Mурine Model of Invasive Pulmonary Aspergillosis following an Earlier Stage, Noninvasive Aspergillus Infection

RYUHEI NAWADA,* RYOICHI AMITANI, EISAKU TANAKA, AKIO NIIMI, KATSUHIRO SUZUKI, TAKAKO MURAYAMA, AND FUMIYUKI KUZE

Department of Infection and Inflammation, Chest Disease Research Institute, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Aspergillus spp. occasionally cause invasive pulmonary aspergillosis following noninvasive infection in patients with underlying bronchopulmonary disorders regardless of their systemic immunological conditions. We developed a murine model of invasive pulmonary aspergillosis following an earlier stage, noninvasive Aspergillus infection. BALB/c mice were inoculated intratracheally with agarose beads containing Aspergillus fumigatus conidia. Two weeks after inoculation, half of the mice were immunosuppressed with cortisone acetate. During a 4-week observation period, the survival rate of infected immunosuppressed mice was significantly lower (P < 0.01) than that of infected nonimmunosuppressed mice. The number of CFU in the lungs gradually decreased in the nonimmunosuppressed mice, whereas a time-related significant increase (P < 0.05) of CFU was demonstrated in the immunosuppressed mice. In the lungs of the nonimmunosuppressed mice, there was marked accumulation of neutrophils, lymphocytes, and macrophages (in this order) around the agarose beads in the bronchi. Aspergillus hyphae were surrounded by the inflammatory cells and did not invade the lung parenchyma. In contrast, in the immunosuppressed mice, Aspergillus hyphae proliferated markedly and invaded the lung parenchyma after immunosuppression. In this model, the two-dimensional extents of the lesions were evaluated with an image-processing system. Time-related increase of the area of peribronchial necrosis in immunosuppressed mice was significant (P < 0.05) after immunosuppression. This model should therefore be useful for investigating the pathophysiology of noninvasive Aspergillus infection and invasive pulmonary aspergillosis and also for clarifying the mechanism of conversion to the invasive disease from the noninvasive stage.

Materials and Methods

Experimental design. Agarose beads containing Aspergillus conidia were inoculated into mice to develop noninvasive Aspergillus infection. To develop invasive aspergillosis from the noninvasive infection, immunosuppression with cortisone acetate was started 2 weeks after inoculation. We monitored the mortality, as well as CFU, histopathology, and the extents of the lesions of the lungs of mice in three groups: infected and immunosuppressed mice, infected and nonimmunosuppressed mice, and noninfected and nonimmunosuppressed mice (Fig. 1).

Animals. Specific-pathogen-free, 6-week-old female BALB/c mice (weighting 16 to 20 g; Japan SLC, Inc., Hamamatsu, Japan) were used in these experiments. The mice were housed five to six in each cage and received standard mouse chow (CE-2; Clea Japan Inc., Tokyo, Japan) and water, to which was added 0.3 mg of tetracycline hydrochloride (Sigma Chemical Co., St. Louis, Mo.) per ml.

Fungus. A. fumigatus AF-1 strain was originally isolated at our institute from the sputum of a patient with chronic necrotizing pulmonary aspergillosis. This strain had been stored at −80°C in 10% glycerol and cultured as needed before experiments.

Production of agarose beads with and without conidia (3, 19). A. fumigatus was cultured on potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 30°C for 5 days. A. fumigatus conidia were harvested with sterile physiological saline containing 0.05% Tween 80. The conidia were then washed with sterile physiological saline and resuspended at a concentration of 2 × 10^8/ml. Ten milliliters of this suspension was mixed with the same amount of 7.8% potato dextrose agar solution kept at 50°C. Ten milliliters of this mixture, which contained 10^6 A. fumigatus conidia per ml, was then dripped into 50 ml of heavy mineral oil (Sigma) kept at 30°C in a flask and stirred vigorously with a magnetic bar. The flask was immediately cooled with crushed ice, while stirring continued for 5 min. During this time, agar droplets solidified into beads. The oil bead slurry was washed twice with 0.5% sodium deoxycholate (Nacalai Tesque Inc., Kyoto, Japan) in physiological saline and then washed three times with sterile physiological saline. The agarose beads were resuspended in sterile physiological saline at a concentration of 10^9/ml at room temperature, and used for inoculation.

Agarose beads without conidia were also prepared with 3.9% potato dextrose agar solution as described above.

