aspergillosis. This suggests that AFLEI may be useful for treating aspergillosis, so we investigated the properties of AFLEI and treatment efficacy in immunocompromised mice.

Materials and Methods

Fungal strain

A. flavus was identified and isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis.

Purified elastase and elastase inhibitor (AFLEI)

Elastase from *A. flavus* was purified by the method of Hasegawa *et al.*[8]. Elastase inhibitor from *A. flavus* (AFLEI) was purified by the method of Okumura *et al.*[14].

Assay for elastolytic activity

Elastolytic activity of the elastase was assayed by the method of diazocoupling measuring p-nitroanilide (p-NA) released from 50 mM Succinyl L-alanyl- L-alanyl-L-alanyl p-nitroanilide (STANA) in DMSO, which was used as the substrate. To determine the extent of STANA digestion, 0.1 m*l* of the enzyme solution and 0.9 m*l* of 50 mM Tris-HCl buffer, pH 7.5, and 20 μl of 50 mM STANA solution were incubated for 60 min at 37℃. The reaction was stopped by adding 1 m*l* of 10% trichloroacetic acid (TCA). Then 0.2 m*l* of 0.1% sodium nitrite, 0.5% ammonium sulfamate and 0.1% N-1-naphtyletylenediamine dihydrochloride were added to the reaction solution and the color developed was read at 550 nm.

Assay for elastase inhibitory activity

Fifty microliters of elastase inhibitor solution (crude enzyme) was mixed with the purified elastase from *A. flavus* and incubated for 15 min at 37℃ prior to the addition of the STANA substrate. Then 0.9 m*l* of 50 mM Tris-HCl buffer, pH 7.5, and 20 μl of 50 mM STANA solution were added and incubated for 60 min at 37℃. The reaction was stopped by adding 1 m*l* of 10% TCA. Two-tenths m*l* of 0.1% sodium nitrate, 0.5% ammonium sulfamate, and 0.1% N-1-naphthylethylenediamine dihydrochloride were then added to the solution and the color developed was read at 550 nm.

Elastase inhibitor from *A. flavus*

Elastase inhibitor was obtained by the following method. *A. flavus* spores (2×10^8 cfu) were cultured in 100 ml of RPMI 1640 (Sigma Chemical Co., Ltd., St. Louis, MO, USA)) or synthetic medium of 1% yeast carbon base (YCB) (Difco Lab., Detroit, MI, USA) containing 1% nitrogen source (casamino acid, proteose peptone, tryptone peptone, skim milk (Difco), polypepton (Nihon Pharmaceutical Co., Ltd., Tokyo), casein (Nacalai Tesque, Inc., Kyoto), gelatin (Merck KGaA, Darmstadt, Germany), keratin (Katayama Chemical Industry, Ltd., Osaka) or elastin (Sigma), respectively. The cultures were incubated at 37℃ for 10 days.

Western blot analysis

Western blot analysis was performed essentially as described previously[20]. Samples [AFLEI (0.5 mg), elastase (0.3 mg), the mixture (AFLEI and elastase) and culture filtrate] were run on an SDS-PAGE in 15% gels and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Co., MA, USA). After blocking with Tris-buffer saline containing 3% skim milk and 0.05% tween 20, the membranes were incubated sequentially with primary antibody and horseradish peroxydase-conjugated antibody. Bands were detected by chemiluminescence (ECL, GE Healthcare UK Ltd., England). The peptide fragment with C-E-K-E-A-Q-F-V-K-Q-E-I-G-Q-P-Y-T of the AFLEI polypeptides was synthesized and antiserum specific for the oligopeptide was obtained by immunization of rabbits with the peptide coupled to keyhole limpet haemocyanin using benzidine. The resulting anti-AFLEI peptide antibody was used at a dilution of 1:1000.

Immunocompromised mice

Immunocompromised mice were prepared by the method of Hasegawa *et al.*[11]. White SPF (specific pathogen free) mice (18～20 g) of ddy strain were injected i.v. with cyclophosphamide (100 mg/kg/time) three times every second day to prepare the immunocompromised mice, and the animals then inhaled the spores. Ten immunocompromised mice were exposed to spores in each experiment.

Infection model

A chamber (1-liter Erlenmeyer flask with two side arms) similar to that described by Piggot and Emmons[21] was used to expose mice to inhalation of an aerosol of *A. flavus* spores (Fig. 1). Sterile potato dextrose agar (100 m*l*) in the bottom of the chamber was inoculated with the spores. After incubation for 4 days at 37℃, a thick layer of the sporulating fungus completely covered the agar surface. Mice were introduced into the side arms of the chamber, and spores were dispersed from the culture below by pumping 200 m*l* of air (in about 15 sec) through a tube. Mice inhaled spores for 1 min, and were observed daily until death. AMB was injected peritoneally and AFLEI intravenously after mice had inhaled the spores.

Histological study of injection of purified elastase and AFLEI

White rats (180～200 g) of the Wistar strain were anesthetized, then purified elastase or a mixture of

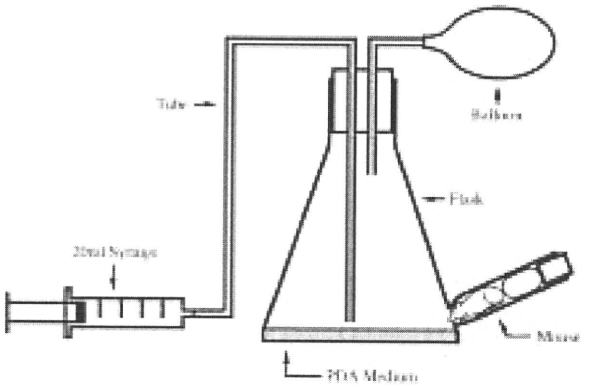


Fig. 1. Inhalation method for exposing mice to spores of *A. flavus*.

