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PATHOGENESIS OF TRICHOMONAS VAGINALIS: INVESTIGATIONS IN A MODIFIED MOUSE MODEL AND THE INTERACTION WITH LACTOBACILLUS ACIDOPHILUS

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By

Therese McGrory

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

In most normal healthy women vaginal Lactobacillus is a predominating organism and numbers of this organism become reduced in the presence of Trichomonas vaginalis infection. Recent work with a mouse model of T. vaginalis indicated that only 25% of mice harbour Lactobacillus spp. and T. vaginalis introduced in the vagina persisted for about 7 days after which it was rapidly eliminated. In an attempt to establish a better model of intravaginal infection that resembles human disease we established Lactobacillus acidophilus in estrogenized Balb/c mice. Rates of T. vaginalis infection in this group were then compared to a control group of mice who were not treated with L. acidophilus. The addition of L. acidophilus did not significantly alter the resident mouse vaginal flora. The interaction observed in the model between T. vaginalis and L. acidophilus was then examined in in-vitro competitive assays between the two organisms. Interesting was the deleterious effect of T. vaginalis on L. acidophilus seen in the combined cultures with the reduction in bacteria seen at a much greater rate than in matched controls. Finally, this modified model was employed in preliminary immunological studies of T. vaginalis infection. Mice immunized with T. vaginalis prior to intravaginal challenge were less likely to develop infection than controls.

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This thesis is dedicated to my parents.

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LIST OF ABBREVIATIONS

ABTS 2.2'-Azino-di-[3-athyl-benzthiazolinsulfonate(6)]

CDF cell-detaching factor

CDC Centers for Disease Control (USA)

CNA colistin sulfate and nalidixic acid

coag -ve coagulase negative

ELISA Enzyme Linked Immunosorbent Assay

FBS fetal bovine serum

IgA immunoglobulin class A

IgG immunoglobulin class G

IM intramuscular inoculation

INOC inoculation

INTRAVAG intravaginal inoculation

LA *Lactobacillus acidophilus*

Lacto Lactobacillus species

MRS Lactobacilli MRS broth

NBCS newborn calf serum

PBS phosphate buffered saline

PEA phenyl-ethyl alcohol

STD sexually transmitted disease

Tsoy tryptic soy broth

TV *Trichomonas vaginalis*

TYI Diamond TYI-S-33 medium

INTRODUCTION

TRICHOMONAS VAGINALIS:

GENERAL BACKGROUND

Since 1836, when Donne first described Trichomonas vaginalis, much information has been accumulated regarding this flagellated protozoan, the only member of the Trichomonas genus (T. hominis, T. tenax, T. vaginalis) that parasitizes man (Jirovec and Petru, 1968; Honigberg, 1978).

The structure, including the fine structure, of T. vaginalis has been thoroughly examined by several methods including light microscopy (Honigberg and King, 1964), scanning electron microscopy (Warton and Honigberg, 1979) and freeze-fracture electron microscopy (Honigberg et al., 1984). T. vaginalis varies in size and shape in fresh and in fixed and stained preparations as well as with varying physiochemical conditions (Honigberg, 1978). In axenic cultures, grown in liquid media such as Diamonds- TYI (Diamond, 1957), the parasite is pear-shaped or ovoid, ranges in size from 10-30 μm, has four anterior flagella, an anteriorly located elongate nucleus, an undulating membrane that extends to two thirds of the body length and an axostyle

that projects from the posterior surface of the parasite (Jirovec and Petru, 1968; Honigberg, 1978).

Vaginal infections with T. vaginalis occur frequently in an estimated 5 million women in the United States (Jarecki-Black et al., 1988) and 180 million persons worldwide (Brown, 1972) are infected anually. These figures are reflective of the segment of the population attending STD clinics, and are characteristic of reports on the epidemiology of T. vaginalis infection. In fact, these figures are thought to be gross underestimations of the actual number of people infected with this organism.

T. vaginalis primarily infects the squamous epithelium in the genital tract (Nielson and Nielson, 1975) and in women can occur both intravaginally and extravaginally (Wallin et al., 1981). Both the clinical signs and the symptoms associated with trichomoniasis vary according to strain differences in virulence and differences in individual host susceptibility and immune response. There is some evidence that fewer T. vaginalis organisms can be obtained from infected women during menstruation (Demes et al., 1988), and that prevalence of trichomoniasis is lower in women who use oral contraceptives that in those who do not (Krieger et al., 1985). These reports demonstrate other factors, such as nutrient sources and the influence of hormones, that may regulate the ability of T. vaginalis to establish infection.

Asymptomatic infections occur with a frequency of 9% to 56 % (Krieger, 1981; Catterall, 1972; Brown, 1972; Jirovec and Petru, 1968; Wisdom and Dunlop, 1965). Furthermore, symptoms may vary over time evidenced by the fact that approximately one-third of asymptomatically infected women will develop frank vaginitis within the next six months (Krieger, 1981, Rein, 1978). Among women with trichomoniasis the prevalence of clinical features vary with a frequency as described below: vaginal discharge is a common complaint occurring in 50% - 75% of women (Oriel et al., 1976; Wisdom and Dunlop, 1965; Fouts and Kraus, 1980; Hughes et al, 1966); however, it is not always described as malodorous with only approximately 10% of women presenting with this symptom (Gardner, 1981; Grys, 1973). Vulvovaginal irritation is common with dyspareunia (10%-50%) (Hughes et al, 1966) and dysuria (30%-50%) (Rein, 1978; Clark and Solomons, 1959) described by approximately one third to one half of patients. T. vaginalis has also been implicated in adverse pregnancy outcome (Grice, 1974; Pilawski and Malecha, 1983) and is considered a predictor of low birth weight (Cotch, 1991), premature rupture of membranes (Draper et al.,1991), premature delivery and post-partum endometritis (Cotch, 1991). A recent report documenting the ability of T. vaginalis to rupture membranes in an in-vitro system lends support to these observations (Draper et al., 1991).

The first clinical description of T. vaginalis in men was published in 1884 when Marchand identified these organisms in

the male urinary tract (Weston and Nicol, 1963). It is widely accepted that T. vaginalis is transmitted mainly by sexual intercourse and there are very few documented cases of other modes of transmission (Kreiger, 1990; Su-Lin, 1982). Only a few reports have documented information regarding male urogenital trichomoniasis (Short et al., 1984). Infection in the male urethra with T. vaginalis either occurs far less frequently than trichomoniasis in women or it is more difficult to detect (Street et al., 1982) There are wide variations in the reported frequencies of symptomatic and asymptomatically infected men (Catterall, 1960; Willcox, 1960; Kuberski, 1980); however, the majority of studies show that male sexual contacts of women infected with T. vaginalis are asymptomatic (Jirovec and Petru, 1968; Kuberski, 1980). Non-gonococcal urethritis is the most common clinical symptom associated with T. vaginalis infection in men (Kreiger, 1990). Other conditions that have also been associated with infection include prostatitis (Kuberski, 1980), epididymitis (Liston and Lees, 1940; Fisher and Morton, 1969), balanoposthitis (Watt and Jennison, 1960; Wilson and Ackers, 1980) and infertility (Tuttle et al., 1977).

Neonatal trichomoniasis is acquired while passing through an infected birth canal, and as such it represents a non-sexual transmission of infection. Furthermore, studies have shown that trichomonads are able to survive for short periods (3 to 6 hours) on inanimate objects (Su-Lin, 1982).

Contaminated fomites may then be responsible for indirect or non-sexual infection in children (Komorowska et al., 1962); however, infections with T. vaginalis in children, most of which are self-limited, are not always congenital and may also be markers of sexual abuse.

ASSOCIATION OF T. VAGINALIS WITH OTHER GENITAL PATHOGENS:

Despite the fact that trichomoniasis is one of the most common sexually transmitted diseases, it has not received appropriate attention from the scientific community. Reasons for the lack of interest in trichomoniasis may be due in part to the traditional view that infection with T. vaginalis is a nuisance disease and the organism a harmless inhabitant of the vagina (Jirovec and Petru, 1968; Honigberg, 1978). Furthermore, morbidity associated with trichomoniasis was poorly characterized and it was not directly associated with any significant mortality. Although T. vaginalis is now recognized as a significant urogenital pathogen its mechanisms of pathogenesis are not yet clearly understood and little is known about the relationships betwen T. vaginalis and other microorganisms.

In 1943, Allen and Baum suggested an association between trichomoniasis and pelvic inflammatory disease. This association has received renewed interest since it has been

recognized that T. vaginalis can migrate to the upper genital tract (Mardh and Westrom, 1970) and has the ability to carry infective organisms on its surface (Keith et al., 1986). Studies linking trichomoniasis and cervical neoplasias (Honigberg et al., 1984) suggest an oncogenic potential of T. vaginalis. Trichomoniasis is also frequently associated with other sexually transmitted diseases including those caused by Chlamydia trachomatis (Nielsen and Nielsen, 1975) and human papillomavirus (HPV) (Rodgerson, 1972). Furthermore, there have been reports of the acquisition and retention of viruses by T. vaginalis (Pindak et al., 1989) and an association between T. vaginalis infection and HIV seropositivity (Cameron and Padian, 1990). The numerous associations outlined above suggest that the protozoan infection could be a novel means of transmission of bacteria and viruses. This theoretical possibility necessitates further study into the role of the trichomonad as a potential vector for the transmission of other pathogens.

THERAPY:

Although T. vaginalis infections occasionally resolve spontaneously, in many cases they become chronic infections that require treatment. In 1954 a substance with antitrichomonal activity was isolated (Despois et al., 1956)

and later marketed as the nitroimidazole derivative metronidazole. Since the advent of metronidazole treatment, drug resistant strains of T. vaginalis have emerged (Robinson, 1962; Kulda et al., 1982; Somshor and Romanowski, 1991) with variable levels of resistance. Other explanations for metronidazole treatment failures include drug malabsorption (Davis et al., 1984) or local inactivation of metronidazole by bacteria (McFadean et al., 1969). Furthermore, since its introduction in 1960, there have been concerns regarding the drug's potential toxicity and long-term safety. Mutagenic compounds including acetamide, which is a hepatocarcinogen in rats, have been identified in patients treated with metronidazole (Speck et al., 1976; Koch et al., 1981). Nitroimidazole compounds have been shown to produce chromosomal aberrations in bacteria (Voogd et al., 1974; Legator et al., 1975) and in some patients exposed to high doses of the drug (Mitelmann et al., 1980). Despite these concerns the high prevalence of trichomoniasis and the absence of an alternative treatment necessitate the use of nitroimidazole derivatives as therapy for T. vaginalis infections.

An attempt at vaccine therapy, as an alternative to nitromidazole chemotherapy for the treatment of trichomoniasis, was introduced in Switzerland and is currently in use in some countries. The vaccine was developed using aberrant strains of Lactobacillus acidophilus isolated from

women with vaginitis and later marketed as SolcoTrichovac. Several clinical trials report a reduction in the incidence of reinfection in vaccinated women (Harris et al., 1984; Litschgi, 1983) and elimination of the infection (Ngumbi and Nyakeri, 1984), with cure rates as high as 84% to 90% (Ruttgers and Lorenz. 1982). The principle for using these strains of Lactobacillus in the development of the vaccine was that they possessed cross-reacting antigens to T. vaginalis and thus stimulated the production of protective antibodies against T. vaginalis. Despite documented support for this report (Bonilla-Musoles, 1984; Stojkovic, 1984), other researchers have shown, using several different serological tests, that there is no antigenic similarity between T. vaginalis and L. acidophilus (Gombosova et al., 1986). Furthermore antiserum to L. acidophilus failed to inhibit T. vaginalis cytoadherence or host cell killing (Alderete, 1988). The claims of the efficacy of the SolcoTrichovac vaccine have been met with much skepticism in the scientific community. Aside from the fact that many of the vaccine studies were uncontrolled and therefore their results difficult to interpret, the evidence that cross-reaction between the bacteria and T. vaginalis is unlikely negates the putative scientific explanation or principle behind the vaccine.

INTERACTION OF T. VAGINALIS WITH OTHER MICROFLORA:

As outlined earlier, infections in the vagina respond to external stimuli such as changes in hormone and nutrient levels. As well, hormonal levels affect the vaginal microflora and the resident bacterial species fluctuate continually over the course of the menstrual cycle. The effect of T. vaginalis infection on the composition of normal microflora can only be recognized once the endogenous vaginal flora has been studied in detail. In 1892, Doderlein published the first extensive study on human vaginal microflora. At that time the vaginal flora was considered homogeneous, consisting only of "Doderlein bacillus". Those Gram positive rods are now recognized as members of tne genus Lactobacillus. Although other studies reported heterogeneous flora in the vagina the additional bacteria were initially deemed pathogenic and considered markers representing an unhealthy state (Larsen and Galask, 1980). Eventually, numerous organisms, including potentially pathogenic bacteria were isolated from normal vaginas and recognized as endogenous flora. Over the last century numerous detailed qualitative studies of vaginal flora have been reported. Generally, the vaginal microflora of healthy women consists of various aerobic and anaerobic bacteria, frequently dominated by Lactobacillus sp. (Redondo-Lopez et al., 1990; Larsen and Galask, 1982).

Lactobacilli are found in the vaginal flora of approximately 54%-96% of women sampled (Larsen and Galask, 1980; Hill et al., 1984; Redondo-Lopez et al., 1990). The few quantitative studies that have been done demonstrate a variation in concentration of bacteria from 10^5 to 10^{11} organisms/mL of secretion (Bartlett and Polk, 1984; Sautter and Brown, 1980), with isolation of Lactobacilli in mean concentrations of 10^8 organisms/mL of secretion, the predominant species recovered from the vagina (Eschenbach, 1984; Hamman, 1987).

Although the basic vaginal flora in women has been well documented (Brown, 1978; Larsen and Galask, 1980; Redondo-Lopez et al., 1990), the role of flora in genital disease has not been established. A predominance of Lactobacilli and an acidic vaginal pH (less than 4.5) are considered markers of a healthy vagina (Hillier, 1991). However, the reduction in Lactobacilli and elevation in pH do not accompany all vaginal infections. Studies examining the vaginal flora changes in Candida albicans infections showed continued predominance of Lactobacilli and maintenance of vaginal pH in the acidic range (Drake et al, 1980). Conversely, Candida vaginitis, following antibiotic treatment, has been attributed to the loss of the protective Lactobacillus population in the vagina (Redondo-Lopez et al., 1990).

