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Allergic Effects of Airborne Fungal Conidia on BALB/c Mice

Madhu Manikpuri, Rajesh Anand and Bhupendra N. Tiwary*

Department of Biotechnology, Guru Ghasidas Vishwavidyalaya, Bilaspur – 495009, Chhattisgarh, India; tiwarybn8@gmail.com

Abstract

Airborne fungal spores are one of the major causes of human respiratory disorders that are responsible for allergic reactions, asthma and respiratory tract infections. Determination of total and specific serum Immunoglobulin E (IgE) level has been widely applied to the diagnosis of allergic diseases including allergy caused by fungi. The present study was carried out to assess the allergenic potential of dominant airborne fungal spores and its impact on the respiratory system. The BALB/c mice were sensitized intranasally with the conidial suspension of six selected fungal stains and the allergic potential was evaluated on the basis of serum IgE, differential blood cell counts and histopathology of the lung tissues from mice receiving post sensitization doses. A significant increase in the total serum IgE level was observed in the serum of all mice as compared to the control. Amongst the fungi used in the study, *Aspergillus terreus* showed the maximum increase in the level of serum IgE of treated animals. The differential cell count revealed the highest increase in the eosinophil count in mice treated with *Aspergillus terreus* and *Penicillium* sp. The histopathological analysis of the lungs of treated animals showed increased infiltration of blood cells and thickening of airway epithelia. The observation of the study indicated that spores of *Aspergillus terreus* have greater allergic potential than all other airborne fungi.

Keywords: Aeroallergens, Allergens, Allergic Rhinitis

1. Introduction

Exposures to fungal spores can pose a significant concern to human health, such as invasive diseases, allergic sensitization, hypersensitivity pneumonitis, and asthma[1]. For centuries, fungi have been known to be associated with hypersensitivity reactions in humans[2]. In recent years, the prevalence of allergic diseases has increased worldwide[3]. Fungal spores are one of the important aeroallergens and are presently perceived as one of the critical reasons for respiratory hypersensitivities[4]. It has been estimated that around 2-6% of the human populations are sensitive to fungal spores[5]. The most common allergens are derived from several genera, including *Aspergillus*, *Penicillium*, *Curvularia*, *Coccidioides*, *Fusarium*, etc[6]. The allergic reaction is an altered and accelerated response of the body's immune system to a foreign substance to which a person has been sensitized by earlier exposure[7]. The allergens are a subset of antigens that stimulate a type I immediate, Immunoglobulin E (IgE)-mediated hypersensitivity on the exposure after sensitization.[4] On account of atopic sensitization, the allergen can cause asthma, allergic rhinitis or conjunctivitis, urticaria, or atopic dermatitis[6]. The primary route of exposure is inhalation of allergic fungal spores where they mediate the first contact with the immune system[8]. IgE has long been considered as the major molecular component of atopic diseases, including asthma. Clinical studies have revealed a close association

*Author for correspondence

between asthma and serum IgE levels[9–11]. In allergic reactions, on exposure to an antigen, the plasma cells produce IgE which binds to high-affinity IgE receptors on mast cells and basophils. The binding of IgE to cells triggers the activation of these cells after cross-linking by antigen, therefore, initiating the allergic reaction. Activated mast cells release key molecules in anaphylactic and immediate-type allergic reactions[12]. Furthermore, mast cells have been shown to produce the T helper 2 (Th2)-type cytokines[13]. Interleukin 4 (IL-4), produced by these cells may induce B cell to produce IgE and similarly, IL-5 produced by these cells act as a primary factor for eosinophil activation and differentiation. IgE receptors have also been demonstrated on eosinophils which are essential cells in the allergic asthmatic response[4]. Animal models of allergic diseases have been investigated to find insights about the immunological responses that follow the fungal challenge and shows potential relevance to human diseases[5,7]. The animals develop an allergic response with an elevated level of serum IgE, prominent eosinophilia in lung and development of airway hyperresponsiveness[14]. Many of these studies are based on a single exposure with few studies using repeated exposures and even fewer studies utilizing inhalation exposures[15,16]. In the present study we used an immune-competent murine model (BALB/c mice) with repeated inhalation exposures of conidial suspensions of different fungal strains. The BALB/c mice were primarily sensitized with a selected dose of conidial suspension and subsequently challenged with the same antigen through the airways. The rationale of this study was to ascertain the effects of sensitization doses of fungal conidia and to assess the allergic responses of these airborne fungal spores on the respiratory system.

