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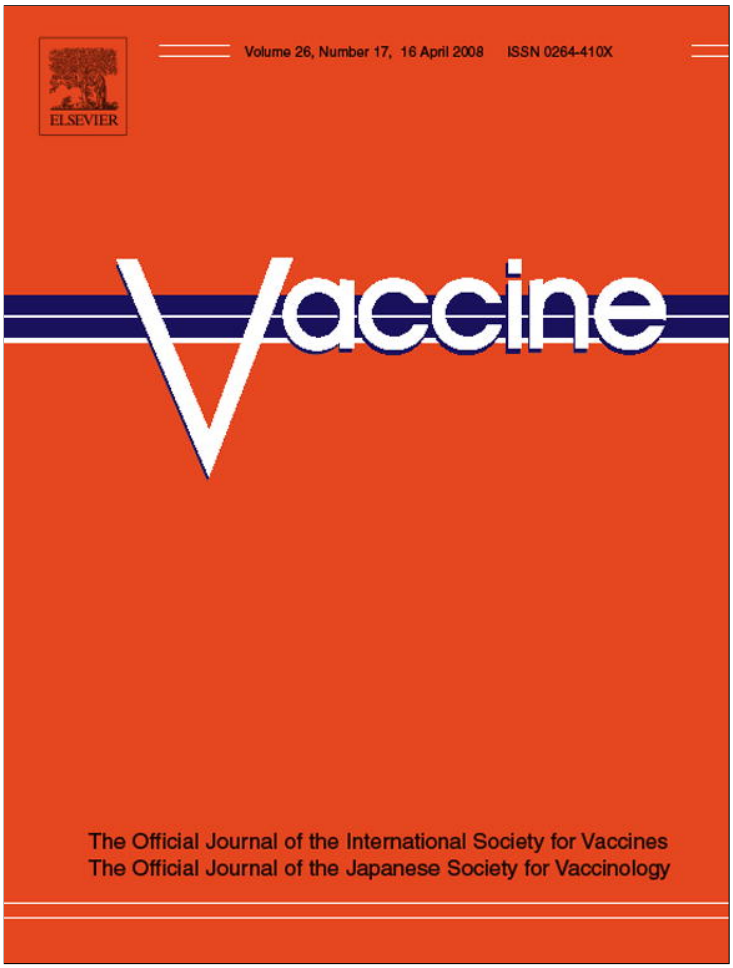

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In vitro and in vivo effectiveness of egg yolk antibody against *Candida albicans* (anti-CA IgY)

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Summary We prepared anti-*Candida albicans* antibody in chicken egg yolk (anti-CA IgY) and investigated its in vitro and in vivo effectiveness. Anti-CA IgY significantly reduced the adherence capacity of *C. albicans* to FaDu cells (human pharynx carcinoma cells) in a dose-dependent manner. The protective efficacy of anti-CA IgY was investigated in experimentally induced oral candidiasis in immunosuppressed mice. Oral administration of anti-CA IgY significantly reduced the number of *C. albicans* and the scores of the tongue lesions. Moreover, anti-CA IgY reduced the colonization of *C. albicans* in mice organs. These results indicate that anti-CA IgY has a protective effect against the oral candidiasis of experimentally infected mice and reduces the dissemination of *C. albicans*. Putting together, these results indicate that anti-CA IgY is effective against *C. albicans*. This effect might be due to the blocking of the binding of *C. albicans* to the host cells. Therefore, anti-CA IgY might be considered as a prophylactic immunotherapy or possibly an adjunctive antifungal therapy.
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Introduction

Candida albicans is a member of the indigenous microbial flora of the gastrointestinal tract, mucocutaneous membranes, and oral cavity in healthy humans [1–3]. Although *C. albicans* rarely causes infections in healthy human without predisposing factors, immunosuppressed patients can suffer from mucosal, cutaneous, or systemic candidiasis [4]. *C. albicans* is also a potential pathogen and a frequent cause of complicating systemic infections and mortality in patients under chemotherapy for cancer [4–6], immunosuppressive therapy [7], or prolonged antibiotic therapy [8]. Oropharyngeal candidiasis is the most common opportunistic infection associated with oral injuries [9] and hyposalivation [10,11]. The expression of *C. albicans* virulence in the oral cavities is strongly correlated with impairment of the immune system, particularly in patients with HIV infection [12–14]. Oral thrush is a common form of the oropharyngeal candidiasis and its clinical features include white patches appearing

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as discrete lesions on the buccal mucosa, throat, tongue, and gum linings that develop into confluent pseudomembranes resembling milk curds [15]. Long-term treatment of oropharyngeal candidiasis with antifungal therapy such as fluconazole, itraconazole, and ketoconazole sometimes leads to the emergence of drug-resistant *C. albicans* [14].

