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Short communication

# Active and passive immunization against oral *Candida albicans* infection in a murine model

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**Background/aims:** Clinical and laboratory studies are consistent with a major role for cell-mediated immunity in recovery from oral infection with *Candida albicans*, but the role of humoral immunity remains controversial. The purpose of this study was to establish the relative contributions of cellular and humoral immunity to protection against oral candidiasis in a murine model, and to determine whether host responses could be enhanced by different immunization strategies.
**Results:** Active oral immunization was protective in BALB/c and CBA/CaH mice, reducing both fungal burden and duration of infection after secondary challenge, whereas systemic immunization failed to protect against subsequent oral challenge. *Candida*-specific IgM was the predominant antibody detected in serum following both primary and secondary oral challenge; however, *Candida*-specific salivary IgA was not detectable. Immunization by passive transfer of either lymphocytes or immune serum did not confer any significant protection against oral infection in either susceptible or resistant mouse strain.
**Conclusion:** The data demonstrate a possible role for mucosa-associated immunity following active immunization by the oral route, most likely exerted by local T lymphocytes resident in the oral mucosa, but there was no evidence to support a role for humoral immunity in protection against oral candidiasis.

Key words: immunization; mucosal immunity; oral candidiasis; T cells

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Mucosal infections caused by *Candida albicans* are commonly encountered in medical and dental practice. Clinical presentations include oral infection in patients with dental prostheses, and oral candidiasis in patients with depressed immune defenses, such as those with HIV+/AIDS and transplant recipients. The underlying feature most frequently associated with the various clinical presentations of mucosal candidiasis is an impairment of cell-mediated immune responses. HIV+/AIDS patients, in particular, demonstrate a reduction in $CD4^+$ T cells (20, 29), so it can be inferred that protective responses are dependent on the presence and functionality of $CD4^+$ T lymphocytes. There has been little clinical evidence for a role for humoral immunity in oral candidiasis (13, 22, 24); however, Matthews et al. (26) have speculated that in AIDS patients, spread of the yeast from the oral cavity and dissemination throughout the body may be inhibited by the presence of an antibody to a 47 kDa protein of *C. albicans*.

Most mouse models of candidiasis have focused on the systemic infection, in which the primary effector cell responsible for eradicating the yeast is the neutrophil (18). The role of T cells in recovery from primary infection has not been fully elucidated (2, 7), but they are essential for the development of acquired immunity against the infection (9). However, $CD4^+$ T lymphocytes have been shown to be essential for recovery from primary oral *C. albicans* infection in mice (14). Clearance is mediated by T-cell augmentation of macrophage and neutrophil activity (15). The role of humoral immunity in oral candidiasis is unclear, and most of what is known about protective antibodies to *Candida* has been derived from the systemic model (27, 30, 32).

Attempts have been made to protect against systemic candidiasis by both active and passive immunization, with variable success (1, 28, 30). Ashman & Papadimitriou (4) have shown that passive transfer of serum from mice recovering from initial systemic challenge protected against

intravenous re-infection, but the magnitude of the protective effect was dependent on mouse strain. They postulated that T-cell-mediated augmentation of phagocytosis was the dominant process of recovery during primary infection, whereas antibody produced as a consequence of this exposure was responsible for the protection against systemic re-infection (4).

The purpose of this study was to examine the role of active and passive immunization against an oral *Candida albicans* infection in a murine model.

## Material and methods

### Mice

Specific pathogen-free BALB/c (H-$2^d$) and CBA/CaH (H-$2^k$) euthymic female mice, 6–8 weeks of age, were purchased from the Animal Resources Centre, Perth Australia. These mice undergo routine microbiological screening and do not harbor *C. albicans* in the gut. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Queensland, and were carried out in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 1997. Mice were housed in standard cages, and provided with food and water *ad libitum*.

