A Vaccine and Monoclonal Antibodies That Enhance Mouse Resistance to Candida albicans Vaginal Infection

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We previously reported that a vaccine composed of liposome-mannan complexes of Candida albicans (L-mann) stimulates mice to produce protective antibodies against disseminated candidiasis. An immunoglobulin M (IgM) monoclonal antibody (MAb), B6.1, specific for a β-1,2-mannotriose in the complexes protects against the disease, whereas MAb B6 does not. In the present study, the vaccine and MAbs B6.1 and B6 were tested for the ability to protect against Candida vaginal infection, established by intravaginal (i.vg.) inoculation of yeast cells in mice maintained in pseudoestrus. Fungal CFU in each vagina was determined to assess the severity of infection. Mice vaccinated before infection developed about 62% fewer vaginal CFU than nonimmunized controls. Naïve mice that received polyclonal antiserum (from vaccinated mice) i.vg. before infection had 60% fewer CFU than controls. The serum protective factor was stable at 56°C, but C. albicans cells absorbed this factor. Mice given MAb B6.1 i.vg. after infection was established had fewer Candida CFU in vaginal tissue than control mice given buffer instead of antibody. MAbs B6.1 and B6 given intraperitoneally before infection protected mice, but MAbs preabsorbed with yeast cells did not. MAb B6.1 also protected against C. tropicalis vaginal infection, but MAb B6 did not. The protective activities of MAbs B6.1 and B6 appeared to be specific because an irrelevant IgM carbohydrate-specific MAb and an irrelevant IgG protein-specific MAb were not protective; also, MAb B6.1 did not affect development of vaginal chlamydial infection. These studies show that an appropriate antibody response, or administration of protective antibodies, can help the host to resist Candida vaginal infection.

Vaginal candidiasis, a mucosal infection caused by Candida species (39), is one of the most common infections in women (41). An estimated 75% of all females experience at least one episode of the disease during their lifetime (40). In the United States, there are approximately 13 million cases of vaginal candidiasis annually (34). C. albicans is the most common etiologic agent (14, 22), but other Candida species such as C. tropicalis, C. glabrata, and C. parapsilosis also cause the disease (22, 30).

Topical and/or oral administration of antifungal drugs is used for the prevention and treatment of vaginal candidiasis (2, 5, 41). In otherwise healthy individuals, however, antifungal drugs are used after the onset of disease; thus, these patients must suffer symptoms before seeking therapy, and in some the disease will recur after discontinuation of the drug (22, 30). Newly developed triazoles have been beneficial in prevention and treatment of candidiasis; however,azole-resistant strains of C. albicans are emerging (9, 36, 42), and prolonged preventive use of antifungal drugs in healthy individuals is unwarranted. These problems led us to consider alternative preventive and therapeutic approaches.

Host immunological defenses that protect against Candida vaginal infection are not well defined and may involve both cell-and antibody-mediated mechanisms. Vaginal immunization with C. albicans protected pseudoestrous mice against experimental vaginal infection (11), and local cell-mediated immunity may have a role in host defense against this condition (16). The role of a specific antibody in host defense against Candida vaginitis is not well understood because patients with this condition are likely to have antibodies of various isotypes in vaginal secretions (15, 31, 37). Cassone et al. (8) showed, however, that antibodies, apparently against mannans and secretory aspartyl proteinases of C. albicans, mediated protection against infection in ovariectomized and estrogen-treated rats.

In previous work we focused on the role of antibodies to Candida in host defense against disseminated candidiasis. Vaccination with liposome-encapsulated C. albicans surface mannan (L-mann) provoked a protective antibody response against disseminated disease due to either C. albicans or C. tropicalis (16). A monoclonal antibody (MAb), B6.1, specific for the Candida mannan, enhanced resistance of normal and SCID mice against disseminated candidiasis (16) and had a protective effect in neutropenic mice (17). A second MAb, B6, did not show protective activity (16, 17). Both MAbs are immunoglobulin M (IgM), and both agglutinated yeast cells (16). MAb B6.1 is specific for a β-1,2-mannotriose (18), which is an acid-stable part of the complex (unpublished data).