The few studies that have been performed to examine the bacterial flora associated with trichomoniasis have reported

variable results (Nicoli et al., 1981; Mason et al., 1982; McLellan et al., 1982; deLouvois et al., 1975; Hite et al., 1947; Von Eicher, 1968). However, all of these reported a decrease in the prevalence of Lactobacilli. Jirovec and Petru (1968) described four phases in the development of trichomoniasis. The first phase "Trichomoniasis acuta" occurs shortly after infection. It is recognized by decreases in the number of epithelial cells and Lactobacilli and an increase in trichomonads and leukocytes. Following the first phase is "Culminating Trichomoniasis" characterized by many trichomonads, leukocytes and bacteria with a simultaneous absence of Lactobacilli and epithelial cells. In "Chronic Trichomonaisis", the third phase, there are many epithelial cells, few leukocytes and a variety of bacteria. Trichomonad numbers begin to fluctuate and Lactobacilli are still absent. In the final stage "Latent Trichomoniasis" normal numbers of Lactobacilli and epithelial cells are re-established while few leukocytes and trichomonads remain. These drastic changes in the normal physiologic state of the vagina during trichomoniasis suggest a role for Lactobacilli in the development and progression of disease. The mechanisms involved in the displacement of Lactobacilli have not been elucidated.

LACTOBACILLI:

Trichomonas vaginalis normally requires a pH of 6.0 to 6.5 to proliferate in vitro; however it is able to establish infection in the acidic (pH 4.5) environment of the female vagina. In trichomoniasis vaginal pH appears to rise from less than 4.5 to greater than 5.0 (Hanna et al, 1985) along with a major reduction in the numbers of vaginal Lactobacillus. Lactobacilli are involved in maintaining the acidic vaginal pH and its protective function against invading pathogens (Redondo-Lopez et al., 1990; Eschenbach et al., 1989); however, the mechanisms of protection by indigenous Lactobacilli are not completely understood (Redondo-Lopez et al., 1990). Several mechanisms have been proposed and are currently being investigated.

POSSIBLE CONTROL MECHANISMS:

Low vaginal pH has long been considered a primary mechanism controlling the composition of the vaginal ecosystem. Lactobacilli produce lactic acid which may contribute to vaginal acidity. Furthermore in-vitro growth inhibition of Gardnerella (Skarin and Sylwan, 1986) and E.

coli (Tramer, 1966) have been attributed to lactic acid production by Lactobacilli. However, fatty acids produced by vaginal epithelial cells and secreted into the vagina may be crucial to the maintenance of an acidic vaginal pH (Preti and Higgins, 1975). Additionally, vaginal pH does not always correlate with the presence or absence of Lactobacilli (Redondo-Lopez et al., 1990). As such, the contribution of Lactobacillus for the acidic pH of the vagina and its use as a protective mechanism are not yet confirmed.

Another possible protective mechanism employed by Lactobacilli is competition for adherence. Lactobacilli adhere to uroepithelial cells and inhibit, by steric hindrance, the invading pathogens from colonizing the vagina (Chan et al., 1985). Studies have indicated a clear relationship between adherence and colonization in the vagina (Gibbons et al., 1978; Smith, 1977). Furthermore, in-vitro reports have documented the ability of Lactobacilli to interfere with uropathogenic bacterial colonization of uroepithelial cells (Chan et al., 1984; Reid et al., 1987), and of the urinary tract of mice (Reid et al., 1989), and rats (Reid et al., 1985). The type of bacteria that colonize the vaginal epithelial cells is influenced by many factors including nutritional status; hormonal levels, other flora present in the vagina, vaginal pH, and antibiotic therapy (Mardh and Westrom, 1976; Zawaneh et al., 1979). Studies have indicated that the relative adherence of organisms correlates

with their natural host tropisms such that both indigenous and pathogenic bacteria are found in a narrow range of hosts (Gibbons et al., 1978). Furthermore, different species of bacteria, and even different strains of the same species, such as Lactobacilli, adhere with different avidity. All of these factors must be taken into account in an investigation of competitive exclusion. Failure to account for these variables may be responsbile for some of the lack of agreement seen in reports published on this subject. In an in-vitro investigation pre-incubation of WISH cells with Lactobacillus fermentum resulted in complete or partial inhibition of adherence of T. vaginalis to the monolayers (Martinotti et al., 1986).

Although some investigators have reported that Lactobacilli interfere with the adherence of other bacteria to epithelial cells (Sobel et al., 1981a), other studies have shown that, at certain pH, some bacteria adhere equally well (Sobel et al., 1981b) or outcompete Lactobacilli in adherence (Mardh and Westrom, 1976, Wood et al., 1985). Thus there are conflicting reports suggesting that Lactobacilli have a limited ability to inhibit adherence of invading pathogens or to employ competitive exclusion as a protective mechanism in vivo.

Finally, the protective mechanism may be via Lactobacillus secreted products possessing antimicrobial activities such as hydrogen peroxide (H_2O_2), bacteriocins, and

other inhibitory proteins. A report supporting this view demonstrated that H_2O_2-producing Lactobacilli were found in 96% of normal women, as compared to only 6% of women with bacterial vaginosis (Eschenbach et al., 1989). Some investigators believe that a lack of H_2O_2-producing Lactobacilli may predispose a woman to bacterial vaginosis and that bacterial vaginosis may actually lead to a further decrease in H_2O_2-producing Lactobacilli (Klebanoff et al., 1991). Women with H_2O_2-producing Lactobacilli are seven times less likely to develop bacterial vaginosis than women without H_2O_2-producing Lactobacilli (Hillier, 1991).

Bacteriocins, another inhibitory substance produced by various bacteria including Lactobacillus sp., are active over a narrow spectrum of closely related species (Barefoot and Klaenhammer, 1984). Conversely, inhibitory proteins or bacteriocin-type compounds are active against a wide antibacterial spectrum and have been reported to antagonize Clostridium (McCormick and Savage, 1983), Neisseria (Morin et al., 1980) and other gram-positive and gram-negative species (Mehta et al., 1983). Other inhibitory substances called lactocidins (Vincent et al., 1959) are considered unrelated to bacteriocins (Reeves, 1968) and show bacteriostatic activity against other strains. Together these proteinaceous inhibitors may control potential urogenital pathogens of the vagina in a very specific manner.

Clearly these mechanisms do not confer protection to the vagina individually but rather cooperate to maintain a healthy environment. An in-vitro study that supports this theory was performed to determine if the protective effect of Lactobacillus could be overcome by either changes in pH or the introduction of high concentrations of bacteria (Hiller, 1991). Results from this study showed that at very low pH the Lactobacillus is inhibitory, however, as pH rises the inhibitory properties of the Lactobacilli are lost. Thus the conclusions that can be drawn are that changes in vaginal pH and introduction of high concentrations of bacteria could alter normal vaginal flora.

IN-VITRO INTERACTIONS OF T. VAGINALIS WITH OTHER MICROFLORA:

There appear to be interactions between T. vaginalis and other microorganisms in-vitro. Obviously, information acquired from in-vitro systems cannot be directly correlated to a clinical case, and no in-vitro model has yet been designed that can mimic in-vivo conditions (Wood et al., 1985). As such, although interactions have been examined to some extent, our knowledge is limited.

In a report by Szreter (1979), T. vaginalis lived longer when grown with living cultures of Micrococcus sp., Staphylococcus aureus, or Streptococcus faecalis. However,

survival time was shortened when Proteus vulgaris or Pseudomona aeruginosa was added. Killed organisms had no significant effect on the growth of T. vaginalis. Conversely, another study reported that while trichomonads, in the presence of S. aureus and Escherichia coli had an increased growth rate, they also had an accelerated rate of extinction compared to axenic cultures (Horwatt, 1985). In these experiments, when combined with bacterial cultures, T. vaginalis achieved peak concentrations earlier than the axenic cultures, however, concentrations were lower than those of controls. Similar trends in T. vaginalis growth were observed in Candida albicans coincubation assays (Kurnatowska and Horwatt, 1983).

A limited number of reports have been published documenting the growth of T. vaginalis in in-vitro competitive assays with Lactobacillus sp. One of these reports observed that in the presence of a high concentration of Lactobacilli, T. vaginalis did not grow well (Soszka and Kuczynska, 1977). It is interesting to note that the same experiment also showed that when a high concentration of T. vaginalis was incubated with Lactobacillus the latter became involutional in form. The presence of atypical forms of Lactobacilli in trichomoniasis have since been described in other reports (Milovanovic et al., 1983). Many of the co-incubation studies outlined above considered only the effect on T. vaginalis growth and did not report the likely subsequent

effect on bacterial growth. Studies investigating T. vaginalis interactions in-vitro have reported that the trichomonads are actively phagocytic protozoa that ingest and degrade other microorganisms. Reports have confirmed that Neisseria gonorrhoeae (Francioli et al., 1983, Street et al., 1984), Mycoplasma hominis (Street et al., 1984), Staphylococcus aureus (Szreter and Tymoczko, 1989), and Streptococcus faecalis (Szreter et al., 1987) are ingested, killed and digested by T. vaginalis. Ingestion of Chlamydia trachomatis could not be demonstrated but its survival was decreased when mixed with T. vaginalis (Street et al, 1984). The in-vitro systems clearly do not approximate the complexity of the vaginal ecosystem but these studies may contribute to our rudimentary knowledge of the interaction of T. vaginalis with other microflora.

ANIMAL MODELS OF T. VAGINALIS INFECTION:

Although the specific features of host-parasite interaction in animal models would differ considerably from infections in the human genitourinary tract, in-vivo models present another venue for investigations of pathogenesis in trichomoniasis. T. vaginalis infections have been studied in animals by subcutaneous, intraperitoneal, intramuscular and

intravaginal routes. Subcutaneous and intraperitoneal routes have been widely used for evaluating the pathogenicity of the organism. Honigberg established a correlation between clinical presentations of the vaginal disease and the corresponding abscess size in a subcutaneous mouse assay (Honigberg et al., 1966; Kulda et al., 1970). Intravaginal infections of lab animals have the potential to simulate the manifestations found in human urogenital trichomoniasis, however, investigators have experienced much difficulty in paralleling the human vagina in experimental animals (Kulda, 1990). Several attempts at intravaginal models of infection have utilized monkeys (Street et al., 1983), guinea pigs (Kazanowska et al., 1983), hamsters (Jirovec and Petru, 1968; Honigberg, 1978), rats (Jirovec and Petru, 1968; Honigberg, 1978) and mice (Cappuccinelli et al., 1974; Coombs et al., 1986). Of all these non-human mammals, mice have been the most widely used and have provided the most information concerning experimental infections with T. vaginalis. Intravaginal inoculations in mice have been successfully accomplished using three different modes of inoculation:

1. Inoculation of parasites from axenic cultures
2. Serial passage of trichomonads from mouse to mouse using a primary culture of vaginal wash as inoculum (Coombs et al., 1987).
3. Sexual transmission by mating males with infected and non-infected females (Cappuccinelli et al., 1974).

Due in part to the technical difficulties associated with establishing intravaginal infections, previous attempts to produce intravaginal growth of T. vaginalis have met with limited success and have yielded variable results. Investigators have agreed that pre-treatment of animals with estrogens appears to be essential for establishment of infection (Cappuccinelli et al., 1974; Coombs et al., 1986; Gardner et al., 1987). Rodents undergo a hormonally regulated cyclic variation called the estrous cycle which is similar to the human menstrual cycle. The stage of the estrous cycle at the time of inoculation has been shown to influence the outcome of other genital infections including Campylobacter fetus (Schurig et al., 1974) and Neisseria gonorrhoeae (Braude et al., 1978). It is not known what factors are directly responsible for the enhancement of infection with T. vaginalis in estrogenized animals. The indirect effect of increased glycogen content in vaginal mucosa as well as the changes in normal flora, number of neutrophils and modifications of epithelial cells (Braude et al., 1978, Corbeil et al., 1985) are the apparent factors deemed responsible for alterations in susceptibility.

A direct effect of estrogens on T. vaginalis is unlikely since estradiol was found to be injurious to the parasite in-vitro (Glebski, 1969). A more recent report from Garber et al. (1991) showed that β-estradiol did not affect the growth of T. vaginalis in-vitro; however, it did cause a reduction in

production of the cell detaching factor (CDF). CDF, a virulence factor found in the extracellular filtrate of T. vaginalis grown in cell culture, causes cell detachment in-vitro (Garber et al., 1989). Furthermore, production of CDF has been shown to correlate with clinical presentation (Garber and Lemchuk-Favel, 1990). As such, although estrogens would not suppress trichomonad growth, they may decrease symptoms by decreasing CDF activity.

The time between the administration of estrogen and inoculation of the parasites is important for establishment of infection. The highest infection rate is observed when mice are in proestrous/estrous phase when fewer neutrophils or bacteria are present in the vagina (Corbeil et al., 1985).

Major differences exist between the genital flora of women and that of mice, the most obvious of these involves the Lactobacillus sp. Although dominant in a healthy woman's vagina, recent work has shown that Lactobacillus is harboured by only a small percentage of mice (Meysick and Garber, 1992). Understandably, the features of host-parasite interaction in the mouse differ considerably from infections in the human genitourinary tract. However, studies of other animals show the same discrepancies. The minimal amounts of Lactobacilli and neutral pH documented for animals studied (Larsen et al., 1976; Skangalis et al., 1979) suggest that the high numbers of Lactobacilli and low pH are distinct characteristics of the

human vagina. The ease of manipulation and cost efficiency associated with the mouse makes this animal a model of choice.

IMMUNOLOGICAL ASPECTS OF T. VAGINALIS INFECTION:

The early attempts at immunological studies demonstrated that T. vaginalis was antigenically distinct from other human trichomonads (Tokura, 1935). Since 1956 when Schoenherr demonstrated antigenic diversity among different isolates of T. vaginalis many different serotypes have been identified (Kott and Adler, 1961; Teras, 1966). There has been renewed interest in the antigenic diversity of T. vaginalis with the suggestion that a relationship may exist between pathogenicity and antigenic complexity (Su-Lin and Honigberg, 1983). Although no relationship between antigenic structure and high or low virulence could be confirmed, it has since been shown that heterogenicity among T. vaginalis isolates is related to the surface disposition of high molecular weight proteins (Alderete et al., 1985). These same investigators believe that all strains of T. vaginalis possess the full complement of antigens and that heterogeneity could be explained by the failure of individuals to respond to all of the antigens (Alderete et al., 1986). Garber and colleagues (1986) also demonstrated variability in immune response. Their data

reinforces the theory that different individuals respond immunologically to different antigens of T. vaginalis. The antigenic diversity of T. vaginalis has also been demonstrated in geographic regions of the United States (Kreiger et al., 1985). It is interesting to note that in this same study one of the isolates, an organism isolated in 1963, displayed similar antigenic composition to contemporary isolates. The antigenic stability demonstrated in this isolate may prove to be a significant factor in vaccine development.

HUMAN DEFENSE MECHANISMS:

The invasive and cytopathogenic potential of T. vaginalis is demonstrated by its deleterious effects in vaginal infections; nevertheless, it is rarely able to spread out of the human genitourinary system. The inability to extend infection beyond this specific area may be attributed to host defense mechanisms including, non-immunologic factors, immunologic but non specific defense mechanisms and specific immunologic responses (Ackers, 1990).

