Experimental Aspergilloma in the Pleural Cavity of the Rabbit

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ABSTRACT. The number of patients with aspergillosis is presently increasing along with the increase in immunocompromised hosts. Aspergillomas arising from saprophytic proliferation of Aspergillus often occur in preexisting lung cavities. Using many animal models, aspergilloma experiments have been performed to gain some knowledge regarding the pathophysiology, pathogenesis, and treatment of aspergillomas. To date, however, no success has been achieved in making a saprophytic fungus ball. The present report describes the development of an aspergilloma animal model in the rabbit by a two step process; creation of an abnormal pleural cavity by the injection of turpentine oil, followed by inoculation of that pleural cavity with an artificial fungus ball made of A. fumigatus. Although the saprophytic aspergilloma created by this method is not yet completely identical with that in human cases, I believe that this experimental system should prove useful for study of the pathogenesis of aspergilloma.

Key words: experimental aspergilloma — pleurisy due to turpentine oil — Aspergillus fumigatus

The Aspergillus species are molds that are ubiquitous in nature.[1] Aspergillosis is thought to occur as the result of inhaling air-borne Aspergillus spores. Therefore, the lung is the most frequent organ of involvement.[1] Aspergillosis is increasing in frequency, especially with the increase in immunocompromised hosts.[1-3] The number of patients with aspergillomas, which arise from saprophytic proliferation of Aspergillus in a preexistent lung cavity (caused by tuberculosis, a pulmonary cyst, or bronchiectasis), is also increasing.[1,4-6] Although many experiments have been performed on animal models of aspergilloma, nobody has yet succeeded in creating a saprophytic fungus ball. I carried out experiments to induce aspergilloma in animals using methods that would fulfill two major objectives; the creation of an abnormal pleural cavity and the inoculation of an artificial fungus ball.

MATERIALS AND METHODS

White male rabbits weighing 2 kg were used in the experiments. The Aspergillus fumigatus PT-7 strain (obtained from Pfizer Co., Ltd.),[7] isolated from a pulmonary aspergilloma patient, was used. The spores of the A. fumigatus PT-7 strain were cultured at 37°C for five days on Sabouraud's agar.

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Then an artificial fungus ball was prepared in vitro with the A. fumigatus PT-7 strain according to the method of Hisauchi (Fig. 1).[8] Small colonies were prepared by stationary culturing of little spores of A. fumigatus at 27°C for two days in a flask containing 200 ml of Sabouraud's liquid medium. A fungus ball, about 10 mm in diameter, was prepared by shaking the culture of one pipetted small colony at 37°C at 110 strokes per minute for one day in another flask containing 300 ml of Sabouraud's liquid medium. The artificial fungus ball created by this method is shown in Fig. 2A,B. Active proliferation of the hyphae of A. fumigatus was recognized.

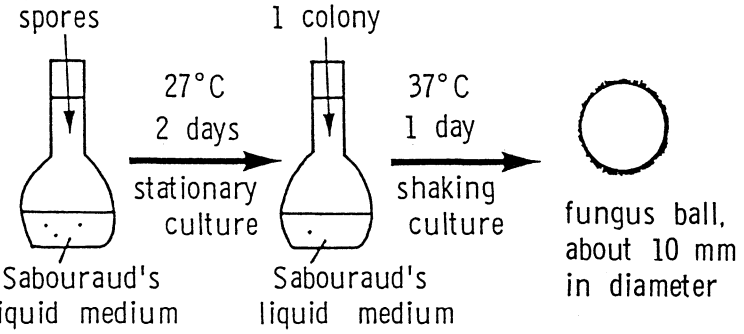

Fig. 1. The artificial fungus ball was prepared in vitro with the A. fumigatus PT-7 strain, according to the method of Hisauchi.

