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INDUCIBLE IMMUNITY TO TRICHOMONAS VAGINALIS  
IN A MOUSE MODEL OF VAGINAL INFECTION

A Thesis Submitted to the  
School of Graduate Studies  
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Master of Science  
Department of Microbiology and Immunology  
Faculty of Medicine

By

Mary Cherian Abraham

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UNIVERSITÉ D'OTTAWA  
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## ABSTRACT

*Trichomonas vaginalis* is a protozoan parasite that causes a common sexually transmitted disease, trichomoniasis. Despite its high prevalence, the pathogenesis and immunogenicity of *T.vaginalis* are not well understood. Because currently available therapies are not always effective in treatment or in disease control and because of the impact of *T.vaginalis* on the health of women, a vaccination strategy could be an effective method of prevention. We studied the protective effects of subcutaneous immunization with *T.vaginalis* using a mouse model of vaginal infection. Balb/c mice were either immunized with *T.vaginalis* suspended in Freund's complete adjuvant (FCA) at day -56 and boosted with Freund's incomplete adjuvant (FIA) at day -28, or immunized only at day -28 with either Tv/FCA or Tv/FIA. Control mice were sham immunized and boosted with phosphate-buffered saline suspended in FCA and FIA respectively. At day 0 all mice were intravaginally inoculated with *T.vaginalis*. Mice immunized and boosted had significantly less intravaginal infection and had an elevated serum and vaginal antibody response compared to single immunization or control mice. Immunization with whole *T.vaginalis* appears to confer protection against intravaginal challenge with *T.vaginalis* in this animal model of infection.

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## TABLE OF CONTENTS

	PAGE
ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	ii
TABLE OF CONTENTS.....	iii
LIST OF FIGURES.....	v
LIST OF TABLES.....	vii
LIST OF ABBREVIATIONS.....	viii
<b>INTRODUCTION</b>	
<u>TRICHOMONAS VAGINALIS</u> .....	1
ANIMAL MODELS OF <u>T.VAGINALIS</u> INFECTION.....	15
IMMUNOLOGIC ASPECTS OF TRICHOMONIASIS.....	17
RATIONALE.....	29
OBJECTIVES.....	32
<b>MATERIALS AND METHODS</b>	
STRAINS.....	33
INTRAVAGINAL INOCULATION OF MICE.....	34
IMMUNIZATION EXPERIMENTS.....	36
RE-INFECTION EXPERIMENTS IN METRONIDAZOLE	
TREATED <u>T.VAGINALIS</u> INFECTED MICE.....	41
MOUSE VAGINAL WASHES AND TAIL BLEEDS.....	43
ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA).....	44
STATISTICAL ANALYSIS.....	46
ANIMAL CARE.....	46

**RESULTS**

OPTIMIZATION OF ELISA.....	47
OPTIMUM INOCULATION SIZE OF <u>T.VAGINALIS</u>	
FOR IMMUNIZATION.....	47
IMMUNIZATION REGIMEN OF <u>T.VAGINALIS</u> .....	57
IMPORTANCE OF BOOSTING.....	65
METRONIDAZOLE TREATMENT & RE-INFECTION.....	73
<b>DISCUSSION</b>	
IMMUNIZATION WITH <u>T.VAGINALIS</u> IN OUR MOUSE	
MODEL OF INFECTION.....	75
SIZE OF INOCULATION TO <u>T.VAGINALIS</u>	
FOR IMMUNIZATION.....	76
IMMUNIZATION REGIMEN AND ROLE OF ADJUVANT.....	77
MULTIPLE IMMUNIZATION ON PROTECTION.....	83
CONCLUSION.....	88
LIST OF REFERENCES.....	89
APPENDIX.....	102

## LIST OF FIGURES

1.	Mouse anti- <i>T.vaginalis</i> standard curve.....	48
2.	Optimum inoculation size of <i>T.vaginalis</i> for Immunization: Survival analysis.....	53
3.	Optimum inoculation size of <i>T.vaginalis</i> for immunization: Kinetics of IgG serologic response to <i>T.vaginalis</i> upon immunization and infection.....	54
4.	Optimum inoculation size of <i>T.vaginalis</i> for Immunization: Kinetics of vaginal IgG response to <i>T.vaginalis</i> upon infection.....	55
5.	Optimum inoculation size of <i>T.vaginalis</i> for immunization: Kinetics of vaginal IgA response to <i>T.vaginalis</i> upon infection.....	56
6.	Immunization regimens and role of adjuvant: Survival analysis.....	61
7.	Immunization regimens and role of adjuvant: Kinetics of IgG serologic response to <i>T.vaginalis</i> upon immunization and infection.....	62
8.	Immunization regimens and role of adjuvant: Kinetics of vaginal IgG response to <i>T.vaginalis</i> upon infection.....	63
9.	Immunization regimens and role of adjuvant: Kinetics of vaginal IgA response to <i>T.vaginalis</i> upon infection.....	64

10.	Multiple immunization and protection: Survival analysis.....	69
11.	Multiple immunization and protection: Kinetics of IgG serologic response to <i>T.vaginalis</i> upon immunization and infection....	70
12.	Multiple immunization and protection: Kinetics of vaginal IgG response to <i>T.vaginalis</i> upon infection.....	71
13.	Multiple immunization and protection: Kinetics of vaginal IgA response to <i>T.vaginalis</i> upon infection.....	72

## LIST OF TABLES

1.	Dosage optimization.....	38
2.	Immunization regimen and role of adjuvant.....	39
3.	Role of multiple immunization.....	40
4.	Does vaginal infection and treatment induce protection.....	42
5.	Optimum inoculation size of <u>T.vaginalis</u> For immunization.....	52
6.	Immunization regimen and role of adjuvant.....	60
7.	Multiple immunization on protection.....	68
8.	Metronidazole treatment and protection.....	74

## LIST OF ABBREVIATIONS

ABTS	2.2'-Azino-di-[3-athyl-benzthiazolinsulfonate(6)]
CDF	Cell-detaching factor.
CPE	Cytopathic effects.
ELISA	Enzyme linked immunosorbent assay.
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant.
HIV	Human immunodeficiency virus.
IgA	Immunoglobulin class A
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
La	<i>Lactobacillus acidophilus</i> .
MRS	Lactobacillus MRS broth
NBCS	Newborn calf serum
PBS	Phosphate buffered saline.
PMN	Polymorphonuclear neutrophils
STD	Sexually transmitted disease.
SDS	Sodium dodecyl sulphate
Sc	Subcutaneous immunization
Tv	<i>Trichomonas vaginalis</i>
TYI	Diamond TYI-S-33 medium
ug	Microgram
um	Micrometer

## INTRODUCTION

### TRICHOMONAS VAGINALIS:

#### GENERAL BACKGROUND

*Trichomonas vaginalis*, the causative agent of sexually transmitted trichomoniasis throughout the world, was first described by Donne in 1836. This flagellated trophozoite protozoan parasite belongs to the family Trichomonadidae. *T. vaginalis* is the only pathogenic member of the three trichomonad species (*T.hominis*, *T.tenax*, and *T.vaginalis*) that parasitize humans, and it inhabits the genito-urinary system (Brown 1972; Honigberg 1978; Jirovec and Petru 1968).

*T. vaginalis* is a pleomorphic organism and environmental conditions appear to affect its morphology. The morphology of this parasite is often variable in living, and in fixed stained preparations as well as with varying physicochemical conditions (Honigberg 1978). In axenic cultures, such as in Diamonds- TYI medium, the organism appears pear-shaped or ovoid, ranging in size from 10-30 um, with four anterior flagella, an undulating membrane and a long axostyle, which bisects the cell longitudinally, protruding through the posterior end (Jirovec and Petru 1968; Honigberg 1978; Lossick and Kent 1991; Heine and McGregor 1993). The motion of the

flagella and the undulating membrane gives *T. vaginalis* its characteristic jerky, non-directional movement. *T. vaginalis* has a large nucleus, characteristic of other eukaryotic cells, a highly developed golgi complex (Heine and McGregor 1993) but lacks mitochondria and can exist in anaerobic conditions (Krieger 1981; Muller 1990).

For many decades, *T. vaginalis* was thought to be a commensal of the urogenital tract. However, now its role as a pathogen of the human urogenital tract is undisputed. The reasons why this protozoan deserves renewed and increased attention in biomedical research are numerous. Trichomoniasis is an important sexually transmitted disease (STD) of world wide distribution. It has been estimated that annually, 5 million women in the United States (Jarecki-Black et al., 1988) and 180 million women worldwide are infected with *T. vaginalis*. (Brown 1972; Thomson and Gelbert 1989). Despite the prevalence of *T. vaginalis* infection, its pathogenic mechanisms have not been well characterised. Trichomoniasis is not a self-limiting disease and the extent and nature of sequelae remain largely undefined (Alderete 1987). *T. vaginalis* in women has been associated with an increased incidence of low birth weight, (Cotch 1991; Lossick and Kent 1991) premature rupture of membranes (Draper et al., 1991; Heine and McGregor 1993; Lossick and Kent 1991), premature delivery and post-partum endometritis (Cotch 1991; Heine and McGregor 1993). In vitro

studies of Draper et al. (1991) support the observations on the ability of *T.vaginalis* to rupture amniotic membranes. Association of *T.vaginalis* with an increased predisposition to HIV infection has already been documented (Laga et al., 1993; 1994).

The clinical presentation of trichomoniasis ranges from acute to asymptomatic infection. Nearly 50% of the women harboring this parasite are asymptomatic (Krieger 1981) with one-third of them developing frank vaginitis within six months (Rein 1990; Wolner-Hansen et al., 1989). How the clinical presentation of the disease can vary from a totally asymptomatic vaginal infection to florid vaginitis is not clear. *T. vaginalis* primarily infects the mucosal squamous epithelium of the urogenital tract (Nielsen and Nielsen 1975) occurring both intravaginally and extravaginally in women (Wallin et al., 1981).

It is not known whether inflammatory response seen in trichomoniasis is due to close contact between *T.vaginalis* and the vaginal epithelium, due to cellular products secreted by the organisms, due to the host response to mucosal infection, or a combination of factors. *T.vaginalis* causes a cytotoxic effect on cell culture monolayers, and this effect is dependent upon the inoculum size and length of time that *T.vaginalis* is in contact with the monolayer (Alderete and

Pearlman 1984; Garber et al., 1987; Garber et al., 1989a; Krieger et al., 1984). Garber et al.(1989a) demonstrated the presence of a secretable cytotoxin or cell detaching factor (CDF) of *T.vaginalis*. This detaching factor causes a cytopathic effect (CPE) with disruption of the cell culture monolayer in a similar manner to the effects seen when *T.vaginalis* is in direct contact with the monolayer. Aside from CDF, multiple proteases have been found in cell lysates of *T.vaginalis* (Coombs and North 1983; Lockwood et al., 1984). Protease activity has been implicated as having a role in *T.vaginalis* adherence to epithelial cells (Arroyo and Alderete 1989), suggesting a role of protease in pathogenesis. Garber and Lemchuk-Favel (1989b) were able to isolate two extracellular cysteine proteases from the cell free filtrate of *T.vaginalis*. Very recently, Provenzano and Alderete (1995) proposed the potential role of *T.vaginalis* cysteine proteases in degrading secretory immunoglobulins as well as their role in enhancing the infection rate and pathogenicity of other concomitant coinfecting STD pathogens, including HIV. Factors in the vaginal microenvironment including menstrual blood, pH, oxidation-reduction potential, hormonal levels and other microbes may be associated with pathogenicity.

**CLINICAL PRESENTATION:**

In women, the most common complaint associated with trichomoniasis and an acute inflammatory response, is a heavy vaginal discharge and vulvovaginal irritation (Fouts and Kraus 1980; Rein 1990). Vaginal and cervical erythema, also termed "strawberry cervix" is noted in many patients (Rein 1990; Wolner-Hanssen 1989). As mentioned previously, *T.vaginalis* has also been associated with increased prenatal morbidity.

*T.vaginalis* is also found in men even though it seems to be more often asymptomatic. In chronic cases there may be a slight itching sensation and purulent discharge may occasionally be observed. Purulent discharge and inflammation from the external meatus are characteristic of acute trichomonas urethritis and prostatitis (Kuberski 1978; 1980). Complications due to *T.vaginalis* infection might develop including balanoposthitis, urethral stricture, cystitis, epididymitis and sterility (Jarecki-Black et al., 1988; Paulson et al., 1985; Tuttle et al., 1977). Non-gonococcal urethritis is the most common clinical symptom associated with *T.vaginalis* infection in men (Kreiger 1990; Kuberski 1980).

**TRANSMISSION:**

It is widely accepted that *T.vaginalis* is transmitted by

sexual intercourse (Kreiger 1990; Rein 1990) and there are very few documented cases of other modes of transmission. Neonatal trichomoniasis is acquired during vaginal delivery (Bramley 1976; Neistein et al., 1984; Kurnatowska 1990) and has been reported in about 5 percent of female babies born to infected mothers (Neistein et al., 1984; McLaren et al., 1983). The organism is sensitive to desiccation and survives in various body fluids such as urine, semen, vaginal exudates (Whittington 1957; Lossick 1990) or on moist objects like sponges, towels, toilet seats etc. (Whittington 1951). Such contaminated objects (fomites) may also be responsible for indirect or non-sexual transmission (Lossick 1990).

#### **DIAGNOSIS:**

Accurate diagnosis is necessary for specific treatment of trichomoniasis. Routine clinical diagnosis is dependent on microscopic identification of the parasite in wet mount preparations by characteristic morphology. Other diagnostic methods include the use of cultures and a variety of cytologic smears (Heine and McGregor 1993; Manson et al., 1976; Krieger et al., 1988).

Serological methods for diagnosis of *T.vaginalis* infection have been performed for a number of years. Serologic tests include complement fixation, precipitation, agglutination,

hemagglutination, fluorescent antibody technique, gel diffusion, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). These have been developed for detection of antitrichomonal antibodies in serum, vaginal secretions or semen samples (Alderete 1984 ; Yule et al., 1987). Polymerase chain reaction based methods in *T.vaginalis* diagnosis offer a high degree of sensitivity by the ability to amplify and detect target sequences (Rubino et al., 1991; Riley et al., 1992). Diagnosis of infection in asymptomatic women and men with non-gonococcal urethritis was considered a problem until Garber et al.(1987) developed a culture technique employing a McCoy cell system which could detect *T.vaginalis* growth with an inoculum as low as under  $10^2$ /mL.

#### **TREATMENT:**

The drug of choice for treatment of trichomoniasis is metronidazole (Hager et al., 1980; Dykers 1975). Since metronidazole treatment has become available, drug resistant strains of *T.vaginalis* have emerged with variable levels of resistance (Kulda et al., 1982). Furthermore, a carcinogenic potential of metronidazole was reported based on animal studies (Speck et al., 1976; Koch et al., 1981) and mutagenic effects in bacteria (Voogd et al., 1974; Legator et al., 1975), resulting in some controversy on reservation for use in treatment. Because of the prevalence of metronidazole

resistance and a concern regarding the safety of this drug, new control strategies such as vaccination are required to control *T.vaginalis* infection.

#### **INTERACTION OF T.VAGINALIS WITH OTHER MICROFLORA:**

Although *T.vaginalis* is now recognized as a significant urogenital pathogen as mentioned previously, the mechanism of its pathogenicity is not yet clearly understood. One important factor that must be taken into account in understanding the pathogenicity of *T.vaginalis*, is the role of normal microbial flora in protecting against, or facilitating infection with *T.vaginalis*. The vagina and its unique microflora form a finely balanced ecosystem. The harsh vaginal environment influences the microbial types present, and the microflora in turn affects the vaginal environment (Redondo-Lopez et al., 1990). The ecosystem changes under pressure from a variety of external stimuli such as hormonal levels and fluxes, pregnancy and delivery, trauma, coitus, and birth control methods (Jorma Paavonen 1983; Larsen and Galask 1982; Mehta 1982; Redondo-Lopez 1990).

The vaginal flora is a dynamic and closely interrelated system, which may also be influenced by other factors such as glycogen content of epithelial cells, glucose and vaginal pH. The first extensive study of the vaginal flora was reported by

Doderlein in 1892, who identified the prevalence of lactobacilli in the normal vaginal flora. Later qualitative and quantitative studies of the vaginal flora have demonstrated that many other facultative and obligate anaerobic organisms are present in high concentrations (Larsen and Galask 1980). Lactobacilli dominate the facultative genital tract flora as indicated by their presence in most women, being found in 70% to 96% of female patients (Larsen and Galask 1982). Various species of lactobacilli are present in the vaginal flora, and diphtheroid bacilli comprise most of the remaining facultative gram-positive rods in the female genital tract. Gram-positive cocci are the second most frequently observed bacterial morphologic forms in the normal vagina. Catalase-positive, coagulase-negative staphylococci (*Staphylococcus epidermidis* and *Staphylococcus saprophyticus*) are isolated from the vagina of 62% of healthy women. *Micrococcus varians* (strict aerobes), *Streptococcus* species, and *Enterococcus* species are isolated from the vagina of 30% to 40% of healthy women (Larsen and Galask 1980; 1982; Redondo-Lopez 1990). Gram-negative rods occur at low incidence with *Escherichia coli* most frequently isolated from the female genital tract (Jorma Paavonen 1983; Larsen and Galask 1982). Some common representatives of anaerobic organisms include gram-positive cocci (*Peptococcus asaccharolyticus* and *Peptostreptococcus anaerobius*), Lactobacillus species, Bifidobacterium, Eubacterium, and Bacteroides species,

including a low prevalence of *B.fragilis* (Larsen and Galask 1980;1982). Generally, the vaginal microflora of healthy women consists of various aerobic and anaerobic bacteria, frequently dominated by lactobacillus species.

*L.acidophilus* is generally accepted to be the most frequently isolated lactobacillus species from the normal human vagina based on early studies. However, other studies fail to agree on which species predominate. Giorgi et al.(1987) suggested that *L.cripatus*, *L.jensenii*, *L.fermentum* and *L.gasseri* were the predominant vaginal species. This seems likely to be due to the various methods employed to identify the lactobacilli (Redondo-Lopez et al., 1990; McGroarty 1993). Research is currently applying molecular biological techniques for species identification of lactobacilli, but until these techniques are perfected and standardized, the predominant species of lactobacilli will remain controversial.

