Antifungal Antibiotic Benanomicin A Increases Susceptibility of *Candida albicans* to Phagocytosis by Murine Macrophages

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Benanomicin A is an antifungal antibiotic produced by *Actinomadura spadix*. In the present study, we investigated the effect of benanomicin A on the phagocytosis of *Candida albicans* by murine peritoneal macrophages and on the cell-surface hydrophobicity (CSH) of *C. albicans*. Although pretreatment of macrophages with benanomicin A had no effect on the phagocytosis, addition of benanomicin A to the culture of macrophages and *Candida* cells increased the susceptibility of *Candida* cells to the phagocytosis by the macrophages. Pretreatment of *Candida* cells with benanomicin A also increased the susceptibility of *Candida* cells to the phagocytosis. When *Candida* cells were mixed with benanomicin A, the antibiotic bound irreversibly to *Candida* cells. These data suggest the possibility that the increased susceptibility of *Candida* cells to the phagocytosis is mediated by the binding of benanomicin A to *Candida* cells. Examination of physicochemical property of *Candida* cell surface showed that the CSH of *Candida* cells significantly decreased by the treatment with benanomicin A. Thus, binding of benanomicin A to *Candida* cells may induce biochemical/physicochemical alternation of the surfaces, so that they become more susceptible to phagocytosis by murine macrophages. These properties of benanomicin A, along with its antifungal activity, seem to be beneficial in the treatment of fungal infections.

Benanomicin A is a benzo[*a*]naphthacenequinone antifungal antibiotic produced by *Actinomadura spadix* MH193-16F[1,2]. It has potent *in vitro* antifungal activity against a wide range of pathogenic fungi and has therapeutic efficacy in mice infected with *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*[3]. Studies on the interaction of benanomicin A with yeast cells as well as with various yeast mannans have demonstrated that benanomicin A selectively binds to cell wall mannans of *C. albicans* and other susceptible yeasts[4]. Therefore, it was believed that the cell wall mannan or other cell wall components and drug interaction may be essential for benanomicin A to exert its antifungal action[4]. The mannan is a major cell wall antigenic component of *C. albicans* and has diverse immunological properties[5]. There is evidence that virulence of *C. albicans* is related to the chemical structure of cell wall mannan and that some mannans or mannoproteins play a critical role in adherence of *C. albicans* to host tissues[6,7]. Therefore, it seems likely that binding of benanomicin A to cell wall mannan may modulate the biological characteristics of the surface of *C. albicans* and lead to altered interaction with host cells.

It is generally accepted that not only polymorphonuclear leukocytes but also macrophages play a key role in the host defense against systemic *C. albicans* infection through phagocytosis of invading fungi[8]. Besides the antifungal activity of benanomicin A, a possible modulatory activity of benanomicin A on phagocytosis of *Candida* cells by phagocytes is of particular interest, because such activity, if it exists, may boost the therapeutic effects of the antibiotic. In the present study, therefore, we examined how benanomicin A modifies the phagocytosis of *C. albicans* by macrophages.

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Materials and Methods

Chemicals

Benanomicin A sodium salt was purified from the culture of *Actinomadura spadix* as described previously[1] and dissolved in phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO). Endotoxin contamination was less than one ng per one g of the antibiotic as measured by the *Limulus* ES Test Wako (Wako Pure Chemical Ind., Ltd., Osaka).

Microorganisms

C. albicans TIMM1768 was grown for 24 hours at 37°C in yeast extract-polypeptone-dextrose broth and washed three times with PBS. For preparation of formalin-killed cells, yeasts were killed with 1% formalin at 4°C for 24 hours and then washed ten times with PBS. Both killed and viable yeasts were suspended in RPMI1640 medium containing 10% (V/V) heat-inactivated fetal bovine serum (FBS; Flow Laboratory Inc., VA), 50 units/ml penicillin, 5 μg/ml streptomycin sulfate, and 2 mM glutamine. The number of yeasts was determined with a hemocytometer.

Macrophages

Thioglycollate-elicited peritoneal macrophages were prepared from male C3H/He mice, 8～10 weeks of age, obtained from Clea Japan Inc., Tokyo, Japan as described previously[9]. The elicited cells were washed with PBS and resuspended in 10% FBS-RPMI1640 medium at a concentration of 5×10^5 cells/ml. One ml of the cell suspension was dispensed into 24-well flat-bottom tissue culture plates containing a 13 mm round Celldesk (Sumitomo Bakelite Co., Ltd., Tokyo) in each well, and the plates were incubated for two hours at 37°C in 5% CO_2. Non-adherent cells were removed by washing with prewarmed PBS and then the Celldesks were transferred to a new well containing 0.5 ml of 10% FBS-RPMI1640 medium with antibiotic.

