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ORIGINAL ARTICLE

Evaluation of the correlation between tissue reaction and cytokines patterns induced by *Alternaria alternate* in mice

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Abstract *Alternaria alternata* is well-known as a source of allergenic components in the cell wall and cytoplasm of conidia and hyphae that cause respiratory allergic disorders. The purpose of this study was to evaluate tissue reaction and Th_2 cytokines in mice exposed to *A. alternata*. *A. alternata* was cultured, and fungal extract was prepared by freeze–defreeze and sonication methods. BALB/c mice in one group were sensitized by two intraperitoneal injections of *A. alternata* extract and then intra-nasally challenged with spores suspended in sterile normal saline solution, and in another group, mice only received spores intra-nasally. Blood sampling and necropsy were performed at 1 and 72 h after spore inhalation. Histopathology demonstrated an inflammatory response with cells including lymphocytes, macrophages, neutrophils and eosinophils present and mucus hypersecretion in the lungs and airway epithelial cell hyperplasia and necrosis observed in sensitized and non-sensitized mice. Sera were analyzed by ELISA to determine serum levels of Interleukin (IL)-4 and IL-13 in immediate response and late-phase reaction, respectively. Increasing Th_2 cytokine (IL-4 and IL-13) levels in the sera was also observed in the sensitized and challenged mice. The results showed that exposure to extract and then spores of *A. alternata* induced rapid and highly elevated production of IL-4 and IL-3. These cytokines were associated with respiratory histopathological changes.

Keywords *Alternaria alternate* · IL-4 · IL-13 · Lung · Macrophage

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Introduction

Alternaria species are common indoor and outdoor air-borne fungi, which have been identified as causative agents of respiratory allergic disorders in humans and domestic animals (Downs et al. 2001; Dye et al. 2005). *Alternaria* produces many different proteins that function as allergens (Sanchez and Bush 2001). Immediate (type 1) hypersensitivity results from the activation of the Th_2 subset of CD4+ helper T cells by environmental antigens, leading to the production of IgE antibodies, which become attached to mast cells. When these IgE molecules bind the antigen (allergen), the mast cells are triggered to release mediators that transiently affect vascular permeability and induce smooth muscle contraction in various organs and may stimulate more prolonged inflammation (the late-phase reaction). When individuals who were sensitized by previous exposure to an allergen are re-exposed, the allergen binds to multiple specific IgE molecules on mast cells, usually at or near the site of allergen entry (Kumar et al. 2007). Often, the IgE-triggered reaction has two well-

defined phases: (1) the immediate response, characterized by vasodilatation, vascular leakage and smooth muscle spasm, usually evident within 5 to 30 min after exposure and subsiding by 60 min; and (2) a second, late-phase reaction that usually sets in 2 to 8 h later and may last for several days and is characterized by inflammation (Kumar et al. 2007). Mould allergy diagnoses are performed with fungal extracts consisting of a complex mixture of proteins, glycoproteins, polysaccharides and other substances.

The main purpose of this study was to evaluate the role of *A. alternata* extract and spores in inducing immunopathological reactions in mice.

Materials and methods

This study was performed on 36 female, 4 to 6 weeks, BALB/c mice obtained from the Razi Vaccine and Serum Research Institute. The animals were divided into three groups, which were kept and isolated from each other to prevent cross-contamination.

Alternaria alternata was cultured on Sabouraud glucose agar (Merck Co., Darmstadt, Germany) at 28°C for 1 week. Subsequently, fungal cultures were grown for 4 weeks in flasks containing 300 ml of Sabouraud glucose broth media at 28°C. Fungal extracts were prepared by freeze–defreeze and sonication methods. Extracts were centrifuged for 45 min at 14,000×*g* at 4°C. Protein concentration was measured by microassay Bradford method and extracts were stored at −20°C. Mice in group 1 were sensitized to *A. alternata* by two intraperitoneal injections of extract on days 0 and 7. On day 12, all sensitized (group 1) and non-sensitized (group 2) mice were challenged intranasally with 1×10^6 *A. alternata* spores suspended in sterile normal saline solution. Control mice (group 3) received only sterile normal saline solution in the same volume and via the same routes.

Blood samples were obtained directly from the heart at 1 and 72 h after intranasal challenge immediately prior to necropsy. Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) to determine serum levels of Interleukin (IL)-4 and IL-13 in immediate response and late-phase reaction, respectively. Assessment of these protein levels were performed by using a standardized sandwich ELISA technique.

Tissues for histopathological examination were collected at necropsy. Lungs were fixed in 10% buffered formalin, embedded in paraffin, and 5 µm sections were cut. Sections were stained with haematoxylin and eosin (H&E) for light microscopy examination of the lung inflammation.