2. Material and Methods

2.1 Fungal Cultures and Preparation of Conidial Suspensions

Six most frequent airborne fungal strains (*Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus terreus*, *Alternaria alternata,* and *Penicillium* sp.) found during the sampling of air in and around Bilaspur city (C.G.), India was selected for the study[17]. The study was done to see the allergic effects of these fungal isolates using an animal model (BALB/c mice). All the strains were cultured on Potato Dextrose Agar (PDA) medium at a temperature of 28±2°C for 5 days. After incubation conidia were harvested by flooding the culture with Phosphate Buffer Saline-Tween-20 (0.01% (v/v) sterile polyoxyethylene sorbitan monooleate, Merck) solution (PBS-T), gently scraped with the aid of a sterile needle and collected in sterile vials[18]. The conidia were enumerated by direct count using the Neubauer hemocytometer and suspension of desired concentrations was prepared for each strain using sterile PBST. The viability of conidia of all the fungal strains was determined by germination of spores on PDA. Freshly prepared conidial suspensions were used in the experiment.

2.2 Animals

Male BALB/c mice (aged 8 to 9 weeks) were procured from the Columbia Institute of Pharmacy, Raipur (C.G.) and were maintained as per the guidelines of IAEC (Institutional Animal Ethics Committee). Prior to starting the administration of fungal conidia, the animals were acclimatized for one week by providing standard food and water ad libitum in the animal house.

2.3 Experimental Groups

Altogether seven groups were made for the animal studies (four animals each group): Group I, II, III, IV, V, VI (treated) and VII (Control). Animals of group I to VI were treated with a selected conidial suspension while the control group was administered with PBS-T (Table 1).

2.4 Determination of Lethal Dose of Fungal Conidia and Selection of the Sensitization Dose

In order to determine the number of mold spores, required for the induction of an immune response, BALB/c mice of each group were treated intranasally with different concentration of the conidial suspension of selected fungal strains. After dosing, the lethal dose was determined by observing the mortality rate of animals in each group.

2.5 Sensitization and Allergic Studies

The animal groups were sensitized with their respective fungal strains using the optimized lethal dose of fungal spores (Table 1). Under a low dose of halothane, mice were placed in an upright position and inoculated intranasally with 50µl (25µl per nare) of the conidial suspension. The mice were then held in the upright position for two minutes to allow for complete instillation of the administered dose. Control group was inoculated with 50µl (25µl per nare) of PBS-T.

Table 1. The doses of different fungal strains used for the sensitization

Group	Fungal strain	Inoculum concentration (Conidia/50µl PBST)
I	*Aspergillus niger*	3.5×10^7/50µl
II	*Penicillium* sp.	2.0×10^4/50µl
III	*Alternaria alternata*	1.7×10^6/50µl
IV	*Aspergillus terreus*	2.5×10^7/50µl
V	*Aspergillus flavus*	1.1×10^7/50µl
VI	*Aspergillus fumigatus*	1.5×10^7/50µl
VII	Control	50µl PBST

The procedure was re-iterated four times on a weekly basis (Day 1, 8, 15 and 22). The control groups were examined in parallel. The treated and control mice were then examined for total serum IgE (weekly), leukocyte in blood and histological parameters of the lung tissue.

2.6 Quantification of Total Serum IgE

Blood samples were collected from the retroorbital plex of all the experimental and control mice. The amount of serum IgE produced in response to the fungal conidia was determined by Enzyme-Linked Immunosorbent Assay (ELISA), using the total mouse IgE kit (Sigma Aldrich). The sandwich ELISA protocol as described in the kit were followed for the determination of total IgE in the animal serum. The quantities of serum IgE were calculated using the software MasterPlex ReaderFit (Hitachi Software Engineering America Ltd., USA), applying the five parameter best curve fit method.

2.7 Leukocyte Determination in Mice Blood

Leukocyte counts in the blood samples of sensitized and control mice were determined in the blood smear stained with Wright's stain through microscopic examinations. The smear was evaluated for each cell of the leukocyte species; lymphocytes, monocytes, neutrophils, basophils, and eosinophils.