Phagocytosis

Phagocytosis experiments were carried out by using macrophage monolayers formed on the Celldesks. Macrophages were infected with 5×10^6 cells of *C. albicans* for 30 minutes at 37°C. The infectivity ratio was 10 yeasts per macrophage. After infection, the macrophage monolayers were rinsed vigorously in cold PBS to remove nonphagocytosed *C. albicans* cells, fixed with methanol, and stained with Giemsa stain. Two hundred macrophages on each Celldesk were examined for phagocytosis by a light microscope. The percentage of macrophages ingesting one or more yeasts was calculated as follows: number of macrophages ingesting yeasts/number of macrophages counted $\times 100$. The mean number of *C. albicans* cells ingested per phagocytosing macrophage was represented as the phagocytic index.

In some experiments, latex beads (Sekisui Chemical Co., Ltd., Osaka) were also used for phagocytosis experiments. Macrophages were infected with 2.5×10^7 particles of the latex beads for 30 minutes at 37°C. The infectivity ratio was 50 particles per macrophage. After infection, the percentage of macrophages ingesting one or more particles was calculated as described above in the phagocytosis experiment using *C. albicans*.

Cell Surface Hydrophobicity

The cell surface hydrophobicity (CSH) of *C. albicans* cell was determined exactly as described previously[10]. In the present study, *n*-hexadecan was used as non-polar solvent.

Benanomicin A Extraction

The amount of bound benanomicin A to *Candida* cells was determined spectrophotometrically after extraction with dimethyl sulfoxide (DMSO). In brief, *Candida* cells (2×10^7 cells) in 1.0 ml of PBS containing 0.5 mM $CaCl_2$ were incubated at 37°C for appropriate incubation periods in the presence of 100 μg/ml of benanomicin A. The treated cells were washed 3 times with PBS. The bound benanomicin A was extracted with 1.0 ml of DMSO. The concentration of benanomicin A in the extracts was determined spectrophotometrically by measuring absorbance at 490 nm in comparison with a standard benanomicin A solution[1].

Statistics

Student's *t*-test was used to determine statistical probability.

Results and Discussion

Table 1 shows the results of experiments which were conducted to determine whether benanomicin A affects the phagocytosis of *C. albicans* by murine macrophages. When the antibiotic and formalin-killed *Candida* cells were simultaneously added to macrophage monolayers,

Table 1. Effect of benanomicin A on the phagocytosis of formalin-killed *Candida albicans* by murine macrophages.

Benanomicin A (μg/ml)	Phagocytosis (%)	Phagocytic Index
0	37±1.9	2.8±0.1
1	45±3.0	3.4±0.4
3	53±0.9**	3.5±0.1**
10	57±1.3**	3.9±0.4*
30	79±3.3**	4.2±0.3**

Macrophage monolayers were incubated with formalin-killed *C. albicans* at a macrophage: *Candida* ratio of 1:10 at 37°C for 30 minutes in the presence of benanomicin A. Phagocytosis and phagocytic index were determined as described in Materials and Methods. The data represent the mean±standard deviation for triplicate cultures. *: $P<0.05$, **: $P<0.01$ compared with benanomicin A untreated group.

3 μg/ml or higher concentrations of benanomicin A significantly enhanced the phagocytosis of *Candida* cells in a dose dependent manner as determined by an increase in the phagocytic index as well as percentage of phagocytosis. As shown in Table 2, a similar dose-dependent enhancing effect of benanomicin A on phagocytosis was also demonstrated when *Candida* cells were pretreated with benanomicin A at a concentration of 3 μg/ml or above and then washed to remove the free antibiotic before adding to macrophage monolayers. In contrast, benanomicin A had no effect on the phagocytic function of macrophages when macrophage monolayers were preincubated with the antibiotic at concentrations up to 30 μg/ml for one hour at 37°C, washed to remove free antibiotic and then phagocytosis of yeasts by macrophages was determined (data not shown). These data indicate that benanomicin A may primarily act on yeasts, not macrophages, to result in more phagocytosis. Such a possible primary action of benanomicin A on the target cells regarding phagocytosis was supported by the following study using latex beads. That is, treatment of macrophages and latex beads with benanomicin A (up to 30 μg/ml) for one hour did not result in any enhancement of the latex bead phagocytosis compared with benanomicin A untreated cultures (data not shown). Thus, the augmenting effect of benanomicin A on phagocytosis may be specific to target cells like *Candida*, not latex beads, and may not be due to an effect on macrophages, which is supported by other studies on cytokine and reactive oxygen production by macrophages. That is, the treatment of macrophages with benanomicin A, up to 50 μg/ml, did not alter the production of interleukin 1 and hydrogen peroxide (unpublished data). It seems likely that benanomicin A has only a minimum effect, if any, on macrophage functions, including phagocytic activity. It seems more probable that benanomicin A may alter the surface nature of target yeasts directly so they can be more phagocytosed by macrophages.

