

## 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 vaginalis* or *Lactobacillus rhamnosus* were more frequent and persistent than those in mice pre-treated with *Lactobacillus gasseri* or *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.<sup>1</sup> An estimated eight million new *T. vaginalis* infections occur annually in the United States<sup>2</sup> but are 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),<sup>3</sup> Bangladesh,<sup>4</sup> and New Guinea<sup>5</sup> are reported to have trichomoniasis. Although women in industrialized countries are perceived to have lower rates of *Trichomonas* infection,<sup>6</sup> 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*.<sup>7,8</sup> While half of *T. vaginalis* infected women are asymptomatic,<sup>9</sup> trichomoniasis has been associated with adverse pregnancy outcomes including premature rupture of the membranes, preterm labor, and low birth weight infants.<sup>10–12</sup> As demonstrated for other STDs characterized by vaginal inflammation, there is an increased rate of HIV acquisition associated with *T. vaginalis* infection.<sup>13</sup>

An Institute of Medicine report recommends a “multifaceted approach to prevention” of STDs including “developing more effective interventions”.<sup>14</sup> One intervention that would allow women more control over their potential acquisition of STDs is a prophylactic intravaginal microbicide effective against HIV and other STDs. New formulations and compounds with broad spectrum anti-STD activity should be considered as possible solutions.<sup>14,15</sup> The Designed Antibiotic Peptide (DAP) used in this study is a synthetic peptide antibiotic similar to naturally occurring cecropins<sup>16</sup> or defensins.<sup>17</sup> DAPs have a broad spectrum of *in vitro* activity for a variety of pathogens including *Neisseria gonorrhoea*, *Gardnerella*, *Candida*, *Chlamydia*, *Staphylococcus*, *Pseudomonas*, and *Stenotrophomonas*.<sup>18</sup> This new class of wide spectrum antibiotic has not been tested as an intravaginal microbicide.

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*.<sup>19</sup> *Lactobacillus acidophilus* pre-infected mice have been reported to achieve a greater frequency and duration of *T. vaginalis* infection.<sup>20</sup> 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<sup>21</sup> 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<sup>-1</sup>; 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.<sup>19</sup> 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* (49540) and maintained as recommended by ATCC. Isolates were cryopreserved in 10% glycerol, and fresh cultures reinitiated 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% CO<sub>2</sub>. 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 Rhogosa 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.<sup>22</sup> *Trichomonas vaginalis* were maintained in modified TYM<sup>23</sup> 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  $3 \times 10^6$  trophozoites.

**Demonstration of *T. vaginalis* Infection.** The vagina of each mouse was washed repeatedly with 30 µL room temperature TYM without antibiotics. The wash was pipetted into 12 mL TYM additionally supplemented with 50 µg mL<sup>-1</sup> ciprofloxacin (Bayer), 30 µg mL<sup>-1</sup> clindamycin phosphate (Upjohn), and 40 µg mL<sup>-1</sup> fluconazole (Roerig).<sup>7</sup> 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: FAKKFAKKFKKFAKKFAKFAFAF) with *in vitro* activity against a variety of bacterial and protozoan pathogens.<sup>18</sup> This peptide was synthesized and authenticated by Mass Spectroscopy and HPLC by Multiple Peptide Systems (San Diego, CA). Metronidazole injection USP (5 mg mL<sup>-1</sup>) 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<sup>-1</sup> metronidazole gel (v/w) 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 trichomonocidal activity *in vitro* by a previously described 96-well plate method.<sup>24</sup> Drugs dissolved in water directly, without prior

compounding, killed *T. vaginalis* at 4–8 µg mL<sup>-1</sup>. After compounding into gel, *T. vaginalis* were killed at 16–32 µg mL<sup>-1</sup> 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 hr 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 ( $2 \times 10^7$  CFU). Forty-eight hr after the last *Lactobacillus* pre-treatment (on day 12), each mouse received  $3 \times 10^6$  *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 microbicide 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  $3 \times 10^6$  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.

