of *A. fumigatus*-exposed and non-exposed mice. Clear changes in airway histopathology were only observed in mice that were older than 5 months of age and had been exposed to *A. fumigatus* for 16 days. No differences were observed between exposed and non-exposed mice that were 2 months

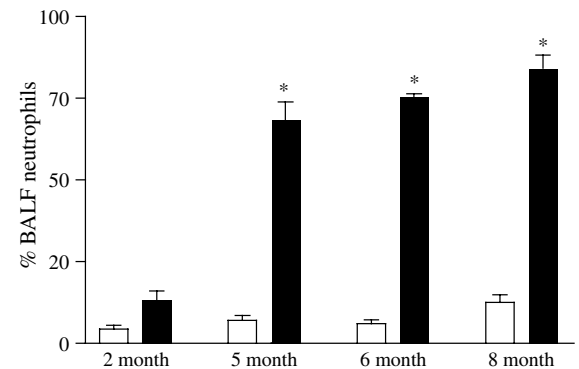


Figure 1. Percentage of neutrophils in bronchoalveolar lavage after being exposed to sterile hay (open bar) or moldy hay (*Aspergillus fumigatus*) (closed bars) for 16 days ($n = 8$ mice per group). Each bar represents the mean ± SD of the relative percentage of neutrophils that were obtained from different groups of mice (* $P < 0.05$ comparing sterile hay to moldy hay exposure).

Porcentaje de neutrófilos en el lavado broncoalveolar después de ser expuesto al heno estéril (barra abierta) o heno con esporas (*Aspergillus fumigatus*) (barras cerradas) durante 16 días ($n = 8$ ratones por grupo). Cada barra representa la media ± desviación estándar de la proporción relativa de los neutrófilos que se obtuvieron de los diferentes grupos de ratones (* $P < 0,05$ se compara el heno estéril con la exposición del heno con esporas).

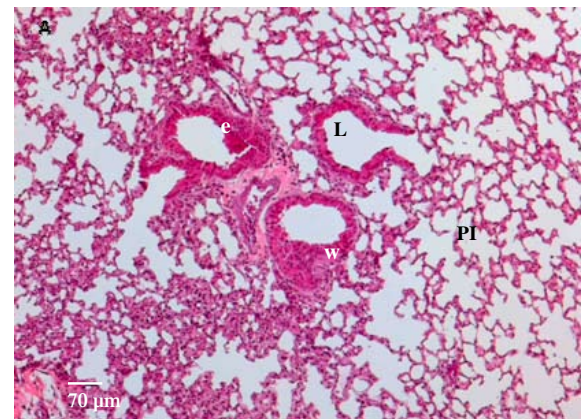


Figure 2. Representative lung histology of one mouse aged (5 month-old) that was exposed to sterile hay for 16 days. The bronchiole contains no inflammatory cell influx within the wall (W), the lumen (L), peribronchial tissue (PI), and the surface epithelium. (e) The lining of the bronchiolar lumen is thin and composed of cuboidal, ciliated and non-ciliated cells. Hematoxylin-eosin stained.

Histología representativa de pulmón de un ratón de 5 meses de edad que se ha expuesto al heno estéril durante 16 días. El bronquiolo no contiene células inflamatorias en la pared (W), lumen (L), tejido peribronquial (PI), y la superficie del epitelio. (e) El revestimiento del lumen bronquial es delgado y está compuesto de células cúbicas, ciliadas y no ciliadas. Tinción Hematoxilina-eosina.

of age. The luminal surface epithelium in the bronchiolar airways of unaffected, non-exposed mice was composed of thin cuboidal epithelial cells. These cells were mostly

