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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 for the formation of biofilms, and the microorganisms in the biofilms serve 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 tropicalis* IFO0618, *Candida dubliniensis* CD36 and CD57, *Candida parapsilosis* ATCC22019 and FRCP-0201, *Candida glabrata*

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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 spectrophotometer.

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% CO_2 and used at 95% confluence in all experiments.

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 (OD_{660} = 1.0). The wells were washed with PBS-T (0.05% Tween 20 in PBS, PBS-T) and blocked with 150 μl 1.0% (w/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 Laboratory Japan, Tokyo, Japan) at 405 nm. The background (control) was defined in wells coated without IgY. All samples were tested in triplicate.

Effects of anti-*C. albicans* IgY on cell growth of *Candida* strains: Cell suspensions of *C. albicans* SC5314 and 0 or 2 mg/ml of anti-*C. albicans* IgY or 2 mg/ml control IgY were mixed and incubated in YPD or PBS for 24 h at 37°C in aerobic conditions. The absorbance at 660 nm was measured at 0, 1, 3, 6 and 24 h after incubation. To confirm visually the specificity of the anti-*C. albicans* IgY, 2 mg/ml of anti-*C. albicans* and 2 mg/ml of control IgY were applied to cell suspensions of *C. albicans* SC5314 cultivated in YPD or RPMI1640 with 10% fetal bovine serum (FBS); and incubated aerobically for 60 min at 37°C. The cells treated with anti-*C. albicans* IgY and control IgY were washed three times using sterile PBS and mixed with 1/1,000 diluted FITC-conjugated rabbit anti-chicken IgY antibodies (ANASPEC, 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 OD_{660} = 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 RPMI1640, and 2 mg/ml anti-*C. albicans* IgY was added to the 96-well microtiter plate wells. The chemically defined RPMI1640 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 titers were 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

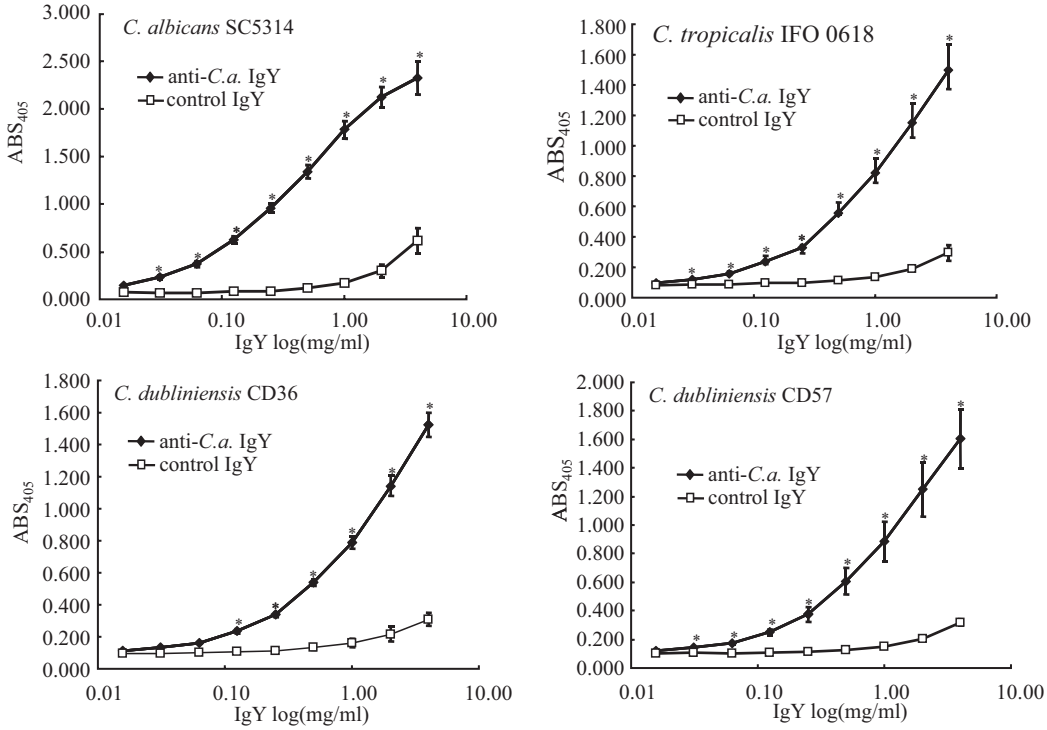

Fig. 1. ELISA antiboby titer of anti-*C. albicans* IgY. Various protein concentration (0.0, 0.063, 0.0125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) of anti-*C. albicans* IgY or control IgY were applied to 96-well microtiter plates coated with *C. albicans* SC5314, *C. tropicalis* IFO0618, *C. dubliniensis* 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).