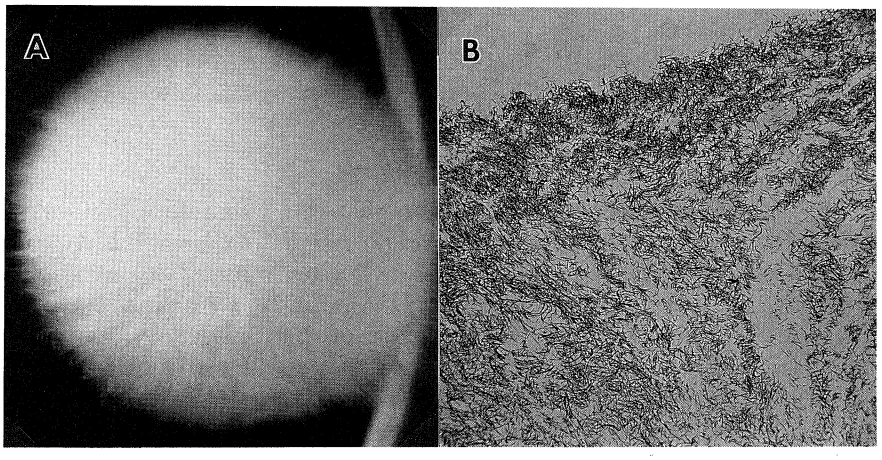

Fig. 2. The artificial fungus ball (macroscopically (A), under A microscope ×40 (B)).

Using the method of Sahn et al.,[9] artificial pneumothorax was produced by puncturing the right pleural cavity of rabbits anesthesized with pentobarbital sodium at the rate of 20 mg/kg of body weight with an 18-gauge needle, followed by the injection of 0.3 ml of turpentine oil in 0.7 ml of saline solution to induce chemical pleuritis (Fig. 3A,B). Collapse of the lung and thickness of the pleura were recognizable macroscopically (Fig. 3A). A severe fibrous thickness of the pleura was recognized microscopically (Fig. 3B).

The third week after the injection of turpentine oil into the pleural cavity, an incision about 2 cm long was made in the intercostal space parallel to a rib. One artificial fungus ball was directly inoculated into the pleural cavity with

sterilized tweezers and the skin was closed with silk sutures. In order to prevent adhesion of the pleura, 10 ml of saline was injected into the pleural cavity every week.

The rabbits were sacrificed and the right thorax was opened at 3 days, and 1, 2, 3, 4, 8, and 12 weeks after inoculation of the artificial fungus ball. Observation of the pleural surface, isolation of fungus from the lesions, and a histological study of the lesions were performed. Grading of the lesions was done based on the following parameters: 1) the degree of inflammatory response in the pleura and fungus ball; 2) the thickness of the pleura; 3) degree of Aspergillus proliferation. Double diffusion method[10] was used to detect serum antibody against Aspergillus. The double diffusion plates from Mercia Diagnostics Ltd. were used and somatic antigen and culture filtrate antigen were employed as antigens against Aspergillus fumigatus.

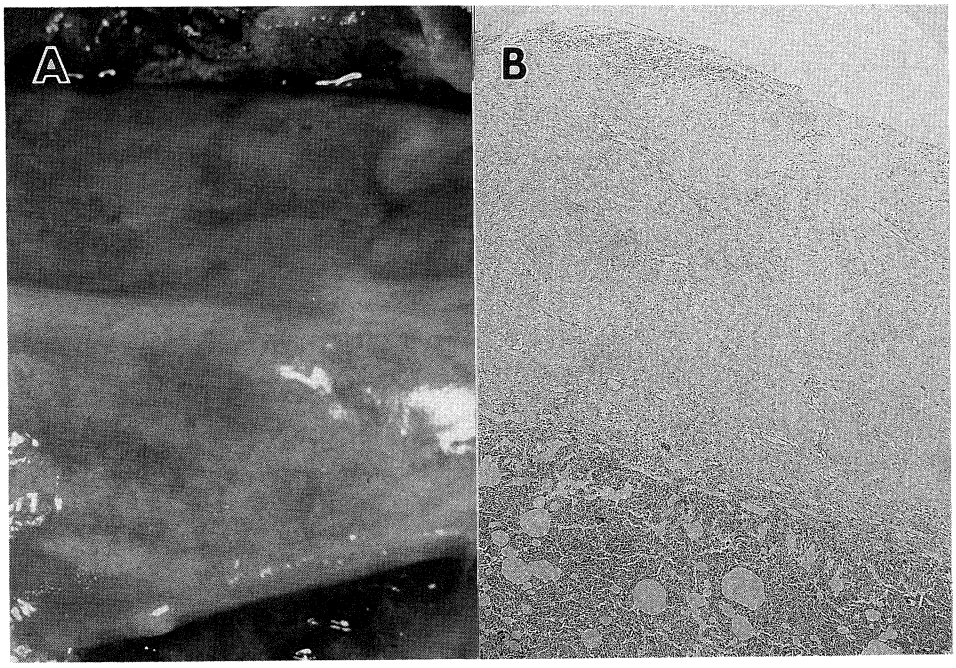

Fig. 3. Chemical pleuritis was produced by injection of turpentine oil into the right pleural cavity of the rabbit, according to the method of Sahn et al. (macroscopically (A), under a microscope ×40 (B)).