### Oral infection

*C. albicans* isolate 3630, derived from a patient with cutaneous candidiasis, was obtained from the Australian Medical Mycology Reference Laboratory, and grown in Sabouraud broth for 48 h at room temperature with continuous agitation. Mice were challenged orally with $10^8$ live yeasts in 20 µl phosphate-buffered saline (PBS), delivered by pipette, as described previously (15). The infection was monitored by swabbing the oral cavity on days 1, 4, 8, and 14 with sterile cotton swabs moistened with sterile PBS, and plating on Sabouraud agar plates. Agar plates were incubated for 48 h at 37°C. All inoculation and sampling procedures were conducted under halothane anaesthesia using an inhalation apparatus and a scavenging system. Colony forming units (cfu) were counted on Sabouraud agar plates, and the counts were transformed into scores correlating with the level of recoverable yeast from the oral cavity as described previously (15). The scoring system used was as follows:

0 = No detectable yeasts
1 = 1–10 cfu/plate
2 = 11–100 cfu/plate
3 = 101–1000 cfu/plate
4 = 1000+ cfu/plate.

### Active immunization of mice

Mice were primed either systemically by intravenous injection of $3 \times 10^5$ *C. albicans* in 200 µl PBS or orally with $10^8$ viable yeast cells in 20 µl PBS by pipette, and rested for 4–6 weeks. Primed mice were subsequently challenged orally with $10^8$ *Candida* yeasts, and monitored as described above.

### Passive immunization by adoptive lymphocyte transfer

Immunized donor animals were primed orally or systemically as described above. Spleens were removed from donor mice 4–6 weeks after immunization, and spleen cells were obtained as described previously (16). Naive inbred BALB/c and CBA/CaH mice were intravenously injected with $3 \times 10^7$ lymphocytes from either orally or systemically immunized isogenic animals. After receiving the immune lymphocytes, the mice were challenged orally with $10^8$ yeasts, and monitored as described earlier.

### Passive immunization by serum transfer

Blood was collected by direct heart puncture from mice immunized via the oral route only, 4–6 weeks after immunization. This has been shown to be the time required to elicit appropriate antibody responses against a wider variety of antigenic determinants (Hu et al., submitted). Serum was clarified by centrifugation at 10,000 ***g*** for 15 min at 4°C, and heat inactivated at 56°C for 30 min. Serum from naïve mice was treated in the same manner, and used in control mice. Immune serum 250 µl was injected i.v. into test animals, after which they were challenged orally with $10^8$ *Candida* yeasts 2 h later. Control animals received naïve serum.

### *Candida* antigen preparation and protein estimation

*C. albicans* strain 3630 was incubated in Sabouraud broth for 48 h at room temperature. $10^9$ *C. albicans* cells were pelleted, washed twice with 10 ml sterile water, resuspended in an equal volume of protein extraction buffer, and mixed with an equal volume of 0.4 mm glass beads prewashed in 1 M HCl and coated with Sigmacote (Sigma-Aldrich, St. Louis, MO). Yeast cells were disrupted by vortexing the mixture for 15 min at 4°C, and for a final 1 min after the addition of 0.1% sodium dodecyl sulfate (SDS) to ensure complete cell lysis. The supernatant was clarified, and protein estimation was carried out using the BCA Protein Assay Reagent Kit according to the manufacturer's instructions (Pierce Chemical, Rockford, IL).

### ELISA for *Candida*-specific immunoglobulin in serum and saliva

Peripheral blood was collected by direct heart puncture from mice immunized via the oral route only. Serum was clarified

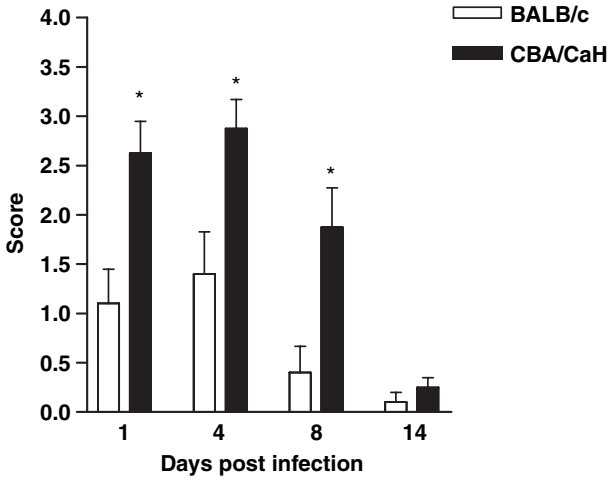


*Fig. 1.* Oral infection in BALB/c and CBA/CaH mice after inoculation with $10^8$ *C. albicans* yeasts. Bars represent scores (mean ± SEM) for a minimum of 10 mice/group. Each experiment was repeated at least twice. Oral infection was more severe in CBA/CaH mice compared to BALB/c (* $P < 0.05$).

