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**INVESTIGATION INTO THE POTENTIAL INVOLVEMENT OF
APOPTOSIS IN THE PATHOGENIC MECHANISMS OF
THE PROTOZOAN PARASITE *TRICHOMONAS VAGINALIS***

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 Biochemistry, Microbiology, & Immunology

Submitted by
Shannon Hayward-McClelland

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ABSTRACT

The protozoan parasite *Trichomonas vaginalis* is the causative agent of the common sexually transmitted disease trichomoniasis. *T. vaginalis* infection varies greatly with respect to clinical severity and, while not life-threatening, can lead to a number of serious urogenital complications. Despite a great deal of research, the exact pathogenic mechanisms employed by the parasite in its attack on the human urogenital tract remain largely undefined. It is likely, given the wide variations in symptomology seen in those infected with *T. vaginalis*, that the parasite employs a number of different pathogenic mechanisms. These mechanisms include the ability to evade host immune responses, cellular adhesion, haemolysis, the excretion of soluble factors, and interaction with the resident urogenital flora. *In vitro* studies have shown that *T. vaginalis* causes a number of pathological changes in host cells and effectively destroys a variety of host cell monolayers. It has also been documented that many microorganisms, including a number of protozoan pathogens, are capable of inducing apoptosis in host cells. Our hypothesis tied together these two areas and proposed that *T. vaginalis* would induce apoptosis in host cells as part of its pathogenic assault. This hypothesis was tested in an established co-culture system; previous research demonstrated that the parasite destroyed McCoy cell monolayers. The TUNEL assay was used to identify cells undergoing apoptotic DNA fragmentation as a result of co-incubation with *T. vaginalis*. Early experiments suggested that apoptosis may have been occurring in the McCoy monolayers, but this was later disproved with the help of a sandwich ELISA to detect DNA/histone fragments that characteristically result from apoptosis. Instead, the cells that had originally been identified as apoptotic were found to be adherent trichomonads. While McCoy cell apoptosis in response to co-culture with *T. vaginalis* was not observed, *T. vaginalis* did, over time, cause detachment of almost all monolayer cells, and also caused irreparable damage in a portion of these cells. Induction of McCoy monolayer detachment was determined to be contact-independent, given that trichomonad culture supernatants had a similar effect.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di- [3-ethylbenzthiazoline sulfonate]
AIDS	acquired immunodeficiency syndrome
Apaf-1	apoptotic protease-activating factor
ATCC	American Type Culture Collection
Ca²⁺	calcium ion
CAM	camptothecin
caspase	cysteinyl aspartate-specific proteinase
CDC	Centers for Disease Control and Prevention
CDF	cell detaching factor
cfu	colony forming units
CHO	Chinese hamster ovary
CO₂	carbon dioxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EIA	enzyme-linked immunoassay
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GST	glutathione transferase
³H	tritium
Hep-2	human epithelial
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus

HSV	herpes simplex virus
IAP	inhibitor of apoptosis protein
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
ISNT	<i>in situ</i> nick translation
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
KH₂PO₄	potassium dihydrogen phosphate
L	liter
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
MRS	deMan, Rogosa, and Sharpe (lactobacilli media)
N	normal
NaCl	sodium chloride
Na₂HPO₄	sodium monohydrogen phosphate
NaOH	sodium hydroxide
NH₂	amino group
nm	nanometer
O₂	oxygen
OH	hydroxyl group
PBS	phosphate buffered saline
P:C	parasite to target cell ratio

PFA	paraformaldehyde
PI	propidium iodide
POD	peroxidase
PVC	polyvinyl chloride
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
STD	sexually transmitted disease
TNFR	tumor necrosis factor receptor
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated 2'- deoxyuridine-5'-triphosphate (dUTP) nick end labeling
Tv	<i>Trichomonas vaginalis</i>
TYI	Diamond's TYI-S-33 medium (Trypticase, yeast extract, iron- serum)
U	units
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
WHO	World Health Organization
z-IETD-fmk	z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone
z-VAD-fmk	z-Val-Ala-Asp(OMe)-fluoromethylketone

INTRODUCTION

Trichomoniasis: Significance and Epidemiology

Trichomoniasis is a sexually transmitted disease (STD) of global importance (World Health Organization, 1995) and it is caused by the protozoan parasite *Trichomonas vaginalis*. While once considered to be a disease of little importance, trichomoniasis has now been accepted to have economic, cultural and medical repercussions (Hook, 1999). With an annual incidence of more than one hundred and seventy million cases, it has become the most prevalent non-viral STD (World Health Organization, 1995). The same WHO document shows that eight million new cases of *T. vaginalis* infection are reported each year in North America alone. Given that up to fifty percent of infections may exist without any symptoms (Fouts and Kraus, 1980), these infection rates are likely underestimated. Asymptomatic infections are especially problematic because carriers may transmit the parasite unknowingly to others (Nicoletti, 1961). *T. vaginalis* is an obligate parasite that is almost always transmitted via sexual contact (Heine and McGregor, 1993). Although the organism lacks a cystic stage, it has been shown to survive outside its host for a number of hours (Honigberg, 1978; Lossick, 1990a). While it is theoretically possible for urogenital trichomonads to be transmitted non-sexually, there has been no documentation of this happening (Heine and McGregor, 1993). Trichomoniasis has a cosmopolitan distribution and cases have been documented throughout all continents, races, and socio-economic strata. It has been suggested, however, that certain populations are more affected by the disease than others. Risk factors for the acquisition of trichomoniasis appear to include multiple sexual partners, low education and income, a previous history of STDs, and the non-use of contraceptives (Lossick, 1990a; Heine and McGregor, 1993). The true prevalence of trichomoniasis is difficult to determine since sampling is not random and is biased by the fact that most surveys involve those people visiting STD clinics with symptoms of *T. vaginalis* infection or other STDs (Lossick,

1990a). Unfortunately, *T. vaginalis* is not a reportable disease in most countries, so accurate data with respect to disease morbidity and infection trends are difficult to collect (Lossick, 1990a).

Clinical Picture of Urogenital Trichomoniasis in Women

T. vaginalis primarily infects the squamous epithelium of the female urogenital tract. The infection is multi-focal and parasites can be detected in the vagina, cervix, urethra, bladder, and in the Skene's, Bartholin's, and periurethral glands (Honigberg, 1978; Wolner-Hanssen *et al.*, 1989; Krieger, 1995). Trichomoniasis is generally a disease of the reproductive years, and is rarely reported before puberty or after menopause (Honigberg, 1978). The incubation period is generally in the range of a few days to a month; infection may persist for a protracted period of time. Clinical presentation in women can range from asymptomatic to flagrant vaginitis to a chronic carrier state (Wolner-Hanssen, 1989; Rein, 1990; Heine and McGregor, 1993). In addition, symptoms can vary over time in a given patient. Rein (1990) has suggested that over thirty percent of asymptotically infected women become symptomatic within a period of six months. While the phenomenon of disparity among patient clinical profiles is not completely understood, the explanation likely involves differences in the inherent virulence of individual strains and also variation in host susceptibility (Rein, 1990).

The prevalence of specific symptoms in *T. vaginalis*-infected women differs greatly from study to study. Acute trichomoniasis has been reported to present with any or all of the following symptoms: vaginal discharge, vaginitis, vulvitis, vulvovaginal irritation or pruritis, dyspareunia, and dysuria (Rein, 1990). Vaginal, and less frequently, vulvular erythema has also been found in women infected with *T. vaginalis*. Colpitis macularis or "strawberry cervix", which is created by capillary dilatation and punctate hemorrhages on the vaginal walls and the cervix, can be detected by the naked eye in only two percent of cases, but can be found by colposcopy in as many as

ninety percent of *T. vaginalis*-infected women (Fouts and Kraus, 1980; Wolner-Hanssen *et al.*, 1989; Krieger *et al.*, 1990; Heine and McGregor, 1993). In general, the pH of the vagina also changes through the course of infection, with a rise from the normal 4.5 to greater than 5.0 (Rein and Chapel, 1975; Rein, 1990).

Trichomoniasis symptoms and parasite load have been shown to be cyclical and tend to worsen during menstruation (Rein and Chapel, 1975; Spiegel, 1990; Heine and McGregor, 1993).

Menstrual blood may serve as a stimulant or nutrient for the parasites. The elevation in vaginal pH, with a coincident decrease in oxidation-reduction potential seen during menses, may also enhance growth of anaerobic microorganisms like *T. vaginalis* (Spiegel, 1990).

T. vaginalis infection can lead to a number of serious complications in women. These include premature rupture of the placental membranes, premature labour, low birth weight infants, adnexitis, pyosalpinx, endometritis, atypical pelvic inflammatory disease (Heine and McGregor, 1993), cervical cancer (Wolner-Hanssen *et al.*, 1989; Viikki *et al.*, 2000), cervical erosion (Rein and Chapel, 1975) and infertility (Rein, 1990). It has also been suggested that infection with urogenital trichomonads can increase the risk of transmission of other STDs such as herpes simplex virus (HSV) (Pindak *et al.*, 1989) and human immunodeficiency virus (HIV) (Cameron and Padian, 1990; Laga *et al.*, 1993, 1994).

Clinical Picture of Urogenital Trichomoniasis in Men

Although male trichomoniasis has generally been considered asymptomatic, it has been concluded that thirty to fifty percent of all men with trichomonal infections show signs of mild symptomatic disease. These symptoms include urethral discharge, urethritis, and prostatitis (Krieger, 1995). The infection has been linked to infertility and a variety of other complications

and inflammatory diseases of the male genitourinary tract (Krieger, 1995).

It has not been determined with any certainty why the clinical picture of infection is so different in males and females. Zinc, which has been shown to be present in the prostatic fluid, may be cytotoxic to *T. vaginalis* and hence limit infection in men (Krieger and Rein, 1982). The reducing environment of the vagina may also contribute to the symptoms seen in women, via the activation of certain pathogenic mechanisms. The oxidative environment of the male genital tract may, on the other hand, inhibit such mechanisms (Alderete and Provenzano, 1997).

Detection and Diagnosis of *T. vaginalis* Infection

Diagnosis of trichomoniasis can be difficult due to the fact that many of the symptoms mimic those of candidiasis and bacterial vaginosis (Rein and Holmes, 1983) or of other STDs (Jirovec and Petru, 1968). Furthermore, some of the “hallmark” signs of *T. vaginalis* infection, like the “strawberry cervix”, are actually observed in only a minority of patients (Fouts and Kraus, 1980). Diagnosis should therefore be made based on clinical presentation and the positive identification of trichomonads in the urogenital tract via a laboratory investigation.