C. albicans expresses several virulence factors that required for the establishment of candidiasis such as adhesion to the host cells, phenotypic switching, and germ tube formation [16]. Adhesion of the organism to mucosal epithelium is a prerequisite for colonization and is, therefore, regarded as the initial step in the process leading to infection. Moreover, adhesion and colonization of the organism to oral epithelium can serve as a reservoir for disseminated infections, such as pneumonia, and gastrointestinal infection [17]. Furthermore, adhesion to endothelium and extracellular matrix (ECM) components are required for dissemination of *C. albicans* [18].

Limited antifungal drug choices and the potential risk of the emergence of the drug-resistant *C. albicans* strains [19,20] besides the lack of safe and reliable vaccines to confer protective immunity against fungal infection [4] indicate the need for adjunct therapeutic strategies. The use of specific antibodies as an adjunct to antifungal drugs can be considered one approach. However, the role of specific antibodies in controlling the dissemination of *C. albicans* is controversial. Some investigators have reported that specific antibodies increased the resistance to systemic candidiasis [21,22]. On the other hand, other researchers have reported that systemic immunization of mice against *C. albicans* did not confer any protection against oral candidiasis [23]. The failure of systemic immunization to confer protection on oral cavity may be related to the circulation patterns of the lymphocytes, which directed to the systemic rather than the mucosal regions [24]. Chicken egg yolk has been recognized as an inexpensive alternative antibody source, and passive immunization with egg yolk immunoglobulin (IgY) has shown therapeutic value against rotavirus, parvovirus, *E. coli*, *S. typhimurium*, *S. mutans*, *H. pylori*, and *P. gingivalis* [25—32]. In this study, we investigated the in vitro and in vivo efficacy of chicken egg yolk antibody prepared against *C. albicans*. Anti-CA IgY reduced the adhesion activity of *C. albicans* to human cells. Furthermore, anti-CA IgY reduced the oral candidiasis and the systemic dissemination in immunosuppressed mice model of oral candidiasis.

Materials and methods

Organism and antigen preparation

C. albicans JCM 1542 [3] was stored at −80 °C in YPD (yeast extract, 1%; polypeptone, 2%; and dextrose, 2%) broth (SIGMA—ALDRICH, Inc., St. Louis, MO, USA) containing 10% glycerol until the experiment was performed. The organism was cultured in YPD broth for 24 h at 37 °C with orbital shaking at 100 rpm. The fungal cells were harvested by centrifugation for 10 min at 8000 rpm at 4 °C, washed twice with sterile phosphate-buffered saline (PBS; pH 7.2). Cells were resuspended in PBS and sonicated for 10 min in an ice bath. The sonicated cells were then dialyzed against PBS. The protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad laboratories, CA, USA).

Anti-*C. albicans* egg yolk antibody (anti-CA IgY) preparation

For the egg antibody production, 5-month-old White leghorn hens (strain Hyline W36; GHEN Corporation, Gifu, Japan) in conventional isolated poultry housing were immunized according to the method described by Yokoyama et al. [28]. Briefly, the vaccine was prepared by mixing 0.5 mg of *C. albicans* antigen with 0.5 ml emulsion oil containing 5% Arlacel 80 (Maine Biological Laboratories, Waterville, ME, USA) and hens were immunized by injecting 0.5 ml to each of the breast muscles. Six weeks after the initial immunization, a booster was given in the same manner. Eggs from the immunized hens were harvested daily from the second week till the sixth week after the booster and stocked at 4 °C. Egg yolk was separated carefully from the albumin and yolk membrane. The yolk was then pooled, homogenized, and filtrated through Teflon filter cloth. Partially purified specific IgY powder was prepared from the egg yolk by ammonium sulfate precipitation [25]. Then, the precipitated IgY was suspended in PBS, dialyzed, and freeze-dried in a Labconco freeze-drying machine (Labconco LL-12, Labconco Corp., Kansa, MO, USA) and a solution containing 10 mg/ml was prepared in PBS. Control IgY powder was prepared from the egg of non-immunized hens by the same method.