by centrifugation at 10,000 ***g*** for 15 min at 4°C, and stored at −20°C until analyzed. Saliva was collected by injecting mice subcutaneously with 20 µg/ml carbachol (carbamylcholine chloride, Sigma-Aldrich) to stimulate salivation. Carbachol has been shown not to affect the concentration of salivary IgA in stimulated saliva, in contrast to pilocarpine (10). Saliva samples were clarified as above and stored with 20 µl of 10 mM phenyl methyl sulfonyl fluoride (Sigma-Aldrich) at −20°C until analyzed.

Serum samples were tested for the presence of *Candida*-specific IgG1, IgG2a, and IgM, while saliva samples were tested for IgA. Briefly, 96-well microtiter plates were coated with *Candida* antigen diluted in borate-buffered saline (50 µl.ml$^{-1}$), and incubated for 2 h at room temperature on a plate shaker. The wells were washed five times with 250 µl/well PBS, and blocked with 200 µl/well 5% fetal calf serum-PBS Tween (FCS-PBST) for 2 h at 37°C. The plates were washed again five times with 200 µl/well PBST. 50 µl/well of appropriately diluted serum or saliva in 1% FCS-PBST was added, and incubated for 2 h at 37°C. Plates were washed again with PBST, followed by the addition of 50 µl/well of horseradish peroxidase-conjugated detecting antibody diluted in 1% FCS-PBST at an optimal concentration (Goat Anti-Mouse IgA(α); 1 : 2000, Goat Anti-Mouse IgG1(γ); 1 : 2000, Goat Anti-Mouse IgG2a(γ); 1 : 2000, Goat Anti-Mouse IgM (µ); 1 : 3000, Caltag Laboratories, Burlingame, CA). The plates were incubated overnight at 4°C, washed with PBST, and developed for 15 min with tetramethylbenzidine-peroxidase substrate (TMB Peroxidase EIA Substrate Kit, Bio-Rad, NSW, Australia) at room temperature. The reaction was stopped by adding 0.5 M $H_2SO_4$, and plates were read at 450 nm, and analyzed using appropriate software (MICROPLATE MANAGER Version 2.0.2, Bio-Rad, Hercules, CA). The titer of antibody (in arbitrary units) was determined as the reciprocal of the dilution that resulted in an $OD_{450}$ of 0.1 units minus the background.

### Statistics

Quantitative data were analyzed using *t*-test and one way analysis of variance (GRAPHPAD PRISM Version 2.01, GraphPad Inc, San Diego, CA).

## Results

### Primary infection in BALB/c and CBA/CaH mice

BALB/c and CBA/CaH mice were inoculated orally with $10^8$ *C. albicans* yeasts and monitored for 14 days. Both strains were colonized with the yeast, although the severity of the infection was significantly greater in CBA/CaH compared to BALB/c mice ($P < 0.05$) at all time points except day 14 (Fig. 1).

### Active immunization

Both the severity and duration of oral colonization were decreased following oral immunization ($P < 0.01$), especially in the more susceptible CBA/CaH strain. The infection in both strains was cleared by day 8 (Fig. 2). Systemic immunization did not protect against oral challenge. In contrast, the severity of the oral infection in BALB/c mice was significantly exacerbated

