

***Candida albicans*, *Cryptococcus neoformans* or *Aspergillus fumigatus* Induces an Antifungal Activity in Mouse Serum, which is Different from Transferrin**

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ABSTRACT. It has been reported that administration of *Candida albicans* into mouse induces an antifungal activity in serum, which has been identified as transferrin. In the present study, we show that not only *C. albicans*, but also other fungus such as *Cryptococcus neoformans* or *Aspergillus fumigatus* similarly can induce an antifungal activity in mouse serum. This antifungal activity was inhibited by the addition of ferrous ion, indicating that the growth inhibition of *C. albicans* was due to deficiency of ferrous ion, which may be caused by transferrin. Indeed, addition of transferrin in an *in vitro* assay system using RPMI1640 culture medium inhibited the growth of *C. albicans*, *C. neoformans* or *A. fumigatus*. However, when *C. albicans* was grown in RPMI1640 medium with 10% fetal bovine serum (FBS), transferrin was unable to inhibit the growth of *C. albicans*, in sharp contrast, when *C. albicans* treated mouse serum was added instead of FBS, the growth of the organism was inhibited. Similar results were obtained when *C. neoformans* or *A. fumigatus* was used. Taken together, the results suggest that antifungal activity induced by *C. albicans*, *C. neoformans* or *A. fumigatus* was not due to transferrin but likely due to other unknown serum proteins, which may cut off the source of iron for the growth of these fungi.

KEY WORDS: antifungal activity, *Aspergillus*, *Candida*, *Cryptococcus*, *Fungus*.

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Invasive mycoses have increasingly been recognized as a leading cause of death in cancer patients, haemopoietic stem-cell transplant recipients, and other highly immunocompromised patients. Aspergillosis and candidiasis are the adverse prognostic and most important intractable diseases among fungal infections and their mortality rate has been reported to be about 40 to 70% [12, 13, 18, 32]. Antifungal chemotherapy is often used for the treatment of invasive mycoses. However, antifungal chemotherapy is not often effective for invasive mycoses when the host immune system is significantly suppressed. Although immune therapy particularly vaccination is recently getting popular for the treatment of invasive mycoses, vaccination is not always effective to certain patients with feeble immunogenic responses [5, 10, 25, 26]. Therefore, treatment measures which can stimulate immunity in these patients or which is effective even for immunosuppressed patient, for example, antifungal substances associated with host defenses, have been paid much attention [14, 17].

Transferrin [8, 23], lactoferrin [1, 7, 16] and calprotectin [1, 19, 24] are well known antifungal substances associated with innate immunity in humans and animals. Transferrin and lactoferrin, which are present in blood and in mucus secretions, respectively, are chelating proteins sequestering iron while calprotectin, which is present in neutrophil, is a chelating protein for calcium and zinc. Among these host protective substances, transferrin has been well studied and it is indeed one of the most important protective factors for microbial infection [6]. It has high affinity for iron, result-

ing in restriction of free iron to insufficient level for the growth of microbes including fungi. Watanabe *et al.* reported that intraperitoneal administration of formalin-killed *C. albicans* in mouse induces transferrin in serum [31]. Furthermore, both Watanabe *et al.* [30] and Okawa *et al.* [15] have confirmed that the substance present in mouse peritoneal ascitic fluid which inhibited the growth of *C. albicans* is transferrin. Subsequently, Ubukata *et al.* also found the substance, which is most likely to be transferrin in ascites of tumor-bearing mice [28]. However, there were subtle differences between microstructural observations of *C. albicans* treated with the ascites and transferrin under the scanning electron microscopy, indicating that there might be some differences between treatment with transferrin and with ascites [28]. Furthermore, it is currently not known whether administration of other fungi in mice can similarly induce antifungal activity in their sera like in the case of *C. albicans*.

In this study, we therefore examined whether other fungi such as *C. neoformans* and *A. fumigatus* in addition to *C. albicans* were able to induce antifungal activity in mice sera. Our results further indicate that antifungal activity present in mouse serum may be transferrin independent.

MATERIALS AND METHODS

Strains and growth media: *C. albicans* strains IFM40009, Ca-15 and KE-2, *C. neoformans* strain IFM49624 and *A. fumigatus* strain IFM40835 were used in this study. The fungal strains IFM40009, IFM49624 and IFM40835 were kindly provided by Medical Mycology Research Center, Chiba University and the strains Ca-15 and KE-2 were kindly provided by Shionogi & Co., Ltd. (Osaka, Japan). *C.*

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albicans and *C. neoformans* were cultured on Sabouraud Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 35°C for 24 hr. *A. fumigatus* was cultured on Potato Dextrose Agar (Nissui Pharmaceutical Co., Ltd.) at 30°C for 7 days. Each strain was stored in 1 ml of Yeast Nitrogen Base (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) including 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) at -80°C before use.