The traditional method of *T. vaginalis* detection has been the direct microscopic examination of motile trichomonads in vaginal secretions. This technique is simple and quick, but has been shown to have widely varying specificity, depending on the collection of the specimen, the amount collected, and the number of motile parasites present; characteristic *T. vaginalis* motility can be lost rapidly. The sensitivity of the wet mount preparation ranges from thirty-eight to eighty-two percent (McMillan, 1990) and the technique requires the presence of at least 10^4 organisms per mL to give positive results.

T. vaginalis can also be detected by microscopic observation of fixed specimens. A variety of staining techniques has been reviewed in the literature (see McMillan, 1990 for details and references). These techniques include Gram-, Papanicolaou-, Romanowsky-, Acridine orange-, silver, Diff-Quick, and Periodic acid-Schiff staining. While these procedures are quick and permanent, their diagnostic specificity and sensitivity is questionable, even when the slides are reviewed by a skilled technician (Lossick and Kent, 1991). One limiting factor to consider is the fact that the typical morphological characteristics of the trichomonads may be lost during staining and fixation (Bhatt *et al.*, 1996).

Having the ability to amplify the number of trichomonads from an original sample, the broth culture technique (with a high degree of sensitivity (ninety-two to ninety-five percent)) has been dubbed the “gold standard” for diagnosis of *T. vaginalis* infection (Bhatt *et al.*, 1996).

Interpretation is simple and only a few hundred trichomonads/mL are required to initiate growth (Garber *et al.*, 1987). The drawbacks to this technique include: elevated cost associated with the procedure, lack of media on hand in many labs, and a delay in diagnosis to allow for growth of the specimen (Bhatt *et al.*, 1996). Other researchers have used cell-culture technique to recover trichomonads from clinical samples. Garber *et al.* (1987) found that the cultivation of *T. vaginalis* in McCoy cell monolayers was extremely sensitive (detecting as few as three organisms per mL), but this method is not routinely used because of the associated inconvenience and higher cost.

More sophisticated methods for the diagnosis of *T. vaginalis* infection have been introduced, but few are in routine laboratory usage due to the fact that they are not always cost-effective. These *T. vaginalis*-detecting techniques include latex agglutination (Carney *et al.*, 1988), specific dot-enzyme immunoassays (Gombosova and Valent, 1990), direct immunofluorescence assays (Krieger *et al.*, 1988; McMillan, 1990), and monoclonal-based enzyme-linked immunoassay

(EIA) (Lisi *et al.*, 1988). Other diagnostic procedures rely on detection of anti-trichomonal antibodies (Street *et al.*, 1982; Sibau *et al.*, 1987) or trichomonal DNA (Rubino *et al.*, 1991; van Der Schee *et al.*, 1999) in a given sample.

Treatment of *T. vaginalis* Infection

Prior to 1959, people infected with *T. vaginalis* relied on local topical preparations to relieve some of the symptoms of trichomoniasis. Protracted infection and re-infection was commonplace, however, because topical treatments did not penetrate to all sites harbouring trichomonads, and were unsuitable for treatment of infected men (Lossick and Kent, 1991).

In the early 1960s, the treatment of trichomoniasis was radically altered with the introduction of metronidazole (α -hydroxyethyl-2-nitroimidazole), a 5-nitroimidazole derivative of a *Streptomyces* spp. antibiotic, azomycin. Metronidazole is the only nitroimidazole currently available in North America, but other structurally related nitroimidazoles with varying pharmacokinetic and therapeutic activities, including tinidazole, secnidazole, ornidazole and mebendazole, are available elsewhere (Rossignol *et al.*, 1984; Lau *et al.*, 1992; Mathisen and Finegold, 1992; Gillis and Wiseman, 1996).

A number of oral metronidazole treatment regimens for *T. vaginalis* infection have been described, and with proper compliance, most are equally effective in both males and females (Lossick, 1982; Lossick, 1990b). The Center for Disease Control (CDC) guidelines currently suggest that metronidazole be given orally in a single 2 g dose or 250 mg twice a day for seven days (CDC, 1998). Successful treatment of infection is observed in ninety to ninety-five percent of cases; simultaneous treatment of sexual partners increases the success rate (CDC, 1998).

Metronidazole enters the *T. vaginalis* cell by a process of passive diffusion. The drug, itself inactive, is then reduced anaerobically in the hydrogenosome by pyruvate-ferredoxin oxidoreductase to yield short-lived nitro radical intermediates. A concentration gradient is formed, allowing more metronidazole to enter the cell. The highly reactive intermediates damage the parasite's DNA (Ings *et al.*, 1974; Muller, 1981; Moreno *et al.*, 1984; Edwards, 1993). The cell responds rapidly with the cessation of cell division and motility; within eight hours, cell death occurs (Nielson, 1976).

Metronidazole treatment is curative in a large majority of cases, but occasionally, treatment failure can occur. A variety of explanations have been put forth to try to explain refractory cases of trichomoniasis. Once non-compliance and re-infection were eliminated, researchers proposed ineffective delivery of the drug to the urogenital area, inactivation of the drug by vaginal bacteria (McFadzean *et al.*, 1969; Ingham *et al.*, 1979; Edwards *et al.*, 1979), and incomplete drug absorption (Kane *et al.*, 1961) as possible explanations for treatment failure.

Finally, it was proposed that while these explanations were possible, the likely reason for treatment failure was that *T. vaginalis* isolates could possess true metronidazole resistance (Lumsden *et al.*, 1988). In 1989, the CDC estimated that five percent of all *T. vaginalis* isolates possessed metronidazole resistance of varying degrees (Narcisi and Secor, 1996). Resistance can be caused by a number of different mutational changes in the *T. vaginalis* genome and can affect both aerobic and anaerobic mechanisms of metabolism (Upcroft and Upcroft, 2001). In aerobic metronidazole resistance, the transcription of the ferredoxin gene is diminished, thereby hampering the cell's ability to activate the drug (Quon *et al.*, 1992). In examples of anaerobic resistance, the activities of pyruvate-ferredoxin oxidoreductase and hydrogenase are either impaired or absent (Kulda *et al.*, 1993).

Host Defense Mechanisms

There exists a complicated host defense system, which plays an important role in restricting a *T. vaginalis* infection to the genitourinary compartment. Prior exposure induces no real host protection against re-infection, despite the presence of an initial measurable host immune response (humoral and cellular) (Honigberg, 1986; Ackers, 1990). Why protection does not occur is largely unknown, but may involve induction of an inappropriate or low-grade response or may be related to immune evasion by the parasite itself.

The host defense network is comprised of three components: nonimmunologic factors (zinc concentration and iron availability), innate immune responses (complement, neutrophils, natural antibodies, and macrophages), and acquired immune responses (antibody production and cell-mediated immunity) (Landolfo *et al.*, 1980; Demes *et al.*, 1987; Ackers, 1990; Shaio and Lin, 1995).

While the specific host immune response remains the least well-defined aspect of *T. vaginalis* immunology, many researchers have documented the presence of circulating serum and local cervicovaginal antibodies (IgG, IgA, and IgM) to *T. vaginalis* (Mason, 1979; Su, 1982; Street *et al.*, 1982; Alderete, 1984; Cogne *et al.*, 1985; Sibau *et al.*, 1987 and Sharma *et al.*, 1991). There is limited evidence of what effect these antibodies may have on the parasite. According to Honigberg (1986), antibody titers in *T. vaginalis* infection progressively decline after eradication of the parasite by treatment. Within six to twelve months, all antibodies disappear from circulation, leaving the host with no specific immune defense. The existence of cell mediated immunity in response to *T. vaginalis* infection has also been documented, but its role remains largely undefined (Yano *et al.*, 1983; Mason and Patterson, 1985).

***Trichomonas vaginalis*- The Organism**

Taxonomy

The eukaryotic, aerotolerant anaerobic (Paget and Lloyd, 1990) protozoan parasite *T. vaginalis* was first identified by Donné (Donné, 1836). The taxonomic status of *T. vaginalis* can be described as: Subkingdom Protozoa, Phylum Sarcomastigophora, Subphylum Mastigophora, Class Zoomastigophorea, Superorder Parabasalidea, Order Trichomonadida, Family Trichomonadidae, and Subfamily Trichomonadinae (Schmidt and Roberts, 1981; Marquardt and Demaree, 1985; Honigberg, 1990).

Morphology

There have been a number of studies into *Trichomonas* structure and morphology. Both light and electron microscopic examinations of the organism have contributed to the current body of information. The shape of *Trichomonas vaginalis* tends to be variable depending upon whether the specimen is fixed or living. The method of fixation itself can influence the shape and size of the trichomonad (Honigberg and King, 1964). On average, *T. vaginalis* has been shown to be approximately 10 μm in length and 7 μm in width (Honigberg and King, 1964) (Figure 1a-d). Shape tends to be more uniform among trichomonads grown in non-living axenic culture than in those found in vaginal secretions. In axenic culture, they tend to be ovoid, ellipsoidal, or spheroidal (See Figure 1e). *In vivo*, however, *T. vaginalis* tends to be more ameboid with distinct pseudopodial extensions (Heath, 1981; Arroyo *et al.*, 1993) (Figure 1f).

Regardless of its shape, the *T. vaginalis* cell bears many interesting surface and internal features. One of the most striking features of the trichomonad is its collection of flagella (Figures 1a-f, 2a and 2b). Emerging from the anterior pole of the cell are the four anterior flagella which originate

Figure 1: Scanning Electron Micrographs Illustrating Surface Morphology of *T. vaginalis*

The anterior flagella (a.fl.) can be seen emerging from the periflagellar canal (p.c.) in both the dorsolateral (a) and dorsal (b-d) views of *T. vaginalis*. The recurrent flagellum (r.fl. in d) emerges from the periflagellar canal posterior and dorsal to the anterior ones. The wall of the canal is reinforced by the pelta (pe. in d). The margin of the undulating membrane (u.m. in b) is made up of the attached portion of the recurrent flagellum (inner component) and the outer accessory filament (ac.f.)(outer component) (d). (Reproduced, with permission, from Warton and Honigberg, 1979).

T. vaginalis grown to late logarithmic phase in suspension media possesses an elipsoidal, ovoid shape (e) while a transformation to an ameboid, “fried-egg” appearance can be observed in the same isolate after only five minutes of contact with vaginal epithelial cells (f). (Reproduced from Arroyo *et al.*, 1993 with permission of publisher).