In this study, we tested the ability of the L-mann vaccine and the MAbs to enhance resistance of mice to Candida vaginal infection. All of these reagents showed protective effects.

MATERIALS AND METHODS
Organism and culture conditions. C. albicans CA-1, previously characterized as a serotype A strain by use of rabbit anti-C. albicans serum developed by Hasenclever et al. (19, 20), is a serotype B strain according to the Candida-Check system (Iatron Laboratories Inc., Tokyo, Japan). C. tropicalis CT-4 is from our stock culture collection. Species classification was confirmed by API 20C yeast identification strips (BioMerieux Vitek, Inc., Hazelwood, Mo.), and this strain was used in a previous study (16). Stock cultures were stored at −20°C. New yeast suspensions were started each week from the stock cultures and grown as hydrophobic stationary-phase yeast cells in glucose-yeast extract-peptone broth at 37°C as previously described (21). Yeast cells were harvested from the broth cultures by centrifugation, washed in cold (0 to 4°C) sterile deionized water, suspended to the desired yeast cell concentration in cold sterile Dulbecco's

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phosphate-buffered saline (DPBS; Sigma Chemical Co., St. Louis, Mo.), and used immediately to infect the test mice. Mice. BALB/c female mice obtained from either The Jackson Laboratory (Bar Harbor, Maine) or Charles River Laboratories (Kingston, N.Y.) were used at 6 to 9 weeks of age. Three days before infection with C. albicans, each mouse received a subcutaneous (s.c.) injection of estradiol valerate (0.5 mg per mouse; Sigma) suspended in sterile sesame oil (Sigma) to induce pseudoestrus (12, 13). Chlamydia infection experiments are described below. In all experiments, mice were maintained in accordance with institutional regulations in an Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility at Montana State University (MSU).

MAbs. MAbs B6.1 and B6 were isolated and characterized as previously described (16). They were produced in serum-free medium (HB101) that was concentrated and prepared as 0.5 and 0.2 ml, respectively. In addition to the various MAb preparations, the starting concentration of MAb S9 was estimated at 4.6 mg/ml. An irrelevant IgG MAb, termed 54.1 and specific for human neutrophil cytochrome b (4) (a generous gift from Jim Burritt and Al Jesaitis, MSU), was used as an additional negative control at a starting concentration of 5.0 mg/ml, as estimated by the above methods. MAb S9 and S4.1 were used in experiments at the same concentrations as MAbs B6.1 and B6.

All antibody preparations were tested for the presence of endotoxin contamination by the Limulus amebocyte lysate test (E-Toxate kit; Sigma). This test was performed according to the manufacturer's instructions. L-mann vaccine. The L-mann vaccine was prepared as previously described (16). In brief, a C. albicans cell surface mannan-enriched fraction was isolated by a β-mercaptoethanol treatment of yeast cells and encapsulated into multilamellar liposomes. Control liposomes (L-DPBS) were prepared by adding diluent buffer (DPBS) instead of mannan during the preparation as previously described (16). Control and test liposomes were identical with respect to microscopic appearance.

Passive transfer of active immunization. Before vaginal infection with C. albicans, mice were immunized with the L-mann vaccine by intravenous (i.v.) injection once a week for a total of 5 weeks as previously described (16). Each injection consisted of 0.2 ml of L-mann, which contained 178 μg of the enriched mannan fraction. Control mice received L-DPBS or buffer (DPBS) alone. Four days after the fifth immunization, all mice were given estradiol s.c. Three days later, they were infected with C. albicans (5 × 10⁵ yeast cells) intravaginally (i.v.g.) by gentle insertion of a thin micropipette tip into the vaginal tract and delivery of 10 μl containing the desired number of the yeast cells. Two days after the infection, the animals were sacrificed and CFU of vaginal tissue was determined as indicated below.