Cell preparation: *C. albicans*, *C. neoformans* and *A. fumigatus* strains were taken out from -80°C storage, thawed and centrifuged at $1,600 \times g$ at 4°C for 20 min. Supernatant was discarded and cells were washed with 15 ml of sterile normal saline by centrifugation at $1,600 \times g$ at 4°C for 20 min. The washing process was repeated thrice. Finally, cells were suspended in 1 ml of sterile saline and boiled for 1 hr or treated with formaldehyde (2% final conc.) at 4°C for 24 hr. Formaldehyde treated cells were washed five times with 15 ml of sterile saline as described above and finally cells were suspended in 1 ml of sterile saline. Total number of cells was counted by using a haemocytometer. For further experimentations either heat-killed or formaldehyde-killed fungal cells were used.

Preparation of sera from fungus-injected mice: Either live or killed *C. albicans*, *C. neoformans* or *A. fumigatus* fungal strain was diluted with sterile saline and used for this purpose. In each case 0.2 ml of 10^5 , 10^6 , 10^7 or 10^8 cells/ml was injected into each 5-week-old male ICR mouse (Clea Japan, Inc., Tokyo, Japan) through tail vein. ICR mice were bred in clean isolators until sacrifice. Blood was collected from each mouse by cardiac puncture under ether anesthesia after 48 hr of post-administration of live or killed fungal cells. Collected blood sample was transferred to a Sepaclean tube (Eiken Kizai Co., Ltd., Tokyo, Japan) and centrifuged at 4°C at $1,600 \times g$ for 15 min to separate serum. Serums were stored at -20°C for future use.

In vitro antifungal activity assay: In a 96-well plate containing RPMI1640 medium with and without 10% FBS. One hundred eighty μ l of *C. albicans* strain Ca-15 (1×10^3 cfu/ml), *C. neoformans* strain IFM49624 (1×10^3 cfu/ml) or *A. fumigatus* strain IFM40835 (1×10^4 cfu/ml) was inoculated in each well followed by incubation at 35°C for 48 to 72 hr under 5% CO₂ in air in the presence or absence of various concentrations of respective mouse serum, which was prepared as described above. Antifungal titer was expressed as serum concentration that caused 50% inhibition of the growth. *C. albicans* strain Ca-15 (1×10^3 cfu/ml) was cultured in RPMI1640 medium with various concentrations of ferrous chloride (0.2 μ M-25 μ M) in absence or presence of the mouse serum. *C. albicans* strain Ca-15 (1×10^3 cfu/ml) was also cultured in RPMI1640 medium with various concentrations of transferrin (0.25 μ g/ml-128 μ g/ml), lactoferrin (0.25 μ g/ml-128 μ g/ml) or calprotectin (0.125 μ g/ml-16 μ g/ml) in presence or absence of 10% FBS. Fungal growth was monitored by a spectrophotometer (ARVO 1420 Multi-label Counter, Perkin Elmer Japan Co. Ltd.) measuring the optical density (OD) of a culture at 625 nm [OD₆₂₅]. Mini-

mum inhibitory concentration (MIC) of each substance added was determined by 50% fungal growth inhibition. Statistical analysis was performed by a 2-way ANOVA followed by Dunnett's post hoc tests.

RESULTS

Induction of antifungal activity: It has been reported that antifungal activity was induced in mouse serum by administration of formalin-killed *C. albicans*. Therefore, first we examined that whether antifungal activity of mouse serum could be induced by intravenous injection of formalin-killed as well as heat-killed *C. albicans* cells. As shown in Fig. 1, administration of formalin-killed or heat-killed *C. albicans* strain KE-2 into mice significantly induced antifungal activity in sera against the *C. albicans* strain Ca-15 in a dose-dependent manner. To test whether this phenomenon was specific for the *C. albicans* strain KE-2 only, other formalin-killed strains including *C. albicans* strain IFM40009, *C. neoformans* strain IFM49624 and *A. fumigatus* strain IFM40835 were intravenously administered into mice and antifungal activity induced in each mouse serum was measured. While induction of antifungal activity was much higher in the case of *C. albicans*, the other fungal strains, *C. neoformans* and *A. fumigatus*, were also able to induce similar antifungal activity in the mouse serum (Table 1).

