USE OF INTRAVAGINAL MICROBICIDES TO PREVENT ACQUISITION OF TRICHOMONAS VAGINALIS INFECTION IN LACTOBACILLUS-PRETREATED, ESTROGENIZED YOUNG MICE

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Abstract. D2A21, a novel peptide antibiotic has in vitro activity against a wide spectrum of sexually transmitted diseases (STD). In this study we tested the hypothesis that intravaginal D2A21 would interfere with acquisition of Trichomonas vaginalis infection in a modified mouse model. T. vaginalis infections of estrogenized young mice pre-treated with Lactobacillus gasseri or Lactobacillus rhamnosus were more frequent and persistent than those in mice pre-treated with Lactobacillus acidophilus. One hundred percent T. vaginalis infection was achieved for 2–4 days post-challenge when intravaginal L. rhamnosus pre-treatments were given to estrogenized mice 48 hr prior to a single T. vaginalis challenge. Estrogenized mice pre-treated with L. rhamnosus were pre-medicated with intravaginal placebo gel, 0.5% or 2% D2A21 gel, or 500 µg/mL metronidazole gel prior to T. vaginalis challenge. Both 2% D2A21 and metronidazole gels were significantly more efficacious (10% or none infected) than placebo gel (53% infected) in preventing vaginal T. vaginalis infections in mice.

INTRODUCTION

Twelve million new cases of sexually transmitted diseases (STDs) are reported to the Centers for Disease Control every year in the United States. An estimated eight million new T. vaginalis infections occur annually in the United States but not reportable. In addition, trichomoniasis may be the most common non-viral STD in the world. Forty to 46% of women in developing countries including Natal (South Africa), Bangladesh, and New Guinea are reported to have trichomoniasis. Although women in industrialized countries are perceived to have lower rates of Trichomonas infection, certain groups continue to be highly infected. Thirty percent of indigent, predominantly African American women seen in our urban emergency department and 47% of pregnant inmates in New York City were culture positive for T. vaginalis. While half of T. vaginalis infected women are asymptomatic, trichomoniasis has been associated with adverse pregnancy outcomes including premature rupture of the membranes, preterm labor, and low birth weight infants. As demonstrated for other STDs characterized by vaginal inflammation, there is an increased rate of HIV acquisition associated with T. vaginalis infection.

An Institute of Medicine report recommends a “multifaceted approach to prevention” of STDs including “developing more effective interventions”. One intervention that would allow women more control over their potential acquisition of STDs is a prophylactic intravaginal microbiode effective against HIV and other STDs. New formulations and compounds with broad spectrum anti-STD activity should be considered as possible solutions. The Designed Antibiotic Peptide (DAP) used in this study is a synthetic peptide antibiotic similar to naturally occurring cecropins or defensins. DAPs have a broad spectrum of in vitro activity for a variety of pathogens including Neisseria gonorrhoea, Gardnerella, Candida, Chlamydia, Staphylococcus, Pseudomonas, and Stenotrophomonas. This new class of wide spectrum antibiotic has not been tested as an intravaginal microbiode.

A mouse model of vaginal trichomoniasis would provide an economical test of efficacy if it could be adapted for testing of intravaginal microbicides. Young estrogenized mice are more susceptible to vaginal infection with T. vaginalis. Lactobacillus acidophilus pre-infected mice have been reported to achieve a greater frequency and duration of T. vaginalis infection. Using a different T. vaginalis isolate and similar methods, we were able to produce only transient T. vaginalis infections in mice. Recent studies using molecular methods to classify Lactobacillus species suggest that L. acidophilus may not be the most common lactobacillus in the “normal” human vaginal flora. Therefore we examined several other Lactobacillus species from the human vaginal flora for their ability to enhance the vaginal receptivity of estrogenized mice for colonization by T. vaginalis. Finally we prepared drug-in-gel-formulations with efficacy against T. vaginalis in vitro and used the modified mouse model of trichomoniasis to test these formulations for their ability to prevent T. vaginalis colonization of susceptible mice.

