A Protective Mechanism in Lungs of Rats Experimentally Infected with *Aspergillus fumigatus*

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Aspergillus fumigatus **is associated with invasive disease aspergillosis in immunocompromised individuals. The major aim of this study was to investigate the biochemical and immunological responses of male Wistar rats against** *A. fumigatus* **experimentally-induced pulmonary fungal infection. Nostril experimental exposure of male Wistar rats to a high dose of** *A. fumigatus* **freeze-dried preparation for only 24 hr resulted in a significant increase in levels of catalase, nitric oxide and lipid peroxide in lung homogenates, compared to those of the control animals. However, the oxidative status of the lungs of rats challenged with killed fungus did not change significantly, except for the stimulation in the level of lipid peroxide. IgG level was significantly elevated only in rats that received two low doses of fungus, compared to unexposed animals ($p < 0.005$). Examining the lung of rats exposed to** *A. fumigatus* **revealed no abnormal changes, except for pus in bronchial lumen spaces and per bronchial inflammation. Histologically, large numbers of granuloma cells were evident in the lungs of challenged rats, while no granuloma formation was evident in the lungs of rats exposed to killed fungus.**

KEYWORDS : *Aspergillus fumigatus*, **Granuloma, Immunoglobulin G**

Aspergillus infections are among the most feared opportunistic infections in humans. *Aspergillus* fungi are ubiquitous in nature; thus, exposure to their spores must be a common event. *Aspergillus* is saprophytic in soil and on many kinds of decaying organic matter. *A. fumigatus* is the most common species isolated from human infections, and it is often associated with invasive aspergillosis in immunosuppressed patients [1]. Aspergillosis is an invasive disease of the lungs, although colonization of other organs can occur. The infection is serious and can often prove fatal due to the difficulty of diagnosis and poor prognosis [1-5].

In healthy individuals, anatomical barriers and the components of the immune system including serum complement, antibodies, phagocytes and cell-mediated immunity generally provide protection against infection [6]. However, the host immune defense mechanisms against aspergillosis are unclear.

Reactive oxygen species (ROS) are essential components of the defensive mechanism against fungus infection [7-9]. Nitric oxide (NO) production in mammals is regulated by various stimuli and plays several roles, ranging from homeostatic control of arteriolar pressure to immunomodulant effects [10, 11]. NO is also an important antimicrobial agent [12]. Although susceptibility is not universal, NO-related antimicrobial activity has been shown against a wide range of pathogenic microorganisms including parasites, viruses, bacteria and fungi [13].

We have studied the expression of inducible nitric oxide synthetase (iNOS) and arginine metabolism in lung of mouse exposed to *Fusarium kyushuense* [14]. The present study was undertaken to understand more fully the mechanism of protection from *A. fumigatus* pulmonary infection in a rat model.

Materials and Methods

Animals. Forty male Wistar rats, 8-weeks-of-age, weighing 180~200 g were maintained in a controlled environment of temperature, humidity and light. They were fed a commercial rat chow and tap water.

Fungal strain. *A. fumigatus* was isolated and identified from a patient with bronchial asthma. The isolate was maintained on 2% corn meal agar for 21 days at $28 \pm 2^\circ$C. After the incubation period, the fungus was filtered though cheese cloth (muslin) and washed several times with deionized water and freeze-dried. Prior to infection, 0.2 g of fungus was suspended in 1 mL of sterile cold 0.9% saline and homogenized manually in a glass homogenizer [15].

Experimental infection. Rats were divided into four groups (n = 10 per group). The first group (group I) of animals received 40 μL of 0.9% saline by drop-wise addition into a nostril under mild anesthesia and served as the control. Group II similarly received 900 μg of *A. fumigatus*
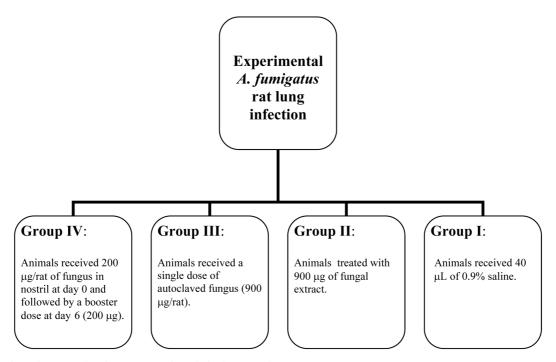
Fig. 1. Design of *Aspergillus fumigatus* rat lung infection experiment.

extract suspended in 40 μL of 0.9% saline. Group III similarly received a single dose of autoclaved *A. fumigatus* (900 μg/rat). Group IV similarly received *A. fumigatus* (200 μg/rat) day 0 of the experiment and followed by another application of the same amount of fungus at day 6 (Fig. 1). After 24 hr of treatment, rats were killed and lung, spleen and blood specimens were collected.

Determination of NO in lung homogenate. One half of each lung was homogenized in ice-cold physiological saline using an electrical homogenizer. The extract was filtered through a filter membrane and the clear filtrate was used in the determination the of level of NO [16]. Briefly, equal volume of lung homogenate and Griess reagent (1% sulfanilamide/0.1% nephthylethelene diamine/2.5% phosphoric acid) was mixed and incubated at room temperature for 10 min. NO concentration was determined spectrophotometrically at 520 nm using sodium nitrite as a standard.