Next, to see if a live fungal strain can induce more antifungal activity in the mouse serum, we injected live cells of *C. albicans* strain IFM40009, *C. neoformans* strain IFM49624 or *A. fumigatus* strain IFM40835 into mice. As shown in Table 2, each live fungus strain was able to induce very strong antifungal activity in mouse serum. Moreover, antifungal activity induced in the mouse serum by live *C. albicans* strain KE-2 inhibited not only the growth of homologous strain of *C. albicans* but also other fungi used in this study, namely, *C. neoformans* and *A. fumigatus* (data not shown).

Effect of ferrous ion on the growth inhibition activity of mice serum: It is well known that antifungal activity induced in mouse serum by the administration of *C. albicans* was due to transferrin [31]. Therefore, we investigated if this antifungal activity was inhibited by ferrous ion. Since live *C. albicans* strain KE-2 induced highest antifungal activity in mouse serum (data not shown), the *C. albicans* strain Ca-15 was cultured in RPMI1640 medium with 10% mouse serum obtained from live *C. albicans* strain KE-2 treated mouse. Presence of such mouse serum inhibited the growth of *C. albicans* strain Ca-15 in RPMI1640 medium. However, as expected, this growth inhibition of *C. albicans* strain Ca-15 could be abolished by addition of ferrous ion in the medium in a dose-dependent manner (data not shown). The result suggests that deficiency of ferrous ion might be a likely factor for growth inhibition of *C. albicans* strain Ca-15 in presence of activated mouse serum.

Effect of antifungal chelating proteins on the growth of various fungi: Since addition of ferrous ion abolished antifungal activity of fungus activated mouse serum, we asked

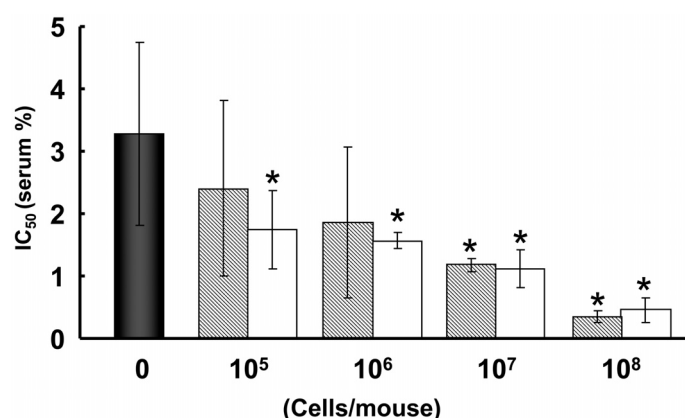


Fig. 1. Growth inhibition of *C. albicans* strain Ca-15 by mouse serum collected after injection with formalin- (open bar) or heat-killed (hatched bar) *C. albicans* strain KE-2. Mice were injected by various concentrations of formalin- and heat-killed *C. albicans* strain KE-2 via tail vein and serum was collected after 48 hr of injection. Various concentrations of serum was incubated at 35°C in 5% CO₂ in air with *C. albicans* strain Ca-15 (1×10^3 cells/ml) in RPMI1640 medium containing 10% FBS. After 48 hr of incubation, serum concentration inhibiting 50% growth of *C. albicans* strain Ca-15 (IC₅₀) was measured by a spectrophotometer (OD₆₂₅). IC₅₀ was represented by means \pm S.D. (n=4). The serum from saline-administered mouse was used as a control (black column). * p<0.05 vs control.

Table 1. Anti-*Candida* activity of mouse serum obtained by the injection of formalin-killed *C. albicans*, *C. neoformans* or *A. fumigatus*

Number of cells injected	IC ₅₀ (serum %)		
	<i>C. albicans</i> IFM40009	<i>C. neoformans</i> IFM49624	<i>A. fumigatus</i> IFM40835
0	2.52 \pm 0.72	2.52 \pm 0.72	2.52 \pm 0.72
1 \times 10 ⁵	2.58 \pm 0.68	1.83 \pm 0.91	2.68 \pm 0.77
1 \times 10 ⁶	1.60 \pm 0.14*	1.94 \pm 0.14	2.53 \pm 0.71
1 \times 10 ⁷	0.68 \pm 0.16*	2.15 \pm 0.45	1.98 \pm 0.71
1 \times 10 ⁸	0.25 \pm 0.02*	0.91 \pm 0.04*	1.45 \pm 0.03*