MATERIAlS AND METHODS

Mice. Three to four week old (10–13 g) female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN), were injected IP with 0.1 mL of 500 µg estrogen (Premarin; 5 mg mL⁻¹; Ayerst Laboratories, Philadelphia, PA) on the day of receipt (day 1) and every 48 hr throughout the experiments. The estrogen pre-treatment of mice induces an estrus-like state including a vaginal epithelium similar to that of humans and increased susceptibility to T. vaginalis infection. We found that adult mice were refractory to T. vaginalis infection even if pre-treated with estrogen. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals as approved by the Institutional Animal Care and Use Committee.

Lactobacillus. Four species of Lactobacillus originally isolated from the human vaginal flora were obtained from American Type Culture Collection (ATCC): L. acidophilus (4356), L. gasseri (4963), L. rhamnosus (casei) (7469) and...
**L. vaginalis** (ATCC 49540) and maintained as recommended by ATCC. Isolates were cryopreserved in 10% glycerol, and fresh cultures reintiated at the beginning of each experiment.

**Mouse pre-treatment with lactobacilli.** A 300 mL volume of MRS Lactobacillus broth was inoculated with 10^6 colony forming units (CFU) of Lactobacillus sp. and incubated 14 hr at 37°C, 5% CO2. The organisms were harvested, pooled and washed once in broth. The cells were re-suspended in a small volume of MRS broth forming a thick slurry and 30 µL of this suspension was given to each mouse intravaginally (about 10^10 CFU/mouse). For demonstration of Lactobacillus infection the vagina of each mouse was rinsed with 30 µL MRS, the washings added to 3 mL MRS and Rhogosan Agar plates streaked for isolation and colony counts.

**Trichomonas vaginalis.** The strain used in infection studies (ATCC 30235) was isolated from a patient with vaginal discharge, marked inflammation, heavy cellular degeneration, chronic cervicitis with mild dysplasia and identified as pathogenic by a subcutaneous mouse assay.**2** Trichomonas vaginalis were maintained in modified TYM**1** or cryopreserved in 5% DMSO and stored at -70°C. A fresh aliquot of T. vaginalis was grown from this stock for each experiment. Logarithmic growth phase trophozoites were washed and re-suspended in TYM without antibiotics so that mice received a single vaginal instillation of 15 µL T. vaginalis suspension containing approximately 3 x 10^7 trophozoites.

**Demonstration of T. vaginalis Infection.** The vagina of each mouse was washed repeatedly with 30 mL room temperature TYM without antibiotics. The wash was pipetted into 12 mL TYM additionally supplemented with 50 µg mL^-1 ciprofloxacin (Bayer), 30 µg mL^-1 clindamycin phosphate (Upjohn), and 40 µg mL^-1 fluconazole (Roerig).7 Adding the vaginal wash to a tube of TYM was sufficient to dilute any remaining medication (30 µg D2A21/mouse or 7.5 µg metronidazole/mouse) to non-toxic concentrations in vitro. The cultures were incubated at 37°C for a week after inoculation and checked daily for the presence of T. vaginalis using an inverted microscope.

**Development, formulation and in vitro testing of intravaginal microbicides.** The Designed Antimicrobial Peptide (DAP) antibiotic, D2A21, is a Demegen patented peptide (amino acid sequence: FAKFKAFKFKKFQKFKAF) with **in vitro** activity against a variety of bacterial and protozoan pathogens.18 This peptide was synthesized and authenticated by Mass Spectroscopy and HPLC by Multiple Peptide Systems (San Diego, CA). Metronidazole injection USP (5 mg mL^-1) was obtained from Baxter Healthcare Corporation (Deerfield IL). A hydrophilic gel was used to compound gels for use in these studies. A 0.5 or 2% w/v D2A21 peptide solution was prepared in normal saline and 3.25% w/v hydroxyethylcellulose NF polymer slowly added to the peptide solution with constant stirring. The gel was stored at 5°C until used. Metronidazole solution for injection was used to formulate a 500 µg mL^-1 metronidazole gel (w/v) by mixing the liquid into a weighed portion of the placebo gel (identically prepared without drug). The drug-containing and drug-free (placebo) gels were assayed for trichomonadical activity in vitro by a previously described 96-well plate method.22 Drugs dissolved in water directly, without prior compounding, killed T. vaginalis at 4–8 µg mL^-1. After compounding into gel, T. vaginalis were killed at 16–32 µg mL^-1 concentrations of D2A21 or metronidazole. The placebo gel alone was non-toxic to T. vaginalis at concentrations comparable to those used in the drug-containing gels.