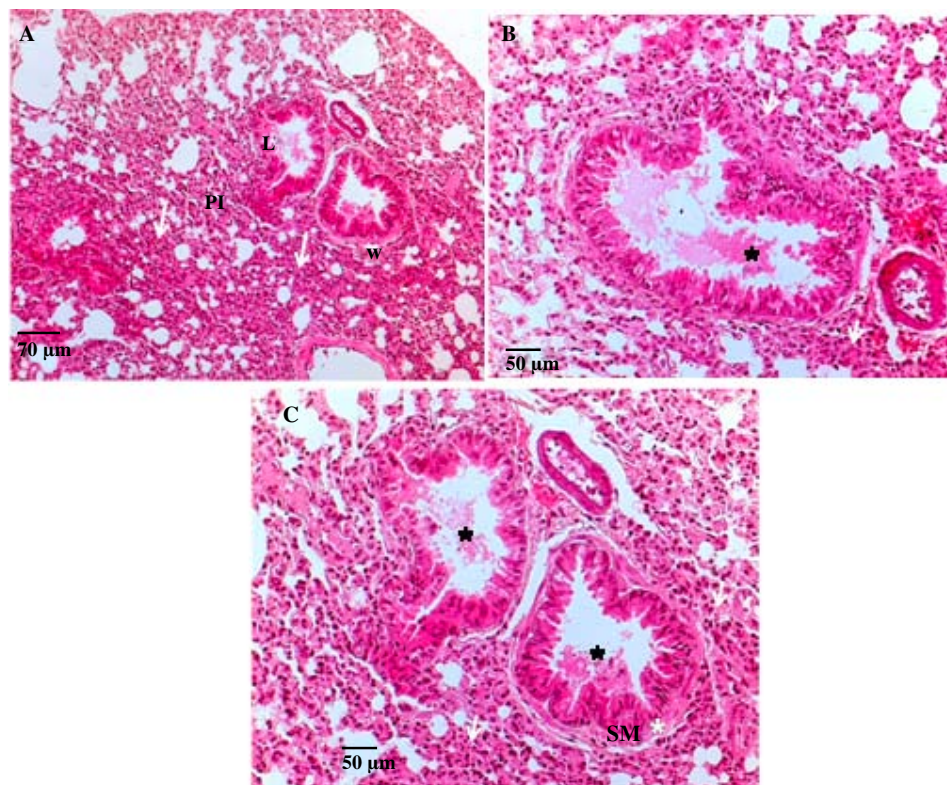


Figure 3. Representative lung histology of mice (5 and 8 month-old) that were exposed to moldy hay (*Aspergillus fumigatus*) for 16 days. (A) Severe atelectasia and peribronchiolar and alveolar infiltration by inflammatory cells (white arrows). (B) Peribronchial infiltration by polymorphonuclear neutrophils and lymphoplasmacytic cells (white arrows). The bronchiolar lumen was stenosed by cells debris (asterisk). (C) Peribronchial infiltration of polymorphonuclear neutrophils and lymphoplasmacytic cells (white arrows). The bronchiolar lumen was stenosed by cells debris (black asterisk) and extensive folding of the airway epithelium was observed. Extensive folding is a sign of smooth muscle (SM) contraction (white asterisk). Hematoxylin-eosin stained.

Histología representativa de pulmón de ratones (5 y 8 meses de edad) que fueron expuestas a heno con esporas (*Aspergillus fumigatus*) durante 16 días. (A) atelectasia grave con infiltración peribronquial y alveolar por células inflamatorias (flechas blancas). (B) Infiltración peribronquial por neutrófilos polimorfonucleares neutrófilos y células linfoplasmáticas (flechas blancas). El lumen bronquial se observa estenosado por detritus celulares (asterisco). (C) se observa una infiltración peribronquial con polimorfonucleares neutrófilos y células linfoplasmáticas (flechas blancas). El lumen bronquial se observa estenosado por detritus celulares (asterisco negro) y una exfoliación del epitelio respiratorio. Esta exfoliación es un signo de contracción (SM) músculo liso (asterisco blanco). Tinción Hematoxilina-eosina.

non-ciliated clara cells and ciliated cells that were either with or without a few intervening mucus cells (figure 2). We observed pathological signs in the lungs of animals exposed to *A. fumigatus*. The bronchiolitis observed in animals exposed to *A. fumigatus* was characterized by significant peribronchiolar and perivascular mononuclear and neutrophilic infiltration, accumulation of intraluminal bronchiolar mucus and serofibrinous exudates. Moreover, there is extensive folding of the airway epithelia, which is a sign of airway smooth muscle contraction and smooth muscle hypertrophy. This inflammatory response was observed predominantly in the peribronchiolar interstitial tissues and often extended into the lamina propria and luminal surface epithelium of the affected airways. We also observed infiltration of inflammatory cells into the alveolar space (figures 3A, 3B and 3C).