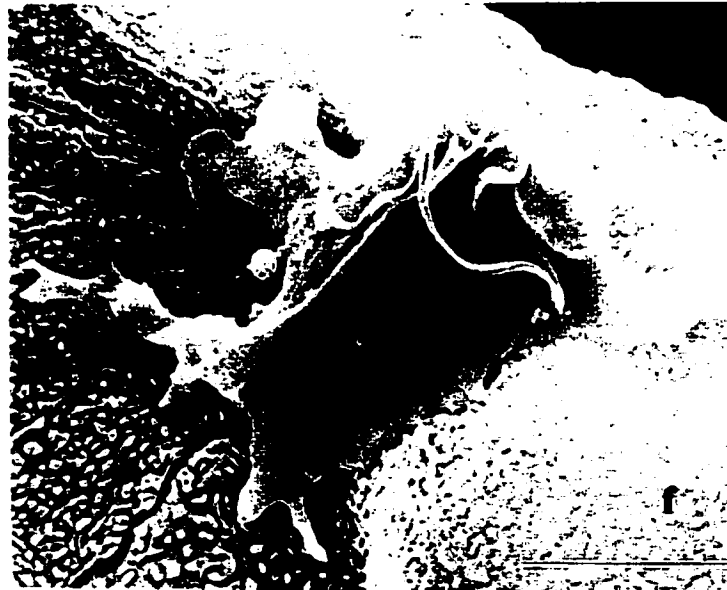
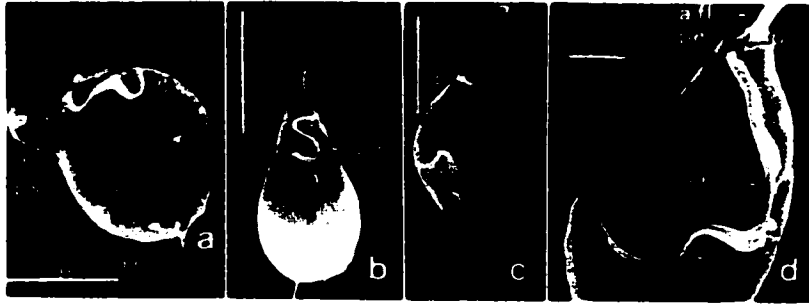
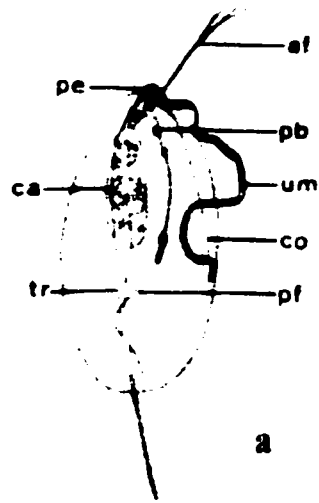


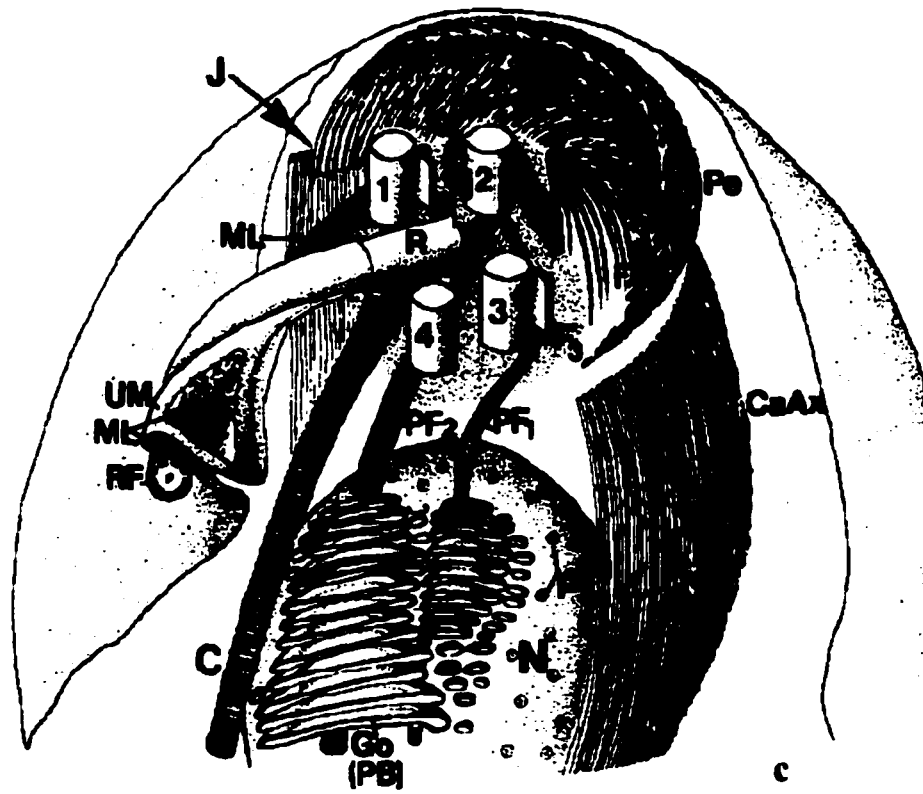
Figure 2: Diagrams of *T. vaginalis* Illustrate Important Internal and External Features

Left (a) and right (b) views of *T. vaginalis* highlight all the mastigont organelles: these include the anterior flagella (af), pelta (pe), V-shaped parabasal body (pb) with unequal arms, and parabasal filaments (pf), undulating membrane (um), along with the capitulum (ca), and trunk (tr) of the axostyle. (Reproduced, with permission, from Honigberg and Brugerolle, 1990).

(c) Schematic diagram of *T. vaginalis* karyomastigont system (including the nucleus). The kinetosomal complex, which includes the parallel kinetosomes (#1 to #4) of the anterior flagella and the kinetosome R of the recurrent flagellum (RF) can be seen clearly. The filaments or lamellae (F₁, F₂, F₃, and X) connect the kinetosomes with one another or with other organelles, and anchor the complex to various areas of the cytoplasm. The costa (C) supports the undulating membrane (UM) along the dorsal surface of the cell. The undulating membrane (UM) is composed of a dorsal fold of the cytoplasmic membrane (which encloses the marginal lamella (ML)) and of the attached recurrent flagellum (RF). The parabasal apparatus is made up of parabasal filaments (PF₁ and PF₂) and a parabasal body (PB) (two Golgi complexes (Go)). The peltar-axostylar consists of the pelta (Pe), which supports the wall of the periflagellar canal from which emerge the flagella, and of the axostyle. The pelta and axostyle are connected along the peltar-axostylar junction (J). The anterior portion of the axostyle, the capitulum (CaAx), can be seen here. Finally, the nucleus (N), with an envelope containing many pores (P), is apposed to the dorsal concave surface of the capitulum. (Reproduced, with permission, from Honigberg and Brugerolle, 1990).



10
μm



in the kinetosomal complex (Figure 2c). Slightly posterior and dorsal to where the anterior flagella originate, the kinetosomal complex gives rise to the undulating membrane and the supporting costa (Figure 2c). The undulating membrane, is made up of the outer accessory filament (an extension of the outer membrane and the cytoplasm of the organism) and a fifth flagellum (termed the recurrent flagellum). Together, they extend a portion of the way along the body of the trichomonad. The recurrent flagellum ends with the undulating membrane and does not continue beyond as a free posterior flagellum. *T. vaginalis* derives its characteristic corkscrew motility from the action of the flagella and the undulating membrane (Honigberg, 1978; Honigberg and Brugerolle, 1990).

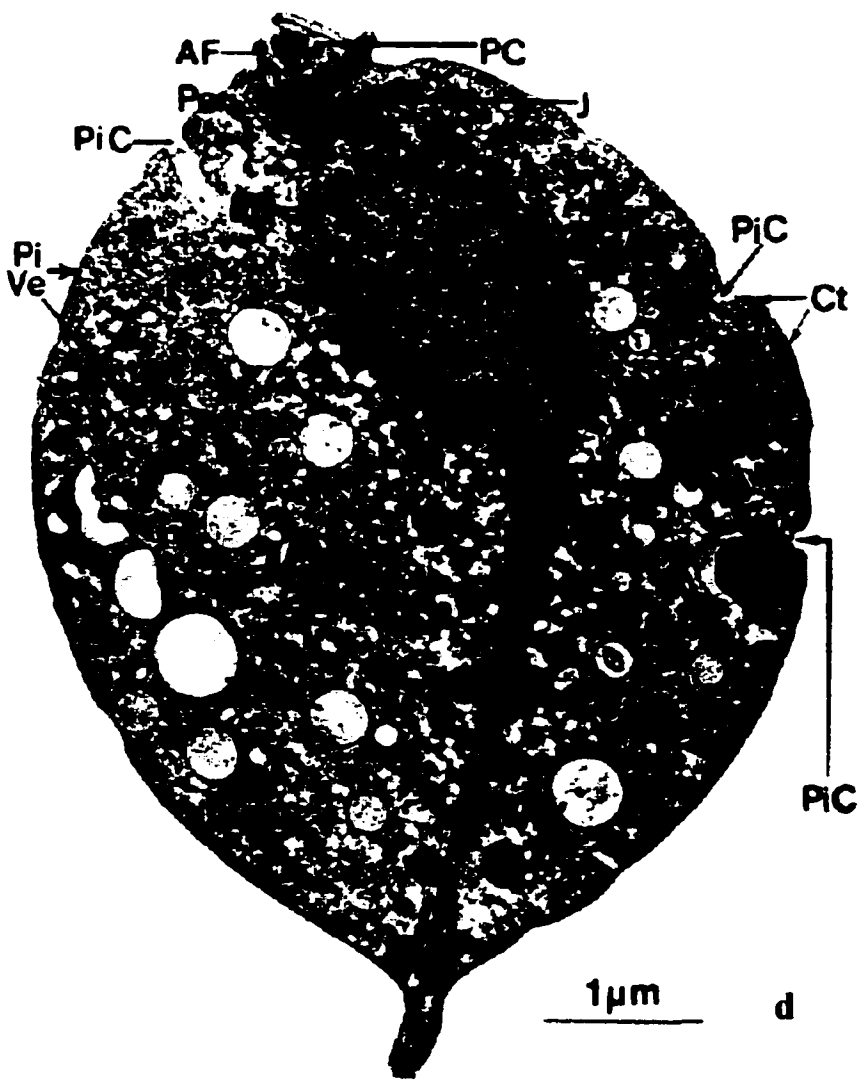
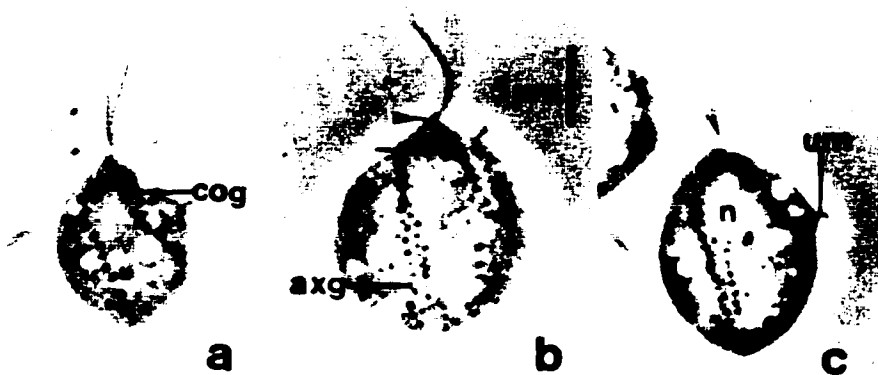
Located anteriorly, an elongated nucleus with dimensions of approximately 4 μm by 2 μm , can be found with the left ventral surface next to the spatulate axostylar capitulum. At its anterior end, the capitulum is connected to the pelta, a crescent-shaped structure that engulfs the area from which the anterior flagella protrude (Figure 2c). The capitulum continues, posterior to the nucleus, and narrows to give rise to a slender rod-like axostylar trunk down the centre of the cell. The axostyle protrudes through the posterior end of the cell body and eventually tapers to a point that is believed to play a role in anchoring the trichomonad to host tissues (Figure 3d). The nucleus is also surrounded by endoplasmic reticulum (Figure 3d). Situated dorsally and to the right of the nucleus, there can be found a parabasal apparatus. Typically V-shaped, it consists of a well-developed parabasal body (two Golgi complexes) and two parabasal filaments linking the apparatus to the kinetosomal complex. Each Golgi complex consists of stacks of cisternae surrounded by vesicles (Honigberg and Brugerolle, 1990) (Figure 2c).