In vitro adhesion inhibition activity of anti-CA IgY

The adhesion inhibition assay was based on the protocol of Alberti-Segui et al. [33]. In our assay, FaDu cells (human pharynx carcinoma cell line; ATCC HTB-43) were plated in a 24-well tissue culture plate in Minimum essential medium (Nissui, Japan) with Earle's salts and non-essential amino acids and supplemented with L-glutamine, sodium pyruvate, and 5% fetal bovine serum. Cells were incubated at 37 °C in 5% CO_2 tension for 24 h or until a confluent monolayer was formed. At the same time, one colony of *C. albicans* was inoculated into 10 ml of YPD broth and incubated overnight at 37 °C with 100 rpm orbital shaking. On the day of the assay, overnight suspension of *C. albicans* was collected by centrifugation at 3000 rpm, 4 °C for 10 min and washed 3 times with PBS. *C. albicans*—IgY mixture was prepared as follows. *C. albicans* cells were adjusted to be 1×10^6 CFU/ml in MEM. The same volume of anti-CA IgY solution (10 mg/ml) was added to *C. albicans* suspension. The mixture was incubated for 1 h at 37 °C. Then, FaDu cells were carefully washed 3 times with PBS to remove cell growth media and then 0.5 ml of *C. albicans*—IgY mixture was added to each well. After 1 h of incubation at 37 °C in 5% CO_2 tension, the plate was washed 3 times with PBS to remove non-adherent fungal cells. FaDu cells were then lysed by 0.5 ml of 0.1% Triton X-100. After that, the plates were centrifuged at 2000 rpm for 5 min, the supernatant was removed by aspiration and pellets containing the adherent fungal cells were resuspended in 1 ml of PBS. To count the adherent fungal cells, serial 10-fold dilutions were made in PBS and 0.1 ml of each dilution was inoculated on YPD agar (2 plates for each dilution). The

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agar plates were incubated overnight at 37 °C and *C. albicans* CFUs were counted. Control IgY-treated and non-treated wells were used as control. The results shown are the average of 3 independent experiments. In a separate experiment, we examined the dose-dependent adhesion inhibition efficacy of anti-CA IgY. Different concentrations of anti-CA IgY (0.0, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mg/ml) were used. *C. albicans* challenge dose was 1×10^6 CFU/ml. The protocol was performed as described previously. The results shown are the average of 5 independent experiments.

Mouse challenge test

Six-week-old SPF female ICR mice (SLC, Inc., Japan) were used. Animals were kept in cages housing 5 mice/cage and given *ad libitum* access to food and water. The mouse model of oral candidiasis was based on the protocol of Takakura et al. [15] with some modifications. Mice were immunosuppressed with three subcutaneous inoculations of prednisolone (Wako Pure Chemical Industries, Ltd., Japan) at a dose of 100 mg/kg body weight in day −1, 1, and 3 after *C. albicans* infection. Tetracycline hydrochloride (Wako Pure Chemical Industries, Ltd., Japan) was given in drinking water everyday starting one day before *C. albicans* infection at concentration 0.83 mg/ml to disturb the normal bacterial flora in the oral cavity and facilitate the colonization of *C. albicans* [15]. Animals were anesthetized by an intramuscular injection with 50 μl of 2 mg/ml chlorpromazine chloride (Wako Pure Chemical Industries, Ltd., Japan) in each femur. Mice infection was performed by soaking of small cotton pads in the *C. albicans* suspension (3.7×10^6 cell/ml) and then the entire mouth cavity was swabbed. Anti-CA IgY was applied to mice in the form of gel suspension (50 mg/ml). Anti-CA IgY gel was prepared by dissolving of 0.5 g anti-CA IgY powder into 10 ml distilled water then 0.4 g of a neutral gel powder consisting of dextrin, pectin, and pH adjuster (Food Care IFA, Japan) was added and well mixed for 1 min to form a homogeneous gel. Mice were given IgY gel (100 μl/mice) into the mouth by using a wide blunt needle twice a day until the end of the experiment. The control infected and non-infected groups received control IgY gel. The body weight of mice was recorded once a day till the end of the experiment. Five mice per group were sacrificed at days 1, 3, 5, and 7 after infection by cervical dislocation. The tongue lesions were macroscopically evaluated. The lesions evaluation was indicated by a lesion score from 0 to 4 on the basis of the extent and severity of the lesions on the tongue surface as follows: 0, normal; 1, small vesicles-like white spots; 2, vesicles-like white spots; 3, loss of tongue epithelium; 4, white patches-like pseudomembrane. Microbiological evaluation of the progression of the oral and disseminated infection was carried out as follows. Individual mice were dissected and tongue, lungs, kidneys, and intestine were aseptically removed, weighted, and mechanically homogenized in sterile PBS with a glass tissue grinder. Tissue homogenates were then serially diluted in sterile PBS. From each dilution 0.1 ml was plated on Candida GE agar (Nissui, Japan). The number of colony-forming units (CFU), expressed as CFU/G tissue, was determined after 24 h of incubation at 37 °C.

