Original Article

Effect of Serum Components on Biofilm Formation by Aspergillus fumigatus and Other Aspergillus Species

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SUMMARY: Biofilm production by microorganisms is critical for their pathogenicity. Serum promotes biofilm production by Aspergillus fumigatus; however, its effects on other Aspergillus spp. have not been reported. We analyzed biofilm formation by five Aspergillus spp., i.e., A. fumigatus, A. flavus, A. niger, and A. terreus, and examined the effects of serum/serum proteins such as fetal bovine serum (FBS), fetuin A, and bovine serum albumin (BSA) on hyphal growth, hyphal branching, and extracellular matrix (ECM) formation. The antifungal susceptibility of A. fumigatus isolates that formed biofilms was also examined. All serum/serum proteins promoted the growth of all these fungal species; growth promotion was most evident with FBS, followed by fetuin A and BSA. This effect was most evident in case of A. fumigatus and least evident in case of A. terreus. Electron microscopy showed thick ECM layers surrounding fungal cell walls after culture with FBS, particularly in A. fumigatus. An increase in hyphal branching caused by fetuin A was the highest in case of A. fumigatus and A. nidulans. Biofilm-forming A. fumigatus showed resistance to most antifungal agents, although a synergism of micafungin and amphotericin B was suggested. Our results indicate that serum promotes biofilm formation, including thick ECM, by many Aspergillus spp., particularly A. fumigatus, and that this may be closely related to its virulence.

INTRODUCTION

Aspergillosis is the most serious fungal infection in many countries, including Japan (1). This disease is often refractory to treatment with antifungal agents, resulting in a high mortality rate. The most common causative agent is Aspergillus fumigatus, although cases caused by other Aspergillus spp., i.e., non-fumigatus Aspergillus, are increasing (2).

Recent studies indicated that A. fumigatus adheres to biotic and abiotic surfaces to form a biofilm (3,4), and within this biofilm, these fungi are surrounded by an extracellular matrix (ECM). Loussert et al. showed that although the composition of ECM may differ depending on the disease state, A. fumigatus forms a biofilm surrounded by ECM both in aspergillosis and in invasive aspergillosis, which is thought to protect this fungus from the host defense system and from the harmful effects of antifungal agents (5). Our group previously indicated that serum as well as fetuin A, a serum glycoprotein, promotes both hyphal growth and biofilm formation of A. fumigatus (6). However, the details of biofilm formation by Aspergillus spp., particularly by non-fumigatus Aspergillus, and the effects of serum have not been investigated.

Thus, in the present study, we evaluated the biofilm-forming capacity of 5 representative pathogenic Aspergillus spp., i.e., A. fumigatus, A. flavus, A. nidulans, A. niger, and A. terreus, and compared the biofilm structures of these species and the effects of serum/serum proteins. Furthermore, to determine a way to deal with intractable fungus forming thick biofilms, we examined the activity of various antifungal agents used either alone or in various combinations.

MATERIALS AND METHODS

Strains and culture conditions: We used the 5 most common pathogenic Aspergillus spp., i.e., A. fumigatus, A. flavus, A. nidulans, A. niger, and A. terreus. For each species, type strains or clinical isolates with typical mycological characteristics were selected (Table 1). All the isolates, A. fumigatus (3 strains), A. flavus (3 strains), A. nidulans (3 strains), A. niger (3 strains), and A. terreus (3 strains), were stored and maintained in the culture collection of the Medical Mycology Research Center, Chiba University. Identification of all these strains was reconfirmed by both mycological and genetic analyses. Fungi were grown on potato dextrose agar
Biofilm Formation of *Aspergillus* spp.

Table 1. *Aspergillus* species used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>IFM 41204</td>
<td>Cattle lung, Finland</td>
</tr>
<tr>
<td></td>
<td>IFM 49896</td>
<td>Lung, Japan</td>
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</tr>
<tr>
<td></td>
<td>IFM 41933</td>
<td>Unknown, USA</td>
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<td><em>A. flavus</em></td>
<td>IFM 48054T</td>
<td>Lung, Japan</td>
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<tr>
<td></td>
<td>IFM 61573</td>
<td>Unknown, USA</td>
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<td><em>A. nidulans</em></td>
<td>IFM 51356</td>
<td>Bronchium, Japan</td>
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<td></td>
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<tr>
<td></td>
<td>IFM 61956</td>
<td>Sputum, Japan</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>IFM 54290</td>
<td>Blood, Japan</td>
</tr>
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<td></td>
<td>IFM 55890T</td>
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</tr>
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<td></td>
<td>IFM 61468</td>
<td>Sputum, Japan</td>
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<tr>
<td><em>A. terreus</em></td>
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<tr>
<td></td>
<td>IFM 47796T</td>
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<td></td>
<td>IFM 61863</td>
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</table>

IFM, Institute for Food Microbiology (at present, Medical Mycology Research Center, Chiba University), Chiba, Japan; ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory; T, type strain.