Mice were inserted nose down into two side arms. Spores from culture grown in the bottom of the flask were dispersed by injection of air through the tube.

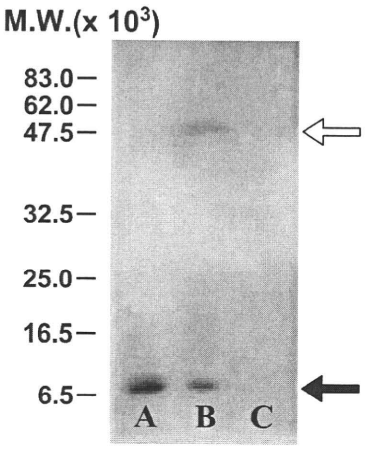
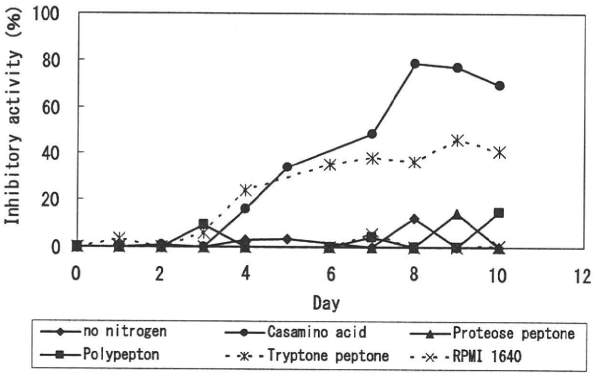


Fig. 3. Western blot analysis of AFLEI (A), mixture of AFLEI and elastase (B) and elastase (C).

A white arrow is the mixture of AFLEI and elastase (lane B) and a black arrow is AFLEI (lane A and lane B). Elastase from *A. flavus* was not detected (lane C).

purified elastase and AFLEI was injected to the lungs. The rats were killed 24 hr after injection and their lungs were removed.

Resected lungs were fixed with neutral buffered formalin processed by routine methods. Paraffin-embedded tissue blocks were cut into 4 μm-thick sections. After deparaffinization, the sections were stained with Hematoxylin and Eosin (HE). We evaluated the degree and sites of hemorrhage, the necrosis of pulmonary alveoli and the infiltration of inflammatory cells.



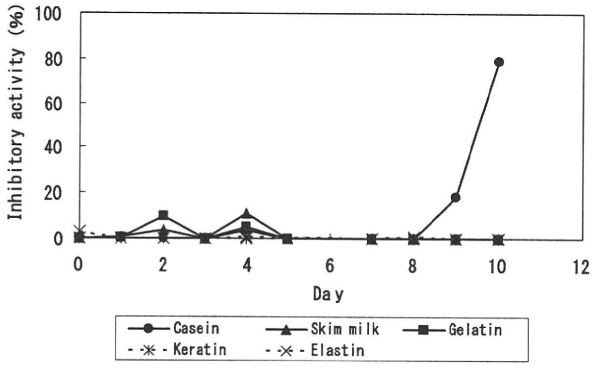


Fig. 2. Elastase inhibitory activity of culture filtrate from *A. flavus*.

Results

Differences in production of elastase-inhibiting substances according to nitrogen source

Changes in the production of elastase inhibitor were examined by varying nitrogen sources in culture media (Fig. 2). *A. flavus* was grown in each of the culture media. Using RPMI 1640 medium, casamino acid, tryptone peptone, proteose peptone or polypepton, inhibitory activity was detected in culture supernatant for casamino acid (16% inhibition) and tryptone peptone (24% inhibition) from day 4 onward. With casamino acid, the greatest inhibitory activity (79% inhibition) was seen on day 8, and with tryptone peptone, the greatest inhibitory activity (49%) was seen on day 9. When skim milk proteins, gelatin, keratin and elastin were used as nitrogen sources, the highest inhibition rate was a low 11%. The only substance that showed strong inhibitory activity was casein, for which the inhibition rate was 79% on day 10.

Detection of AFLEI by Western blot analysis

The Western blot method using anti-AFLEI antibody was used to detect AFLEI and the mixture of purified elastase and AFLEI (Fig. 3). A band was seen at a molecular weight of approximately 7,500 for AFLEI alone, and bands at molecular weights of

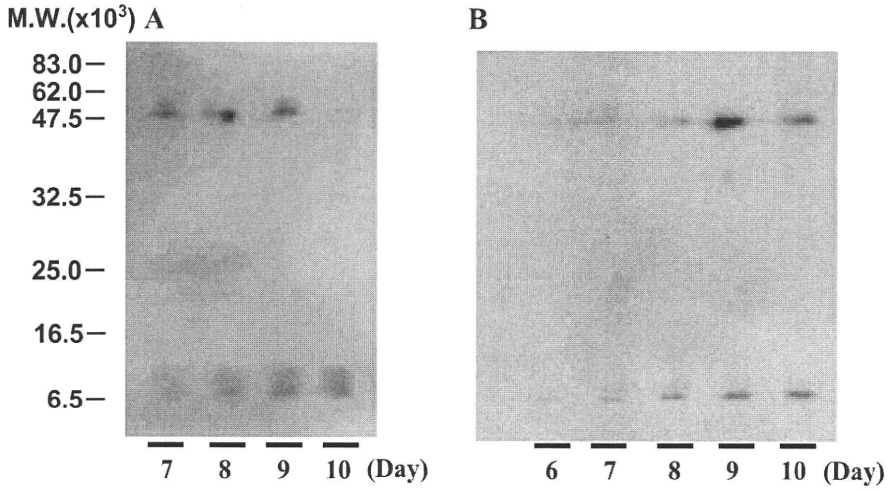


Fig. 4. Western blot of YCB-casamino acid and tryptone peptone medium.
A: YCB-casamino acid medium, B: YCB-tryptone peptone medium

