Preparation of Anti-*Candida Albicans* Antibodies in an Egg-Laying Hen and Their Protective Efficacy in Mice

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ABSTRACT

Limited antifungal drugs, their high toxicity, and the emergence of resistant *Candida albicans* strains indicate the need for novel therapeutic strategies. The objectives of this study were to prepare antibodies against *C albicans* in an egg-laying hen and evaluate the efficacy of these antibodies in preventing the dissemination of *C albicans* in BALB/c mice.

An egg-laying hen was immunized with a mixture of heat-killed *C albicans* yeast cells and germ tubes. Egg yolk immunoglobulin (IgY) was extracted and purified from the yolk of eggs laid before and after immunization. Anti-*C albicans* IgY antibodies in the extracts were detected by enzyme-linked immunosorbent assay. The protective efficacy of post-immunization IgY was tested by injecting groups of BALB/c mice with a lethal dose of *C albicans* and a variable regimen of pre- or post-immunization IgY. The results indicated the following: 1) all the mice that received either pre- or post-immunization IgY survived at 7 days post-challenge compared with 20% of those in the untreated controls; 2) mean candidal colony forming units (CFU/mg) of kidney tissue were $25 \times 10^8 \pm 5.4 \times 10^8$ in untreated mice and $10 \times 10^3 \pm 1.4 \times 10^3$ in mice treated with pre-immunization IgY; no *C albicans* colonies could be detected in all challenged mice that were treated with post-immunization IgY; 3) multiple abscesses were observed in kidneys obtained from mice treated with pre-immunization IgY. No abscesses were seen in kidneys obtained from mice injected with post-immunization IgY.

Post-immunization IgY might be considered a prophylactic agent or possibly an adjunct to antifungal therapy. Pre-immunization IgY appeared to contain factors that prolonged survival, but did not prevent dissemination of the fungus.

INTRODUCTION

Candida albicans is a benign commensal in the majority of healthy individuals and can be isolated from the normal flora of the oral cavity, gastrointestinal tract, skin, and urogenital tract. However, a change from the normal physiologic or immune state, or microbial flora of the host, allows *C albicans* to increase in number and invade tissues. Disseminated candidiasis is a serious disease that often results in death, even in patients treated with antifungal agents.[1-3]

The role of specific antibodies in controlling the dissemination of *C albicans* is controversial. It has been reported that there was no increase in resistance to *C albicans* challenge in rabbits given *C albicans* hyperimmune serum.[4] Moreover, T- and B-cell–deficient severe combined immune deficiency (SCID) mice were as resistant to systemic *C albicans* infection as were immunocompetent mice.[5,6] In contrast, a number of reports indicated that specific antibodies increased the resistance to systemic candidiasis.[7-10] The disparity between findings regarding the role of humoral immunity has been attributed to a number of factors.[11] One of which is the ability of *C albicans* to undergo reversible transition between budding, pseudohyphal, and hyphal forms.[12] Such transitions might lead to changes in antigenic structure of the organism. Thus, antibodies produced against one form might not be effective against another form.

Limited antifungal drug choices and their high toxicity as well as the potential risk of the emergence of drug-resistant *C albicans* strains indicate the need for novel therapeutic strategies. The use of specific antibodies as an adjunct to antifungal drugs can be considered one approach.

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 enterotoxigenic *Escherichia coli, Salmonella enterica* serovar Typhimurium, rotavirus, Staphylococcal enterotoxin B, *Streptococcus mutans,* and *Helicobacter pylori.*[13-15]

The aims of this study were to prepare IgY antibodies against *C albicans* by immunizing an egg-laying hen with a combination of yeast and germ tube cells and to determine whether these antibodies prevented disseminated candidiasis in BALB/c mice.