In order to determine in detail the possible mechanism of how benanomicin A-*Candida* cell interact regarding phagocytosis, the binding of benanomicin A to *Candida* cells was examined. For this purpose, formalin-killed yeasts were used, since viable yeasts may metabolize benanomicin A during incubation. Figure 1A shows time-dependent binding kinetics of benanomicin A to formalin-killed *Candida* cells at 37°C. As seen in the

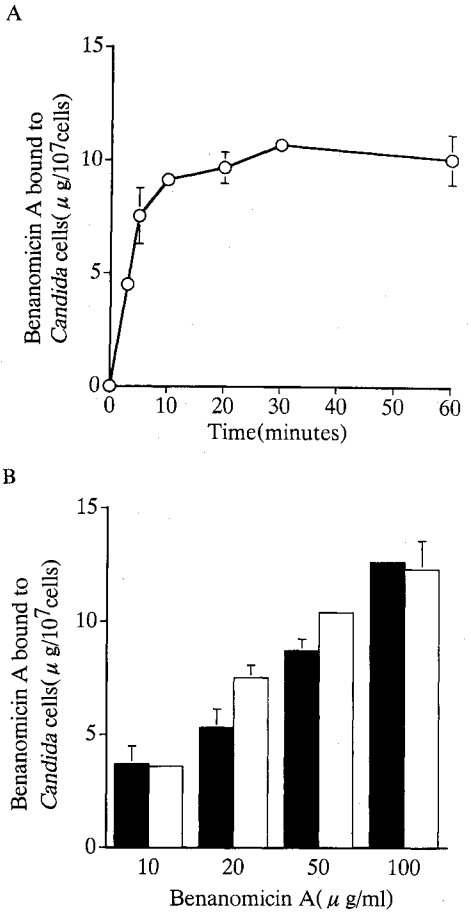
Table 2. Effect of pretreatment of formalin-killed *C. albicans* with benanomicin A on phagocytosis by murine macrophages.

Benanomicin A (μg/ml)	Phagocytosis (%)	Phagocytic Index
0	49±2.9	3.1±0.2
3	57±0.6*	3.4±0.2
10	63±1.8**	3.7±0.2*
30	60±2.3**	5.4±0.4**

Candida cells (10^7 cells/ml) were treated with benanomicin A at 37°C for 60 minutes, and then the cells were washed three times with PBS and suspended in RPMI1640 medium. They were added to the wells containing macrophage monolayers at a macrophage : *Candida* ratio of 1 : 10, and incubated at 37°C for 30 minutes. Phagocytosis and the phagocytic index were determined as described in Materials and Methods. The data represent the mean±standard deviation for triplicate cultures. *: $P<0.05$, **: $P<0.01$ compared with benanomicin A untreated group.

Fig. 1. Kinetics (A) and dose-response (B) effects of benanomicin A on *C. albicans*.

■; Live *Candida* cells; □; killed *Candida* cells.



A: Formalin-killed *Candida* cells (2×10^7 cells) in 1.0 ml of PBS containing 0.5 mM $CaCl_2$ were incubated at 37°C in the presence of benanomicin A (100 μg/ml). At the indicated time, the treated cells were harvested, washed 3 times with PBS, and then extracted with 1.0 ml of DMSO.

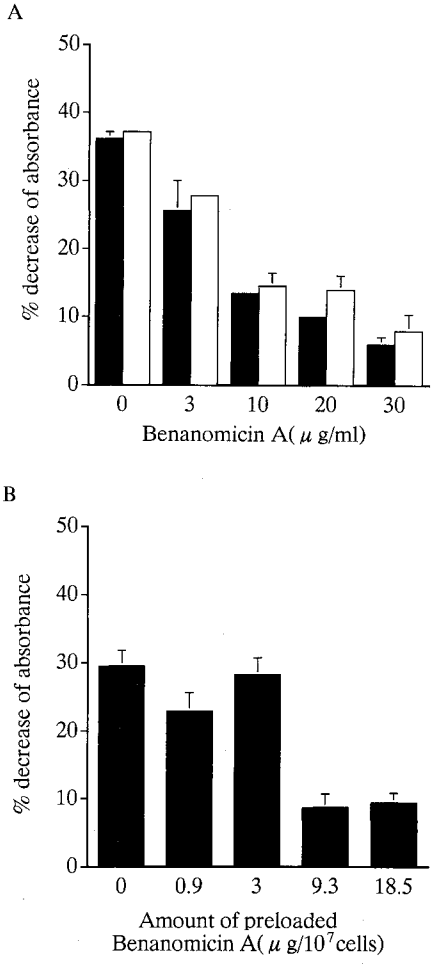
B: Formalin-killed or live *Candida* cells (2×10^7 cells/ml) were incubated with various concentrations of benanomicin A at 37°C for 30 minutes in the same conditions as described above and the cell-bound benanomicin A was extracted. The data represent the mean±standard deviation for triplicate cultures.