ANTIBODY LEVELS IN BALF AND SERUM

BALF from 5-, 6- and 8-month-old mice, which had been exposed to *A. fumigatus*, exhibited a significant increase in fungus-specific IgE, IgG1 and IgG2a antibodies when compared to control mice. However, there was no significant difference in the levels of *A. fumigatus*-specific antibodies in BALF from 2-month-old mice (figure 4). *A. fumigatus* IgE levels in the serum from the exposed mice in all age groups were significantly greater than the serum levels in the control mice (figure 5A). However, serum IgG1 levels were not significantly different (figure 5B). Serum IgG2a levels were only significantly different between the control mice and exposed mice that were older than 6 months of age (figure 5C).

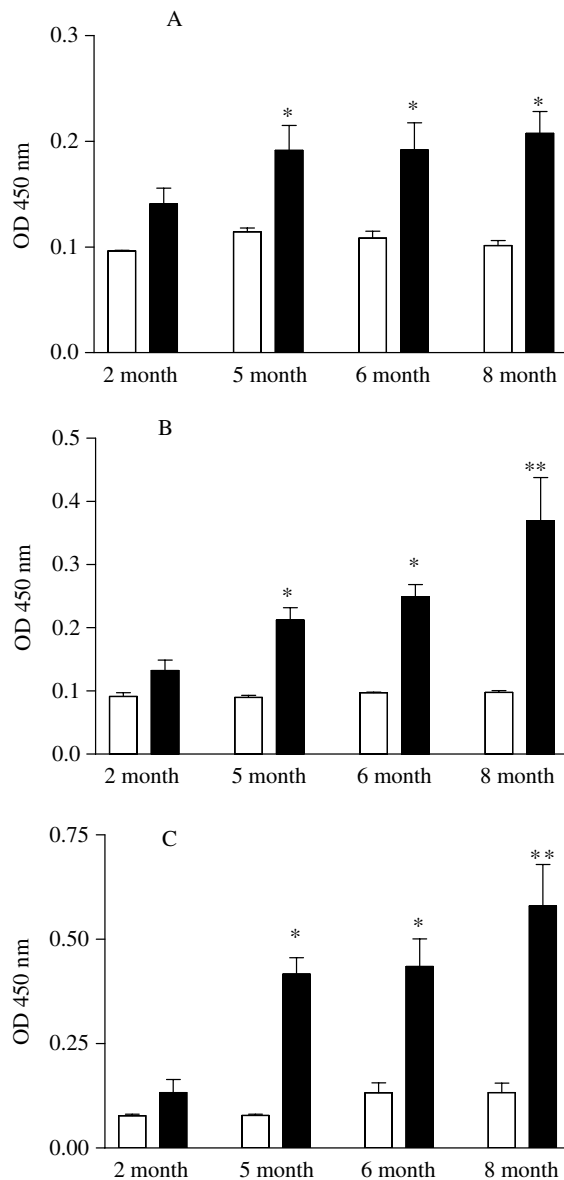


Figure 4. Level of specific IgE (A), IgG1 (B) and IgG2a (C) against *Aspergillus fumigatus* in BALF from mice that were exposed to either sterile hay (open bars) or moldy hay (closed bars), 16 days after allergen exposure (n = 8 mice per group). Each bar represents the mean of the optical density (OD), which was obtained from each of the different groups of mice (*P < 0,05; ** P < 0,01 comparing sterile hay to moldy hay exposure).