Physical and nutritional barriers are two of the non-immunologic host defense mechanisms that may mediate the action of trichomonads. Zinc has also been proposed as a factor limiting infection in men. Normal men have concentrations of prostatic zinc lethal for most isolates of T. vaginalis (Krieger and Rein, 1982); however, there are men

with suboptimal levels of zinc. As well there are zinc-resistant parasites. Other investigators have suggested another mechanism involving iron uptake by the organism. Lactoferrin is an important iron source at the site of infection; however the inability of T. vaginalis to cause parasitemia results from its inability to bind transferrin and thus acquire iron from the bloodstream (Peterson and Alderete, 1984). In a recent extension of this work, the investigators determined that the levels of trichomonad adherence to epithelial cells were modulated by iron such that the presence of lactoferrin enhanced the adherence of the parasite (Lehker et al., 1991).

Other naturally-occurring defense mechanisms include complement, neutrophils, and natural antibodies. Gillin and Sher (1981) first stated that T. vaginalis activates complement by the alternative pathway. The activation of complement enables the polymorphonuclear neutrophils to surround, fragment and phagocytose pieces of T. vaginalis in-vitro (Rein et al., 1980). Furthermore, a secreted by-product of T. vaginalis metabolism is chemotactic for neutrophils but unrelated to activation of complement (Mason and Forman, 1980). Conversely, a more recent report has discussed the effect of secreted neutrophil products on the migration of motile organisms. This data suggests that migration away from neutrophil products may be a means by which trichomonads evade the host immune defenses (Styrt et al., 1991). It is not

known whether these effects seen in vitro actually play an important role in the human response to infection. Spontaneous cytotoxicity against T. vaginalis has been demonstrated in both unstimulated murine macrophages (Landolfo et al., 1980) and healthy volunteers' peripheral blood cells deemed to be of the monocyte macrophage lineage (Mantovani et al., 1981).

Natural antibody has been detected in healthy human sera; however, neither its origin nor its role in host defense mechanisms are known (Reisenhofer, 1963). Some explanations for the presence of these antibodies include cross-reaction with normal flora, infection with commensal trichomonads or genetic predisposition (Samuels and Chun-Hoon, 1964). All of the above mentioned defense mechanisms would be active in a primary infection as they do not require previous exposure to the organism.

The role of acquired immunity in human trichomoniasis is not clearly understood. Previous exposure to T. vaginalis, unlike infection with Giardia lamblia, does not appear to confer any gradual acquisition of protection (Ravidin and Guerrant, 1982). Repeated infections with T. vaginalis may occur without significant decreases in either duration of infection or intensity of symptoms as one might expect in the presence of a specific immune response.

In-vitro studies and animal models have provided some evidence of specific immunologic defense mechanisms.

Honigberg (1970), reported that pretreatment of mice with sera from immunized rabbits or infected humans, resulted in significantly less pathologic changes compared to controls when T. vaginalis was grown within the mouse peritoneal cavity. The mechanism for this effect is not completely understood but it would suggest a protective effect of antibody. A more recent investigation showed that monoclonal antibodies raised against T. vaginalis can kill the antigen-positive parasite independent of complement activation (Alderete and Kasmala, 1986). As outlined earlier, non-stimulated macrophages derived from Balb/c mice can kill the parasites in-vitro (Landolfo et al., 1980). Interestingly, it was later demonstrated that macrophage cytotoxicity could be enhanced by pre-incubation with T-cells collected from mice immunized against T. vaginalis (Martinotti et al., 1986) or treatment with lymphokines (Ryu et al., 1990).

Many reports have been published confirming the existence of serum antibodies IgG, IgA and IgM (Chipperfield and Evans, 1972) in human trichomoniasis. However they appear to have little effect in controlling infection in vivo. One might expect that circulating antibody would have very little effect on a non-disseminating parasite and that protection would more likely be conferred by a local antibody. However, although antitrichomonal antibody has been demonstrated in human cervicovaginal secretions by various immunological methods (Alderete, 1984; Street et al., 1982; Ackers et al., 1975; Su-

Lin, 1982), once again there is very little evidence that it is harmful to the parasite. There is some evidence that specific cell mediated immunity may be instrumental in eliminating infection (Yano et al., 1983); however the data is scant and further work must be done in this area before definite conclusions can be drawn.

IMMUNOPROPHYLAXIS:

Protection by vaccination with Lactobacillus, an organism thought to have cross-reacting antibodies to T. vaginalis, was described earlier in this review. Vaccination via injection of living and heat-killed trichomonads has been attempted in mice and appeared to result in protection in some cases (Kelly and Schnitzer, 1952; Schnitzer and Kelly, 1953; Kelly et al., 1954; Baba, 1958). A more recent attempt at protection from infection by exposing mice to the trichomonad prior to a second challenge also achieved considerable success (Martinotti et al., 1977). These reports stated that protection did not appear to correspond to serum antibody level and the actual mechanism of protection in these cases is not completely understood. Finally, a few patients with severe cases of trichomoniasis, unresponsive to chemotherapy, were inoculated with heat killed T. vaginalis intravaginally (Aburel et al., 1963). This approach appeared successful; 40

of the 100 women receiving treatment were cured and another 49 lost all symptoms. This appears to be the only published study demonstrating the potential of a protective immune response (Ackers, 1990).

RATIONALE:

While the symptoms associated with trichomoniasis have been well characterized, the mechanisms associated with the pathogenesis of this disease are not clearly understood. An in-vitro model of T. vaginalis could provide insight into the pathophysiology and immunology of this disease.

In chronic latent trichomoniasis in women there is a major reduction to total loss of the Lactobacillus population. This change in the normal physiologic state of the vagina due to trichomoniasis prompted us to study this apparent relationship between Lactobacillus and T. vaginalis in a mouse model. It was then important to determine the effect of this addition on the resident mouse vaginal microflora.

This change in population levels during trichomoniasis suggest that T. vaginalis alters the Lactobacillus population; however, the mechanism employed by the protozoa to mediate this reduction has not yet been defined. In-vitro competitive assays between T. vaginalis and L. acidophilus could provide insight into the interactions between these two organisms.

The importance of creating a more consistent and sustained model of intravaginal T. vaginalis infection that plausibly resembles human disease, lies in its use as a tool in further studies. As such, preliminary experiments were performed using the model in an attempt to elucidate the immunology of T. vaginalis infections.

OBJECTIVES:

1. To establish a more consistent and sustained mouse model for T. vaginalis intravaginal infection.

2. To study the interactions between L. acidophilus, T. vaginalis and vaginal flora in the modified mouse model.

3. To examine the interaction between L. acidophilus and T. vaginalis in in-vitro competitive growth assays.

4. To employ the modified model in preliminary immunological studies of T. vaginalis infection.

MATERIALS AND METHODS

STRAINS:

Isolates of T. vaginalis were obtained from vaginal secretions from women. Organisms were grown in 5% CO_2 at 37 ^{0}C at a 45 ^{0} angle, in glass, screw-capped tubes (16 by 125 mm) in 10 mL of Diamond's TYI-S-33 medium, pH 6.2 (Diamond et al, 1957) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco Laboratories, Life Technologies Inc. Grand Island, N. Y.), 100 U/mL penicillin, 100 µg/mL streptomycin (Penicillin-Streptomycin Solution Gibco Laboratories) and 2.5 µg/mL Amphotericin B (Gibco Laboratories) as previously described (Garber et al., 1987). Cultures were passaged every 2-3 days. Axenic cultures were mixed with an additional 10% heat inactivated FBS and 10% dimethyl sulfoxide and stored at -70 ^{0}C. For these experiments a well characterized isolate #263 (Garber et al., 1989) derived from a woman with symptomatic vaginitis was used.

L. acidophilus (ATCC 4356) was purchased from the American Type Culture Collection, Rockville, Md. Organisms were grown in 5% CO_2 at 37 ^{0}C in glass, screw-capped tubes (16

by 125 mm) in Bacto Lactobacilli MRS Broth (MRS) (Difco Laboratories, Detroit) or on MRS plates (Bacto Lactobacilli MRS Broth with an additional 1.5% Bacto Agar) (Difco Laboratories). Axenic cultures were mixed with 10% glycerol and stored at -70 ^{0}C.

INTRAVAGINAL INOCULATION OF BALB/C MICE:

L. acidophilus

Two days prior to inoculation of Lactobacillus a subcutaneous injection of 0.05mL of Delestrogen (Estradiol Valerate 10mg/mL Squibb Canada, Montreal, Quebec) (Coombs et al., 1986) was performed on the flanks of 22-24g female BALB/c mice (Charles River, Montreal, Quebec). Immediately before inoculation the stage of estrus of each mouse was determined by vaginal smear according to established criteria (Fox and Laird, 1970).

Mice were inoculated intravaginally using an Eppendorf pipet with 10^{10} L. acidophilus/mL on two consecutive days (total inoculated volume 20μL on two consecutive days).

Inoculum preparation

Prior to inoculation a one litre quantity of MRS broth was inoculated with 0.1% of a pure culture of L. acidophilus and incubated overnight at 37 ^{0}C in 5% CO_2. Organisms were then harvested by centrifugation for 10 minutes at 5,000 x 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 L. acidophilus a standard curve of optical density at 650 nm in a spectrophotometer (Beckman DU-88) versus colony forming units/mL was established. Serial 10-fold dilutions from the original sample were made in PBS. The number of colony forming units of L. acidophilus was determined by spreading 100 µL of each suspension on MRS plates which were incubated at 37 °C. Bacterial colony forming units were counted at 48 hrs for confirmation of the concentration of L. acidophilus inoculated. Media control mice were inoculated with 20 µL of MRS without L. acidophilus.

Vaginal washes were performed using 50 µL pre-warmed MRS in a disposable Eppendorf pipet. Once the tip was inserted into the vagina the fluid was injected and aspirated several times until turbid. Confirmation of infection with L. acidophilus was determined by culturing vaginal washes in MRS broth supplemented with 5 µg/mL ciprofloxacin (Squibb Canada) and 180 µg/mL cefoxitin (Mefoxin, Merck, Sharp & Dohme) and incubated at 37 °C for 24-48 hours. One wash was performed on mice to determine the success of L. acidophilus infection in mice prior to T. vaginalis inoculation. Vaginal washes for L. acidophilus were discontinued once mice had been inoculated with T. vaginalis.

T. vaginalis

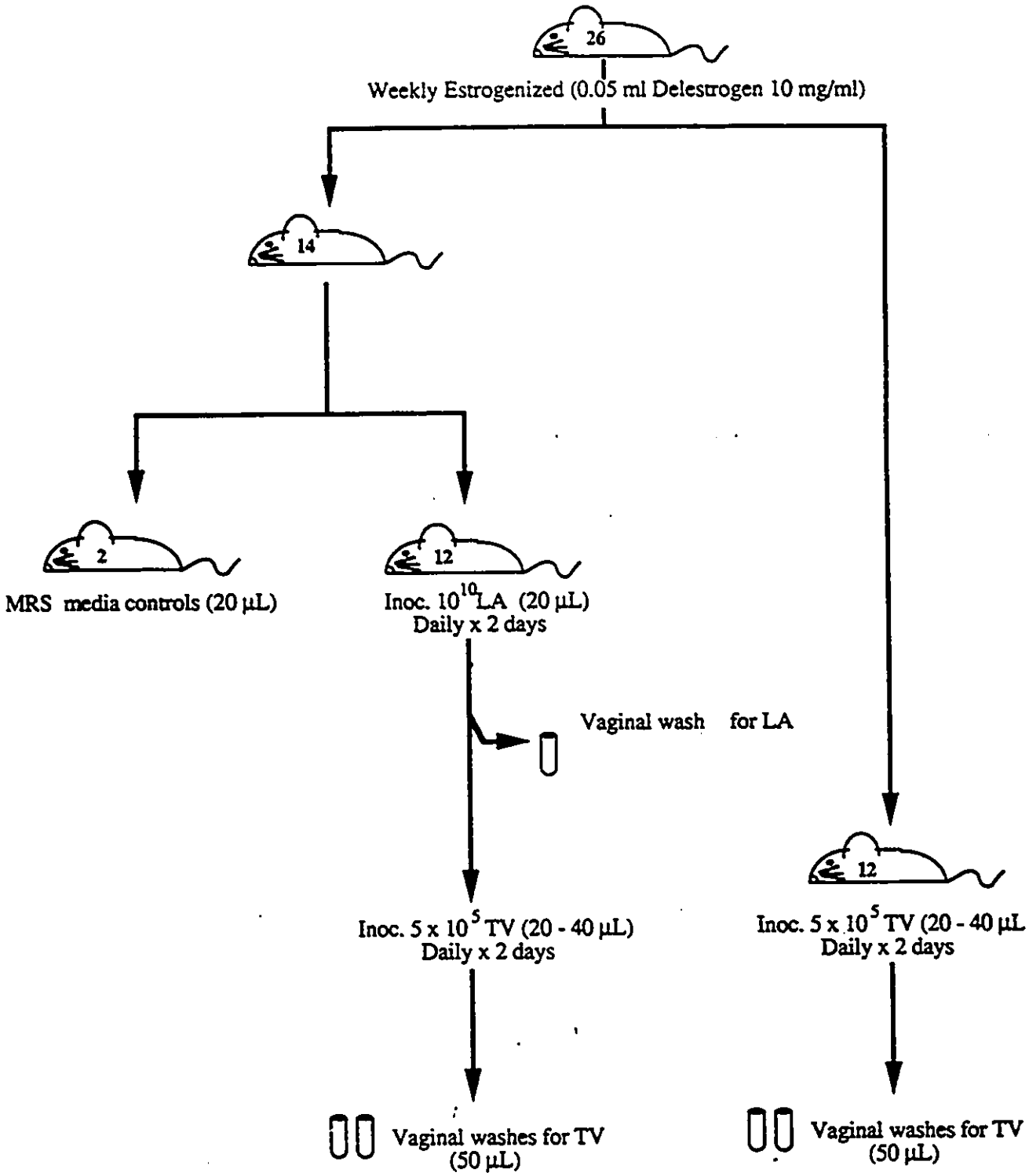
A similar procedure to that described above was used for T. vaginalis inoculations. Two days prior to T. vaginalis inoculation mice were injected for a second time subcutaneously with 0.05 mL Delestrogen one week after the first injection. On two consecutive days 5 x 10^5 log phase T. vaginalis were administered intravaginally to each BalB/c mouse (total inoculated volume 20 µL on two consecutive days). Prior to inoculation, trichomonads were harvested by centrifugation for 10 minutes at 140 x g in a Sorvall GLC-1 centrifuge, washed three times in PBS and the final pellet resuspended in TYI supplemented with 10% FBS and 0.32% Bacto Agar as previously described (Meysic and Garber, 1992)

Vaginal washes were performed with 50 µL pre-warmed TYI supplemented with 10% FBS in a disposable Eppendorf pipet. The tip was inserted into the mouse vagina and the contents dispelled and aspirated until turbid. Duration of infection with T. vaginalis was determined by culturing vaginal washes in glass screw-capped tubes in 10 mL pre-warmed TYI supplemented with 10% FBS, 300 U/mL penicillin, 300 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 300 µg/mL kanamycin and 10 µg/mL gentamicin. Antibiotics were incorporated to overcome bacterial contamination. Cultures were incubated in 5% CO_2 at 37 ^{0}C at a 45 ^{0} angle and examined daily for the presence of motile T. vaginalis using an inverted microscope.