One of the most consistent features of the *T. vaginalis* cell is the collection of granules termed the paracostal and paraxostylar granules (Figure 3a-d). The paracostal granules are found on either side of the filamentous costa, while the paraxostylar granules are located in three rows that

Figure 3: Light Microscopy and Transmission Electron Microscopy Illuminate Key Internal Structures in the *T. vaginalis* Cell

(a-c) Motile organisms under the light microscope show nuclei (n), anterior flagella, undulating membrane (um), as well as paraxostylar (axg) and paracostal (cog) granules or hydrogenosomes. Note the parallel rows of the paraxostylar granules, which constitute a diagnostic characteristic of *T. vaginalis*. The flagella emerge from the periflagellar canal (arrowhead in **b**). The region of the parabasal apparatus is also visible (small arrow in **b**). (Reproduced, with permission, from Honigberg and Brugerolle, 1990).

(d) A transmission electron micrograph of the organism. The anterior flagella (AF) originate from the periflagellar canal (PC), which is supported by the pelta (Pe). The nucleus (N) is lodged in the depression of the axostylar capitulum (CaAx). Endoplasmic reticulum (ER) can be found in the nuclear region. The axostylar capitulum narrows into the axostylar trunk (TrAx), which projects beyond the posterior surface of the cell. Hydrogenosomes (H) are lined up along the axostylar trunk, while vesicles (V) can be seen throughout the cytoplasm. The cell surface is marked with depressions leading to pinocytotic canals (PiC). Note the cell coat (Ct) visible along the right side of the cell. Pinocytotic vesicles (PiVe) can be found in the cytoplasm. (Reproduced, with permission, from Honigberg and Brugerolle, 1990).



parallel the axostyle, from its anterior originating point to the posterior area where it emerges from the cell body. These granules can be seen in living trichomonads and in many stained preparations. Most of the paraxostylar and paracostal granules are in fact electron-dense hydrogenosomes, which have been shown to play a vital role in cell metabolism (Honigberg and King, 1964; Honigberg, 1978; Honigberg and Brugerolle, 1990). The hydrogenosome is a double membrane-bound organelle; unlike mitochondria, the inner membrane of the hydrogenosome does not form cristae (Muller, 1988). Being a primitive eukaryotic organism, *T. vaginalis* lacks mitochondria and it is the function of the hydrogenosomes to carry out analogous metabolic functions (Muller, 1993). Studies have shown that the major role of these organelles is the anaerobic conversion of pyruvate to acetate, malate, CO₂, and H₂. This process is accompanied by the substrate-level phosphorylation of ADP to ATP (Muller, 1988).

The trichomonad cell membrane is covered with a thin cell coat. Structural and metabolic studies have indicated that endocytosis encompasses both phagocytosis and pinocytosis. Throughout the cytoplasm there can be found food vacuoles of varying sizes. Pinocytic canals and pinocytic vesicles can be seen at or near the trichomonad cell surface (Honigberg and Brugerolle, 1990) (Figure 3d).

Reproduction and Life Cycle

Like many other protozoan parasites, *T. vaginalis* exists only in the trophozoite stage and lacks a true cystic stage (Honigberg and Brugerolle, 1990). When faced with unfavourable environmental conditions, trichomonads may round up and internalize their locomotor organelles, forming what have been described by some as “pseudocysts”. Since these forms have not been found to give rise to normal motile trophozoites, however, they may represent a degenerate form of the organism (Honigberg and Brugerolle, 1990).

As in all trichomonads, reproduction in *T. vaginalis* occurs via a process termed cryptopleuromitotic division. This process involves longitudinal binary fission without the concomitant disappearance of the nuclear membrane (Brugerolle, 1975). The process begins with the appearance of a duplicate kinetosome and flagellum as well as the development of two attractophores (described as “bell-clapper shaped” organelles) which give rise to the poles of division. Originating from these attractophores, microtubules join to form one or more bundles. The extranuclear spindle created by these bundles is termed the paradesmose. Chromosomal microtubules, also originating from the attractophores, attach to the nuclear chromosomal kinetochores or centromeres. Elongation of the extranuclear spindle leads to the separation and migration of the daughter mastigont systems. During telophase, two daughter nuclei are formed. Once the daughter cells have separated, each produces the necessary organelles to replace those lost in the division process (Brugerolle, 1975; Honigberg and Brugerolle, 1990).

Nutritional Requirements and Cultivation

T. vaginalis is considered to be an aerotolerant anaerobe (Paget and Lloyd, 1990) that has become optimally adapted to survive in the vaginal milieu where high levels of CO₂ and traces of O₂ are the norm. *T. vaginalis* is also an obligate parasite that must glean many necessary nutrients from its surroundings; it lacks the ability to synthesize many of these macromolecules, acquiring them instead from vaginal secretions (Huggins and Preti, 1981) or via the ingestion of bacterial and host cells (Francioli *et al.*, 1983; Street *et al.*, 1984). Given the paucity of *de novo* synthesis of such essential nutrients as carbohydrates (ter Kuile, 1994; ter Kuile and Muller, 1995), amino acids (Rowe and Lowe, 1986; Zuo *et al.*, 1995), fatty acids (Roitman *et al.*, 1978), vitamins (Hollander and Leggett, 1985), iron (Gorrell, 1985), pyrimidines and purines (Heyworth *et al.*, 1982; Wang and Cheng, 1984; Harris *et al.*, 1988; Wang, 1990), appropriate *in vivo* culture media must therefore incorporate all these factors.

In keeping with this, Diamond (1986) outlined the essential components of complex liquid media for the axenic cultivation of trichomonads: (1) peptones (combinations of peptides and amino acids), (2) liver or yeast extracts which provide a source of B vitamins, purines and pyrimidines, (3) maltose or glucose which act as the main source of fermentable carbohydrates, (4) buffer, (5) reducing compounds, (6) agar to aid in the maintenance of low redox potential, (7) serum providing essential lipids and fatty acids, and (8) trace metals. Addition of an iron supplement, in particular, favours trichomonad growth. In axenic culture, *T. vaginalis* will grow in a relatively wide range of pH, but tends to flourish at a pH of 6.0 to 6.3 (Diamond, 1986).

Strain Heterogeneity

Much research has been conducted in the area of antigenic differences among isolates of *T. vaginalis*. Given that many of these studies focused on the development of serologic tests for trichomoniasis, antigenically defined populations of *T. vaginalis* have come to be referred to as serotypes (Ackers, 1990). It has been estimated that there exist between two and eight different serotypes (Honigberg, 1978; Garber *et al.*, 1986). These distinct "antigenic types" were demonstrated by precipitation, agglutination, complement fixation, and hemagglutination tests. Honigberg (1978) proposed that, for the most part, the number of antigenic types discovered tends to correlate with the number of strains examined.

A number of studies (Alderete *et al.*, 1985, 1986a, 1986b; Garber *et al.*, 1986) have indicated that the antigenic heterogeneity observed in *T. vaginalis* is a product of the surface location of a group of high molecular weight proteins, a number of which have been identified and analyzed. Interestingly, they also found that all isolates possess the capability to synthesize the same complement of highly immunogenic proteins; it is their deposition onto the cell surface that separates the strains.

***T. vaginalis* Pathogenesis**

Even though *T. vaginalis* has become one of the most intensely-studied members of the trichomonad family and gives rise to a common STD, the pathogenic mechanisms whereby the protozoan invades the human urogenital tract, establishes residency there and induces pathologic effects are largely undefined. Given the wide gamut of clinical symptoms seen in trichomoniasis sufferers, it is unlikely that any single mechanism could be responsible. More likely, there are many mechanisms in the pathogenic arsenal of the parasite. Research to date suggests that these mechanisms include (1) the ability to evade host immune responses, (2) cellular adhesion, (3) haemolysis, (4) the excretion of soluble factors like extracellular proteinases and cell detaching factor (CDF), and (5) interaction with the normal urogenital flora.

Immune Evasion

T. vaginalis has developed specialized mechanisms that allow it to survive and flourish despite the best efforts of the host immune system. The ability of *T. vaginalis* to evade destruction by the host immune system and to survive in such a hostile and variable environment is an important element of its pathogenesis.

One way in which *T. vaginalis* manages to evade the host immune response is via the degradation of host antibodies. It has been documented that all trichomonal isolates synthesize numerous cysteine proteinases of varying molecular weights (Coombs and North, 1983; Lockwood *et al.*, 1987; Neal and Alderete, 1990; Alderete *et al.*, 1991). Provenzano and Alderete (1995) demonstrated that cysteine proteinases produced by *T. vaginalis* possess the ability to degrade human IgG, IgM, and IgA. They were able to detect immunoglobulin-degrading proteinases in the vaginal washes of patients with trichomoniasis, and went on to confirm proteolytic activity against immunoglobulin in the vaginal washes, suggesting *in vivo* importance. The production of

antibody-degrading proteinases by *T. vaginalis* appears to be constitutive and is independent of vaginal iron status. This may be particularly advantageous since it allows for parasite resistance to antibody regardless of the host immune status at the time of infection (Provenzano and Alderete, 1995).

