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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. nidulans*, *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 aspergilloma 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

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Table 1. *Aspergillus* species used in this study

Species	Strain	Source
	IFM 41204	Cattle lung, Finland
A. fumigatus	IFM 49896	Lung, Japan
	IFM 58689 (ATCC MYA-3626)	Unknown, USA
	IFM 41933	Pleural, Japan
A. flavus	IFM 48054^T (RIB 1427, NRRL 1957)	Unknown
	IFM 61573	Lung, Japan
	IFM 51356	Bronchium, Japan
A. nidulans	IFM 57839^T (ATCC 10074, IMI 126691, NRRL 187)	Unknown, Belgium
	IFM 61956	Sputum, Japan
	IFM 54290	Blood, Japan
A. niger	IFM 55890^T (ATCC 16888, IMI 050566, NRRL 326)	Unknown, USA
	IFM 61468	Sputum, Japan
	IFM 47756	Sputum, Japan
A. terreus	IFM 47796^T (ATCC 10071, IMI 017294, NRRL 255)	Soil, USA
	IFM 61863	Ear canal, Japan

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).

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% CO_2 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% CO_2 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 μm 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 1.5×10^5/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 μl of phosphate-buffered saline (PBS). Fungi in each well were stained with 200 μl/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 μl/well of 90% ethanol. The eluates were then transferred to a new microplate and the absorbance ($OD_{600\ nm}$) 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 fungus/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 micafungin (MCFG) with amphotericin B (AMPH-B), MCFG with voriconazole (VRCZ), and VRCZ with AMPH-B, were determined for *A. fumigatus* that formed a biofilm. The *A. fumigatus* 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 μg/ml, 0.25 to 64 μg/ml, and 0.03 to 8 μg/ml, respectively. The final concentration of conidia was set to be 1.5×10^5/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 $\leqq$ 0.5: synergy; 0.5 < FICI $\leqq$ 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 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-

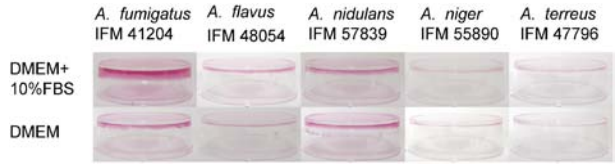

Fig. 1. (Color online) Effect of serum on the fungal growth of *Aspergillus* spp. Conidia from 5 representative *Aspergillus* spp., *A. fumigatus* IFM 41204, *A. flavus* IFM 48054, *A. nidulans* IFM 57839, *A. niger* IFM 55890, and *A. terreus* IFM 47796, were cultured with 10% FBS at 37°C for 30 h in tissue culture dishes for the formation of biofilm. Biofilm formed on each dish was visually examined after removal of medium. Three strains were examined for each species and pictures of a representative isolate for each species are shown. The dishes were turned upside down to reveal the biomass adherent to the dish bottom, and its thickness.

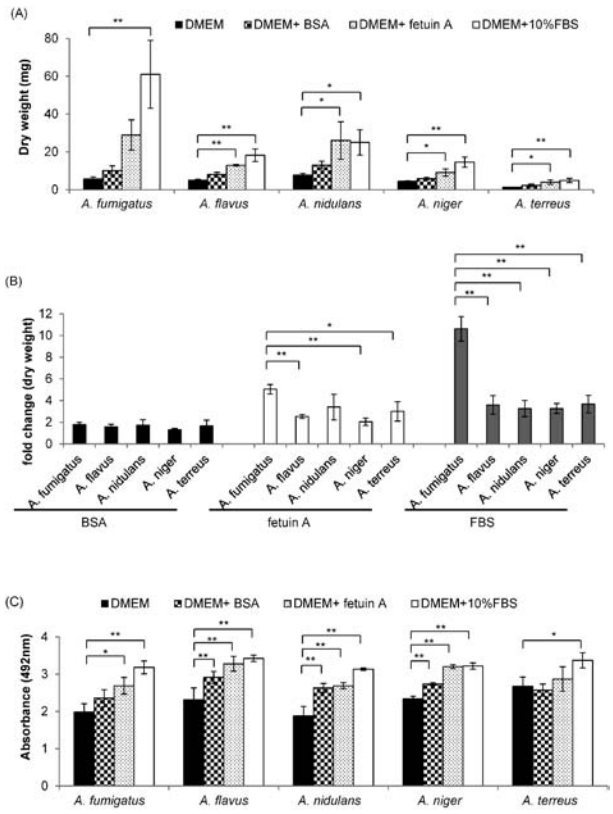

Fig. 2. Measurement of the effect of serum and serum proteins on fungal growth. Strains of 5 different *Aspergillus* spp. were cultured in the presence of BSA, fetuin A, and 10% FBS at 37°C for 30 h, and growth was quantified by measuring dry weight (A) and by XTT assay (C). The ratio of dry weight cultured 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 (*, $P < 0.05$; **, $P < 0.01$).