Since the first description of lactobacilli by Doderlein it has been widely assumed that lactobacilli normally present in the vagina, protect against the overgrowth of potentially pathogenic indigenous flora and exogenous pathogens. The pathogenesis of bacterial vaginosis is thought to first involve the elimination or reduction of antibacterial activity of the indigenous vaginal lactobacilli (Skarin and Sylwan

1986; Redondo-Lopez et al., 1990). Lactobacilli inhibit the in-vitro growth of organisms associated with bacterial vaginosis including Gardnerella, Mobiluncus, Peptostreptococcus and Bacteroides species (Skarin and Sylwan 1986; Redondo-Lopez et al., 1990). There have been numerous reports of the in-vitro ability of lactobacilli, to inhibit the growth of potential pathogens by the release of metabolic byproducts such as fatty acids, hydrogen peroxide, hydrogen, hydroxyl ions and ammonia, as well as bacteriocins and bacteriocin-like substances (Redondo-Lopez et al., 1990; McGroarty 1993; Mardh and Soltesz 1983).

Low vaginal pH is believed to be a primary mechanism controlling the composition of the human vaginal microbial-flora. Lactic acid and other fatty acids produced by lactobacilli contribute to the maintenance of a low vaginal pH which is thought to be an important control mechanism preventing colonisation by pathogenic organisms. Fatty acids produced by vaginal epithelial cells probably play a more prominent role in maintaining the low vaginal pH (Redondo-Lopez et al., 1990; McGroarty 1993).

Aside from lactic acid production, lactobacilli have demonstrated the ability to excrete other compounds with antimicrobial activity including hydrogen peroxide ( $H_2O_2$ ) and inhibitory proteins (Hillier et al., 1992; Mardh 1991). In-

vitro studies have revealed that the released  $H_2O_2$  can be used for autoinhibition and for inhibition of adjacent microorganisms either alone or via a peroxidase-mediated system (Klebanoff et al., 1991). Inhibition of a variety of bacteria by  $H_2O_2$  producing lactobacilli such as *Salmonella typhimurium*, *E.coli*, *Clostridium perfringens* (Gilliland and Speck 1977a) and *L.acidophilus* (Gilliland and Speck 1977 b; Klebanoff et al., 1991) have been described. In-vitro inhibition of HIV by high concentrations of  $H_2O_2$  producing *L.acidophilus* strains and equivalent inhibition by low concentrations of *L.acidophilus* in combination with peroxidase and a halide have been described by Klebanoff and Coombs (1991).

Bacteriocins are proteinaceous bactericidal substances produced by bacteria, with a narrow spectrum of activity, inhibiting strains of the same or closely related species. Such proteinaceous inhibitors have been identified and characterised in lactobacilli (Barefoot and Klaenhammer 1984; Redondo Lopez et al., 1990). Lactobacilli are also known to produce a variety of inhibitors that broadly resemble bacteriocins but do not act against the producing strain (McGroarty 1993; Redondo-Lopez et al., 1990). Lactobacilli also adhere to uroepithelial cells and may inhibit by steric hinderance of adhesion of potential pathogens from colonising the vagina. This may constitute the protective effect of

lactobacilli.

#### VAGINAL FLORA IN TRICHOMONIASIS:

Although the basic vaginal flora in women has been well documented (Larsen and Galask 1982; Redondo-Lopez et al., 1990; Larsen 1993), the role of bacterial flora in genital tract infections has not been established. Understanding the role of commensal flora in pathogenicity of *T.vaginalis*, such as the mechanisms involved in colonisation may provide targets for approaches in designing effective control strategies. A predominance of lactobacilli and an acidic vaginal pH (less than 4.5) are considered part of a healthy vaginal environment (Larsen 1993). *T. vaginalis* has been reported to alter the normal physiological state of the human vagina drastically during infection. In trichomoniasis, the principal alterations appear to be a reduction in the lactobacillus population, a rise in anaerobic flora and an increase in vaginal pH to > 5.0 (Hanna et al., 1985; Holst et al., 1987; Spiegel 1990). The mechanisms involved in the disruption of the lactobacillus population and alteration of the vaginal flora have not yet been elucidated.

Jirovec and Pertu (1968) described four stages in the development of trichomoniasis based on the severity of infection. 1) "*Trichomonas acuta*" is the first phase which

occurs shortly after contact with the organism. In the vaginal secretions there are fewer epithelial cells, with the decrease in number of lactobacilli, while the trichomonads and leukocytes are increased, without significant alterations in the microflora. 2) The second phase referred to as "Culminating trichomoniasis", is characterised by numerous trichomonads and leukocytes, pleomorphic bacteria, absence of lactobacilli and the presence of few epithelial cells. 3) In "Chronic trichomoniasis", there are many epithelial cells, few leukocytes and a variety of bacteria other than lactobacilli. *T.vaginalis* is present in varying numbers. 4) The final phase, "Latent trichomoniasis", shows normal number of lactobacilli with many epithelial cells along with few leukocytes and trichomonads. The relevance of this classification is not known, whether there is an actual progression from stage 1 to stage 4 is still disputed. It is not known whether this progression would be controlled by virulence factors expressed by the infecting strain of *T.vaginalis*, by the host response or both. Furthermore, the mechanisms involved in the disruption of the lactobacillus population and alterations to the vaginal flora in general have not yet been elucidated. In this regard, an animal model may be useful to study the in-vivo interactions between *T.vaginalis* and the endemic vaginal flora even though, the specific features of the host-parasite interaction in animal models would differ from infections in the human genitourinary tract. An in-vivo model may also help

to study the virulence markers and effects of different pathogenic strains of *T.vaginalis*.

#### **ANIMAL MODELS OF T.VAGINALIS INFECTION:**

Several attempts have been made at establishing an animal model for *T.vaginalis* infection. The laboratory mouse (Cappuccinelli et al., 1974; Coombs et al., 1986) has been the animal most widely used. Rats (Jirovec and Pertu 1968; Honigberg 1978), guinea pigs (Kazanowska 1983), hamsters (Jirovec and Pertu 1968; Honigberg 1978), and monkeys (Street et al., 1983) have also been employed. The inoculation routes most frequently used were intraperitoneal, subcutaneous, intramuscular and intravaginal. Intravaginal infection of experimental animals with *T.vaginalis* are likely to provide manifestations that are more similar to those found in human urogenital trichomoniasis. However, investigators have experienced difficulties in artificially replicating the human vagina owing to differences in hormonal control and uncharacterised differences in immune response to infectious agents.

Mice were first used for intravaginal infection by Cappuccinelli et al. (1974) and the establishment of intravaginal infection with *T.vaginalis* in mice treated with estrogens was successfully accomplished. Later, many studies

have agreed that pretreatment of mice with estrogens is essential for establishing intravaginal infection (Meysick and Garber 1992, McGrory and Garber 1992; Coombs 1986). The vaginal epithelium is exposed to estrogen during most of the menstrual cycle in humans (Larsen 1993). *T.vaginalis* can settle only in an estrogenized environment (Jirovec and Petru 1968). The same study also showed that lactobacillus colonisation of the vagina is related to the estrogen effect on the vaginal epithelium. However, the factors responsible for the enhancement of infection with *T.vaginalis* in estrogenized animals are not known. The factors such as glycogen deposition on the vaginal epithelium, modification of vaginal epithelial cells, as well as changes in the normal flora and number of neutrophils (Corbeil et al., 1985) may have an indirect effect on enhancement of infection. The highest infection rate is observed when the mice are in proestrous/estrous phase when fewer neutrophils or bacteria are present in the vagina (Corbeil et al., 1985).

Meysick and Garber (1992) have studied the normal mouse vaginal flora and the effects of *T.vaginalis* infection on population dynamics. Features of host-parasite interactions in the mouse differ considerably from those of human genitourinary tract. Low prevalence of lactobacilli and neutral pH in the mouse vagina (Larsen et al., 1976; Skangalis et al., 1979) differ from the high carrier rates of

lactobacilli and lower pH characteristic of the human vagina. In an attempt to alter the mouse vagina to parallel the situation found in the women, McGrory and Garber (1992) introduced *L. acidophilus* in the vagina of Balb/c mice prior to inoculating the mice with *T. vaginalis*. This resulted in a significantly more consistent and sustained *T. vaginalis* infection. Their studies have also shown that the addition of *L. acidophilus* did not otherwise significantly alter the resident mouse vaginal flora. It has been well documented that trichomoniasis in women is characterized by reduction in number or total loss of lactobacilli (Jirovec and Petru 1968), which was also observed by McGrory et al. (1994).

Intravaginal infection of nonhuman primates with *T. vaginalis* may yield results that are more relevant to human trichomoniasis. Studies in squirrel monkeys (Gardner et al., 1987; Street et al., 1983) with *T. vaginalis* have been successful. As in the case of the mouse model, the vaginal pH of the squirrel monkey is neutral and the flora does not include lactobacilli. Furthermore, hormonal status of the primates appeared important in the successful establishment of infection.

**IMMUNOLOGIC ASPECTS OF TRICHOMONIASIS:**

Considering the high incidence of trichomoniasis in women in North America and world wide, the knowledge of the pathogenesis and host immune responses to infection with *T.vaginalis* is limited. The first attempt at antigenic analysis of *T.vaginalis* were concerned with establishing the organism as a valid species, antigenically distinct from other human trichomonads (Honigberg 1970; 1978). Later work was more concerned with the demonstration of antigenic differences between different isolates of the same species. Several groups have addressed the question of antigenic diversity of *T.vaginalis* and the results have shown that, each strain possesses unique antigens (Su-Lin and Honigberg 1983). Alderete and his colleagues (1985, 1986a and 1986 b) studied the extent and nature of heterogeneity among representative *T.vaginalis* isolates and revealed that antigenic heterogeneity among *T.vaginalis* isolates is dependent upon the surface disposition of highly immunogenic protein antigens. Their data also suggested phenotypic variation among all heterogeneous isolates and also for all clones and subpopulation derived from the heterogeneous isolates. Studies by Garber et al. (1986) also demonstrated the antigenic heterogeneity of *T.vaginalis* and showed that different individuals appear to respond immunologically to different *T.vaginalis* antigens.

At present only two conclusions appear to be reasonably firmly established; 1) that *T.vaginalis* isolates possess a large number of common antigens and a much smaller number that are shared with other Trichomonadidae, and 2) that antibodies raised against *T.vaginalis* recognize not only common but also strain-specific antigens (Ackers 1990). Whether this represents genuine antigenic diversity or variability of immune responsiveness, and whether this phenomenon is relevant for the development of specific serodiagnostic tests, is not clear.

#### **EFFECT OF IMMUNE MEDIATORS ON T.VAGINALIS IN-VITRO:**

It has long been observed that *T.vaginalis* and other trichomonads are lysed by normal human or animal serum (Honigberg 1970; 1978; Gillin and Sher 1981). *T.vaginalis* activates complement by the alternative pathway as was reported by Gillin and Sher (1981). Later, Demes et al. (1987) documented that complement activated by the alternative pathway is responsible for the lysis of *T.vaginalis* in serum. The question of whether specific antibody can enhance lysis in the classic pathway is under investigation: The studies of Shaio et al. (1993) indicated that both, enhanced classical complement pathway activation and an alternative complement pathway activation facilitate *T.vaginalis* lysis. It has also been shown that monoclonal antibodies raised against

*T.vaginalis* and *T.foetus* do possess effector functions, that antibody can apparently kill antigen-positive parasites in a complement-independent manner (Alderete and Kasmala 1986). To date, no evidence has been found for the direct killing of *T.vaginalis* organisms by cytotoxic T cells. However, both macrophages and neutrophils have been shown to kill this parasite, while the effect of eosinophils has not been reported (Landolfo et al., 1980; Mantovani et al., 1981). The killing of *T.vaginalis* by polymorphonuclear neutrophils in-vitro was described by Rein et al., (1980) and Shaio and colleagues in 1991. The process was shown to depend on the presence of oxygen and complement, but not specific antibody. The authors suggest that alternative pathway activation leads to the formation of C3b that binds to the surface of the parasite, which in turn leads to the binding of the parasite to neutrophils through the C3b receptors.

#### **HUMAN DEFENSE MECHANISMS:**

Human vaginal trichomoniasis, although it can be unpleasant and may possibly be associated with long-term side effects, is never fatal. *T.vaginalis* is highly site specific and occurs almost exclusively in the genital tract. However, the fact that *T.vaginalis* infection is not a systemic disease may be attributable to host defense mechanisms (Ackers, 1990). These include nonimmunologic factors, immunologic but

nonspecific defense mechanisms, and specific immunologic responses.

#### **NONIMMUNOLOGIC HOST DEFENSE MECHANISMS:**

Nonimmunologic host defense mechanisms that may protect against trichomoniasis include physical, nutritional and/or other nonimmunologic barriers. The zinc content of prostatic secretions is thought to be an important nonspecific defense against urinary tract infection in men. The antibacterial activity of the prostatic fluid of humans, dogs, and rats is proportional to its zinc concentrations (Kreiger and Rein 1982A; Fair et al., 1976; Graves and Gardner 1993; Heine and McGregor 1993). In the case of trichomoniasis zinc has been suggested as a trichomonocidal component of prostatic fluid. The concentration found in normal men is lethal for most isolates of *T.vaginalis*. However, the existence of both zinc resistant parasites and men with below normal levels of prostatic zinc has been reported (Kreiger and Rein 1982 A and B). Other investigators have suggested that *T.vaginalis* is limited in its growth by the ability to acquire iron (Peterson and Alderate 1984; Graves and Gardner 1993). *T.vaginalis* resides within the human urogenital tract, where it is likely that key nutrients such as lipids and iron may be limiting factors. Lactoferrin is an important iron source at the site of infection. It has recently been suggested that the level of trichomonal adherence to epithelial cells was modulated by

iron such that the presence of lactoferrin enhanced the adherence of the parasite (Lehker et al., 1990 ; 1991). Thus, iron availability from lactoferrin may be an environmental signal that triggers *T.vaginalis* to modulate the amount of adhesin proteins, thereby enabling the recognition and binding to the host epithelial cell. Even more importantly, given the high requirement for iron by this organism, low iron conditions at the site of infection and down regulation of adhesin synthesis may result in the parasite migrating toward more iron rich sites. The end result of such a strategy would be the persistence of this protozoan within the vagina, despite the flushing action of mucosal secretions, desquamation of the mucosal epithelium and overall nutrient limitations and fluxes (Lehker et al., 1991).

The vertebrate host has evolved two mechanisms of immunity. The first and phylogenetically older mechanism is the nonspecific protection provided by complement alone or in concert with phagocytes such as neutrophils and macrophages. The nonspecific factors are genetically determined and are present in every healthy individual. The second mechanism which evolved later, is the specific immune response involving antibodies and lymphocytes. The specific immune response occurs as a reaction of the host to a particular antigenic stimulus (Roitt 1992).

**NONSPECIFIC IMMUNOLOGIC DEFENSE MECHANISMS:**

In trichomoniasis, nonspecific immunologic defense mechanisms include complement, neutrophils, and natural antibodies. *T.vaginalis* is capable of activating complement via the alternative pathway (Gillin and Sher 1981). Using several different methods, the authors demonstrated the involvement of the complement system in the destruction of the parasite in the serum. The evidence of the role of complement in killing of *T.vaginalis* in serum was supported by the experiments of Shaio et al. 1993. Their results also support the killing of trichomonads through enhanced antibody-dependent classical pathway of complement activation in-vitro but, very little is known about the role of complement in vaginal secretions (Demes et al., 1987). The activation of complement enables the polymorphonuclear neutrophils to surround, phagocytose and fragment *T.vaginalis* in to pieces (Rein et al., 1980; Shaio et al., 1991). Chemotaxis is the first step for neutrophils to phagocytose invading microorganisms. Therefore, the presence of chemoattractants will enhance phagocytosis by neutrophils. Manson and Forman (1980) reported a heat-labile chemoattractant produced by *T.vaginalis* in the presence of human serum. Shaio et al. (1992) made an attempt to identify the nature of the chemoattractant in the supernatant collected from neutrophils

activated by *T.vaginalis*, and is most likely leukotriene B4. Other investigators have described 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).

Cytotoxicity of macrophages against *T.vaginalis* has been recognized by many investigators. Spontaneous cytotoxicity against *T.vaginalis* has been demonstrated in both unstimulated murine macrophages (Landolfo et al., 1980) and from healthy volunteer peripheral blood cells predominantly of the monocyte macrophage lineage (Mantovani et al., 1981). Studies of Rye et al. (1990) and Yoon et al. (1991) indicated that unstimulated mouse peritoneal macrophages are cytotoxic for *T.vaginalis* and that the addition of lymphokines increases the cytotoxicity by activating macrophages, to more efficiently kill *T.vaginalis*.

Natural antibody has been detected in healthy human sera. The origin of these natural antibodies in human sera is not known, but has been attributed to cross-reaction with commensal trichomonads (Ackers 1990). The role if any, of natural antibodies in human defense mechanisms is not known, but specific natural antibody is known to be able to play a stimulatory role in the activation of complement and may

enhance complement mediated lysis, perhaps by acting as opsonins (Shaio et al., 1991).

#### **SPECIFIC IMMUNOLOGIC DEFENSE MECHANISMS:**

Although individuals infected with *T.vaginalis* develop both a humoral and a cellular immune response to the organism, their role in human trichomoniasis is not clearly understood. Many tests have confirmed the existence of specific anti-*T.vaginalis* IgM, IgG and IgA in serum, but have shed no further light on their protective function if any (Wos and Walt 1986; Cogne et al., 1985; Mathews and Healy 1983; Sibau et al., 1987; Street et al., 1982; Garber et al., 1986). Honigberg (1987) showed that serum from infected persons can protect mice against the destructive effects of intraperitoneally infected *T.vaginalis* by reducing the extent and severity of the damage caused by the parasite. However, serum from noninfected persons has no protective effect. Studies of Alderete and Kasmala (1986) have also shown that monoclonal antibodies raised against *T.vaginalis* can kill the antigen-positive parasite independent of complement activation. An increase in the number of IgM, IgG and IgA secreting plasma cells was observed in the endocervix following infection with *T.vaginalis*, *N.gonorrhoeae* and *Candida albicans* (Chipperfield and Evans 1972), with greatest increases in IgM bearing cells in patients with

trichomoniasis.