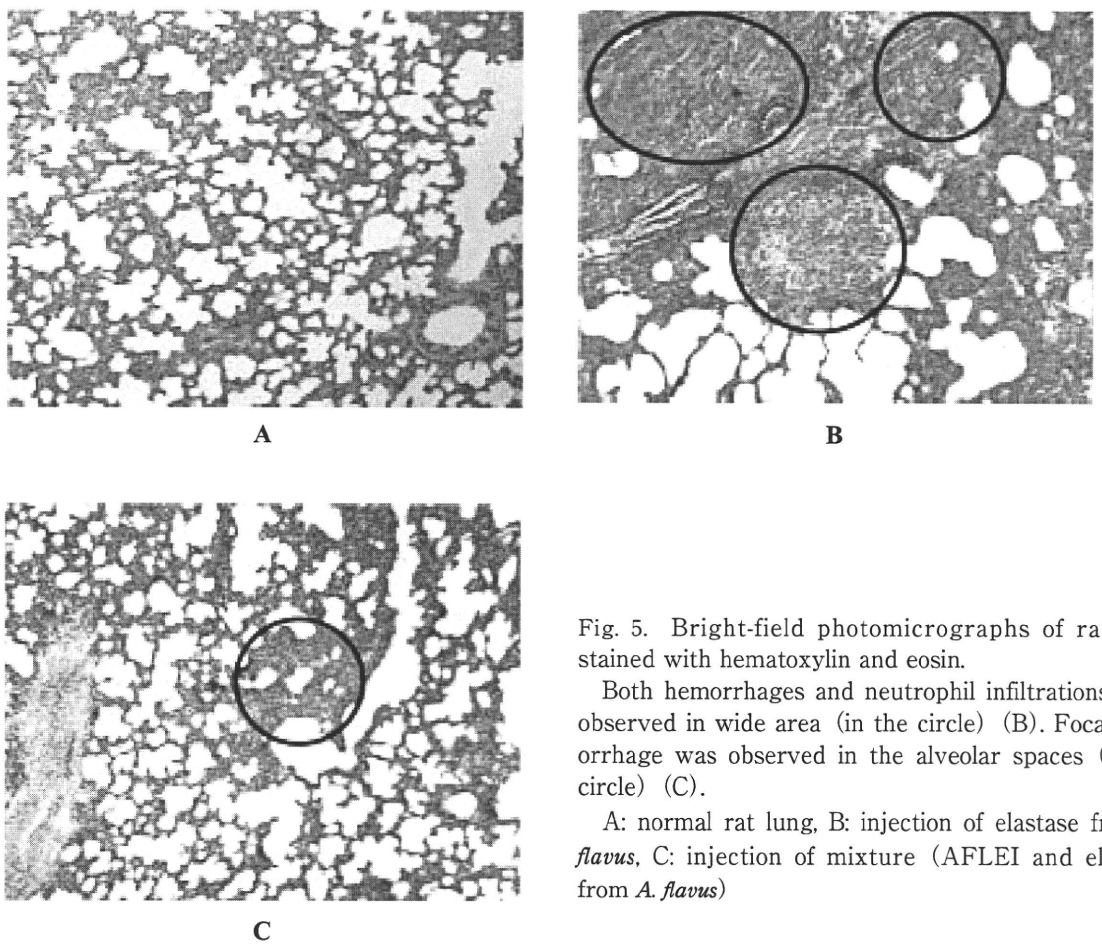


Fig. 5. Bright-field photomicrographs of rat lung stained with hematoxylin and eosin.

Both hemorrhages and neutrophil infiltrations were observed in wide area (in the circle) (B). Focal hemorrhage was observed in the alveolar spaces (in the circle) (C).

A: normal rat lung, B: injection of elastase from *A. flavus*, C: injection of mixture (AFLEI and elastase from *A. flavus*)

approximately 7,500 and 48,000 were seen for the mixture of purified elastase and AFLEI. As the molecular weight of purified elastase is 40,000, the band at around 48,000 was thought to represent the AFLEI-purified elastase mixture.

When examining the effect of nitrogen sources on AFLEI production, culture supernatants for casamino acid and tryptone peptone, which showed inhibitory activity, were examined by Western blot analysis (Fig. 4). Examination showed the presence of bands at molecular weights of approximately 7,000 (low molecular weight band) and 50,000 (high molecular weight

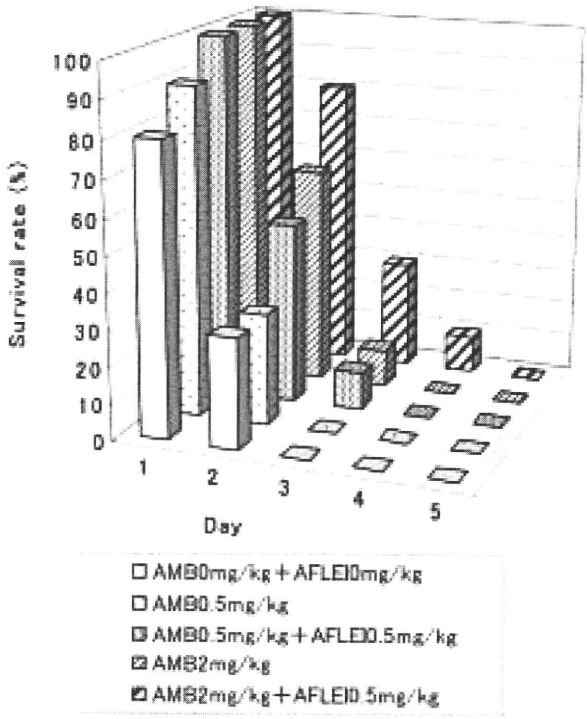


Fig. 6. Survival rate of immunocompromised mice infected by inhalation of spores of *A. flavus*.