Nivel de IgE (A), IgG1 (B) y IgG2a (C) específicas contra *Aspergillus fumigatus* en BALF de los ratones que fueron expuestos a heno estéril (barras abiertas) o al heno con esporas (barras cerradas), 16 días después de exposición al alérgeno (n = 8 ratones por grupo). Cada barra representa la media de la densidad óptica (DO) que se obtuvo de cada uno de los diferentes grupos de ratones (*P < 0,05, ** P < 0,01 se compara la exposición del heno estéril con heno con esporas).

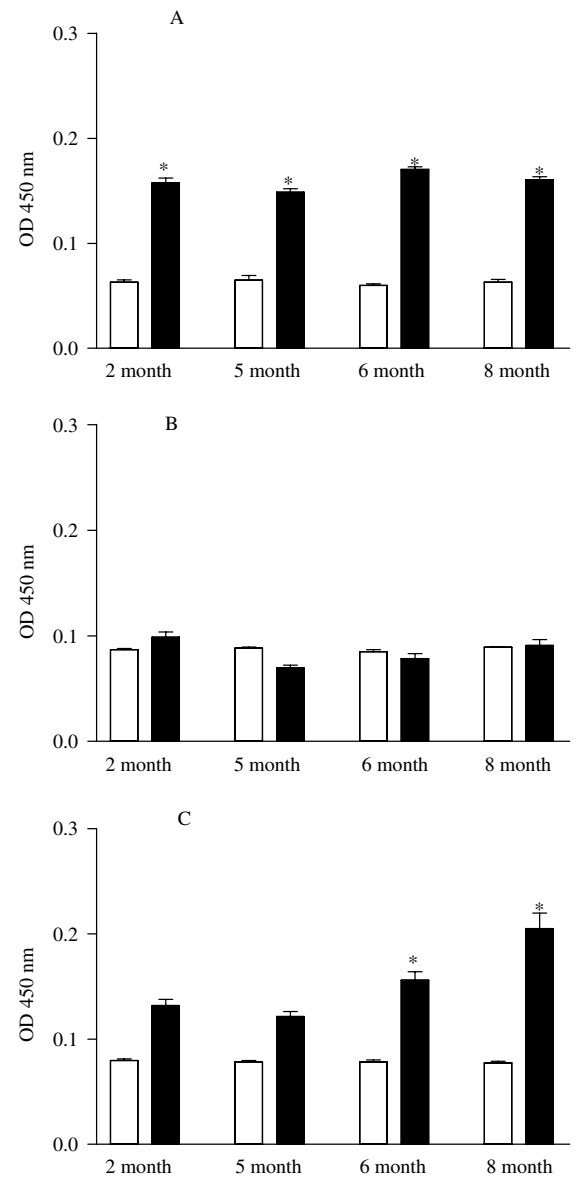


Figure 5. Level of specific IgE (A), IgG1 (B) and IgG2a (C) against *Aspergillus fumigatus* in the serum from mice that were exposed to either sterile hay (open bars) or moldy hay (closed bars), 16 days after allergen exposure (n = 8 mice per group). Each bar represents the mean of the optical density (OD), which was obtained from each of the different groups of mice (*P < 0,05, ** P < 0,01 comparing sterile hay to moldy hay exposure).

Nivel de IgE (A), IgG1 (B) y IgG2a (C) específicas contra *Aspergillus fumigatus* en sueros de los ratones que fueron expuestos a heno estéril (barras abiertas) o al heno con esporas (barras cerradas), 16 días después de exposición al alérgeno (n = 8 ratones por grupo). Cada barra representa la media de la densidad óptica (DO) que se obtuvo de cada uno de los diferentes grupos de ratones (*P < 0,05, ** P < 0,01 se compara la exposición del heno estéril con heno con esporas).

RECURRENCE OF SIGNS ASSOCIATED WITH RESPIRATORY DISTRESS

Only older mice (5 to 8 months of age) exhibited clear signs of respiratory distress, such as tachypnea and audible sibilance, 16 days after exposure to *A. fumigatus*. The distressed animals displayed a different behavior when compared to control or young mice. The distressed mice also showed a tendency to huddle into groups and remain still. These animals recovered 7 to 8 days after being relocated into a remission environment. After this period, the experimental animals were exposed again, as previously described, to *A. fumigatus*. However, the mice showed signs of respiratory distress four days after exposure. Same as before, the animals recovered when they were placed in a remission environment. Antigen exposure was repeated one additional time, 2 weeks after the final observation of signs associated with respiratory distress. This time, the onset of respiratory distress was faster (one day after exposure). Once again, the animals recovered when placed in a remission environment.