**Modification of the trichomoniasis mouse model for testing of intravaginal microbicides.** Four species of Lactobacillus or sterile medium (control) were instilled into the vaginas of groups of five estrogenized mice on two successive days and the vaginal content cultured for lactobacilli three days later. Mice were inoculated with T. vaginalis on the fifth day following Lactobacillus pre-treatment and cultured for T. vaginalis three days later. The following week, the same mice were twice re-treated with the same Lactobacillus species as before and re-challenged with T. vaginalis 48 hr after the last Lactobacillus pre-treatment. Mice were cultured for T. vaginalis infection for six days beginning 48 after T. vaginalis challenge (Figure 1).

**Optimization of T. vaginalis persistence.** Based on the results of the previous experiment, mice were given four Lactobacillus pre-treatments using the two species of Lactobacillus that had greater effects on mouse susceptibility to T. vaginalis. Estrogenized mice housed in two groups of six were inoculated intra-vaginally four times (days 4, 5, 9, and 10) with one of the two Lactobacillus species (Figure 2). Each mouse received 30 µL of Lactobacillus sp. slurry (2 x 10^7 CFU). Forty-eight hr after the last Lactobacillus pre-treatment (on day 12), each mouse received 3 x 10^6 T. vaginalis intra-vaginally in a 15 µL volume. Forty-eight hr after the T. vaginalis inoculation (on day 14) and for a week thereafter, each mouse’s vagina was cultured for the presence of T. vaginalis.

**The effect of intravaginal microbicides on the acquisition of T. vaginalis infection by mice in a modified model of trichomoniasis.** The microbicidal testing used the modifications to the mouse model established in the first series of experiments (see Results, Figures 1 and 2). Estrogenized mice were pre-treated with L. rhamnosus four times, on days 4, 5, 9, and 10. The experimental groups of mice (10 per group in each replicate) received placebo (gel without drug), Metronidazole gel, 0.5% D2A21 gel or 2% D2A21 gel (Table 1). Each animal received a specified gel by insertion of a 2 mm diameter stick bearing approximately 15–20 µL of gel into their vagina and gently smearing by twisting prior to removal. Animals were challenged with T. vaginalis (on day 12), five minutes after gel pre-treatment, by pipetting 15 µL T. vaginalis suspension (approximately 3 x 10^6 trophozoites) into the vagina. Mice were cultured for T. vaginalis beginning 48 hr after T. vaginalis challenge (days 14–22). This set of experiments was replicated three times.