MATERIALS AND METHODS

Organism

C albicans was isolated from the throat of a patient in the intensive care unit. The isolate was confirmed to be *C albicans* by the germ tube test and the API 20 C AUX kit (biomérieux, Marcy-L'Etoile, France). The LD50 of this isolate in BALB/c mice was determined to be 12.10×10^6 organisms.[16]

Immunization of an Egg-Laying Hen

Subcultures of *C albicans* incubated on Sabouraud dextrose agar at 37ºC for 24 hours were harvested, counted, and suspended in phosphate buffered saline (PBS, pH 7.5, 0.01 M) to contain 10×10^6 cells/mL. Germ tubes were obtained by incubating *C albicans* colonies in serum at 37ºC for 3 hours. After being centrifuged and washed, these cells were counted and suspended in PBS to contain 10×10^6 cells/mL. Both cell suspensions were heated at 80ºC for 30 minutes. Lack of viability was tested by plating on Sabouraud dextrose agar and incubating at 37ºC for 24 hours. A suspension of 5×10^6 yeast cells and 5×10^6 germ tube cells emulsified in Freund's complete adjuvant was injected subcutaneously into an egg-laying hen.

A booster containing 5×10^6 heat-killed yeast cells and 5×10^6 heat-killed

Table 1. Survival of Mice Challenged With *Candida Albicans* and *C Albicans* Colony Forming Units (CFUs) per Milligram of Kidney

	Group A	Group B	Group C	Group D	Group E	Group F
Intra-peritoneal injection	Ag*	Ab† followed by Ag 2 hours later	Ag and Ab given simultaneously	Ag followed by Ab 2 hours later	Ag followed by daily Ab injection‡	Ag followed by pre-immunization IgY injection 2 hours later§
Survival 7 days post challenge	1/5 (20%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Mean *C albi-cans* CFU/mg of kidney at death or on day 7	25×10^8 ± 5.4×10^8	<1 CFU	<1 CFU	<1 CFU	<1 CFU	10×10^3 ± 1.4×10^3

*Ag; $2LD_{50}$ of *C albicans* live yeast cells (24.2×10^6).
†Ab; 10 mg/0.5 mL of post-immunization IgY.
‡For 5 days.
§0 mg/0.5 mL.
Each group consisted of 5 mice.

germ tube cells emulsified in Freund's incomplete adjuvant was administered subcutaneously 15 days following primary immunization.

Extraction of IgY

IgY was purified from the yolk of 5 eggs laid before immunization and from eggs laid on day 5, 12, 14, 21, and 25 after primary immunization, using EGGstract IgY Purification System (Promega Corporation, Madison, WI). All the post-immunization extracts were pooled, dialyzed, and freeze-dried using Freeze Dry/Shell Freeze System (Labconco, Kansas City, MO). A solution containing 20 mg/mL in PBS was prepared. The same procedure was used for the pre-immunization extracts.

Protective Effect of Post-Immunization IgY

Thirty female 8-week-old BALB/c mice were randomly divided into 6 groups. The injection regimen of post- and pre-immunization IgY and challenge with 2LD50 of *C albicans* is provided in Table 1. The mice were monitored for 7 days and the time of death in each group was recorded. Survival differences between the groups were calculated for statistical significance using Statcalc Epi Info Version 6 (Centers for Disease Control and Prevention, Atlanta, GA). Fisher exact 1-tailed and 2-tailed *P* value calculation formulas were used. $P<0.05$ was considered to be significant.

Gross Morphology of Kidneys and Determination of Colony-Forming Units (CFUs)

The kidney is a target organ in experimental disseminated candidiasis.[17] The kidneys obtained from the mice that died and the mice that were killed after the 7-day monitoring period were removed and photographed. The kidneys from all mice were then homoge-

