Original Article

Effects of IgY against Candida albicans and Candida spp.: Adherence and Biofilm Formation

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SUMMARY: The fungal pathogen Candida albicans is an opportunistic fungal pathogen that causes oral and vaginal mucosal infections as well as systemic disease. The ability of C. albicans to adhere to host surfaces is positively correlated with its pathogenicity. We prepared a polyclonal anti-Candida albicans antibody in chicken egg yolk (anti-C. albicans IgY) and investigated its in vitro effectiveness in preventing C. albicans adherence and biofilm formation. Anti-C. albicans IgY significantly reduced the adherence of C. albicans SC5314 to human oral epithelial cells in a dose-dependent manner. The same effect was also observed in other Candida spp. including C. albicans serotype A and B. Further, the IgY inhibited biofilm formation of C. albicans in medium without serum, but the inhibition was slightly restored in medium conditioned with 10% serum. The data indicate that anti-C. albicans IgY cross-reacted with various Candida spp. and may have a protective effect against oral candidiasis and reduce the dissemination of Candida spp. This effect may be due to the blocking of the binding of Candida spp. to the host cells. However, the blocking did not play a role when Candida formed a germ tube in the presence of serum. Therefore, anti-C. albicans IgY may be considered as a prophylactic immunotherapy or possibly an adjunctive antifungal therapy under limited conditions.

INTRODUCTION

Most bacteria and fungi that exist in humans as surface-attached communities are called biofilms, and such communities usually affect human health. The tissues are the substrates on which biofilms form, and such communities are called biofilms, and such communities are known as reservoirs to continuously seed an infection. The fungal pathogen Candida albicans is an opportunistic fungal pathogen that causes oral and vaginal mucosal infections as well as systemic disease (1). The ability of C. albicans to adhere to host surfaces is positively correlated with its pathogenicity (2). It produces adherent biofilms on a variety of different surfaces in vitro (3-6). Biofilm formation begins with surface adherence of the yeast form, which grows to yield a basal layer. The basal layer cells include some hyphae, or long tubular chains of cells, which extend to yield an upper layer that is almost exclusively hyphae. As the biofilm matures, it produces an extracellular matrix containing predominantly carbohydrate and protein (7-9).

Adherence is a critical property for biofilm microbial cells, with multiple adhesion molecules functioning in successful biofilm formation. Specific adherence to the protein surface is provided by several surface adhesins of Candida. Recent reports have demonstrated that antibodies with defined specificities to these surface adhesins show different degrees of protection against systemic and mucosal candidiasis (10-12). Secretory immunoglobulin A (sIgA) is thought to play a central role by inhibiting Candida adherence to host cells (13-15). Complex mixtures of antibodies having different specificities such as those found in salivary sIgA are shown to decrease adhesion of C. albicans to the host surface but do not inhibit germination (16). Therefore, the use of antibodies as an adjunct to antifungal drugs may be considered one approach to protecting against candidiasis.

Chicken eggs are known as an inexpensive and convenient source for mass production of specific antibodies (17). Specific egg yolk immunoglobulin (IgY) can be produced in egg yolk by immunizing hens with specific antigens. IgY is isolated in large quantities from the yolk by simple methods without distress to the birds (18), and has been used extensively for the treatment and prevention of various infections in animals and humans with mixed success (19-26). In particular, polyclonal anti-C. albicans antibody in chicken egg yolk prevented C. albicans from adhering to oral epithelial cells where the effect depended on the density of the infection (27). However, the IgY was induced by immunization with the C. albicans yeast form and included antibodies against various antigens. In general, the activity and diversity of IgY against Candida spp. are not well understood. The objective of this study was to evaluate the efficacy of a specific IgY against C. albicans and other Candida strains to develop an alternative therapy for candidiasis.

MATERIALS AND METHODS

Yeast strains: C. albicans SC5314 (serotype A), NIH207 (serotype A) and NIH792 (serotype B), Candida tropicalisIFO00618, Candida dubliniensis CD36 and CD57, Candida parapsilosis ATCC22019 and FRCP-0201, Candida glabrata...
850821 and CBS138 were used. All strains were provided by Dr. Masakazu Niimi from the National Institute of Infectious Diseases. For use in experiments, all organisms were grown in liquid Yeast Peptone Dextrose (YPD: 2% Bacto peptone, 2% dextrose and 1% Yeast extract) broth aerobically at 37°C; and washed three times in phosphate-buffered saline (PBS). Then they were suspended to the appropriate concentration in PBS.