Statistical analysis

The data of the adhered *C. albicans* colonies were analyzed by the Student's *t*-test. The data of the dose–response, the relative body weight (loss/gain), and the $\log_{10}$ CFU of *C. albicans* isolated from mice organs were analyzed by ANOVA test. The tongue lesions scoring data were analyzed by Mann–Whitney test. *P* values of <0.05 were considered significant.

Results

In vitro adhesion inhibition activity of anti-CA IgY

In order to investigate the activity of anti-CA IgY, we performed an in vitro adhesion assay and examined the adherence ability of the anti-CA IgY-treated *C. albicans* to FaDu cells. Anti-CA IgY-treated *C. albicans* showed significant reduction in the adherence capacity when compared with the non-treated *C. albicans* ($P < 0.001$) (Fig. 1A and B). On the other hand, the control IgY-treated *C. albicans* did not show any significant reduction in the adhesion ability. Almost similar results were obtained with different *C. albicans* challenge doses (data not shown). In addition, we examined the dose–response efficacy of anti-CA IgY. The inhibition of *C. albicans* adhesion was directly correlated with the IgY concentration (Fig. 1C). These results indicated the adhesion activity of *C. albicans* was reduced after the incubation of *C. albicans* with anti-CA IgY and the reduction of the adhesion activity was correlated to the IgY concentration. The in vitro results indicate that anti-CA IgY has anti-adhesion activity against *C. albicans*.

Efficacy of anti-CA IgY against *C. albicans* infection in a mouse model of oral candidiasis

In order to determine whether anti-CA IgY plays a role in the reduction of oral candidiasis in immunosuppressed mice model, mice were administrated anti-CA IgY gel twice a day. At days 1, 3, 5, and 7 after infection 5 mice were sacrificed and tongue lesions were scored based on the severity and the number of the white patches on the tongue (Fig. 2). Furthermore, colonization of *C. albicans* in mice tongue was examined by counting the recovered *C. albicans* CFU/g. The control non-infected group showed neither tongue lesions nor *C. albicans* colonization. Both anti-CA IgY-immunized group and control IgY-immunized group did not show any lesions at day 1 after infection. At day 3 after infection, 4 out of 5 mice of the anti-CA IgY-immunized group showed lesions with the average score 1.7 ± 0.82. At day 5 after infection, 2 out of 5 mice of the anti-CA IgY-immunized group showed mild lesions with the average score 0.2 ± 0.42 and at day 7 after infection, only 1 out of 5 mice showed mild lesions. On the other hand, in the case of control IgY-immunized group, all mice showed tongue lesions at day 3 (average score 2.7 ± 0.92) and at day 5 (average score 2.7 ± 1.08) after infection. At day 7 after infection, 3 out of 5 mice showed tongue lesions with the average score 1.3 ± 1.18. Statistical analysis showed significant differences ($P < 0.05$)

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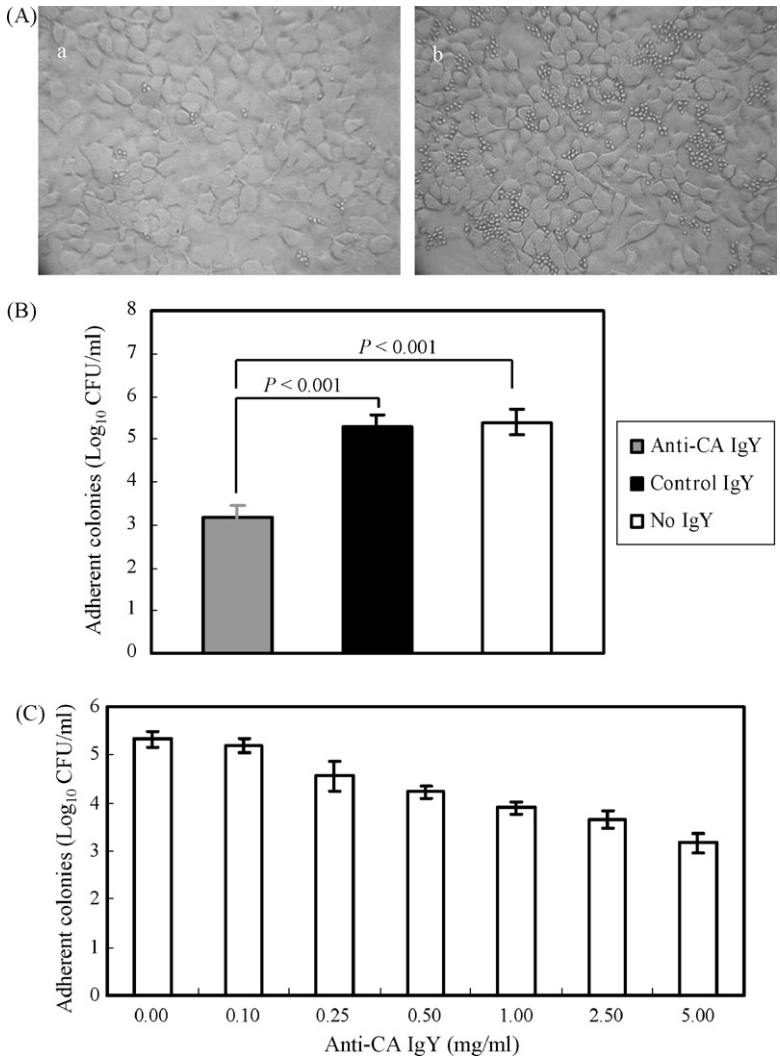