Various concentrations of mice sera were incubated with *C. albicans* strain Ca-15 (1×10^5 cells/ml) at 35°C in 5% CO₂ in air in RPMI1640 medium containing 10% FBS. After 48 hr of incubation, serum concentration inhibiting 50% growth of *C. albicans* strain Ca-15 (IC₅₀) was measured by a spectrophotometer (OD₆₂₅). IC₅₀ was represented by means \pm S.D. (n=4). The serum from saline-administered mouse was used as a control. * p<0.05 vs control.

whether the antifungal activity was due to transferrin. *C. albicans* strain Ca-15, *C. neoformans* strain IFM49624 or *A. fumigatus* strain IFM40835 were cultured in RPMI1640 medium with or without 10% FBS in the presence of various concentrations of transferrin. When fungi were cultured in the absence of 10% FBS, transferrin was able to inhibit the growth of *C. albicans* strain Ca-15, *C. neoformans* strain IFM49624 or *A. fumigatus* strain IFM40835 (Table 3). The range of MIC values was 4 to 8 μ g/ml. Interestingly, however, in the presence of 10% FBS in the medium, transferrin did not inhibit the growth of any of the three fungi used in this study for which antifungal activity was demonstrated by

mouse serum (Tables 1 and 2) indicating that the antifungal activity induced in mice serum was not due to transferrin (Table 3). It might be due to other serum proteins since heating the mouse serum at 100°C for 20 min abolished its antifungal activity (data not shown). Similarly, we explored the possibility that antifungal activity of lactoferrin and calprotectin, which are also known as antifungal proteins due to their chelating property with ferrous ion and with calcium or zinc, respectively. As shown in Table 3, lactoferrin and calprotectin demonstrated low MIC values as transferrin showed. Similarly, lactoferrin and calprotectin did not inhibit the growth of all three fungi in presence of 10% FBS.

Table 2. Anti-*Candida* activity of mouse serum obtained by infecting with live *C. albicans*, *C. neoformans* or *A. fumigatus*

Number of cells injected	IC ₅₀ (serum %)		
	<i>C. albicans</i> IFM40009	<i>C. neoformans</i> IFM49624	<i>A. fumigatus</i> IFM40835
0	3.17 ± 0.37	3.17 ± 0.37	3.17 ± 0.37
1 × 10 ⁵	1.02 ± 0.07*	2.85 ± 1.24	4.54 ± 1.80
1 × 10 ⁶	0.16 ± 0.07*	3.56 ± 2.41	1.40 ± 0.38
1 × 10 ⁷	ND	1.14 ± 0.24*	0.84 ± 0.52*
1 × 10 ⁸	ND	0.35 ± 0.05*	0.25 ± 0.06*

Various concentrations of mice sera were incubated with *C. albicans* strain Ca-15 (1 × 10³ cells/ml) at 35°C in 5% CO₂ in air in RPMI1640 medium containing 10% FBS. After 48 hr of incubation, serum concentration inhibiting 50% growth of *C. albicans* strain Ca-15 (IC₅₀) was measured by a spectrophotometer (OD₆₂₅). IC₅₀ was represented by means ± S.D. (n=5). The serum from saline-administered mouse was used as a control. * p<0.05 vs control.

Table 3. Antifungal activity of serum chelating proteins on *C. albicans*, *C. neoformans* or *A. fumigatus* in absence or presence of 10% fetal bovine serum (FBS)

Organism	Chelating Protein	MIC (μg/ml)	
		RPMI1640	10%FBS+RPMI1640
<i>C. albicans</i> Ca-15	Transferrin	4	>128
	Lactoferrin	8	>128
	Calprotectin	1	>16
<i>C. neoformans</i> IFM49624	Transferrin	8	>128
	Lactoferrin	4	>128
	Calprotectin	1	>16
<i>A. fumigatus</i> IFM40835	Transferrin	4	>128
	Lactoferrin	8	>128
	Calprotectin	1	>16

Various concentrations of chelating proteins were incubated with *C. albicans* (1 × 10³ cells/ml), *C. neoformans* (1 × 10³ cfu/ml), or *A. fumigatus* (1 × 10⁴ cfu/ml) at 35°C in 5% CO₂ in air in RPMI1640 medium in presence or absence of 10% FBS. After 48 hr of incubation, growth inhibition of *C. albicans* strain Ca-15 was measured by a spectrophotometer (OD₆₂₅) and minimum inhibitory concentrations (MICs) of mouse serum was determined (n=3).