Another important means of immune evasion is in the ability of *T. vaginalis* to coat itself with host plasma proteins, rendering the protozoan virtually invisible to the host immune response (Peterson and Alderete, 1982). Various assays and experiments have indicated that these proteins are bound, some loosely, others avidly, to the trichomonad surface via specific membrane sites (Peterson and Alderete, 1982). Specific and non-specific immune responses could be effectively blocked if the parasite is camouflaged in host plasma proteins.

Alderete and Garza (1984) have suggested that *T. vaginalis* may also elude the host immune response through the secretion of highly immunogenic antigens. They reasoned that the continuous release of these membrane protein antigens might neutralize the host's anti-*T. vaginalis* immune response by blocking antibody or cytotoxic lymphocyte receptors and hence interfering with specific antibody or cell-mediated killing.

Rendon-Maldonado *et al.* (1998) used transmission and scanning electron microscopy to show that *T. vaginalis* has the ability to internalize and degrade host leukocytes. Researchers found that both strains studied (one highly virulent, the other moderately virulent) were capable of internalizing the host cells, with the process occurring more rapidly in the more virulent strain. This may provide *T. vaginalis* with both an efficient means of nutrient acquisition as well as a way of counter-attacking the host immune response.

Another method of trichomonal immune evasion lies in the organism's capacity for complement avoidance. It has been well documented that *T. vaginalis* activates the alternative complement pathway (Gillin and Sher, 1981). Research has shown, however, that fresh isolates differ with respect to their sensitivity to complement-mediated killing (Demes *et al.*, 1988). Furthermore, it was demonstrated that during most of the menstrual cycle, *T. vaginalis* encounters very little complement activity. Demes *et al.* (1988) found an almost total lack of complement activity in cervical mucus samples studied. They also noted that while complement activity does increase with the onset of menses, menstrual blood possesses considerably less complement activity than does serum. Therefore, it appears that *T. vaginalis* colonizes a ecological niche where complement is not a real factor until the onset of menses, at which point, the trichomonad implements an iron-mediated system which protects it from complement-mediated killing. With increasing levels of iron in the menstrual blood, *T. vaginalis* upregulates synthesis of proteinases which are capable of degrading the C3 portion of host complement. This helps to prevent complement-mediated killing of the parasite and thus allows persistence of infection (Alderete *et al.*, 1995c).

These tactics further demonstrate how *T. vaginalis* is able to elegantly respond to changing environmental factors in its bid to survive in the vaginal environment. They also reinforce the genuine complexity of the parasite-host relationship.

Surface Adhesins and Adherence to Host Cells

The surface of a given *T. vaginalis* cell plays a vital role in its interaction with the surrounding environment. On the surface of the cell can be found a myriad of proteins and other molecules, some of which have been shown to play a part in the parasite's adherence to epithelial cells in the human vagina. It has been reported by a number of researchers (Heath, 1981; Alderete and

Pearlman, 1984; Krieger *et al.*, 1985; Graves and Gardner, 1993; Alderete *et al.*, 1995a) that cytoadherence of the trichomonad to host epithelial cells is essential for the effective colonization and persistence of the pathogen. Alderete and Garza (1985) described the parasite-epithelial cell association as time, temperature and pH-dependent in nature. They also found that *T. vaginalis* adhered with more avidity and possessed a higher degree of cytotoxicity to epithelial cell lines than they did to fibroblast cell lines. This finding is noteworthy because it parallels the situation seen *in vivo* where virulent trichomonads predominantly parasitize the vaginal epithelium.

According to Alderete *et al.* (1995a), cytoadherence, in its simplest form, involves the interaction of molecules on the surface of the microbe (adhesins) with specific molecules on the host cell surface (receptors). Trichomonal adherence is ligand-receptor in nature (Alderete and Garza, 1988) and depends upon four distinct surface proteins. These proteins have been designated with names reflecting their respective molecular masses: AP65, AP51, AP33, and AP23 (Arroyo *et al.*, 1992; Alderete *et al.*, 1995b; Engbring *et al.*, 1996). Anti-adhesin antibodies have been shown to specifically recognize and bind to their respective protein. Further, these antibodies inhibited the binding of live trichomonads to epithelial cells and protected the host cells from contact-dependent cytotoxicity (Arroyo *et al.*, 1992). The same study showed that pretreatment of epithelial cells with purified adhesins had a similar inhibitory effect on adherence.

Gene expression of the four adhesins has been shown to be regulated in a coordinated fashion at the transcriptional level. Lecker *et al.* (1991) and Ryu *et al.* (2001) have suggested that this regulation is, at least in part, under the control of environmental iron levels. Alderete *et al.* (1995b) estimate that such regulation may be a mechanism by which *T. vaginalis* adapts to the constantly changing environmental conditions found in the vaginal compartment.

Downregulation of adhesins in response to a limited exogenous supply of iron may allow trichomonads to migrate more freely toward sites that are richer in iron. This would allow the

parasite to persist despite the flushing action of the mucosal secretions, desquamation of the mucosal epithelium, and a general shortage of nutrients (Alderete *et al.*, 1995a).

Surface adhesins are very sensitive to proteinases (Alderete and Garza, 1988; Arroyo *et al.*, 1992), yet strangely enough, cysteine-proteinase activity is a prerequisite for effective cytoadherence (Arroyo and Alderete, 1989; Arroyo and Alderete, 1995; Alvarez-Sanchez, 2000; Mendoza-Lopez *et al.*, 2000). The exact nature and functions of the surface proteinases are unknown, but it has been proposed that they may carry out some unmasking function. Alderete *et al.* (1995a) suggest that they contribute to adherence by degrading the proteins which protect the adhesins on the parasite surface.

While the exact details have not been completely elucidated, it appears that there exists a sophisticated signal transduction system in the *T. vaginalis* cell. The system initiates a number of changes in the parasite as it adheres to a host cell, the first being a transformation in the shape of the parasite. Arroyo *et al.* (1993) found that the typical ellipsoidal “pear-shape” (with a rough surface, four anterior flagella, a lateral undulating membrane and posterior axostyle) of the *in vitro*-grown *T. vaginalis* (Figure 1e) gave way rapidly, after contact with a vaginal epithelial cell, to a thin, flat amoeboid shape (with a smooth surface) (Figure 1f). The amoeboid shape seemed to allow the parasite to maximize the area of adhesion to the surface of the target cell. These “amoebic” parasites formed pseudopodia and filopodia that were able to interdigitate at specific sites on the plasma membrane of the vaginal epithelial cell. The trichomonads attached to the target cell in such an orientation that the side opposite the undulating membrane always faced the host cell, with the flagella and undulating membrane remaining free (Alderete and Garza, 1985). Once the morphological transformation had taken place, however, the axostyle was no longer apparent (Arroyo *et al.*, 1993). Rasmussen *et al.* (1986) demonstrated that once the morphological transformation occurred, the amoeboid *T. vaginalis* were found to contain a dense

network of microfilaments in the area of the parasite in contact with the target epithelial cell. Krieger *et al.* (1985) supported the notion that the microfilaments concentrated at the site of contact played a role in the adherence to and parasitism of the target cell, in that inhibitors of microfilament and microtubule assembly dramatically reduced the cytopathic effects of trichomonads on Chinese Hamster Ovary (CHO) cell monolayers.

The second contact-dependent alteration was increased synthesis of the four surface adhesin proteins (AP65, AP51, AP33, and AP23), possibly strengthening attachment to the target cell, and thus allowing for more efficient parasitism of the target cell. Furthermore, the contact-initiated enhancement of surface adhesin synthesis may allow for the translocation of adhesins to the expanding filopodia and pseudopodia as they attach to newly-recognized contact sites on the target cell (Arroyo *et al.*, 1993). It was noted that the morphological transformation to an ameboid form occurred in response to contact with vaginal epithelial cells (the *in vivo* target cell), but not in response to contact with HeLa epithelial cells. The increase in adhesin synthesis, thought to result from a signal separate from the signal to change shape (Arroyo *et al.*, 1992), however, occurs in response to contact with either vaginal or HeLa epithelial cells (Arroyo *et al.*, 1993). The fact that increased adhesin synthesis occurs as a result of contact with HeLa epithelial cells may suggest that it is the interaction itself, between the adhesins and their receptors, which provides the signal for synthesis upregulation (Arroyo *et al.*, 1992, 1993).

Arroyo *et al.* (1993) also showed that while a given vaginal epithelial cell was originally parasitized by just one parasite, it was quickly covered by numerous trichomonads with multiple membrane interdigitations adjacent to one another. Alderete *et al.* (1995a), in reference to this study, have suggested that perhaps the original adherent trichomonad recruits other parasites to the target cell via the generation of a chemoattractant type of signal.

Other surface molecules have been implicated in the adherence mechanisms of *T. vaginalis*. Silva Filho *et al.* (1988) report that *T. vaginalis* displays, on its surface, laminin-binding proteins which may aid in attachment to target cells. These receptors are thought to recognize the glycoprotein laminin, which is located in the basement membranes of the urogenital tract and is believed to promote cellular adhesion, differentiation, shape, and motility (Silva Filho *et al.*, 1988). Crouch and Alderete (1999) have suggested that *T. vaginalis* may persist at sites in the vaginal compartment beneath the epithelial surface via interaction with laminin and fibronectin. They hypothesize that this may explain the non-self-limiting nature of trichomoniasis in the face of exfoliating vaginal epithelial cells from the vaginal epithelium. They have noted that receptors on the trichomonad surface interact with both laminin and fibronectin, and propose that these are independent of the four adhesin proteins.

In vitro pathological studies have shown that *T. vaginalis* possesses the ability to destroy monolayers and does so via an ordered process of events (Hogue, 1943; Christian *et al.*, 1963; Farris and Honigberg, 1970; Heath, 1981; Alderete and Pearlman, 1984; Alderete and Garza, 1985; Silva Filho and de Souza, 1988). Essentially, when first introduced into a co-culture with monolayer cells, trichomonads swim freely in the growth media, and eventually settle onto the target cells. Once *T. vaginalis* establishes direct contact with the target/host cells, the pathological effects can be observed. Small holes or “plaques” in the monolayer can be seen as the monolayer cells detach from areas where the trichomonads have adhered (the timing of this process varies depending on the target cell type, trichomonad virulence and concentration, and other culture conditions). As the parasites infiltrate more of the monolayer, these plaques grow in size until eventually the entire monolayer detaches. These plaques may have the same etiology as the hemorrhagic lesions seen in the cervix and vaginal mucosa in severe trichomoniasis (Christian *et al.*, 1963).