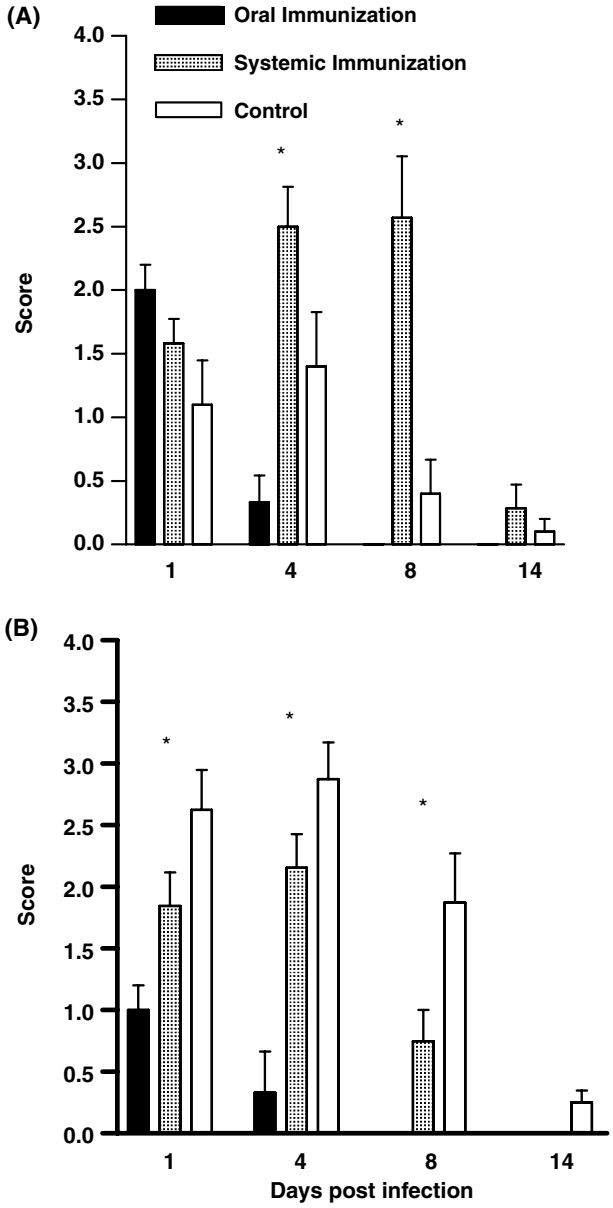


*Fig. 2.* Oral infection after active immunization in BALB/c (A) and CBA/CaH (B) mice. Mice were immunized either orally or systemically, then challenged orally with $10^8$ *C. albicans* yeasts. Bars represent scores (mean ± SEM) for a minimum of 10 mice/group. Each experiment was repeated at least twice. Control mice were not immunized. Oral immunization decreased the severity and duration of infection in both mouse strains, while systemic immunization was non protective, and exacerbated the infection in BALB/c mice (* $P < 0.01$).