(PDA; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) plates at 25°C for 1 week for conidiation. Conidia were harvested using 0.05% Tween 20 and then suspended in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 1.67 × 10^5/ml and then transferred to a tissue culture dish and cultured at 37°C with 5% CO2 for 30 h for biofilm formation. Controls were prepared in DMEM without FBS. After removing the medium, the biofilms that had formed on each dish were visually examined for comparisons.

**Biofilm formation for visual observations:** *Aspergillus* conidia were resuspended in DMEM that contained 10% fetal bovine serum (FBS; Life Technologies Corp., Gaithersburg, MD, USA) at a final concentration of 1.67 × 10^5/ml and then transferred to a tissue culture dish and cultured at 37°C with 5% CO2 for 30 h for biofilm formation. Controls were prepared in DMEM without FBS. After removing the medium, the biofilms that had formed on each dish were visually examined for comparisons.

**Fungal growth by dry-weight measurements:** The effects of FBS, bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan), and fetuin A from fetal bovine blood (Wako) on fungal growth were examined. Each of these was added to a conidia suspension in DMEM in 3 dishes with 9-cm diameter for each isolate. The final concentrations used were 10% FBS, 10 mg/ml BSA, and 2 mg/ml fetuin A. The concentrations of these serum proteins were decided to achieve the same concentrations in 10% FBS/DMEM and were used throughout the experiments. After culture at 37°C with 5% CO2 for 30 h, fungal communities were harvested by filtering through Miracloth (Merck Biosciences, Whitehouse Station, NJ, USA). Cells were weighed after lyophilization for dry-weight measurements. Each experiment was conducted in triplicate.

**Fungal metabolic activity by XTT assay:** An XTT assay can determine the metabolic activity of microorganisms and is used as another method to determine microbial growth. Conidia were cultured at a final concentration of 1.67 × 10^5/ml, and fungal growth in various media was determined by the XTT assay for comparisons. This method exclusively assesses only metabolically active, viable fungi in contrast to dry-weight measurements that determine the total weights of fungi, including their attached structures. This assay was performed as reported previously (7). Hyphal growth in the presence of BSA, fetuin A, or FBS was quantified by measuring the absorbance at 492 nm with a microplate reader (SUNRISE Rainbow RC-R; Tecan Systems, Inc., San Jose, CA, USA). The XTT absorbance value, after normalizing for background absorbance, was used to assess the effects of serum proteins versus negative controls without these proteins. Each isolate was tested in triplicate.

**Fungal growth evaluations by electron microscopy:** For ultrastructural analysis of biofilm production by scanning electron microscopy (SEM), specimens were fixed using an osmium vapor technique. After osmium fixation, samples were allowed to dry in a desiccator and then subjected to platinum deposition. All the specimens were mounted on specimen stubs, sputter coated, and viewed under an S-3400N scanning electron microscope (Hitachi, Tokyo, Japan).

For analysis by transmission electron microscopy (TEM), fungal communities were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed for 1 h in the same buffer that contained 1% osmium tetroxide, and then embedded in epoxy resin. Ultrathin sections were cut, picked up on 400-mesh grids (8), and observed with a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan).

**Hyphal branching visualization by Fungiflora Y staining:** Each isolate was cultured in DMEM that contained 2 mg/ml of fetuin A at 37°C for 30 h and then stained to visualize hyphal branching. Fungiflora Y (Technicon International, Inc., Tokyo, Japan), which specifically stains β-linked polysaccharides, was used to stain hyphae and for counting their branches. The staining procedure was according to the manufacturer’s instructions. An Axio Imager A1 equipped with AxioCam MRc (Carl Zeiss AG, Jena, Germany) was used to acquire images, which were used to determine the the fre-
quency of branching. The number of branches was counted for 50 \( \mu m \) from the tip of each hypha in a cluster. A total of 30 hyphae were examined for each species.