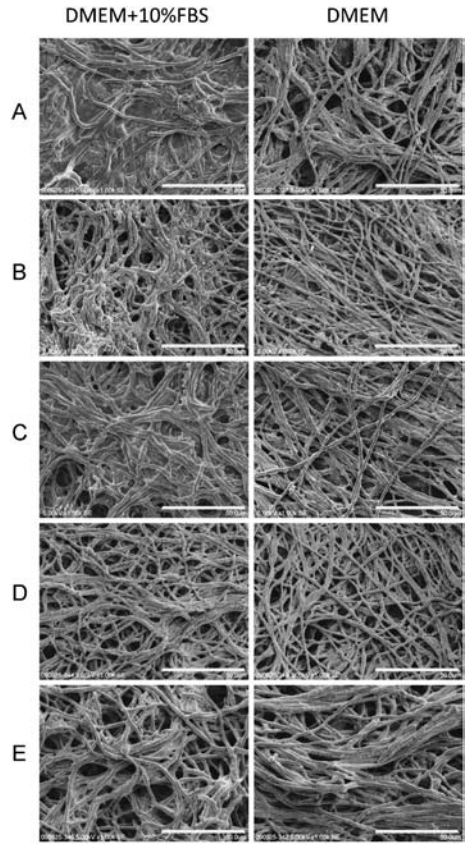

Fig. 3. Scanning electron microscopy (SEM) images of *Aspergillus* spp. forming biofilm. Conidia from 5 representative *Aspergillus* spp., (A) *A. fumigatus* IFM 41204, (B) *A. flavus* IFM 48054, (C) *A. nidulans* IFM 57839, (D) *A. niger* IFM 55890, and (E) *A. terreus* IFM 47796, were cultured at 37°C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

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 ($P <$

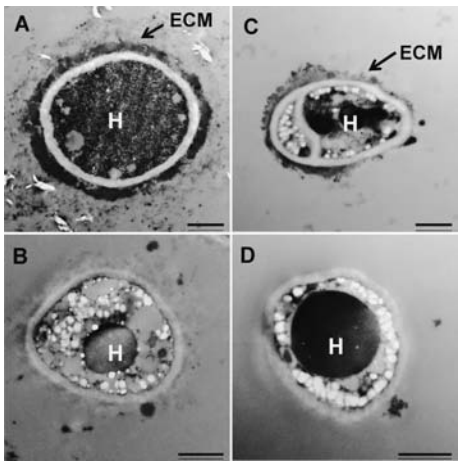

Fig. 4. The effect of FBS on extracellular matrix (ECM) formation by *A. fumigatus* and *A. terreus* in biofilms seen on TEM images. *A. fumigatus* IFM 41204 and *A. terreus* IFM 47796 were cultured at 37°C for 48 h with or without FBS and then processed. Thick ECM surrounding the hyphal cell wall was 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 indicats cross section of hypha. Scale bars indicate 1 μm.

