Inducible Immunity to \textit{Trichomonas vaginalis} in a Mouse Model of Vaginal Infection

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We studied the protective effect of subcutaneous immunization with \textit{Trichomonas vaginalis} in a mouse model of vaginal infection. BALB/c mice were immunized with various doses of \textit{T. vaginalis} ($4.5 \times 10^5$, $9 \times 10^6$, and $1 \times 10^7$ organisms per ml) suspended in Freund’s complete adjuvant 56 days prior to vaginal infection and were given booster injections of the same doses of \textit{T. vaginalis} in Freund’s incomplete adjuvant 4 weeks later. Control mice were immunized and given booster injections of phosphate-buffered saline suspended in Freund’s complete and incomplete adjuvants. The mice were tail bled and vaginal washes were performed at weekly intervals for 4 weeks to determine the isolation of \textit{T. vaginalis} and the serum and vaginal antibody reactivity. Mice which had been immunized and given booster immunizations had significantly fewer intravaginal infections and had increased serum and vaginal antibody responses compared with those of control mice ($P < 0.01$). Mice that were vaginally infected, treated with metronidazole, and then reinfected vaginally did not develop protective immunity. Subcutaneous immunization with whole \textit{T. vaginalis} organisms appears to confer protection against intravaginal challenge with \textit{T. vaginalis}, protection which is not achieved as a result of prior vaginal infection.

\textit{Trichomonas vaginalis} is a protozoan parasite that afflicts an estimated 5 million women in the United States (24), and 180 million people worldwide (7, 8, 44) are infected annually. The disease may range from asymptomatic to severe, and the pathogenic factors associated with the disease presentation are not well understood (26, 38). An increased predisposition to disease may range from asymptomatic to severe, and the estimated 5 million women in the United States (24), and 180 million people worldwide (7, 8, 44) are infected annually. The role of cell-mediated immunity in human trichomoniasis is not clearly understood. Previous exposure to \textit{T. vaginalis}, unlike infection with \textit{Giardia lamblia}, does not appear to confer protection. Repeated infections with \textit{T. vaginalis} may occur without a significant decrease in either the duration of infection or the intensity of symptoms as might be expected in the presence of a specific immune response (1, 23).

Serologic tests have confirmed the presence of specific anti-\textit{T. vaginalis} immunoglobulin M (IgM), IgG, and IgA, but with no specific protective role being identified to date (10, 18, 40, 41, 45). Although antitreichomonal antibody has been demonstrated in human cervicovaginal secretions by various immunological methods (31, 37, 41, 42, 46), there is no evidence that local vaginal immunoglobulins play a role in protection from \textit{T. vaginalis} infection. The role of cell-mediated immunity in vivo has also not been well defined (30, 46).

In this report, we demonstrate that it is possible to achieve significant protection against \textit{T. vaginalis} infection with subcutaneous immunization with whole-cell \textit{T. vaginalis} in a mouse model of vaginal \textit{T. vaginalis} infection.

\textbf{MATERIALS AND METHODS}

\textbf{Strains.} \textit{T. vaginalis} isolates obtained from vaginal secretions of women with acute vaginitis were grown as previously described (19) in 10 ml of Diamond’s TYI-S-33 medium (TYI), pH 6.2 (14), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories, Life Technologies Inc., Grand Island, N.Y.), 10 U of penicillin per ml, 100 \(\mu\)g of streptomycin per ml (penicillin-streptomycin solution, Gibco Laboratories), and 2.5 \(\mu\)g of amphotericin B (Gibco Laboratories) per ml and incubated in 5\% CO\textsubscript{2} at 37\°C. Cultures were passaged every 2 to 3 days. A well-characterized isolate, 263 (17), recovered from a woman with symptomatic vaginitis was used for these experiments.