A number of researchers have assessed the viability of detached monolayer cells following contact with *T. vaginalis*, and have shown that irreversible damage and death of the target cell is the norm (Hogue, 1943; Heath, 1981; Alderete and Pearlman, 1984; Krieger *et al.*, 1985; Rasmussen *et al.*, 1986). For example, Alderete and Pearlman (1984) demonstrated that exposure of monolayer cultures of human urogenital and vaginal, human epithelial, normal baboon testicular, and monkey kidney cells to *T. vaginalis* resulted in disruption of monolayers. Via Trypan blue exclusion and ³H-thymidine release they also showed that irreversible damage to the cells comprising these monolayers took place. Mirhaghani and Warton (1996) showed, with electron microscopic analysis, that direct contact between trichomonads and amnion membrane epithelial cells resulted in the destruction of the target cells, and eventually led to the desquamation of the epithelial layer from the basement membrane.

Haemolysis

It has been well documented that *T. vaginalis* possesses the ability to bind and lyse human erythrocytes (Krieger *et al.*, 1983; Dailey *et al.*, 1990; Lehker *et al.*, 1991; Fiori *et al.*, 1993). Furthermore, it has been shown that this β -haemolytic activity is correlated with virulence in patients, animal models, and tissue culture (Krieger *et al.*, 1983). The clinical observation that trichomoniasis is frequently exacerbated during menses may be due to increased substrate and therefore enhanced levels of haemolytic activity (Krieger *et al.*, 1983).

Given the fact that *T. vaginalis* does not possess the ability to synthesize, *de novo*, a number of molecules necessary for survival, the parasite must obtain these nutrients from exogenous sources. Erythrocytes and other host cells may therefore become an attractive source of nutrients for the trichomonad. *T. vaginalis* lacks the necessary enzymatic machinery for the biosynthesis of lipids (Peterson and Alderete, 1984; Beach *et al.*, 1990, 1991), so the β -haemolytic activity of

trichomonads may be a very important means of nutrient acquisition. Haemolysis of erythrocytes also provides *T. vaginalis* with a supply of iron, which is integral to its growth and multiplication (Lehker *et al.*, 1990; Lehker and Alderete, 1992; Fiori *et al.*, 1993) and is a limiting factor in the vaginal environment.

Haemolysis is a contact-dependent process. Fiori *et al.* (1993) demonstrated that haemolysis occurs when trichomonads are co-cultured with target cells, but not when they are separated by a semi-permeable membrane. The process is also sensitive to temperature and pH. Dailey *et al.* (1990) showed that haemolysis is optimal at 37°C and at a level of acidity reflecting the pH range (5.0-6.0) found in the vagina during trichomoniasis.

Erythrocyte lysis is mediated by protein receptors found on the surface of both the red blood cells and the trichomonads. Fiori *et al.* (1993) identified five trichomonad surface adhesins that facilitate the binding of the *T. vaginalis* cell to the host erythrocyte. Molecular mass determination has shown that three of the five polypeptides are identical to the adhesins found by Arroyo *et al.* (1992) (AP65, AP51, and AP33). The two other surface proteins defined by Fiori *et al.* (1993) possessed molecular masses of 140 and 42 kDa. On the basis of this work, Fiori *et al.* (1993) have divided the haemolytic process into at least three distinct steps. Firstly, the trichomonad recognizes and adheres to the target erythrocyte via the adhesins described previously. Secondly, the parasite releases toxic molecules that are believed to form functional pores in the target membrane. It has been suggested that these toxic molecules are soluble perforin-like proteins (Fiori *et al.*, 1993). While in contact with the target erythrocyte, *T. vaginalis* has also been shown to cause disruption of the target cell cytoskeleton via the degradation of the molecule spectrin. It is believed that when the parasite is in close intimate contact with the erythrocyte, it transfers a 30 kDa proteinase effector into the target cell, which then suffers cytoskeletal disruption (Fiori *et al.*, 1997). (It has been suggested that the same type

of proteinase could be used by *T. vaginalis* to induce cytoskeletal disruption in nucleated target cells as well. This would leave the host cell membrane more vulnerable to lysis by perforin proteins released by the trichomonad (Fiori *et al.*, 1999). Finally, the *T. vaginalis* cell detaches from the erythrocyte prior to target cell lysis (Fiori *et al.*, 1993). Apolipoprotein CIII-specific receptors on the trichomonad surface enable it to bind and incorporate lipids from the lysed erythrocyte (Peterson and Alderete, 1984). As well, *T. vaginalis* has specific surface receptors that allow it to bind and internalize iron-containing molecules like haemoglobin (Lehker *et al.*, 1990).

Proteinases

T. vaginalis has the ability to produce a variety of different proteinases (Coombs and North, 1983; Lockwood *et al.*, 1987). It has been shown that *T. vaginalis* lysates contain proteinases and that the parasite secretes, (both *in vitro* and *in vivo*), a variety of proteinases (Alderete *et al.*, 1991). Neale and Alderete (1990) identified twenty-three distinct proteinase activities in their evaluation of fresh and cultured *T. vaginalis* isolates. They demonstrated that some proteinases were differentially expressed and underwent phase variation, suggesting that they were regulated by environmental factors.

It seems reasonable that some of the proteinases produced by *T. vaginalis* play a role in the pathogenesis of the organism. In order for cysteine proteinases to be functional, they must first be activated by reducing agents. Alderete and Provenzano (1997) have shown that the human vagina possesses a reducing environment sufficient for the activation of *T. vaginalis* proteinases, supporting the hypothesis that they play a role *in vivo*.

It has been established that while the pH of vaginal secretions in healthy individuals is less than or equal to 4.5 (Paavonen, 1983; Larsen, 1993), a rise in pH to greater than 5.0 correlates with *T. vaginalis* infection (Hanna *et al.*, 1985; Rein, 1990). The proteinases characterized by Garber and Lemchuk-Favel (1989) were found to be active over a wide pH range (4.0-8.0), suggesting a potential role for proteinases in the establishment of infection. As has been discussed in the preceding sections, cysteine proteinases appear to be involved in different aspects of *T. vaginalis* pathogenesis, including adherence to host cells (Arroyo and Alderete, 1989; Arroyo and Alderete, 1995; Alvarez-Sanchez, 2000; Mendoza-Lopez *et al.*, 2000), cytoskeletal disruption in erythrocytes (Fiori *et al.*, 1997) and in nucleated target cells (Fiori *et al.*, 1999), and immune evasion via immunoglobulin degradation (Provenzano and Alderete, 1995). Lecker and Sweeney (1999) have demonstrated another interesting pathogenic role played by *T. vaginalis* cysteine proteinases. They showed that secreted trichomonad proteinases were able to degrade mucin, the framework molecule of mucus, which helps to form a physical barrier to microbial colonization in the human urogenital tract.

Cell Detaching Factor (CDF)

Hogue (1943) first proposed that *T. vaginalis* may produce soluble cytotoxic mediators *in vitro* via a contact-independent mechanism when she reported that cell-free filtrates caused severe pathology in tissue culture. The issue has been debated ever since, but evidence is accumulating in support of this hypothesis (Farris and Honigberg, 1970; Pindak *et al.*, 1986; Silva Filho and de Souza, 1988; Garber *et al.*, 1989; Garber and Lemchuk-Favel, 1990; Pindak *et al.*, 1993). Heath (1981) suggested that *T. vaginalis* likely employs both contact-mediated and contact-independent pathogenic mechanisms. In the *in vivo* setting, the importance of contact-mediated pathogenesis is readily apparent. Adhesion of trichomonads to the vaginal epithelium is important in the disruption of the superficial layers of the squamous epithelium. Heath (1981) observed, however,

that many infected women display a notable increase in the sub-epithelial vascularity of the cervicovaginal walls which is frequently out of proportion to the numbers and location of trichomonads adhering to the epithelium. This idea is supported by the findings of Nielson and Nielson (1975) who employed electron microscopy to look at biopsied vaginal epithelial cells from patients with trichomoniasis. These findings may suggest that *T. vaginalis* is able to exert pathogenic effects on host cells without actually coming into contact with them.

A number of researchers have examined contact-independent *T. vaginalis* cytotoxicity and have verified the original findings. Many of these studies give direct evidence showing cytopathic effects in culture cells in response to incubation with cell-free filtrates of *T. vaginalis* (Farris and Honigberg, 1970; Pindak *et al.*, 1986; Silva Filho and de Souza, 1988; Garber *et al.*, 1989; Garber and Lemchuk-Favel, 1990; Pindak *et al.*, 1993). Others have used a semi-permeable filter system which prevents contact between the trichomonads and the culture cells, but permits passage of media. Garber and Bowie (1990) used this system and concluded that trichomonad pathology still occurs in the absence of contact.

Pindak *et al.* (1986) first designated the trypsin-like component of cell free filtrate, which caused rounding and detachment of a number of different types of monolayer cells, as cell detaching factor (CDF). CDF was also shown to prevent attachment of fresh culture cells to the culture plate. It has been demonstrated that although the monolayer cells become detached, they remain viable for an extended period of time, and are even capable of re-forming a monolayer if washed and transferred to fresh media (Pindak *et al.*, 1986; Garber *et al.*, 1989).

The role of CDF in pathogenesis is unknown, but the *in vitro* detaching of monolayer cells may mirror the *in vivo* observation that patients with trichomoniasis often display sloughing of the vaginal epithelial cells (Garber *et al.*, 1989). Garber and Lemchuk-Favel (1990) have concluded

that CDF activity correlates strongly with clinical disease and thus may be an important marker of virulence in *T. vaginalis*. Cell-free filtrates of *Pentatrichomonas hominis*, a non-pathogenic gastrointestinal trichomonad, do not display any CDF activity, also suggesting that this component is relevant to an isolate's virulence mechanism (Garber *et al.*, 1989).

CDF has been identified as a metabolic product of *T. vaginalis*; lysate of the parasite is inactive, suggesting that the factor is not an internal component (Pindak *et al.*, 1986). It is a heat and acid labile 200 kDa glycoprotein (Pindak *et al.*, 1986; Garber *et al.*, 1989). Garber *et al.* (1989) found that the amount of CDF in growth filtrate varied with duration of trichomonad growth, the initial inoculum of *T. vaginalis*, and the pH of the filtrate at the time of harvesting. Research has indicated that, due to its acid lability, CDF activity is influenced by the pH of the surrounding media (Pindak *et al.*, 1986; Garber *et al.*, 1989). Garber *et al.* (1989) have found that CDF is active from pH 5.0 to pH 8.5, with peak activity at 6.5. Despite the fact that low pH (<5.0) causes the inactivation of CDF, the compound may still play a role in the *in vivo* setting. While the normal pH of the healthy vaginal compartment stays at or below 4.5, the pH rises to greater than 5.0 with *T. vaginalis* infection. Thus, once an infection has been established, the altered vaginal pH becomes permissive for the functioning of CDF (Garber *et al.*, 1989). CDF activity may also be affected by vaginal estrogen concentration. Garber *et al.* (1991) found that while estrogen has no real effect on *T. vaginalis* growth or on the activity of CDF, it does inhibit the production of CDF by the parasite. The diminished levels of estrogen found in the vaginal environment around the time of menses may allow for augmented production of CDF, leading to the exacerbation of the disease (Garber *et al.*, 1989).