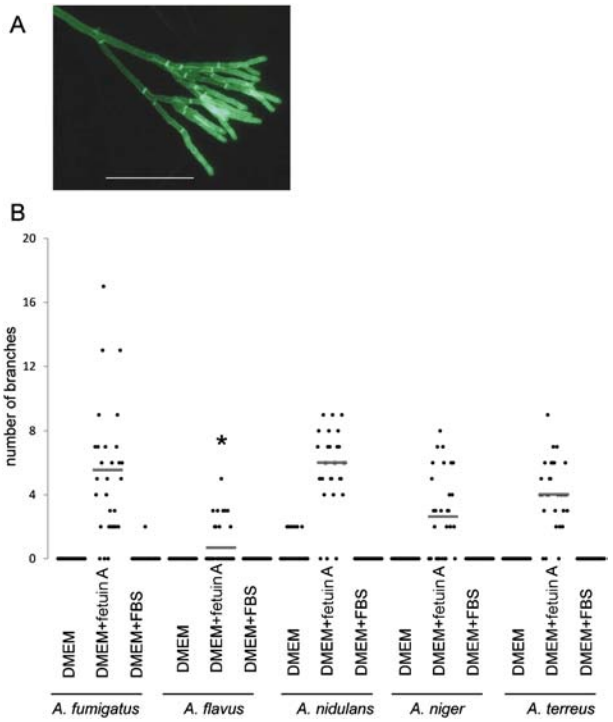

Fig. 5. (Color online) Frequency of hyphal branching and the effect of serum/serum proteins. The fungi were cultured in DMEM, fetuin A (2 mg/ml), and DMEM with 10% FBS at 37°C for 30 h, and then stained with Fungiflora Y to visualize hyphal branching. (A) Images of *A. fumigatus* IFM 41204 hyphae cultured in DMEM with 2 mg/ml fetuin A. Frequent barnching 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* (*, $P < 0.01$).

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

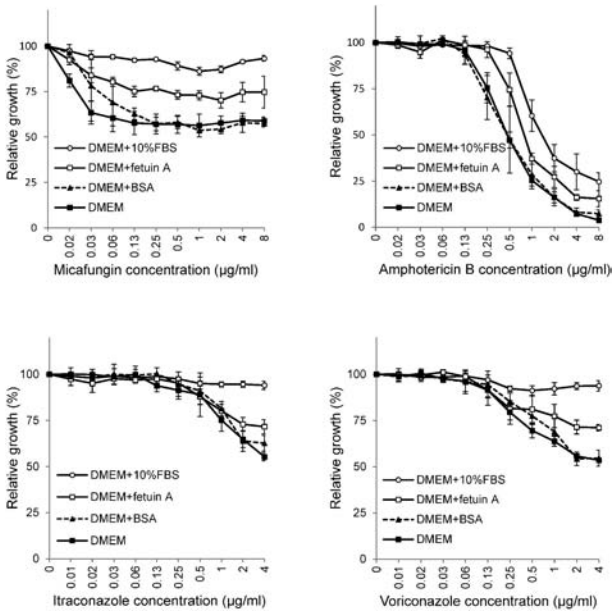

Fig. 6. Antifungal susceptibility of *A. fumigatus* forming biofilms. A representative isolate of *A. fumigatus* (ATCC MYA-3626, the QC isolate designated in CLSI M38A2 microdilution method) was used in this experiment. *A. fumigatus* conidia suspended in DMEM, DMEM with 10% FBS, DMEM with 10 mg/ml of 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.

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-

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)

	$SMIC_{50}$ (μg/ml)			$SMIC_{50}$ (μg/ml)		$SMIC_{50}$ (μg/ml)		$SMIC_{50}$ (μg/ml)	
	VRCZ	MCFG	AMPH-B	MCFG/AMPH-B	FICI	MCFG/VRCZ	FICI	VRCZ/AMPH-B	FICI
DMEM + FBS	>64	>64	2	4/0.5	0.281	>64/>64	—	2/1	0.531
DMEM + fetuin A	64	>64	2	4/0.125	0.094	16/16	0.5	1/0.5	0.266
DMEM	32	16	0.5	4/0.125	0.5	4/0.25	0.07	4/0.125	0.313

$SMIC_{50}$, 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.

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 MCFG and VRCZ, 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 obserbed 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

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 a thick ECM may block the antifungal activity of these drugs. AMPH-B was rather exceptional in this regard because it still showed some activities when fungi were cultured with FBS. This implies that this drug may still exert some activity against biofilm-forming aspergilli in vivo. The effects of biofilms on the antimicrobial effects of drugs has been well investigated for bacterial infections (19) and for some fungal infections such as candidiasis (20,21), although much remains to be clarified for aspergillosis. In candidiasis, AMPH-B and candins, such as MCFG and caspofungin (CPFG), are the only drugs that are known to be effective against biofilm-forming *Candida albicans* (22,23), whereas in aspergillosis their efficacy remains controversial (24,25). In the present study, an antifungal effect of AMPH-B against biofilm-forming aspergilli was indicated as seen in candidiasis, but MCFG activity was not evident. This 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 anidulafungin and VRCZ for invasive aspergillosis patients, although there was a tendency for some favorable effects with this combination, which suggested a possible synergistic effect (28). In the present study, we examined 3 combinations: AMPH-B with MCFG, VRCZ with MCFG, and AMPH-B with VRCZ. All of these showed some degree of synergism, although the fungi with FBS-promoted biofilms were highly resistant, for which the combination of AMPH-B and MCFG 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 study results showed that the serum 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 of 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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