Many reports have been published confirming the existence of antitrichomonal antibodies in human cervicovaginal secretions by various immunological methods, and IgG and IgA antibodies were detected in the majority of reports (Street et al., 1982; Lisi et al., 1986; Su-Lin 1982; Yano et al., 1982). However, the inability to find significant amounts of local antibodies in either infected men or male partners of infected women suggests that local antibodies do not play a significant role in protecting males who are exposed repeatedly to urogenital trichomonads (Ackers et al., 1978). There is also no evidence to support that local vaginal immunoglobulin plays a role in protection from *T.vaginalis* infection.

In general, little evidence is available on the potential of either the systemic or local antibody response in protection against *T.vaginalis* infection. Several reports describe a cell-mediated immune response including the production and effects of lymphokines by sensitized T cells in the presence of *T.vaginalis* antigen as well as a delayed-type hypersensitivity reaction (Yano et al., 1983; Manson and Patterson 1985; Landolfo et al., 1980). However, the data is scant and further work must be done in this area before drawing any definite conclusions. Thus, the role of acquired immunity in human trichomoniasis is not clearly understood.

Previous exposure to *T.vaginalis* does not appear to confer any acquisition of protection. Repeated infection with *T.vaginalis* may occur without significant decrease in either duration of infection or intensity of symptoms as one might expect in the presence of specific protective immune response (Ackers 1990).

#### **VACCINATION AGAINST TRICHOMONIASIS:**

Vaccination can be a very effective method of disease prevention. Different vaccination strategies are currently available for controlling infectious diseases. A killed or attenuated strain of the organisms is used to stimulate a protective immune response. Alternatively, another substance that carries a cross-reacting antigen and which will stimulate a cross-protective immune response can serve the same function (Gordon, 1994). The latter principle was used in the development of a vaccine for trichomoniasis called Solco-Trichovac (Ngumbi et al., 1984). The hypothesis proposed was that the vaccine could induce cross-reacting antibodies against abnormal lactobacilli and *T.vaginalis*, without adversely affecting the growth of normal lactobacilli in the vagina. However, the efficacy and mode of action of this vaccine has not yet been adequately determined (Lorenz and Ruttgers 1983; Gombosova et al., 1986) because of the lack of properly controlled clinical trials. Additional data suggested that the basic hypothesis behind Solco-Trichovac is incorrect

because there has been no detectable cross-reaction between the lactobacillus used and *T.vaginalis* (Gombosova et al., 1986).

Studies of Aburel et al.(1963) used another immune-therapy type approach and met with some success. They inoculated heat killed *T.vaginalis* intravaginally in infected women and showed some clinical improvement. Honigberg (1987) summarised several studies of protective immunity to *T.vaginalis*. In some studies, employment of live or killed parasites induced active immunity in mice. Utilization of hyperimmune sera from rabbits and from patients infected with the parasite also induced protective immunity in mice. Vaccination of mice with supernatant fluid of sonicated trichomonads simultaneously via the intraperitoneal and intravaginal routes met with 80% protection (Martinotti et al., 1977). Even though vaccination strategies are feasible, immunoprophylactic approaches to *T.vaginalis* are in their infancy.

**RATIONALE:**

*Trichomonas vaginalis* is a major cause of the sexually transmitted trichomoniasis throughout the world. The disease is associated with a broad range of clinical symptoms ranging from a relatively asymptomatic carrier state to a well defined symptomatic condition. An epidemiologic association of HIV infection, as well as increased prenatal morbidity are some factors that illustrate the possible importance of this STD as a co-factor in other disease. Little is known about the pathogenesis and immunogenicity of *T.vaginalis*. As a preliminary step, an in-vivo model of immune response to *T.vaginalis* could provide insights into the immunogenicity of this disease.

Because of the potential side effects of metronidazole and limited potential of the current drug therapy of choice, novel strategies are needed for the control of *T.vaginalis* infection. Vaccination may provide an effective alternative method of prevention of this infection. It is therefore important to study the potential role of immunization of *T.vaginalis* in our established (McGrory and Garber 1992) mouse model of infection.

As the efficacy of the current lactobacillus vaccine, Solco-

Trichovac, is no longer accepted scientifically, vaccine development for trichomoniasis must take a different direction, perhaps by using *T.vaginalis* itself. Successful immunizations with the addition of an adjuvant typically result in potent immune responses, which are maintained for longer period of time. Such an immunization with *T.vaginalis* may open a way to control trichomoniasis. A great deal of work must be performed before a vaccine for vaginal trichomoniasis could be available for human use. Therefore, issues of inoculum size, boosting effects, serum and vaginal antibody are all important in determining the feasibility of an immunization strategy.

## **HYPOTHESIS**

Prior exposure of the immune system to *T.vaginalis*, coupled with an adjuvant, enhances its ability to respond again to this antigen. Thus responses to second and subsequent exposure to *T.vaginalis* should be enhanced and qualitatively different from the primary, natural responses. This immune response to *T.vaginalis* would protect the host against subsequent infection with *T.vaginalis*.

## OBJECTIVES

1. To study the role of immunization with *Trichomonas vaginalis* in a mouse model of vaginal infection.
2. To determine if the serum and vaginal washes contain anti-*Trichomonas vaginalis* antibodies, after immunization.
3. To determine the optimum inoculation size of *Trichomonas vaginalis* required for effective immunization.
4. To study the role of multiple immunization (boosting) on protection.

## MATERIAL AND METHODS

### STRAINS:

A well characterized isolate #263 (Garber et al., 1989) recovered from a woman with symptomatic vaginitis was used for these experiments. The organism was grown as previously described (Garber et al., 1987) in 10 mL of Diamond's TYI-S-33 medium (TYI), pH 6.2 (Diamond et al., 1978) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories, Life Technologies Inc. Grand Island, N.Y.), 100 U/mL penicillin, 100 ug/mL streptomycin (penicillin-streptomycin solution Gibco Laboratories) and 2.5 ug/mL amphotericin B (Gibco Laboratories) and incubated in 5% CO<sub>2</sub> at 37°C. Cultures were passaged every 2-3 days. Axenic cultures were stored at -70°C after being mixed with an additional 10% heat-inactivated FBS and 10% dimethyl sulfoxide.

*L.acidophilus* (La) (ATCC 4356) was purchased from the American Type Culture Collection, Rockville, Md. and cultured in Bacto Lactobacilli MRS broth (Difco Laboratories, Detroit, Michigan) or on MRS plates (Difco Laboratories) and incubated in 5% CO<sub>2</sub> at 37°C. Cultures were passaged every 2-3 days.

Axenic cultures in MRS broth were stored in 10% glycerol at -70°C until required.

#### **INTRAVAGINAL INOCULATION OF MICE:**

##### ***L.acidophilus*:**

Two days prior to inoculation with lactobacillus (day -9), all mice received a subcutaneous injection of 500uL of Delestrogen (Estradiol Valerate 10mg/mL; Squibb Canada, Montreal, Quebec) (Cooks et al., 1984; McGrory and Garber 1992). Immediately prior to inoculation with lactobacilli, the estrus cycle of each mouse was defined as previously described (Fox and Laird 1970). Vaginal smears were stained with methylene blue and cells were examined based on a detection of changes in vaginal epithelium during the cycle.

Prior to inoculation, one litre quantity of MRS broth was inoculated with 0.1% of a pure culture of *L.acidophilus* and incubated overnight at 37°C in 5% CO<sub>2</sub>. 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. Mice were inoculated intravaginally with an Eppendorf pipet using 20uL of 10<sup>10</sup> *L.acidophilus*/mL on two consecutive days (days -7 and -6). Negative control mice were inoculated with 20uL of MRS without *L.acidophilus*

Vaginal washes were performed using 50uL of pre-warmed MRS, which was injected and aspirated into the vagina several times until turbid. Successful infection with *L.acidophilus* was determined by culturing vaginal washes in MRS broth supplemented with 5ug/mL ciprofloxacin (Squibb Canada) and 180 ug/mL cefoxitin (Mefoxin; Merck, Sharp & Dohme) and incubated at 37°C for 24-48 hr prior to *T.vaginalis* inoculation.

***T.vaginalis*:**

Two days prior to *T.vaginalis* infection (day -2), mice were infected with a second subcutaneous dose of 500uL of Delestrogen. *T.vaginalis* was 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 (Meysick and Garber 1992). All groups of mice were inoculated intravaginally with 20uL of  $2.5 \times 10^7$  Tv/mL on two consecutive days (days 0 & 1).

**ANIMALS:**

BALB/c mice employed in this study were obtained from Charles River Co., Montreal, Canada. They were housed in plastic cages and given food and water. Mice used in this experiment were 22 to 24g in weight.

**IMMUNIZATION EXPERIMENTS:****Inoculum preparation.**

*T.vaginalis* 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. The final pellet was resuspended in PBS, cells were counted and adjusted to the final concentration, 100uL of the cell preparation suspended in an equal volume of Freund's Complete Adjuvant (FCA) (total volume 200uL) were used for immunization. The same amount of the cell preparation suspended in an equal volume of Freund's Incomplete Adjuvant (FIA) was used for boosting.

As a vaccine control, one litre quantity of MRS broth was inoculated with 0.1% of a pure culture of *L.acidophilus* and incubated overnight at 37°C in 5% CO<sub>2</sub>. Organisms were then harvested by centrifugation for 10 minutes at 5,000 X g, at 4°C in an Omnifuge RT (Baxter, Canlab), washed three times in PBS pH 7.2 and adjusted to a final concentration of 10<sup>7</sup> *L.acidophilus*/mL. 100uL of the cell preparation suspended in an equal volume of FCA and FIA was used for immunization and boosting.

**SUBCUTANEOUS IMMUNIZATIONS:**

Seventy eight (26/ group) mice were subcutaneously immunized in each side of abdominal area with either 4.5 x 10<sup>5</sup>, 9 x 10<sup>6</sup>

or  $10 \times 10^7$  Tv/mL suspended in FCA (at day -56) and 4 weeks later (day -28) boosted with the same dosage in FIA. An inoculation protocol was designed (Table 1) to determine the optimum inoculation size of *T.vaginalis* required for effective immunization. In order to confirm the optimized inoculum regimen, 12 mice were immunized and boosted with  $9 \times 10^6$  Tv/mL suspended in FCA and FIA, respectively (Table 2). Another set of 32 mice (16/group) were immunized only at day -28 with either Tv/FCA or Tv/FIA (Table 3). Another group of mice was immunized and boosted with *L.acidophilus* suspended in FCA and FIA, respectively (Table 2). Control mice included sham vaccinated mice, immunized with PBS (pH 7.2) in FCA and FIA or mice immunized with  $9 \times 10^6$  Tv/mL in the absence of adjuvant. A third group included unimmunized controls and a final group of negative controls. 48 days after primary immunization, mice were inoculated intravaginally with *L.acidophilus* and 1 week later infected with *T.vaginalis*. At weekly intervals for 4 weeks, mice were tail bled and vaginal washes were performed.

TABLE 1

Optimum inoculation size of *T.vaginalis* for immunization.  
Study protocol outlining the time course for subcutaneous  
immunization and intravaginal challenge with *T.vaginalis*.

- Sc Tv = Subcutaneous immunization of *T.vaginalis*
- FCA = Freund's complete adjuvant.
- FIA = Freund's incomplete adjuvant.
- La =  $10^{10}$  *L.acidophilus* inoculated intravaginally on two  
consecutive days (total inoculated volume 20uL each  
day)
- Tv =  $5 \times 10^5$  *T.vaginalis* inoculated intravaginally on two  
consecutive days (total inoculated volume 20uL each  
day)

**DOSAGE OPTIMIZATION:**

Mouse strain: Balb/c.  
*T.vaginalis* isolate #: 263  
Number of Mice:120

**Experimental Protocol:**

**A) Immunization Schedule:**

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Group 1 ( $4.5 \times 10^5$ Tv)	Group 2 ( $9 \times 10^6$ Tv)	Group 3 ( $10 \times 10^7$ Tv)	Group 4 PBS
DAY - 56 0.1CC Tv + 0.1CC FCA	DAY - 56 0.1CC Tv + 0.1CC FCA	DAY - 56 0.1CC Tv + 0.1CC FCA	DAY - 56 0.1CC PBS + 0.1CC FCA
DAY - 28 0.1CC Tv + 0.1CC FIA	DAY - 28 0.1CC Tv + 0.1CC FIA	DAY - 28 0.1CC Tv + 0.1CC FIA	DAY -28 0.1CC PBS + 0.1CC FIA

-----/

**B) Infection Schedule:**

DAY - 9: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY -7/-6: All mice inoculated with  $1 \times 10^{10}$  La

DAY -2: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY 0/1: All mice challenged with  $2.5 \times 10^7$  Tv/ml

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At weekly intervals for 4 weeks (days 7, 14, 21, 28)  
Tail bleeds and vaginal washes obtained from all mice.

**c) Naive Controls:**

18 Mice Designated as Naive Controls:	No Sc Tv
4 Mice Designated Negative Controls:	No Sc Tv No La, No Tv

TABLE 2

Immunization regimen and role of adjuvant.  
Study protocol outlining the time course for subcutaneous immunization and intravaginal challenge with *T.vaginalis*.

- Sc Tv = Subcutaneous immunization of *T.vaginalis*
- FCA = Freund's complete adjuvant.
- FIA = Freund's incomplete adjuvant.
- La =  $10^{10}$  *L.acidophilus* inoculated intravaginally on two consecutive days (total inoculated volume 20uL each day)
- Tv =  $5 \times 10^5$  *T.vaginalis* inoculated intravaginally on two consecutive days (total inoculated volume 20uL each day)

**IMMUNIZATION REGIMEN & ROLE OF ADJUVANT:**

Mouse strain: Balb/c.  
T.vaginalis isolate #: 263  
Number of Mice:44

**Experimental Protocol:**

**A) Immunization Schedule:**

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Group 1 ( $9 \times 10^6$ Tv)	Group 2 PBS	Group 3 ( $9 \times 10^6$ Tv)	Group 4 ( $1 \times 10^7$ La)
DAY - 56 0.1CC Tv + 0.1CC FCA	DAY - 56 0.1CC PBS + 0.1CC FCA	DAY - 56 0.2CC Tv No adjuvant	Day -56 0.1CC La+ 0.1CC FCA
DAY - 28 0.1CC Tv + 0.1CC FIA	DAY - 28 0.1CC PBS + 0.1CC FIA	DAY - 28 0.2CC Tv No adjuvant	Day -28 0.1CC La+ 0.1CC FIA

-----/

**B) Infection Schedule:**

DAY - 9: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY -7/-6: All mice inoculated with  $1 \times 10^{10}$  La

DAY -2: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY 0/1: All mice challenged with  $2.5 \times 10^7$  Tv/ml

-----/

At weekly intervals for 4 weeks (days 7, 14, 21, 28)  
Tail bleeds and vaginal washes obtained from all mice.

**c) Naive Controls:**

8 Mice Designated as Naive Controls: No Sc Tv

4 Mice Designated Negative Controls: No Sc Tv  
No La, No Tv

TABLE 3

Role of multiple immunization on protection.  
Study protocol outlining the time course for subcutaneous immunization and intravaginal challenge with *T.vaginalis*.

- Sc Tv = Subcutaneous immunization of *T.vaginalis*
- FCA = Freund's complete adjuvant.
- FIA = Freund's incomplete adjuvant.
- La =  $10^{10}$  *L.acidophilus* inoculated intravaginally on two consecutive days (total inoculated volume 20uL each day)
- Tv =  $5 \times 10^5$  *T.vaginalis* inoculated intravaginally on two consecutive days (total inoculated volume 20uL each day)

**ROLE OF MULTIPLE IMMUNIZATION:**

Mouse strain: Balb/c.  
T.vaginalis isolate #: 263  
Number of Mice:80

**Experimental Protocol:**

**A) Immunization Schedule:**

Group 1 ( $9 \times 10^6$ Tv)	Group 2 ( $9 \times 10^6$ Tv)	Group 3 ( $9 \times 10^6$ Tv)	Group 4 PBS
DAY - 56 0.1CC Tv + 0.1CC FCA	-	-	DAY - 56 0.1CC PBS + 0.1CC FCA
DAY - 28 0.1CC Tv + 0.1CC FIA	DAY - 28 0.1CC Tv + 0.1CC FCA	DAY - 28 0.1CC Tv + 0.1CC FIA	DAY -28 0.1CC PBS + 0.1CC FIA

**B) Infection Schedule:**

DAY - 9: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY -7/-6: All mice inoculated with  $1 \times 10^{10}$  La

DAY -2: All mice treated subcutaneously with 0.05cc Delestrogen.

DAY 0/1: All mice challenged with  $2.5 \times 10^7$  Tv/ml

At weekly intervals for 4 weeks (days 7, 14, 21, 28)  
Tail bleeds and vaginal washes obtained from all mice.

**c) Naive Controls:**

12 Mice Designated as Naive Controls:	No Sc Tv
4 Mice Designated Negative Controls:	No Sc Tv No La, No Tv

**RE-INFECTION EXPERIMENT:****IN METRONIDAZOLE TREATED T.VAGINALIS INFECTED MICE:**

To compare the response of subcutaneous immunization to the effect of a prior vaginal infection in preventing a subsequent vaginal infection, 18 mice were given orally 10mg/mL metronidazole (250mg tablet) 14 days after intravaginal inoculation with *T.vaginalis*. Metronidazole tablets were dissolved in dd H<sub>2</sub>O and 100uL administered with an Eppendorf pipet. Control mice included, 9 PBS treated mice, 9 untreated mice and 4 negative controls. 3 weeks later, mice were again subjected to vaginal infection with *T.vaginalis* (Table 4). At weekly intervals, vaginal washes were performed for the recovery of *T.vaginalis* and serum was collected by tail bleeds.

TABLE 4

Re-infection experiment.  
Experimental protocol outlining the time course for  
metronidazole treatment and intravaginal challenge with  
*T.vaginalis*.

- La =  $10^{10}$  *L.acidophilus* inoculated intravaginally on two  
consecutive days (total inoculated volume 20uL each  
day)
- Tv =  $5 \times 10^5$  *T.vaginalis* inoculated intravaginally on two  
consecutive days (total inoculated volume 20uL each  
day)

**RE-INFECTION EXPERIMENT:**

Mouse strain: Balb/c.  
T.vaginalis isolate #: 263  
Number of Mice:40

**Experimental Protocol:**

DAY -35: All Mice La intravag x 2 days  
/-----/  
\  
DAY- 28: All Mice Tv intravag x 2 days  
/-----/  
\  
DAY- 14: Metronidazole treatment (10mg/ml)  
/-----/  
\  
DAY - 7: All Mice La intravag X 2 days  
/-----/  
\  
DAY 0: Groups 1-4 Tv intravag X 2 days  
/-----/  
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At weekly intervals for 4 weeks (days 7, 14, 21, 28)  
Tail bleeds and vaginal washes obtained from all mice.