**Antifungal susceptibility tests for Aspergillus spp. forming biofilms:** Thick biofilms tend to hinder the activity of antifungal agents in some fungal infections such as candidiasis. However, for aspergillosis, the effects of a biofilm are unknown. To examine the effects of thick biofilms on the antifungal susceptibility of aspergilli, *A. fumigatus* isolates were used because they were found to form thick biofilms in a pilot study. The conidia of each isolate were suspended at 1.5 \( \times 10^5/ml \) in DMEM alone, DMEM with 10\% FBS, DMEM with 10 mg/ml of BSA, and DMEM with 2 mg/ml of fetuin A and then cultured in 96-well microplates at 37\°C with 5\% CO\(_2\) for 30 h. Following this, an antifungal agent was added to each well to achieve the desired drug concentration, and the plates were incubated for an additional 24 h. After this, the supernatants were removed and the wells were washed with 200 \( \mu l \) of phosphate-buffered saline (PBS). Fungi in each well were stained with 200 \( \mu l \)/well of 0.4\% (wt/vol) crystal violet (Wako) for 15 min. Excess staining solution was rinsed off with PBS three times. Crystal violet that had bound to the formed biofilm mass was solubilized by adding 250 \( \mu l \)/well of 90\% ethanol. The eluates were then transferred to a new microplate and the absorbance (OD\(_{560nm}\)) reading for each well was determined with the microplate reader.

To confirm that all these isolates had normal susceptibility to the antifungal agents used, the susceptibility of each isolate was determined on the basis of CLSI 38A2 (9). None of the isolates used in the present study showed any increase in minimum inhibitory concentrations (MICs).

When the activity of an antifungal agent is determined at various drug concentration ratios, antifungal activity may be weakened as the number of fungi increases, which interferes with accurate determination of the antifungal activities. To avoid these errors, a larger number of conidia (i.e., 10 times higher than that in the other experiments) was used in a preliminary study; this confirmed that the effect of this ratio was negligible with this experimental design (data not shown).

**Synergistic effects of antifungal agents on biofilm-forming fungi:** Using the crystal violet assay, the synergistic effects of several antifungal combinations, including miconafungin (MCFG) with amphotericin B (AMPH-B), MCFG with voriconazole (VRCZ), and VRCZ with AMPH-B, were determined for *A. flavus* that formed a biofilm. The *A. flavus* reference strain (ATCC MYA-3626) was used as a representative isolate for the present study. All antifungal drugs were provided as standard powders. VRCZ (Sigma-Aldrich) and AMPH-B (Sigma-Aldrich) were dissolved in 100\% dimethyl sulfoxide (DMSO). MCFG, which was a gift from Astellas Pharma Inc. (Tokyo, Japan), was dissolved in sterile water. The ranges of the working concentrations for MCFG, VRCZ, and AMPH-B were 0.5 to 64 \( \mu g/ml \), 0.25 to 64 \( \mu g/ml \), and 0.03 to 8 \( \mu g/ml \), respectively. The final concentration of conidia was set to be 1.5 \( \times 10^5/ml \), as done in the previous susceptibility study. As described previously (10,11), sessile MIC\(_{50}\) (SMIC\(_{50}\)) was used to evaluate the antifungal activity of these drugs against *Aspergillus* biofilms. The effect of an antifungal combination was determined on the basis of the fractional inhibitory concentration index (FICI), which was defined as follows (12,13): FICI \( \leq 0.5 \): synergy; 0.5 < FICI \( \leq 4 \): indifference; FICI > 4: antagonism.

**Statistical analysis:** Results are given as means \pm standard deviations (SDs). As appropriate, statistical comparisons were made by one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparisons test. \( P \)-values less than 0.05 were considered statistically significant.

**RESULTS**

**Comparisons of biofilm formation:** Fungi were cultured in DMEM with 10\% FBS to determine biofilm formation by each species of *A. fumigatus* and non-*fumigatus* *Aspergillus*. These biofilms were visually examined. We observed that all the isolates used had formed biofilms that adhered to the tissue culture dishes, although biofilm thickness varied significantly among these species. The thickest biofilm was produced by *A. fumigatus*, followed by *A. nidulans*, *A. flavus*, and *A. niger* in a descending order; *A. terreus* produced the least thick biofilm. For *A. fumigatus*, massive amounts of a sticky transparent material around the hyphae were evident (Fig. 1).