Inoculation of agarose beads. Each mouse was anesthetized by the intraperitoneal injection of 0.075 mg/g of body weight of pentobarbital (Abbott Laboratories Inc., North Chicago, Ill.). Diethyl ether (via inhalation) was also used as needed. After anesthesia was produced, the trachea was exposed aseptically by ventral cervical skin incision. Twenty microliters of the agarose bead suspension (containing 2 × 10^3 agarose beads) was injected into the trachea with a 26-gauge needle and a small syringe.

Immunosuppression. Cortisone acetate (Sigma) was suspended in sterile distilled water at a concentration of 12.5 mg/ml, and 0.01 ml/g of body weight of this
suspension (equivalent to 125 mg of cortisone acetate per kg of body weight) was injected subcutaneously into each mouse daily. This immunosuppressive regimen was begun on day 14 (inoculation of beads, on day 0) and continued until day 20.

Survival study. First, we evaluated the mortality of mice inoculated with agarose beads containing \textit{A. fumigatus} conidia (group 1 [\( n = 41 \)]) and mice inoculated with agarose beads containing no conidia (group 2 [\( n = 30 \)]). These two groups were monitored for 2 weeks after inoculation, and their survival rates were compared.

Second, to evaluate the mortality caused by \textit{A. fumigatus} infection with and without immunosuppression, the mice that survived for 2 weeks after inoculation of agarose beads containing \textit{A. fumigatus} conidia were divided into two groups (equal numbers in each group) on day 14. One group was immunosuppressed, as described above (group A), and the other group was not immunosuppressed (group B). The mice were immunosuppressed by the daily administration of cortisone acetate subcutaneously for 7 days (s.c. \( \times 7 \) days). These three groups were observed for 4 weeks after the beginning of immunosuppression. In the histopathological study and the measurement of CFU, the mice inoculated with agarose beads were killed on the days indicated by the thick black arrows.

To measure CFU of \textit{A. fumigatus} in the lungs of nonimmunosuppressed mice, BALB/c mice (\( n = 41 \)) were infected, without immunosuppression, as described above. The mice were killed on day 0 (about 1 h after inoculation) (\( n = 5 \)), day 1 (\( n = 5 \)), day 4 (\( n = 4 \)), day 7 (\( n = 7 \)), day 15 (\( n = 4 \)), day 28 (\( n = 4 \)), and day 42 (\( n = 4 \)). Eleven of 41 mice died during the observation period (on days 1 [\( n = 3 \)], 4 [\( n = 4 \)], 6 [\( n = 1 \)], 7 [\( n = 2 \)], and 9 [\( n = 1 \)]).

To measure CFU of \textit{A. fumigatus} in the lungs of immunosuppressed mice, other BALB/c mice (\( n = 31 \)) were infected and immunosuppressed as described above. The mice were killed on days 15 (\( n = 4 \)), 18 (\( n = 4 \)), 21 (\( n = 4 \)), and 24 (\( n = 4 \)). Fifteen of 31 mice died during the observation period (on days 4 [\( n = 3 \)], 5 [\( n = 2 \)], 8 [\( n = 1 \)], and 9 [\( n = 2 \)] and on days 10, 11, 12, 15, 20, 23, and 24 [one mouse each]).

The left lung of each killed mouse was removed and weighed. The lungs were then homogenized with Excel Auto Homogenizer (Nihonseiki Kaisha, Ltd., Tokyo, Japan) at 15,000 rpm for 12 min, and 10-fold dilutions of homogenates were prepared. Five hundred microliters of each solution was seeded on a potato...
infiltration of neutrophils, lymphocytes, and macrophages) and by necrotic lesions were calculated.

Statistical analysis. Survival rates were calculated by the Kaplan-Meier method and compared with the log rank test and the generalized Wilcoxon test. Values for log (CFU/gram) were expressed as means ± standard deviations. The percentages of cross-sectional areas of the inflammatory lesions and necrotic lesions were expressed as means ± standard deviations. The significance of the time-related increase of these values was tested by one-way analysis of variance and the Kruskal-Wallis test. A P value of less than 0.05 was considered significant.

RESULTS

Survival rates. The survival curves of infected mice inoculated with agarose beads containing A. fumigatus conidia (group 1 [n = 41]) and noninfected mice inoculated with agarose beads containing no conidia (group 2 [n = 30]) are shown in Fig. 2A. Nine of 41 mice in group 1 died within 2 weeks after inoculation with agarose beads containing A. fumigatus conidia. The cumulative mortality of group 1 on day 14 was 22.0%. Two of 30 mice in group 2 died within 2 weeks after inoculation with agarose beads containing no conidia. The cumulative mortality of group 2 on day 14 was 6.7%. There was no significant difference between the survival rates of these two groups by the log rank test and by the generalized Wilcoxon test.