Table 1 Th_2 cytokine detection in serum after 1 h

Cytokine group	IL-4 (pg/ml)	IL-13 (pg/ml)
Controls	1.99±0.16	2.92±0.31
Nonsensitized mice	2.79±0.2[a]	5.12±1.1
Sensitized mice	8.99±1[a]	19.92±2.5[a]

Data expressed as mean ±1 standard error

[a] Statistically significant at $P\leq0.05$

Results

Analysis of the Th_2 cytokines

As shown in Tables 1 and 2, low levels of IL-4 and IL-13 were observed in the control group. At both 1 and 72 h after receiving spores, plasma IL-4 and IL-13 were significantly increased in the sensitized mice compared with the control group.

In non-sensitized animals, the levels of IL-4 were significantly higher at 1 h after intranasal inoculation versus controls. Similar result was observed in IL-13 levels at 72 h after receiving spores in non-sensitized mice.

Histopathological findings

In the control group, pulmonary histology showed no signs of inflammation. Histological examination of lung sections from sensitized mice demonstrated interstitial pneumonia and oedema (Fig. 1). Non-sensitized animals showed minimal inflammation foci which were devoid of macrophages, eosinophils and neutrophils, and no mucus hyper-secretion 1 h after receiving spores, but at 72 hours, inflammatory cells including lymphocytes, macrophages, neutrophils and eosinophils were observed. In addition, hyper-production of mucus in airways was observed at this time.

Mice sensitized and challenged with the spores showed an important interstitial pneumonia and oedema at both 1 and 72 h after intranasal inoculation. A strong accumulation of lymphocytes, macrophages, neutrophils and eosinophils was observed, but mucus hyper-secretion was only seen 72 h after spore exposure (Figs. 2, 3 and 4). However, the inflammation was of much greater intensity in the sensitized mice

Table 2 Th_2 cytokine detection in serum after 72 h

Cytokine groups	IL-4 (pg/ml)	IL-13 (pg/ml)
Controls	1.99±0.16	2.92±0.31
Nonsensitized mice	2.71±0.31	4.47±0.5[a]
Sensitized mice	9.85±1.17[a]	3.36±2.48[a]

Data expressed as mean ±1 standard error

[a] Statistically significant at $P\leq0.05$

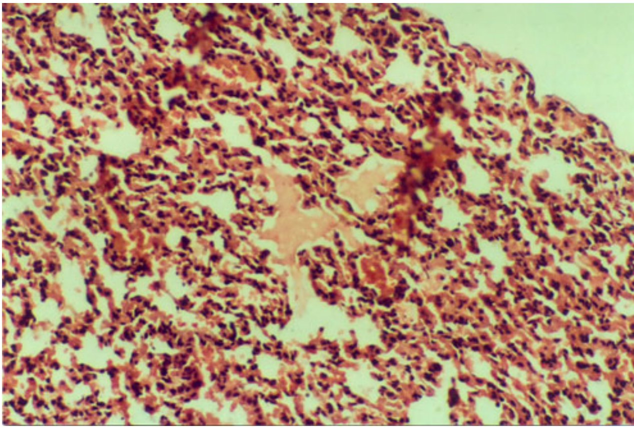

Fig. 1 Lung tissue; acute and local oedema with hyperaemia (H&E, ×200)

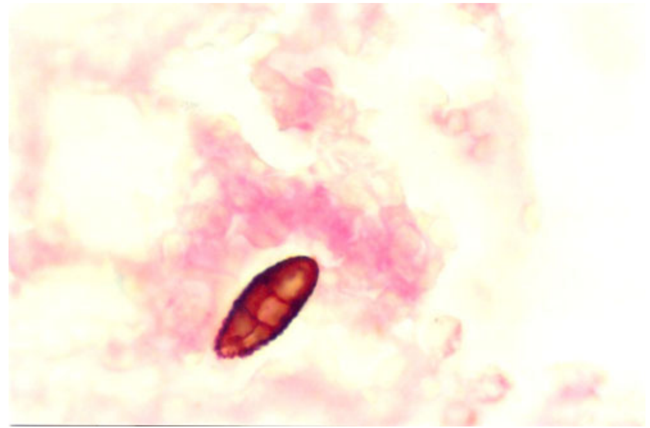

Fig. 3 Presence of the *A. alternate* spore into the alveoli (H&E, ×1,000)

compared with non-sensitized. Epithelial alterations were also noted in the airways of sensitized and non-sensitized mice at 72 h after spore challenge. These changes included airways epithelial cells hyperplasia and necrosis.

Discussion

The Th_2 cells are responsible for essentially all the reactions of immediate hypersensitivity. Several cytokines are important for the late-phase reaction such as IL-4 and IL-5, which amplify the Th_2-initiated immune reaction and IL-13, which stimulates epithelial cells mucus secretion (Kumar et al. 2007). IL-4 stimulates B cells specific for the allergen to undergo heavy chain class switching to IgE and to secrete this isotype. IL-13 acts on epithelial cells and stimulates mucus secretion (Assenmacher et al. 1994; Bober et al. 1995; Zhu et al. 1999; Fallon et al. 2001). IL-3 and IL-4 are closely related cytokines they also have overlapping biologic profiles (Zhu et al. 1999; Fallon et al. 2001).