**Controls:**

9 Mice designated as Naive controls.	No Metro
4 Mice designated as Negative controls.	No Metro No La, No Tv

**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 as long as live trichomonads were not visible on 2 consecutive examinations. Vaginal washes were performed with 50uL pre-warmed TYI supplemented with 10% FBS. The tip was inserted into the mouse vagina and the contents dispelled and aspirated until turbid. Wash material was collected in pre-warmed TYI supplemented with 10% FBS, 300ug/mL penicillin, 300ug/mL streptomycin, 2.5ug/mL amphotericin B, and 10ug/mL gentamicin, incubated in glass, screw-capped tubes, in 5% CO<sub>2</sub> at 37°C at a 45° angle and examined daily for the presence of motile *T.vaginalis* using an inverted microscope.

A second vaginal wash was done (3 days after first vaginal wash) with 50uL PBS. Material was collected and centrifuged for 10 minutes at 14,000 X g in a Heraeus Biofuge A Microfuge. The supernatant was transferred to sterile microfuge tubes and stored at -70°C.

Both, TYI and saline vaginal washes were performed on a weekly basis for four weeks following the intravaginal inoculations with *T. vaginalis*. Mice were tail bled for serological observation by collecting 15-20 uL of blood from

each mouse in non-heparinized/Blue Coded Tip Microhematocrit Capillary Tubes. Samples were centrifuged for 2 minutes in a Micro-Hematocrit centrifuge (Model MB, International Equipment Co.), and serum was collected in 1.5mL microfuge tubes and stored at  $-70^{\circ}\text{C}$ .

#### ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA):

##### ANTIGEN PREPARATION :

Axenic cultures of *T.vaginalis* stored at  $-70^{\circ}\text{C}$  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 *T.vaginalis* in the logarithmic growth phase was harvested by centrifugation for 10 minutes at  $140 \times g$  in a Sorvall GLC-1 centrifuge, washed three times in PBS (pH 7.2). The final pellet was resuspended in 10 mL PBS containing 1% sodium dodecyl sulphate (SDS). The suspension was sonicated three times for 30 seconds with 10-15 seconds pauses at maximum amplitude on ice. The suspension was then centrifuged at  $50,000 \times g$  at  $4^{\circ}\text{C}$  for 60 minutes and the pellet discarded. Protein concentration was determined by the Biorad protein assay (Bio Rad, Richmond Lab), aliquoted and stored at  $-70^{\circ}\text{C}$ .

Ninety-six well round bottom plates (Nunc Polysorb U96), were coated with 10ug *T.vaginalis* antigen suspended in PBS (pH

7.2); plates were wrapped in foil and stored at 4°C for a maximum of one week before use in the ELISA.

#### **ELISA TECHNIQUE:**

Antigen coated plates were thawed and washed three times with washing buffer (PBS, 0.1% Tween 80). Test sera were diluted 1:200 in dilution buffer (PBS, 0.1% new born calf serum, 0.1% Tween 80) and 100uL was applied to each well in serial 2-fold dilution from 1:200 to 1:25600. Each serum sample was assayed in duplicate on two separate plates. After 60 minutes of incubation at room temperature, plates were washed three times with washing buffer and 100 uL of 1:1000 diluted (5% new born calf serum, 0.1% Tween 80) goat anti-mouse IgG peroxidase conjugate (Tago, Burlingame, CA) was added to each well. The plates were incubated at room temperature for 60 minutes and then washed three times with washing buffer. Colour development was performed with 100uL of 0.36 mM 2-2'-Azino-di-[3-ethyl-benthiazoline-6-sulfonate] (ABTS) (Boeringer Mannheim) and 30% H<sub>2</sub>O<sub>2</sub> (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. Vaginal wash samples were diluted 1:1 in dilution buffer PBS (0.1% NBCS, 0.1% Tween 80) and 100uL volumes were then applied in serial 2-fold dilutions to *T.vaginalis* antigen coated plates. In addition to the assays with goat anti-mouse IgG, these samples were also assayed with 100uL of goat-anti-mouse IgA peroxidase conjugate (Tago, Burlingame, CA).

#### **STATISTICAL ANALYSIS:**

Where applicable, data were analyzed statistically with a computer program for chi-square test (Mantel-Haenszel Chi-Square Test from Epistat).

#### **ANIMAL CARE:**

All experimental procedures and protocols involving animals were reviewed by, and set with the approval of, the University of Ottawa Animal Care Committee, protocol number OGH-36.

## RESULTS

### OPTIMIZATION OF ELISA

For optimization of ELISA, different concentration of antigen and conjugated IgG were used. Ninety six well plates were coated with 0.6, 1.2, 2.5, 5, 10 and 20 ug of *T.vaginalis* antigen and different dilutions of peroxidase conjugated goat anti-mouse IgG (1:1000, 1:1500, 1:2000), were tested.

It was established that 10ug was the best amount of antigen to use with a conjugate dilution of 1:1000 for anti-IgG (Figure 1). This was tested on both negative control and positive control sera.

### OPTIMUM INOCULATION SIZE OF T.VAGINALIS FOR IMMUNIZATION

In order to determine the size of inoculation of *T.vaginalis* for immunization, Balb/c mice were subcutaneously immunized with varying doses of Tv ( $4.5 \times 10^5$ ,  $9 \times 10^6$ ,  $10 \times 10^7$  Tv/mL) suspended in Freund's complete adjuvant (FCA) 56 days prior to vaginal infection and boosted with the same doses suspended in Freund's incomplete adjuvant (FIA) 4 weeks later (day -28).

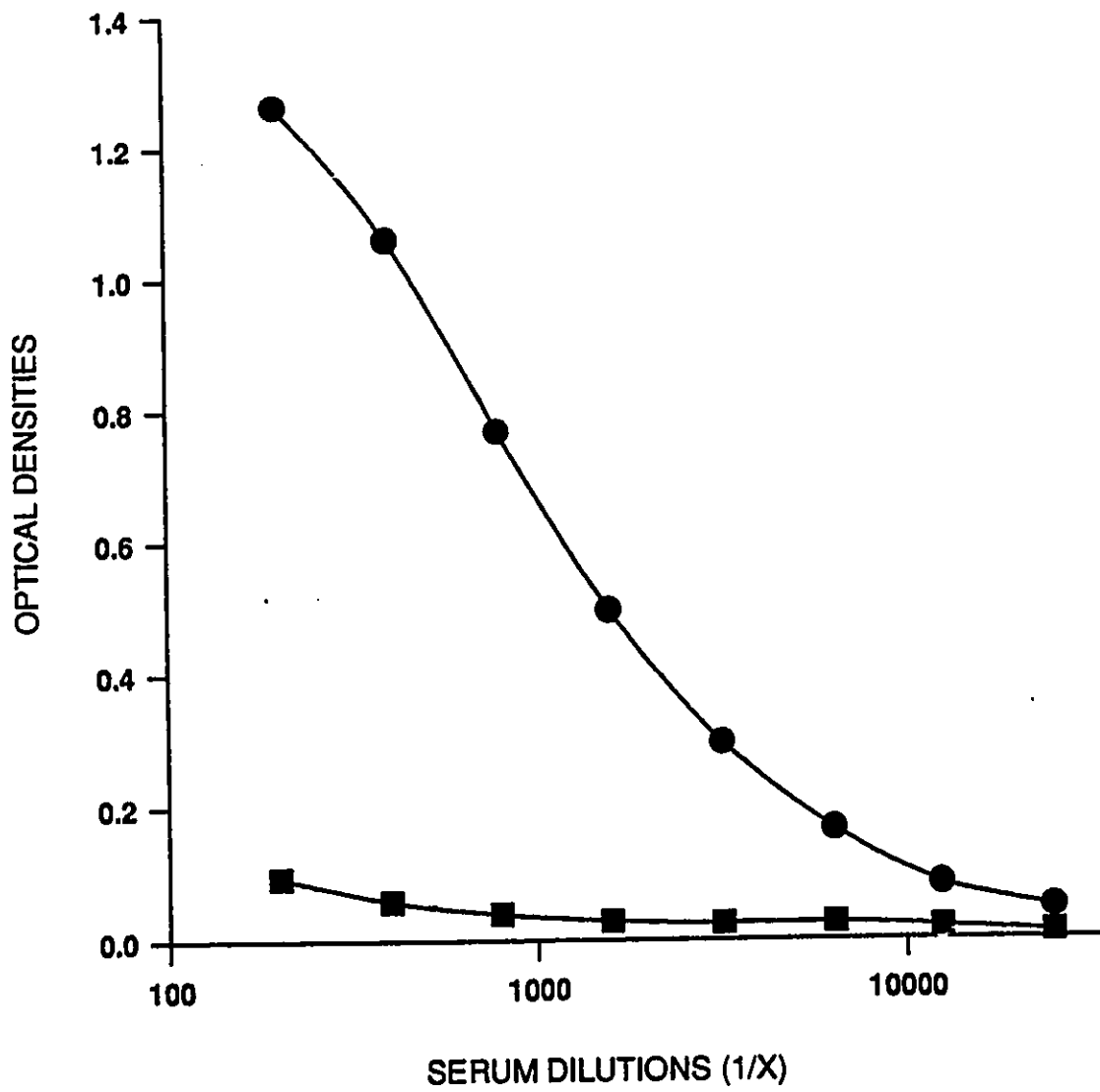
Table 5 depicts the combined results of three separate experimental runs which define the optimal *T.vaginalis* for

**FIGURE 1**

Mouse and *T.vaginalis* standard curve for serum antibody, developed by using 10ug of *T.vaginalis* antigen with a conjugate dilution of 1:1000 for peroxidase conjugated goat anti-mouse IgG.

● Positive control serum.

■ Negative control serum.



immunization dose by measuring the recovery of *T.vaginalis* from vaginal washes. The mice that were immunized and boosted with higher doses of *T.vaginalis* showed less intravaginal infection compared to mice immunized and boosted with a lower dose of *T.vaginalis*, adjuvant control or naive control mice. Seven days after intravaginal inoculation with *T.vaginalis*, only 6 of 25 mice immunized with  $10 \times 10^7$  Tv/mL, and 6 of 25 mice immunized with  $9 \times 10^6$  Tv/mL were intravaginally infected with *T.vaginalis*. Twenty eight days post intravaginal inoculation, *T.vaginalis* could not be recovered from vaginal washes in 95.5% of mice immunized with the  $9 \times 10^6$  Tv/mL dose and 100% of mice immunized with the  $10 \times 10^7$  Tv/mL dose. Twelve of 26 mice immunized and boosted with  $4.5 \times 10^5$  Tv/mL were infected seven days after intravaginal inoculation with *T.vaginalis*. At 28 days post infection 7 mice were still infected. Sixteen of the 17 mice in the PBS immunized group and 14 of the 16 in the naive control group were intravaginally infected at day 7. Eight of the 14 mice in the PBS immunized group and 7 of 15 mice in the naive control groups were still intravaginally infected 28 days post intravaginal inoculation. There was a statistically significant decrease in the recovery of *T.vaginalis* in mice immunized with  $4.5 \times 10^5$  Tv/mL ( $p < 0.01$ ),  $9 \times 10^6$  Tv/mL ( $p < 0.0001$ ) and  $10 \times 10^7$  Tv/mL ( $p < 0.0001$ ) compared to naive controls (Figure 6). Statistically significant decrease in the recovery of *T.vaginalis* was also noticed in mice immunized with  $9 \times 10^6$  Tv/mL ( $p < 0.01$ ) and  $10 \times 10^7$  Tv/mL

( $p < 0.01$ ) compared to mice immunized with  $4.5 \times 10^5$  Tv/mL. No significant difference in recovery of *T.vaginalis* in over 28 days was observed between naive controls and sham vaccinated mice ( $p = 0.99$ ). The six negative control animals had no detectable *T.vaginalis*.

#### ANTIBODY RESPONSE IN SERUM AND VAGINAL WASHES

Figure 3 illustrates, the influence of immunization on serologic IgG response of mice to *T.vaginalis* detected by ELISA. The PBS immunized mice group, naive control group and negative control group showed low IgG response throughout the experiment. The groups of mice immunized with  $9 \times 10^6$  Tv/mL and  $10 \times 10^7$  Tv/mL were showing higher IgG response compared to the group immunized with  $4.5 \times 10^5$  Tv/mL or control mice. Immunization with *T.vaginalis* antigen in adjuvant induced an increased serum IgG response following challenge with an intravaginal inoculation of *T.vaginalis*.

Figures 4 and 5 represent IgG and IgA responses in vaginal washes of immunized mice upon challenged with *T.vaginalis*. A pronounced vaginal IgG response was observed in mice immunized with  $9 \times 10^6$  Tv/mL and  $10 \times 10^7$  Tv/mL, but a low IgG response in mice immunized with  $4.5 \times 10^5$  Tv/mL. All control groups showed low IgG reactivity to *T.vaginalis* antigen. Vaginal IgA response was not as pronounced as in the case of vaginal IgG.

Mice immunized with the two higher concentrations of *T.vaginalis* antigen showed a slight elevation 14 days after infection. Similar to the control groups, immunization with low dose antigen did not induce a detectable increase in vaginal IgA response. These experiments provide some evidence that previous exposure to *T.vaginalis* through subcutaneous immunization results in elevated response of antibodies in vaginal washes and serum, particularly subsequent to a vaginal infection.

TABLE 5. Recovery of *Trichomonas vaginalis* in  
 Mouse Vaginal Washes  
 (Optimum Inoculation Size of Tv for Immunization)

<u>Mouse Group</u>	<u>Days of Culture (positive)</u>			
	<u>+7</u>	<u>+14</u>	<u>+21</u>	<u>+28</u>
Group 1 (n=26) 4.5x10 <sup>5</sup> Tv/mL	12/26	10/25*	8/25*	7/25*
Group 2 (n=26) 9x10 <sup>6</sup> Tv/mL	6/25*	5/25*	3/24*	1/24*
Group 3 (n=26) 10x10 <sup>7</sup> Tv/mL	6/25*	4/23*	1/21*	0/21*
Group 4 (n=18) PBS	16/17*	13/16*	9/15*	8/14*
Group 5 (n=18) No Immunization	14/16*	12/16*	10/15*	7/15*
Group 6 (n=6) Neg. Control.	0/6	0/6	0/6	0/6

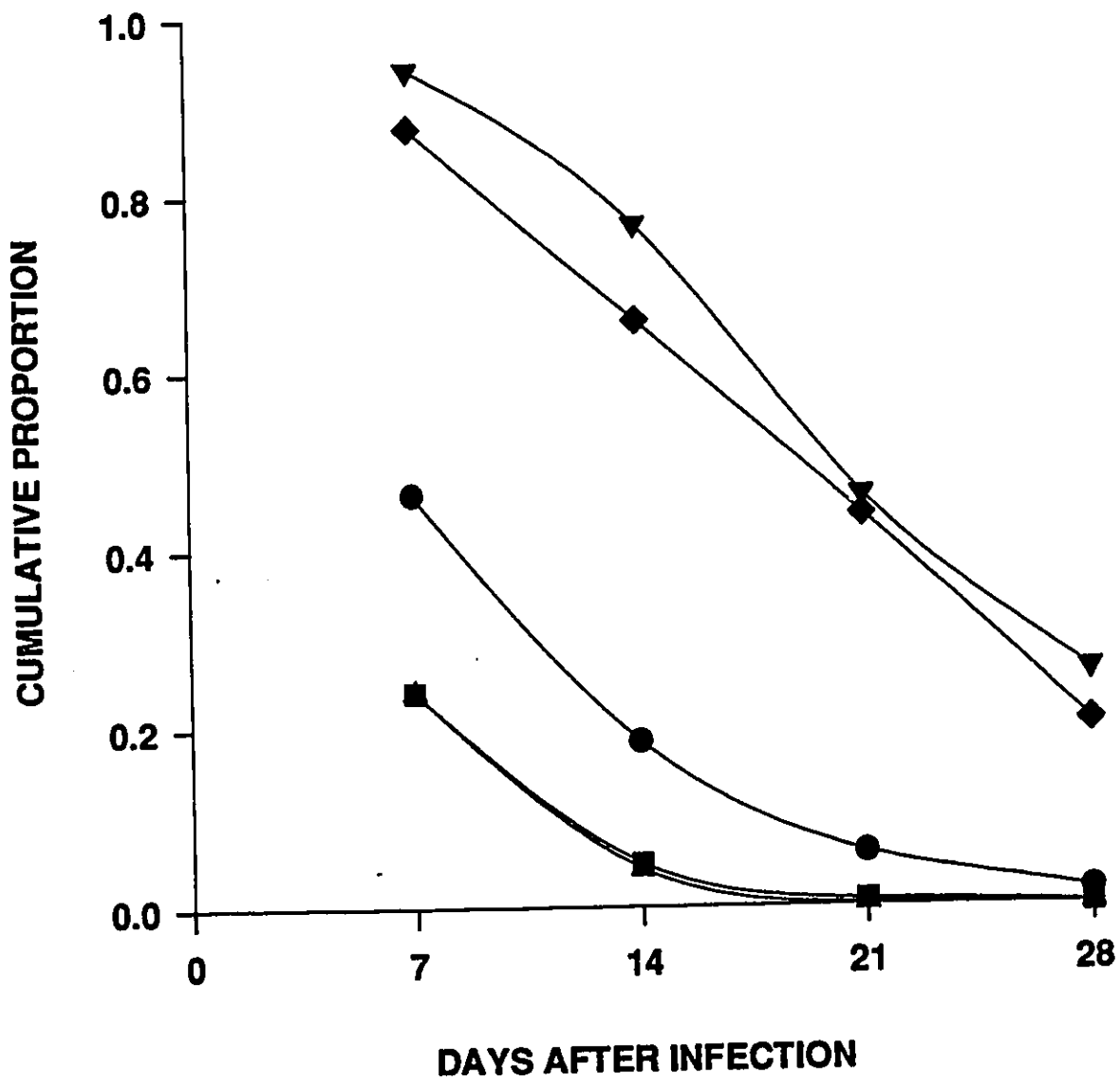
Tv = *T. vaginalis*

\* Changes in the denominator of the different columns are accounted for by unrelated death of mice.