TABLE 1  
Effect of intravaginal gel medications in preventing *Trichomonas vaginalis* infection of mice

Pre-treatment group	Three replicate experiments			Total	
	Number of <i>T. vaginalis</i> infected mice/group <sup>5</sup>			Infected mice/total group <sup>5</sup>	Percent infected
	A	B	C		
Placebo gel <sup>1</sup>	3/10	6/10	7/10	16/30	53
Metronidazole gel <sup>2,*</sup>	0/10	0/9	0/9	0/28	0
2% D2A21 gel <sup>3,*</sup>	0/10	0/9	3/9	3/28	10
0.5% D2A21 gel <sup>4</sup>	3/10	1/9	5/9	9/28	32

\* Significantly different from placebo by Fisher's exact test.

<sup>1</sup> 0.15 µg gel (without drug) pre-treatment/mouse

<sup>2</sup> 500 µg metronidazole mg<sup>-1</sup> gel, 0.15 µg/mouse (0.75 µg metronidazole dose/mouse)

<sup>3</sup> 2% D2A21 gel (wt/wt), 0.15 µg/mouse (3 µg D2A21 dose/mouse)

<sup>4</sup> 0.5% D2A21 gel (wt/wt), 0.15 µg/mouse (0.75 µg D2A21 dose/mouse)

<sup>5</sup> Mice received a  $3 \times 10^6$  *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* chal-

lenged 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* 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 ( $P = 1.82 \times 10^{-6}$ ) 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

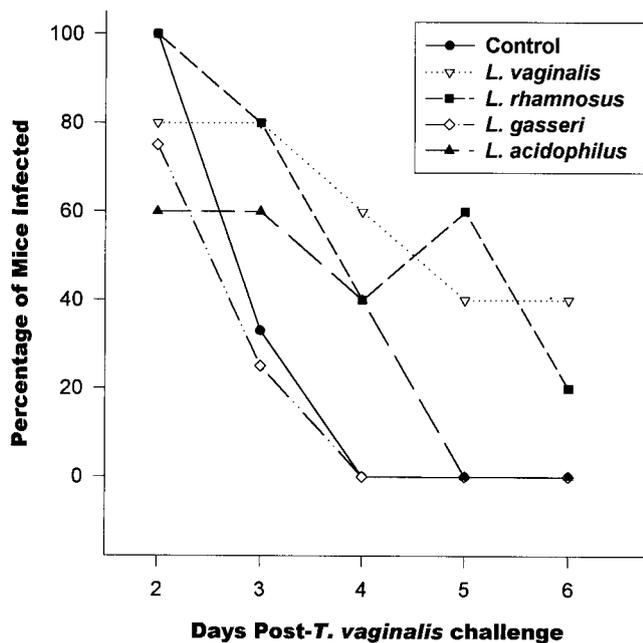


FIGURE 1. Effect of *Lactobacillus* pre-treatment on *Trichomonas vaginalis* infection of mice. Estrogenized mice pre-infected four times with four *Lactobacillus* species over a two week period were challenged with *T. vaginalis* 48 hr after the last lactobacillus pre-treatment and cultured for *T. vaginalis* beginning two days post-inoculation. Control animals received sterile medium pre-treatment prior to challenge.