\textit{Lactobacillus acidophilus} ATCC 4356 was purchased from the American Type Culture Collection, Rockville, Md., and cultured in Bacto Lactobacilli MRS broth (Difco Laboratories, Detroit, Mich.) or on MRS plates (Bacto Lactobacilli Culture Collection, Rockville, Md., and cultured in Bacto Lactobacilli MRS plates, which were incubated at 37\°C. Cultures were passaged every 2 to 3 days.

\textbf{Animals.} BALB/c mice (22 to 24 g) utilized in this study were obtained from the Charles River Co., Montreal, Quebec, Canada.

\textbf{Intravaginal inoculation of mice.} (i) \textit{L. acidophilus}. On day −9, 2 days prior to inoculation with lactobacilli, all mice received a subcutaneous injection of 0.050 ml of estradiol valerate (Delestrogen; 10 mg/ml; Squibb Canada, Montreal, Quebec, Canada) (11, 35). Immediately prior to inoculation with lactobacilli, the estrus cycle of each mouse was defined as previously described (16).

A 1-liter volume of MRS broth was inoculated with 0.1% of a pure culture of \textit{L. acidophilus} and incubated overnight at 37\°C in 5\% CO\textsubscript{2}. Organisms were then harvested by centrifugation for 10 min at 5,000 × g at 4\°C in an Omnifuge RT (Baxter, Canlab) and washed three times in phosphate-buffered saline (PBS) (pH 7.2). The final pellet was resuspended in MRS broth. For quantitation of \textit{L. acidophilus}, a standard curve of optical density at 650 nm in a spectrophotometer (Beckman DU-88) versus CFU per milliliter was established. Serial 10-fold dilutions from the original sample were made in PBS. The number of CFU of \textit{L. acidophilus} was determined by spreading 100 \(\mu\)l of each suspension on MRS plates, which were incubated at 37\°C. Bacterial CFU were counted at 48 h for confirmation of the concentration of \textit{L. acidophilus} inoculated. Mice were inoculated intravaginally with an Eppendorf pipette with 20 \(\mu\)l of a culture containing \(10^{10}\) \textit{L. acidophilus} organisms per ml on two consecutive days (days −7 and −6). Negative-control mice were inoculated with 20 \(\mu\)l of MRS broth without \textit{L. acidophilus} (35).

Vaginal washes were performed using 50 \(\mu\)l of prewarmed MRS broth. Successful infection with \textit{L. acidophilus} was determined by culturing of vaginal washes in MRS broth supplemented with 5 \(\mu\)g of ciprofloxacin (Squibb Canada).
per ml and 180 μg of cefoxatin (Mefoxin; Merck, Sharp & Dohme, Montreal, Quebec, Canada) per ml and incubation at 37°C for 24 to 48 h prior to T. vaginalis inoculation.

(ii) T. vaginalis. Two days prior to T. vaginalis infection (day −2), mice were injected intravaginally with a second subcutaneous dose of 50 μl of estradiol valerate. T. vaginalis was harvested by centrifugation for 10 min at 140 × g in a Sorvall GLC-1 centrifuge and washed three times in PBS, and the final pellet was resuspended in TYI supplemented with 10% FBS and 0.32% Bacto Agar (36). All groups of mice were inoculated intravaginally with 20 μl of 2.5 × 10^7 T. vaginalis organisms per ml on two consecutive days (days 0 and 1).

Immunization. T. vaginalis was grown to log phase in TYI and harvested by centrifugation for 10 min at 140 × g in a Sorvall GLC-1 centrifuge. The pellet was washed three times in PBS by resuspension and centrifugation. The final pellet was resuspended in PBS, cells were counted and adjusted to the final concentration, and 100 μl of the cell preparation suspended in an equal volume of Freund's complete adjuvant (FCA) (total volume, 200 μl) was used for immunization. The same amount of the cell preparation suspended in an equal volume of Freund's incomplete adjuvant (FIA) was used for booster immunization. T. vaginalis is no longer motile and will not grow once mixed with FCA or FIA.