Figure 1 Efficacy of IgY on adherence of *C. albicans* to human cells. (A) FaDu cells inoculated with *C. albicans* pretreated with anti-CA IgY (a) and control IgY (b). (B) Number of *C. albicans* recovered from the cell culture inoculated with various *C. albicans* preparation. Anti-CA IgY-treated *C. albicans* showed significantly reduction in the adherence activity in the comparison with control IgY-treated and non-treated *C. albicans*. No significant difference was observed between control IgY-treated and non-treated *C. albicans*. Data shown are mean ± S.D. of 3 independent experiments. (C) The dose-dependent adhesion inhibition activity of anti-CA IgY. The number of the adherent *C. albicans* was indirectly correlated with the concentration of anti-CA IgY.

in the tongue lesions score of anti-CA-immunized group in the comparison with the control IgY-immunized group (Fig. 2B).

No *C. albicans* was recovered from the tongue homogenates of the non-infected mice. *C. albicans* was recovered from the mice tongues in both anti-CA IgY- and control IgY-immunized groups. In the anti-CA IgY-immunized group, the average counts were 5.25 ± 0.40, 6.05 ± 0.90, 5.31 ± 0.71, and 5.05 ± 0.77 $\log_{10}$ CFU/g at days 1, 3, 5, and 7 after infection, respectively. On the other hand, in the control IgY-immunized mice, CFUs of the recovered *C. albicans* were higher than those of the anti-CA IgY-immunized group and the average counts were 5.82 ± 0.54, 6.65 ± 0.27, 6.41 ± 0.46 and 6.15 ± 0.34 $\log_{10}$ CFU/g at days 1, 3, 5, and 7, respectively. Statistical analysis showed significant differences in the number of the recovered *C. albicans* cells between the two groups (Fig. 3).

As shown in Fig. 4, the non-infected group showed weight loss after prednisolone injection. The maximum weight loss was at day 1 after the third prednisolone injection and reached 3.42 ± 1.17 g. The mice slowly started to regain their body weight at day 2 (3.40 ± 1.17 g), day 3 (3.32 ± 0.84 g), and day 4 (3.14 ± 0.81 g) after prednisolone injection. Similarly, anti-CA IgY-immunized group showed gradual decrease in the body weight in the period from day 1 to day 5 after infection. The maximum weight loss was at day 5 after infection (day 2 after the third prednisolone injection) and reached 3.64 ± 0.97 g. The mice slowly started to regain their body weight at day 6 (3.45 ± 1.13 g) and day 7 (3.18 ± 1.23 g) after infection. Statistical analysis did not show any differences in the relative body weight gain/loss between the control negative group and anti-CA IgY-immunized group. On the other hand, the control IgY-immunized group expressed severe