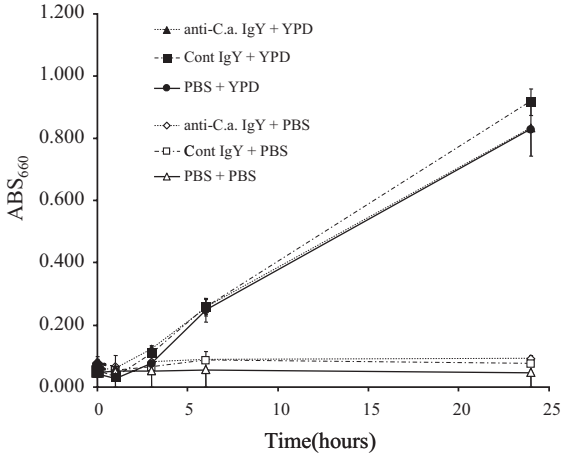

Fig. 2. Effects of anti-*C. albicans* IgY on *C. albicans* growth. Cell suspensions of *C. albicans* SC5314 were mixed with 0 or 2 mg/ml anti-*C. albicans* IgY or 2 mg/ml control IgY; and incubated in YPD or PBS for 24 h. The absorbance at 660 nm was measured at 0, 1, 3, 6 and 24 h after incubation. Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays.

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

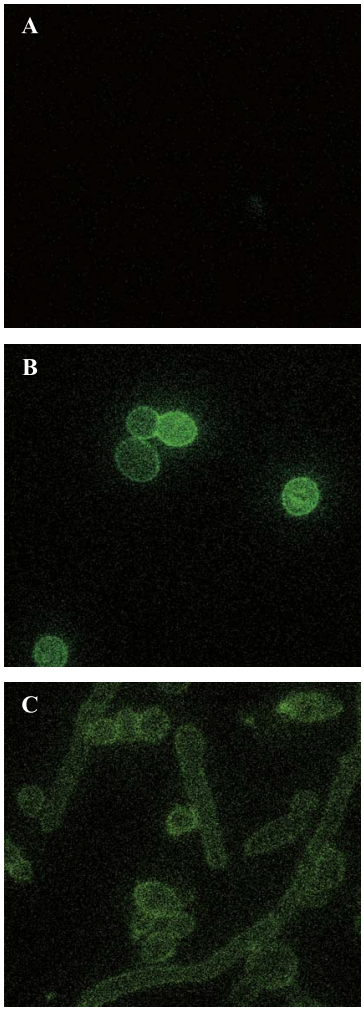

Fig. 3. Photograph of *C. albicans* with FITC-conjugated antibody. *C. albicans* SC5314 was treated with control IgY (A) or anti-*C. albicans* IgY (B). *C. albicans* formed germ tube was treated with anti-*C. albicans* IgY (C). After washing with PBS, the cells were mixed with FITC-conjugated rabbit anti-chicken IgY antibodies. Fluorescence photograph of *C. albicans* treated with antibodies were representative in three independent experiments.

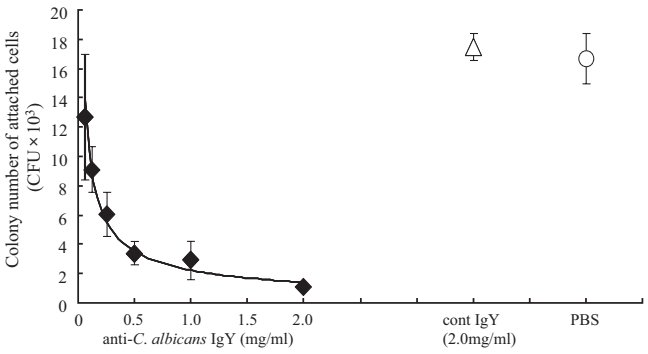

Fig. 4. Effects of anti-*C. albicans* IgY on *C. albicans* adherence. *C. albicans* SC5314 was mixed with 0.0, 0.063, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml anti-*C. albicans* IgY, 2.0 mg/ml control IgY or PBS; and applied onto the epithelial cells (Ca9-22). The cell suspension detached using 0.05% trypsin-EDTA 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