The mice that survived for 2 weeks after inoculation with agarose beads containing A. fumigatus conidia were divided into two groups. One group (group A [n = 16]) was immunosuppressed, and the other group (group B [n = 16]) was not immunosuppressed. The mice that survived for 2 weeks after inoculation with the agarose beads containing no conidia were immunosuppressed (group C [n = 28]). The survival curves of these three groups are shown in Fig. 2B. The cumulative mortality of group A 4 weeks after the beginning of immunosuppression (6 weeks after the inoculation) was 56.4%. The cumulative mortalities of group B and group C were 12.5 and 0%, respectively. The survival rate of group A was significantly lower than those of group B and group C by the log rank test and by the generalized Wilcoxon test (P < 0.01).

A. fumigatus CFU. The log (CFU/gram) values over time are shown in Fig. 3. In the nonimmunosuppressed mice, CFU gradually decreased after inoculation. This result showed that A. fumigatus was gradually killed but that they could still be isolated, even 6 weeks after inoculation, in the nonimmunosuppressed mice.

In the mice immunosuppressed with cortisone acetate 2 weeks after inoculation, CFU began to increase 4 days after the beginning of immunosuppression and continued to increase during the next 7 days. This result showed that A.
fumigatus proliferated in the lungs of the cortisone-treated mice. The increase of log (CFU/gram) after the beginning of immunosuppression was significant by the Kruskal-Wallis test ($P = 0.0279$). The 3-day cultures of the lung specimens on both chocolate agar plates and blood agar plates were all negative.

**Histopathological findings.** The histopathological findings for the lung specimens of the infected nonimmunosuppressed mice are summarized in Table 1. On day 1, slight neutrophil accumulation was seen around the agarose beads in the lungs of all the infected mice. Small *A. fumigatus* hyphae were seen in the agarose beads. No or little infiltration of inflammatory cells was seen in the peribronchial region (Fig. 4A and B). On day 4, moderate to marked neutrophil accumulation was seen around the agarose beads. Slight lymphocyte accumulation was seen in the peribronchial region in some areas in three of four specimens. *A. fumigatus* hyphae were seen in the agarose beads, but not outside the beads (Fig. 4C). On day 7, marked neutrophil accumulation was seen around the agarose beads inside the bronchi. Lymphocyte accumulation was seen in the peribronchial region. The accumulation of foamy macrophages

![Image of histopathological findings](http://jcm.asm.org/)

**FIG. 4.** Lung sections of nonimmunosuppressed mice inoculated with the agarose beads containing *Aspergillus* conidia. (A and B) Two views of one section from a mouse killed 1 day after the inoculation. Small hyphae were seen in the agarose beads. Neutrophil accumulation was seen around the beads. Slight infiltration of inflammatory cells was seen in the peribronchial region. (C) Section from a mouse killed 4 days after the inoculation. Marked neutrophil accumulation was seen around the agarose beads. Slight lymphocyte accumulation was seen in the peribronchial region. (D) Section from a mouse killed 28 days after the inoculation. Marked accumulation of lymphocytes and macrophages was seen around the agarose beads. The sections were stained with hematoxylin and eosin. Magnifications, ×40 (for panels A and D), ×100 (for panel C), and ×200 (for panel B).

**TABLE 2.** Histopathological findings for the lungs of infected immunosuppressed mice

<table>
<thead>
<tr>
<th>Day$^a$</th>
<th>No. of mice</th>
<th>Accumulation of$^b$:</th>
<th>Damage to bronchial walls$^c$</th>
<th>Growth of hyphae out of beads$^d$</th>
<th>Necrosis around bronchi$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophils</td>
<td>Lymphocytes</td>
<td>Foamy macrophages</td>
<td>+</td>
</tr>
<tr>
<td>15 (1)</td>
<td>4</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18 (4)</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21 (7)</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>–/+</td>
<td>+/++</td>
</tr>
<tr>
<td>24 (10)</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>–/+</td>
<td>+/++</td>
</tr>
</tbody>
</table>