Passive transfer of PABs to determine if antibodies in the sera from vaccinated mice could be used to protect the vaccinated mice from vaginal infection. Polyclonal antisera (PAs) were obtained from L-mann-vaccinated mice and pooled. The pooled PAb had an agglutinin titer of 40 to 80 (16) and was either immediately stored at –20°C, heated at 56°C for 30 min, and stored at –20°C or absorbed with 50 ml of C. albicans yeast cells and stored at –20°C. A control sample was taken by carrying out an additional control, one mouse group received normal mouse serum (NMS) obtained from mice that were given only DPBS. Three days after being given estradiol s.c., naive mice were divided into groups of four; each group received one of the following treatments (30 μl per mouse): 6) intraperitoneal antibody given by the i.p. route as described above, but these animals were not infected with C. albicans. The amount of antibody given to mice was usually the same as indicated above, but some groups of mice received a 16-fold-higher dose of MAbs B6.1 before vaginal lavage. The vaginal lavage fluid from each animal was collected by washing the vagina with sterile DPBS (400 μl in total) 4 h after the infection. The wash fluid was used to determine relative susceptibility or resistance to vaginal infection, the number of yeast cells was collected by vaginal lavage with cold DPBS (400 μl per mouse), and the vaginal wash swab was placed on blood agar plates and incubated for 24 h at 37°C. In brief, a C. albicans cell surface mannan-enriched fraction was isolated by a β-mercaptoethanol treatment of yeast cells and encapsulated into multilamellar liposomes. Control liposomes (L-DPBS) were prepared by adding diluent buffer (DPBS) instead of mannan during the preparation as previously described (16). Control and test liposomes were identical with respect to microscopic appearance.

Passive transfer of MAbs by the i.v. or intraperitoneal (i.p.) route. The preventive effects of MAbs B6.1 and B6 were examined by the same injection schedules as above for experiments on PAs. Intravaginal doses of either MAb for the first (i.e., 4 h before infection) and second (i.e., 20 h after infection) treatments were 35 and 10 μg per mouse in volumes of 30 and 10 μl, respectively. Control mice received equivalent volumes of the DPBS diluent.

The therapeutic effect of MAbs B6.1 was also tested by the i.v. route. Mice were given estradiol s.c., and 3 days later these pseudoestrus mice were infected with C. albicans (5 × 10⁵ yeast cells/mouse) i.v.g. At 4 and 24 h after infection, the animals were given 35 and 10 μg, respectively, of MAb B6.1. Forty-eight hours after infection, the vaginal CFU were measured and evaluated as indicated below.

In some experiments, groups of mice received the antibody or diluent i.p. instead of i.v.g. In this test, the antibodies were given again in two doses at 4 h before and 20 h after infection, as described above and previously for experimental disseminated candidiasis (16). The doses of antibodies for the first and second doses were approximately 100 and 40 μg respectively. In addition to the various MAbs preparations, the hybriDoma serum-free medium (HHI101) that was concentrated and prepared as for antibody production was tested for its effect on vaginal infection. The material contained was collected by washing the vagina with sterile DPBS. The cells were suspended in 200 μl of cold DPBS. The washed yeast cell pellet from the first wash was suspended in 50 μl of goat anti-mouse IgM (μ-specific), 10 μg/ml; Sigma, St. Louis, Mo.) in a microcentrifuge tube. The mixture in the tube was plunged into ice and incubated for 30 min. The fungal cells were washed three times in 1 ml of cold DPBS, suspended in 500 μl of fluorescein isothiocyanate-conjugated rabbit anti-goat IgG (anti-whole molecule; 3 mg/ml; Sigma, diluted 1:200 in DPBS; Sigma), incubated in an ice bath for 30 min and washed three times as described above. Evidence of antibody on the fungal cell surface was determined by examination of the cells by confocal fluorescence microscopy (Nikon DVC-250 scanning confocal argon-krypton laser [488-nm excitation wavelength] microscope, equipped with a 60× oil immersion lens, numerical aperture 1.4, as obtained from Bio-Rad, Hercules, Calif.).