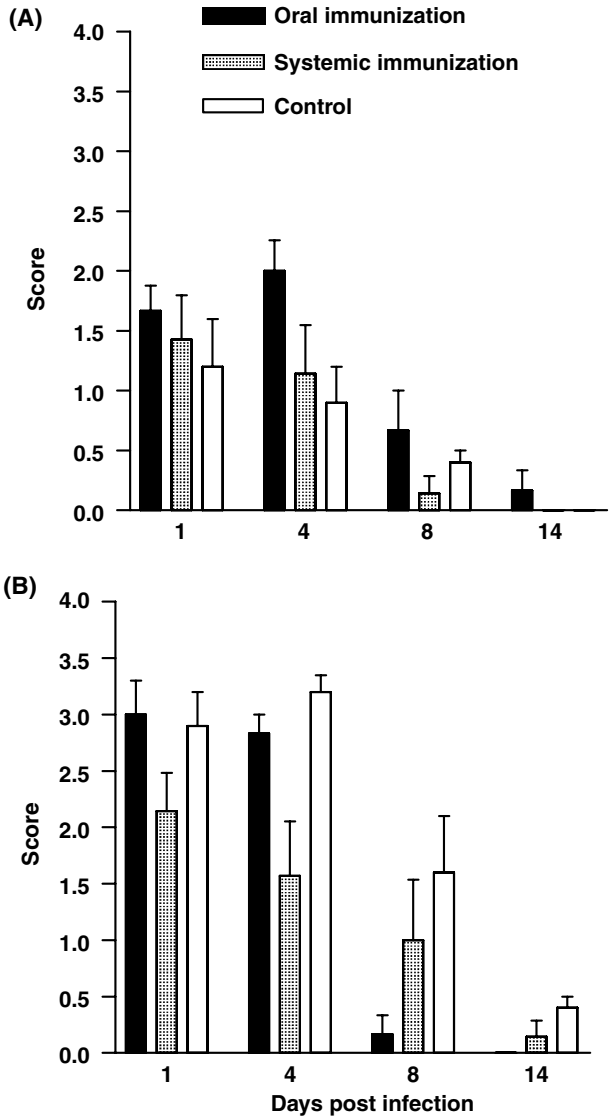


*Fig. 3.* Oral infection after adoptive lymphocyte transfer of BALB/c (A) and CBA/CaH (B) mice with immune spleen lymphocytes taken from donor mice 4–6 weeks after either oral or systemic immunization. Bars represent scores (mean ± SEM) for a minimum of 10 mice/group. Each experiment was repeated at least twice. Control mice received naïve lymphocytes from mice of the same genetic background. Transfer of either orally or systemically immunized lymphocytes had no significant effect on the severity or duration of oral infection in either mouse strain compared to control mice.

compared to control mice ($P < 0.01$) (Fig. 2A).

#### Passive immunization by adoptive transfer of lymphocytes

Neither oral nor systemically primed lymphocytes had any significant effect on the severity or duration of the infection in either mouse strain compared to control mice that received naïve lymphocytes, except on day 8 in CBA/CaH mice ($P < 0.05$) (Fig. 3). There was no difference in the magnitude of oral infection between control mice that received naïve lymphocytes and those that received no lymphocytes, as seen in the primary challenge (data not shown).

#### Passive immunization by serum transfer

Passive transfer of orally immunized serum did not significantly affect the fungal load or the duration of the infection in either mouse strain compared to animals that did not receive serum (Fig. 4).

#### *Candida*-specific immunoglobulin titer after primary and secondary oral challenge

IgM was the predominant antibody class found in BALB/c (500 units on day 4) and CBA/CaH (400 units on day 14) after primary infection. There was some IgG1 present on days 8 (220 units) and 14 (180 units) in BALB/c mice, and days 4 (80 units) and 14 (100 units) in CBA/CaH mice. No IgG2a was detected at any time point in either strain tested. After a secondary oral challenge, IgM was again the predominant antibody detected in the serum of these mice. IgM antibody titers were higher than those observed following the primary infection (1500 units compared with 500 units in BALB/c, and 750 units compared to 400 units in CBA/CaH). IgG1 was detected only in CBA/CaH mice (220 units on day 4, and 650 units on day 14), but IgG2a was found in very small amounts (50–100 units) after secondary immunization in both BALB/c and CBA/CaH mice on day 14. No salivary IgA was detected above baseline levels in either strain following primary or secondary challenge (data not shown).

## Discussion

BALB/c and CBA/CaH mice have been shown to be resistant and susceptible, respectively, to systemic candidiasis (3), and the present study shows that the same pattern holds true for oral candidiasis. The pattern of oral colonization observed in BALB/c mice was similar to that reported previously by our group (14, 16). Histopathological examination of the oral tissues revealed small numbers of yeasts attached to the oral mucosa (16), but hyphal penetration of the epithelium was rare. CBA/CaH mice developed a more severe infection and were more heavily colonized with the yeast compared to BALB/c mice, but there was no difference in the rate of clearance between the two strains. The importance of the genetic background had previously been demonstrated in T-cell-deficient mice (14) in that CBA/CaH nude mice consistently developed a more severe oral infection than BALB/c nude mice.