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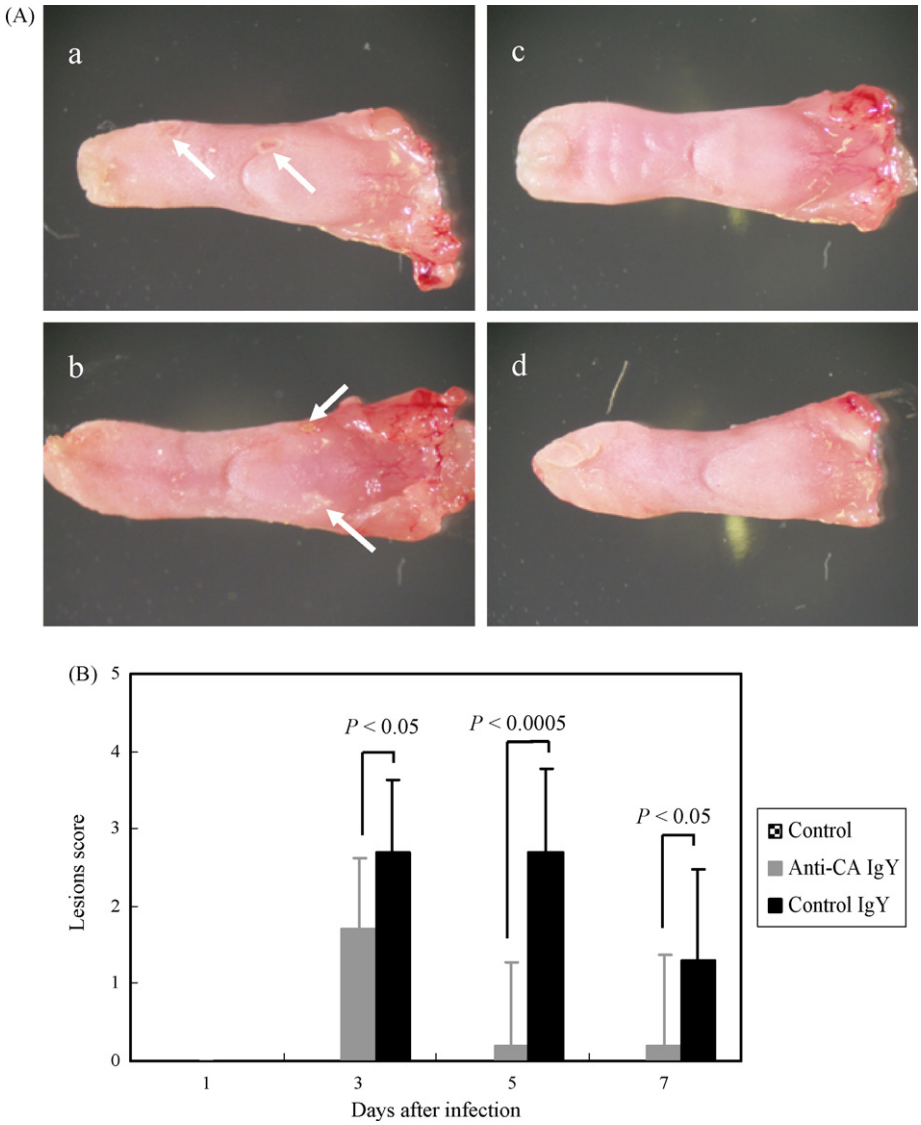

Figure 2 Protective efficacy of IgY against oral candidiasis. (A) Tongues from mice challenged with *C. albicans* and treated with control IgY or anti-CA IgY. The tongue lesions were severe and ranged from large white spots to detachment of tongue epithelium in the control IgY-treated mice (a) and (b). The tongue lesions were mild and consisted mainly from small white spots on the tongue surface in the anti-CA IgY group (c) and (d). (B) Average of the tongue lesion scores. The tongue lesions of anti-CA IgY group were lower than those of the control IgY group. Statistically, there was significant reduction of the tongue lesions (severity and size of the lesions) in the anti-CA IgY group. Data shown are mean ± S.D. for 5 mice at each point of examination in two independent experiments.

weight loss from day 1 after infection till the end of the experiment (day 7) and mice did not regain their body weight. Statistical analysis showed significant differences in the body weight of the control IgY-immunized group in the comparison with the control negative and anti-CA IgY-immunized groups at days 2, 3, 4, 5, 6, and 7 after infection.

No *C. albicans* recovered from lungs, kidneys, and intestine of the non-infected mice. *C. albicans* was recovered from lungs in 60%, 80%, 80% and 20% of anti-CA IgY-immunized mice at days 1, 3, 5, and 7, respectively. In the case of control IgY-immunized group, *C. albicans* was recovered from all mice at all points of examination. The CFUs/g of *C. albicans* recovered from anti-CA IgY-immunized group was lower than those recovered from the control IgY-immunized group with significant differences (Fig. 5A). From kidneys, *C. albicans* was not recovered at day 1 after infection in both anti-CA IgY- and control IgY-immunized groups. *C. albicans* was recovered from 20% of anti-CA IgY-immunized mice at days 3, 5, and 7 after infection. On the other hand, *C. albicans* was recovered from kidneys of 60% of the control-IgY-immunized mice at days 3, 5 and 7 after infection (Fig. 5B). From intestine, *C. albicans* was recovered from both anti-CA IgY- and control IgY-immunized groups at all points of examination. However, statistical analysis showed significant differences in the recovered CFUs/g between anti-CA IgY- and control IgY-immunized groups (Fig. 5C).