## FIGURE 2

Optimum inoculation size of *T.vaginalis* for immunization.  
Survivorship analysis estimating probability of recovery of  
*T.vaginalis* over time.

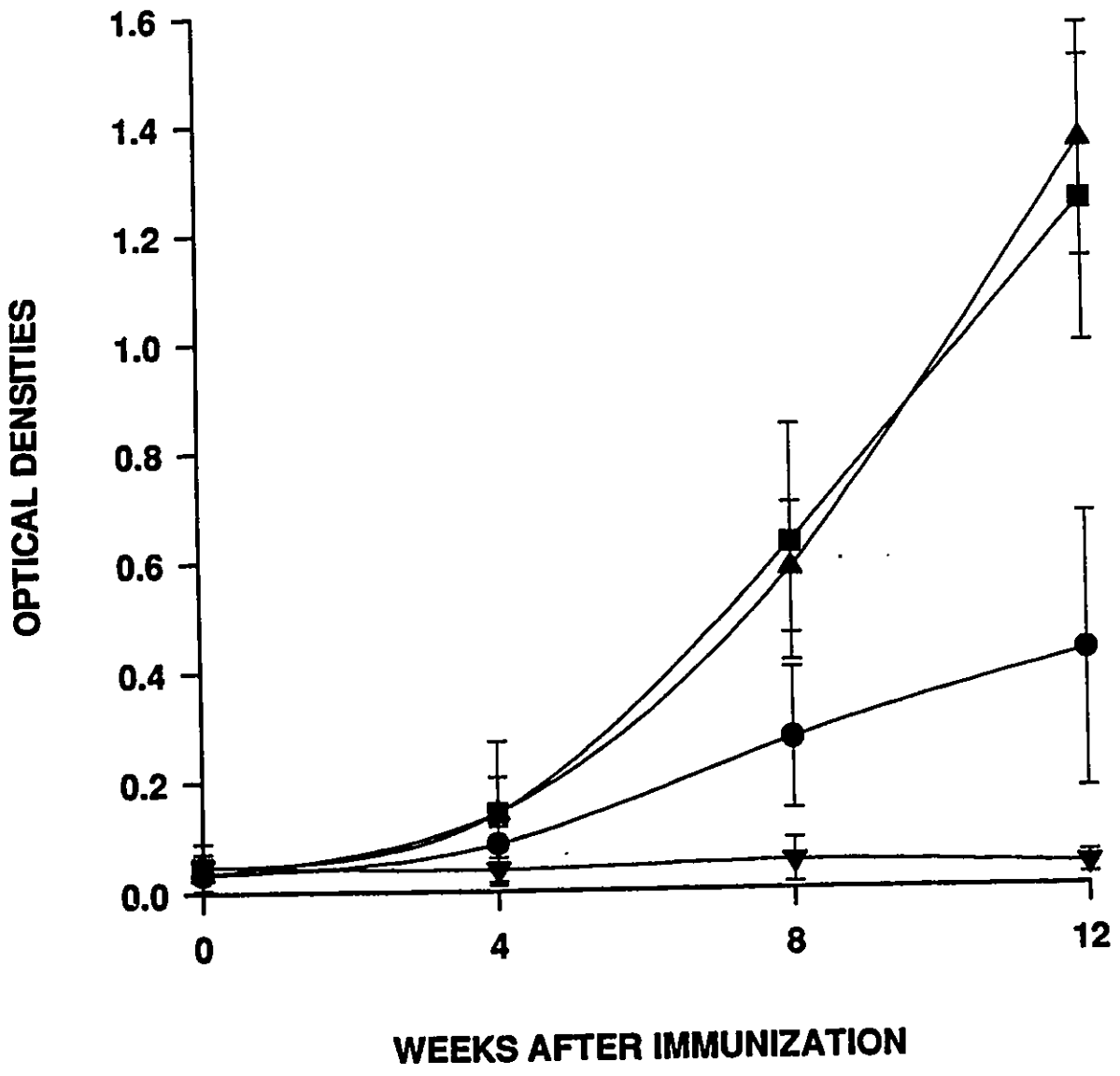
- Group of mice immunized and boosted with  $4.5 \times 10^5$  Tv/mL.
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL.
- ▲ Group of mice immunized and boosted with  $1 \times 10^8$  Tv/mL.
- ▼ Group of mice immunized and boosted with PBS.
- ◆ Group of unimmunized mice (naïve control).



## FIGURE 3

Optimum inoculation size of *T.vaginalis* for immunization.  
Kinetics of IgG serologic response to *T.vaginalis* as measured  
by ELISA.

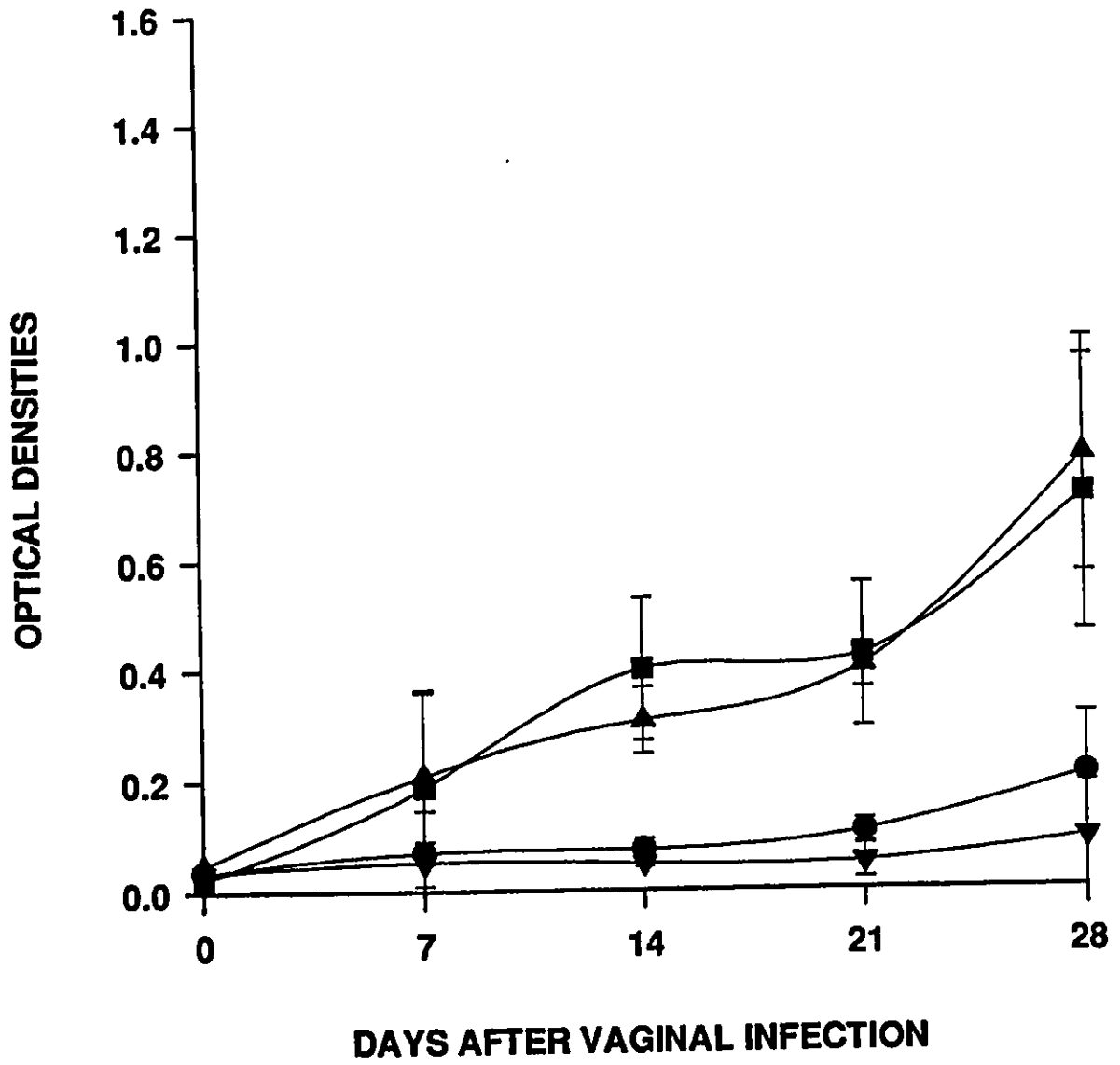
- Group of mice immunized and boosted with  $4.5 \times 10^5$  Tv/ml.
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/ml.
- ▲ Group of mice immunized and boosted with  $10 \times 10^7$  Tv/ml.
- ▼ Group of mice immunized and boosted with PBS.



## FIGURE 4

Optimum inoculation size of *T.vaginalis* for immunization.  
Kinetics of vaginal IgG response to *T.vaginalis* as measured  
by ELISA.

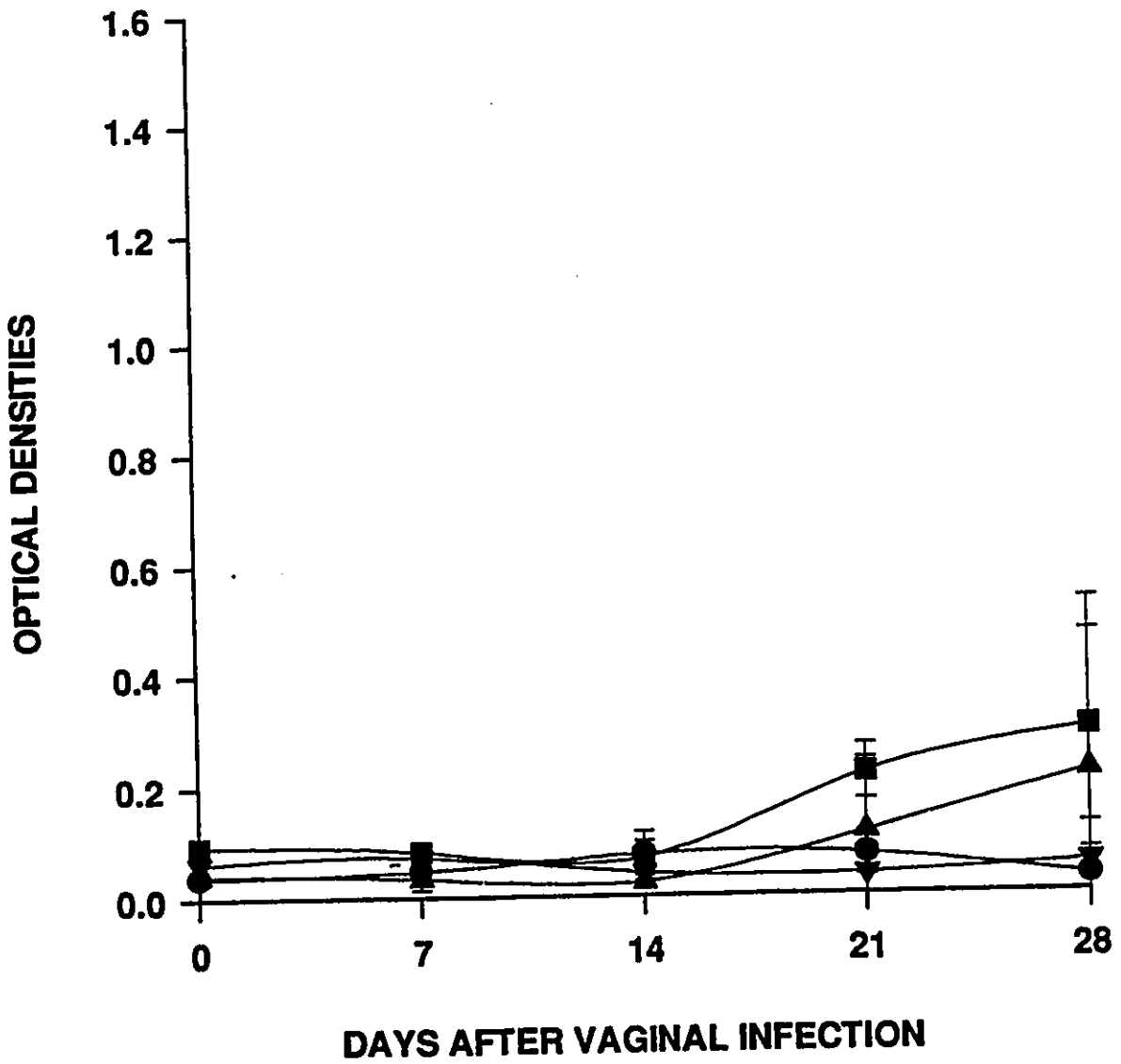
- Group of mice immunized and boosted with  $4.5 \times 10^5$  Tv/mL.
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL.
- ▲ Group of mice immunized and boosted with  $1 \times 10^8$  Tv/mL.
- ▼ Group of mice immunized and boosted with PBS.



## FIGURE 5

Optimum inoculation size of *T.vaginalis* for immunization.  
Kinetics of vaginal IgA response to *T.vaginalis* as measured  
by ELISA.

- Group of mice immunized and boosted with  $4.5 \times 10^5$  Tv/mL.
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL.
- ▲ Group of mice immunized and boosted with  $1 \times 10^8$  Tv/mL.
- ▼ Group of mice immunized and boosted with PBS.



**IMMUNIZATION REGIMEN OF T.VAGINALIS.**

We determined the optimal immunization regimen with *T.vaginalis* as measured by observing the influence of subcutaneous immunization with  $9 \times 10^6$  Tv/mL on antibody response to intravaginal challenge, and on the ability of the trichomonads to establish infection in the vagina. The combined results of two separate trials examining the recovery of *T.vaginalis* from cultures of vaginal washes are presented in Table 6. The number of mice infected with *T.vaginalis* after intravaginal inoculation is presented as the ratio of mice infected to the total number of mice inoculated. The mice immunized and boosted with *T.vaginalis* suspended in Freund's adjuvant were less likely to establish intravaginal infection with *T.vaginalis* compared to the other group of mice. Seven days after intravaginal inoculation, *T.vaginalis* was recovered in 3 of 12 mice in the test group and all 3 cleared the infection 28 days post intravaginal inoculation. Hence the mice that were immunized and boosted with trichomonad suspended in Freund's adjuvant either did not become infected with *T.vaginalis* upon intravaginal challenge or cleared the infection after 21 days. Basically, all mice in sham inoculated group were infected by day 7. Even after 28 days of post intravaginal inoculation, 4 of 6 PBS immunized mice and 2 of 4 immunized with *T.vaginalis* but without adjuvant were infected. Similarly, 4 of 7 mice immunized with

*L.acidophilus* and 5 of 7 naive control mice were still infected by 28 days of post infection. Statistically, *T.vaginalis* was not recovered from the vaginal washes of mice immunized with *T.vaginalis* suspended in Freund's adjuvant prior to vaginal infection compared to naive controls ( $p < 0.0001$ ). No significant difference in recovery of *T.vaginalis* over 28 days was observed between naive controls and sham vaccinated mice ( $p = 0.99$ ), mice immunized without adjuvant ( $p = 0.87$ ) and mice immunized with *L.acidophilus* ( $p = 0.73$ ). This indicates that significant protection and/or clearance only occurs in the mice that received immunization with *T.vaginalis* in the adjuvant (Figure 6). The four negative control mice had no detectable *T.vaginalis* indicating that *T.vaginalis* is not found naturally in mice.

#### ANTIBODY RESPONSE IN SERUM AND VAGINAL WASHES

To define the serologic response to *T.vaginalis* infection and immunization, all groups of mice were tail bled 56 and 28 days prior to infection, the day of infection (day 0) and 28 days post infection by ELISA (Figure 7). In the test group of mice, there was a pronounced IgG response and there was further elevation of IgG response after intravaginal challenge. The IgG response in mice previously immunized with subcutaneous *T.vaginalis* increased over time. This experiment provided some evidence that previous exposure to *T.vaginalis*

suspended in adjuvant results in elevation of IgG response to intravaginal inoculation with *T.vaginalis*. In the sham immunized group, the serologic IgG response was negligible, and the IgG antibody response remained low throughout the experiment, even after vaginal infection.

Figures 8 and 9 represent antibody reactivity detected by ELISA in the vaginal washes 7, 14, 21 and 28 days post intravaginal infection. Only the group of mice immunized and boosted with *T.vaginalis* in adjuvant showed an increased vaginal IgG response (Figure 8) whereas all the control groups showed low antibody response throughout the period. Even though the vaginal IgA response of the test group was not as elevated as serum IgG or vaginal IgG, there was a gradual rise in vaginal IgA after 14 days of infection. The vaginal IgA response (Figure 9) of all control groups were low. In our assay, mice previously exposed to *T.vaginalis* suspended in Freund's adjuvant showed an increased vaginal antibody response as compared to the control groups of mice.