Animals were observed daily until death. Ten immunocompromised mice were used in each experiment.

band) beginning from day 7 for the casamino acid supernatant and from day 6 for the tryptone peptone supernatant. Given previous findings, the low molecular weight band was surmised to be AFLEI and the high molecular weight band to represent the mixture of AFLEI and elastase. With both casamino acid and tryptone peptone, the low molecular weight band was very faint on days 6 and 7 and became more distinct over time. Clarity of this band was virtually identical for the 2 substances on days 9 and 10. It was found that the AFLEI band increased in density relative to the number of days remaining in the culture broth. This indicates that there is a direct relation between the time of incubation in the culture broth and the amount of elastase inhibitor produced. The high molecular weight band for casamino acid was nearly equally distinct on days 7-9, but was fainter on day 10. The high molecular weight band for tryptone peptone became clearer each day through day 9 and faint on day 10.

Histological study of purified elastase and the effect of AFLEI

Purified elastase 20 μg was injected into the rat's bronchus and after 24 hr, bleeding was noted in the extracted pulmonary tissues (Fig. 5). Bleeding was demonstrated in a gross pulmonary specimen in comparison with the normal, and focal hemorrhage was observed in the alveolar spaces in many areas.

Both these hemorrhages and neutrophil infiltrations were observed not in focal area but over a wide area. Hemorrhaging in the bronchioles and fibrinous exudate with large amounts of fibrin in the alveoli was also observed. In the group of rats injected with AFLEI and refined elastase, on the other hand, only a small mount of hemorrhaging was observed in the small focal area for a small number of animals.

Effectiveness in an animal model of disease

Mice that were immunocompromised by cyclophosphamide treatment were made to inhale *A. flavus* spores, and the efficacy of AFLEI was examined (Fig. 6). Survival rate in mice not administered the treatment was 80% on day 1 and 30% on day 2. All mice had died by day 3. Similar survival rates were seen with administration of the antifungal agent AMB at 0.5 mg/kg. When AMB 0.5 mg/kg was administered in combination with AFLEI 0.5 mg/kg, survival rate was 100% on day 1, 50% on day 2, and 10% on day 3, and all animals had died by day 4. When AMB alone was administered at 2.0 mg/kg, changes in survival rates resembled those seen with combined administration of AMB at 0.5 mg/kg and AFLEI at 0.5 mg/kg. With administration of AMB 2.0 mg/kg in combination with AFLEI 0.5 mg/kg, further improvements in survival rate were seen: to 80% on day 2, 30% on day 3, and 10% on day 4. All animals had died by day 5. There were no differences in the lungs of the dead mice in the experiments. Significant difference was examined by Log-rank test, but none was recognized. However, the results show that combined administration of the antifungal agent AMB and AFLEI tended to increase survival. The same experiment was repeated, and similar results showing improved survival with combined administration of AFLEI were obtained.

Discussion

As lung tissue contains large amounts of elastin, we focused on elastase as a factor of infection. We purified elastases from clinical isolates of *A. flavus*[8] and *A. fumigatus*[10] and examined the properties of the elastases. During the purification process, we found that *A. flavus* produces an AFLEI, and we described its properties[14,15]. The present investigation examined the conditions under which AFLEI is produced, detected AFLEI using anti-AFLEI antibody, and examined changes in survival rate for infected mice.

Culturing was performed using various nitrogen sources and production of elastase inhibitor was exam-

ined. When proteins were used as the nitrogen source, inhibitory activity was weak. Use of casamino acid and tryptone peptone as nitrogen sources resulted in strong inhibitory activity, indicating that low molecular weight peptides or free amino acids were necessary for the production of elastase inhibitor. When casein was used as the nitrogen source, strong inhibitory activity was seen on day 10. This suggests that elastase degraded the protein, resulting in the formation of degradation products such as low molecular weight peptides and consequent production of elastase inhibitor. Examination of culture supernatant by Western blot analysis showed a low molecular weight band (approx. molecular weight, 7,000) and a high molecular weight band (approx. molecular weight, 50,000). Molecular weights indicated that the low molecular weight band was AFLEI and the high band was a mixture of AFLEI and elastase. As the high molecular weight band was seen before or at the same time as the low band, AFLEI may act as a regulator of *A. flavus* elastase. It is assumed that the intensity of the high band is dependent on the quantity of produced elastase (Fig. 4).

Injury to rat lung tissue caused by purified elastase and the inhibitory effect of AFLEI on such injuries were examined. Pathological examination of the lungs administered purified elastase showed changes such as extensive bleeding, infiltration of inflammatory cells such as neutrophils, and effusion of a fibrin-like substance. Activity of purified elastase was inhibited when mixed with AFLEI, resulting in very little bleeding and inflammatory cell infiltration, and the difference compared with the use of purified elastase alone was striking. This showed that AFLEI strongly inhibited pathological changes caused by elastase, such as bleeding, inflammatory cell infiltration and effusion of fibrin-like substance. As purified elastase from *A. fumigatus* caused similar pathological changes[11] and activity of this purified elastase was also inhibited by AFLEI[14], AFLEI may inhibit pathological changes caused by purified elastase from *A. fumigatus*. The purified elastase from *A. flavus* used in the present investigation had a molecular weight of 40,000 and pI 8.6. Consequently, these enzymes display strong degrading effects on fibrinogen and collagen (Types I, III and IV)[9]. AFLEI strongly inhibited the activity of the elastase[14]. Type Ⅳ collagen is the main component of the basal membrane of blood vessels and alveoli, and the bleeding seen on histopathological examination was thought to have been caused by injury to the basal membrane of these structures. Moreover, elastase had an injurious effect locally at the infection site, and was thought to be in some way related to the pathogenicity manifested by the tissue infiltration and inflammation exhibited by *A. flavus*. Taken together, these findings suggest that AFLEI inhibits tissue infiltration and inflammation seen with aspergillosis and thereby weakens pathogenicity.