DISCUSSION

RAO in horses is a far more complex condition than allergic diseases, such as insect bite hypersensitivity and atopic asthma in humans (Art *et al* 2008). Advances have been made in the understanding of the immunological and clinical aspects of RAO in horses. However, it is not yet possible to understand the precise mechanism involved in RAO immunopathology. For this reason, the goal of this study was to generate an experimental mouse model of RAO that closely mimics the condition in horses, which focused on the different stages of exacerbation. Generally, experimental models of various pathophysiological processes are limited because they typically do not recapitulate the disease processes that occur in the original species. However, murine models have contributed to the understanding of the pathogenesis of many diseases and the development of new therapies. Studies on the immunological mechanisms and factors that contribute to the generation of airway inflammation in RAO-affected horses have been hampered due to size of the animal, the relatively long lifespan and the lack of suitable immunological tools. The advantages of mouse models include the availability of large numbers of animals, ease of handling and a large number of immunologic tools. Although there are anatomical differences between the murine lung and the horse lung, our results demonstrate that exposure to *A. fumigatus* induces airway inflammation in older mice. This inflammatory response is characterized by recurrence upon antigen re-exposure, enhanced polymorphonuclear neutrophil levels in BALF, and histological features that are similar to those described for RAO in horses (Kaup *et al* 1990^{a,b}, McGorum *et al* 1993, Robinson 2001, Kleiber

et al 2004). This increased immune response is specifically localized to the respiratory tract.

In RAO-affected horses, the infiltration of inflammatory cells in the airway and alveoli is diffuse throughout the lung. Thus, BAL is utilized to diagnose RAO because the inflammatory cells that are recovered should be representative of the overall population of cells in any specific lobular part and is therefore valid for the diagnosis of heaves (McGorum *et al* 1993^a). Neutrophilic inflammation is the most consistent abnormality that is present in BALF from RAO-affected horses (Derksen *et al* 1989, McGorum *et al* 1993^a). Neutrophilia in BALF from horses with RAO is positively correlated with airway hyperactivity (Derksen *et al* 1985, Dixon *et al* 1995, Fairbairn *et al* 1993, Robinson 2001). The presence of large numbers of neutrophils in the BALF from animals that were older than five months is an important finding of our study. Our results are coincident with those of a clinical study of RAO-affected horses. Furthermore, older animals that were exposed to *A. fumigatus* had a higher percentage of neutrophils in the BALF (60% or more), when compared to 2-month-old animals (20%). In horses, animals that are older than 5 years of age are more frequently affected by RAO compared to younger animals, and the prevalence of RAO increases with age (Leguillette 2003). Côté and War (2003) suggest that the incidence of RAO increases until the animals reach 7 to 10 years of age. Once the horses reach this age, the incidence of RAO remains fairly constant. The same phenomenon was observed in our murine model, where airway inflammation and respiratory distress were observed in animals that were older than 5 months of age. However, in our clinical observations, we have found that older horses suffering from RAO sometimes develop clinical exacerbations but do not exhibit a high increase of neutrophils in the BALF. We presume that this finding occurs because these animals are in a more advanced stage of the airway remodeling. Airway smooth muscle remodeling in RAO-affected horses may be involved in airway hyperresponsiveness and chronic lung function impairment through mechanism that are comparable to human asthma (Herzberg *et al* 2006).

On the other hand, the main histopathological lesion observed in RAO-affected horses is bronchiolitis, which is an accumulation of peribronchiolar lymphocytes that is accompanied by an increase in intraluminal neutrophils (Kaup *et al* 1990^{a,b}, Lugo *et al* 2006, Robinson *et al* 1996). The mice in our study developed histopathological changes that are coincident with RAO.

