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.v.g.) 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.v.g. 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.v.g. 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 preventative use of antifungal drugs in healthy individuals is unwarranted. These problems led us to consider alternative preventative 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 pseudoeastrogen 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 defined. Women 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 mannan 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-labile component in the phosphomannoprotein complex of the cell wall (38). MAb B6 is specific for a mannan epitope in the 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 hydrophilic 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...
to vaginal infection due to \textit{C. tropicalis} was also determined. Dosages of antibodies and injection schedules prior to infection were identical to those for the above experiments except that each animal was infected i.v.g. with 10^7 \textit{C. tropicalis} yeast cells in a 10-μl volume.

**Assessment of resistance and susceptibility to \textit{Candida} vaginal infection.** To determine the relative susceptibility of to vaginal infection, the number of \textit{Candida} CFU per gram of vaginal tissue was determined as previously described for kidney CFU measurements (16, 17, 33). In brief, the entire vagina was removed from each infected mouse, weighed, and homogenized in 1 ml of DPBS with a hand-held glass tissue homogenizer. Appropriate dilutions of the vaginal tissue homogenates were plated onto Mycobiotic agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 48 h later, \textit{Candida} colonies were counted. A minimum of four mice per group was tested, and each experiment was repeated at least three times.

**\textit{C. trachomatis} genital infections.** As an additional test for specificity, MAbs B6.1 and B6 were tested for protective effects against \textit{C. trachomatis} infection in mice. Vaginal infection with the mouse pneumonitis \textit{C. trachomatis} strain was performed as described previously (27), with the following modifications. Three groups of five mice received 2.5 mg of Dep-provera (medroxy-progesterone acetate) subcutaneously at 10 and 3 days prior to vaginal infection. Four hours before and 20 h after infection, mice were injected i.p. as described above with either MAAb B6.1, MAAb B6, or DPBS. The course of infection was monitored by swabbing the vaginal tract at various times after infection and enumerating inclusion-forming units by isolation on HeLa cell monolayers. Inclusions were visualized by indirect immunofluorescent staining (44).

**Protection of antibody in vaginal fluid.** Mice received MAAb B6.1 or B6 by the i.p. route and were infected i.v.g. as described above. Control mice received DPBS instead of antibody. For immunofluorescence detection of antibody, vaginal fluid containing Candida cells was collected by vaginal lavage with cold DPBS (400 μl per mouse) 4 h after the second dose of antibody. The \textit{C. albicans} cells present in the vaginal fluid were washed twice with 400 μl of cold DPBS. The washed yeast cell pellet from the second wash was suspended in 50 μl of goat anti-mouse IgG (μ-chain specific; 1:200 dilution) in 200 μl of PBS. Sigma) 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; 3.0 mg/ml; 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 MAAb B6.1 or B6 by the i.p. route as described above, but these animals were not infected with \textit{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 MAAb 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 last dose of antibody. The course of infection was monitored by swabbing the vaginal tract, and the number of \textit{C. albicans} was clarified by centrifugation at 300 × g. In some cultures, the 400-μl vaginal wash was concentrated to approximately 50 μl by use of a C30 concentrator (Pierce, Rockford, Ill.) and centrifugation at 5,000 × g for 20 min. Forty microliters of each preparation was mixed with 10 μl of a 10^-4 m dilution of MAb B6.1 and B6 were tested for protective effects against \textit{C. trachomatis} infection in mice. Vaginal infection with the mouse pneumonitis \textit{C. trachomatis} strain was performed as described previously (27), with the following modifications. Three groups of five mice received 2.5 mg of Dep-provera (medroxy-progesterone acetate) subcutaneously at 10 and 3 days prior to vaginal infection. Four hours before and 20 h after infection, mice were injected i.p. as described above with either MAAb B6.1, MAAb B6, or DPBS. The course of infection was monitored by swabbing the vaginal tract at various times after infection and enumerating inclusion-forming units by isolation on HeLa cell monolayers. Inclusions were visualized by indirect immunofluorescent staining (44).