Kothary *et al.*[22] administered corticosteroid to immunocompromised mice and obtained results showing that, compared with non-elastase-producing strains, elastase-producing strains were more lethal and mycelia proliferation and associated necrosis more pronounced, suggesting elastase to be a significant pathogenic factor. Moreover, Kolattukudy *et al.*[23] irradiated neutrophil-deficient mice, and these mice were transnasally administered *A. fumigatus* conidia. In strains with the elastase-deficiency mutation, the mortality rate decreased. AMB is effective for aspergillosis and used as the first choice but it has a strong side effect. In the present infection experiment, mice that were immunocompromised by treatment with cyclophosphamide were made to inhale spores, and comparison of the effectiveness of AMB with that of AMB in combination with AFLEI showed a trend toward increased survival with this combined treatment. In this investigation, a single dose was administered. An investigation in which dosing frequency is increased or AFLEI dose is increased to 1 mg/kg may show further increases in survival. Use of proteolytic enzyme inhibitors in actual treatment has been reported as effective[16-19]. We expect that AFLEI is likely to be found effective in treating aspergillosis, and speculate that *Aspergillus* produces the inhibitor for regulation of produced elastase. More extensive research is required to investigate the elastase inhibitor and its biological relationships in order to fully understand the function of elastase inhibitor in *A. flavus*.

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Efficacy of SPK-843, a Novel Polyene Antifungal, in Comparison with Amphotericin B, Liposomal Amphotericin B, and Micafungin against Murine Pulmonary Aspergillosis▽

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SPK-843, a new polyene antifungal, exhibited dose-dependent efficacy on murine pulmonary aspergillosis models. SPK-843 doses of higher than 1.0 mg/kg of body weight exhibited no renal toxicities and a tendency toward better survival prolongation than the estimated maximum tolerated doses of amphotericin B (Fungizone) (1.0 mg/kg) and liposomal amphotericin B (AmBisome) (8.0 mg/kg).

Pulmonary aspergillosis in immunocompromised patients is a major clinical concern. Amphotericin B (AMB; Fungizone) was the most commonly used drug, but it has severe side effects (9). Azoles and echinocandins (7) have fewer side effects than those of AMB, but they frequently fail in therapy because these agents are not entirely satisfactory alternatives due to limitations in spectrum (8, 12). Consequently, more-effective antifungal agents with broad spectrum of action and reduced toxicity are required.

SPK-843 is a new polyene antifungal, which is a water-soluble diascorbate salt from SPA-S-752, an amide derivative of partricin A produced by a mutant strain of *Streptomyces aureofaciens*. Clinical trials to clarify the therapeutic efficacy of SPK-843 for deep-seated mycoses are now being performed. SPK-843 is reported to possess in vitro inhibitory activity comparable to or better than that of AMB against *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. (6). The pharmacokinetics of SPK-843 was analyzed, and the drug was found to possess a suitable profile for its therapeutic effect (1, 2). In this study, we evaluated the efficacy of SPK-843 compared to those of AMB, liposomal AMB (L-AMB) (AmBisome), and micafungin in experimental pulmonary aspergilloses.

AMB deoxycholate (Bristol-Myers Squibb K.K., Tokyo, Japan), L-AMB (Fujisawa Healthcare, Inc., Deerfield, IL), sodium micafungin (MCFG [Funguard]; Fujisawa Pharmaceutical Co., Osaka, Japan), and SPK-843 (Kaken Pharmaceutical Co., Tokyo, Japan) were used in this study. The MICs of the antifungal agents against challenge strains were determined by the microdilution method according to Clinical Laboratory Standards Institute (CLSI) M38-A (4). In the MIC measure-

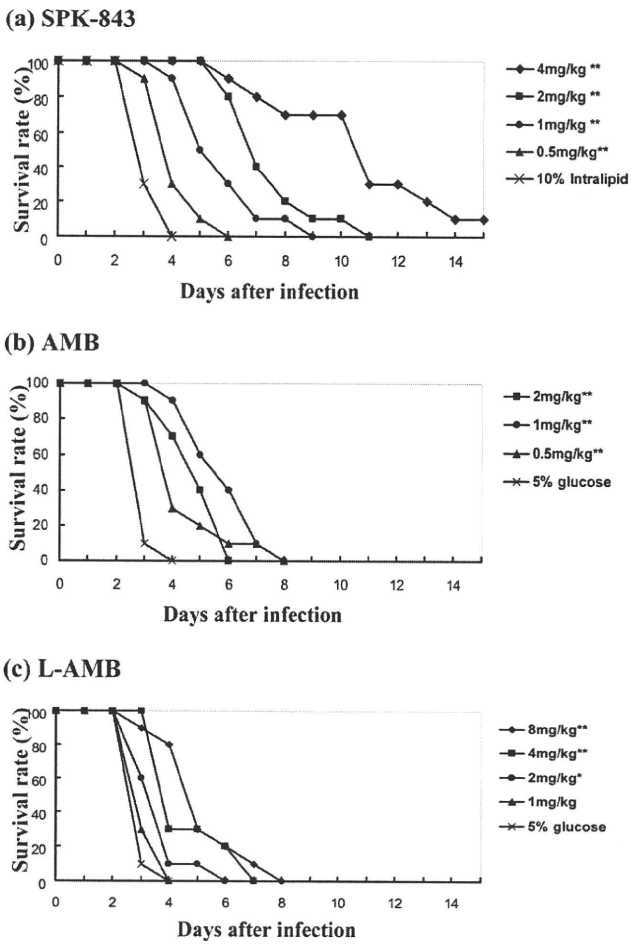

FIG. 1. Effect of SPK-843 (a), AMB (b), and L-AMB (c) on survival curves of mice with pulmonary infection caused by *Aspergillus fumigatus* (*, $P < 0.05$; **, $P < 0.01$; compared to vehicle controls; log rank test).