A. fumigatus has been shown to be important in the etiology of RAO (Mcpherson *et al* 1979, Derksen *et al* 1988, MacGorum *et al* 1993^{a,b}). This fungus produces small conidia and spores that can remain suspended in the air for long periods of time. These factors increase the probability of the mold being inhaled by stabled horses (Jackson *et al* 2000). Moreover, this allergen is present, in large amounts, in the fodder of Chilean horse farms (Araya

and Zaror 1995). RAO in horses is different from ABPA in humans. In addition to bronchospasms, mononuclear cell infiltration into the lungs, and increased levels of *A. fumigatus*-specific IgE antibodies, human patients usually exhibit blood and lung eosinophilia (Greenberger *et al* 2002). The blood and lung eosinophilia is an aspect that is shared with the animal models used to study this human disease (Grüning *et al* 1997, Kurup *et al* 1999, Hogoboam *et al* 1999). Under our experimental conditions, the bronchiolitis observed in the mice was characterized by neutrophil infiltration with a low presence of eosinophils. These findings are similar to the bronchiolitis that occurs in RAO-affected horses.

Compared to healthy animals, horses affected with RAO have significantly higher levels of *A. fumigatus*-specific IgE and IgG antibodies in their BALF (Halliwell *et al* 1993, Schmallenbach *et al* 1998). Our results show that the immune response against *A. fumigatus* allergen, in mice, is characterized by increased levels of antigen-specific IgE, IgG1 and IgG2a antibodies in the BALF. However, the serum levels of these antibody isotypes did not differ between *A. fumigatus* exposed and non-exposed mice, with the exception of IgE and IgG2a. IgE and IgG2a were significantly different in 6- to 8-month-old mice. The same phenomenon occurs in RAO-affected horses (Schmallenbach *et al* 1998, Eder *et al* 2000), confirming that local and systemic immune responses are likely regulated independently of one another. Our findings, which show that IgE and IgG1 are present in the local airways of mice, are in agreement with previously published results that have shown similar findings in RAO-affected horses (Halliwell *et al* 1993, Schmallenbach *et al* 1998, Eder *et al* 2000). In our study, we show that animals exposed to *A. fumigatus* showed a significant increase in serum IgE levels. However, this increase was not observed for IgG1. Although IL-4 stimulates B cell switching to both IgE and IgG1, differences exist in the kinetics of isotype switching in the mouse. The kinetic differences are based on the concentrations of IL-4 that are required to induce isotype switching and the requirements for co-stimulation (Gascan *et al* 1991, Mandler *et al* 1993). These observations may partly explain the increased serum IgE levels, compared to the levels of IgG1, in mice exposed to *A. fumigatus*.

The increased serum and BALF IgE levels suggest that a type I hypersensitivity response may be involved in the pathogenesis of *A. fumigatus*. Also, the increase in the levels of IgG2a in the BALF from mice that were exposed to allergen is compatible with a Th1 response (Takaoka *et al* 2001). These findings mimic the conditions typically observed in RAO-affected horses that have elevated levels of IgGa and IgGb isotypes (Halliwell *et al* 1993). Moreover, IgG2a can activate the complement cascade, which may partly explain the increase in neutrophils observed in the BALF of mice that were exposed to *A. fumigatus*. In equines, RAO is characterized by neutrophilic bronchiolitis, which is considered evidence of a type III response that

results from antigen-antibody complex formation and the subsequent activation of the complement cascade. These events, in turn, cause a release of the anaphylatoxin peptides, C3a and C5a (Lavoie *et al* 2001, Franchini *et al* 2000). A recent study that used IL-4, IFN- γ and IL-12 KO mice showed that the respective cytokines play an important role in the inflammatory process. However, each of these cytokines contributes differentially to the inflammatory process and their individual contributions are based on the route of immunization (Fischer *et al* 2007). In addition, some groups have reported different outcomes of the inflammatory process depending on whether the antigen was delivered orally or nasally. Depending on the route of administration, the resulting inflammation can mimic either eosinophil- or neutrophil- mediated asthma-like diseases (Kurup *et al* 2001). Here, the challenge of mice with *A. fumigatus*-contaminated bedding resulted in a significant increase in neutrophils in the BALF and changes in normal lung histology, which is similar to the condition that occurs when horses are exposed to moldy hay.