To determine the activity of these cytokines following *A. alternata* extract and spores challenges to the mice in this study, serum levels of IL-4 and IL-13 were quantified by ELISA.

Hogaboam et al. (2000) described a model of persistent airway hyperactivity, goblet cell hyperplasia and subepithelial fibrosis that is initiated by the intratracheal introduction of *Aspergillus fumigatus* spores into the airways of mice previously sensitized to *A. fumigatus*. They examined the cytokine profile associated with spore groups and observed that the inflammatory response in the sensitized group was characterized by significant increases in IFN-γ, IL-4, TGF-β and IL-13.

In a separate study by Havaux et al. (2005), a new mouse model of allergic lung inflammation was developed using the spores of *A. alternata* and *Cladosporium herbarum*. Their results demonstrated that *A. alternata* and *C. herbarum* spores have the ability to induce a type-2 antibody response characterized by production of polyclonal IgE immunoglobulins and specific IgG_1 antibodies. Since the production of

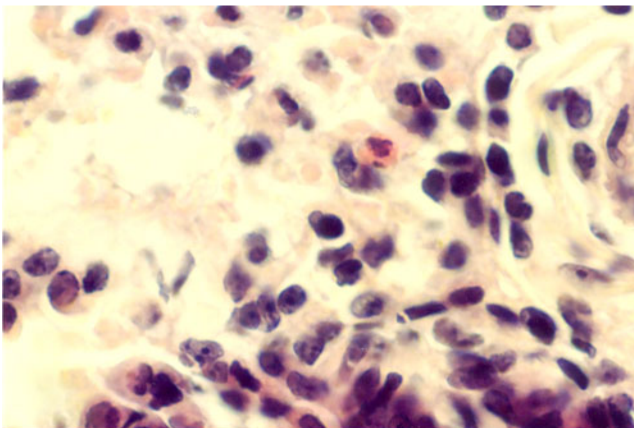

Fig. 2 Infiltration of lymphocytes, macrophages and eosinophils in interstitial space and into the alveoli (H&E, ×1,200)

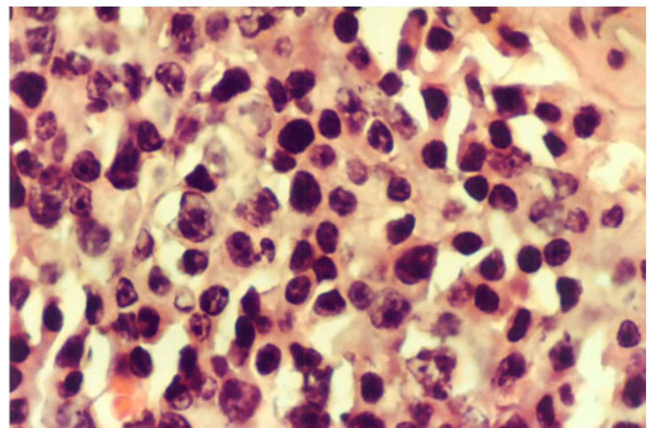

Fig. 4 Infiltration of macrophages, lymphocytes and eosinophils with oedema into the lung tissue (H&E, ×1,000)

IgE by B cells is a T-cell dependent phenomenon requiring the production of Th_2 cytokines, fungal spores not only induce activation of B cells but also of Th_2 cells. In sensitized mice, intranasal challenges with the spores induced the recruitment of already activated Th_2 cells into the lungs, their local re-activation and finally the secretion of IL-4, IL-5 and IL-13. In their study, the lung inflammation induced in fungal spore sensitized and challenged mice was characterized by the appearance in the bronchoalveolar lavages of high numbers of macrophages, neutrophils, eosinophils and a smaller population of lymphocytes.

In this study, we revealed that in sensitized and non-sensitized mice, inhalation of *A. alternata* spores leads to elevation of Th_2 cytokines levels including IL-4 and IL-13 compared with controls and these increases were higher in sensitized mice. We also demonstrated that *A. alternata* spores induce the development of airway hyper-reactivity, interstitial pneumonia, pulmonary inflammation with inflammatory cells including lymphocytes, macrophages, neutrophils and eosinophils, and after 3 days, mucus hyper-secretion and airway epithelial alterations including hyperplasia and necrosis. These findings are compatible with the known ability of IL-13 and IL-4 to induce airway epithelial hypertrophy, mucus hyper-secretion, and goblet cell hyperplasia. This provides a plausible mechanism for the airway obstruction, hyper-production of mucus and goblet cell hyperplasia that are commonly seen in Th_2-polarized inflammatory responses in the airway and GI tracts (Zhu et al. 1999; Fallon et al. 2001). These reactions were more intense in sensitized compared with non-sensitized BALB/c mice.

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