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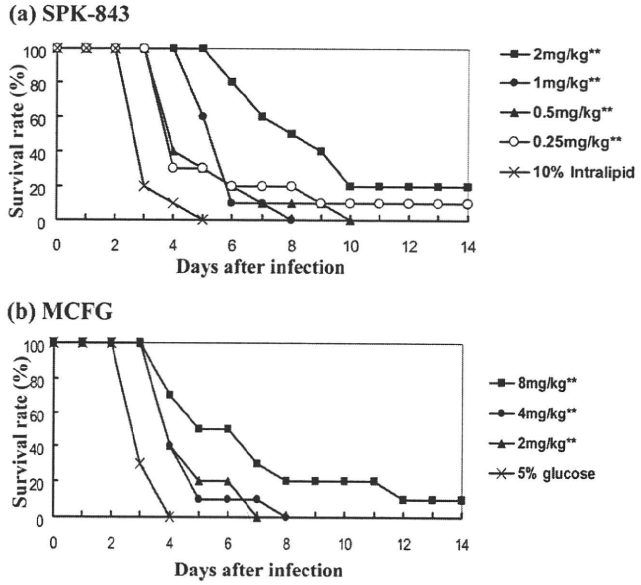

FIG. 2. Effect of SPK-843 (a) and MCFG (b) on survival curves of mice with pulmonary infection caused by *Aspergillus fumigatus* ($**$, $P < 0.01$; compared to vehicle controls; log rank test).

ment, SPK-843 and MCFG were dissolved in water, and AMB (Sigma-Aldrich K.K., Tokyo, Japan) was dissolved in dimethyl sulfoxide.

In experimental aspergilloses, *Aspergillus fumigatus* MF-13 (MIC for SPK-843, 0.5 μg/ml; MIC for AMB, 0.25 μg/ml; MIC for MCFG, 0.0156 μg/ml), which was obtained from the Nagasaki University Hospital, and *A. niger* TIMM 2814 (MIC for SPK-843, 0.0625 μg/ml; MIC for AMB, 0.25 μg/ml) and *A. flavus* TIMM 0057 (MIC for SPK-843, 0.25 μg/ml; MIC for AMB, 0.5 μg/ml), which were obtained from Teikyo University, were used for infection (11). The strains were subcultured on potato dextrose agar (Nissui Pharmaceutical Co., Tokyo, Japan) at 30°C for 6 or 7 days, and the conidia were harvested with sterile saline containing 0.05% Tween 80 and diluted with sterile saline for inhalation. Six-week-old male DBA/2N mice (Charles River Inc., Yokohama, Japan) were used (3, 5). Animals were given drinking water containing 250 mg/800 ml tetracycline (Nacalai Tesque Inc., Kyoto, Japan) throughout the experiment to prevent bacterial infection (10). Three days before infection, mice were subjected to immunosuppression by subcutaneous injection of 50 to 60 mg/kg of body weight of triamcinolone acetonide (Bristol-Myers K.K., Tokyo, Japan) (3). About 60 to 80 mice at a time were confined in an aerosol apparatus (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan) and inhaled with a 10-ml conidial suspension at concentrations of 5×10^8 to 9×10^8 cells/ml by a glass nebulizer at 1-kg/cm^2 pressure for 30 min. Infected mice received intravenous treatments of the drugs or the vehicles ($n = 10$) once daily for 5 days, starting on the next day after inhalation. AMB, L-AMB, and MCFG were dissolved in 5% glucose, and SPK-843 was dissolved in 10% lipid emulsion (10% Intralipid; Terumo Co., Tokyo, Japan) according to clinical preparations. The surviving mice were monitored and analyzed by log rank testing. Each experiment was repeated twice to confirm the reproducibility of results. To investigate renal toxicity, nonin-

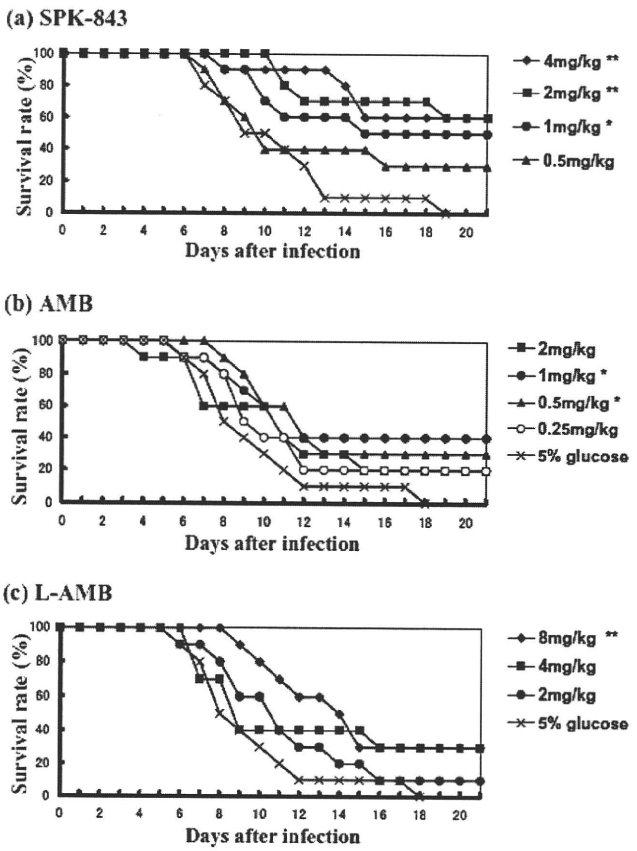

FIG. 3. Effect of SPK-843 (a), AMB (b), and L-AMB (c) on survival curves of mice with pulmonary infection caused by *Aspergillus flavus* ($*$, $P < 0.05$; $**$, $P < 0.01$; compared to vehicle control; log rank test).

fected immunosuppressed mice received intravenous treatments of SPK-843 or AMB once daily for 5 days and sacrificed 2 days after the last administration. Renal histopathological damages were compared in kidneys stained with periodic acid-Schiff stain. The experimental procedures followed the ethical rules from Kaken Pharmaceutical Co. and Nagasaki University Laboratory Animal Center.