Mice were subcutaneously immunized in each side of the abdominal area with either 4.5 × 10^5, 9 × 10^5, or 1 × 10^6 T. vaginalis organisms per ml suspended in FCA (at day −56) and 4 weeks later (day −28) were given booster injections of the same dosage in FIA. An inoculation protocol outlining the time course for subcutaneous immunization and intravaginal challenge with T. vaginalis was designed. Control mice included sham-vaccinated mice immunized with PBS (pH 7.2) in FCA and FIA. Other groups of mice included unimmunized controls and a group of negative controls (no manipulation). Forty-eight days after primary immunization, mice were inoculated intravaginally with L. acidophilus (day −1). One week later they were intravaginally infected with T. vaginalis. At weekly intervals for 4 weeks post-vaginal infection, mice were tail bled and vaginal washes were performed. The experimental protocol was repeated three times, with the results batched for analysis.

To evaluate the effect of vaginal infection on immune response, another group of mice was orally administered 100 μl of metronidazole (10 mg/ml) 14 days after intravaginal inoculation with T. vaginalis. Two weeks later, the mice were again subjected to vaginal infection with T. vaginalis.

Mouse vaginal washes and tail bleeds. The duration of infection with T. vaginalis was determined by culturing vaginal washes in TYI supplemented with 10% FBS and antibiotics. Infection was considered to be present until live trichomonads were not visible in two consecutive examinations. Vaginal washes were performed with 50 μl of prewarmed TYI supplemented with 10% FBS by repeated aspiration until turbid. Wash material was collected in prewarmed TYI supplemented with 10% FBS, 300 μg of penicillin per ml, 300 μg of streptomycin per ml, 2.5 μg of amphotericin B per ml, and 10 μg of gentamicin per ml, incubated in glass screw-cap tubes in 5% CO2 at 37°C for 24 to 48 h prior to incubation in glass screw-cap tubes in 5% CO2 at 37°C for 24 to 48 h prior to incubation. Naive control mice were still intravaginally infected 28 days post-intravaginal inoculation. There was a statistically significant decrease in the recovery of T. vaginalis from mouse vaginal washes from 95.5% of mice immunized with 4.5 × 10^5 organisms per ml compared with that of naive controls. Doses of both 9 × 10^5 (group 2) and 1 × 10^6 T. vaginalis organisms per ml resulted in significantly decreased recovery of T. vaginalis compared with immunization with 4.5 × 10^5 organisms per ml (group 1) (P < 0.01). No significant difference in recovery of T. vaginalis in over 28 days was observed between naive controls and sham-vaccinated mice (P = 0.99). The six negative-control animals had no detectable T. vaginalis.

**RESULTS**

BALB/c mice were subcutaneously immunized with various doses of T. vaginalis organisms (4.5 × 10^5, 9 × 10^5, and 1 × 10^6/ml) suspended in FCA 56 days prior to vaginal infection and were given boosters of the same doses suspended in FIA 4 weeks later (day −28).