The complete protocol for the intravaginal inoculation of mice is outlined in Figure 1. All mice, including controls received identical Delestrogen treatments on two occasions one week apart. Two control mice per experiment were inoculated with uninfected MRS broth only. The remaining mice were divided into two groups: one group received both L. acidophilus and T. vaginalis inoculations one week apart; the other group acted as controls for the addition of the L. acidophilus and were not inoculated with the bacteria in the first week. These mice were then inoculated with T. vaginalis in the second week as outlined above.

FIGURE 1

Lactobacillus acidophilus and *Trichomonas vaginalis* inoculation procedure where LA = *L. acidophilus* and TV = *T. vaginalis*


26
Weekly Estrogenized (0.05 ml Delestrogen 10 mg/ml)
14
2
MRS media controls (20 µL)
12
Inoc. 10^{10} LA (20 µL)
Daily x 2 days
Vaginal wash for LA
12
Inoc. 5×10^5 TV (20 - 40 µL)
Daily x 2 days
Inoc. 5×10^5 TV (20 - 40 µL
Daily x 2 days
Vaginal washes for TV
(50 µL)
Vaginal washes for TV
(50 µL)

SAMPLING OF MOUSE VAGINAL FLORA:

Mouse vaginal flora was sampled with 50 μL 0.9% NaCl, as vaginal wash, using an Eppendorf pipet as previously described (Meysick, K., and G. E. Garber, J. Parasitol., in press). The tip was inserted into the vagina and the wash aspirated several times until turbid. The 50 μL wash was then divided into two equal aliquots, 25 μL was added to 2.5 mL of T-soy broth (Difco Laboratories, Detroit, Michigan) and the remaining 25 μL added to 2.5 mL of pre-reduced Bacto Fluid Thioglycollate Broth enriched for anaerobes with 1% hemin and 1% vitamin K (Difco Laboratories). T-soy broths were incubated at 37 ^{0}C for 12-16 hours. Enriched thioglycollate broths were incubated in anaerobic chambers (PML Microbiologicals) at 37 ^{0}C for 4 days.

For isolation of aerobic organisms, T-soy broths were streaked after incubation on four different plates including general, differential and selective media: Blood agar, Columbia CNA agar, MacConkey CS agar and Chocolate agar (Difco Laboratories). All plates were incubated aerobically in 5% CO_2 at 37 ^{0}C for 24 hours. After primary plating and incubation individual colonies from each plate were picked and subcultured onto Blood agar and incubated at 37 ^{0}C for 24 hours. Colonies from secondary plating were identified by standard methods of morphological characteristics, Gram stain,

hemolysis pattern, catalase, and oxidase (Bacto oxidase Differentiation Disks, Difco Laboratories) reactions. Final identification was determined with the Multi-Scan system (Microscan Pos BP Combo Panel Type 5, Neg BP Combo Panel Type 5 and Autoscan-4 with DMS, American Micro-Scan, West Sacramento, California) for both Gram positive cocci and Gram negative rods.

For isolation of anaerobic organisms enriched Thioglycollate broths were sampled on two separate occasions, once at 48 hrs after initial incubation and again 4 days after initial incubation by streaking onto three different pre-reduced media: Columbia CNA agar, CDC-PEA agar, and Brucella Blood agar (Difco Laboratories). All plates were incubated anaerobically at 37 ^{0}C for 48 hours. After primary plating and incubation individual colonies from each plate were picked and subcultured onto reduced CDC-PEA agar plates and incubated anaerobically at 37 ^{0}C for 48 hours. Secondary plating of isolates with different colony morphology was also done on Chocolate agar and Blood agar and incubated aerobically in 5% CO_2 at 37^{0}C for 24 hours to differentiate between obligate and facultative anaerobes or microaerophilic organisms. Colonies from anaerobic purity plates were identified using standard methods including morphological characteristics, Gram stain, hemolysis patterns, catalase, and oxidase reactions. Final identification was determined using biochemical testing with

An-Ident kits (Analytical Profile Index, Analytab Products, New York).

IN-VITRO COMPETITIVE ASSAYS:

T. vaginalis isolate 263 was grown to log phase in TYI supplemented with 10% FBS without antibiotics. Simultaneously, a pure culture of L. acidophilus was grown to log phase in MRS broth. Both the L. acidophilus and the T. vaginalis were centrifuged and washed separately, as previously described, and suspended in sterile PBS. The trichomonads were counted by trypan blue exclusion using a hematocytometer and the bacterial concentration determined spectrophotometrically at 650 nm.

Appropriate dilutions of both organisms were made in glass screw capped tubes (25 x 150 mm), in pre-warmed TYI supplemented with 10% FBS without antibiotics. Two separate ratios of T. vaginalis (TV) to L. acidophilus (LA) were used: 10^4 TV/mL to 10^5 LA/mL, LA/TV 10:1 ratio and LA/TV 1:1 ratio (LA/TV 10^4 & LA/TV 10^5) along with matched controls (TV 10^4/mL, TV 10^5/mL, LA). A control (LA) in MRS was also included. All tubes were incubated in 5% CO_2 at 37 ^{0}C.

Samplings to monitor changes in T. vaginalis and L. acidophilus growth patterns were made every 8 hours. For T. vaginalis sampling a 100 μL aliquot was removed from each tube

with a sterile Eppendorf pipet. Appropriate 10-fold dilutions of the original sample were made in PBS so that viable trichomonads could be counted by trypan blue exclusion using a hematocytometer, as previously described. Quantitation of L. acidophilus was performed at each sampling by 10-fold dilutions in PBS of a 100 μL sample from each tube. Appropriate dilutions were spread on MRS plates and incubated at 37 °C. Bacterial colonies were counted after 48 hours incubation.

Every 2 hours the pH of 10 μL sample aliqouts was determined by pH paper readings (Accutint Indicator Paper pH range 3.9-5.4 and 5.0-6.6, Anachemia Chemicals Ltd., Montreal,Quebec). Sterile 1N and 3N NaOH were added in 50 μL - 200 μL amounts, when required, to maintain the pH of all tubes, including control and experimental ratios, between pH 5.0 and pH 7.0.

IMMUNOLOGICAL STUDIES

INTRAMUSCULAR INOCULATION OF T. VAGINALIS:

T. vaginalis isolate 263 was grown to log phase in TYI and harvested by centrifugation for 10 minutes at 140 x g in a Sorvall GLC-1 centrifuge. The pellet was washed three times in PBS by resuspension and centrifugation, as previously described. The final pellet resuspended in PBS was adjusted

to a final concentration of 1.8×10^6 T. vaginalis/ml and then loaded in 0.5mL amounts into 1mL syringes. The inocula were injected intramuscularly into the shaved flanks of 22-24 g female BalB/c mice. Control mice received intramuscular injections of 0.5mL PBS only.

An inoculation protocol was designed (Figure 2) to determine the effects of intramuscular inoculation of T. vaginalis on the ability to infect mice intravaginally with T. vaginalis. Three different groups of six mice each were intramuscularly inoculated with the pathogen at days -28, -14 and -7. Another six mice served as controls and were not previously exposed to T. vaginalis through intramuscular inoculation prior to intravaginal inoculations. At day -7, on the same day as the last set of intramuscular inoculations, all four groups of six mice were subjected to the intravaginal inoculation regimen outlined below.

Two days prior to inoculation (day 9) mice received a subcutaneous injection of 0.05mL of Delestrogen. Immediately before intravaginal inoculation with L. acidophilus the stage of estrus of each mouse was determined by vaginal smear using the method of Fox and Laird. Mice were inoculated intravaginally, using an Eppendorf pipet, with 10^{10} L. acidophilus/ml on two consecutive days, as previously described.

FIGURE 2

Immunological studies protocol outlining the time course for intramuscular and intravaginal inoculations of mice.

IM TV= intramuscular inoculation of 0.5 mL of 1.8 X 10^6 TV/mL

LA = 10^{10} L. acidophilus inoculated intravaginally on two consecutive days (total inoculated volume 20μL each day)

TV = 5 X 10^5 T. vaginalis inoculated intravaginally on two consecutive days (total inoculated volume 20μL each day)

IMMUNOLOGICAL STUDIES PROTOCOL

ALL MICE: PRE-BLEED
PRE VAGINAL WASH
ESTROGENIZED 2 DAYS PRIOR TO INTRAVAGINAL INOCULATIONS OF BOTH TRICHOMONAS VAGINALIS AND LACTOBACILLUS ACIDOPHILUS

2 MICE DESIGNATED NEGATIVE CONTROLS: NO IM TV
NO LA, NO TV

TIME COURSE---------->

GROUP1	GROUP2	GROUP3	GROUP4	CONTROLS
M1-M6	M7-M12	M13-M18	M19-M24	NO IM
-28days	-14days	-7days	NO IM	
IM TV	IM TV	IM TV		

\----------/
DAY -7:
ALL MICE LA INTRAVAG X 2 DAYS

\----------/
DAY 0:

GROUPS 1-4 TV INTRAVAG X 2 DAYS

\----------/

AT WEEKLY INTERVALS FOR 4 WEEKS (DAYS 7,14,21,28)
ALL MICE TAIL BLEEDS & VAGINAL WASHES

Five days later, two days prior to T. vaginalis intravaginal inoculations, all mice were again estrogenized, by subcutaneous injection of Delestrogen. Following this, at day 0, mice received 5 x 10^5 log phase T. vaginalis administered intravaginally on two consecutive days, as previously described.

An additional two mice, designated negative controls, were estrogenized and intravaginally inoculated with L. acidophilus in parallel with the mice described above. However, they were not exposed to T. vaginalis through either intramuscular or intravaginal inoculations.

MOUSE VAGINAL WASHES AND TAIL BLEEDS:

Duration of infection with T. vaginalis was determined by culturing vaginal washes in TYI medium supplemented with 10% FBS and antibiotics. Infection was considered to be present until no live trichomonads were visible on 2 consecutive daily examinations. Vaginal washes were performed with 50 µL pre-warmed TYI supplemented with 10% FBS without antibiotics, with an Eppendorf pipet. The medium was inserted into the vagina with the pipet and ejected and aspirated several times until turbid. Wash material was collected in pre-warmed TYI supplemented with 10% FBS and antibiotics, incubated in glass, screw-capped tubes (16 x 125mm) in 5% CO_2 at 37 ^{0}C at a 45 ^{0}

angle and examined daily in an inverted microscope for live trichomonads.

A second vaginal wash was done with 50 μL 0.9% NaCl. This wash material was collected and expelled into 1.5 mL Microfuge tubes. These tubes were then centrifuged for 10 minutes at 14,926 x g in a Heraeus Biofuge A microfuge. The supernatant was transferred to sterile Microfuge tubes and stored at -70 ^{0}C until required for Enzyme Linked Immunosorbent Assays (ELISA). The pellet was resuspended in pre-warmed TYI supplemented with 10% FBS and antibiotics, incubated in 5% CO_2 at 37 ^{0}C at a 45 ^{0} angle and examined daily for live trichomonads as outlined above for the vaginal wash samples.

Both TYI and saline vaginal washes were performed on a weekly basis for four weeks following the intravaginal inoculations of T. vaginalis. At the time of vaginal washings mice were also bled from the tail. Approximately 20 μL of blood was collected from each mouse in non-heparinized/ Blue Coded Tip Microhematocrit Capillary Tubes and then centrifuged for 2 minutes in a Micro-Hematocrit centrifuge (Model MB, International Equipment Co.). The serum was collected in 1.5 mL Microfuge tubes and stored at -70 ^{0}C until required for ELISA.

PREPARATION OF ANTIGEN FOR ELISA PLATES:

Isolates of T. vaginalis were obtained from vaginal secretions from women. Axenic cultures of T. vaginalis, stored at -70 °C were rapidly thawed and maintained in TYI supplemented with 10% FBS and antibiotics. Cultures were passaged every 2-3 days with a minimum of three subcultures before use in the ELISA.

An axenic culture of trichomonads in the logarithmic growth phase was harvested by centrifugation for 10 minutes at 140 x g in a Sorvall GLC-1 centrifuge, washed twice in PBS (pH 7.2), followed by washing twice in carbonate buffer (pH 9.6). The final pellet was resuspended in carbonate buffer and adjusted to a concentration of 2 x 10^5 cells/mL. 100 µL aliquots of this suspension were dispensed into each of 96 wells of round bottom plates (Nunc Polysorb U96), wrapped in parafilm, and incubated at 37 °C overnight. Plates were then wrapped in foil and stored at -70 °C until required for ELISA as previously described (Sibau et al., 1987).

ELISA TECHNIQUE:

Antigen-coated plates were thawed at 37 °C and washed three times with washing buffer (PBS, 0.1% tween 80). Test

sera were diluted 1:200 in dilution buffer (0.1% bovine serum albumin, PBS, 0.1% tween 80) and 100 μL volumes were then applied in 2-fold dilution series to antigen pre-coated plates. Each serum sample was assayed in duplicate on two separate plates. Specimen dilution was necessary to obtain sufficient sample volumes for subsequent testing. After 60 minutes of incubation at 37 ^{0}C plates were washed three times in washing buffer and 100 μL of goat anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma Chemical Co., St. Louis, Missouri) diluted 1:1000 in dilution buffer was added to each well. The plates were incubated at 37 ^{0}C for 45 minutes then washed three times with washing buffer. Colour development was performed with 100 μL of 0.36 mM 2-2'-Azino-di-[3-athyl-benzthiazolinsulfonate(6)] (ABTS) (Boehringer Manheim) and 0.03% H_2O_2 (Fisher Scientific, Ottawa, Ontario) dissolved in citrate buffer (0.1M citrate and 0.02M sodium phosphate, pH 4.25). After incubation at room temperature for 25 minutes the plates were read spectrophotometrically at 405 nm on a Biorad Microplate Reader. Positive and negative serially diluted controls were included with each plate.

The ELISA described above was also used for assays of mouse vaginal washes with the following modifications. Vaginal wash samples were diluted 1:50 in dilution buffer and 100 μL volumes of 2-fold dilutions of 1:50 to 1:400 were added to antigen pre-coated plates. In addition to the assays with goat anti-mouse IgG these same samples were also assayed with

100 μL of goat anti-mouse IgA (whole molecule) peroxidase conjugate (Sigma Chemical Co., St. Louis, Missouri).

Since no T. vaginalis specific IgG or IgA could be detected in the ELISA of mouse vaginal washes, total IgA and IgG capture assays were performed on the mouse vaginal washes to see whether the washes contained detectable levels of either immunoglobulin.