**Preparation of IgY**: Anti-*C. albicans* IgY was acquired by immunization of chickens with the yeast form, which was provided by GHEN Corporation (Tokyo, Japan) as a purified powder. A solution containing 4 mg/ml was prepared in PBS. Control IgY was prepared from the eggs of non-immunized hens. Fat-free egg yolk powder was purified for IgY using the ammonium sulfate precipitation method. The protein concentration was determined using the BioRad protein assay method (BioRad, Hercules, Calif., USA) based on the Bradford method. One milligram per milliliter of bovine serum albumin (IWAI, Tokyo, Japan) was used as the reference protein. The absorbance at 620 nm after a 30-min reaction with Bradford’s solution was measured using a spectro-photometer.

**Epithelial cells**: The human oral squamous carcinoma cell lines, Ca9-22 and HSC-2, were purchased from the Japanese Collection of Research Bioresources in Health Science Research Resources Bank (Tokyo, Japan). They were maintained in Minimal Essential Medium Eagle’s (SIGMA ALDRICH Corp., St. Louis, Mo., USA) containing 10% fetal bovine serum supplemented with 6 mg/ml L-glutamine, penicillin and streptomycin. They were grown on 24-well plates at 37°C in a humidified environment containing 5% CO2 and used at 85% confluence. Ca9-22 and HSC-2 were maintained in Minimal Essential Medium Eagle’s (SIGMA ALDRICH Corp., St. Louis, Mo., USA) containing 10% fetal bovine serum supplemented with 6 mg/ml L-glutamine, penicillin and streptomycin. They were grown on 24-well plates at 37°C in a humidified environment containing 5% CO2 and used at 85% confluence.

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**Antibody titration**: Enzyme-linked immunosorbent assay (ELISA) was used to determine the titer of the specific antibody. Each well of a 96-well polystyrene plate was coated overnight at 4°C with 100 μl of whole yeast in PBS (OD660 = 1.0). The wells were washed with PBS-T (0.05% Tween 20 in PBS, PBS-T) and blocked with 150 μl 1.0% (v/v) skim milk in PBS-T for 1 h at 37°C. After three washes with PBS-T, various protein concentrations (0.032, 0.063, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) of IgY were added to the wells; and the plates were incubated for 1 h at 37°C. The plates were washed three times, and alkaline phosphatase-conjugated goat IgG polyclonal anti-chicken IgY (ABCAM PLC, Cambridge, UK) in PBS-T (1:5,000 dilution) was added. After five washes with PBS-T, bound antibodies were detected after adding 100 μl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubating for 60 min at 37°C. The optical densities were determined using a microplate reader (Multiskan Bichromatic, JET, Sun Jose, Calif., USA) for 60 min at 37°C. The cells were washed three times using sterile PBS and observed using a confocal laser scanning microscope (Olympas, Tokyo, Japan).

**Effects of anti-*C. albicans* IgY on adherence of *Candida* strains**: Absorbance at 660 nm was measured to adjust the yeast concentration to OD660 = 1.0. The yeast was mixed with 0.006, 0.0125, 0.25, 0.5, 1 or 2 mg/ml IgY and 2 mg/ml control IgY for 60 min at 37°C and added to the epithelial cells on a 24-well plate. After 60-min incubation, yeasts adhering to the epithelial cells were separated from free yeasts by washing three times with PBS. Then, 1 ml 0.05% trypsin-EDTA was added to each well, and the plates were incubated for 10 min at room temperature. The detached cell suspensions were collected in 0.5% trypsin-EDTA using the pipetting technique, and spread on the YPD agar plate using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain). After incubation for 24 h at 37°C under aerobic conditions, the number of colonies on the plates was counted and compared to those on the plates that did not have IgY.

**Effects of anti-*C. albicans* IgY on biofilm formation of *C. albicans***: Biofilm formation by *C. albicans* SC5314 was assayed using a method described previously (28,29), with some modification. *C. albicans* incubated for 24 h at 37°C in YPD broth was adjusted to OD = 0.5 at 660 nm, harvested by centrifugation and washed in PBS two times. The *C. albicans* suspension was diluted with RPM1640, and 2 mg/ml anti-*C. albicans* IgY was added to the 96-well microtiter plate wells. The chemically defined RPM1640 medium containing minimal (0.2%) glucose with or without 10% FBS was used as the nutrient-poor condition for the biofilm formation assay. After incubation for 24 h at 37°C, biofilms formed in wells were washed with sterile PBS two times. Biofilm formation was tested using the XTT assay at 492 nm. XTT reduction has been widely used to measure biofilm activity and allows the detection of small differences in metabolic activity between strains (30-32).