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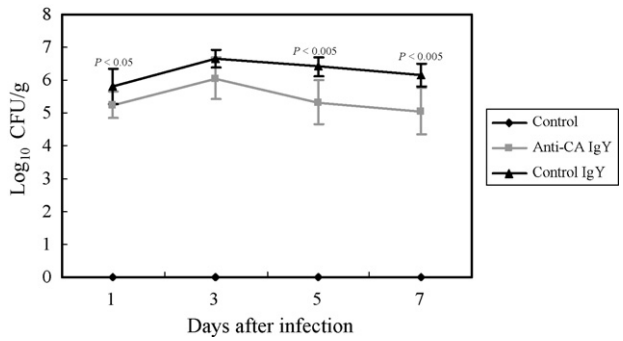

Figure 3 Effect of IgY on the colonization of *C. albicans* in the mice tongue. The anti-CA IgY group showed reduction in the yield of viable *C. albicans* count from the tongue. There was significant difference in the recovered *C. albicans* between the anti-CA IgY group and the control IgY group at days 1, 5, and 7 after infection. Data shown are mean ± S.D. for 5 mice at each point of examination in two independent experiments.

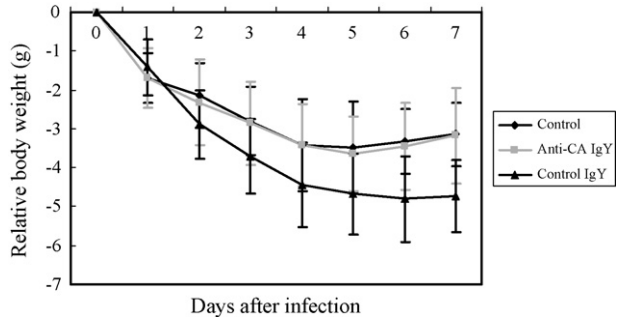

Figure 4 Effect of IgY on the mice body weight after oral infection of *C. albicans*. All mice groups expressed weight loss after inoculation of the prednisolone. The control IgY group expressed weight loss until the end of the experiment. The control negative and anti-CA IgY groups slowly started to regain their body weight before the end of experiment. Significant differences in the relative gain/loss of the body weight were observed in the period from day 2 to day 7 after infection. Data shown are mean ± S.D. of two independent experiments.

Discussion

C. albicans is a constituent of the normal microbial flora that colonizes the mucocutaneous surfaces of the oral cavity, gastrointestinal tract, and vagina in human and many animals [1]. Long-term treatment with antifungal drugs has a potential risk of the emergence of drug-resistant strains [4,14,34]. Therefore, there is an urgent need to develop new preventive strategies and alternative forms of treatment. In the past few years, passive immunization with antibodies has been used as immunotherapy [33,35—42]. Moreover, chicken egg yolk immunoglobulin has been recognized as an alternative antibody source and they showed therapeutic values against several microorganisms [25—32].

In this study, we have investigated the effect of anti-CA IgY on the capacity of *C. albicans* to adhere to human cells. The anti-CA IgY expressed significant reduction ($P < 0.005$) of the adhesion activity of *C. albicans* to human cells. The reduction of the adhesion activity was directly related to the