2.8 Histological Examinations

The dissected lungs of treated mice were removed and fixed in 10% neutral buffered formalin (10% formalin containing 4g sodium dihydrogen orthophosphate monohydrate and 6.5g disodium hydrogen orthophosphate anhydrous per liter of solution), and embedded in paraffin. Ultrathin sections (5µm thick) were stained with hematoxylin and counterstained with eosin. The stained sections were viewed at different magnification under the light microscope equipped with a digital camera (Leica DM IL LED).

3. Statistics

Standard errors and deviations were calculated and two-way ANOVA at a significance level of 0.05 was also performed using the software Graph Pad Prism 7.

4. Results

Mice exposed to fungal spores under this protocol exhibited airway hyper responsiveness, increased IgE production and abnormal blood cell count when compared to the control.

4.1 Lethal Dose

The lethal dose of fungal conidia of each strain was determined by observing the mortality of experimental animals (Table 2).

4.2 Total Serum IgE Value

The result revealed a marked increase in total serum IgE levels in all treated groups compared to the control group. The level of serum IgE increased on day 14 in all the groups of mice challenged for the first time after sensitization dose. However, on the 21st day (two

Table 2. Determination of lethal dose in terms of the mortality rate of treated animals

Animal groups	Fungal strain	% mortality	Lethal dose	Sensitization dose
I	*Aspergillus niger*	100	3.5×10^{8}/50 µl	3.5×10^{7}/50µl
II	*Penicillium sp.*	100	2.0×10^{5} /50 µl	2.0×10^{4}/50µl
III	*Alternaria alternata*	50	3.5×10^{6}/50 µl	1.7×10^{6}/50µl
IV	*Aspergillus terreus*	100	2.5×10^{8}/50 µl	2.5×10^{7}/50µl
V	*Aspergillus flavus*	100	1.1×10^{8}/50 µl	1.1×10^{7}/50µl
VI	*Aspergillus fumiatus*	100	1.5×10^{8}/50 µl	1.5×10^{7}/50µl

Table 3. Amount of total serum IgE (ng/ml) produced in response to fungal conidia challenge

Animal groups	Fungal strain	Total serum IgE (ng/ml)		
		Day 14	Day 21	Day 28
I	*Aspergillus niger*	195.36±6.75	144.17±2.68	153.66±3.50
II	*Penicillium* sp.	187.12±81.81	163.07±1.42	105.72±2.53
III	*Alternaria alternata*	121.23±11.32	105.95±1.85	110.30±0.80
IV	*Aspergillus terreus*	174.24±56.67	170.04±1.53	203.37±8.58
V	*Aspergillus flavus*	118.92±3.66	75.77±0.20	118.09±1.48
VI	*Aspergillus fumigatus*	112.79±1.43	67.09±1.26	202.41±9.66
VII	PBST (Control)	77.8±18.91	98.58±5.90	98.40±8.99

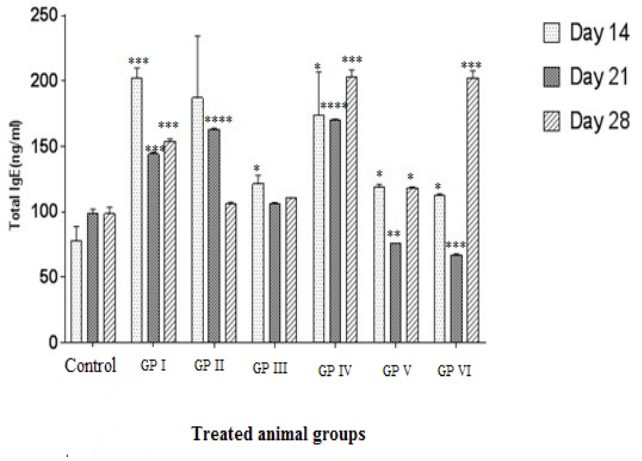


Figure 1. Total serum IgE (ng/ml) in mice treated with fungal conidia.

doses post sensitization) the entire group continued the increasing trend except the groups V (*A. flavus*) and VI (*A. fumigatus*). Surprisingly, after the third dose (post sensitization) only *A. niger*, *A. terreus*, and *A. fumigatus* demonstrated a significant increase in the IgE level (Table 3, Figure 1).