In other experiments, vaginal fluid was collected from mice that received MAbs B6.1 or B6 by the i.p. route as described above, but these animals were not infected with C. albicans. The amount of antibody given to mice was usually the same as indicated above, but some groups of mice received a 16-fold-higher dose of MAbs B6.1 before vaginal lavage. The vaginal lavage fluid from each animal was collected by washing the vagina with sterile DPBS (400 μl in total) 4 h after the infection. The wash fluid was used to determine relative susceptibility or resistance to vaginal infection, the number of yeast cells was collected by vaginal lavage with cold DPBS (400 μl per mouse), and the vaginal wash swab was placed on blood agar plates and incubated for 24 h at 37°C. In brief, a C. albicans cell surface mannan-enriched fraction was isolated by a β-mercaptoethanol treatment of yeast cells and encapsulated into multilamellar liposomes. Control liposomes (L-DPBS) were prepared by adding diluent buffer (DPBS) instead of mannan during the preparation as previously described (16). Control and test liposomes were identical with respect to microscopic appearance.

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were added to the wells, which were then incubated at RT for 1 h and washed as described above. The substrate was prepared by dissolving 25 mg of o-phenylenediamine (Sigma) in 25 ml of 0.1 M citric acid solution (pH 5.0) and then adding 10 \( \mu l \) of 30% hydrogen peroxide. One hundred microliters of substrate was added to each well, the plate was incubated at RT for color development, the reaction was stopped by addition of 100 \( \mu l \) of 10% HSO\(_4\) per well, and the results were read at 490 nm in a microtiter plate reader (model 450; Bio-Rad). As a positive control, MAb B6.1 was used under conditions identical to those described above. A known amount of MAb B6.1 (23.5 \( \mu g \)) was serially diluted in TBS-BSA to determine the sensitivity of the ELISA method for antibody detection.

Statistical analyses. In all cases, the number of animals per group was 5, and averages and standard errors were computed from independent observations run at least two times. Statistical significance of differences between test and control groups was determined by Student’s \( t \) test.

RESULTS

Depending on the route of treatment, i.v. or i.p., the final vaginal Candida CFU count varied. For each experimental protocol, however, the numbers of CFU were highly reproducible within each group. Furthermore, the vaccine and antibodies had a similar magnitude of effect on CFU values.

Prophylaxis potential of the L-mann vaccine against Candida vaginal infection. L-mann-vaccinated mice showed greater resistance to vaginal infection than DPBS control mice (Fig. 1). Vaccinated animals challenged with the yeast cells had \((110 \pm 38) \times 10^3\) CFU/g of vaginal tissue, while mice that received DPBS instead of the vaccine had \((240 \pm 44) \times 10^3\) CFU/g (mean ± standard error). The difference between vaccinated and unvaccinated mice was significant \((P < 0.01)\). The vaginal count from L-DPBS control mice was \((280 \pm 38) \times 10^3\) CFU/g, which was slightly higher than that from DPBS controls, but the differences were not significant \((P > 0.3)\).

Polyclonal antiserum given i.v.g. transfers enhanced resistance. Pooled PAbs from L-mann-vaccinated mice enhanced resistance of mice against vaginal infection (Fig. 2). Treatment of mice with unheated PAb, heated PAb (H-PAb), C. albicans-absorbed PAb (A-PAb), or NMS (negative control) i.v.g. and challenge with C. albicans i.v.g. resulted in CFU per gram of vaginal tissue of \((250 \pm 24) \times 10^4\) for PAb, \((260 \pm 37) \times 10^4\) for H-PAb, \((740 \pm 120) \times 10^4\) for A-PAb, and \((690 \pm 150) \times 10^4\) for NMS (mean ± standard error). When the CFU values for both PAB- and H-PAB-treated mice were compared to values for NMS-treated control mice, the differences were significant \((P < 0.01)\). Antiserum preabsorbed with C. albicans yeast cells did not confer resistance (Fig. 2).