Two replicated experiments for *A. fumigatus*, one comparing SPK-843 to AMB and L-AMB (Fig. 1) and another comparing SPK-843 to MCFG (Fig. 2), were performed under the same conditions. In the *A. fumigatus* infection, all the vehicle-treated mice died within 4 days after the infection, and the pathological examination confirmed that they died with invasive aspergillosis. The administration of SPK-843 and AMB at doses of 0.5 mg/kg or higher and those of L-AMB at doses of 4.0 mg/kg or higher significantly prolonged the survival of infected mice compared to the vehicle-treated mice (Fig. 1). Compared with the administration of MCFG (Fig. 2), the administration of SPK-843 at 0.25 to 1 mg/kg resulted in survival prolongation comparable to that seen for MCFG at 2.0 to 4.0 mg/kg, and the efficacy of SPK-843 at 2.0 mg/kg is comparable to that of MCFG at 8.0 mg/kg. In *A. flavus* infection (Fig. 3) and *A. niger* infection (Fig. 4), SPK-843 had dose-dependent efficacy on survival prolongation at doses of 1.0 mg/kg or higher for *A. flavus* and 0.25 mg/kg or higher for *A. niger*. The high dose of

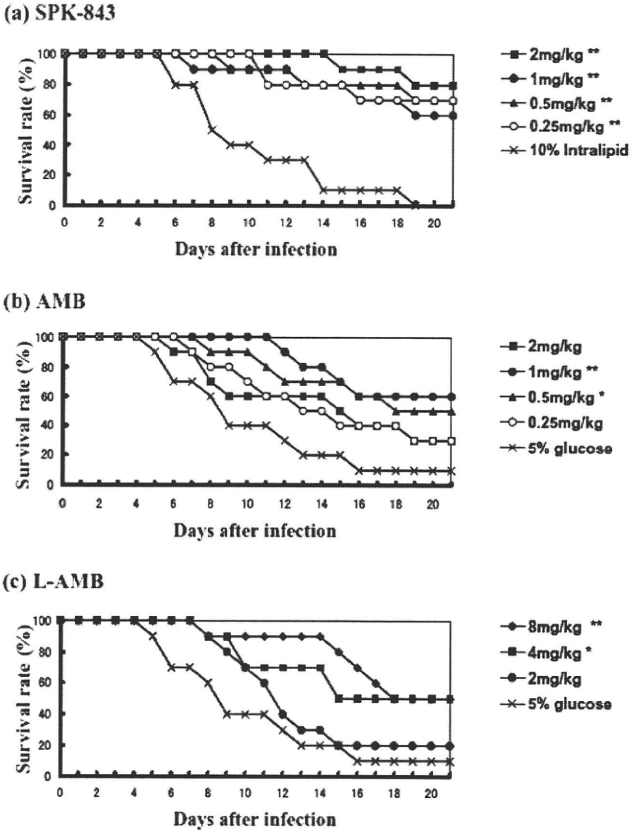

FIG. 4. Effect of SPK-843 (a), AMB (b), and L-AMB (c) on survival curves of mice with pulmonary infection caused by *Aspergillus niger* (*, $P < 0.05$; **, $P < 0.01$; compared to vehicle control; log rank test).

SPK-843 (4.0 mg/kg for *A. flavus*, 2.0 mg/kg for *A. niger*) exhibited better efficacy than AMB (1.0 mg/kg) and L-AMB (8.0 mg/kg).

In all tested aspergilloses, AMB at 2.0 mg/kg was less effective at prolonging survival than at 1.0 mg/kg, suggesting some toxicity. Survival prolongations at 8.0 mg/kg of L-AMB were no more than those at 4.0 mg/kg. In the kidneys of mice treated with AMB at 1.0 mg/kg, tubular cell necrosis and cast formation were observed, suggesting kidney damage. No significant histopathological lesions, however, were found for daily SPK-843 treatment of 1.0 mg/kg or 4.0 mg/kg. The dose-dependent renal toxicity of AMB is well known. SPK-843 is likely to be less toxic against kidneys than is AMB.

In these experiments, at doses higher than 1.0 mg/kg, SPK-843 exhibited dose-dependent efficacy with a tendency toward better efficacy than 1.0 mg/kg of AMB or 8.0 mg/kg of L-AMB. SPK-843 exhibited in vitro activity comparable to or better than that of AMB against three *Aspergillus* species used for the infection models, reflecting comparable efficacy at relatively low doses against the aspergilloses. SPK-843 at 1.0 mg/kg or less was as effective as AMB at the same dose but without renal toxicities. Doses of SPK-843 of higher than 1.0 mg/kg exhibited a tendency toward better survival prolongation than AMB, without renal toxicities. The data obtained in the present study are encouraging for further studies of SPK-843.

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High Mortality in Invasive Aspergillosis: What We Need to Know for Determination of Poor Prognosis and Next Countermeasures

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(See the article by Nivoix et al. on pages 1176–84)

Two major articles that provide definitions and guidelines regarding invasive aspergillosis (IA) have recently been published in *Clinical Infectious Diseases* [1, 2]. These definitions and guidelines were updated from their previous versions, which were published in 2002 and 2000, respectively. Such frequent updates (occurring within 8 years) are required because of the complexity of aspergillosis and the introduction of newer anti-*Aspergillus* drugs, such as voriconazole, echinocandins, and lipid formulations of amphotericin B. These changes also mean that there has been an evolution in the techniques used for the precise and rapid diagnosis of aspergillosis and that newer data are available to inform treatment decisions, although the pace of change has been gradual.