**Statistical analysis.** Significance of Lactobacillus data comparisons was determined with the Kruskal Wallis test and multiple comparisons were made with Dunn’s test as the data were not normally distributed. The final animal infection experiments tested the hypothesis that the microbicidal gel treatments were more effective than placebo in preventing T. vaginalis infection. Results were analyzed using a one-tailed Fisher’s exact test in a pair-wise comparison of the three treatments with placebo.
Treatment and cultured for challenged with T. vaginalis prior to challenge. Inoculation. Control animals received sterile medium pre-treatment times with four Lactobacillus infection of mice. Estrogenized mice pre-infected four vaginalis 2% D2A21 gel 3, *0.5% D2A21 gel 4 Placebo gel 1 286 LUSHBAUGH AND OTHERS Lactobacillus species and culturing for earlier while they still remained infected with Lactobacillus environment was pre-treated with several Lactobacillus Three days post-inoculation, significantly more L. rhamnosus and L. vaginalis were recovered than L. gasseri or L. acidophilus. No lactobacilli were recovered from the control group. Few mice became infected when inoculated with T. vaginalis on the fifth day following Lactobacillus pretreatment. We postulated that inoculating mice with T. vaginalis earlier while they still remained infected with Lactobacillus and culturing for T. vaginalis infection just 48 hr after challenge might improve infection rates. The same mice re-treated with the same Lactobacillus species and T. vaginalis challenged sooner were studied. This time, two days post-infection most of the mice were T. vaginalis infected (Figure 1). Trichomonas vaginalis infection persisted for 6 days post-inoculation in 40–60% of mice pre-treated with L. rhamnosus or L. vaginalis. Mice that were not pre-treated (control) or mice that were pre-treated with L. acidophilus or L. gasseri had T. vaginalis infections that self-cured in two or three days post-inoculation. We hypothesized that four factors contributed to improved mouse receptivity and duration of T. vaginalis infections in estrogenized mice: 1) the presence of Lactobacillus species; 2) the number of times the Lactobacillus pre-treatments were given (four not two); 3) the scheduling of pre-treatments in relation to T. vaginalis challenge (just 48 hr prior); and 4) the timing of T. vaginalis culturing (48 hr. after challenge). Optimization of T. vaginalis persistence. The two species of Lactobacillus (L. rhamnosus and L. vaginalis) that had the greatest effects on mouse susceptibility to T. vaginalis were used to pre-treat mice for T. vaginalis challenge given in 48 hr (Figure 2). Trichomonas vaginalis infection persisted for four days post-challenge in 100% of mice pre-treated with L. rhamnosus and in 67% of mice pre-treated with L. vaginalis. Half of the mice pre-treated with L. rhamnosus remained infected seven days; 17% of mice pre-treated with L. vaginalis were infected at the same time point. One L. rhamnosus pre-treated mouse remained infected 10 days post T. vaginalis challenge. This success in extending T. vaginalis infection to ten days led us to use the revised protocol to test intravaginal microbicides in the mouse model.

The effect of intravaginal microbicides on the acquisition of T. vaginalis infection by mice in a revised model. Lactobacillus rhamnosus was selected as pre-treatment for use in the revised mouse model as it increased T. vaginalis persistence. Three replicate intravaginal microbicide experiments were conducted on mice receiving four L. rhamnosus pre-treatments (Figure 2). In each replicate, four groups of ten estrogenized mice were pre-medicated five minutes prior to a single T. vaginalis challenge with either placebo gel, metronidazole gel, or one of two concentrations of D2A21 in gel (Table 1). Using Fisher’s exact test, both metronidazole (P = 1.82 × 10⁻⁶) and 2% D2A21 (P = 0.0055) gel treatments were significantly more effective than placebo in preventing acquisition of T. vaginalis infection (Table 1). All three mice that became infected after treatment with 2% D2A21 occurred in the third replicate experiment (C) performed after the formulated drug had been stored three

### TABLE 1

Effect of intravaginal gel medications in preventing Trichomonas vaginalis infection of mice

<table>
<thead>
<tr>
<th>Pre-treatment group</th>
<th>Number of T. vaginalis infected mice/group*</th>
<th>Infected mice/total group*</th>
<th>Percent infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Placebo gel¹</td>
<td>3/10</td>
<td>6/10</td>
<td>7/10</td>
</tr>
<tr>
<td>Metronidazole gel²*</td>
<td>0/10</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>2% D2A21 gel³**</td>
<td>0/10</td>
<td>0/9</td>
<td>3/9</td>
</tr>
<tr>
<td>0.5% D2A21 gel⁴</td>
<td>3/10</td>
<td>1/9</td>
<td>5/9</td>
</tr>
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</table>