Table 1 depicts the combined results of three separate experimental runs which define the optimal T. vaginalis immunization dose by measuring the recovery of T. vaginalis from vaginal washes. The mice that were given immunizations and boosters of the higher two doses of T. vaginalis showed less intravaginal infection than mice given immunizations and boosters of the lower dose of T. vaginalis or the adjuvant and naive control mice. Seven days after intravaginal inoculation with T. vaginalis, only 6 of 25 mice immunized with 1 × 10^6 T. vaginalis organisms per ml (group 3) and 6 of 25 mice immunized with 9 × 10^5 organisms per ml (group 2) were intravaginally infected with T. vaginalis. Twenty-eight days post-intravaginal inoculation, T. vaginalis could not be recovered from vaginal washes from 95.5% of mice immunized with the dose of 9 × 10^5 organisms per ml (group 2) or from 100% of mice immunized with 1 × 10^6 organisms per ml (group 3). Twelve of 26 mice given immunizations and boosters of 4.5 × 10^5 T. vaginalis organisms per ml (group 1) were infected 7 days after intravaginal inoculation with T. vaginalis, and at 28 days postinfection, 7 mice were still infected. Sixteen of the 17 mice in the PBS-immunized group and 14 of the 16 in the naive control group were intravaginally infected at day 7. Eight of the 14 mice in the PBS-immunized group and 7 of 15 mice in the naive control group were still intravaginally infected 28 days post-intravaginal inoculation. There was a statistically significant decrease in the recovery of T. vaginalis in mice immunized with 4.5 × 10^5 (group 1) (P < 0.01), 9 × 10^5 (group 2) (P < 0.0001), and 1 × 10^6 (group 3) (P < 0.0001) T. vaginalis organisms per ml compared with that of naive controls. Doses of both 9 × 10^5 (group 2) and 1 × 10^6 (group 3) organisms per ml resulted in significantly decreased recovery of T. vaginalis compared with immunization with 4.5 × 10^5 organisms per ml (group 1) (P = 0.01). No significant difference in recovery of T. vaginalis in over 28 days was observed between naive controls and sham-vaccinated mice (P = 0.99). The six negative-control animals had no detectable T. vaginalis.

**Antibody reactivity in serum and vaginal washes.** Figure 1 represents the influence of immunization on the serologic IgG response of mice to T. vaginalis as detected by ELISA. The
PBS (sham)-immunized mice group, naive (unimmunized) control group, and negative (no manipulation)-control group all showed similar weak IgG responses throughout the experiment. The groups of mice immunized with $9 \times 10^6$ and $1 \times 10^8$ T. vaginalis per ml showed stronger IgG responses than the group immunized with $4.5 \times 10^5$ organisms per ml or the control mice. Immunization with T. vaginalis antigen in adjuvant induced an increased serum IgG response which was further enhanced following intravaginal challenge with T. vaginalis. No serum IgA response was detected.

Figures 2 and 3 represent IgG and IgA responses in vaginal washes of immunized mice upon challenge with T. vaginalis. A pronounced vaginal IgG response was observed in mice immunized with $9 \times 10^6$ and $1 \times 10^8$ T. vaginalis organisms per ml, but only a weak IgG response was observed in mice immunized with $4.5 \times 10^5$ organisms per ml or the control mice. All control groups showed low levels of IgG reactivity to T. vaginalis antigen. The vaginal IgA response was not as pronounced as the vaginal IgG response. Mice immunized with the two higher concentrations of T. vaginalis antigen showed a slight elevation in vaginal IgA levels 14 days after vaginal infection. Similar to the control groups, immunization with the lower dose of antigen did not induce a detectable increase in vaginal IgA response. These experiments provide some evidence that previous exposure to T. vaginalis through subcutaneous immunization results in an increased response of antibodies in vaginal washes and serum, particularly subsequent to a vaginal infection.

Single immunization with $9 \times 10^6$ T. vaginalis organisms per ml in either FCA or FIA at day −56 or −28 resulted in a lower infection rate than that of control mice but with less protection than even two doses of $4.5 \times 10^5$ organisms per ml, and in mice that received only a single immunization, an anamnestic response was not demonstrated upon vaginal infection (data not shown).

Table 2 depicts the recovery of T. vaginalis from vaginal washes of vaginally infected mice who were treated with metronidazole and then reinjected vaginally. Even 28 days post-intravaginal challenge, 47% of metronidazole-treated mice, 55.5% of mice sham treated with PBS, and 62.5% of untreated mice were culture positive for T. vaginalis (P = not significant). The repeated vaginal infection did not induce an antibody response in either serum or vaginal washes in these mice.
Subcutaneous immunization with whole-cell *T. vaginalis* washes and *T. vaginalis* clearance from animals immunized with the lower dose of *T. vaginalis* (35), this study demonstrates that it is possible to achieve significant protection against *T. vaginalis* infection. Compared with the unimmunized mice, those mice that were immunized with the lower dose of *T. vaginalis* also showed significant but lesser protection. This observation is in agreement with similar studies of Cripps et al. (13), in which higher immunizing doses of *Pseudomonas aeruginosa* were necessary to enable clearance of the organism.