The dry weights of the fungal communities for each *Aspergillus* sp. are shown in Fig. 2A and 2B. Compared with culture in DMEM alone, the dry weight increased by approximately 50\% for all the species after 30 h of culture after adding BSA to the growth medium. Compared with culture in DMEM alone, the addition of fetuin A resulted in a 2–3-fold increase in dry weight after 30 h for non-*fumigatus* spp., and *A. fumigatus* showed a 5-fold increase in dry weight. Statistical analysis showed that *A. fumigatus* had a significantly higher growth rate than the other species (\( P < 0.05 \)), except for *A. nidulans*. Compared with culture in DMEM alone, the most pronounced effect was observed after adding FBS, which resulted in an increase of approximately 3–4-fold in dry weight after 30 h for non-*fumigatus* spp. In contrast, *A. fumigatus* showed a 10-fold increase, which was significantly greater than that ob-

![Fig. 1. (Color online) Effect of serum on the fungal growth of *Aspergillus* spp. Conidia from 5 representative *Aspergillus* spp., *A. fumigatus* IFM 41204, *A. flavus* IFM 48054, *A. nidulans* IFM 57839, *A. niger* IFM 55890, and *A. terreus* IFM 47796, were cultured with 10\% FBS, DMEM with 10\% FBS, DMEM with 10\% FBS, DMEM with 10\% FBS, and DMEM with 10\% FBS, respectively.](image)
Fig. 2. Measurement of the effect of serum and serum proteins on fungal growth. Strains of 5 different Aspergillus spp. were cultured in the presence of BSA, fetuin A, and 10% FBS at 37°C for 30 h, and growth was quantified by measuring dry weight (A) and by XTT assay (C). The ratio of dry weight cultured with serum or serum proteins/dry weight with DMEM alone was calculated and shown (B). The experiment was repeated for three times for each isolate for dry-weight measurement and XTT assay. The results were expressed as mean values ± SD of 9 separate experiments for each Aspergillus sp. Statistical analysis was performed with ANOVA followed by Bonferroni’s correction for multiple comparisons (*, P < 0.05; **, P < 0.01).

Fig. 3. Scanning electron microscopy (SEM) images of Aspergillus spp. forming biofilm. Conidia from 5 representative Aspergillus spp., (A) A. fumigatus IFM 41204, (B) A. flavus IFM 48054, (C) A. nidulans IFM 57839, (D) A. niger IFM 55890, and (E) A. terreus IFM 47796, were cultured at 37°C for 48 h with or without FBS in DMEM, and were processed for SEM. Scale bars indicate 50 µm.

Biofilm Formation of Aspergillus spp.

served in any other species (P < 0.01). The growth of A. terreus was the slowest.

When assessed with the XTT assay, the growth of various Aspergillus spp. was significantly enhanced in the presence of BSA, fetuin A, and FBS, and in most cases, statistical analysis did not indicate any significant differences among these species (Fig. 2C).

**ECM formation assessed by electron microscopy:**

ECM formation by Aspergillus spp. was assessed by SEM and TEM. SEM (Fig. 3) showed a thick membranous structure among the hyphae of A. fumigatus when cultured in DMEM with 10% FBS, which was considered to be ECM, while cells cultured in DMEM alone formed only a trace amount of this structure. A. nidulans also formed a thick membranous structure, although the extent appeared to be slightly less than for A. fumigatus. Compared with A. fumigatus and A. nidulans, other Aspergillus spp. formed materials with similar architectures but with much smaller amounts of ECM both in DMEM with 10% FBS and DMEM alone.

Two representative species, A. fumigatus and A. terreus, were examined using TEM for more detailed structural analysis. For A. fumigatus, the cell wall was surrounded by a thick electron-dense material when cultured with FBS (Fig. 4A). In contrast, when cultured without FBS, this fungus formed a very thin layer exclusively of electron-dense ECM (Fig. 4B). Compared with A. fumigatus, A. terreus produced only a thin light layer of ECM when cultured with FBS (Fig. 4C) and did not form ECM in DMEM alone (Fig. 4D). These TEM images were compatible with the findings using SEM.