These reports suggest that contact-independent factors likely play a part in the *T. vaginalis* pathogenic repertoire. A number of researchers question the importance of contact-independent mechanisms, however, and have reported a lack of such pathology in their experiments (Kotcher

and Hoogasian, 1957; Christian *et al.*, 1963; Kulda, 1967; Alderete and Pearlman, 1984; Krieger *et al.*, 1985; Rasmussen *et al.*, 1986). Given that CDF displays sensitivity to acid concentration, some (Pindak *et al.*, 1986; Garber *et al.*, 1989; Garber and Bowie, 1990; Pindak *et al.*, 1993) have suggested that perhaps *T. vaginalis* metabolic end-products interfered with these experiments by lowering the pH to levels (<5.0) that rendered CDF inactive. Garber *et al.* (1989) showed that monolayer cells die rapidly when exposed to pH levels below 5.0 which is in contrast to the effects induced by CDF (i.e. rounding and detachment, but not cell death). Garber and Bowie (1990) acknowledged that contact-dependent mechanisms play an important role in trichomonad pathogenesis, but suggested that some of the contact-dependent cytotoxicity seen in previous research could be attributed to low pH levels. These same low pH levels would prevent contact-independent cytotoxicity by inactivating CDF. Support for this idea came when, using a filter system to keep the cells separate, Garber and Bowie (1990) rigidly controlled the pH in a co-culture of *T. vaginalis* and McCoy cells. They found that McCoy cells detached but remained viable. When the cells were cultured in contact with one another, monolayer cells did die eventually, but pH adjustment led to a decreased rate of target cell death (Garber and Bowie, 1990).

Interaction with Vaginal Flora

The vaginal flora of a healthy woman consists of a wide variety of anaerobic and aerobic bacterial genera and species, but the most dominant is the facultative, microaerophilic, anaerobic genus *Lactobacillus* (Redondo-Lopez *et al.*, 1990). It has been shown that lactobacilli possess many properties and produce a number of metabolites that are important in the maintenance of the normal vaginal environment (Redondo-Lopez *et al.*, 1990). Another hallmark of a healthy human vaginal environment is an acidic pH (4.5 or less). However, once the cervicovaginal region is colonized with *T. vaginalis*, there is almost always a rise in the pH to levels where the parasite

can thrive and multiply more easily (pH of 5.0 or greater) (Hanna *et al.*, 1985; Rein, 1990).

Another pathological change associated with *T. vaginalis* infection is the reduction in numbers or even total loss of resident lactobacilli (Jirovec and Petru, 1968). How *T. vaginalis* eliminates the resident lactobacilli is not well understood, but *in vitro* studies have demonstrated that *T.*

vaginalis does have a deleterious effect on *L. acidophilus* in co-cultures (McGrory *et al.*, 1994).

While the exact mechanisms have not been elucidated, *T. vaginalis* has been shown to phagocytize lactobacilli *in vitro* (Juliano *et al.*, 1991; Rendon-Maldonado *et al.*, 1998). Similarly, trichomonad-secreted metabolites (such as CDF or proteinases) have been shown to inhibit lactobacilli growth *in vitro* (McGrory and Garber, 1992; McGrory *et al.*, 1994). Either or both of these mechanisms may occur *in vivo*. They both lead to the removal of a factor that helps to maintain a pH that is unhealthy for the colonizing trichomonads, and phagocytosis may provide a source of nutrition for the parasite.

Apoptosis-A Potential Pathogenic Mechanism Employed by *T. vaginalis*?

The apoptotic process is known to serve many vital functions, such as cellular deletion during embryonic development, balancing mitosis in continuously regenerating tissues, hormone-dependent involution in the adult, immune system maturation, selective deletion of inappropriate immune cells, and a number of other physiological processes (Allen *et al.*, 1997). Dysregulation of apoptosis can be dangerous, however, and has been linked to a wide variety of diseases and conditions. A lack of apoptosis may constitute one possible route to carcinogenesis or may be involved in the development of some autoimmune disorders. Conversely, elevated levels of apoptosis have been linked to neurodegenerative conditions like Alzheimer's and to the T cell destruction characteristic of AIDS (Wyllie, 1997).

A number of microorganisms can induce apoptosis dysregulation in host cells. In fact, it has been documented to be an important aspect of pathogenesis for many viruses, bacteria, and protozoa. Some viruses have been shown to induce apoptosis in host cells. Examples include coxsackievirus B3, an enterovirus in the family *Picornaviridae* (Carthy *et al.*, 1998), and HIV (Badley *et al.*, 1998). Many other viruses express anti-apoptotic proteins that effectively slow or block the apoptotic cascade (Young *et al.*, 1997) possibly leading to enhanced viral survival. Such viral interference with the apoptotic process may promote cancer (Young *et al.*, 1997; Tschopp *et al.*, 1998). Tschopp *et al.* (1998) have outlined a variety of viruses, including herpesviruses, poxviruses, papovaviruses, adenoviruses, and baculoviruses, that encode a number of immunomodulatory molecules that are capable of interfering with the host's apoptotic signaling pathways.

Researchers have described a number of bacteria that survive, in part, via the ability to induce apoptosis in host immune cells. Some do so by producing pore-forming proteins that induce subtle biochemical and physiological changes in the cell, which lead to apoptosis. Bacterial pathogens that produce such toxins include *Actinobacillus actinomycetemcomitans*, *Staphylococcus aureus*, and *Escherichia coli* (Chen and Zychlinsky, 1994). Other bacteria, such as *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae*, induce apoptosis in host immune cells by producing toxins that enter into the host cells and inhibit protein synthesis (Chen and Zychlinsky, 1994). Monack *et al.* (1996) have shown that *Salmonella typhimurium* induces apoptosis in host macrophages by causing "membrane ruffling" upon entry into the cell. Hersh *et al.* (1999) have shown that *Salmonella* spp. induce apoptosis in host macrophages by binding directly to caspase-1. Group A *Streptococcus pyogenes* has been found to induce apoptosis in host epithelial cells by initiating mitochondrial dysfunction (Nakagawa *et al.*, 2001).

Apoptotic dysregulation has also been documented in cells exposed to various protozoan pathogens. Toure-Balde *et al.* (1995, 1996, 2000) have shown that *Plasmodium falciparum*, a causative agent of malaria, induces apoptosis in human mononuclear cells. Researchers have found that *Trypanosoma cruzi*, (which causes Chagas' disease) evades the host immune response by inducing apoptosis in T cells (DosReis *et al.*, 1995; Lopes *et al.*, 1995) and in B cells (Zuniga *et al.*, 2000). Das *et al.* (1999) found that experimental infection with the intracellular protozoan parasite *Leishmania donovani* leads to selective deletion of host CD4+ T cells, which results in a decrease in the secretion of interleukin-2 and interferon-gamma. Conversely, Moore and Matlashewski (1994) found that *Leishmania donovani* inhibits macrophage apoptosis, thus facilitating the spread of infection by boosting the number of host cells available for parasitization, and by augmenting the number of infected macrophages available for uptake by the sandfly vector. They believe that the parasites do this by inducing host macrophages to secrete a soluble factor that may act in an autocrine manner to prevent apoptotic cell death (Moore and Matlashewski, 1994).

These examples help to illustrate the importance of apoptosis in the pathogenic complement of numerous different microorganisms, including a number of protozoan pathogens. It was proposed, therefore, that *T. vaginalis* might possess the ability to induce apoptosis in its pathogenic attack on monolayer cells in an *in vitro* co-culture system. This relationship has not been investigated before, but seems plausible given the wide scope of the elements incorporated in the pathogenic arsenal of *T. vaginalis*.

Defining Apoptosis

Apoptosis generally takes place in scattered single cells and is considered to be a form of self-directed cellular suicide. It is characterized by a specific set of biochemical and morphological

changes in the cell. The term was first used by Kerr *et al.* in 1972 when they noted that morphologically similar cell deaths could be detected in many pathological conditions as well as in normal tissue.

Morphologically, the apoptotic cell shrinks and becomes denser. The chromatin becomes pyknotic and is packed into smooth masses against the nuclear membrane. This phenomenon leads to margination of the nuclear chromatin such that it takes on a distinctive half-moon, or sickle-shape. During the condensation phase, there is a breakdown of cell-cell interactions as the cell undergoing apoptosis is isolated from its neighbours (Bursch *et al.*, 1990). There is little or no swelling of the cellular organelles. The nucleus may break into small pieces (karyorrhexis) and the cell often emits extensions which tend to break off and become apoptotic bodies. These apoptotic bodies, formed via the “budding phenomenon”, often contain pyknotic nuclear fragments (Majno and Joris, 1995). Interestingly, there appears to be no leakage of cellular contents during the budding process (Bursch *et al.*, 1990). Once they have separated from the main apoptotic body, the smaller buds are free to be phagocytized by macrophages or by neighbouring cells. If budding does not occur, the cell may shrink into a single compact, spherical mass (Majno and Joris, 1995).

Biochemically, DNA is fragmented by endogenous endonucleases via a two-stage process. Initially, the DNA is chopped up into large fragments (50-300 kb in size) and then ultimately a portion of the DNA is further fragmented into oligo- and mononucleosomal-size fragments (Arends *et al.*, 1990; Kokileva, 1994; Walker *et al.*, 1994; Zhivotovsky *et al.*, 1994). Walker and Sikorska (1997) have suggested that depending on the cell type, DNA fragmentation is likely controlled by either Ca^{2+} ions or a decrease in pH. They also stated that while DNA degradation into oligonucleosomes is not essential for apoptosis, all cells must undergo degradation of DNA into large fragments. Another biochemical hallmark of apoptosis is the implementation of

changes in the plasma membrane. One such change, namely the translocation of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane, has been well documented (Allen *et al.*, 1997). Researchers have suggested that this, as well as a number of other surface markers, likely play an important role in the recognition of the apoptotic cell by professional phagocytes.