Catalase activity assay. Catalase activity was determined in the lung homogenate as previously described [17]. The activity of one unit of catalase was defined as the amount of enzyme that decomposes 1 μmol of hydrogen peroxide (H_2O_2) per minute, and the specific activity was expressed as mmol/min/mg protein

Lipid peroxidation. Lipid peroxide level as thiobarbituric acid reactive species was assayed in lung homogenates by the measuring malondialdehyde (MDA) content using 1, 1′, 3, 3′-tetraethoxy-propane as the standard [18].

Determination of total protein. Total soluble protein concentration in the homogenate of lung was determined as previously described [19].

Determination of IgG concentration in rat serum. The level of IgG in the rat serum was determined by using a previously described radial immunodiffusion method [20].

Histology. The lung was removed and half was immediately fixed in 10% (v/v) neutral buffered formalin. After dehydration in a graded ethanol series and clearing with xylene, the material was embedded in paraffin and 8 μm-thick sections were stained with hematoxylin and eosin for light microscope observation.

Statistical analysis. The statistical analysis was carried out using SPSS (SPSS Inc., Chicago, IL, USA) [21]. All data are expressed as mean ± SE. The statistical differences were analyzed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Experimental infection of rats with different doses of freeze-dried *A. fumigatus* preparations was successfully established (data not shown). Then, a high dose of *A. fumigatus* (900 μg/rat) was used to examine the biochemical and immunological responses of the host against the fungal exposure. Rats were first exposed to a single high dose of fungal preparation (group II) and with the same dose of killed fungus (group III), while the group IV of

rats were treated with two minimal doses of fungal preparation (200 μg/rat, on days 0 and 6) (Fig. 1).

Determination of the oxidative status of male Wistar rats after inhalation of *A. fumigatus* demonstrated that exposure of male Wistar rats to 900 μg of *A. fumigatus* for only 24 hr resulted in a significant increase in the level of catalse, NO and lipid peroxides in lung homogenates, compared to control rats (Figs. 1~3). The same dose of killed fungus showed a stimulatory effect on the level of lipid peroxide compared to the control group, while no significant changes were observed in the level of catalase and NO. Moreover, the oxidative status of male Wistar rats exhibited non-significant changes when treated with a low booster dose on day 6 compared to the control group (Table 1).

Group IV rats that received two low doses of fungus within a week exhibited a significant elevation in IgG level compared with controls ($p < 0.005$). No statistical difference was seen between groups I, II and controls (Fig. 2).

Examining the lung of rats administered *A. fumigatus* intranasally after 24 hr revealed no abnormal changes, except for the existence of pus cells in bronchial lumen spaces, and peribronchial inflammation including lymphocytes, neutrophiles and macrophages (Fig. 3B). However, rat lungs

Table 1. Catalase activity, NO level and MDA level in lung homogenates of rats infected with *Aspergillus fumigatus*

Group	Catalase activity (nmol/g)	NO level (nmol/g)	MDA level (nmol/g)
Group 1 (control)	16 ± 1.0	19 ± 1.2	4.5 ± 0.5
Group II	20 ± 2.0	28 ± 2.0	14.5 ± 1.3
Group III	17 ± 1.5	17 ± 1.6	9.0 ± 0.9
Group IV	15 ± 1.0	18 ± 1.5	5 ± 0.4

Group 1, Animals received 40 μL of 0.9% saline; Group II, Animals treated with 900 μg of fungal extract; Group III, Animals received a single dose of autoclaved fungus (900 μg/rat); Group IV, Animals received 200 μg/rat of fungus in nostril at day 0 of experiment and followed by a booster dose (200 μg) at day 6.
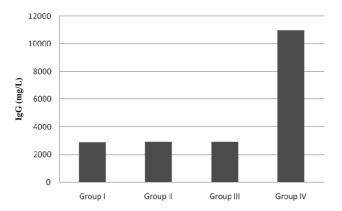NO, nitric oxide; MDA, measuring malondialdehyde.

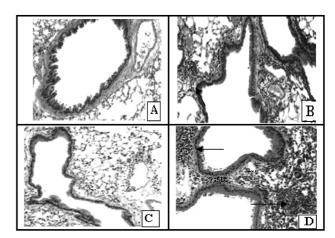**Fig. 2.** IgG level in serum of male Wistar rats experimentally infected with *Aspergillus fumigatus*.

Fig. 3. H&E-stained lung sections (×66) from *Aspergillus fumigatus* exposed rats. Normal control (A), 24 hr exposure to 900 μg of live (B) and killed *A. fumigatus* (C) and day 6 booster exposure of 200 μg of *A. fumigatus* (D). Note the presence of *A. fumigatus* in mononuclear cells (arrows) adjacent to the inflamed airway in (B) and (D).

challenged after 6 days of the first exposure contained large numbers of granuloma (Fig. 3D), which contained numerous cell fragments that had been phagocytized by macrophages. Inflammatory cells consisted of histiocytes, lymphocytes and a few polymorphonuclear leuckocytes in addition to Langhan's giant cells. However, examination of lung tissues of rats exposed to the autoclaved *A. fumigatus* preparation for 24 hr revealed normal cells without granuloma formation, although infiltration for immune cells included macrophages, neutrophiles and lymphocytes.