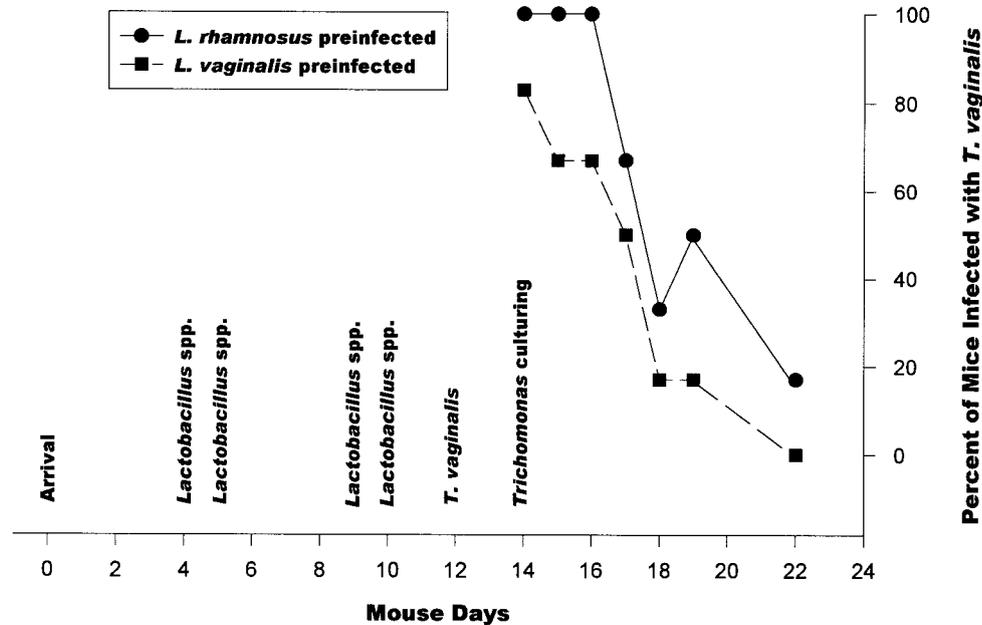


FIGURE 2. Effect of *Lactobacillus* spp. on *Trichomonas vaginalis* infection rate. Two groups of six estrogenized mice pre-infected four times with one of two *Lactobacillus* species (*Lactobacillus* spp.) over a two week period were challenged with *T. vaginalis* 48 hr after the last lactobacillus pre-treatment (*T. vaginalis*) and cultured for *T. vaginalis* beginning two days later (*Trichomonas* culturing). The percentage of *T. vaginalis*-positive mice is plotted against days (Mouse Days) according to the *Lactobacillus* pre-treatment each group received.

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 microbicidal gel with activity for a variety of STD agents. The ideal intravaginal microbicide would be "female controlled and effective against all STDs".<sup>14</sup> 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.<sup>25</sup> Recently, several investigators reported that *L. acidophilus* is not commonly found in the normal human vaginal flora but several other *Lactobacillus* species are.<sup>21,26,27</sup> 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  $\mu\text{g mL}^{-1}$  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.<sup>19,20,28-34</sup> Coombs and others reported that some estrogenized mice were capable of sustaining vaginal *T. vaginalis* infections for as long as 92 days, but not all mice nor most *T. vaginalis* isolates were as successful.<sup>19</sup> The inability to produce frequent persisting infections in estrogenized, lactobacillus pre-treated mice could be related to the *T. vaginalis* isolate that was used. The isolate used in our studies was cryopreserved early in its isolation history and reported to be pathogenic in a sub-cutaneous mouse assay.<sup>35</sup> Other freshly isolated, more virulent, *T. vaginalis* strains might prove more effective in future experiments.

Prevention of STDs is different than treating established STDs. 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.<sup>7,36</sup> 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.<sup>7,36</sup> Oral metronidazole will remain standard treatment for trichomoniasis<sup>37</sup> although alternative therapies are required to eradicate metronidazole-resistant strains.<sup>38</sup> A metronidazole gel is presently used for treatment of bacterial vaginosis (BV)<sup>39</sup> 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.<sup>18,40,41</sup> The mean minimum cidal concentration of different DAPs on these organisms was about 20 µg mL<sup>-1</sup>, with a range of 2–64 µg mL<sup>-1</sup> (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,<sup>42</sup> favors loss of lactobacilli,<sup>43</sup> and possibly increases acquisition of HIV infection.<sup>44</sup> 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.<sup>45</sup> 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<sup>46</sup> and cellulose acetate phthalate<sup>47</sup> 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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