figure, the binding of benanomicin A to *Candida* cells was initiated rapidly and reached a maximum level within 10 minutes. The kinetic study of benanomicin A binding to formalin-killed *Candida* cells at 4°C was also tested. There was no significant difference in the amount of bound benanomicin A between the incubation temperature at 37°C and 4°C (data not shown). Figure 1B shows the difference of benanomicin A binding to yeasts using live *vs.* formalin-killed *Candida*. Either live or killed yeasts were treated with various concentrations of benanomicin A for 30 minutes at 37°C and then the benanomicin A extracted from the yeasts after vigorous washing with PBS. The treatment of *Candida* cells with 10 μg/ml of benanomicin A, which corresponds to the effective concentration for augmentation of phagocytosis by pretreatment as shown in Table 2, caused a marked binding of benanomicin A to both live and killed *Candida* cells. There was no difference in benanomicin A binding between live *vs.* killed cells in a similar dose-dependent manner. These results suggest that the binding of benanomicin A to *Candida* cells may not involve any energy-dependent biochemical process.

The physicochemical properties of *Candida* cell surface are important in macrophage-*Candida* interaction regarding phagocytosis, since CSH of *Candida* cells affects adherence of *Candida* cells to host tissues[14]. This prompted us to study the effect of benanomicin A on the CSH of *Candida* cells. Figure 2A shows that CSH of formalin-killed and live *Candida* cells significantly decreased in the presence of benanomicin A. The CSH of benanomicin A-pretreated and washed *Candida* cells was also examined. Figure 2B shows that the CSH of the benanomicin A treated *Candida* cells, loaded with more than 9.3 μg benanomicin A/10^7 cells in benanomicin A free medium, was markedly lowered in comparison with that of benanomicin A untreated cells. These result suggest that benanomicin A molecules are irreversibly bound to the cell wall of *Candida* cells and as a result their CSH is lowered. This physicochemical effect of benanomicin A may be correlated with the increased susceptibility of *Candida* cells pretreated with benanomicin A to phagocytosis by macrophages.

None of the other antifungal antibiotics or compounds studied to date have been reported to have activity that makes *Candida* cells more vulnerable to phagocytosis by macrophages as observed with benanomicin A. In fact, in the previous study, no such effect was found with amphotericin B or fluconazole which were used as reference drugs (unpublished data). The precise mechanism of this unique action of benanomicin A toward *C. albicans* still remains to be elucidated. Since it has been reported that phagocytosis of none-opsonized *C. albicans* cells by macrophages is mediated by the mannose and/or glucan receptors[11~13], the alteration of the physicochemical property of cell wall mannan or mannoprotein of *Candida* cells caused by binding of benanomicin A to enhance the recognition of *Candida* cells by macrophages through the mannose or glucan-receptors is likely. The ability of *Candida* cells to adhere to host tissues is considered to be important in colonization or pathogenesis of this pathogenic yeast[15,16]. In this regard, the hydrophobic *Candida* cells have been

Fig. 2. CSH of *C. albicans* cells treated with benanomicin A.
■; Live *Candida* cells, □; killed *Candida* cells.



A: Formalin-killed or live *Candida* cells (10^7 cells/ml) were incubated for 10 minutes at 37°C with various dose of benanomicin A. Then the CSH of *Candida* cells was measured in the presence of benanomicin A.

B: Live *Candida* (10^8 cells/ml) were incubated at 37°C for 30 minutes in the presence of benanomicin A (0, 10, 30, 100, or 300 μg/ml). The cells were washed to eliminate unbound benanomicin A, suspended in PBS at 10^7 cells/ml, and the CSH of *Candida* cells was measured. The amount of bound benanomicin A of washed yeasts was measured spectrophotometrically. The data represent the mean ± standard deviation for triplicate cultures. See the legend to Fig. 1.

proven to be more virulent than hydrophilic cells in mice[17]. Thus, the possible relationship between lowered CSH and increased vulnerability to phagocytosis as observed in benanomicin A-treated *Candida* cells is likely.

In conclusion, whatever the actual mechanism of benanomicin A action by which it can modify the cell surface of *Candida* cells so that they become vulnerable to phagocytosis by murine macrophages, this unique activity of benanomicin A, along with the antifungal activity, seems to be beneficial for the treatment of fungal infections.

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