$^a$ The number of days after the beginning of immunosuppression is shown in parentheses.
$^b$ +, slight accumulation around agarose beads and bronchi; ++, moderate to marked accumulation; –/+ , no or slight accumulation.
$^c$ +, damage to bronchial epithelia; +/++, damage to bronchial epithelia or all layers of bronchial walls.
$^d$ –, no hyphal growth out of beads; +, growth only in bronchi; ++, growth in lung parenchyma; ++++, growth only in bronchi or in lung parenchyma.
$^e$ –, no necrosis; +, peribronchial necrosis; ++, lung parenchymal necrosis.
neutrophil accumulation was almost the same as that on day 7. On day 28, neutrophil accumulation was almost the same as that on days 7 and 14, but the marked accumulation of lymphocytes and foamy macrophages was seen (Fig. 4D). On day 42, the accumulation of neutrophils and lymphocytes decreased somewhat. Giant cells were seen surrounding the agarose beads in some areas in two of four specimens. In the specimens from days 7 through 28, some bronchi were damaged in association with severe inflammation. *A. fumigatus* hyphae did not invade the lung parenchyma surrounded by neutrophils during the 6-week observation period. No inflammatory change indicative of *Aspergillus* infection was seen in organs other than the lungs in these nonimmunosuppressed mice.

The histopathological findings for the infected immunosuppressed mice are summarized in Table 2. On day 15 (1 day after the beginning of immunosuppression), the histopathological findings were similar to those for the nonimmunosuppressed mice. On day 18 (4 days after the beginning of immunosuppression), neutrophil accumulation was still moderate. *A. fumigatus* hyphae began to proliferate out of the agarose beads but did not invade the lung parenchyma (Fig. 5A and B). On day 21 (7 days after the beginning of immunosuppression), the accumulation of neutrophils, lymphocytes, and macrophages was less than that on days 15 and 18. *A. fumigatus* hyphae proliferated moderately and invaded the lung parenchyma in some specimens. Damage to the bronchial walls was moderate. Peribronchial necrosis was seen at this time (Fig. 5C and D). On day 24 (10 days after the beginning of immunosuppression), *A. fumigatus* hyphae proliferated markedly. All layers of involved bronchi were severely damaged. Peribronchial necrotic lesions, which included the hyphae, had formed (Fig. 5E). The proliferation of the hyphae and the degree of necrosis increased progressively through the immunosuppres-
sive regimen. No inflammatory change indicative of Aspergillus infection was seen in organs other than the lungs in the immunosuppressed mice. These results suggest that cortisone acetate suppressed the defense mechanism against *A. fumigatus* and that *A. fumigatus* hyphae gradually proliferated out of the agarose beads, invading the lung parenchyma.

In the mice inoculated with the agarose beads containing no conidia, slight neutrophil accumulation interspersed by a few mononuclear cells was seen around the agarose beads (indicated by the arrow). The section was stained with hematoxylin and eosin. Magnification, ×100.

Aspergillosis lesions. The time-related changes in the area occupied by the inflammatory and necrotic lesions are shown in Fig. 7. Figure 7A shows the changes in the nonimmunosuppressed mice, and Fig. 7B shows the changes in the immunosuppressed mice. In the nonimmunosuppressed mice, the area of the inflammatory lesions gradually increased up to 4 weeks after inoculation, decreasing somewhat at 6 weeks. No necrotic lesions were seen in the nonimmunosuppressed mice. In the immunosuppressed mice, on the other hand, the area of the inflammatory lesions that had already developed in 2 weeks after the inoculation began to decrease with immunosuppression. However, during the period of 4 through 10 days after the beginning of immunosuppression, the area of the inflammatory lesions increased again. Small necrotic lesions were first demonstrated 1 day after the beginning of immunosuppression, and the area of the necrotic lesions increased progressively thereafter. The time-related increase of this value was significant by analysis of variance (P = 0.0008) and by the Kruskal-Wallis test (P = 0.0143).

**DISCUSSION**

Although invasive pulmonary aspergillosis usually develops in immunocompromised patients (1, 13, 23), *Aspergillus* spp. occasionally cause relatively mild invasive pulmonary disease in patients who are not severely immunocompromised but have underlying bronchopulmonary disorders (2, 8, 16). Binder et al. (2) speculated that *Aspergillus* organisms first colonize the damaged airways or cavities in which the defense mechanisms against microorganisms are impaired, and this may lead to the invasive disease. It has also been reported that invasive pulmonary aspergillosis developed in patients with aspergilloma (16, 17). Noninvasive *Aspergillus* infection, such as aspergilloma, may precede invasive pulmonary aspergillosis in much higher incidence than we recognize. However, the pathophysiology and pathogenesis of this type of invasive pulmonary aspergillosis are still poorly understood. Although several experimental models have previously been reported, most were slightly increased proliferation of *A. fumigatus* hyphae and bronchial damage. Congestion was also seen in the lung interstitium. In the mice inoculated with agarose beads containing no conidia, congestion was seen in the lung interstitium, and slight neutrophil accumulation was seen around the beads.