As a control study, an extinct fungus ball was prepared by boiling artificial fungus ball in Sabouraud's liquid medium at 100°C for 30 minutes. Using three rabbits, one extinct fungus ball was inoculated into the pleural cavity of each rabbit. The animals were sacrificed and the right thorax was opened at one, four, and eight weeks after inoculation of the extinct fungus ball, respectively.

RESULTS

The results of the experimental aspergilloma are shown in Table 1. The 18 rabbits used in these experiments, two rabbits for each of the periods from the third day to the fourth week, and four rabbits at the eighth and twelfth weeks were sacrificed and examined. The lesions measured 10×12 mm and 11×12 mm at three days after inoculation of the artificial fungus ball, 6×10 mm and 10×10 mm at 1 week, 9×12 mm and 10×10 mm at 2 weeks, 10×13

TABLE 1. Results of experimental aspergilloma (1)

Rabbit No.	Term from inoculation to autopsy	Inflammatory lesions (size) (mm×mm)	Isolation of Aspergillus	Detection of antibody
1	3 days	10×12	+	−
2		11×12	+	−
3	1 week	6×10	+	−
4		10×10	+	−
5	2 weeks	9×12	+	−
6		10×10	+	−
7	3 weeks	13×10	+	+
8		10×10	+	−
9	4 weeks	13×13	+	−
10		11×12	+	+
11	8 weeks	11×13	+	+
12		8× 7	+	−
13		12×12	+	+
14		10×13	+	−
15	12 weeks	10×11	−	−
16		11×11	+	+
17		11×12	+	+
18		8×10	+	−

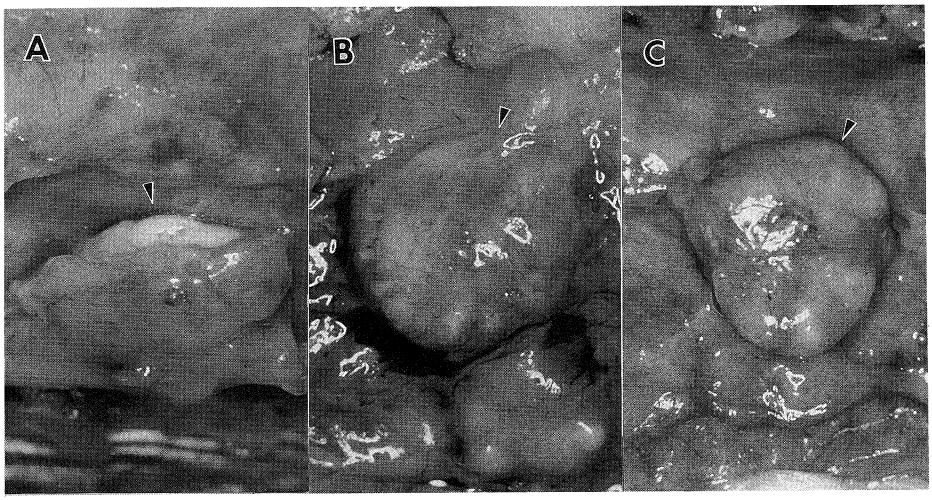

Fig. 4. The surface of the visceral pleura after inoculation of the artificial fungus ball as seen microscopically (A: at 1 week after inoculation of the artificial fungus ball, B: at 4 weeks, C: at 12 weeks).

mm and 10×10 mm at 3 weeks, 13×13 mm and 11×12 mm at 4 weeks, 11×13 mm, 7×8 mm, 12×12 mm and 10×13 mm at 8 weeks, and 10×11 mm, 11×11 mm, 11×12 mm and 8×12 mm at 12 weeks. The size of the lesions changed very little with time. Aspergillus was isolated from the pleural lesions in 17 of 18 rabbits (94.4%), with the exception being one rabbit sacrificed at 12 weeks after inoculation of the artificial fungus ball. Although the serum antigens against the somatic and culture filtrate antigens of A. fumigatus were negative until the third week, they were positive in two of four rabbits at the third and fourth weeks, and in four of eight rabbits at the eighth and twelfth weeks. From the third week after inoculation of the artificial fungus ball, the antibody against A. fumigatus was detected in half of the experimental rabbits. The reason for a negative reading in half of the rabbits was not investigated, but it might be attributed to the method's low sensitivity.