High binding microtiter 96 well flat bottom plates (Costar, Cambridge, MA.) were coated with 0.05 μg/mL non-conjugated goat anti-mouse IgA (Sigma Chemical Co.) suspended in PBS, covered and incubated at 37 ^{0}C overnight, then washed three times with washing buffer. For IgG capture assays 0.05 μg/mL non-conjugated goat anti-mouse IgG (Sigma Chemical Co.) was used to coat the plates. Vaginal wash samples were diluted to a stock concentration of 1:50 in PBS-N (1% newborn calf serum (NBCS) and 0.1% tween 80) then 100 μL volumes of prepared dilutions ranging from 1:50 to 1:6400 were added to coated plates. Plates were covered and incubated at 37 ^{0}C for 30 minutes then washed three times with washing buffer. 200 μL of a 1:1000 dilution of peroxidase conjugated goat anti-mouse IgA (Sigma Chemical Co.) diluted in PBS-N supplemented with an additional 4% NBCS was added to each well. For IgG capture assays a 1:1000 dilution of peroxidase conjugated goat anti-mouse IgG (Sigma Chemical Co.) was used. The plates were covered and incubated for 30 minutes at 37 ^{0}C then washed three times with washing buffer. Colour development was

performed with 100 μL of 0.36 mM ABTS (Boehringer, Mannheim) and 0.03% H_2O_2 (Fisher Scientific) dissolved in citrate buffer. After incubation for 25 minutes at room temperature plates were read spectrophotometrically at 405 nm on a Biorad Microplate Reader.

STATISTICAL ANALYSIS:

Where applicable, data were analyzed statistically with computer programs for chi-square test (Anova from EPISTAT, software programmed by T. L. Gustafson) and 2-way analysis of variance with Scheffe and Student Newman Keuls procedures (Statistical Package for the Social Sciences).

ANIMAL CARE:

All experimental procedures and protocols involving animals were reviewed by and met with the approval of, the University of Ottawa Animal Care Committee, protocol number MI-33.

RESULTS

MODIFICATIONS TO THE INTRAVAGINAL MOUSE MODEL

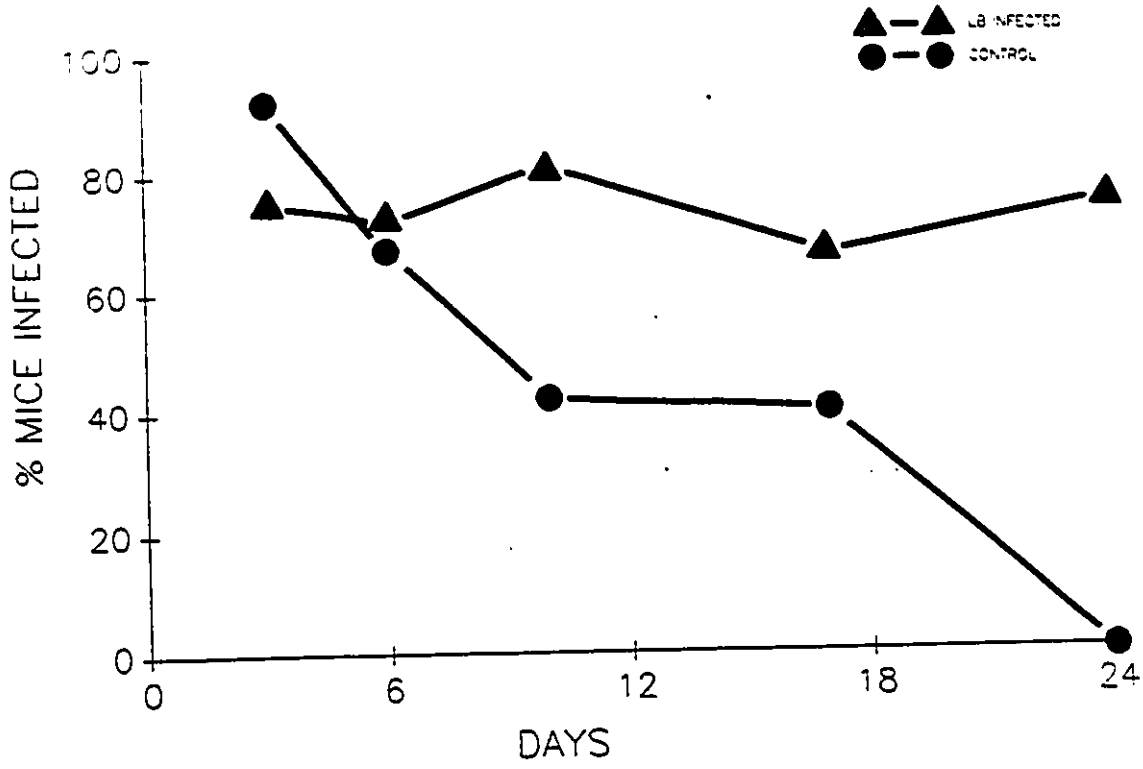
In initial experiments, the rate of T. vaginalis infection in mice pre-treated with L. acidophilus was compared to control mice, not previously inoculated with L. acidophilus. In each of two experiments T. vaginalis infection was determined in 12 Balb/c mice pre-infected with L. acidophilus and in 12 mice that had not been previously infected with the bacteria. Two additional mice, designated media standards, received intravaginal inoculations of media only. FIGURE 3 represents these trials with Graph A representing the first experiment and Graph B representing the second.

Initial T. vaginalis infectivity is similar in the two groups. For example, at day 3, T. vaginalis was detectable in 75% (A) and 83% (B) of L. acidophilus pre-inoculated animals and 92% (A) and 75% (B) in the controls. However, the mice pre-inoculated with Lactobacilli showed a statistically significant increase in duration of infection with T. vaginalis as compared to control mice. For example at day 24 L. acidophilus pre-inoculated animals were (A) 75% and (B) 63% respectively positive for T. vaginalis with controls (A) 0% and (B) 25% positive.

FIGURE 3

Comparison of the percentage of mice infected with T. vaginalis in mice pre-infected with L. acidophilus and controls, not previously inoculated with L. acidophilus. Abbreviations used: LA infected --- Lactobacillus acidophilus pre-infected mice; Controls --- mice not pre-infected with Lactobacillus acidophilus. Graph A represents experiment #1 and Graph B represents experiment #2.

A


LB INFECTED
CONTROL
100
80
60
40
20
0
% MICE INFECTED
0
6
12
18
24
DAYS

B

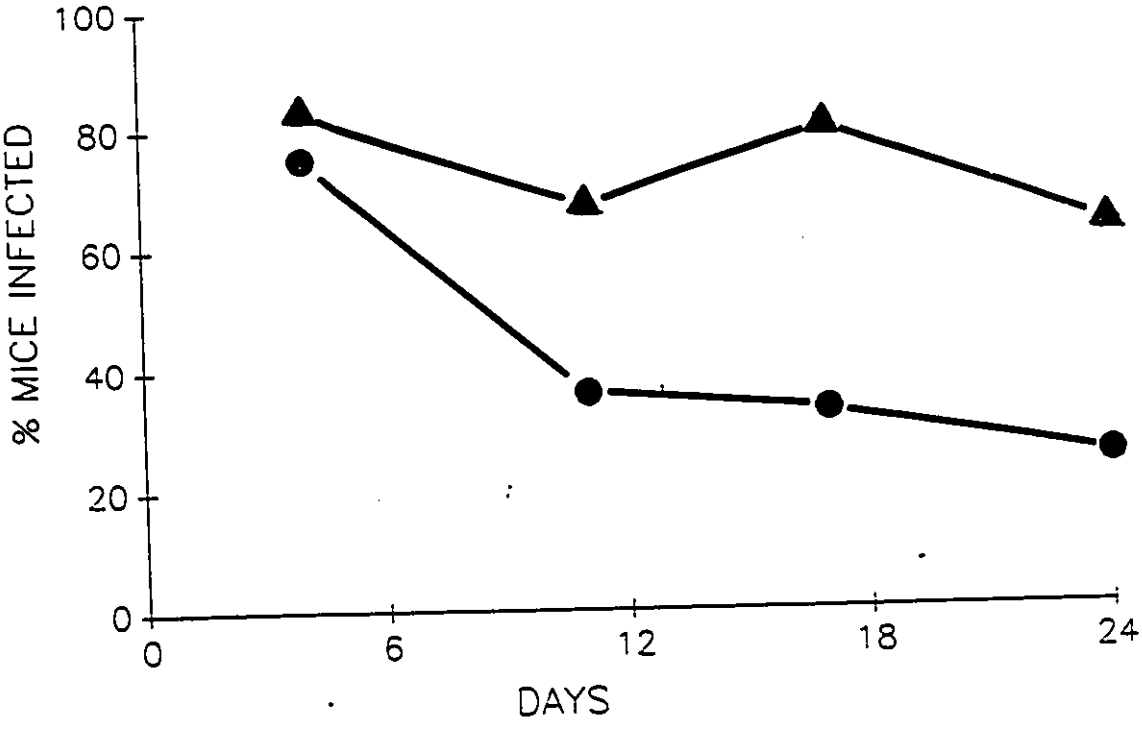

100
80
60
40
20
0
% MICE INFECTED
0
6
12
18
24
DAYS

TABLE 1 shows that the difference between mice pre-inoculated with L. acidophilus and the controls is highly statistically significant at days 11, 17, and 24 samplings ($p=0.03$, $p=0.04$, and $p=0.002$), respectively.

Furthermore, the significant decrease in numbers of mice infected with T. vaginalis after day 7, seen in control mice and previously reported (Meysick and Garber, 1992) is not apparent in the L. acidophilus pre-inoculated mice. No T. vaginalis could be recovered from control mice which had received inoculations of MRS medium only.

Vaginal wash specimens of all mice were examined prior to the T. vaginalis inoculation in order to establish the stage of estrous at the time of inoculation. It has been well documented that estrogenization is required for successful establishment of infection with T. vaginalis. Due to experimental estrogenization, the majority of mice (80%) were in estrous at inoculation. The remaining, relatively small proportion of mice were in metestrous or proestrous.

TABLE 1. Comparison of T. vaginalis infection of mice pre-inoculated with Lactobacillus acidophilus and controls

# Days post-infection	% Mice infected with T. vaginalis		
	LA*pre-infected	Controls	
4	79 (19/24)	83 (20/24)	p=1.0
11	74 (17/23)	37 (9/23)	p=0.03
17	73 (14/18)	25 (7/18)	p=0.04
24	69 (11/16)	11 (2/18)	p=0.002

* LA = L. acidophilus

(X/X)= number of animals infected/ number in group

The apparent inconsistencies seen in the graphs when the percentage of infected mice increased over time are the result of two different phenomena (for example FIG. 3A between days 17 and 24). The first of these involves false negatives which represent mice that were negative for T. vaginalis during a sampling session; however, subsequent samplings were positive for T. vaginalis cultures. As a result mice were designated negative for T. vaginalis only after two sequential negative cultures were detected for that particular mouse. False negatives were not characteristic of one particular group, and were no more frequent in the L. acidophilus pre-inoculated mice than in controls. The second phenomenon is the declining numbers of mice per group over the course of the experiment as a result of occasional death due to the high number of manipulations involved. Once again this was not specific to either group (controls 6 deaths, LA-infected 8 deaths, p=NS).

ALTERATIONS IN RESIDENT MOUSE VAGINAL FLORA

In order to monitor any alterations in vaginal flora as a result of the addition of L. acidophilus or the combined effect of the addition of L. acidophilus and T. vaginalis, the vaginal flora of 33 mice was sampled on three different occasions:

1. Estrogenized mice were sampled immediately prior to intravaginal inoculation with L. acidophilus to establish the baseline mouse vaginal flora.

2. A second sampling was performed one week post-inoculation with L. acidophilus and prior to intravaginal inoculation with T. vaginalis to determine any alterations in vaginal flora as a result of the addition of L. acidophilus.

3. The final sampling was performed one week post-inoculation with T. vaginalis to determine if the combined effect of T. vaginalis and L. acidophilus caused any significant alterations in mouse vaginal flora.

The average number of bacterial species recovered per mouse and the relative frequency of recovery per species are presented in TABLE 2 and TABLE 3, respectively.

TABLE 2. Average number of species isolated per mouse relative to the time of sampling.

TIME OF SAMPLING	AVERAGE # SPECIES/MOUSE	
PRIOR TO INOC	2.49 ± 1.24	
AFTER LA INOC	3.03 ± 1.28	p=0.05
AFTER TV INOC	2.88 ± 1.54	p=NS

LA = L. acidophilus; TV = T. vaginalis

INOC = intravaginal inoculation

n=33

TABLE 3. Vaginal flora in 33 BalB/c mice

Microorganism	Frequency of isolation		
	Control	LA*	LA/TV**
Staphylococcus aureus	53%	53%	71%
Enterococcus faecium	40%	66%	59%
Coag -ve Staphylococcus	29%	59%	33%
Escherichia coli	22%	34%	47%
Enterococcus faecalis	15%	16%	24%
Lactobacillus sp.	30%	66%	18%
Proteus mirabilis	8%	16%	0%
Bacillus sp.	18%	0%	5%
Neisseria subflaviae	0%	6%	6%
Enterobacter aerogenes	0%	3%	6%
Bifidobacterium sp.	0%	3%	5%
Acinetobacter lwoffi	2%	3%	0%
Streptococcus viridans	8%	0%	0%
Grp F Streptococcus	2%	0%	0%
Aeromonas hydrophilia	0%	9%	0%
Clostridium sp.	0%	3%	0%

*LA = L. acidophilus infected, **TV = T. vaginalis infected

The average number of species per mouse in the control samplings (before inoculations with either organism) was 2.49 ± 1.24. This increased slightly to 3.03 ± 1.28 (p=0.05 marginally significant) at the second sampling, which would account for the addition of L. acidophilus, and then fell to 2.88 ± 1.54 (p=NS) with the addition of T. vaginalis.

The most frequently isolated microorganisms included: Enterococcus sp., Staphylococcus aureus, coagulase negative Staphylococcus and Escherichia coli. Although anaerobic culturing techniques were employed the incidence of anaerobic organisms was relatively low. This may reflect a limitation in sampling mouse vaginal flora. The differences between the average number of species isolated per mouse and the frequency of isolation of particular vaginal species were not statistically significant from one occasion of sampling to another.

IN-VITRO COMPETITIVE GROWTH ASSAYS

In-vitro coincubation growth assays at controlled pH were performed with T. vaginalis isolate 263 and L. acidophilus in order to elucidate the interactions between the two organisms. Two separate ratios of T. vaginalis (TV) to L. acidophilus (LA) were used: 10^4 TV/mL to 10^5 LA/mL (LA/TV 10:1 ratio) and 10^5 TV/mL to 10^5 LA/mL (LA/TV 1:1 ratio) along with appropriate TV and LA controls. LA was grown in MRS broth, the usual

medium employed for Lactobacillus growth, as a control, to ensure that the Lactobacillus strain employed exhibited normal growth under the conditions of the experiment. Two trials were performed with all culture combinations and controls set up in triplicate. Results of these trials are presented in FIGURES 4 & 5 with Graph A representing the first experiment and Graph B representing the second.

FIGURE 4

Comparison between the growth of T. vaginalis coincubated with L. acidophilus and matched axenic controls of T. vaginalis. Graph A represents experiment #1 and Graph B represents experiment #2.