**Fetuin A effects on hyphal branching:**

Fetuin A is known to promote hyphal branching by A. fumigatus (6), although its effect on other Aspergillus spp. remains unknown. When examined morphologically, A. fumigatus (Fig. 5A) and A. nidulans showed abundant branching when cultured with fetuin A, followed by A. terreus and A. niger, with A. flavus showing the lowest branching frequency. In contrast, when cultured with FBS or DMEM alone, little hyphal branching was observed regardless of the fungal species (Fig. 5B). This was confirmed by determining the branching frequencies using scatter plots. When cultured with fetuin A, there were 5.2 ± 4 branches/50 µm for A. fumigatus, 5.7 ± 2.4 branches/50 µm for A. nidulans, 4.1 ± 2.1 branches/50 µm for A. terreus, and 2.7 ± 2.6 branches/50 µm for A. niger. For A. flavus, the branching frequency was lowest among all Aspergillus spp. examined (0.9 ± 1.4 branches/50 µm), and this was significantly lower than that for any other species (P < 0.01).
Fig. 4. The effect of FBS on extracellular matrix (ECM) formation by A. fumigatus and A. terreus in biofilms seen on TEM images. A. fumigatus IFM 41204 and A. terreus IFM 47796 were cultured at 37°C for 48 h with or without FBS and then processed. Thick ECM surrounding the hyphal cell wall was evident in A. fumigatus (A, C). (A) A. fumigatus cultured with FBS, (B) A. fumigatus without FBS, (C) A. terreus with FBS, (D) A. terreus without FBS. H indicates cross section of hypha. Scale bars indicate 1 µm.

Fig. 5. (Color online) Frequency of hyphal branching and the effect of serum/serum proteins. The fungi were cultured in DMEM, DMEM with 2 mg/ml fetuin A, and DMEM with 10% FBS at 37°C for 30 h, and then stained with Fungiflora Y to visualize hyphal branching. (A) Images of A. fumigatus IFM 41204 hyphae cultured in DMEM with 2 mg/ml fetuin A. Frequent branching is evident. Scale bar indicates 50 µm. (B) The numbers of hyphal branches within 50 µm from the tip. In each experiment, 10 hyphal tips were measured in each isolate. Fetuin A evidently promoted the branching in all Aspergillus spp., but the effect was significantly weaker in A. flavus (*, P < 0.01).

Fig. 6. Antifungal susceptibility of A. fumigatus forming biofilms. A representative isolate of A. fumigatus (ATCC MYA-3626, the QC isolate designated in CLSI M38A2 microdilution method) was used in this experiment. A. fumigatus conidia suspended in DMEM, DMEM with 10% FBS, DMEM with 10 mg/ml of BSA, and DMEM with 2 mg/ml of fetuin A were cultured in 96-well plates for biofilm formation, and then antifungal agents were added. Viability of fungi in each well was determined by the crystal violet method. Each value is expressed as the ratio of the reading of each well to the one in the control well. Error bars represent SD of 3 separate experiments.

Fig. 7. (Color online) Comparison of hyphal growth rate among serum proteins. The fungi were cultured in DMEM, DMEM with 10% FBS, DMEM with 10 mg/ml of BSA, and DMEM with 2 mg/ml of fetuin A were cultured in 96-well plates for biofilm formation, and then antifungal agents were added. Viability of fungi in each well was determined by the crystal violet method. Each value is expressed as the ratio of the reading of each well to the one in the control well. Error bars represent SD of 3 separate experiments.

0.01). As observed visually, no branching was observed for any of the isolates when cultured with FBS or DMEM only.

Susceptibility to antifungal agents by A. fumigatus after forming biofilms: Results of susceptibility testing showed that the antifungal effect decreased when a fungus was cultured with serum proteins to form biofilms, although the extent varied depending on which serum protein was used for biofilm formation (Fig. 6). This decreased effect was most pronounced with FBS, followed by fetuin A and BSA in a descending order. Susceptibility to AMPH-B was least affected by these serum proteins because it showed the highest activity among all antifungals even when fungi were cultured with these proteins. Fungal growth with 1 µg/ml of AMPH-B was reduced to 25% of the control when fungi were cultured with DMEM alone or with BSA, and it was significantly inhibited to 40% of the control when fungi were cultured with fetuin A. The fungi with FBS-induced biofilms were most resistant, although their growth was suppressed to 65% of the control with 1 µg/ml of AMPH-B.