TABLE 6. Recovery of *Trichomonas vaginalis* in  
 Mouse Vaginal Washes  
 (Immunization regimen & role of adjuvant)

<u>Mouse Group</u>	<u>Days of Culture (positive)</u>			
	<u>+7</u>	<u>+14</u>	<u>+21</u>	<u>+28</u>
Group 1 (n=12) 9x10 <sup>6</sup> Tv/mL+FA	3/12	2/11*	1/11*	0/11*
Group 2 (n=6) PBS+FA	6/6	5/6	4/6	4/6
Group 3 (n=6) 9x10 <sup>6</sup> Tv/mL-FA	4/5*	4/5*	3/5*	2/4**
Group 4 (n=8) 10 <sup>7</sup> LA+FA	6/8	6/8	4/7*	4/7*
Group 5 (n=8) No Immunization	7/7*	6/7*	6/7*	5/7*
Group 6 (n=4) Neg. Control.	0/4	0/4	0/4	0/4

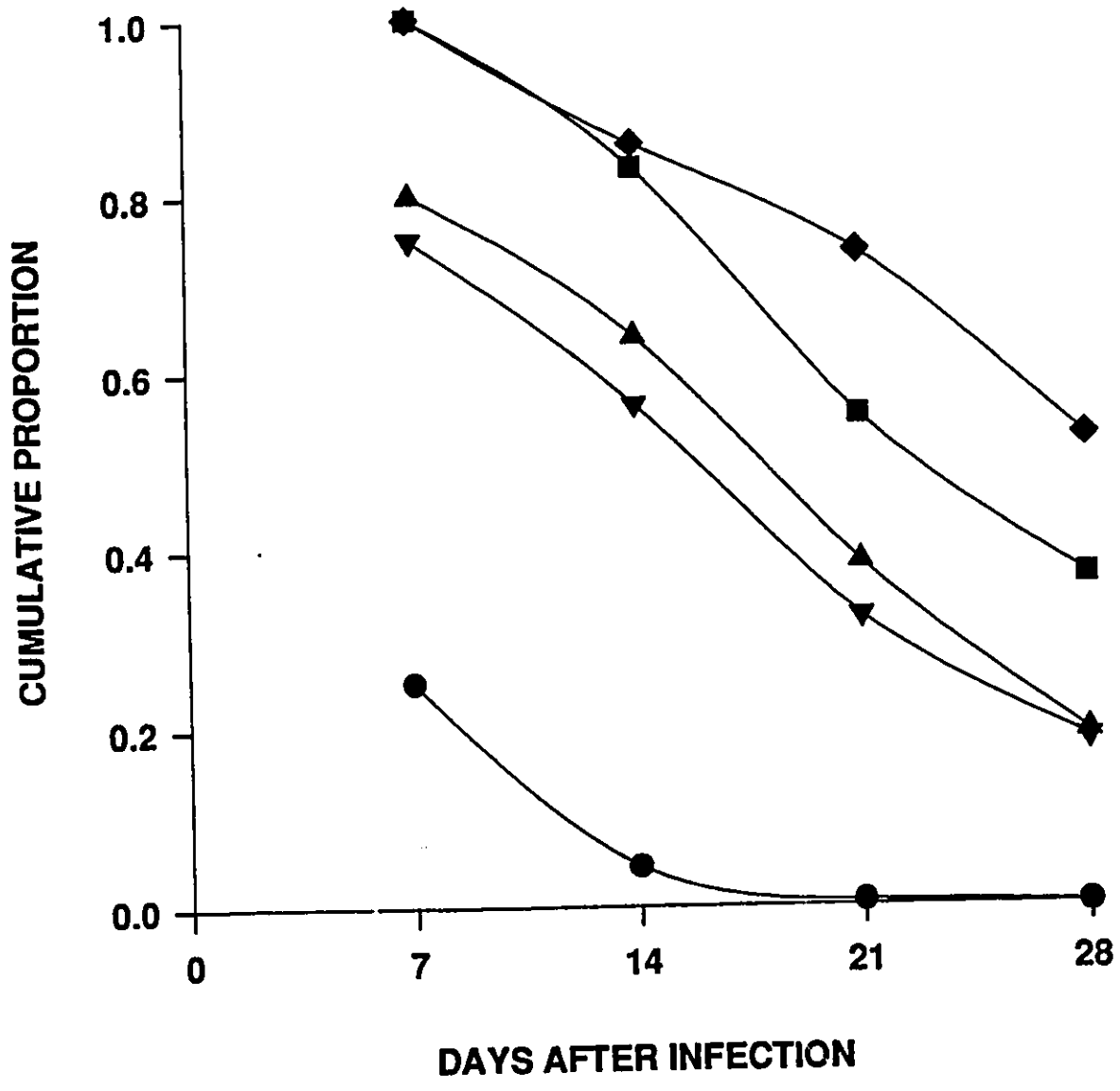
Tv= *T.vaginalis*

\* Changes in the denominator of the different columns are accounted for by unrelated death of mice.

## FIGURE 6

Immunization regimen and role of adjuvant.  
Survivorship analysis estimating probability of recovery of  
*T.vaginalis* over time.

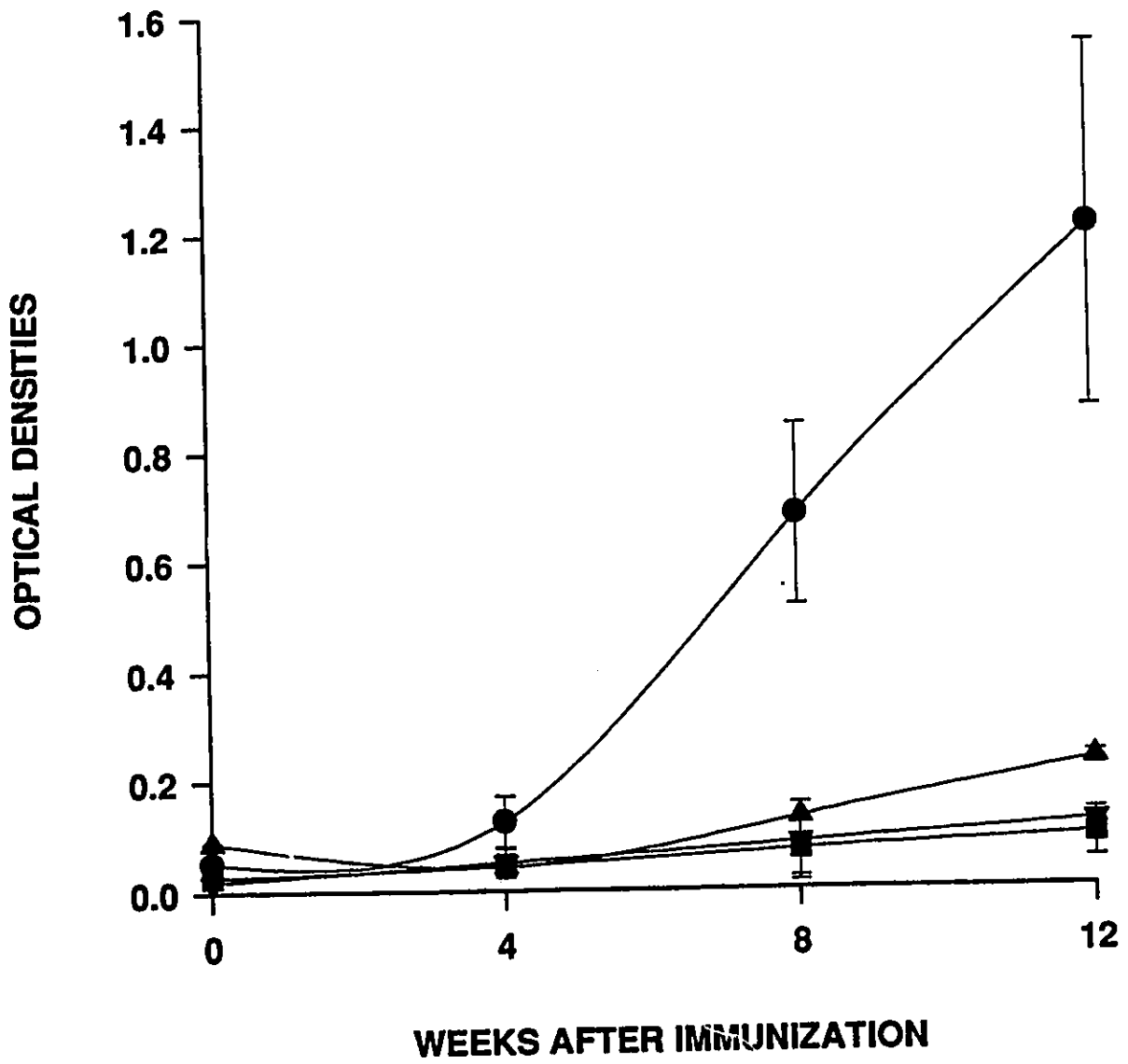
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant.
- Group of mice immunized and boosted with PBS.
- ▲ Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL without adjuvant.
- ▼ Group of mice immunized and boosted with  $1 \times 10^7$  La/mL in Freund's adjuvant.
- ◆ Group of unimmunized mice (naive control).



## FIGURE 7

Immunization regimen and role of adjuvant.  
Kinetics of IgG serologic response to *T.vaginalis* as measured  
by ELISA.

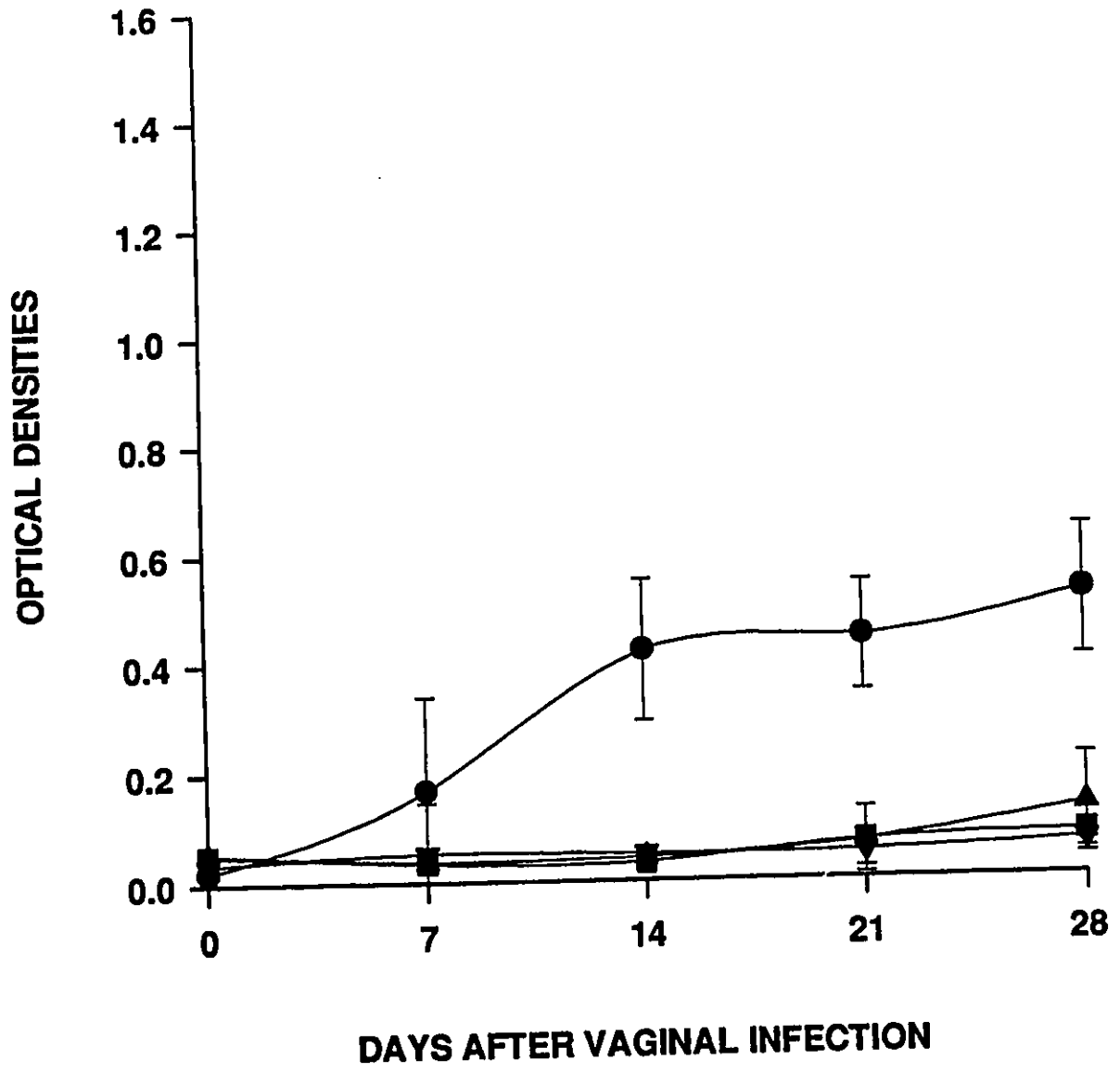
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant.
- Group of mice immunized and boosted with PBS.
- ▲ Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL without adjuvant.
- ▼ Group of mice immunized and boosted with  $1 \times 10^7$  La/mL in Freund's adjuvant.



## FIGURE 8

Immunization regimen and role of adjuvant.  
Kinetics of vaginal IgG response to *T.vaginalis* as measured  
by ELISA.

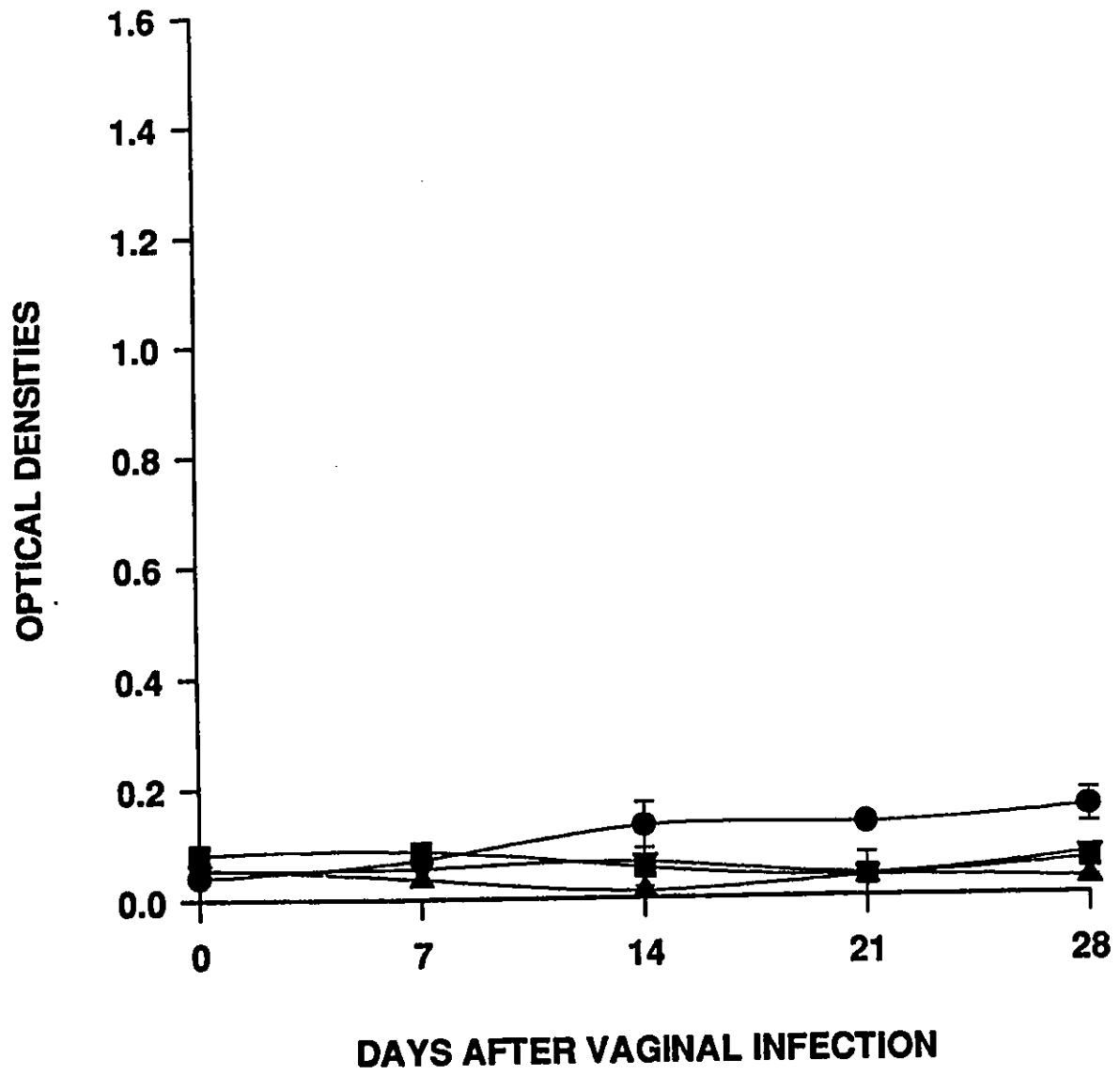
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant.
- Group of mice immunized and boosted with PBS.
- ▲ Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL without adjuvant.
- ▼ Group of mice immunized and boosted with  $1 \times 10^7$  La/mL in Freund's adjuvant.



## FIGURE 9

Immunization regimen and role of adjuvant.  
Kinetics of vaginal IgA response to *T.vaginalis* as measured  
by ELISA .

- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant.
- Group of mice immunized and boosted with PBS.
- ▲ Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL without adjuvant.
- ▼ Group of mice immunized and boosted with  $1 \times 10^7$  La/mL in Freund's adjuvant.



**IMPORTANCE OF BOOSTING:**

To examine the importance of booster inoculation on protection against intravaginal challenge with *T.vaginalis*, mice were immunized with  $9 \times 10^6$  Tv/mL suspended in FCA at day -56 and boosted with FIA at day -28 or immunized only at day -28 with either Tv/FCA or Tv/FIA. Control mice were immunized with PBS suspended in FCA at day -56 and boosted with FIA at day -28.

The combined results of two separate runs examining the recovery of *T.vaginalis* from vaginal washes are shown in Table 7. Mice immunized and boosted with *T.vaginalis* antigen preparation prior to intravaginal challenge were less likely to be infected with *T.vaginalis* than mice receiving a single immunizing dose or mice not previously exposed to *T.vaginalis*. In the immunized group, 7 days after intravaginal challenge, *T.vaginalis* could be recovered from vaginal washes in 5 of the 20 mice but only 1 of 20 immunized mice was still infected 28 days after intravaginal challenge with *T.vaginalis*. The mice that received a single immunizing dose of *T.vaginalis* in FCA, 28 days prior to the intravaginal challenge, 10 of the 16 mice were infected by 7 days of post intravaginal infection, 5 of the 14 mice were still infected on day 28. These results were similar to those in mice receiving a single immunizing dose of *T.vaginalis* in FIA, 28 days prior to the intravaginal

challenge, 6 of 14 mice were infected on day 28. *T.vaginalis* was not recovered from the vaginal washes of mice immunized and boosted with *T.vaginalis* prior to vaginal infection compared to naive controls ( $p < 0.0001$ ). No significant difference in the recovery of *T.vaginalis* over 28 days was observed between naive controls and mice that received only one immunization in FCA ( $p = 0.824$ ), mice that received only one immunization in FIA ( $p = 0.704$ ) and sham immunized mice ( $p = 0.99$ ) (Figure 10). Most of the control mice were infected throughout the period.

Figure 11 represents the reactivity of IgG detected by ELISA in serum samples 0, 4, 8 and 12 weeks post immunization. The group of mice immunized and boosted prior to the intravaginal challenge elicited an elevated IgG response. An increase in antibody reactivity was observed upon booster immunization and vaginal infection when compared to the group of mice that received only a single immunizing dose prior to vaginal infection. In our assay, mice previously immunized with *T.vaginalis* antigen preparation showed an increase in serum *T.vaginalis* IgG reactivity compared to negative control mice which had not previously been exposed to subcutaneous *T.vaginalis*.