In the current study, enhancement of host responses against oral candidiasis was achieved by active immunization of mice via the oral, but not the systemic route. Both the severity and duration of oral colonization were decreased following oral immunization, especially in the more susceptible CBA/CaH strain. Elahi et al. (11) have also demonstrated that oral immunization with *Candida* yeasts induced protective immunity. Immunization decreased the fungal load in the oral cavity, although it did not affect the duration of infection. In contrast, neither immunization via intravenous

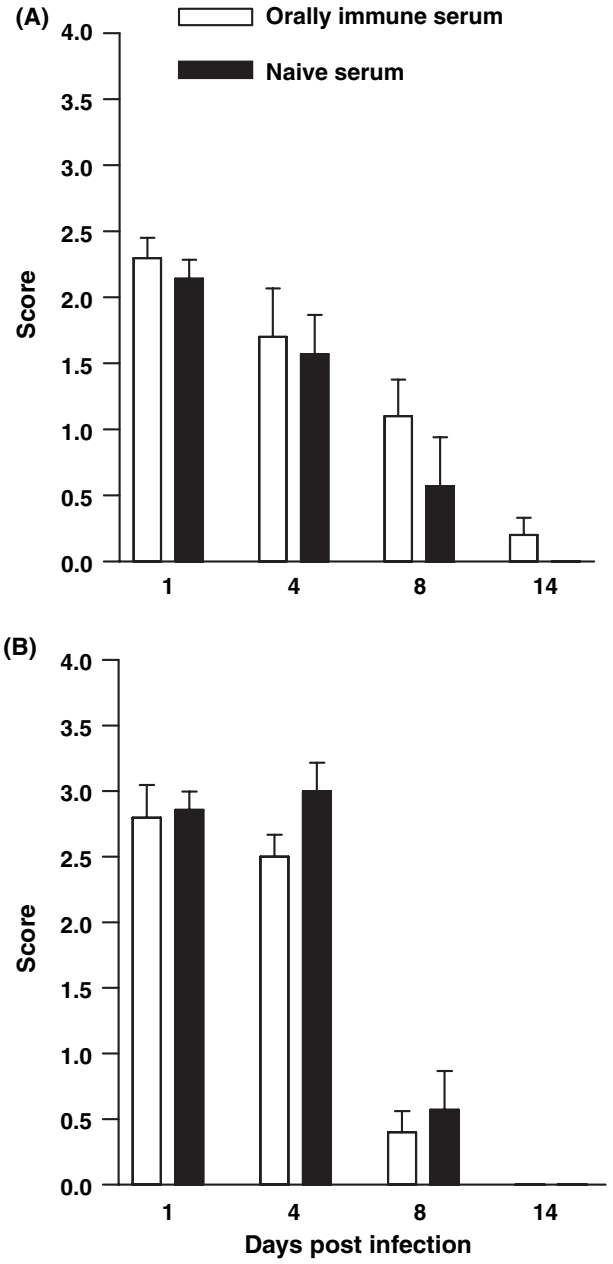


*Fig. 4.* Oral infection after passive immunization of BALB/c (A) and CBA/CaH (B) mice with immune serum from donor mice 4–6 weeks after oral immunization. Bars represent scores (mean ± SEM) for a minimum of 10 mice/group. Each experiment was repeated at least twice. Recipient mice received serum from orally immunized animals, while controls received naïve serum. Passive transfer of orally immunized serum had no significant effect on the severity or duration of oral infection in both mouse strains compared to control mice.

challenge in the current experiments nor via subcutaneous injection as performed by Elahi et al. (11) conferred any protection at the oral mucosal surface. Both these studies suggest a role for local protection conferred by mucosa-associated lymphoid tissue in the clearance of *C. albicans* yeasts from oral cavity. This protection is most likely mediated by T cells resident in the mucosal compartment. $CD4^+$ T cells are present in the lamina propria of these mice (8), and we have previously shown that $CD4^+$ T cells from the systemic circulation can enter the oral tissues in T-cell-deficient mice following reconstitution and exert functional activity (14). Nonetheless, this does not exclude a protective role for $\alpha/\beta$ or $\gamma/\delta$ intraepithelial T lymphocytes (IEL), which are resident in murine oral mucosa and may play a role in the clearance of the yeast from the mucosal surfaces (8, 12, 19). Chakir et al. showed an increased recruitment of $\gamma/\delta$ T cells that coincided with a dramatic decrease in viable *Candida* in the mucosal tissue of mice recovering from the infection (8). Jones-Carson et al. have shown that mice deficient in $\alpha/\beta$ and $\gamma/\delta$ T lymphocytes are more susceptible to mucosal but not systemic candidiasis (19). Some types of IELs are known to possess immunological memory (17), and it is possible that these cells contribute to the rapid eradication of the yeast from the oral tissues by exerting regulatory functions through the secretion of Th1 cytokines (17). The failure of systemic immunization to confer protection on the oral cavity may be related to the circulation patterns of the lymphocytes, which are directed to the systemic rather than the mucosal regions (25).