The effects of the serum proteins were much more evident with itraconazole (ITCZ) and VRCZ than with AMPH-B. These azoles suppressed fungal growth to only 60–70% of the control even at high concentrations of these agents (1–2 µg/ml) when cultured with DMEM alone or with BSA, while fungal growth was suppressed to 75% of the control in fetuin A-induced biofilms and to 90% of the control in FBS-induced biofilms.

The effect of serum proteins on MCFG activity was also evident. When cultured with BSA, fungal growth decreased to 50% of the control at high MCFG concen-
trations. Adding fetuin A or FBS further weakened this effect, with fungal growth being suppressed to only 75% and 90% of the control, respectively. The effect of BSA was only negligible, except with MCFG.

**Synergistic effects of antifungals on** *A. fumigatus* **after forming biofilms:** To assess the synergistic effects of antifungal agents on *A. fumigatus* after it formed biofilms, fungi were exposed to combinations of antifungals and their viability was examined by a crystal violet assay. Synergistic inhibitory activity was the strongest with the combination of MCFG and AMPH-B against the biofilm-forming *A. fumigatus* reference strain regardless of culture with serum or serum proteins (FICI values, 0.094–0.5). In fact, this was the only combination that showed a synergistic effect against fungi with FBS-induced biofilms (Table 2). For the combination of VRCZ and AMPH-B, a synergistic effect was observed when fungi were cultured in DMEM alone or with fetuin A (FICI values, 0.07–0.5), while synergistic activity was not observed when FBS was used. The combination of VRCZ and AMPH-B showed synergistic inhibitory activity against fungi cultured in DMEM alone or with fetuin A (FICI values, 0.266–0.313) but not when FBS was used (FICI value, 0.531). Taken together, AMPH-B showed the strongest antifungal activities for any biofilm-producing condition examined, which was further enhanced by a synergistic effect with MCFG even when FBS was used for biofilm formation.

**DISCUSSION**

Serum is known to promote the growth of *A. fumigatus* (14), although the active component(s) in serum or its effect on non-*fumigatus* *Aspergillus* spp. remains unknown. In the present study, we used 5 representative pathogenic *Aspergillus* spp. and examined the effects of serum (FBS) and serum proteins (BSA and fetuin A) on these fungi. We found that FBS and these 2 serum proteins promoted the growth of these fungal species as assessed either by dry weight or the XTT assay. In general, the promotion of biofilm formation was most evident with FBS, followed by fetuin A and BSA. Toyotome et al., reported the promoting effect of serum and its components on *A. fumigatus* (6); however, studies on non-*fumigatus* spp. are limited. To our knowledge, this is the first report to compare the effects of serum and serum proteins on biofilm formation by various *Aspergillus* spp.

As assessed by the XTT assay, the extent of growth promotion by serum and serum proteins was not significantly different among the fungal species examined. In contrast, fungal biomass determined by dry weight showed significant differences among these species. The promoting effect in terms of dry weight was greatest for *A. fumigatus*, while the other species showed lesser increases. For *A. terreus*, in particular, this increase was the least among all the *Aspergillus* spp. examined.

Visual examinations revealed that *A. fumigatus* produced massive amounts of a thick sticky transparent material in conjunction with hyphal growth. This suggested that FBS promoted the growth of hyphae as well as the production of the thick material that surrounded them and resulted in the increased dry weight of *A. fumigatus* cultures.

Electron microscopy (SEM, TEM) showed the formation of a thick ECM surrounding the cell wall when *A. fumigatus* was cultured with FBS. On the other hand, for *A. terreus*, only an extremely thin layer was observed. This ECM observed with electron microscopy seemed to be closely related to the transparent sticky material around the hyphae that was observed visually. These observations strongly suggested that FBS promoted the production of ECM by *A. fumigatus* most actively among all the *Aspergillus* spp. examined. Considering that *A. fumigatus* has the highest virulence among all *Aspergillus* spp., the capacity to form ECM could be an important factor for the virulence of *A. fumigatus*.

For some microorganisms, their natural growth rate is possibly related to their virulence. However, in our study, *Aspergillus* spp. that formed a thick ECM, such as *A. fumigatus* and *A. nidulans*, tended to grow more slowly than species that formed a thinner ECM. The relationship between the natural growth rate and ECM formation is an interesting issue and needs to be clarified.