4.3 Differential Cell Count

The inflammatory cell response in the blood samples of mice treated with different fungal spores was very similar although the numbers of inflammatory cells were constantly lower in the control group. Intranasal inoculation of spores in mice induced the accumulation of macrophages, neutrophils, eosinophils and a lower amount of lymphocytes and there was a significant increase in the number of blood cells among the animals of treated groups. The result showed that inoculation of the fungal conidia in BALB/c mice led to the accumulation of a high number of neutrophils in all the groups of mice treated with fungal conidia as compared to the control. The minimum number of lymphocytes in blood was observed in animals of GP I, treated with *A. niger*. Among all the groups, the number of neutrophils was highest in the animals of GP IV treated with *A.terreus*. Similarly, an increased number of blood eosinophils were observed in animals of GP IV while minimum numbers were recorded in control (Figure 2).

4.4 Histological Examinations

Histological examination of lung sections from the sensitized and challenged mice demonstrated an accumulation of eosinophils around the vessels and the bronchioles, a thickening of the airway epithelia and the presence of goblet cells in sensitized mice. Non-sensitized mice showed minimal inflammation foci devoid of eosinophils, and epithelial thickening (Figure 3).

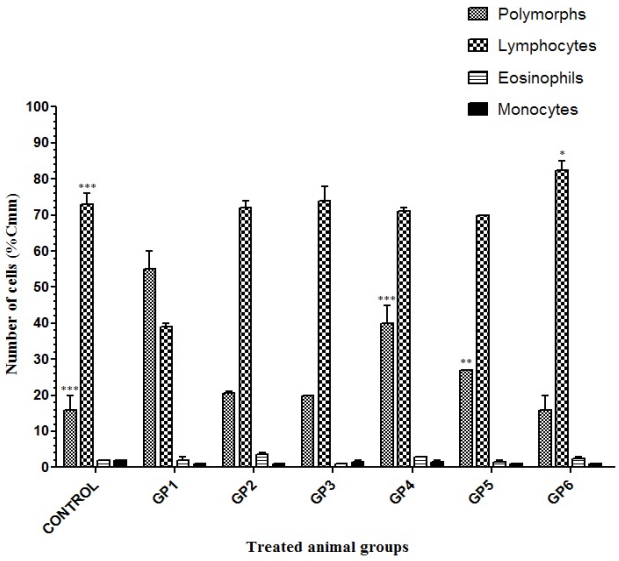


Figure 2. Differential blood cell count of animals of different groups treated with respective fungal conidia.

5. Discussion

Animal models of fungal exposure have provided an understanding of the pulmonary immune mechanisms that mediate fungal clearance and pathogenesis. In this study, we hence described a system that more closely represents typical inhalation exposures to fungal conidia and the pulmonary immune responses in immuno-competent mice following repeated (n = 4) exposures to conidia of different fungi. Mice were exposed four times at weekly interval and harvested 24 hours after the final exposure. Initially, the lethal dose of fungi was determined as described earlier. In the present study animals of all the experimental groups showed a marked increase in serum IgE production in response to post sensitization with the