Oral protection, as determined by *Candida* counts in the saliva of recipient mice, could be achieved by adoptive transfer of mesenteric lymph node cells following intragastric immunization (31), and in light of the active immunization results in the current study, it was expected that lymphocytes isolated from orally immunized mice would be protective against oral challenge in naïve recipients. However, adoptive transfer of immune lymphocytes into naïve recipients failed to confer any significant protection against oral challenge. Neither oral nor systemically immunized lymphocytes had any significant effect on the severity or duration of the infection in either mouse strain. This contrasts with our previous work in T-cell-deficient mice, in which reconstitution with either naïve or immune spleen lymphocytes resulted in the clearance of a chronic oropharyngeal *C. albicans* infection (14).

It is possible that the oral mucosal compartment in immunocompetent mice can not support additional lymphocytes entering the tissues, as previous studies have shown that depletion of lymphocytes in the oral tissues of immunocompetent mice by gamma-irradiation to the head and neck was necessary to demonstrate penetration of $CD4^+$ T cells into the oral cavity (16). Thus, resident intraepithelial T lymphocytes may act to protect the oral tissues in minimally colonized immunocompetent mice, whereas more severe lesions as seen in immunodeficient mice may stimulate recruitment of $CD4^+$ T lymphocytes from the systemic circulation.

In the present study, mice were also passively immunized by transferring immune serum from animals that had recovered from an oral infection into recipients that were subsequently challenged orally. Passive transfer of orally immunized serum conferred no protection against oral challenge in either mouse strain tested. This

is in agreement with other studies showing that transfer of serum from mice immunized intraperitoneally did not lead to oral protection (31). Passively transferred serum from systemically immunized mice did not result in any enhanced protection (Fig. 2), so further studies focused only on oral immunization.

The contribution of humoral immunity to protection against oral candidiasis was assessed by examining *Candida*-specific immunoglobulin production in the sera and saliva of recovering mice. In the present study, IgM was the predominant immunoglobulin detected in the sera of BALB/c and CBA/CaH mice after the primary oral immunization, and to a lesser degree IgG1. No IgG2a was detected in the sera of these mice. A similar pattern was seen after a secondary oral immunization, although levels of IgM and IgG1 were elevated compared to primary immunization, and some IgG2a was seen late in the infection. This is in agreement with levels seen following systemic immunization with *C. albicans* (34).

The significance of salivary IgA in oral candidiasis remains unclear. In this study, salivary IgA was not detected above baseline in either strain of mice following oral immunization. Some workers have reported that salivary antibodies to *C. albicans* were present in high titers in patients with oral candidiasis (6, 13, 21), while others have found reduced titres of *Candida*-specific salivary IgA (5, 24, 23). Salivary IgA has been shown to inhibit adherence of *C. albicans in vitro* to human oral epithelial cells (33); however, it appears that this antibody was not involved in the protection of the oral mucosa against *C. albicans* in our experimental model of infection.

In summary, host responses against oral *C. albicans* infections are enhanced in mice after active oral immunization with the yeast. Induction of an immune response by active immunization via the oral route, but not by systemic immunization, can be protective against *C. albicans* at the mucosal surface. This effect is most likely attributable to local T lymphocytes resident in the oral mucosal tissues. There is no evidence to suggest that humoral immunity plays any role in protection against oral candidiasis in this model of the disease.

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