Looking at vaginal IgG response (Figure 12) at days 7, 14, 21 and 28 days of post intravaginal inoculation, only mice

immunized and boosted with *T.vaginalis* showed an increase in antibody reactivity. A measurable but smaller vaginal IgG response was seen in the groups of mice which received a single immunization, but not in control groups. Vaginal IgA reactivity (Figure 13) in all the groups, immunized and unimmunized were not elevated, only the group of mice immunized and boosted showed a slight elevation.

TABLE 7. Recovery of *Trichomonas vaginalis* in  
 Mouse Vaginal Washes  
 (Multiple Immunization and Protection)

Mouse Group	Days of Culture (positive)			
	+7	+14	+21	+28
Group 1 (n=20) Tv + FCA-> -56 Tv + FIA-> -28	5/20	5/20	3/20	1/20
Group 2 (n=16) Tv + FCA-> -28	10/16	9/15*	7/14*	5/14*
Group 3 (n=16) Tv + FIA-> -28	10/16	8/16	7/16	6/14*
Group 4 (n=12) PBS + FCA-> -56 PBS + FIA-> -28	11/12	8/11*	6/11*	6/10*
Group 5 (n=12) No Immunization	9/11*	8/11*	6/10*	5/10*
Group 6 (n=4) Neg. Control.	0/4	0/4	0/4	0/4

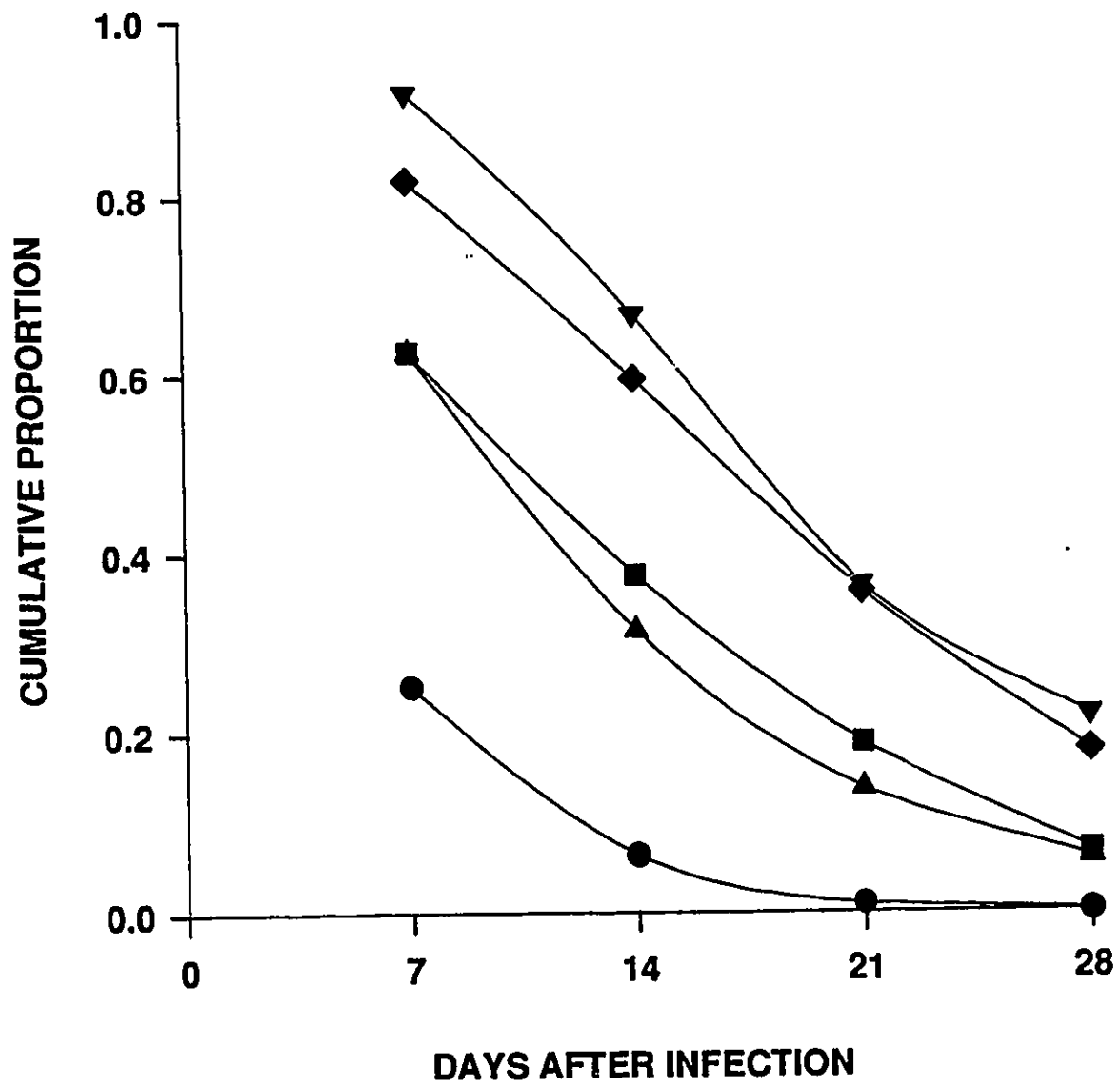
Tv = *T. vaginalis*

\* Changes in the denominator of the different columns are accounted for by unrelated death of mice.

## FIGURE 10

Multiple immunization and protection.  
Survivorship analysis estimating probability of recovery of *T.vaginalis* occurring over time.

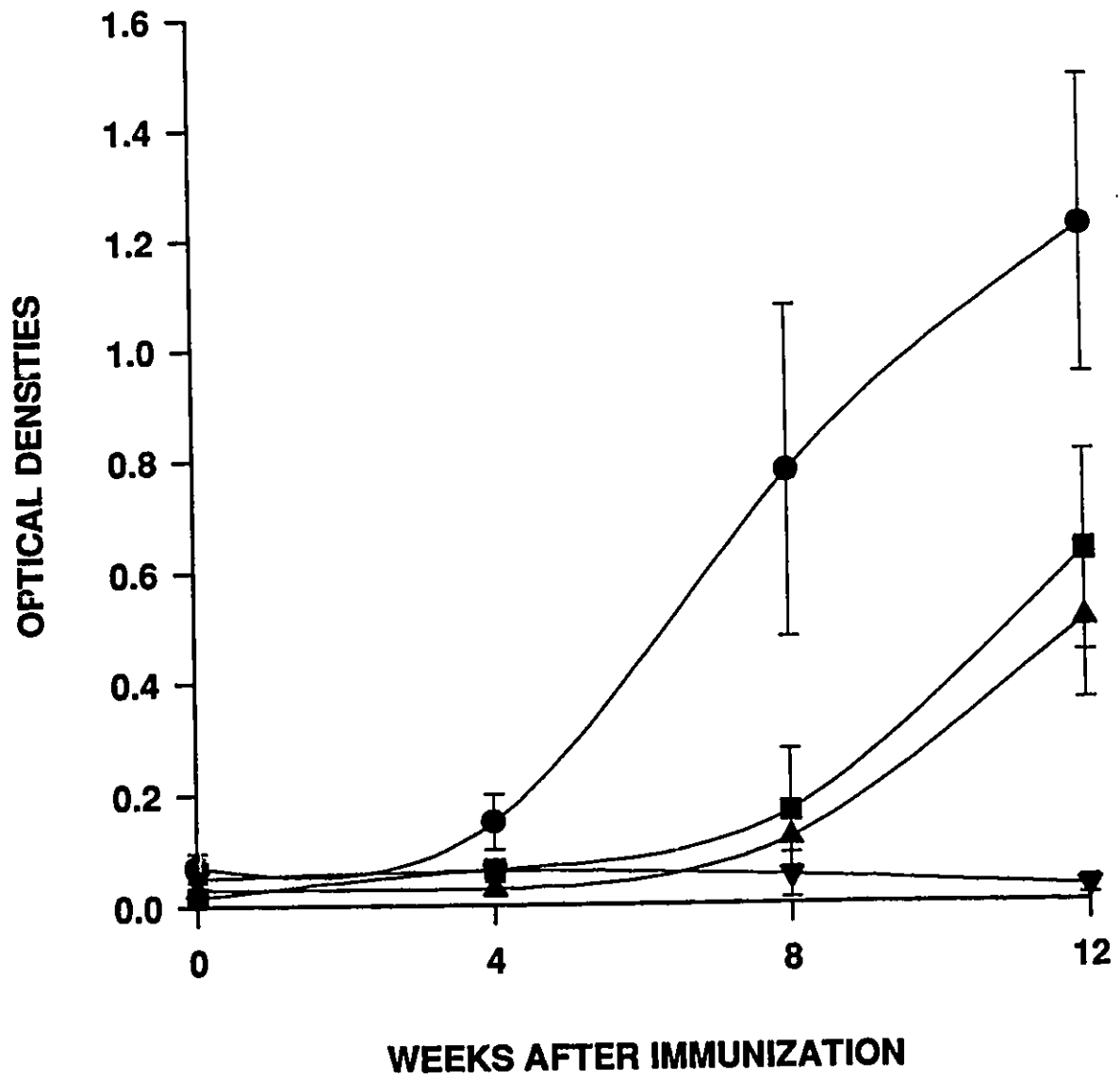
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant at days -56 and -28.
- Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's complete adjuvant at day -28.
- ▲ Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's incomplete adjuvant at day -28.
- ▼ Group of mice immunized and boosted with PBS.
- ◆ Group of unimmunized mice (naive control).



## FIGURE 11

Multiple immunization and protection.  
Kinetics of IgG serologic response to *T.vaginalis* as measured  
by ELISA.

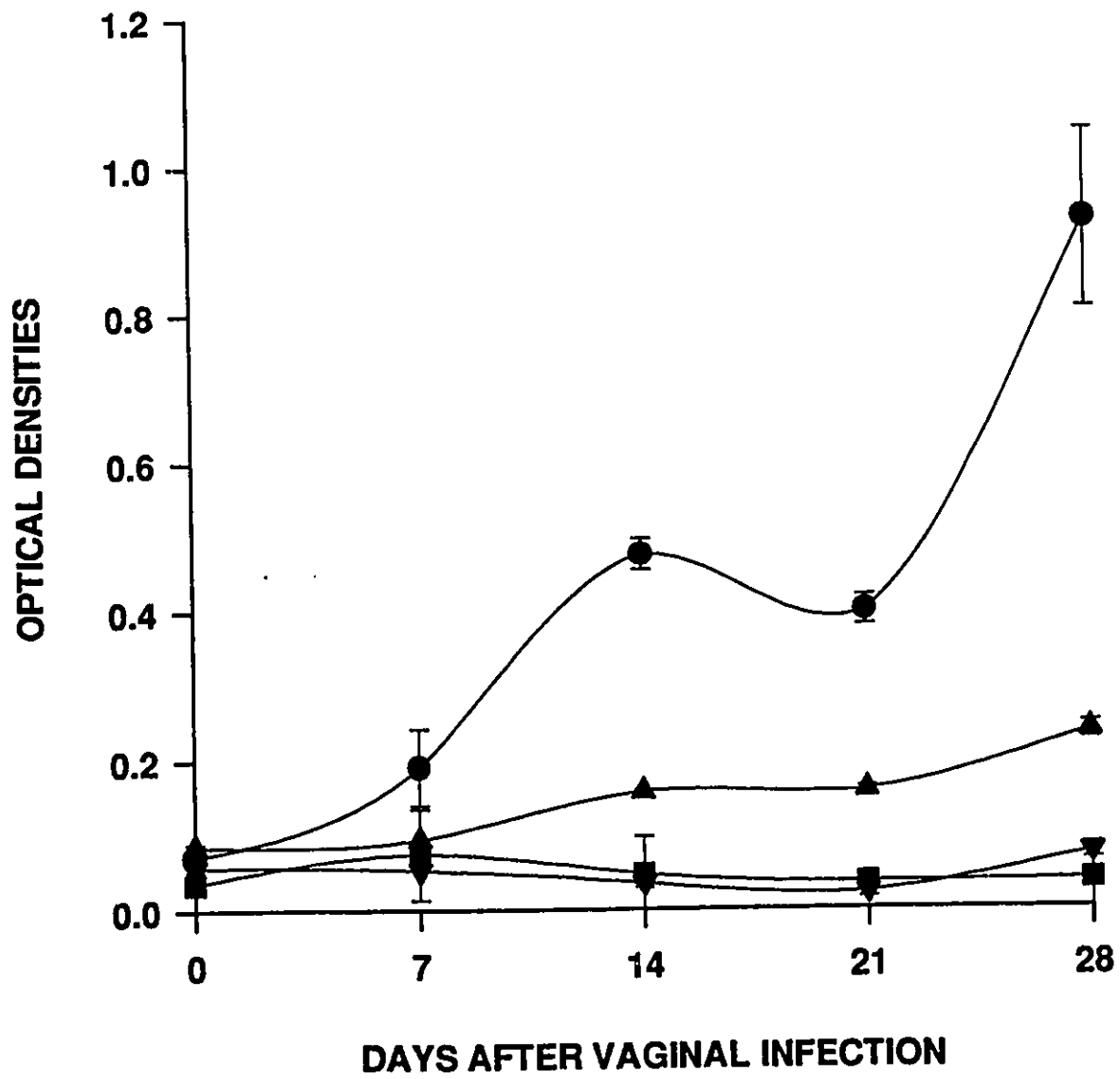
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant, at days -56 and -28 respectively.
- Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's complete adjuvant at day -28.
- ▲ Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's incomplete adjuvant at day -28.
- ▼ Immunized and boosted with PBS.



## FIGURE 12

Multiple immunization and protection.  
Kinetics of vaginal IgG response to *T.vaginalis* as measured  
by ELISA.

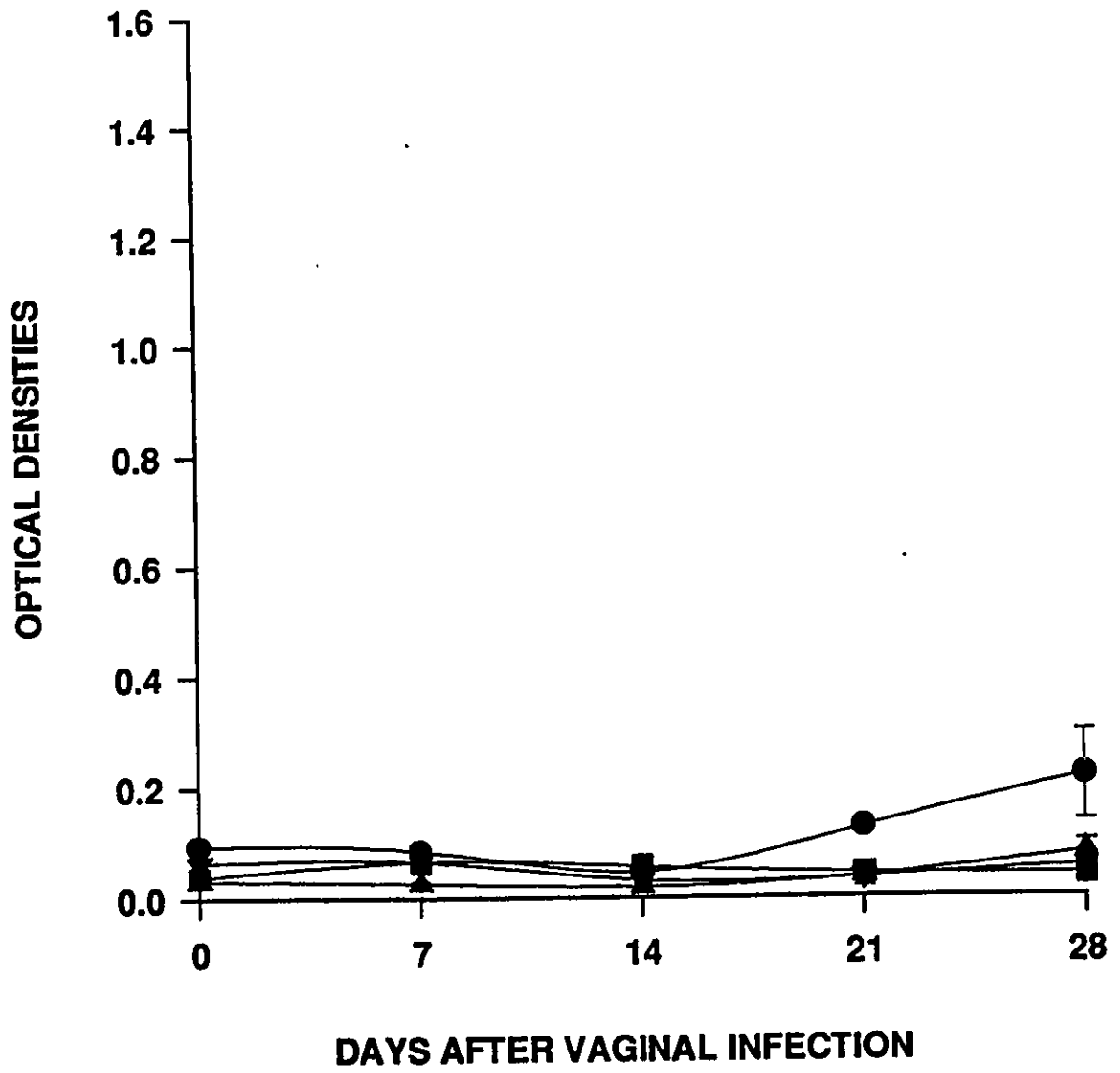
- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant, at days -56 and -28 respectively.
- Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's complete adjuvant at day -28.
- ▲ Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's incomplete adjuvant at day -28.
- ▼ Immunized and boosted with PBS.



## FIGURE 13

Multiple immunization and protection.  
Kinetics of vaginal IgA response to *T.vaginalis* as measured  
by ELISA.

- Group of mice immunized and boosted with  $9 \times 10^6$  Tv/mL in Freund's adjuvant, at days -56 and -28 respectively.
- Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's complete adjuvant at day -28.
- ▲ Group of mice immunized with  $9 \times 10^6$  Tv/mL in Freund's incomplete adjuvant at day -28.
- ▼ Immunized and boosted with PBS.