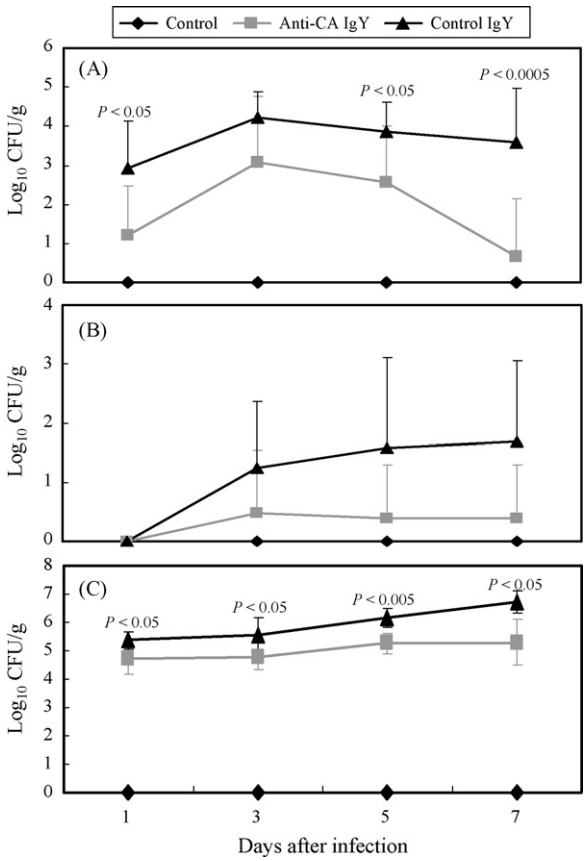

Figure 5 Efficacy of IgY on the dissemination of *C. albicans* in the mice lungs (A), kidneys (B), and intestine (C). The anti-CA IgY group showed significant reduction in the recovered *C. albicans*. Data shown are mean ± S.D. for 5 mice at each point of examination in two independent experiments.

dose of the anti-CA IgY where, the dose—response analyses showed that using anti-CA IgY at 0.25 mg to 5 mg/ml significantly reduced the adhesion activity of *C. albicans* but the lower doses did not show any significant effect. These results indicate that the anti-CA IgY altered the adhesive properties of *C. albicans*. It has been reported that alteration and/or deletion of one or more of *C. albicans* cell-surface adhesions inhibit the adhesion ability of the organism [33,43]. Anti-CA IgY was prepared by immunization of chicken by the whole cell *C. albicans* antigens with high concentration of cell wall antigens. Therefore, our results suggest that the reduction of the adhesion properties of *C. albicans* may be due to alterations of the cell wall adhesions or blocking the binding of *C. albicans* to the host cells. One other possible mechanism is that IgY causes agglutination of the yeast cells, which effectively reduces the number of independent infection unit.

Adherence to the host cells is required for virulence of the mucosal pathogens; therefore, interfering with the adherence of a particular pathogen prevents or delays the colonization and consequently the disease [44]. Several attempts have been made to protect against systemic candidiasis by both active and passive immunization, but only a few have been made to protect against oral candidiasis [23]. In the current study, the protective efficacy of anti-CA IgY

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was investigated in an immunosuppressed mice model of oral candidiasis. The severity of oral candidiasis was estimated both by measuring the number of viable *C. albicans* cells recovered from the mice tongues and by manifestations of tongue lesions [15]. Oral protection was achieved by immunization of mice via the oral route. The results showed that, both the oral colonization of *C. albicans* and the severity of the tongue lesions were decreased in the anti-CA-immunized mice. This reduction may be due to the difficulty of *C. albicans* to adhere to tongue epithelial cells due to impaired adhesion activity.

Data of the *Candida* count in the internal organs is interesting and indicates the possible translocation of *Candida* cells to the internal organs. Takakura et al. [15] indicated the possible translocation of *C. albicans* to the gastrointestinal tract but not to other organs. However, our results indicate the possibility of systemic dissemination of *C. albicans*. This dissemination is due to the reduction of the immune response of the mice by three injections of prednisolone. The protective effect of anti-CA IgY against systemic dissemination of *C. albicans* was demonstrated by the reduction of the body weight loss after treatment with prednisolone and the reduction of *C. albicans* cells in the mice organs as well. The results showed that anti-CA IgY immunization reduced the body weight loss and the number of the CFU of *C. albicans* recovered from lungs, kidneys and intestine. These findings are in agreement with other study indicated that anti-*Candida* IgY can protect mice against lethal *Candida* infection [45]. In our study, a different model was used. Although no death was observed, lower *Candida* loads in the internal organs indicate that IgY protects against systemic dissemination.

The mechanism(s) of action of IgY in the reduction of the dissemination remains to be clarified. Abdelnoor et al. [45] suggested that the protection effect of IgY was through the enhancement of the host immune response. In our study, the repeated prednisolone inoculation reduces the immune response of the mice. Therefore, the reduction of the *C. albicans* dissemination is not due to the protective immunological responses of the mice [46]. However, it might be a reflection of the difficulty of *C. albicans* to invade tissues as a result of altered adhesion activity [22]. Reduction of adhesion might also account for delayed or reduced colonization [33].

In conclusion, we presented evidences for the activity of anti-CA IgY against *C. albicans*. Anti-CA IgY reduced the adhesion ability of *C. albicans* to human cells. Furthermore, it reduced the severity of the tongue lesions and the systemic dissemination. These results suggest that anti-CA IgY could be used as a preventive immunotherapy against oral and disseminated candidiasis. In future, we would like to conduct a clinical trial of anti-CA IgY in patients with oral candidiasis.

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