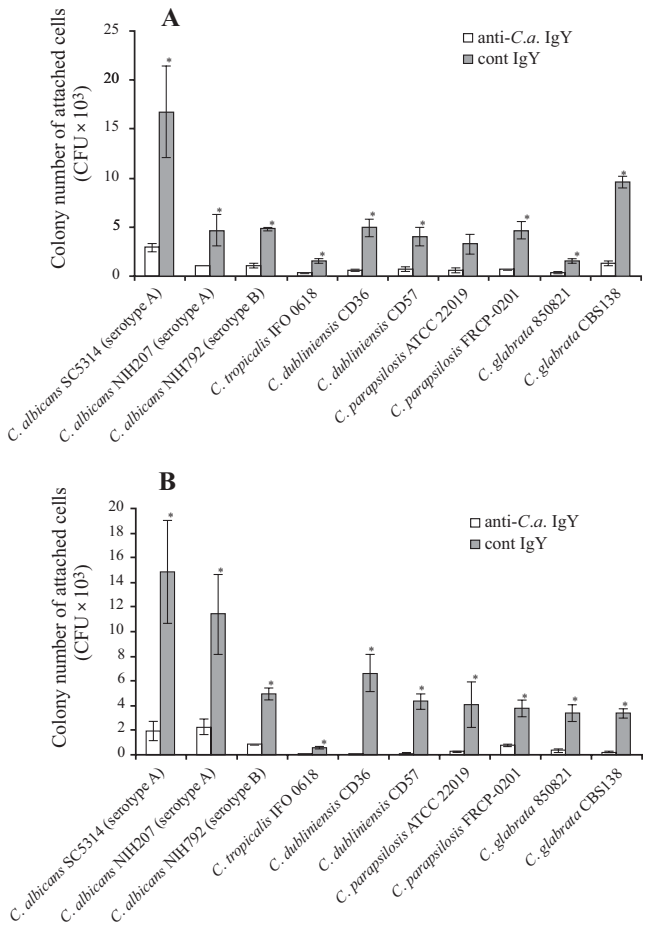

Fig. 5. Effects of anti-*C. albicans* IgY on *Candida* spp. adherence. *Candida* spp. were mixed with 2.0 mg/ml anti-*C. albicans* IgY or 2.0 mg/ml control IgY for 60 min. The treated cells were added onto monolayers of the epithelial cells {(A) Ca9-22 and (B) HSC-2}. The cell suspensions were detached using 0.05% trypsin-EDTA; and 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; and compared to control IgY (*$P < 0.01$).

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*

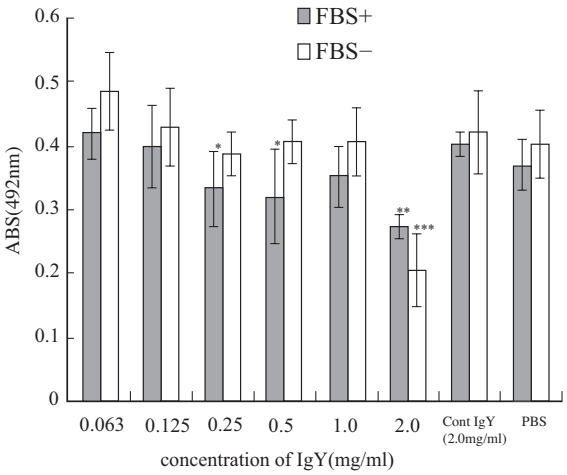

Fig. 6. Biofilm formation of *C. albicans* treated with anti-*C. albicans* IgY. A *C. albicans* SC5314 suspension was added to 0.0, 0.063, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml anti-*C. albicans* IgY, 2.0 mg/ml control IgY or PBS to the wells of 96-well microtiter plates. After incubation for 24 h in PBS or YPD with and without 10% FBS, biofilm formation was observed using microphotography. Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays; and compared to control IgY (*$P < 0.05$, **$P < 0.01$ in condition with FBS, ***$P < 0.01$ in condition without FBS).