MAb B6.1 given i.v.g. has both preventive and therapeutic effects. MAb B6.1 or B6 was given i.v.g. as described above (i.e., before and after infection with yeast cells) to test for a preventive effect (Fig. 3A) or only after an i.v.g. infection to test for a therapeutic effect (Fig. 3B). Mice given MAb B6.1 or B6 before infection had, respectively, about 90 and 65% fewer vaginal CFU than DPBS control mice. The differences in CFU between MAb B6.1-treated or B6-treated mice and the control animals were statistically significant \((P < 0.001\) and \((P < 0.01\), respectively) (Fig. 3A). Mice given MAb B6.1 therapeutically at 4 and 24 h after infection developed 48% fewer CFU than DPBS control animals \((P < 0.02)\). MAb B6 did not have a therapeutic effect at a dose either comparable to that of MAb B6 or 16-fold higher than that of MAb B6.
B6.1 (Fig. 3B) or 16-fold higher (data not shown). In fact, CFU values obtained after therapeutic application of MAb B6 were higher than CFU from DPBS control mice ($P < 0.05$) (Fig. 3B).

**MAb B6.1 given i.p. enhances resistance against vaginal infection due to *C. albicans* and *C. tropicalis***. Mice that received MAb B6.1 i.p. before and after infection with *C. albicans* developed 91% fewer CFU than DPBS control mice ($P < 0.001$) (Fig. 4A). In addition, enhanced resistance against vaginal infection was noted in mice that received either PAb from vaccinated mice or MAb B6. These animals developed 43 and 55%, respectively, fewer CFU compared to DPBS controls. Neither of the two irrelevant MAbs, S9 and 54.1, nor the hybridoma culture medium, HB101, had an effect (Fig. 4A).

**DISCUSSION**

The L-mann vaccine, which was previously shown to induce protective responses in mice against disseminated candidiasis (16, 17), caused enhanced resistance against experimental vaginal infection in mice. The prevailing belief is that Th1-type responses are important in the host defense against vaginal candidiasis (35). Cassone and workers have observed that antitinnan antibodies given i.v.g. to rats enhance their resistance to vaginal infection (8). We confirmed this observation in the mouse model of *Candida* vaginal infection. Our finding that serum from immunized animals conferred protection when given i.p. was, however, surprising because the predominant protective antibody response in vaccinated mice is IgM (reference 16 and our unpublished observations). This observation was strengthened by the finding that the protective factor in the polyclonal immune serum is removed by preabsorption with *C. albicans* yeast cells. Furthermore, MAbs specific for *Candida* mannann epitopes are also effective in enhancing resistance against vaginal infection when given i.p. or i.v.g. As in previous studies on disseminated candidiasis (16, 17), MAb B6.1 was effective in protecting naive animals against vaginal infection. This antibody had protective activity when given before infection and therapeutic activity when given after infection. MAb B6.1 is specific to a 1,2-linked mannotriose, which is an acid-labile component of the phosphomannann complex of *C. albicans* (18). This epitope appears to be a major surface marker, as indicated by confluential distribution patterns revealed by immunofluorescence (17), electron microscopy, and relative abundance data obtained by gel electrophoresis (our unpublished findings). MAb B6.1 protected mice against
vaginal infection due to *C. tropicalis* (Fig. 4), which was expected because this species produces a β-1,2-mannantriose as part of its phosphomannan complex (25) and the antibody also protected animals against disseminated disease (16, 17).