Analysis of the antibody response in serum and vaginal washes revealed that anti-*T. vaginalis*-specific IgG activity was higher in mice immunized with the higher doses than in the unimmunized mice but not mice immunized with the lowest dose (4.5 × 10^7 organisms per ml). There is also a relationship between trichomonad clearance and specific antibody response. However, at a lower immunizing dose, *T. vaginalis* clearance from the vaginal wash samples was also observed but to a lesser degree, and in these mice the specific antibody response was not different from that seen in unimmunized mice. This suggests that the antibody response plays a role in protection; however, this does not preclude other immunologic mechanisms of protection.

The role of acquired immunity in human trichomoniasis is not clearly understood. Chronic infection with the parasite is common, and immune protection from reinfection appears poor (23). One explanation for the absence of a protective immune response in humans, in contrast to our results with mice, may be simply due to antigenic heterogeneity of *T. vaginalis*. However, a successfully treated woman can often be reinfected by her untreated sexual partner, although antigenic homogeneity of the infecting pathogen would be expected.

Antigen presentation may be crucial in inducing an immune response, and a systemic antigen presentation may be advantageous over a local vaginal presentation. This may explain why repeated human vaginal infection did not induce protection or a detectable immune response compared with subcutaneous immunization. Protection was also not demonstrated in mice which were infected vaginally, treated with metronidazole, and then reinfected vaginally. This suggests that the response to infection and immunization protection could be associated with the route of immunization. Also, the immune system of the mouse may respond to a different antigen or may process the antigen in a manner entirely different from that of the human immune system. Mice that were immunized with and given booster injections of *T. vaginalis* in the absence of adenovirus did not show significant protection compared with that of mice immunized with adenovirus (data not shown). Many immunologic studies have shown that previous exposure of the immune system to a pathogen enhances its ability to respond again to that antigen (6, 13, 43). This is not seen in repeated vaginal infection.

In human infection, it appears that the antibody responsiveness of patients is not strong, and the antibody titers are low even in active *T. vaginalis* infection (4, 23). Our study demonstrated that it is possible to achieve a strong response of *T. vaginalis*-specific IgG antibody in mice by booster immunization with *T. vaginalis* suspended in adjuvant.

The presence of antitrichomonal antibody in human cervical vaginal secretions has been confirmed by various immunologic methods, and *T. vaginalis*-specific IgG and IgA antibodies were reported in a majority of these studies (2, 4, 5, 9, 37, 41, 42). However, there is little evidence that it results in protective immunity. Alderete et al. (4) reported that in some cases, trichomoniasis was not associated with detectable vaginal antibody. *T. vaginalis* antibody was detected in vaginal washes only from the group of mice immunized with and given booster injections of *T. vaginalis* suspended in Freund's adjuvant prior to vaginal infection, and these mice showed a further significant IgG response upon vaginal infection. The IgA enzyme immunosassay reactivity was not as high as the IgG reactivity. This result is in agreement with a previous report (20) of the induction of antibodies in genital secretions by utilizing a systemic route of immunization (6, 43). In vitro studies of Alderete and Kasmala (3) have also shown that monoclonal antibodies raised against *T. vaginalis* can kill the antigen-positive parasite independently of complement activation. However, sera from infected patients have been shown to exhibit complement-mediated lytic activity on trichomonads in cultures (22). The role of complement in the immune protection seen has not yet been studied.