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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REFERENCES

1. Odds, F.C. (1988): *Candida* and Candidosis. 2nd ed. Bailliere Tindall, UK.
2. Calderone, R.A. and Braun, P.C. (1991): Adherence and receptor relationships of *Candida albicans*. Microbiol. Rev., 55, 1-20.
3. Kumamoto, C.A. (2002): *Candida* biofilms. Curr. Opin. Microbiol., 5, 608-611.
4. Ramage, G., Saville, S.P., Thomas, D.P., et al. (2005): *Candida* biofilms: an update. Eukaryot. Cell, 4, 633-638.
5. Chandra, J., Patel, J.D., Li, J., et al. (2005): Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. Appl. Environ. Microbiol., 71, 8795-8801.
6. Kuhn, D.M., Chandra, J., Mukherjee, P.K., et al. (2002): Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect. Immun., 70, 878-888.
7. Douglas, L.J. (2003): *Candida* biofilms and their role in infection. Trends Microbiol., 11, 30-36.
8. Baillie, G.S. and Douglas, L.J. (2000): Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J. Antimicrob. Chemother., 46, 397-403.
9. Chandra, J., Kuhn, D.M., Mukherjee, P.K., et al. (2001): Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J. Bacteriol., 183, 5385-5394.
10. De Bernardis, F., Boccanera, M., Adriani, D., et al. (1997): Protective role of antimannan and anti-aspartyl proteinase antibodies in an experimental model of *Candida albicans* vaginitis in rats. Infect. Immun., 65, 3399-3405.
11. Han, Y., Morrison, R.P. and Cutler, J.E. (1998): A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. Infect. Immun., 66, 5771-5776.
12. Matthews, R. and Burnie, J. (2001): Antifungal antibodies: a new approach to the treatment of systemic candidiasis. Curr. Opin. Investig. Drugs, 2, 472-476.
13. Eostein, J.B., Kimura, L.H., Menard, T.W., et al. (1982): Effects of specific antibodies on the interaction between the fungus *Candida albicans* and human oral mucosa. Arch. Oral Biol., 27, 469-474.
14. Fidal, P.L., Jr. (1999): Host defense oropharyngeal and vaginal candidiasis: site-specific differences. Rev. Iberoam Microl., 16, 8-15.
15. Vudhichamnong, K., Walker, D.M. and Ryley, H.C. (1982): The effect of secretory immunoglobulin A on the *in vitro* adherent of the yeast *Candida albicans* to human oral epithelial cells. Arch. Oral Biol., 27, 617-629.
16. San Millan, R., Elguezabal, N., Regulez, P., et al. (2000): Effect of salivary secretory IgA on the adhesion of *Candida albicans* to polystyrene. Microbiology, 146, 2105-2112.
17. Kuroki, M. (1999): Oral passive immunization using chicken egg yolk immunoglobulins against bovine rotavirus and coronavirus infection. Recent Res. Dev. Virol., 1, 95-106.
18. Akita, E.M. and Nakai, S. (1992): Immunoglobulins from egg yolk: isolation and purification. J. Food Sci., 57, 629-634.
19. Kuroki, M., Ikemori, Y., Yokoyama, H., et al. (1993): Passive protection against bovine rotavirus-induced diarrhea in murine model by specific immunoglobulins from chicken egg yolk. Vet. Microbiol., 37, 135-146.