Culture with fetuin A promoted hyphal branching in all *Aspergillus* spp. examined, although the extent of this effect significantly differed by species. This effect was most pronounced for *A. fumigatus* and *A. nidulans*, followed by *A. terreus* and *A. niger*, and it was least pronounced for *A. flavus*. We previously reported that fetuin A promoted hyphal branching as well as hyphal growth by *A. fumigatus* (6). In the present study, we also showed its effect on non-*fumigatus* spp. for the first time. Various functions of fetuin A have been reported, such as inhibiting calcification and the tyrosine kinase activities of insulin receptors (15,16). Serum fetuin A levels were elevated in some bacterial infections (17); however, its precise role in infection remains unclear. Hyphal branching forms a tight hyphal network, which results in the formation of a sturdy

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**Table 2. Interaction of MCFG with AMPH-B, MCFG with VRCZ, or VRCZ with AMPH-B against biofilm of *A. fumigatus* reference strain (ATCC MYA-3626)**

<table>
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<tr>
<th>SMIC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
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<tr>
<td></td>
<td>MCFG</td>
<td>AMPH-B</td>
<td>MCFG/AMPH-B</td>
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<tr>
<td>DMEM + FBS</td>
<td>&gt;64</td>
<td>&gt;64</td>
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SMIC<sub>50</sub>, the sessile MIC, the concentration at which a 50% decrease in absorbance was detected in comparison to the absorbance of an untreated biofilm formed by the same fungal strains.

VRCZ, voriconazole; MCFG, micafungin; AMPH-B, amphotericin B; FICI, fractional inhibitory concentration index.
biofilm; in this regard, fetuin A could promote infection. However, this effect was not evident when fungi were cultured with FBS, which contained a similar amount of fetuin A. One possible explanation is that some other component in FBS altered the metabolism of fungi to promote ECM formation rather than branching, thereby inhibiting its promoting effect on branching. Some previous studies investigated biofilm formation of this fungus and hypothesized that within a certain environment, starvation-caused autolysis of this fungus resulted in the release eDNAs, which aided in the development and maintenance of its biofilm architecture (18). For our experiments, the culture period was very short (30 h) and was not sufficient to induce fungal starvation, which suggests that some other mechanism was involved. Thus, further study will be required.

The most evident suppressive effect of culture with serum proteins on antifungal activities (i.e., resistance to antifungal agents) was shown by culture of fungi with FBS, followed by culture with fetuin A, and lastly culture with BSA. This order of effect was in agreement with the extent of ECM formation, which suggests that AMPH-B could be an option for treating biofilm-forming aspergillosis.

Some reports have shown that a combination of certain antifungals increased their antifungal activity against A. fumigatus in vitro, suggesting that they had a synergistic action (26,27). A synergistic effect on biofilm-forming aspergilli in vitro was shown for the combinations of CPFG with AMPH-B and CPFG with VRCZ by Liu et al. (11). In a clinical study, Marr et al. did not find a statistically significant synergistic effect by amphotericin B or voriconazole against Aspergillus fumigatus biofilms in vitro. Antimicrob Agents Chemother. 2010;65:271-4. One possible explanation is that the fungus with FBS-promoted biofilms were highly resistant, for which the combination of AMPH-B and CPFG showed the most promising results. Antifungal activity in vitro depends on many factors such as the isolate used, culture conditions, and the medium used. Thus, we cannot draw any definitive conclusions on the basis of the results of this study. However, the new findings shown here should provide important clues for further study of the efficacy of combination therapy for intractable, biofilm-forming aspergillosis.

Our results showed that the synergistic effect on biofilm formation and the extent of ECM formation varied tremendously according to the Aspergillus spp. This is of particular interest from a clinical perspective because this biofilm-forming capacity has been suspected to be an important factor for infection persistence. Although some other factors such as gliotoxin have been proposed as important virulence factors (29), no single factor has been shown to play a key role in the pathogenesis of aspergillosis. Our results suggest that the biofilm-forming capacity of A. fumigatus could be another candidate factor for its virulence. Analyzing the production of a thick ECM and its structure should aid in identifying new therapeutic targets and the development of new antifungal agents or combinations.

**Conflict of interest** None to declare.

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