As mentioned above, IgE-mediated type I reactions and type III reactions have been suggested to be involved in the pathogenesis of heaves (Halliwell *et al* 1993, Schmallenbach *et al* 1998, Eder *et al* 2000, Franchini *et al* 2000, Eder *et al* 2001, Lavoie *et al* 2001, Curik *et al* 2003, Künzle *et al* 2007, Tahon *et al* 2009, Moran *et al* 2010^{a,b}). Moreover, recent findings suggest that in addition to the contribution of inflammatory cytokines, such as IL-1 β , IL-8 and IL-17 (Cordeau *et al* 2004, Ainsworth *et al* 2007, Pietra *et al* 2007, Riihimäki *et al* 2008), helper T lymphocytes may contribute to the pathogenesis of heaves through their secretion of either Th1 or Th2 cytokines (Giguere *et al* 2002, Ainsworth *et al* 2003, Horohov *et al* 2005). Resident lung cells have also been implicated in the onset of the airway inflammatory response against *A. fumigatus*. Many resident lung cells express lectin and Toll-like receptors that can interact with *Aspergillus* components. Thus, inhalation of the fungus alone may induce the production of proinflammatory, Th-1 and Th-2 cytokines, which may be sufficient to trigger the onset of respiratory airway inflammation (Rementeria *et al* 2005, Meier *et al* 2003, Berndt *et al* 2007). In our study, the progressive nature of the disease that was induced by fungus aspiration indicates that a specific immune response is needed and that this response is probably modulated by cytokines coming from the innate immune system.

In summary, RAO-affected horses spontaneously develop bronchoconstriction and airway inflammation in response to specific airborne irritant and allergen exposure (Robinson 2001). Clinical manifestation of the disease can be tempered by altering environmental conditions to minimize exposure to the causative agents. Therefore, research using this mouse model for the study of equine RAO can offer the opportunity to approximate the immunologic response to airborne irritants and allergens as well as to evaluate pharmacological therapies. We conclude

that simple exposure of mice to moldy hay induces a spontaneous condition that is similar to RAO in horses, including the recurrent airway inflammation and similar histopathological changes. In due time, this procedure may constitute an experimental model for RAO because the allergic inflammatory reaction we observed here is a very complex phenomenon that accurately mimics RAO in horses. Further studies are warranted to fully understand the pathogenic mechanisms of RAO which, in turn, can lead to new treatment approaches.

SUMMARY

The aim of this study was to determine if mice exposed to *Aspergillus* (*A. fumigatus*) spores develop airway inflammation in a similar manner to Recurrent Airway Obstruction (RAO) in horses. Different groups of 2-, 5-, 6- and 8-month-old Rockefeller (RK) mice were used throughout the experiment. The mice were maintained in the animal house for 16 days and kept in cages containing hay contaminated with *A. fumigatus*. After 16 days of mold exposure, the mice were bled and sacrificed to obtain bronchoalveolar lavage fluid (BALF) or lung tissues for histological analysis. In addition, we measured the levels of *A. fumigatus*-specific antibodies by ELISA. The results demonstrated that inhalation of *A. fumigatus* spores in mice induced an inflammatory response in the lungs that was characterized by peribronchiolar and perivascular mononuclear infiltration and bronchiolar luminal exudates. Moreover, there was extensive folding of the airway epithelia, which is a sign of airway smooth muscle contraction and smooth muscle hypertrophy. In addition, we observed a high number of neutrophils and an increase in IgE, IgG1 and IgG2a antibodies in the BALF from mice that were older than 5 months and were exposed to *A. fumigatus*. These animals recovered from their respiratory distress when they were placed in a remission environment. We conclude that simple exposure of mice to moldy hay induces a condition that is similar to RAO in horses. Therefore, using this mouse model for the study of equine RAO offers the opportunity to approximate the immunologic response to airborne irritants and allergens as well as to evaluate pharmacological therapies.

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