**FIG. 6.** Lung section of a mouse killed 4 days after inoculation of agarose beads containing no conidia. A slight accumulation of neutrophils, interspersed by a few mononuclear cells, is seen around the agarose beads (indicated by the arrow). The section was stained with hematoxylin and eosin. Magnification, ×100.

**FIG. 7.** Portion of the lung area occupied by inflammatory (open circles) and necrotic (closed circles) lesions in the lung sections. Results from nonimmunosuppressed (A) and immunosuppressed (B) mice are shown. The mice were immunosuppressed by the daily administration of cortisone acetate subcutaneously for 7 days (s.c. × 7 days).
models of rapidly progressive invasive aspergillosis or disseminated aspergillosis (5–7, 10, 12, 18, 21), except for a rat model of so-called recurrent pulmonary aspergillosis developed by Niki et al. (14), and an animal model that can simulate invasive pulmonary aspergillosis following noninvasive Aspergillus infection has not yet been developed. In the previous models, animals were immunosuppressed before inoculation of Aspergillus conidia, and invasive disease developed rapidly, almost skipping a noninvasive stage. These previous models were not necessarily suitable for investigating the pathophysiology of noninvasive Aspergillus infection and the pathogenetic mechanisms of invasive pulmonary aspergillosis following the noninvasive stage. In contrast, our model could simulate noninvasive Aspergillus infection in airways, using an agarose bead method that was originally described by Cash et al. (3) and modified by Tanaka et al. (19). Furthermore, it could also simulate invasive pulmonary aspergillosis that developed from noninvasive Aspergillus infection when cortisone acetate was administered. This model made it possible to clarify histopathological features of noninvasive Aspergillus infection and those during the progression to invasive pulmonary aspergillosis from the noninvasive stage.

In the nonimmunosuppressed mice in this model, Aspergillus organisms persisted in the agarose beads in the bronchi for at least 6 weeks after inoculation, and we could observe the natural course of cell dynamics in noninvasive Aspergillus infection. Neutrophil accumulation was first seen after inoculation and was followed by lymphocyte accumulation a few days later and foamy macrophage accumulation 1 to 2 weeks later. The accumulation of inflammatory cells gradually increased until 4 weeks after inoculation and then slightly decreased at 6 weeks after inoculation. In a preliminary study, we found that this inflammatory response finally diminished 3 to 4 months after inoculation (data not shown). Although the defensive role of neutrophils against Aspergillus hyphae has already been recognized (4), the roles of the lymphocytes and macrophages that were seen in the chronic phase of aspergillosis are still unclear. Even noninvasive aspergillosis, such as aspergilloma, in humans is usually accompanied by an inflammatory response. Aspergillus hyphae sometimes proliferate in the cavity wall, and chronic inflammation with lymphocyte infiltration is seen around the cavity wall (11, 22). The histopathology of the nonimmunosuppressed mice in our model seems similar to that of noninvasive Aspergillus infection in humans.

In the immunosuppressed mice of our model, the accumulation of the inflammatory cells gradually decreased and Aspergillus hyphae proliferated out of the agarose beads. The proliferation of the hyphae gradually increased while the immunosuppressive regimen was continued. The bronchial walls were damaged, and the lung parenchyma was invaded by the hyphae. Necrosis was seen in the lung parenchyma around the bronchi, and the area of the necrotic lesions progressively increased after the immunosuppression. We could observe the histopathological changes during the progression of the invasive disease, because the progression of the disease was relatively slow in our model compared with the previous models. We believe that this is a unique advantage of our model and that this model will be helpful for the consecutive analysis of the pathophysiology of invasive pulmonary aspergillosis and the stage of noninvasive Aspergillus infection and also for investigating the mechanisms of development of the invasive disease from the noninvasive stage.

Our model has another advantage over other models. The inflammatory and necrotic lesions were not diffuse, being rather densely scattered around the beads in the lungs. We could quantify the two-dimensional extent of the lesions in the lungs with the image-processing system, because the lesions were seen as nodules in the cross sections. We therefore consider that our model can be used to assess therapeutic effects in terms of the extent of the lesion and mortality.

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REFERENCES