DISCUSSION

It has been reported that administration of formalin-killed *C. albicans* into mouse induces antifungal activity, which has been identified to be transferrin [31]. We also confirmed that not only formalin-killed *C. albicans* but also formalin-killed *C. neoformans* or *A. fumigatus* can induce antifungal activity in mouse serum. Transferrin, which is present in the sera of human and mice, is well known to be a chelating protein for ferrous ion [11]. For example, mice with liver injury induced by D(+)-galactosamine were more susceptible to *Candida* infection due to decreased amount of transferrin [2, 3]. Moreover, mice administrated with excess amount of iron die in early stage of *Candida* infection [2–4]. Thus, transferrin has been thought to play an important role in protection against fungal and bacterial infections [21]. The protective mechanism of transferrin is due to a strong chelating activity for ferrous ion resulting in

the deficiency of ferrous ion required for the growth of microbes including fungi. In addition, anti-*Candida* activity, which was identified as transferrin, was reported to be induced in mouse serum and in ascites of tumor-bearing mouse by administration of formalin-killed *C. albicans* [30]. However, the anti-*Candida* activity found in ascites might not be transferrin because micromorphological change of *C. albicans* caused by the ascites was different from that of transferrin [28]. In this study, therefore, we further investigated if the anti-*Candida* activity induced by *C. albicans* was corresponding to transferrin or other substances. When various concentrations of ferrous ion were added to RPMI1640 medium containing mouse serum but no FBS along with the *C. albicans* strain Ca-15 followed by incubation of the culture, the inhibition of growth of the fungus was decreased in a dose-dependent manner (data not shown). The result suggests that anti-*Candida* activity was probably due to the deficiency of ferrous ion. To see if

chelating proteins such as transferrin and lactoferrin were induced in the serum of mouse administrated with *C. albicans*, the effect of iron-chelating proteins such as transferrin and lactoferrin on the growth of *C. albicans*, *C. neoformans* and *A. fumigatus* were examined. Not only transferrin and lactoferrin but also calprotectin inhibited the growth of these fungi in the absence of 10% FBS (Table 3). However, when 10% FBS was included in the medium, the growth inhibition by these chelating proteins disappeared, even 1.28 mg/ml of transferrin which corresponds to 1.5 times higher concentration of amount present in mouse serum when induced by *C. albicans* were not able to inhibit the growth (data not shown), suggesting that none of the chelating proteins used in this study inhibit the growth of *C. albicans*, *C. neoformans* and *A. fumigatus* in the presence of 10% FBS. Nevertheless, antifungal activity induced in the mouse serum could demonstrate antifungal activity even in the presence of 10% FBS, indicating that the antifungal activity induced was not corresponding to the transferrin or lactoferrin. It is also possible that complement or antibody against these fungi could be responsible for the antifungal activity. But these possibilities can be denied because ICR mice were fed in clean isolators and mouse serum was collected after 48 hr of administration, and incubation of mouse serum at 56°C for 60 min did not eliminate the antifungal activity (data not shown). These data suggest that the antifungal activity could be due to other serum protein, which may contribute in cutting off the source of iron.

There may be another mechanism, which can be a source of iron to fungus in the serum. It has been reported that hemin, one of the heme proteins, can be used as a source of iron [22, 29]. Thus, antifungal activity induced in the mouse serum could be due to the restriction of use of iron originated from heme protein. Not only live fungus such as *C. albicans*, *C. neoformans* and *A. fumigatus* but also formalin- and heat-killed fungi could also induce antifungal activity, suggesting that the substances associated with cell surface of the fungus could induce antifungal activity rather than secreted protein or something like that from fungi [9, 27].

Till now, transferrin is well known as a serum protein which is important for host defense but transferrin can only inhibit the use of free iron and cannot inhibit the all sources of iron for microbes including fungi. Edson *et al.* reported that hemopexin, which can bind heme with high affinity and normally present in the serum could inhibit the growth of *Bacteroides fragilis* [20]. The antifungal activity found in this study might be due to the induction of hemopexin. This possibility is currently under investigation in our laboratory.

In conclusion, not only heat-killed and formalin-killed *C. albicans* but also live and other fungi such as *C. neoformans* and *A. fumigatus* could induce anti-fungal activity in mouse serum, which seems to be different from transferrin but they could be other serum proteins.

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