Infectious diseases are simple in principle and involve a host, a microorganism, and a route of infection. Invasive fungal infections (IFIs), however, are rarely simple, with numerous complicating factors that include: (1) complex immunodeficiencies caused by underlying hematological malignancy, the use of intensive chemotherapy (including immunosuppressive agents and corticosteroids), plus the patient's comorbid condition(s); (2) a lack of specific symptoms and signs of IFI, and (3) difficulty in isolating the pathogen, including *Aspergillus* species, or detecting their specific antigen, such as galactomannan and β-D-glucan, in clinical samples that include blood and bronchoalveolar lavage fluid. Although the major aim of establishing revised definitions of invasive fungal disease was to facilitate the identification of reasonably homogeneous groups of patients, with the goal being to perform appropriate clinical trials and to help communication between international clinicians and researchers [1], such revised definitions also reflect the evolution of diagnostic tools and of our understanding of IFI. The galactomannan and β-D-glucan tests have been extensively investigated for decades as a means of enhancing the reliability of IA diagnosis [3, 4]. In another approach, high-resolution CT has also proven to be a valuable tool [5]. However, no straightforward or exceptional tools exist for the accurate diagnosis of IA. In addition, unraveling all of the factors associated with mortality in IA is quite difficult.

In this issue of *Clinical Infectious Diseases*, Nivoix et al. [6] have attempted to discover the factors associated with overall and attributable mortality in IA. This study analyzed the complexity of 289 aspergillosis cases. The prognostic factors identified in this analysis as correlates of overall mortality were (1) receipt of allogeneic hematopoietic stem cell or solid-organ transplantation, (2) progression of underlying malignancy, (3) prior respiratory disease, (4) receipt of corticosteroid therapy, (5) renal impairment, (6) low monocyte count, (7) dissemination of aspergillosis, (8) diffuse pulmonary lesions, (9) pleural effusion, and (10) proven or probable (as opposed to possible) aspergillosis. Similar factors also predicted an increased attributable mortality, with the following exceptions: pleural effusion and monocyte count had no impact, whereas neutropenia was associated with a higher attributable mortality.

Although the host factors in definitions of IFI are not actual risk factors for IA, allogeneic hematopoietic stem cell transplantation, receipt of corticosteroid therapy, and neutropenia often overlap. These data suggested that using these host factors in the definition of IFI [1] is relatively appropriate. Other factors, such as the

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progression of underlying malignancy, prior respiratory disease, renal impairment, low monocyte count, disseminated aspergillosis, and extended pulmonary lesions are easily understood to affect to the prognosis of IA. However, more-detailed information, such as what kinds of prior respiratory disease affect the prognosis of IA, is worth knowing. Needless to say, early administration of antifungal drugs is extremely important for improving the prognosis of IA [7]. The predicted prognostic factors evaluated in this study [6] will be very useful, not only for estimating the prognosis, but also for making a decision about the early administration of antifungal drugs. However, some caution is warranted. The previous definition of IFI, described by Stevens et al. [8], was applied in the study by Nivoix et al. [6], in which a total of 94 cases were classified as possible cases. Possible cases are usually excluded in clinical trails, because infection by *Aspergillus* species is not highly likely. The inclusion of possible cases by Nivoix et al. [6] makes it difficult to evaluate the true risk factors associated with mortality due to *Aspergillus* infection. In actual clinical settings, unlike in clinical trials for drug registration, physicians use all of the mycological tests, including direct culture and microscopy, as well as indirect serum tests, with the realization that they do not work perfectly (as mentioned earlier). We may wish to treat a possible case of IA to avoid overlooking a potentially fatal disease in a high-risk patient. If we are to avoid excessive preemptive or empirical treatment, it is very important to improve diagnosis and to fill the gap between the definitions of probable and possible cases. It is even better for clinicians to identify which factors are important in distinguishing possible cases from probable or proven cases. Although no such information is provided in this study [6], future research will answer these questions and will hopefully lead to an improvement in the prognosis of IA.

Another novel finding of the study by Nivoix et al. [6] is that the availability of treatment with voriconazole, compared with treatment using other antifungal drugs, has improved the prognosis of IA. The superiority of voriconazole to amphotericin B for the treatment of IA has been already reported [9], and the data in this study [6] provided almost the same result. However, one should recognize that it is not only the newer drugs that have affected the prognosis of IA over the course of this long study period, but that other advances in medical care have also had an impact. It might be obvious that newer agents and a better treatment strategy would definitely affect the prognosis, not only for aspergillosis, but also for all other infectious diseases; however, to be scientifically valid, the results must be evaluated by clinical trials that are strictly designed to compare the effectiveness of the drugs. In this study [6], the clinical backgrounds of the patients were varied, as the authors point out, and one should take into account one notable part of their data: the 12-week overall survival among patients receiving various antifungal drugs was only 52.3%, which is ~20% less than that reported in clinical trials performed in a more rigorous manner [9, 10]. The authors explained that the reason for this discrepancy was that their study, compared with clinical trials, included a greater number of patients with severe illness, such as patients with renal impairment and/or intubation. This meant that IA was associated with a gross survival rate of 52.3% in actual clinical settings, which reminds us that we need better antifungal drugs. Perhaps the newer antifungal drugs that have recently become available will improve the prognosis of IA. A strong and reliable anti-*Aspergillus* drug is not yet fully available, and we need advances in this field. Because current available antifungal drugs are limited, the possibility of improving the prognosis of IA may depend on the administration of combination antifungal drugs and/or on earlier administration. Combination therapy, although widely discussed around the world, has never been evaluated in a large-scale randomized controlled trial, although such a study is in progress [11]. The latest treatment guideline for aspergillosis does not recommend combination therapy as primary therapy. Results of a combination trial are eagerly awaited. On the other hand, rapid and accurate diagnosis that will enable the initiation of intensive treatment is also important.

In conclusion, Nivoix et al. [6] have identified the important factors associated with overall and attributable mortality in IA. In doing so, they have raised the issue of when it is appropriate to start preemptive therapy. The basic strategy for treating infectious diseases—giving appropriate drugs to the right person at the proper time—remains a prime mandate.

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