○ T. vaginalis control (TV 10^4)

△ T. vaginalis control (TV 10^5)

● experimental test 10:1 ratio (LA/TV 10^4)

▲ experimental test 1:1 ratio (LA/TV 10^5)

↑ commencement of pH adjustments

↓ final pH adjustment

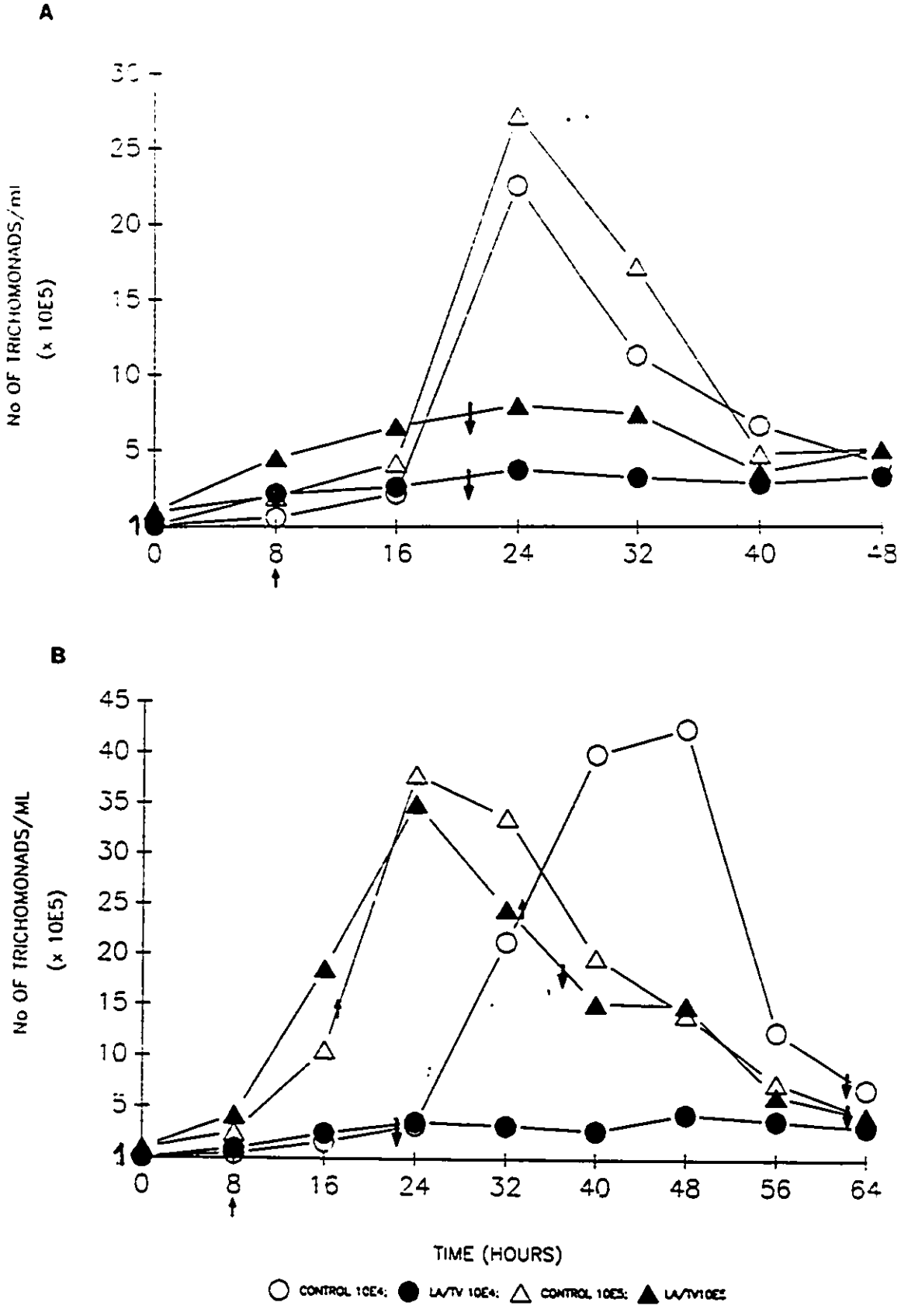
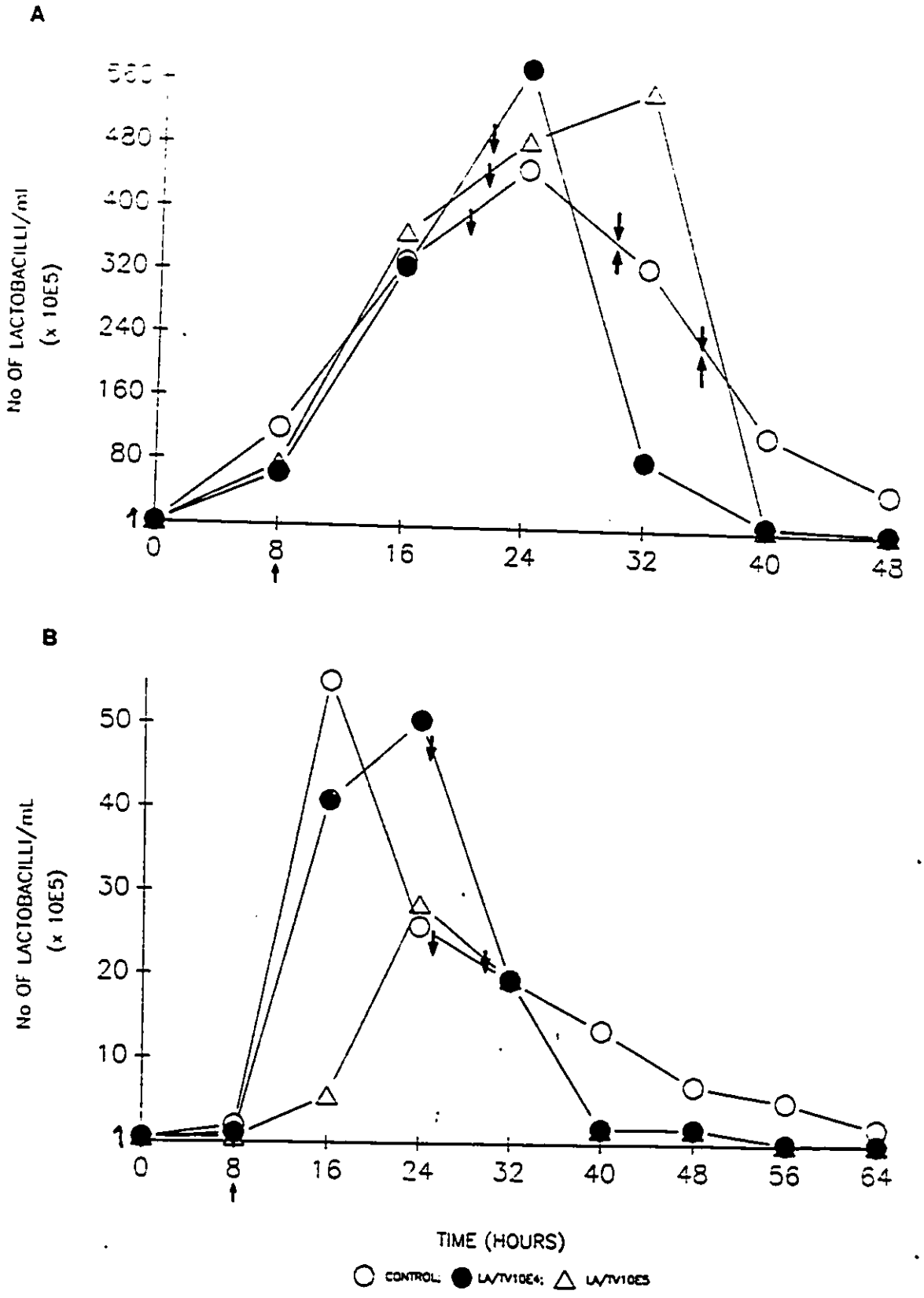

A
No OF TRICHOMONADS/ml
(x 10E5)
30
25
20
15
10
5
1
0
8
16
24
32
40
48
B
No OF TRICHOMONADS/ML
(x 10E5)
45
40
35
30
25
20
15
10
5
1
0
8
16
24
32
40
48
56
64
TIME (HOURS)
CONTROL 10E4:
LA/TV 10E4:
CONTROL 10E5:
LA/TV10E5

FIGURE 5

Comparison between the growth of L. acidophilus coincubated with T. vaginalis and a control L. acidophilus grown without the parasite. Graph A represents experiment #1 and Graph B represents experiment #2.

- ○ L. acidophilus control (LA)
- ● experimental test 10:1 ratio (LA/TV 10^4)
- ▲ experimental test 1:1 ratio (LA/TV 10^5)
- ↑ commencement of pH adjustments
- ↓ final pH adjustment
- ⊸ single pH adjustments


A
No OF LACTOBACILLI/ml
(x 10E5)
480
400
320
240
160
80
1
0
8
16
24
32
40
48
B
No OF LACTOBACILLI/mL
(x 10E5)
50
40
30
20
10
1
0
8
16
24
32
40
48
56
64
TIME (HOURS)
CONTROL
LA/TV10E4
LA/TV10E5

pH ADJUSTMENTS:

The pH of experimental test ratios and the LA control were monitored throughout the experiment because of the possible effect of acidity due to L. acidophilus. In the second experiment the protocol was modified such that the T. vaginalis controls were pH controlled as well as the experimental ratios and the L. acidophilus control. Addition of sterile sodium hydroxide (NaOH) to the media was performed every two hours as required to maintain the pH of the media above 5.0. In the first experiment the requirement for pH adjustments began at 8 hrs and continued every two hours until approximately 22 hrs. Two further adjustments were required, at 30 and 36 hours, to maintain the pH of control Lactobacillus above 5.0. In the second set of experiments pH adjustments commenced at 8 hrs in both test ratios as well as the Lactobacillus control. The adjustments of both test ratios and the Lactobacillus control continued for only 2 to 6 hours after peak concentrations of growth had been attained. The adjustments in the T. vaginalis controls began later in the experiment, generally 8 to 16 hours prior to the attainment of peak concentrations. Two hour adjustments of T. vaginalis controls were continued throughout, until the end of the experiment. In FIGURE 4 & 5 commencement of pH adjustments is signaled on the graphs by an up arrow (↑) and continued every 2 hours until the time point on the curve

marked with a down arrow (↓). Later single pH adjustments are indicated by facing arrows at a given point on the curve (→←). Generally pH adjustments were required from approximately 8 hours after the experiment began until after peak concentrations had been attained. After this time pH adjustments were required only sporadically.

The addition of sterile NaOH to the media to maintain the pH above 5.0 did not have a deleterious effect on the growth of the organisms. Evidence supporting this claim is found by comparing the growth of control TV 10^4 and 10^5 in FIGURE 4 A (no pH control) & B (pH controlled). Similar peak concentrations were attained in both controls in which the first controls were not pH adjusted. However, the time taken to reach the peaks were different.

Growth of T. vaginalis in the presence of L. acidophilus showed various trends as compared to TV controls (FIGURE 4 A&B). Generally, the growth of trichomonads coincubated with the bacteria was slightly higher than matched trichomonad controls for the first 16 hours of the assay. Although this observed trend of initial rapid growth was observed in both concentrations of the test ratios these differences were not statistically significant. This initial rapid growth did not attain as high a peak concentration as seen in controls but rather a more constant level in the number of trichomonads was maintained. In the first experiment peak concentrations achieved by controls were 2.25×10^6 TV/ml and 2.71×10^6 TV/ml

as compared to only 3.21 X 10^5 and 7.42 X 10^5 for the test ratios of LA/TV at 10^4 (10:1) and 10^5 (1:1), respectively. Similar results were generated in the second experiment with 10^4 control achieving a peak concentration of 4.26 X 10^6 compared to 4.38 X 10^5 for the LA/TV 10^4 (10:1) test ratio.

An exception to this trend was seen in the second experiment (FIGURE 4B) where the growth of LA/TV 10^5 (1:1) paralleled its matched control. The peak concentrations achieved by the control and test were 3.79 X 10^6 and 3.50 X 10^6, respectively. It is not completely understood why this discrepancy occurred. It is interesting to note that a concordant change in the growth of Lactobacilli at this test ratio (1:1) was also noted (Figure 5B). The Lactobacilli growth in this particular ratio was slower than that seen in the other LA/TV (10:1) ratio tested. In this case the peak concentration achieved by the bacteria was 2.84 X 10^6 as compared to concentrations of 5.5 X 10^6 and 5.03 X 10^6 in the control and LA/TV 10^4 (10:1) ratio, respectively.

Aside from differences apparent at individual time points there is varied significance in the differences in trichomonad growth trends between test ratios and controls. The obvious differences in peak concentrations attained by test ratios and controls resulted in significant differences in trend ($p=0.01$ to $p=0.03$ by 2 way analysis of variants). Again, with the exception in test ratio LA/TV 10^5 (1:1) where no significant difference was seen.

It is interesting to note that, although the levels of growth attained by experimental test ratios did not equal those of controls, the death rate of trichomonads was no greater in coincubation tubes than in controls. There were no significant differences in the concentrations of trichomonads in controls as compared to the test ratios in the final samplings. The first experiment had final trichomonad concentrations of 5.75×10^5 in controls and 3.69×10^5 in test ratios. The second experiment generated similar results with 5.57×10^5 in controls and 3.69×10^5 in test ratios. As such, although co-incubation with Lactobacilli does result in altered growth of T. vaginalis it does not result in increased trichomonad death.

Growth of L. acidophilus when coincubated with each concentration of T. vaginalis showed far more significant deviations with respect to controls than did the trichomonads (FIGURE 5 A&B). Regardless of the initial concentration of trichomonads included in the test tubes, the bacteria in coincubation tubes grew similarly to controls, in the first 8 to 16 hours with peak concentrations achieved at 16 to 32 hours. In the first experiment, Lactobacillus concentrations reached 4.51×10^7, 5.77×10^7, with 4.87×10^7 for controls. After the attainment of peak concentrations, death was apparent in all tubes. By 32 to 40 hours the concentration of L. acidophilus grown in the presence of T. vaginalis had declined sharply as compared to controls. In the first

experiment, control concentrations remained at 1.27 X 10^7 at the 40 hour sampling while test ratio concentrations had fallen to 7.8 X 10^5 and 6.07 X 10^5 for the 10^4 (10:1) and 10^5 (1:1) test ratios, respectively. The second experiment generated similar results with growth of Lactobacilli in the controls remaining relatively stable during the final samplings of the assay whereas the test ratio concentrations of bacteria fell rapidly. In an eight hour period between 48 and 56 hours in this experiment, the concentration of the control Lactobacilli fell slightly from 6.87 X 10^5 to 5.05 X 10^5 compared to a fall from 1.8 X 10^5 to 1.8 X 10^4 in the test ratio concentrations. The trichomonads appeared to have a deleterious effect on the growth of the Lactobacilli since the death was not as rapid in the control bacteria cultures. These notable effects are represented graphically in FIGURE 5 in which differences in growth after approximately 16 hours clearly show the adverse effect of trichomonad growth on the Lactobacilli.