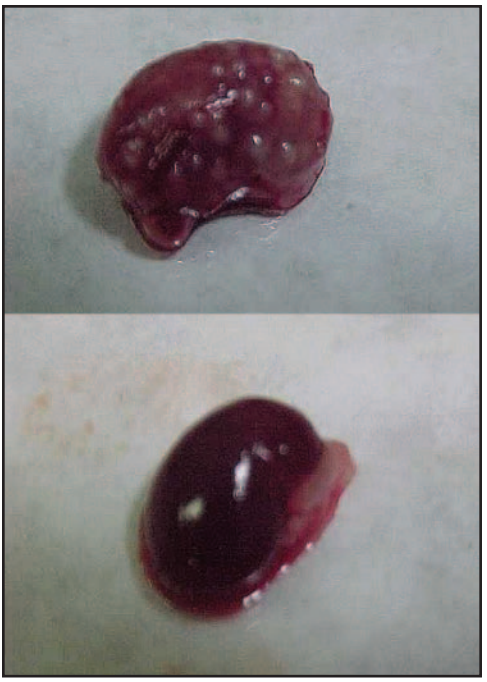

Figure 1. Gross morphology of (A) a kidney from the group receiving 2LD50 of *Candida albicans* and pre-immunization IgY, compared with (B) a normal kidney from the group that received 2LD50 of *C albicans* and post-immunization IgY. Kidney in (A) has pale variegated surface with yellow, tan, and purple areas. Surface has multiple white pinpointed elevated lesions, some of which are confluent, ranging in size from 0.1 to 1 mm. These lesions likely correspond to microabscesses.

nized with PBS in glass tissue homogenizers. The number of candidal CFUs per mg of each kidney was determined using the plate dilution method on Sabouraud dextrose agar. The colonies isolated from each kidney were confirmed to be *C albicans* using the API 20 C AUX kit.

Detection of Anti-C albicans IgY by Enzyme-Linked Immunosorbent Assay (ELISA)

Yeast cells and germ tubes were lysed using PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Fifty microliters of the lysate was added to each well of a 96-well flat-bottomed polystyrene microplate. The plate was incubated overnight at 4ºC. The wells then were washed with PBS, followed by the addition of 50 µL of 1% bovine albumin in PBS to each well, and the plate was allowed to stand for 1 hour at room temperature. The washing step was then repeated, followed by the addition to each well of 25 µL of IgY preparation to be tested (pre- and post-immunization IgY; undiluted, and 1:5 and 1:10 dilutions). The wells were then incubated for 30 minutes at 37ºC followed by washing and the addition of 25 µL to each well of peroxidase conjugated rabbit anti-IgY antibody, diluted in 1:1000 with PBS (original sample concentration of 1 mg/mL). The plate was then incubated for 30 minutes at 37ºC, washed, and 25 µL of 3,3',5,5' tetramethylbenzidine (TMB) was added to each well, and then allowed to stand for 30 minutes in the dark. Twenty-five microliters of 3 M sodium hydroxide were added to each well. The absorbances were read at 450 nm.

RESULTS

Survival Rates of Mice

All the mice in groups that were challenged with 2LD50 of *C albicans* and received pre- or post-immunization IgY according to the regimen shown in Table 1 survived the 7-day period. Four of 5 mice that did not receive IgY died. The Fisher exact 1-tailed *P* value was equal to 0.02 and the Fisher exact 2-tailed *P* value was equal to 0.04.

Gross Appearance of the Kidneys

The gross morphology of a representative kidney obtained from a mouse that had received pre-immunization IgY and a representative kidney obtained from a mouse that had received post-immunization IgY are shown in Figure 1. The latter kidney appeared to be normal. The former kidney appeared to have a pale variegated surface, with yellow, tan, and

Table 2. Absorbance in the ELISA Using Different Dilutions of Pre- and Post-Immunization IgY

IgY Dilution	Absorbance* Pre-IgY	Post-IgY
Undiluted	0.574	2.052
1:5	0.230	1.313
1:10	0.214	1.060

*Mean; samples were tested in duplicates.

purple areas. The surface had multiple white pinpointed elevated lesions that appeared like micro abscesses, ranging in size from 0.1 to 1 mm.

C Albicans CFUs in Kidney

The mean CFU/mg of homogenized kidney of each group is given in Table 1. Challenged mice that did not receive IgY had a mean CFU/mg count of $25 \times 10^8 \pm 5.4 \times 10^8$, challenged mice that received pre-immunization IgY had a mean CFU/mg count of $10 \times 10^3 \pm 1.4 \times 10^3$. No *C albicans* colonies could be detected in all challenged mice that were injected with post-immunization IgY.