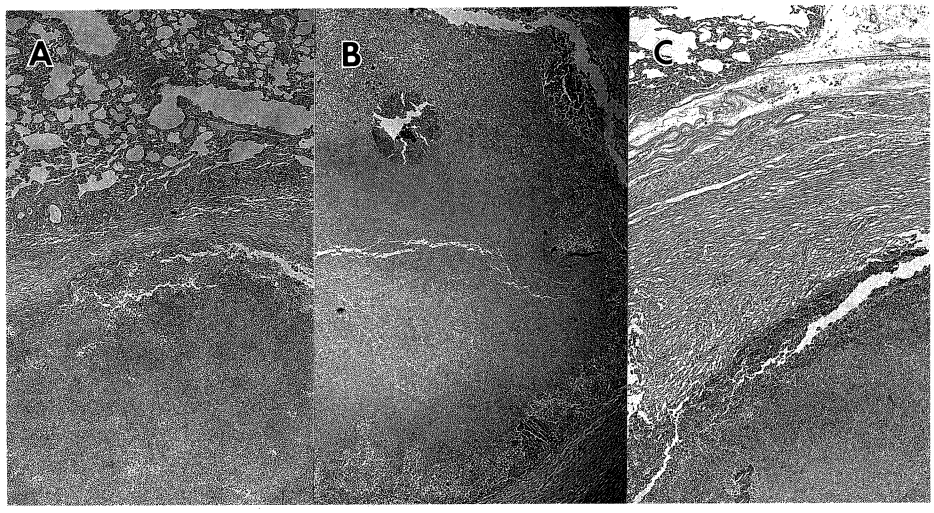

Fig. 5. 40×magnification, PAS stained micrographs (A : at 1 week after inoculation of the artificial fungus ball, B : at 4 weeks, C : at 12 weeks).

The surface of the visceral pleura after inoculation of the artificial fungus ball is visible to the naked eye in Fig. 4 (A : at 1 week after inoculation of the artificial fungus ball, B : at 4 weeks, C : at 12 weeks). The white ball-like lesions are recognizable between the visceral and parietal pleurae. The lesions adhered to the surface of the lung, but separation of the lung and the lesions was easily performed. These characteristics remained unchanged during the observation periods, shown in Fig. 4. A 40× magnification, PAS stained micrograph is shown in Fig. 5 (A : at 1 week after the inoculation of artificial fungus ball, B : at 4 weeks, C : at 12 weeks). The artificial fungus ball was surrounded by fibrous tissue and inflammatory cells invaded the fungus ball. Hyphae were localized in the ball-like lesions, and no hyphae invaded the visceral pleura or the parenchyma of the lung. The degree of inflammatory cells invasion and hyphae did not change over the course of the experiment. The degree of membrane thickness increased with the passage of time. The degree of inflammatory cell invasion was as same as than in the control group (Table 2).

TABLE 2. Results of experimental aspergilloma (2)

Term from inoculation to observation	3 days		1 week			2 weeks		3 weeks		4 weeks			8 weeks					12 weeks			
	1	2	3	4	E	5	6	7	8	9	10	E	11	12	13	14	E	15	16	17	18
pleura																					
cell infiltration	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
thickness	0	0	0	1	1	1	0	2	1	3	1	1	1	2	2	1	1	3	2	3	2
hyphae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mycetoma																					
cell infiltration																					
Mono	1	1	2	2	2	2	2	3	2	3	2	2	3	3	2	3	2	2	3	3	3
PMN	3	3	3	3	2	2	2	2	2	2	2	2	2	1	2	1	1	1	2	1	1
hyphae	2	2	2	2	3	2	1	2	3	2	2	2	2	2	2	2	3	3	2	2	2

E : Extinct fungus ball
Mono: Mononuclear cells
PMN : Polymorphonuclear leukocytes