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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 μl 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 μl of 10% H2SO4 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 μg) 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.vg. 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 magnitudes 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 ± 38) × 105 CFU/g of vaginal tissue, while mice that received DPBS instead of the vaccine had (240 ± 44) × 105 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 ± 38) × 105 CFU/g, which was slightly higher than that from DPBS controls, but the differences were not significant (P > 0.3).

Polyclonal antiserum given i.vg. 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.vg. and challenge with C. albicans i.vg. resulted in CFU per gram of vaginal tissue of (250 ± 24) × 104 for PAb, (260 ± 37) × 104 for H-PAb, (740 ± 120) × 104 for A-PAb, and (690 ± 150) × 104 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.vg. has both preventive and therapeutic effects. MAb B6.1 or B6 was given i.vg. 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.vg. 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

FIG. 1. Vaccination with L-mann enhances protection of mice against vaginal infection. Pseudoestrous mice were vaccinated with L-mann i.v. and challenged with C. albicans (5 × 107 yeast cells) i.vg. Control mice were given L-DPBS or DPBS (diluent) only. Vaginal CFU per gram of tissue were determined by plating on Mycobiotic agar. The L-mann-vaccinated mice developed approximately 62% less CFU than mice that received DPBS (P < 0.01). CFU from L-DPBS control-vaccinated mice were slightly higher than CFU from DPBS controls, but the difference was not statistically significant (P > 0.3). Bars denote standard errors.

FIG. 2. PAb protects mice against Candida vaginal infection. PAb from L-mann-vaccinated mice was administered to pseudoestrous mice i.vg. before and after an i.vg. challenge with C. albicans. The resulting vaginal CFU were compared with CFU from animals H-PAb, A-PAb, or NMS. Mice given the unheated or heated PAb had 60% fewer CFU than animals that received NMS (P < 0.01). Mice that received the absorbed serum developed almost the same number of CFU as the NMS groups. Bars denote standard errors.

FIG. 3. MAb B6.1 given i.vg. has preventive and therapeutic effects. Pseudoestrous mice were given MAb B6.1 i.vg. either before and after an i.vg. challenge with yeast cells (A) or only after infection (B). Mice given MAb B6.1 and those given MAb B6 before and after infection developed about 90 and 65%, respectively, fewer CFU than DPBS control mice (P < 0.001 and P < 0.01, respectively) (A). MAb B6.1 also reduced by 48% the CFU in animals in which vaginal infection was established before MAb treatment (P < 0.02), whereas MAb B6 did not show this therapeutic effect (B), even at a 16-fold-higher dose (data not shown). Bars denote standard errors.
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 control mice. Neither of the two irrelevant MAbs, S9 and 54.1, nor the hybridoma culture medium, HB101, had an effect (Fig. 4A).

The experiment was done as an additional control for vaccine antigenicity (16). Neither MAb B6.1 nor MAb B6 had any effect on the course of a chlamydial vaginal infection. The numbers of infectious chlamydial cells shed throughout the course of infection and the duration of infection were not different among antibody-treated and control groups (data not shown).

**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 T1-type responses are important in the host defense against vaginal candidiasis (35). Cassone and workers have observed that antimannan antibodies given i.v. 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* mannan epitopes are also effective in enhancing resistance against vaginal infection when given either i.v. or i.p.

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 β-1,2-linked mannotriose, which is an acid-labile component of the phosphomannan complex of *C. albicans* (18). This epitope appears to be a major surface marker, as indicated by confluent 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. tropicais (Fig. 4), which was expected because this species produces a β-1,2-mannotriosse 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. tropicais 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 MAb 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 hybridomas were treated 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 indicates 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 (40, 28, 43), the most important considerations may be the titer of the appropriate specific antibody and the antibody isotype.

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