Detection of Anti-*C Albicans* IgY by ELISA

The absorbances obtained when different dilutions of pre- and post-immunization IgY were used are provided in Table 2. In all dilutions used, the absorbances obtained for post-immunization IgY exceeded that of pre-immunization IgY (3.6- to 5.7-fold).

DISCUSSION

Morbidity and mortality rates associated with systemic infections by *C albicans* remain high; the main reason being the difficulties in the treatment of this type of infection. Antifungal drug choices are limited because of their toxicity and the potential risk of the emergence of drug-resistant *C albicans* strains.[18] Currently, there are no vaccines available against *C albicans*, and there is an urgent need to develop prophylactic measures, especially for immunocompromised human hosts, as well as alternative forms of treatment.

This study is the first known attempt to produce antibodies against *C albicans* in egg-laying hens. A distinguishing feature is that the egg-laying hen was immunized with a combination of heat-killed germ tubes (the beginning of hyphae) and heat-killed yeast cells. It has been reported that yeast cells disseminate more effectively and hyphae evade macrophage engulfment and invade endothelial and epithelial cells.[18] Since the egg-laying hen was immunized with a combination of yeast cells and hyphae, it could be assumed that post-immunization IgY contained antibodies directed against epitopes found on both forms.

The post-immunization IgY protected mice against lethal challenge with *C albicans.* All post-immunization IgY-protected mice remained alive at 7 days post-challenge, there was no candidal CFU in kidney tissue, and the gross morphology of the kidneys appeared to be normal. In contrast, pre-immunization IgY prolonged the survival of the mice, but did not prevent dissemination of *C albicans,* as indicated by the presence of candidal CFU in kidney tissue

and the lesions observed in the kidneys. The prolongation of survival of mice in this group might be caused by the presence of specific antibodies in yolk prior to immunization, as indicated by the absorbance readings obtained in ELISA, and/or to the presence of nonspecific protective factors. It might be argued that the adjuvants used in the immunization protocol boosted the nonspecific protective factors present in egg yolk, which could be responsible for the protective effect of post-immunization IgY. However, in another study performed in the American University of Beirut laboratory anti-*Aspergillus fumigatus* antibodies were prepared in egg-laying hens using the same immunization protocol. Post-immunization IgY, but not pre-immunization IgY contained anti-*A fumigatus* antibodies and protected mice against lethal challenge (unpublished data).

The activation of the complement system results in the enhancement of phagocytosis and is thought to be one of the factors involved in the protective potential of anti-*C albicans* antibodies.[19] However, it has been reported that IgY antibodies do not activate the mammalian complement system.[20,21] If the antibodies present in post-immunization IgY enhance phagocytosis by macrophages, they do it independent of complement. This concurs with the report of Cline and Lehrer who indicated that the opsonic property of *Candida* immune serum was attributed to the presence of specific IgG, and not to activation of the complement system.[22]

An egg-laying hen was used as a host for harvesting antibodies instead of mammals such as a rabbit or a horse since chicken IgY can be isolated from the egg yolk in a noninvasive manner that surpasses the painful blood-collecting step. Moreover, a chicken is cheap to maintain, and purifying IgY from the yolk is simpler than purifying IgG from mammalian blood. Another important factor to consider is the quantity of IgY produced in a chicken in 1 month is 10 times the amount produced in a rabbit and is comparable to the amounts produced by large animals.[23,24]

The drawbacks of using IgY in humans are similar to that of the use of other heterologous immune sera. Complexes would be formed if anti-IgY antibodies are present in plasma. These complexes might be cleared resulting in the reduction of the protective effect of the immune IgY. Moreover, some individuals are allergic to eggs; however, it has been reported that the allergens are mainly present in the egg white and not the yolk.[25]

The protective effect of post-immunization IgY encourages the idea of using it as a prophylactic agent in individuals at high risk, and possibly as an adjunct to anti-*C albicans* therapy. Additional work to purify the active component of post-immunization IgY and to standardize the dose to be used is in progress.

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