20. Nguyen, S.V., Umeda, K., Yokoyama, H., et al. (2006): Passive protection of dogs against clinical disease due to canine parvovirus-2 by specific antibody from chicken egg yolk. Can. J. Vet. Res., 70, 62-64.
21. Yokoyama, H., Peraita, R.C., Diaz, R., et al. (1992): Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. Infect. Immun., 60, 998-1007.
22. Yokoyama, H., Hashi, T., Umeda, K., et al. (1997): Effect of oral egg antibody in experimental F18+ *Escherichia coli* infection in weaned pigs. J. Vet. Med. Sci., 59, 917-921.
23. Yokoyama, H., Umeda, K., Peralta, R.C., et al. (1998): Oral passive protective immunization against experimental salmonelosis in mice using chicken egg yolk antibodies apecific for *Salmonella enteritidis* and *S. typhimurium*. Vaccine, 16, 388-393.
24. Nomura, S., Masaoka, T., Kurabayashi, K., et al. (2005): Effect of dietary anti-urease immunoglobulin Y on *Helicobacter pylori* infection in *Mongolian gerbils*. Helicobacter, 10, 43-52.
25. Yokoyama, K., Sugano, N., Rahman, A.K.M.S., et al. (2007): Activity of anti-*Porphylomonas gingivalis* egg yolk antibody against gingipains *in vitro*. Oral Microbiol. Immunol., 22, 352-355.
26. Yokoyama, K., Sugano, N., Shimada, T., et al. (2007): Effects of egg yolk antibody against *Porphylomonas gingivalis* gingipains in periodontitis patients. J. Oral Sci., 49, 201-206.
27. Ibrahim, El-SM., Rahman, A.K.M.S., Isoda, R., et al. (2008): *In vitro* and *in vivo* effectiveness of egg yolk antibody against *Candida albicans* (anti-CA IgY). Vaccine, 26, 2073-2080.
28. Romage, C., Saville, S.P., Wickes, B.L., et al. (2002): Inhibition of biofilm formation by farnesol, a quorum-sensing molecule. Appl. Environ. Microbiol., 68, 5459-5463.
29. Perumal, P., Mekala, S. and Chaffin, W.L. (2007): Role for cell density in antifungal drug resistance in *Candida albicans* biofilms. Antimicrob. Agents Chemother., 51, 2454-2463.
30. Hawse, S.P. and Douglas, L.J. (1994): Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. Infect. Immun., 62, 915-921.
31. Kuhn, D.M. and Ghannoum, M.A. (2004): *Candida* biofilms: antifungal resistance and emerging therapeutic options. Curr. Opin. Investig. Drugs, 5, 186-197.
32. Mukherjee, P.K., Zhou, G., Munyon, R., et al. (2005): *Candida* biofilm: a well-designed protected environment. Med. Mycol., 43, 191-208.
33. Ibrahim, El-SM., Rahman, A.K.M.S., Umeda, K., et al. (2004): Identification of the dialysable serum inducer of germ-tube formation in *Candida albicans*. Microbiology, 150, 3041-3049.
34. Nordhang, M.L., Nesse, L.L., Norcross, N.L., et al. (1994): A field trial with an experimental vaccine against *Staphylococcus aureus* mastitis in cattle. J. Dairy Sci., 77, 1267-1275.
35. Nobile, C.J., Andes, D.R., Nett, J.E., et al. (2006): Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. PLoS Pathog., 2, 63.
36. Sundstrom, P. (2002): Adhesion in *Candida* spp. Cell. Microbiol., 4, 461-469.
37. Lo, H.J., Kohler, J.R., DiDomenico, B., et al. (1997): Nonfilamentous *C. albicans* mutants are avirulent. Cell, 90, 939-949.
38. Kimura, L.H. and Pearsall, N.N. (1980): Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. Infect. Immun., 28, 464-468.
39. Kobayashi, C., Yokoyama, H., Nguyen, S.V., et al. (2004): Effect of egg yolk antibody on experimental *Cryptosporidium parvum* infection in *scid* mice. Vaccine, 23, 232-235.
40. Zhen, Y-H., Jin, L-J., Guo, J., et al. (2008): Characterization of specific egg yolk immunoglobulin (IgY) against mastitis-causing *Escherichia coli*. Vet. Microbiol., 130, 126-133.
41. Zhen, Y-H., Jin, L-J., Li, X-Y., et al. (2009): Efficacy of specific egg yolk immunoglobulin (IgY) to bovine mastitis caused by *Staphylococcus aureus*. Vet. Microbiol., 133, 317-322.
42. Hamada, S., Horikoshi, T., Minami, T., et al. (1991): Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. Infect. Immun., 59, 4161-4167.
43. Smith, D.J., King, W.F. and Godiska, R. (2001): Passive transfer of immunoglobulin Y antibody to *Streptococcus mutans* glucan binding protein B can confer protection against experimental dental caries. Infect. Immun., 69, 3135-3142.
44. Hamada, S., Takada, H., Ogawa, T., et al. (1990): Lipopolysaccharides of oral anaerobes associated with chronic inflammation: chemical and immunomodulating properties. Int. Rev. Immunol., 6, 247-261.