cell infiltration (/HPE×400)
0 : 0
1 : 1~9
2 : 10 ~ 99
3 : 100 ~ 499
4 : 500 ~

hyphae (/HPF×400)
0 : 0
1 : 1 ~ 9
2 : 10 ~ 49
3 : 50 ~

thickness (mm)
0 : ~ 0.24
1 : 0.25 ~ 0.49
2 : 0.50 ~ 0.99
3 : 1.00 ~

DISCUSSION

Aspergillosis is caused by inhalation of the ubiquitous fungus, Aspergillus spores.[1] Therefore, the lung is the most frequently involved organ.[1] In recent years with the increase in immunocompromised hosts, aspergillosis has also increased in frequency.[1-3] The pulmonary infections due to Aspergillus have been classified into three major categories, invasive pulmonary aspergillosis, pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis,[1,6,11,12] or primary, secondary, and saprophytic aspergillosis.[13] It is believed that aspergilloma occurs as a result of saprofitic proliferation in abnormal spaces, such as those caused by tuberculosis, bronchial cysts, and bronchiectasis.[1,4-6,13] It has been reported that an active man inhales 5.7×10^7 spores of all types daily, including Aspergillus spores.[14] Since the spores of Aspergillus are small in diameter (2.0 to 3.5 μm), they may be inhaled easily and, during heavy exposure, may reach the terminal airways and alveloar spaces.[1-14] Most of the spores are caught by the respiratory bronchiole, and removed by bronchial ciliary action. It has also been suggested that the spores that may remain in the lung are phagocytized by alveolar macrophages and polymorphonuclear leukocytes; thus, aspergillosis does not ensue in a healthy man.[14-18]

Many experimental studies of animal models of aspergillosis have been performed to gain some knowledge about the pathogenesis of aspergillosis.[19-25] To date, however, no one has yet succeeded in devising a simple method for making a saprophytic fungus ball. Many experiments have been carried out on animals exposed to aerosols of Aspergillus spores. When aerosols of Aspergillus spores were inhaled by untreated mice[19] or rabbits,[20,21] granulomas formed temporarily and then disappeared. Sawasaki et al.[21] carried out experiments in which Aspergillus spores were directly injected into

the lungs or the trachea of untreated rabbits. They reported that all rabbits developed showed subacute pneumonia. Fatal pulmonary aspergillosis occurred after inhalation of Aspergillus spores in mice or rats pretreated with irradiation,[22] anticancer drugs,[22,25] steroid[23,24,26-28] or alloxan.[24] But formation of aspergilloma was not observed with the inhalation of Aspergillus spores. It is thought that localized destruction of the lung may be necessary for the proliferation of pulmoary aspergilloma. Sawasaki et al.[21] postulated that in "primary aspergilloma" the check-valve mechanism of a fungus mass; growth on an ulcerated bronchial wall and stenosis or occlusion of a pulmonary or bronchial artery, plays an important role in producing cavities with aspergilloma in an area of necrotic pulmonary parenchyma due to fungus, and they performed suitable experiments. The pulmonary artery was ligated with silk thread, the right lower bronchus was reduced by approximately one-third with a loose ligature of silk thread, and a saline suspension of 20 mg of the spores was injected into the distal bronchus. They reported the creation of experimental aspergilloma in animals, but this experimental technique is complicated, and the aspergilloma thus produced differs from the clinical aspergilloma occurring as the result of the saprophytic proliferation of Aspergillus in a preexistent lung cavity.

I theorized that the pleural cavity of rabbits could replicate an abnormal space for the purpose of making a simple experimental model of aspergilloma. As with most animals, the rabbit pleural cavity is a space consisting of two pleurae, the visceral and parietal pleurae, and the two pleurae are separated by a thin layer of fluid.[29,30] Chemical pleuritis was produced by the injection of turpentine oil through a percutaneous puncture of the right pleural cavity of the rabbits. Sahn et al.[9] observed that an injection of turpentine oil into the rabbit pleural cavity produces an exudative pleural effusion during the acute phase, then a decrease in its volume, and finally, adhesion of the pleurae. However, it is known that the formation of aspergilloma dose not occur if disconnected spores are inhalated or injected as previously mentioned. Accordingly, an artificial fungus ball that Hisauchi[8] produced by shaking a culture of Aspergillus spores was used. This artificial fungus ball is a lump of Aspergillus hyphae with the hyphae forming a zonation.[8] It differs from those in aspergilloma patients, which contain cells and necrotizing substances.[31,32] When these two-step experiments were performed, the size of the inoculated fungus balls did not change macroscopically. The lesions were covered with a thin fibrous membrane, adhered to the pleura, and did not have the mobility common to the fungus balls of aspergilloma patients. Aspergillus hyphae and severe invasion of polymorphonuclear leukocytes were present in the fungus balls. Although these histological changes remained until 12 weeks after the inoculation of the artificial fungus balls, there was no invasion of hyphae into the pleura or the parenchyma of the lung. Therefore, an animal model of aspergilloma in the rabbit can be made by simple two-step process; creation of an abnormal space in the pleual cavity by the injection of turpentise oil and inoculation of the affected pleural cavity with an artificial fungus ball. Although these fungus balls did not grow in size as opposed to fungus balls in human cases, where the fungus proliferates and the fungus ball enlarges, I believe this experimental system should prove useful for study of the pathogenesis of aspergilloma.

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