The exact function of IgG in serum and IgG and IgA in vaginal washes and the role they may play in protection are not clear. We have shown a relationship with a humoral immune response and protection from vaginal infection. However, other immune processes may also be involved in protection. FCA is a known stimulant of both cell-mediated and humoral responses (12) and FIA stimulates predominantly humoral immunity (12), suggesting that immunization with *T. vaginalis* suspended in these adjuvants may induce both arms of the

### TABLE 2. Protection and the recovery of *T. vaginalis* from mouse vaginal washes after metronidazole treatment

<table>
<thead>
<tr>
<th>Mouse group and treatmenta</th>
<th>No. of mice with <em>T. vaginalis</em> on the indicated day of culture/total inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+7</td>
</tr>
<tr>
<td>1, metronidazole (n = 18)</td>
<td>12/18</td>
</tr>
<tr>
<td>2, PBS (n = 9)</td>
<td>8/9</td>
</tr>
<tr>
<td>3, no drug (n = 9)</td>
<td>7/85</td>
</tr>
<tr>
<td>4, no treatment (negative</td>
<td>0/4</td>
</tr>
</tbody>
</table>

a All mice in groups 1 to 3 received subcutaneous estradiol valerate and vaginal inoculation of *L. aceratilus* prior to vaginal *T. vaginalis* infection, as described in the text. Group 1 was infected vaginally with *T. vaginalis*, treated with metronidazole, and then reinfected. Group 2 was infected vaginally with *T. vaginalis*, shamtreated with PBS, and then reinfected. Group 3 was not previously infected vaginally (one vaginal infection). Group 4 received none of the above manipulations.

b Total does not equal *n* because of unrelated death of a mouse.

### DISCUSSION

Despite the existence of chemotherapeutic agents, the incidence of trichomoniasis is increasing, and this has led to renewed interest in the immunological response of the host as a means of preventing this infection. Immune responses to infection with *T. vaginalis* have been described, including specific secretory antibody in vaginal secretions (2), IgM and IgG antibody in serum (31, 41), polymorphonuclear cell chemotaxis (32, 33), and phagocytosis (39). Despite these responses, chronic infection with the parasite is common, and immunity to reinfection is poor (1). Also, information regarding host immunological responses to *T. vaginalis* remains inadequate.

With an established mouse model of vaginal *T. vaginalis* infection (35), this study demonstrates that it is possible to achieve significant protection against *T. vaginalis* infection by subcutaneous immunization with whole-cell *T. vaginalis*. This study also shows that protection from vaginal challenge is associated with a specific anti-*T. vaginalis* local and systemic humoral immune response. This response is vaccine dose dependent and is predominantly IgG, with a lesser IgA response.

As a first step, recovery of *T. vaginalis* from mouse vaginal washes and trichomonal clearance from animals immunized with various doses of *T. vaginalis* suspended in adjuvant were compared with those of unimmunized animals. Mice that were immunized with the higher doses showed significant protection. Compared with the nonimmunized mice, mice that were immunized with the lower dose of *T. vaginalis* also showed significant but lesser protection. This observation is in agreement with similar studies of Cripps et al. (13), in which higher immunizing doses of *Pseudomonas aeruginosa* were necessary to enable clearance of the organism.

Analysis of the antibody response in serum and vaginal washes revealed that anti-*T. vaginalis*-specific IgG reactivity was higher in mice immunized with the higher doses than in the unimmunized mice but not mice immunized with the lowest dose (4.5 × 10^7 organisms per ml). There is also a relationship between trichomonad clearance and specific antibody response. However, at a lower immunizing dose, *T. vaginalis* clearance from the vaginal wash samples was also observed but to a lesser degree, and in these mice the specific antibody response was not different from that seen in unimmunized mice. This suggests that the antibody response plays a role in protection; however, this does not preclude other immunologic mechanisms of protection.

The role of acquired immunity in human trichomoniasis is
immune system. Cell-mediated immunity may also be important in the protection (34) against T. vaginalis infection, and its role needs to be further elucidated.

These preliminary immunological studies have opened the door to further investigations into the immune response to T. vaginalis infection. The successful protective immune response seen in mice suggests the possibility of application of a similar approach in the development of a human vaccine.

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REFERENCES