**Statistical analysis**: All data were analyzed using the Statistical Package for SPSS for Windows (version 100; SPSS, Chicago, Ill., USA). The Student’s t test with the Bonferroni Method was used to compare data of treatment with control IgY and anti-*C. albicans* IgY. P-values less than 0.05 were considered to be significant.

**RESULTS**

Antibody titers of IgY to *Candida* spp. were measured using ELISA (Fig. 1). Anti-*C. albicans* IgY reacted to *C. albicans* SC5314, *C. tropicalis* IFO0618, *C. dubliniensis* CD36 and CD57 in a dose-dependent manner where the IgY titer was significantly elevated at concentrations of 0.032, 0.073 or 0.125 mg/ml increasing to 4 mg/ml of antibody (IgY). By contrast, the control IgY to these *Candida* spp. were poor in all tested concentrations, whereas 4 mg/ml of the control antibody reacted only slightly to *Candida* spp. The titers of both antibodies were similar to those of other *Candida* strains (data not shown). Two milligrams per milliliters of IgY showed a stronger response to *Candida* strains than the control IgY and was used in further experiments. Before the *Candida* adherence tests, the effect of IgY was tested to determine whether the antibodies inhibited cell growth of *C. albicans*. Anti-*C. albicans* IgY did not inhibit the cell growth of *C. albicans* in comparison to the cell growth in YPD.
medium containing control IgY or PBS (Fig. 2). *C. albicans* did not grow in PBS containing anti-*C. albicans* IgY, control IgY and PBS. To confirm the reactivity of the anti-*C. albicans* IgY induced by immunization with the yeast form, a reaction assay using a second fluorescence-conjugated antibody was performed and observed by microscopy. The fluorescence activity did not appear in the assay using the control IgY (Fig. 3A). By contrast, significant fluorescence on the yeast form was confirmed using the anti-*C. albicans* IgY (Fig. 3B). Therefore, the reactivity of anti-*C. albicans* IgY to the *C. albicans* yeast form was confirmed. Further, the effect of anti-*C. albicans* IgY on the adherence of *Candida* spp. to monolayers of CA9-22 epithelial cells was observed (Fig. 4). Anti-*C. albicans* IgY inhibited the adherence of *C. albicans* in a dose-dependent manner (from 0 to 2 mg/ml) whereas 2 mg/ml of control IgY and PBS did not inhibit the adherence. Further, anti-*C. albicans* IgY significantly inhibited the adherence of various *Candida* strains including different serotype strains (A and B) of *C. albicans* in comparison with control IgY (Fig. 5A). Inhibition of adherence was also observed in the other epithelial cell line, HSC-2 (Fig. 5B). To measure inhibition effects of anti-*C. albicans* IgY on biofilm formation of *C. albicans*, various concentrations of anti-*C. albicans* IgY were applied into the biofilm formation. Two milligrams per milliliters of anti-*C. albicans* IgY strongly inhibited the biofilm formation of *C. albicans* SC5314 in comparison with 2 mg/ml of control IgY (Fig. 6). Other concentrations of anti-*C. albicans* IgY did not affect the biofilm formation. Therefore, 2 mg/ml of anti-*C. albicans* IgY is a sufficient amount to inhibit biofilm formation. It is known that serum induces germ tube formation (filamentous form) in *Candida* (33). To detect the reactivity of the anti-*C. albicans* IgY to the germ tube, *C. albicans* was cultivated in medium supplemented with 10% FBS and mixed with the antibody. Yeast and filamentous forms were observed in Fig. 3C. The fluorescence activity of the filamentous form was lower than that of the yeast form of *C. albicans* in the assay using the anti-*C. albicans* IgY (Fig. 3C). To determine whether the IgY anti-*C. albicans* antibody affects the biofilm formation including the germ tube formation of *C. albicans*, we performed further experiments. The biofilm formation assay was performed in a medium conditioned with 10% FBS. Slight inhibitory activity by anti-*C. albicans* IgY was observed for the concentrations 0.25 and

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**Fig. 1.** ELISA antibody titer of anti-*C. albicans* IgY. Various protein concentration (0.0, 0.063, 0.0125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) of anti-*C. albicans* IgY or control IgY were applied to 96-well microtiter plates coated with *C. albicans* SC5314, *C. tropicalis* IFO0618, *C. dubliniensis* CD36 and CD57. The titers were determined using a microplate reader at 405 nm. Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays; and compared to control IgY (*P < 0.01).
Fig. 3. Photograph of C. albicans with FITC-conjugated antibody. C. albicans SC5314 was treated with control IgY (A) or anti-C. albicans IgY (B). C. albicans formed germ tube was treated with anti-C. albicans IgY (C). After washing with PBS, the cells were mixed with FITC-conjugated rabbit anti-chicken IgY antibodies. Fluorescence photograph of C. albicans treated with antibodies were representative in three independent experiments.