**METRONIDAZOLE TREATMENT & RE-INFECTION:**

In order to determine whether vaginal infection and treatment induce protection, Balb/c mice were intravaginally infected with *T.vaginalis* and treated with metronidazole before the second intravaginal challenge. Table 4 depicts the recovery of *T.vaginalis* from the vaginal washes of infected and treated mice. Seven days after second intravaginal challenge 12 of 18 mice treated with metronidazole, 8 of the 9 mice treated with PBS and 7 of 8 untreated mice showed *T.vaginalis* culture positivity. Even after 28 days of post intravaginal challenge, 47% of metronidazole-treated mice, 55.5% mice treated with PBS and 62.5% of untreated mice were culture positive for *T.vaginalis*. Serum and vaginal *T.vaginalis* antibody response were not detected in these mice.

TABLE 8. Recovery of *Trichomonas vaginalis* in

## Mouse Vaginal Washes

(Metronidazole Treatment &amp; Protection)

<u>Mouse Group</u>	<u>Days of Culture (positive)</u>			
	<u>+7</u>	<u>+14</u>	<u>+21</u>	<u>+28</u>
Group 1 (n=18) Drug-Metro	12/18	10/18	9/17*	8/17*
Group 2 (n=9) PBS	8/9	6/9	6/9	5/9
Group 3 (n=9) No Drug	7/8*	6/8*	5/8*	5/8*
Group 4 (n=4) Neg. Control.	0/4	0/4	0/4	0/4

\* Changes in the denominator of the different columns are accounted for by unrelated death of mice.

## DISCUSSION

### IMMUNIZATION WITH T.VAGINALIS IN OUR MOUSE MODEL OF INFECTION

Despite the existence of chemotherapeutic agents, the incidence of trichomoniasis is increasing, and this has led to renewed interest in the immunological response of the host to this infection. Immune responses to infection with *T.vaginalis* have been described, including specific secretory antibody in vaginal secretion (Ackers et al., 1975), IgM and IgG antibody in serum (Manson, 1979; Street et al., 1982), polymorphonuclear cell chemotaxis (Manson and Forman, 1980, 1982) and phagocytosis (Rein et al., 1980). Despite these responses, chronic infection with the parasite is common, and immunity to re-infection is poor (Ackers 1990). Also, information regarding host immunological responses to *T.vaginalis* remains inadequate.

The results presented in this study demonstrate that it is possible to achieve significant protection against *T.vaginalis* infection by subcutaneous immunization using whole cell *T.vaginalis* in an animal model. This study also proved that successful immunization can elicit an immune response that is stronger than or different from the immune response elicited by the natural infection. We achieved inducible immunity

which provides protection from vaginal challenge to the trichomonad.

**SIZE OF INOCULATION TO T.VAGINALIS FOR IMMUNIZATION:**

Dose-response experiments were performed in order to determine the optimal dose of *T.vaginalis* for inducing immunization protection and anti-*T.vaginalis* antibodies. As a first step, recovery of *T.vaginalis* in mouse vaginal washes and trichomonad clearance from animals immunized with various doses of *T.vaginalis* suspended in adjuvant was compared with unimmunized animals. Mice that were immunized with the higher doses showed significant protection. Compared to the non-immunized mice, mice that were immunized with the lowest dose also showed significant but a lower level of protection. This observation is also in agreement with the studies of Cripps et al. (1994) who showed the clearance of *Pseudomonas aeruginosa* infection was obtained with the highest immunizing dose.

Analysis of antibody response in serum and vaginal washes revealed that *T.vaginalis* specific IgG reactivity was significantly higher in mice immunized with the higher dose compared to the unimmunized mice but not in mice immunized with the lowest dose. Analysis on trichomonad clearance and specific antibody response revealed that some relationship exists between trichomonad clearance and specific antibody

response. However, at a lower immunizing dose, *T.vaginalis* clearance from the vaginal wash samples were also observed, but to a lesser degree. This is the situation where the specific antibody response was not different from that in unimmunized mice. This study, suggests that antibodies provide some degree of protection, but there may be other mechanisms of protection that do not involve antibody. The exact function of IgG in serum and vaginal washes and the role it may play in protection is not clear. IgA antibody response in vaginal washes were also not elevated. We hypothesise that specific antibodies to *T.vaginalis* in conjunction with other immune processes may be involved in protection. Freund's complete adjuvant is a known stimulant of both cell-mediated and humoral responses (Cooper 1994) and Freund's incomplete adjuvant stimulates predominantly humoral immunity (Cooper 1994) suggesting that immunization with *T.vaginalis* suspended in these adjuvants induces both arms of the immune system. Therefore, cell-mediated immunity may also play an important role in protection against *T.vaginalis* infection.

#### **IMMUNIZATION REGIMEN AND ROLE OF ADJUVANT:**

We examined the ability of immunization with a *T.vaginalis* whole cell antigen preparation to induce protective immunity against subsequent intravaginal challenge with *T.vaginalis*. The results suggested that immunization with Freund's adjuvant

resulted in the induction of protective immunity against infection. Mice immunized with *T.vaginalis* in adjuvant developed high antibody response and were less likely to sustain an infection after vaginal challenge compared to control groups.

The role of acquired immunity in human trichomoniasis is not clearly understood. As mentioned previously, chronic infection with the parasite is common, and immunity to re-infection is poor (Honigberg 1987; Chao et al., 1989). One could speculate that an explanation for the absence of a protective immune response in humans compared to our results as seen in mice is simply due to the fact that immune system of the mouse may respond to a different antigen or process the antigen in an entirely different manner than would the human. Interestingly, mice that were immunized and boosted with *T.vaginalis*, in the absence of adjuvant, did not show significant protection, compared to mice immunized with the adjuvant. Many immunologic studies have shown that previous exposure of the immune system to a pathogen enhances its ability to respond again to that antigen (Bouvet et al., 1994; Thapar et al., 1990; Cripps et al., 1994). In our study, immunization with an adjuvant elicited an immune response that is stronger than and/or different from the immune response elicited by natural infection or by immunization without adjuvant. This response induced protection from vaginal challenge with *T.vaginalis*.

The mechanism of protection in the mouse model, whether cell mediated or humoral, will require much further work before it can be defined.

Examination of the antibody response in serum and vaginal washes in immunized mice following intravaginal challenge with *T.vaginalis* was the second aspect of this immunological investigation. A measurable serologic response was apparent in mice subcutaneously immunized and boosted with *T.vaginalis* suspended in Freund's adjuvant, prior to the intravaginal challenge with the trichomonad. Considerable IgG reactivity was detected after primary immunization, and a booster immunization led to a further elevation in the IgG antibodies. The reactivity of *T.vaginalis* specific IgG was even higher after vaginal infection. An interesting observation was the important role of adjuvant in enhancing the antibody response shown by the mice.

In human infection, it appears that the responsiveness of antibody in patients is not strong and the antibody titers are low even in active *T.vaginalis* infection (Honigberg 1987; Alderete et al., 1991A; Chao et al., 1989). Our study demonstrated that it is possible to achieve a high response of *T.vaginalis* specific IgG in mice by booster immunization with *T.vaginalis* suspended in adjuvant. Adjuvants have been used to augment the immune response in experimental immunology as

well as in practical vaccination for more than 60 years. The mode of action of adjuvants was summarized by Chedid (1985) as "(i) the formation of a depot of antigen at the site of inoculation which is slowly released; (ii) the presentation of antigen to immunocompetent cells; and (iii) the production of different lymphokines, such as various interleukins and tumor necrosis factor".

Many reports have been published confirming the existence of antitrichomonal antibody in human cervicovaginal secretions by various immunological methods and *T.vaginalis* specific IgG and IgA were reported in a majority of reports (Street et al., 1982; Peter et al., 1988; Su-Lin 1982; Ackers et al., 1975; Alderete, 1984). However, there is little evidence that it results in protective immunity. Alderete et al., (1991 A) reported that in some cases, trichomoniasis was not associated with detectable vaginal antibody. Attempts to detect antibody in vaginal washes showed that only the group of mice immunized and boosted with *T.vaginalis* suspended in Freund's adjuvant prior to infection showed a significant IgG response upon infection. The IgA EIA reactivity was not as elevated as the IgG. This result is in agreement with previous studies, reporting the induction of antibodies in genital secretions by a conventional systemic route of immunization (Bouvet et al., 1994; Thapar et al., 1990). Our study observed IgG and IgA titres in vaginal washings after immunizations via

subcutaneous route with *T.vaginalis* in combination with Freund's adjuvant and suggest that immunization might have an important influence on secretory immunoglobulin at mucosal surfaces.

We have determined that immunization with *T.vaginalis* in combination with Freund's adjuvant induced protection against vaginal infection by *T.vaginalis* and an enhanced antibody reactivity in serum and vaginal washes. However, an increased antibody response may not be the only explanation for the protective effect seen in this study. As well, the exact function of these antibodies and the role they may play in protection is not clear. 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. Although evidence exists for both serum and vaginal antitrichomonal antibodies in women infected with *T.vaginalis* (Ackers et al., 1978), neither appeared to protect against reinfection with the trichomonad. However, Honigberg (1970) summarized a report suggesting that serum from infected patients protected mice against *T.vaginalis* infection. The mechanism for this effect was not completely understood. In-vitro studies of Alderete and Kasmala (1986) have also shown that monoclonal antibodies raised against *T.vaginalis* can kill the antigen-positive parasite independent of complement activation.

However, sera from infected patients have been shown to exhibit complement mediated lytic activity on trichomonads in cultures (Holbrook et al., 1982). Passive immunization experiments with mouse sera could be performed to determine to what extent humoral immunity is involved in the observed protection.

It is generally accepted that IgA is an important immunoglobulin in many mucosal secretions in humans and other animals and that, secretory IgA plays an important role in immunity to certain microbial infections of the respiratory and gastrointestinal tracts (Goldblum 1990; Thaper et al., 1990). Chipperfield and Evans (1972) noted an increase of IgA-, IgG-, and IgM- containing plasma cells in endocervical tissues from women with gonorrhoea, trichomoniasis, and candidiasis. Plasma cells of the IgM class were more prominent in trichomoniasis than in the other two infections. Unexpectedly, in our study, the elevation of the IgA antibody response to *T.vaginalis* did not rise as much as the serum IgG antibodies and vaginal IgG antibodies. Possible explanations for the low rate of IgA detection in the vaginal washes in the present study as compared to that of Ackers et al. (1975) and Su-Lin (1982), may be: "i) low IgA antibody levels are present in the vaginal washes that can be demonstrated by the assay used in this study; ii) IgA may be produced by the host only during a certain period in the course of infection and the low

IgA could be a result of the adsorption of the antibody by the organisms". However, there is little evidence that it is harmful or inhibitory to the parasite. Ackers et al. (1975) suggested that low parasite counts in vaginal secretions were associated with the presence of antitrichomonad antibody. Chipperfield and Evans (1972) suggested that the typical absence of the urogenital trichomonad from the cervical mucosa might be caused by the production of antibodies, Such antibodies could prevent invasion of the uterus and fallopian tubes by *T.vaginalis*.

#### **MULTIPLE IMMUNIZATION AND PROTECTION:**

In order to understand the importance of boosting in inducing protective immunity and anti-*T.vaginalis* antibodies, multiple immunization experiments were performed. Looking at the recovery of *T.vaginalis* in mouse vaginal washes and trichomonad clearance, we have seen that, mice which were immunized and boosted prior to the intravaginal challenge were less likely to develop infection and had a significant rate of trichomonad clearance, compared to the mice which received a single immunization or no immunization. In human trichomoniasis previous exposure to *T.vaginalis* does not appear to convey any protection. Repeated infection with *T.vaginalis* may occur without significant difference in either duration of infection or intensity of symptoms as one might

expect if there were a specific immune response (Graves and Gardner 1993; Chao et al., 1989). These results underscore the importance of a booster immunization protocol for the development of protective immunity in our mouse model. Examination of the antibody response in serum and vaginal washes revealed high response of IgG antibodies in mice immunized and boosted with *T.vaginalis*, prior to intravaginal challenge with the trichomonad. Serum IgG antibodies were also raised but to a lesser degree in mice which received only a single dose of immunization, compared to the control mice. However, the level of protection and response of serum IgG in these single immunization animals was found to be much lower, compared to the immunized and boosted animals. Boosting did not alter vaginal IgA but did influence vaginal IgG response.

Protection against experimental *T.vaginalis* infection in mice can be elicited by subcutaneous immunization with whole cell trichomonad. Enhanced protozoal clearance and also pronounced antibody response were seen in immunized and boosted animals, however the mechanism by which a subcutaneous immunization leads to protection is not clear. The role of antibody in trichomonad clearance from vaginal infection is far from clear. Protection due to antibody has been demonstrated in some systems (Honigberg 1970), but not in others (Martinotti et al., 1977). The role played by cell mediated immunity in protection is also important to

consider.

An explanation for the low rate of IgA detection in the vaginal washes, in light of studies with the *Neisseria gonorrhoeae* and *Streptococcus sanguis* (Pault et al., 1977) is that production of IgA protease reduces the IgA activity. Proteinases degrading antibodies have also been found in protozoan parasites (McKerrow et al., 1993). *T.foetus*, the causative agent of fetal wastage in cattle, is known to secrete cysteine proteinases that digest the heavy chain of host IgG (Talbot et al., 1991). A recent report also describes human IgA heavy chain degradation by *Entamoeba histolytica*, another mucosal protozoan parasite (Kelisall and Ravdin 1993). IgG and IgA antibodies to immunogenic *T.vaginalis* surface proteins and to proteinases have been found in sera and vaginal washes of patients with trichomoniasis (Alderete et al., 1991 A and B; Bozner et al., 1992; Sharma et al., 1991). Very recently, Provenzano and Alderete (1995) reported that *T.vaginalis* cysteine proteinases are capable of degrading human immunoglobulins and suggested its possible role in immune evasion and in predisposing the urogenital mucosa to coinfection and pathogenesis. It is noteworthy that no vaginal antibody is detected in some women with trichomoniasis (Alderete et al., 1991 A). This lack of detection of antibody may be directly or indirectly a result of trichomonal Ig-degradation activity that may be associated with the

trichomonal proteinases.

Previous studies also demonstrate that the mucosal immune response in the genital tract is affected by female hormones (Wira and Sullivan 1985; Wira and Sandoe 1980). Studies of Wira and Sullivan (1985) documented that estrogen and progesterone can lower vaginal antibody and secretory antibody component levels in cervical and vaginal secretions. Further studies are needed to identify the role, if any, these factors may play in lowering antibody response in the vaginal washes. In our study, the vaginal washes were pooled from mice in each group because of the small quantities of washes. Thus, washes obtained could contain antibodies from both immune and nonimmune mice. For an accurate detection, analysis of the vaginal washes from individual mice would be necessary.

Despite identical procedures in the immunization, boosting, administration of estrogens, inoculation of *L.acidophilus* and inoculation of *T.vaginalis*, mice showed different response to the antibody reactivity and varied susceptibility to infection. The variations observed in the mice also appear in natural infection. Individual host response to infectious agents is dependent on many factors such as properties of antigen, dose of antigen, site of exposure, hormonal levels, and ongoing immune response in the host (Honigberg 1987).

Regardless of immunization and infection, the death of mice seen in each group was considered as a consequence of the experimental manipulations and associated stress and did not reflect the type of intervention used.

Protection was not demonstrated in mice which were infected vaginally, treated with metronidazole and then reinfected vaginally. This suggests that the response to infection and immunization protection could be associated with the route of immunization. These results also underscore the importance of an immunization protocol for the development of protective immunity in our mouse model.

### CONCLUSION

In conclusion, we have found that subcutaneous immunization with *T.vaginalis* appears to induce protection from intravaginal challenge with *T.vaginalis*. Mice that had received subcutaneous immunization and boosting with *T.vaginalis* prior to intravaginal challenge were significantly less likely to develop infection and had a significantly higher rate of trichomonad clearance, compared to the mice which received only a single immunization or no immunization. A measurable serum and vaginal antibody response was also apparent in mice subcutaneously immunized with *T.vaginalis* prior to vaginal infection. However, the mechanism by which the immunization mediates this protection is not clearly understood. The role of cell mediated immunity may be a factor in the protective response to *T.vaginalis* infection. Passive immunization experiments with anti-*T.vaginalis* mouse sera could be performed to determine the extent of the role of systemic humoral immunity in protection.

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

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## APPENDIX

## TYI S-33 Medium:

Ingredients for 900ml of medium.

783 ml dd H<sub>2</sub>O

Casitone or Casein digest peptone Enzymatic digest of casein. BBL Microbiology system. Becton Dickinson & co. Cockeville MD21030.	18.0 gm
Bacto Yest Extract ("Difco" Certified) Difco Laboratories, Detroit, Michigan.	9.0 gm
Dextrose (Certified A.C.S Anhydrous) Fisher Scientific Company. Fair Lawn, New Jersey 07410	9.0 gm
Sodium Chloride. (Anala R Grade) BDH Chemicals Vancouver.	1.8 gm
Potassium Phosphate Monobasic (A.C.S) Fisher Scientific Company Fair Lawn, New Jersey 07410	0.54 gm
Potassium Phosphate Dibasic Anhydrous (A.C.S) Fisher Scientific Company. Fair Lawn, New Jersey 07410	0.9 gm
L.Cysteine HCL.H <sub>2</sub> O, N.R.C Grand Island Biological Comapany (GIBCO) Grand Island NY 14072	0.9 gm
L.Ascorbic Acid (Vit.C) BDH Chemical Vancouver.	0.18gm
Ferric Ammonium Citrate Mallinckrodt, Inc. St.Louis Missouri 63147	0.02052 gm
pH to 6.2 Autoclave.	
Vitamin Tween NCT 109(#320-1340) 0.1ml Tween 80+ 130ml NCT 109 Vit.	27ml

**Citrate Buffer:**

Citric acid (Fisher)	1.11 g
Na <sub>2</sub> HPO <sub>4</sub> (BDH)	0.117 g
dd H <sub>2</sub> O	80 ml

pH to 4.25 exactly with 1M NaOH then dilute to 100 ml with dd H<sub>2</sub>O.

**Substrate Solution for EIA:**

Citrate Buffer	10 ml
H <sub>2</sub> O <sub>2</sub>	20 ul
ABTS	4 mg
dd H <sub>2</sub> O	10 ml