* Significantly different from placebo by Fisher’s exact test.
¹ 0.15 μg gel (without drug) pre-treatment/mouse
² 500 μg metronidazole mg/g gel, 0.15 μg/mouse (0.75 μg metronidazole dose/mouse)
³ 2% D2A21 gel (wt/wt), 0.15 μg/mouse (5 μg D2A21 dose/mouse)
⁴ 0.5% D2A21 gel (wt/wt), 0.15 μg/mouse (0.75 μg D2A21 dose/mouse)
* Mice received a 3 × 10⁴ T. vaginalis intravaginal challenge 5 min after intravaginal pre-treatment. Number of infected mice divided by the number of mice per group.

### RESULTS

Modification of the trichomoniasis mouse model for testing of intravaginal microbicides. The mouse vaginal environment was pre-treated with several Lactobacillus species and the resulting effects on T. vaginalis colonization and persistence were examined in several small pilot studies. Three days post-inoculation, significantly more L. rhamnosus and L. vaginalis were recovered than L. gasseri or L. acidophilus. No lactobacilli were recovered from the control group. Few mice became infected when inoculated with T. vaginalis on the fifth day following Lactobacillus pretreatment. We postulated that inoculating mice with T. vaginalis earlier while they still remained infected with Lactobacillus and culturing for T. vaginalis infection just 48 hr after challenge might improve infection rates. The same mice re-treated with the same Lactobacillus species and T. vaginalis challenged sooner were studied. This time, two days post-infection most of the mice were T. vaginalis infected (Figure 1). Trichomonas vaginalis infection persisted for 6 days post-inoculation in 40–60% of mice pre-treated with L. rhamnosus or L. vaginalis. Mice that were not pre-treated (control) or mice that were pre-treated with L. acidophilus or L. gasseri had T. vaginalis infections that self-cured in two or three days post-inoculation. We hypothesized that four factors contributed to improved mouse receptivity and duration of T. vaginalis infections in estrogenized mice: 1) the presence of Lactobacillus species; 2) the number of times the Lactobacillus pre-treatments were given (four not two); 3) the scheduling of pre-treatments in relation to T. vaginalis challenge (just 48 hr prior); and 4) the timing of T. vaginalis culturing (48 hr. after challenge).

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months under refrigeration (Table 1). The 0.5% D2A21 gel (P = 0.086) was not significantly different from placebo in preventing T. vaginalis infection.

**DISCUSSION**

The incidence of STDs is rapidly increasing, particularly among women and minorities. One prevention strategy that women could use to control their exposure to STDs is an intravaginal microbical gel with activity for a variety of STD agents. The ideal intravaginal microbicide would be “female controlled and effective against all STDs”. An animal model of trichomoniasis, one of the more common STDs, would be valuable if it were adapted for testing of intravaginal microbicides. Meysick and Garber reported that the mouse vaginal flora usually lacks lactobacilli and pre-infecting estrogenized mice with L. acidophilus enhanced the susceptibility of estrogenized mice to T. vaginalis infection.

Recently, several investigators reported that L. acidophilus is not commonly found in the normal human vaginal flora but several other Lactobacillus species are. We reasoned that other Lactobacillus species from the human vaginal environment might increase susceptibility of mice to *Trichomonas* infection. Initial pilot experiments using small numbers of mice found that vaginal pre-infection with *L. vaginalis* and *L. rhamnosus* supported a higher prevalence and persistence of *Trichomonas* infection than pre-infection with *L. gasseri* or *L. acidophilus*. This suggested a possible correlation between the Lactobacillus species used to condition the mouse vaginal environment and success in establishing *T. vaginalis* infections (Figure 1). In a second experiment, *L. rhamnosus* pre-treatment supported higher *T. vaginalis* infection frequency and persistence than *L. vaginalis* (Figure 2). Long term *T. vaginalis* infection was not achieved with either pre-infection; most *T. vaginalis* infections were self-cured ten days post challenge. Using infection techniques developed in the small pilot studies, three replicate experiments compared the efficacy of placebo gel, metronidazole gel or two concentrations of DAP D2A21 gels to prevent *T. vaginalis* acquisition in 120 L. rhamnosus pre-inoculated estrogenized mice. Intravaginal metronidazole gel at 500 µg mL⁻¹ completely prevented infection of all mice tested. Both the 2% D2A21 and metronidazole gels were also significantly more effective than placebo in preventing mouse infection with *T. vaginalis*. We demonstrated the utility of *L. rhamnosus* pre-inoculated mice for testing the efficacy of microbicides in preventing the acquisition of *T. vaginalis* infections.