Although MAb B6 was not effective in prevention of disseminated candidiasis (16), it could enhance resistance against vaginal infection, albeit not to the same extent as MAb B6.1. MAb B6 did not, however, protect mice against vaginal infection due to *C. tropicalis* even though the antibody reacts with this species. MAb B6 is specific for an acid-stable component of the phosphomannan complex (reference 17 and our unpublished data), but epitope distribution is patchy on the cell surface and appears less concentrated than the B6.1 epitope (18). A possible explanation for the protective activity of MAb B6 against vaginal infection is that the B6 epitope is more exposed due to lowered expression of the acid-labile β-1,2-oligomannosyl residues. In agreement with this hypothesis is the finding that expression of these acid-labile components is reduced when fungal cells are grown in an acidic environment (24, 26). Removing Candida cells from the vaginal tract of infected animals and assessing expression of the B6 and B6.1 epitopes will test this hypothesis.

Since MAb B6 enhanced resistance to vaginal infection, it did not serve as a negative control for these studies. Additional controls were thus added to ensure that protection due to MAbs B6.1 and B6 was specific. First, none of the preparations had detectable endotoxin contamination by the *Limulus* amebocyte lysate test (<0.15 ng/ml). Second, uninoculated hybridoma growth medium, which was concentrated in the same way as the MAb preparations, did not have protective activity. Third, the protective activity of both antibodies was removed by preabsorption with *Candida* yeast cells. Fourth, an irrelevant IgM MAb, specific for a group B streptococcal polysaccharide and prepared exactly like MAbs B6.1 and B6, was not protective. Fifth, neither MAb B6.1 nor MAb B6 protected mice against vaginal infection due to *C. trachomatis*. For this latter experiment, susceptibility to disease was heightened by pretreatment of the mice with progesterone, rather than estradiol as used in the Candida infection model, but the antibodies were ineffective nonetheless.

The mechanism(s) by which the vaccine and MAbs B6.1 and B6 protect against vaginal infection is unknown. Active immunization of mice may well lead to general stimulation of the immune system, but the protective value of immune sera indicated that protective antibodies were produced. In our studies on disseminated candidiasis, MAb B6.1 promoted in vitro neutrophil candidacidal activity (6). Extrapolation of these observations to the in vivo situation may make sense for host defense against disseminated candidiasis but not for resistance against vaginal infection. Hematoxylin-and-eosin-stained tissue sections of infected vaginal tissue did not reveal the presence of many neutrophils (our unpublished findings), and others have provided evidence that these phagocytes may not alter the outcome of experimental vaginal candidiasis (1). In our studies, the vaccine was given i.v. and the MAbs were given either i.v. or i.p. As alluded to above, protection by the i.v.g. route is not surprising, as others have shown that this route is an effective way to protect rats against *Candida* vaginal infection (8). An explanation of protection induced by vaccination is complex, as plasma and secretory antibodies and cell-mediated immune responses may participate in the host defense. We have chosen, instead, to focus on mechanisms by which protective antibodies may be administered i.p. yet exert their effect on the vaginal epithelium.

Protection of mice against vaginal infection by i.p. administration of antibody was an unexpected result. The implication is that MAbs B6.1 and B6 are somehow transported from the peritoneal cavity to the vaginal epithelial surface, but we have thus far failed to find the antibodies in vaginal lavage fluids. We are currently considering the possibility that the antibodies are found in subsurface locations within the vaginal epithelium.

The role of antibodies in host defense against fungal diseases is worth further investigation (7). Strong evidence for protective antibodies against cryptococcosis has been demonstrated (29), and a role for antibodies against blastomycosis has been suggested (23). The important point related to our work is that the presence of *Candida*-specific antibodies in the sera or on the vaginal epithelium of patients with candidiasis does not imply that antibodies are not protective. Indeed, as others have found with experimental cryptococcosis (10, 28, 43), the most important considerations may be the titer of the appropriate specific antibody and the antibody isotype.

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