Fig. 4. Effects of anti-C. albicans IgY on C. albicans adherence. C. albicans SC5314 was mixed with 0.0, 0.063, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml anti-C. albicans IgY, 2.0 mg/ml control IgY or PBS; and applied onto the epithelial cells (Ca9-22). The cell suspension detached using 0.05% trypsin-EDTA were spread on YPD agar plates. After incubation for 24 h, the numbers of colonies on the plates were counted. Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays.

0.5 mg/ml (P < 0.05). The 2 mg/ml concentration of anti-C. albicans IgY significantly inhibited biofilm formation in the medium with FBS (P < 0.01), but the inhibiting activity was weak in comparison with that by 2 mg/ml of anti-C. albicans IgY in the medium without FBS (Fig. 6). PBS did not affect biofilm formation in medium with or without FBS.

DISCUSSION

A number of secretory antibody-mediated mechanisms are operative in the mammary gland including (i) anti-adhesive activity, (ii) opsonization followed by phagocytosis, (iii) toxin neutralization and (iv) antibody-mediated lysis of pathogens (34). This study provided evidence for the anti-adhesive activity of anti-C. albicans IgY (IgA-like) against C. albicans. We found that anti-C. albicans IgY inhibits adherence of C. albicans and also other Candida spp. to monolayers of oral epithelial cells and confirmed that the IgY antibodies cross-reacted with various Candida spp. The IgY induced by immunization with C. albicans may react with various antigens including adhesins from Candida spp. that adhere to epithelial cells. For example, Hwp1 and Als3 are known for their role in host attachment and are the most well characterized C. albicans cell surface proteins (35,36). This is possibly the reason for the inhibition mechanism by anti-C. albicans...
IgY, where the IgY antibody may cross-react with adhesins Hwp1 and Als3 in the yeast form. The effects may also be associated with the inhibition of biofilm formation in the medium without conditioning serum.

However, the biofilm of *C. albicans* in the medium conditioned with 10% FBS was more resistant to anti-*C. albicans* IgY. A non-dialyzable component of serum induces germ-tube formation using the YPD medium supplemented with 10% serum (28,37). The inhibition of adhesion is usually achieved by blocking the adhesins present on the fungal cell wall (16), but for the inhibition of germination there may be another important mechanism because filamentation plays a key role in the adhesion process in biofilm formation (38).

Anti-*C. albicans* IgY induced by immunization with the yeast form is not likely to play an extensive role in the germination of *C. albicans* since it may not include all antibodies to antigens of the filamentous form of *C. albicans* (Fig. 3C). Therefore, the germination might disturb the inhibition by anti-*C. albicans* IgY to biofilm formation in the presence of serum. In contrast to the discrete activity of germination and adhesion to the epithelial cells, anti-*C. albicans* IgY did not exhibit a potent fungicidal effect on *Candida* spp., as it did on *C. albicans*.

Passive immunization therapies against pathogens have been extensively studied (39-41). In the oral cavity, successful passive immunization with IgY against dental caries (e.g., *Streptococcus mutans*) has been reported in a rat model (42,43) and in human subjects (44). Oral passive immunization of anti-*C. albicans* IgY was shown to be effective (27) and significantly reduced the number of *C. albicans* colonies and the scores for tongue lesions. They indicated that this effect may be due to the blocking of the binding of *C. albicans* to the host cells. Here, we demonstrate that anti-*C. albicans* IgY has anti-adherence activity against various *Candida* spp. strains, both when grown in suspension and as a biofilm in the medium without serum. However, these concentrations of IgY did not achieve *Candida* growth inhibition. Chicken egg yolk immunoglobulin is recognized as an antibody source and showed therapeutic values against several microorganisms (19-26). It is possible the anti-*C. albicans* IgY may be used as a preventive immunotherapy against oral and disseminated candidiasis and *Candida* spp. infections. However, the IgY did not completely affect the biofilm formation when *C. albicans* formed germ tubes in the growth medium conditioned with serum. Therefore, treatment with anti-*C. albicans* IgY may be considered a prophylactic immunotherapy or possibly an adjunctive anti-fungal therapy under limited conditions.

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