All vaginal *T. vaginalis* infections of laboratory animals attempted since 1962, including those reported here, have dealt with variability in host susceptibility, *T. vaginalis*-strain infectivity and duration of infection, and prevention of *T. vaginalis* infections. Although a metronidazole-containing gel was effective in preventing acquisition of *T. vaginalis* by mice, two recent studies have shown that metronidazole is ineffective as intravaginal treatment in attempts to cure pre-existing *T.
vaginalis infections in humans. Several explanations have been suggested for the failure of intravaginal treatment for trichomoniasis: retention of infection in peri-vaginal glands, re-infection of the patient though self-inoculation of infected urine, or intercourse with infected partners. Oral metronidazole will remain standard treatment for trichomoniasis, although alternative therapies are required to eradicate metronidazole-resistant strains. A metronidazole gel is presently used for treatment of bacterial vaginosis (BV) and its use might increase treated women’s resistance to acquiring an infection with susceptible T. vaginalis. However, a single-ingredient intravaginal gel with a limited activity spectrum presents little advantage for prophylaxis of a variety of STDs other than trichomoniasis and BV. In contrast with the narrow spectrum of metronidazole, DAPs are synthetic peptide antibiotics with a broad spectrum of activity for a variety of pathogens. The mean minimum cidal concentration of different DAPs on these organisms was about 20 µg mL⁻¹, with a range of 2–64 µg mL⁻¹ (Lushbaugh WB and others, unpublished data). To be effective, an intravaginal microbicide must protect the female partner during every act of intercourse. The effects of frequent or chronic use of intravaginal microbicides on the normal vaginal flora, including lactobacillus species, are still being assessed. Nonoxynol-9 causes inflammation, favors loss of lactobacilli, and possibly increases acquisition of HIV infection. These problems should be considered during the development of new intravaginal prophylactic antibiotics. Metronidazole is teratogenic and mutagenic in vitro. Even though its use during pregnancy has long been questioned, a recent meta-analysis failed to find any relationship between metronidazole exposure during the first trimester of pregnancy and birth defects. DAP peptide antibiotics such as D2A21 have some properties that may favor their use as intravaginal microbicides. Active peptides may have a limited half-life in the vaginal environment that contains peptidases and oxidative products of inflammation. Preliminary studies have shown that some Lactobacillus species are more susceptible in vitro to D2A21 than others. Most L. acidophilus or L. vaginalis were susceptible to 20 µg/mL D2A21 but L. rhamnosus were resistant to D2A21 in vitro (Lushbaugh WB and others, unpublished data). DAPs such as D2A21 may be unique as intravaginal microbicides in the prevention of STDs. Recently, chlorhexidine and cellulose acetate phthalate have also shown promise as new compounds for use as intravaginal microbicides.

We recognize an important shortcoming of the present mouse model: the lack of long-term persistence of T. vaginalis in the mouse vagina such as occurs in human patients. Whether prevention of an infection that is destined to be transient can be correlated with prevention of natural infections in humans remains to be determined and can be answered only by further studies. We are continuing to search for ways to create chronic T. vaginalis infections in mice. Data from this study suggest that the current model is a reasonable approach to test intravaginal microbicide effectiveness.

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