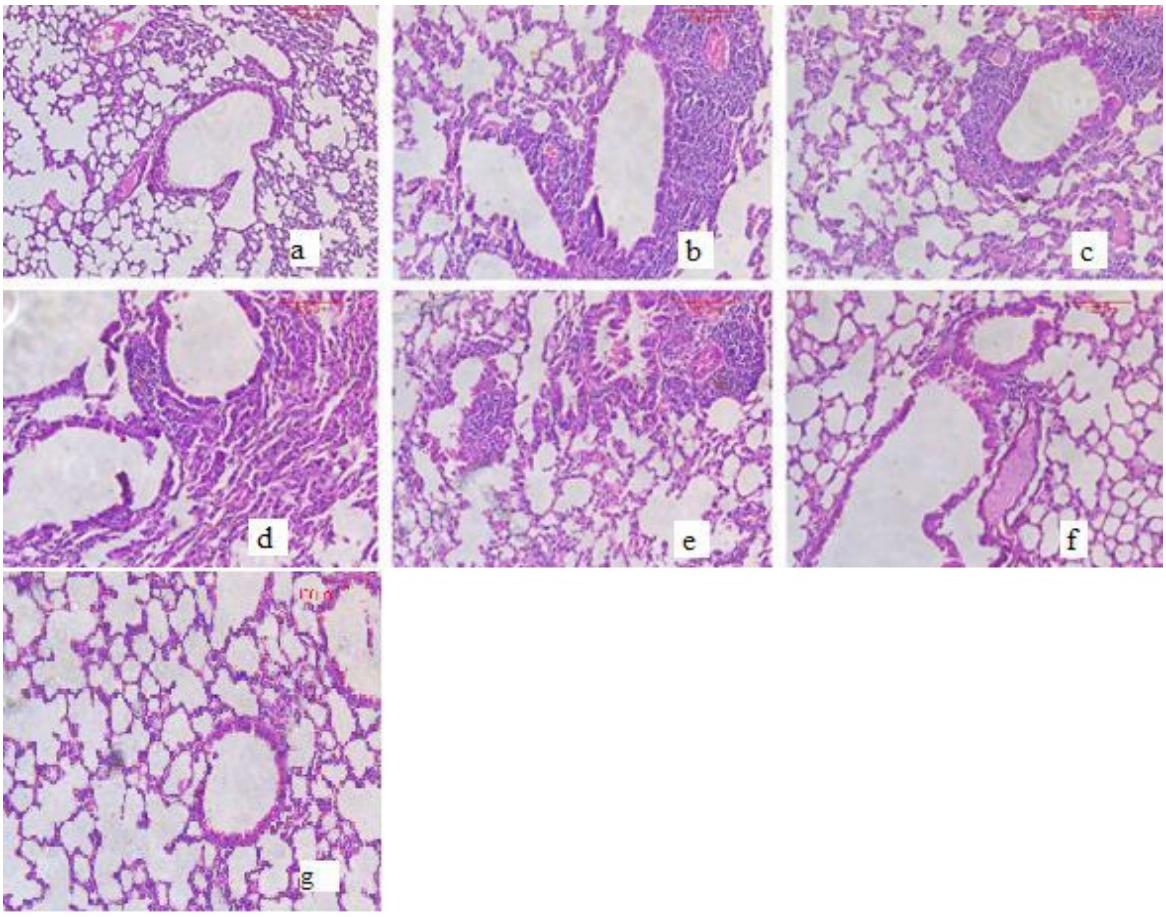


Figure 3. Histological analysis of lung sections of experimental mice.
Lung section: a) GP I mice treated with *A. niger* conidia, b) GP II mice treated with *Penicillium* sp. conidia, c) GP III mice treated with *Alternaria alternata* conidia, d) GP IV mice treated with *A. terreus* conidia, e) GP V mice treated with *A. flavus* conidia, f) GP VI mice treated with *A. fumigatus* conidia, and g) Control

fungal conidia. The high amount of total serum IgE may indicate the prevalence of allergic reactions. All the fungi demonstrated allergic reaction at one or more time points during the present study. How00eveA CccC./mgasr, the maximum allergic reactions were observed in .,the case of *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus fumigatus*. The higher allergic reactions of these fungi might be due to higher sensitization doses used during the study, suggesting the allergy to be dependent on the quantity of allergen introduced to the host. The hallmark of allergic reactions and the one parameter used for screening allergy in patients is the serum IgE level[19]. IgE holds a unique position among immunoglobulins. It is normally present in human serum in extremely small amounts; its serum concentration may increase several hundred folds in response to specific stimuli. The levels are increased in allergic diseases like allergic rhinitis, allergic bronchial asthma, and atopic dermatitis or urticaria. When allergens entered into the body specific B cells are introduced to produce IgE. Allergens combined with IgE to induce an allergic reaction revealing its role as an important objective index for the diagnosis of allergic diseases. Raised IgE levels are strongly associated with atopic disease in humans[20]. The lungs of sensitized mice with fungal conidia showed subtle eosinophilic infiltrations around bronchioles, confirming that the

allergen challenges in this protocol induced acute allergic airway inflammation.

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7. Declaration of Interest

The author reports no conflicts of interest. The authors are responsible for the content and writing of the manuscript.

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