Significant differences in results measured by 2-way analysis of variance were also apparent between the controls and experimental ratios. In the first experiment the difference between the LA controls and the TV/LA 10^4 (10:1) ratio was $p=0.02$ and $p= 0.01$ for the TV/LA 10^5 (1:1) ratio. In the second trial this difference increased to $p=0.002$ between the control and the TV/LA 10^5 (1:1) ratio. At 16 hours the LA/TV 10^4 (10:1) ratio was not significantly

different from the control but a significant difference was seen at 40 hours. In all cases trichomonads had a deleterious effect on the survival of Lactobacilli in coincubation assays. The inconsistency described in the LA/TV 10^4 (10:1) ratio may be due to the addition of excess of NaOH prior to attainment of the peak concentration.

INTRODUCTORY IMMUNOLOGICAL STUDIES

The more consistent and sustained model of intravaginal trichomonad infection outlined above was employed in introductory immunological studies. Immune response to T. vaginalis was measured by observing the influence of immunization on serologic response to intravaginal challenge, and on the ability of the trichomonads to establish infection in the vagina. To measure these parameters twenty-six mice were divided into four groups of six, with the final two mice designated negative controls. Three of the four groups of six mice received intramuscular inoculations of T. vaginalis at days -28, -14 and -7, respectively, and the fourth group of six mice were not previously exposed to T. vaginalis through intramuscular inoculation. Following this, all four groups were inoculated intravaginally using the modified inoculation procedure established in the first part of this project. The

four groups could then be compared in terms of serologic response and status and ability to establish T. vaginalis vaginal infection. The two control mice received neither intramuscular nor intravaginal inoculations of T. vaginalis; however, they were estrogenized and inoculated with L. acidophilus as previously described.

The combined results of two separate trials examining the recovery of T. vaginalis from cultures of vaginal washes are presented in TABLE 4. The number of mice infected with T. vaginalis after intravaginal inoculation is presented as a ratio of mice infected to the total number of mice. The mice inoculated intramuscularly with the trichomonad prior to intravaginal inoculation were less likely to establish infection intravaginally with T. vaginalis than mice not previously exposed to T. vaginalis. For example at the first occasion of sampling, 7 days after intravaginal inoculation with T. vaginalis, only 4 of the 12 mice in the M1-M6 group (those immunized 28 days prior to intravaginal challenge) were intravaginally infected with T. vaginalis.

TABLE 4. RECOVERY OF TRICHOMONAS VAGINALIS IN MOUSE VAGINAL WASHES

	TIME OF SAMPLING IN DAYS			
MOUSE GROUP	+7	+14	+21	+28
Group 1 (M1-M6) IM inoc 28 days prior to IV	4/12	0/12	0/9*	0/9*
Group 2 (M7-M12) IM inoc 14 days prior to IV	3/11*	1/11*	0/10*	0/9*
Group 3 (M13-M18) IM inoc 7 days prior to IV	10/12	6/11*	2/11*	0/10*
Group 4 (M19-M24) no IM inoc prior to IV	11/12	11/12	9/11*	6/8*
Controls (M25/M26) no IM no IV	0/2	0/2	0/2	0/2

IM = intramuscular inoculation of T. vaginalis
IV = intravaginal challenge with T. vaginalis
* = death of mice

For samples taken 28 days after the intravaginal challenge T. vaginalis was not recovered from mice in any of the first three groups, which were immunized by T. vaginalis intramuscular inoculations. While the mice that received intramuscular inoculations of the trichomonad either did not become infected with T. vaginalis upon intravaginal challenge or cleared the infection after a couple of weeks, most (6 of the 8) of the mice not previously exposed to T. vaginalis were still intravaginally infected 28 days post intravaginal inoculation. The two negative control animals had no detectable T. vaginalis. Control mice were also used as serologic negative controls.

The second aspect of these studies involved the serological response to T. vaginalis. FIGURE 6 represents the IgG response of mice to a single intramuscular inoculation of T. vaginalis. The graph represents the levels of IgG antibody measured by an ELISA reaction and expressed as a ratio of the optical density (OD) over the threshold (ratio = OD/threshold). The threshold antibody level was calculated as the control negative (CN) of each plate plus 2 standard deviations (SD) of the mean of all control negative plates in the assay (threshold = CN + 2SD). This graph can be interpreted as the primary response to T. vaginalis antigen and defines the immune status of the mice prior to intravaginal challenge with T. vaginalis.

FIGURE 6

Serologic IgG response of mice to intramuscular inoculations with T. vaginalis measured by ELISA assay.

ratio = OD/threshold

- threshold = CN + 2SD
- OD is the absorbance (optical density value)
- CN is the control negative of the plate
- 2SD is two standard deviations of the mean of all control negatives

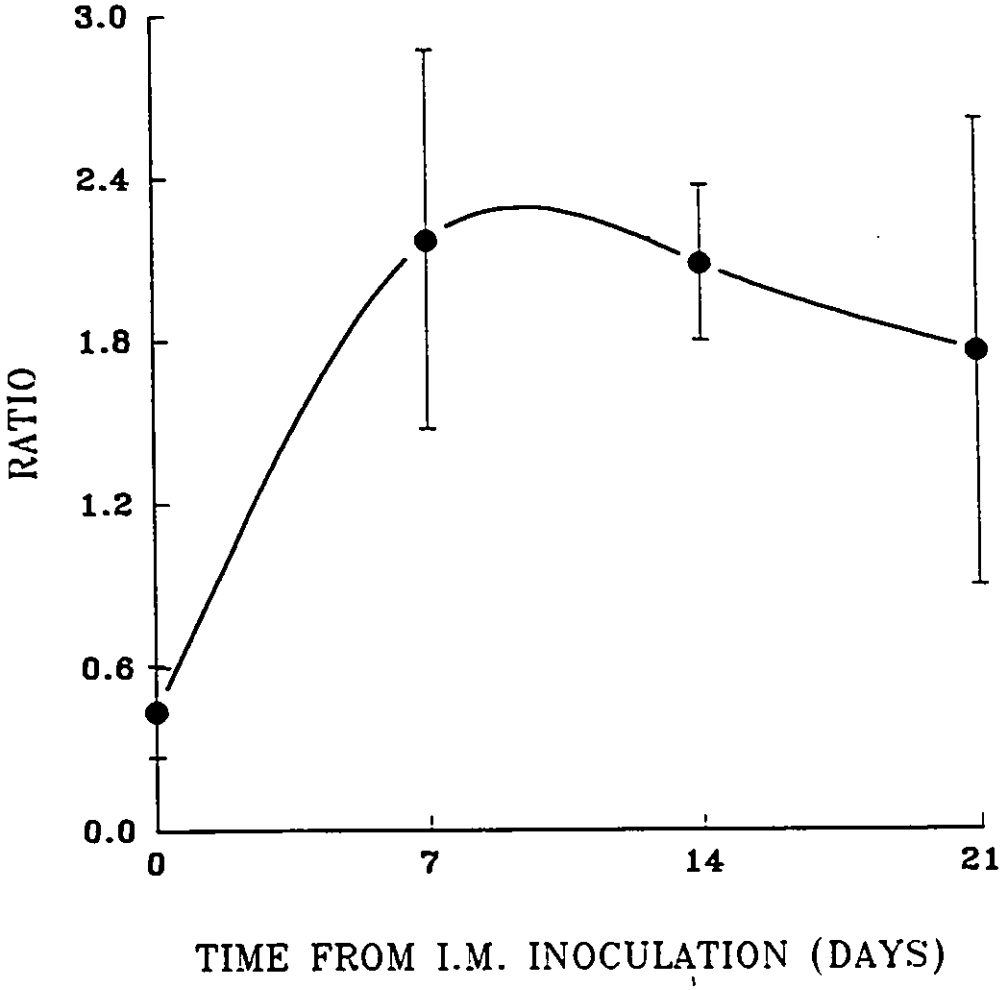

3.0
2.4
1.8
1.2
0.6
0.0
RATIO
0
7
14
21
TIME FROM I.M. INOCULATION (DAYS)

Figure 7 A&B represents the levels of IgG detected by ELISA in serum samples from mouse tail bleeds at days 7, 14, 21 and 28 post intravaginal inoculation. Graph A represents the control groups of mice (those not immunized by intramuscular inoculation of T. vaginalis) and Graph B represents the IgG levels measured in response to vaginal infection with T. vaginalis in mice previously immunized by intramuscular inoculations. These curves represent a secondary immune response to T. vaginalis antigen and thus reflect a more pronounced response than seen with a single exposure to T. vaginalis. These experiments provide some evidence that previous exposure to T. vaginalis through intramuscular inoculation results in elevated levels of IgG in response to intravaginal inoculation with T. vaginalis. Whereas control and single intravaginal exposure IgG antibody levels remained low throughout the 28 day sampling period, the IgG levels in mice previously exposed to trichomonads, by intramuscular inoculation, increased over time. These results reflect those presented from the mouse vaginal wash cultures (TABLE 4); as time goes on and presumably, antibody levels increase, the T. vaginalis infection decreases or is cleared. In our assay mice previously exposed by intramuscular inoculation to T. vaginalis showed an increased IgG response as compared to either negative control mice or mice not previously exposed to the trichomonad.

FIGURE 7

Influence of immunization on serologic IgG response to intravaginal challenge with T. vaginalis. Graph A represents control mice, those not immunized by intramuscular inoculation of T. vaginalis. Graph B represents mice immunized by intramuscular inoculations of T. vaginalis prior to intravaginal challenge.

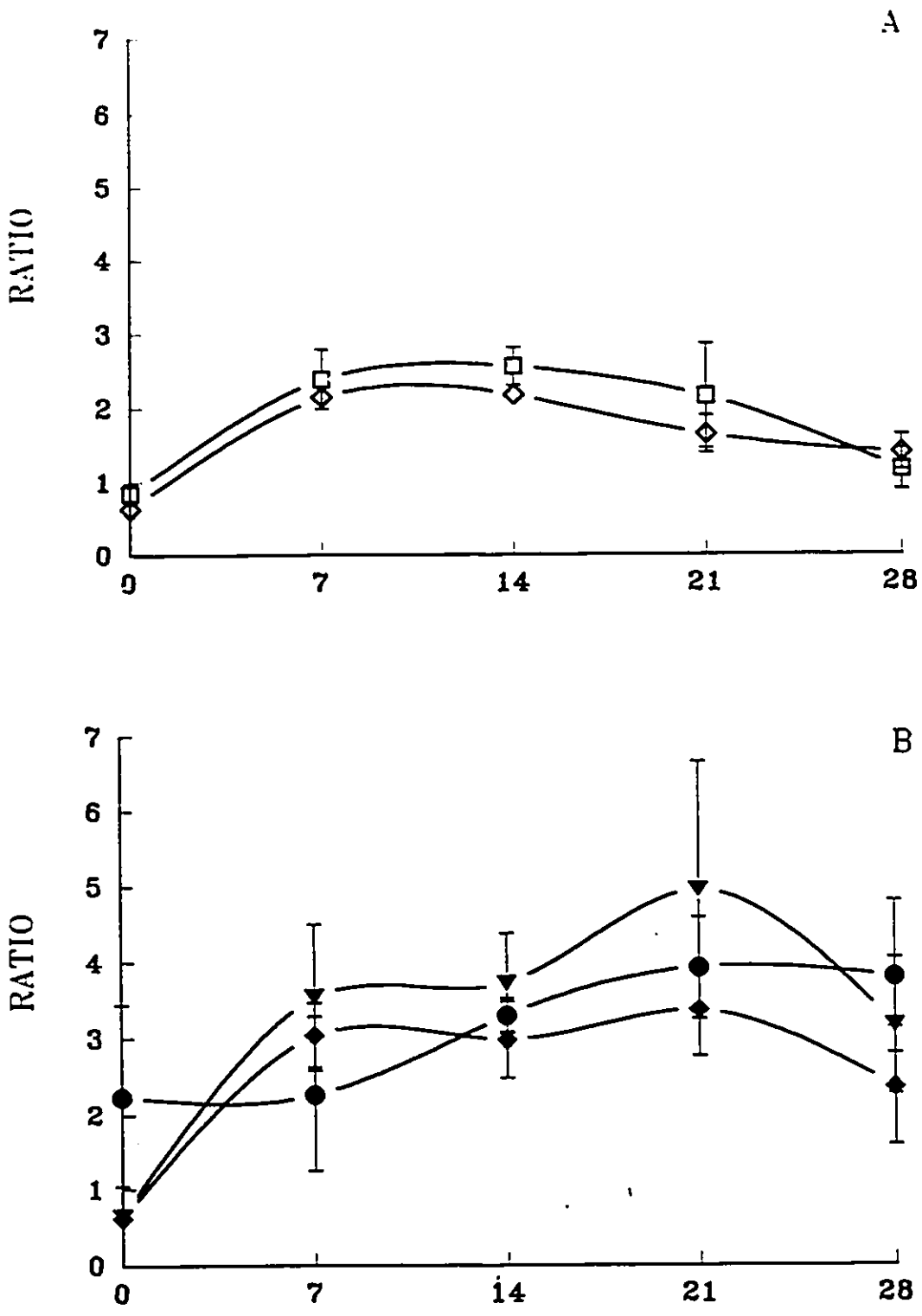
◇ negative control mice not exposed to T. vaginalis

□ mice not intramuscularly inoculated prior to intravaginal challenge with T. vaginalis

● mice intramuscularly inoculated 28 days prior to intravaginal challenge with T. vaginalis

◆ mice intramuscularly inoculated 14 days prior to intravaginal challenge with T. vaginalis

▼ mice intramuscularly inoculated 7 days prior to intravaginal challenge with T. vaginalis


A
RATIO
7
6
5
4
3
2
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7
14
21
28
B
RATIO
7
6
5
4
3
2
1
0
0
7
14
21
28
TIME FROM INTRAVAGINAL INOCULATION
(DAYS)

Attempts to detect secreted IgA and IgG in the vaginal washes were unsuccessful. This may simply be a reflection of the limitations of the technique employed in vaginal wash collections. Assays for total IgG and IgA (non-T. vaginalis specific) were also unsuccessful. Previous experiments have shown that these immunoglobulins are present in the mouse vagina.

DISCUSSION

AN INTRAVAGINAL MOUSE MODEL OF T. VAGINALIS INFECTION

Historically there has been much difficulty in developing a model of the human vaginal ecosystem in experimental animals. The minimal amounts of Lactobacilli and neutral pH documented for animals studied (Larsen et al., 1976; Skangalis et al., 1979) suggest that the high numbers of Lactobacilli and low pH of 4.5 are distinct characteristics of the human vagina. Although the specific features of host-parasite interaction in the mouse differ considerably from infections in the human genitourinary tract, the Lactobacillus inoculated intravaginal model manipulates the vaginal microflora of the mouse to resemble the condition that exists in women.