Discussion

The present study sheds light on the influence of *A. fumigatus* in the oxidative status and immune response of male Wistar rats in addition to histological manifestation of the infection. *A. flavus* has been reported as a potent hepatotoxic and hepatocarcinogenic agent in humans and various animal species [22]. The present results demonstrate that the inhalation of a high dose (900 μg) of *A. fumigatus* for 24 hr leads to the elevation of oxidative stress of male Wistar rats. The activity of NO and lipid peroxide measured as MDA, as well as catalase, have been significantly increased. These findings indicate that ROS has a defensive role against *A. fumigatus* infection at an early stage of infection. ROS are involved in many of the complex interactions between the invading microorganisms and its host [23]. Production of ROS by the host, especially by phagocytes, is a counteractive mechanism aimed at microbial elimination. Phagocytosis and mechanisms of killing of *A. fumigatus* conidia by murine alveolar macrophages have been studied [24]. In that study, the

engulfment of conidia by murine alveolar macrophages took place within 2 hr after infection and the killing process began after 6 hr of phagocytosis and is mediated by ROS.

The antimicrobial activity of NO has been demonstrated by a variety of approaches [10, 11]. Yet, an excess of NO production may become dangerous for the cells. The same observation has been reported in an *in vitro* study [25], in which decreased cell viability was associated with an increase in iNOS activity after the treatment of human erythroid K-562 cells with *A. terreus*.

On the other hand, the present results showed no significant change in the activities of NO and catalase in male Wistar rats exposed to either killed fungus or to low booster dose of *A. fumigatus*, compared to that of the control group. This observation may confirm the antimicrobial role of NO at early stage of infection with living organisms. This might be attributed to the ability of living fungus to produce mycotoxins [26].

On the other hand, the level of IgG in the serum of male Wistar rats exposed to a single dose of either live or killed *A. fumigatus* showed an insignificant change when compared to that of the control group, while the IgG level was dramatically increased in rats after treatment with the second dose of *A. fumigatus*. Thus, it can be concluded that the resistance against fungal infection is carried out at earlier stage of infection with help of immune cells, while the contribution of humoral immunity comes later. It has been reported that the invasive aspergillosis cause a paralysis of immune system in humans [1].

Constitutive NOS and iNOS inhibit the development of several pathogens. This study indicated a high activity for NOS in rat lung exposed to the lung preparation for 24 hr without challenge comparing to the killed fungal preparation and the challenged rats. This might due to the early killing of fungi by alveolar macrophages [27]. Although monocytes, macrophages and neutrophiles can damage fungal hyphae, but the interactions of several potential oxidative and nonoxidative antihyphae mechanisms may define the host ability to limit fungal infections. NOS catalyzes the production of nitric oxide; a mediator of potential importance in numerous physiologic and inflammatory processes in the lung.

Presently, catalase activity was highest in rats exposed to a live fungal extract (G_{II}), followed by rats challenged with killed *A. fumigates* (G_{III}). However, this differs from a previous study [28] that concluded that the antimicrobial and cytotoxic actions of NO are enhanced by other macrophage products such as H_2O_2, cysteine, glutathione, acid or peroxide.

Intranasal administration of *A. flavus* antigen induced hypersensitivity that was characterized by elevated anti-*A. flavus* IgG antibody level in serum. This type of hypersensitivity in rat constitutes a humoral immune reaction [29] where the specific IgG level was increased during the experiment, after the first exposure without challenge, in rats challenged with the living fungi and in rats challenged with killed fungi.

Granuloma was observed only in lung tissue of rats exposed to *A. flavus* freeze-dried preparation and challenged after 6 days of first exposure. Granuloma is a compact organized structure dominated by mononuclear phagocytes. It is formed as a result of an unsuccessful attempt to remove irritants [30]. The presence of multinucleated giant cells is a common feature within the granuloma. It is assumed that these cells play a role in protecting the host from a persistent invader, as doe's grnuloma itself [31]. It has been established that antigen-sensitized T lymphocytes are involved in the formation of granuloma [32]. *In vivo*, granuloma formation is accompanied by monocyte proliferation [33]. Rats exposed to either *A. flavus* freeze-dried for only 24 hr or to killed fungi did not form *in vivo* granuloma. This indicates that the rats cleared the infecting fungal elements like hyphae or spores by different immune cells and different host immune defending enzymes. One complication of pulmonary aspergillosis might be the deposition of calcium oxalate crystals in the tissue [34]. This is most commonly seen with *A. niger* infection and is believed to be caused by the combination of oxalic acid produced by the fungal mycelia with the patient's serum calcium, with the presence of crystals indicative of the chronic and severe nature of the disease.

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