Applying a modification to an existing mouse model we have shown that although initial T. vaginalis infectivity is similar in the two groups, mice pre-inoculated with L. acidophilus show a significantly longer duration of infection

as compared to the mice not pretreated with L. acidophilus. The marked decrease seen after day 7, obvious in the control group and previously reported (Meysick and Garber, 1992) is not apparent in the Lactobacillus treated group.

T. vaginalis normally requires a pH of 6.0 - 6.5 to proliferate in vitro, however it is able to establish infection in the acidic (pH 4.5) environment of the human vagina. In trichomoniasis, vaginal pH appears to rise from less than 4.5 to greater than 5.0 (Hanna et al., 1985) with a major reduction of vaginal Lactobacillus. Lactobacilli involvement in maintaining the acidic vaginal pH and its protective function against invading pathogens has been well documented (Redondo-Lopez et al., 1990).

The relationship between T. vaginalis and Lactobacilli in the vagina is not completely understood. The reduction in the number of Lactobacilli seen in women with trichomoniasis together with the fact that very few mice harbour endogenous Lactobacilli prompted our attempt to pre-inoculate the mouse intravaginally with Lactobacilli prior to inducing T. vaginalis infection. Clearly, Lactobacilli-infected mice have a statistically significant prolongation of T. vaginalis infection (Day 24, $p=0.002$). However, the mechanism for this prolongation has not been explained. We detected no significant change in the overall vaginal bacterial flora. Futhermore, the mechanism employed by T. vaginalis to mediate the reduction of Lactobacillus in the vagina of women with

trichomoniasis has not yet been defined. Two possible mechanisms have been proposed. The invading trichomonads may phagocytose the bacteria, perhaps using them as a nutrient source. T. vaginalis has already been documented to ingest other pathogens (Garcia-Tamayo et al., 1978; Street et al., 1984). However, this first hypothesis is unlikely since the invading trichomonads would not be able to phagocytose enough cells of L. acidophilus in a short time to allow a subsequent rise in pH for the establishment of the parasite. As a consequence the majority of the T. vaginalis inocula would perish in the acidic vagina before phagocytosis of a great number of Lactobacilli could be accomplished.

The second hypothesis involves the effect of T. vaginalis secreted products on Lactobacilli. Several soluble secreted products have been isolated from T. vaginalis cultures: a cell detatching factor, CDF (Garber et al., 1989) and two cysteine-like proteases (Garber et al., 1989). The proteases, active over a wide pH range, may be factors in altering the vaginal environment and establishing infection. The action of the proteases may be directly against the Lactobacillus population possibly disrupting the bacteria's cell wall or may involve an indirect action against Lactobacillus secreted proteins rendering them inactive. If the proteases did act to modify the Lactobacillus directly or indirectly they could account for the reduction in the bacterial population and the rise in pH seen in trichomoniasis. Once the pH had been elevated, the

CDF, active only at pH greater than 5.0 could become functional and result in the characteristic sloughing off of epithelial cells seen in this disease.

In summary, this more reliable and consistent model of intravaginal infection can be useful to study T. vaginalis pathogenesis and the role of the immune response to infection with this parasite. The role of T. vaginalis virulence factors in pathogenesis could also be studied using this model.

ALTERATIONS IN RESIDENT MOUSE VAGINAL FLORA

Experiments monitoring vaginal flora before and after the additions of L. acidophilus and T. vaginalis were performed to detect any alterations in the mouse vaginal flora. In all cases there were no significant alterations in mouse vaginal microflora as a result of the addition of L. acidophilus or the combined effect of the addition of L. acidophilus and T. vaginalis.

The absence of alterations in mouse vaginal flora as a result of the addition of T. vaginalis corroborates the results of Meysick and Garber (1992), the only other published study examining mouse vaginal flora of which we are aware. The frequent isolation of Staphylococcus aureus and absence of anaerobic organisms in the mouse is very different from the normal flora in women which usually consists mainly of

anaerobic species (Larsen and Galask, 1982; Hill et al., 1984). The most notable difference in vaginal flora between mice and women involves the Lactobacillus species. While Lactobacilli are found in the majority of women sampled (Redondo-Lopez et al., 1990), only 25% of mice harbour indigenous Lactobacillus.

It is interesting to note that while trichomoniasis in women results in a decrease in the number of Lactobacilli, the addition of these bacteria to the mouse vagina prior to T. vaginalis inoculation increases the likelihood of infection with the trichomonad. The requirement for Lactobacilli suggests that the presence of the bacteria encourages a particular vaginal environment that is optimal for T. vaginalis infection. This requirement for Lactobacilli contradicts results presented by Hillier (1991) and Eschenbach and colleagues (1989) which suggest that the presence of Lactobacilli in the human vagina induces protection against invading pathogens causing bacterial vaginosis.

Another explanation may be that the environment of the mouse vagina, with its differences from the human vagina in terms of flora and pH may require the presence of the Lactobacilli to establish T. vaginalis infection whereas the woman does not. In this case the importance of the Lactobacilli would be in altering the mouse vaginal ecosystem to resemble that of women. The addition of Lactobacilli to the mouse vagina may result in a reduction in pH from its

usual neutral pH of 6 or 7 to a more acidic pH similar to that found in women. It would be interesting to monitor the mouse vaginal pH before and after the addition of Lactobacillus to examine any alterations. A similar procedure was attempted in our experiments but, the technical problems associated with monitoring pH in such a small area without contaminating the measuring device could not be overcome. Further examination of this question would require the design of a suitable pH probe for the pupose of mouse vaginal measurements.

IN-VITRO COINCUBATION ASSAYS

The addition of L. acidophilus to the mouse model prior to inoculation with T. vaginalis resulted in a more consistent and sustained infection with the trichomonad. It has been well documented that trichomoniasis in women is characterized by a reduction in numbers or total loss of Lactobacilli (Jirovec and Petru, 1968). The reduction in Lactobacilli post infection with T. vaginalis was also seen when monitoring mouse vaginal flora in the modified mouse model. After the addition of T. vaginalis to the mouse vagina the frequency of recovery of Lactobacilli from the vaginal washes decreased from 66% to 18%. The mechanism employed by T. vaginalis to mediate the reduction in Lactobacilli has not yet been

defined. In-vitro experiments monitoring the growth of T. vaginalis and L. acidophilus were performed to examine the interactions between these two organisms.

Preliminary experiments with coincubation assays demonstrated that a reduction in pH to less than 4.5 corresponded to T. vaginalis cell death. In order to examine the interaction between the two organisms over an extended period of time all cultures were pH controlled. Adjustments with sterile sodium hydroxide were made to ensure that the pH did not fall below 5.0 at any time throughout the experiment. Similar adjustments to control cultures ensured that the effects observed throughout the experiment were not the result of pH adjustments.

A single concentration of L. acidophilus was incubated with two different concentrations of T. vaginalis. The trichomonads in the experimental test ratios had slightly higher initial growth rates, however, they achieved significantly lower peak concentrations than their matched controls. The trends observed in trichomonad growth in coincubation assays is characteristic of T. vaginalis growth when coincubated with other bacteria including E. coli, S. aureus and Candida albicans (Horwatt, 1985; Kurnatowska, 1983). These reports document similar patterns in trichomonad growth including initial rapid growth and subsequent lower peak concentrations.

It is important to note that although they achieved early peak concentrations the attrition rate of trichomonads coincubated with Lactobacilli was no greater than that seen in controls. In summary, coincubation of T. vaginalis with L. acidophilus does not contribute to or affect the normal pattern of trichomonad death, when pH is sustained above 5.0, the pH seen in the vagina of women with T. vaginalis infections.

L. acidophilus growth, however, when coincubated with T. vaginalis was significantly different as compared to controls. Initially at both concentrations of T. vaginalis, L. acidophilus initially grew similarly to controls. Following the attainment of peak concentrations the growth of L. acidophilus varied in experimental test ratios and controls. Coincubation of L. acidophilus with T. vaginalis resulted in a slightly more rapid death of bacteria than seen in controls.

T. vaginalis appears to have a deleterious effect on the growth of L. acidophilus in in-vitro competitive assays at controlled pH. The results of these assays appear to reflect the situation apparent in the vagina during trichomoniasis and lend support to the theory of phagocytosis of Lactobacilli by trichomonads. Phagocytosis of the bacteria may not be reflected in the first occasions of sampling up to 16 hrs due to the rapid doubling time of the Lactobacilli compared to that of T. vaginalis. However, the deleterious effect of T. vaginalis on the bacteria becomes apparent from the 32 hr

sampling onward. This theory could also explain the discrepancy seen in the LA/TV 10^5 (1:1) ratio in experiment #2. The trichomonad growth rate was higher than that seen in the LA/TV 10^4 (10:1) ratio and the resultant Lactobacillus growth was lower. These results suggest that a higher initial inoculum of T. vaginalis permits the trichomonads to overcome the Lactobacilli at a more rapid rate.

Why are Lactobacilli required for establishment of T. vaginalis in the mouse model of vaginal infection? One theory may involve T. vaginalis phagocytosis of the bacteria as an additional nutrient source (Francioli et al., 1983; Street et al., 1984). The second theory also relates to the nutritional requirements of T. vaginalis. As previously explained estrogenization has been shown to be a critical factor in achieving T. vaginalis infection in mice. Estrogens are thought to enhance vaginal cell glycogen levels and create a nutritionally rich environment (Coombs et al., 1986). Furthermore, levels of glycogen and pH are inversely related. This suggests that at the higher, more neutral vaginal pH of the mouse the glycogen levels may be lower than those in the acidic vagina of women. Addition of lactic acid producing Lactobacilli to the mouse vagina may contribute to a lowering of mouse vaginal pH to a level similar to that found in women (Mardh and Soltesz, 1983). Perhaps it is the lower vaginal pH and the resultant higher glycogen levels that potentiate infection with T. vaginalis in the modified mouse vagina.

Then, as the trichomonads phagocytose the bacteria and metabolize the glycogen the pH of the vagina rises to the more neutral levels seen in trichomoniasis.

PRELIMINARY IMMUNOLOGICAL STUDIES

Although interesting in its own right, the creation of a model of infection is intended to provide a vehicle for the study of various aspects of infection. Once we had modified the existing mouse model we were interested in employing it in introductory immunological studies. Despite identical procedures in the administration of estrogens, inoculation of L. acidophilus and inoculation of T. vaginalis, mice show varied susceptibility to T. vaginalis infection. The variations in infectivity observed in the mice also appear in infection of women. Individual host susceptibility is dependent on many factors such as inoculum size, hormonal levels, stage of estrous and the host immune response.

Immunological studies were conducted on two levels. The first of these examined the ability to infect mice intravaginally with T. vaginalis once they had been immunized via intramuscular inoculations. The results suggested that a previous exposure to T. vaginalis induced protection from challenge with the trichomonad. As such, mice that had intramuscular inoculations of T. vaginalis prior to intravaginal inoculations were less likely to develop

infection than mice that were only exposed for the first time to the trichomonad through intravaginal inoculation. Previous intravaginal exposure to T. vaginalis in women does not appear to confer any gradual acquisition of protection. Repeated infections occur without significant decreases in either duration of infection or intensity of symptoms (Ravidin and Guerrant, 1982) but whether protection is strain specific is not known. One could speculate that an explanation for the absence of a protective immune response as seen in mice is simply due to antigenic differences in the organisms being compared. Also, the immune system of the mouse may respond to a different antigen or process the antigen in an entirely different manner than would the human. The mechanism of protection in the mouse model whether cell mediated or humoral will require much further work before it can be defined.

The second level of study in the preliminary immunological investigation involved an examination of the serologic response in mice following intravaginal challenge with T. vaginalis. A measurable serologic response was apparent in mice intramuscularly and intravaginally inoculated with T. vaginalis. Furthermore, the results provide evidence of a more pronounced immune response to reinfection with T. vaginalis. Although an increased IgG response was apparent in mice immunized with T. vaginalis it is not deemed singly responsible for the protective effect apparent in these studies. In an earlier report Martinotti and colleagues

(1977) found that protection in mice does not appear to correspond to serum antibody titre. A similar situation exists in human T. vaginalis infections. Serum antitrichomonal antibodies in urogenital trichomoniasis, including IgA, IgM and four IgG subclasses, are involved in human antitrichomonal antibody response (Cogne et al, 1985). Although evidence exists for both serum antibodies and antitrichomonal antibody in vaginal secretions of women infected with T. vaginalis (Ackers et al., 1978), neither appears to protect against reinfection with the trichomonad. However, sera from infected patients have been shown to exhibit complement-mediated lytic activity on trichomonads in cultures (Holbrook et al., 1982) and to protect against experimental trichomoniasis in rodents (Teras, 1966). Passive immunization experiments with mouse sera could be performed to determine to what extent humoral immunity is involved in the observed protection.

The increase in levels of IgG following the inoculation procedure was apparent not only in mice exposed to T. vaginalis but also in negative control mice which received neither intramuscular nor intravaginal inoculations of T. vaginalis. These slight increases in levels of IgG may be a reflection of the non-specific activation of the immune response previously reported with L. acidophilus inoculations (Alderete, 1988). Some investigators of the SolchoTrichovac vaccine proposed that its action was the result of cross-

reacting antigens to T. vaginalis present in some strains of Lactobacilli (Bonita-Musoles, 1984; Stojkovic, 1984). However, Gombosova (1986) later demonstrated that there was no antigenic similarity between T. vaginalis and L. acidophilus. This phenomena could also represent a technical problem with the whole cell T. vaginalis ELISA employed in these studies.

The inability to detect IgA and IgG in mouse vaginal washes do not negate the presence of these immunoglobulins, rather they are a reflection of the technological limitations in the sampling procedure or the antisera used to detect them.

These preliminary immunological studies have opened the door to further investigations into the immune response to T. vaginalis infection. Successful detection of antibodies in vaginal secretions as well as other classes of serum antibodies may provide further insight into the mechanisms of protection involved in trichomonad infections. The role of cell mediated immunity may be a crucial factor in the immune response to T. vaginalis infection.

CONCLUSIONS

In completing our objectives we have determined that the addition of L. acidophilus improved the mouse model in terms of T. vaginalis infectivity without significantly affecting resident vaginal flora. The reduction of L. acidophilus as seen in women with trichomoniasis was observed in the in-vitro coincubation assays; however, the mechanism T. vaginalis employs to mediate this reduction is still not completely understood. Several theories for the interaction between Lactobacilli and T. vaginalis were proposed including the effects of pH, glycogen levels, phagocytosis and T. vaginalis secreted products. While the preliminary immunological studies appear to have raised many more questions than they have answered, these investigations provide a basis for further studies of the immune response to T. vaginalis. The protective immune response seen in mice suggests promise for the application of such studies to vaccine development and clinical use.

Although this work has added to our knowledge of T. vaginalis infections, there remain many unanswered questions concerning T. vaginalis pathogenesis. Clearly, further study is required for a more complete understanding of the pathogenic mechanisms of T. vaginalis, its interaction with other microflora and the importance of host immune response in controlling or eliminating infection.

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