The process of apoptosis is under genetic control and can be instigated by an internal genetic clock (as in programmed cell death), or conversely, can be induced by extracellular agents such as hormones, cytokines, killer cells, microorganisms, or a variety of different chemical or physical agents (Majno and Joris, 1995). Apoptosis can occur very rapidly, and for this reason tends to be relatively unobtrusive in tissue sections. It is generally suggested that apoptosis does not induce an inflammatory response, but this may not be completely accurate. It has been well documented that the apoptotic bodies resulting from cellular suicide are often recognized and phagocytized by macrophages (Majno and Joris, 1995). This would indicate that the dying cells must release some form of chemoattractant, allowing professional phagocytes to find their target. It could be that since apoptotic cells often die singly, and not *en masse*, the level of chemoattractant emitted attracts only a few nearby macrophages and does not induce a large-scale inflammatory response (Majno and Joris, 1995).

Often, the term programmed cell death is used as a synonym for apoptosis, but this is misleading. There are many situations where a programmed cell death occurs by a mechanism other than apoptosis. Programmed cell death merely indicates that a genetic clock selects a given time for the death of certain cells, while the genetic program for apoptosis specifies the means by which the suicide will occur (Wick, 1994; Hockenberry, 1995; Majno and Joris, 1995).

The Caspase Family of Cysteine Proteinases

The central effectors of the apoptotic process are the cysteiny aspartate-specific proteinases (caspases). The caspase gene family has thus far been shown to include at least fourteen mammalian members, of which eleven human enzymes have been identified (Nicholson, 1999). Caspases generally function in an ordered enzymatic cascade. In the typical caspase cascade, the upstream caspases (initiator caspases including caspases-6, -8, -9, and -10) are activated by association with one or more caspase adapters. This initial activation step has an important regulatory role and is controlled by a complex network of pro- and antiapoptotic proteins. Once the initiator caspase is transformed to its active state, it processes and activates one or more downstream caspases (executioner or effector caspases-usually caspases-2, -3, and -7). The active effector caspases then cleave various cellular substrates, which leads to the ultimate apoptotic demise of the cell (Chang and Wang, 2000).

Caspases are first synthesized as inactive precursors, or procaspases. All procaspases contain a highly conserved protease domain. The protease domain is comprised of a large subunit (17-21 kDa) and a small enzyme subunit (10-14 kDa). The procaspases also contain a prodomain or NH₂-terminal peptide of varying lengths; the prodomain can be longer than one hundred amino acids in the initiator caspases or may contain fewer than thirty amino acids in the effector caspases. Some procaspases contain a brief linker between the small and large subunits. A two-step proteolytic processing mechanism gives rise to a mature caspase tetramer (a homodimer of the large and small heterodimers arranged in twofold rotational symmetry) (Rathmell and Thompson, 1999; Chang and Yang, 2000).

One of the most remarkable enzymatic properties of the caspases is their specificity with respect to substrate binding. Caspases recognize a short tetrapeptide sequence within targeted

polypeptides and have demonstrated an absolute requirement for an aspartic acid residue in the P₁ position (Thornberry, 1997; Nicholson, 1999). The variability in residue preference for the P₂-P₄ (P₄ in particular) sites has helped researchers to group the caspases in the following manner: the group I caspases (including caspases-1, -4 and -5) prefer substrates with bulky hydrophobic residues at P₄; the group II enzymes (including caspases -2, -3, and -7) prefer aspartic acid at the P₄ site; group III caspases (including caspases-6, -8, and -9) bind preferentially to branched-chain aliphatic amino acids at the P₄ site. While their substrate preferences have not been as well defined, phylogenetic evidence suggests that caspases-11, -12, and -13 resemble the group I caspases, and caspase -10 is similar to the group III caspases (Rathmell and Thompson, 1999).

There are at least three different mechanisms for caspase activation in the mammalian cell. The first, called recruitment activation, involves the interaction of a procaspase in an oligomeric activating complex via its prodomain. An example of this kind of activation is the recruitment of procaspase-8 to an oligomeric activation complex following ligation of the surface CD95 (Fas) receptor. The second mechanism, transactivation, occurs when one caspase is activated by another. An example would be the cleavage and activation of a downstream effector caspase by an initiator caspase that has recently undergone activation by recruitment. The third mechanism, where a caspase initiates its own activating cleavage, is termed autoactivation (Nicholson, 1999; Wolf and Green, 1999; Kruidering and Evan, 2000).

In mammalian cells, there are two fundamental ways by which a caspase cascade is known to be initiated. The first is called the intrinsic pathway and is generally thought to occur in cells that have been damaged or neglected. Essentially, cytochrome *c*, under the control of the Bcl-2 family proteins, is released from the mitochondria; cytochrome *c* then binds and activates apoptotic protease-activating factor (Apaf-1). Apaf-1 binds and activates caspase-9, which, in turn, activates caspase-3, initiating the downstream caspase cascade (Porter and Janicke, 1999;

Rathmell and Thompson, 1999; Chang and Yang, 2000). The second mechanism is the extrinsic pathway, which can occur following the binding of surface death receptors. The most thoroughly described surface receptors belong to the tumor necrosis factor receptor (TNFR) family. The TNFR family includes apoptosis-inhibiting and apoptosis-promoting receptors. A well-characterized apoptosis-promoting receptor, CD95 (Fas), can directly recruit and activate caspases-8 and -10 via its intracellular death domain. Once initiated, the upstream caspases can then proceed to bind and activate the downstream effector caspases (Rathmell and Thompson, 1999; Chang and Yang, 2000).

The substrates associated with the downstream effector caspases are varied and numerous; researchers have identified more than sixty mammalian caspase substrates. The ultimate consequences of substrate cleavage include the impairment of homeostatic and repair processes, the inactivation of apoptosis inhibitors, the disassembly of structural integrity, induction of morphological alterations, and the labeling of the dying cell for phagocytosis. To do this, the caspases may inactivate the normal function of a substrate, activate the substrate via influence on regulatory domains, alter the function of the target protein, or become directly involved in the proteolysis of the structural components of the cell (Nicholson, 1999).

Regulation of the complex apoptosis cascade involves both apoptosis-promoting and apoptosis-inhibiting proteins. Apoptosis inhibitors may be produced endogenously by the cell. The inhibitor of apoptosis (IAP) molecules, which carry out their function either by binding directly to the caspases or by influencing signal transduction pathways, are important examples of endogenous caspase inhibitors. Viruses have also been shown to produce caspase inhibitors that prevent host cell death, thus allowing for viral survival and propagation (Chang and Yang, 2000). In the laboratory, several classes of reversible and irreversible peptide-based caspase inhibitors have been designed for use in various apoptosis models. Reversible inhibitors include aldehydes,

nitriles, and ketones. Irreversible inhibitors include chloromethylketones, fluoromethylketones, and diazomethylketones. The peptide portions of these compounds determine their caspase-binding preference (Thornberry, 1997).

Hypothesis and Objectives

Hypothesis

T. vaginalis causes a number of pathological changes in host cells and effectively destroys a variety of host cell monolayers *in vitro*. The pathology induced by *T. vaginalis in vitro* likely mirrors the *in vivo* situation, where host tissues display trichomonad-induced lesions and cell sloughing. It has been well established that *T. vaginalis* uses haemolysis of erythrocytes and phagocytosis of urogenital flora as an effective nutrient acquisition system. Many microorganisms, including a number of protozoan pathogens, possess the ability, as part of their pathogenic arsenal, to induce apoptosis in host cells. Our hypothesis is that *T. vaginalis* induces apoptosis in host cells during the course of infection. We speculate that it may be to the parasite's advantage to induce apoptosis in host tissues or target monolayer cells. The break down into apoptotic bodies of a target cell might make it a more suitable candidate for phagocytosis, thus facilitating the salvage of a variety of nutrients (carbohydrates, amino acids, fatty acids, vitamins, and iron) and nucleic acids which *T. vaginalis* cannot synthesize.

Objectives

Using an *in vitro* system (McCoy cell monolayer), the objective was to look at the possible induction of apoptosis by *T. vaginalis*. The fragmented DNA characteristic of apoptosis would be detected with the use of TUNEL and ELISA assays. If apoptosis was confirmed, the mechanism of induction would be investigated by examining the involvement of caspases, and by

determining if the process was contact-dependent or independent. The co-culture nature of the experiments would also allow us to examine the contact-dependent and independent interaction of *T. vaginalis* and McCoy cell monolayers with respect to pathology other than the induction of apoptosis.

MATERIALS AND METHODS

Cell Culture

Growth and Maintenance of McCoy Cells

The monolayer cells used in the co-culture experiments were mice fibroblast (McCoy) cells that had been used extensively in our lab over the past decade. McCoy cells were grown at 37°C / 5% CO₂ in Minimum Essential Medium (Gibco BRL-Life Technologies Inc. Grand Island, NY) adjusted to a pH of 7.5 and supplemented to provide the following concentrations: 2 mM L-glutamine (Gibco-BRL), 7 mM sodium bicarbonate (Fisher Scientific, Whitby, ON), 24 mM glucose (Fisher Scientific), 8 µg gentamicin (Gibco-BRL) per mL, 25 U nystatin per mL. Heat inactivated fetal bovine serum (FBS) (Gibco-BRL) was added at the end to provide 10% of the total volume of the media. This media will be referred to as CMGA (Garber *et al.*, 1987)

McCoy monolayers were regularly grown in 75 cm³ and 25 cm³ vent cap plastic tissue culture flasks with 0.2 µm membrane cap (Corning, Fisher Scientific). Every two to three days, media in the flasks was decanted, cells were trypsinized (0.25% Trypsin (Gibco-BRL)), resuspended in CMGA, spun at 500 x g for five minutes and then were transferred into fresh media.

For co-incubation experiments, McCoy cells were first cultured in flasks, then transferred to four-well Nunc Lab Tek II Chamber Slides (Gibco-BRL). These consisted of four sterile plastic chambers attached to a glass slide. Normally, 1x10⁵ McCoy cells, in 1 mL of CMGA, were placed in each chamber and were allowed to form a monolayer over forty-eight hours. Healthy cells were firmly attached to the glass slide after twenty-four hours, at which point, the media was replenished.

Growth and Maintenance of Jurkat Cells

Jurkat cells (clone E6-1 (T cell leukemia, human) ATCC TIB-152) were cultured in plastic tissue culture flasks in RPMI 1640 medium (Gibco-BRL) supplemented to yield the following concentrations: 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate (Gibco-BRL). Heat inactivated FBS was added to make up 10% of the final culture media volume. Cells were enumerated and sub-cultured every two to three days in order to maintain the cell density between 10^5 and 10^6 cells per mL.

Culture of Microorganisms

Trichomonas vaginalis

The *T. vaginalis* isolates used in these experiments were originally harvested from the vaginal secretions of a female patient suffering from vaginitis. An isolate designated "202" was employed for these experiments. In a ranking of clinical isolates, "202" has been shown to cause moderate pathology in patients (Garber and Lemchuk-Favel, 1990). Organisms were grown in glass screw-capped tubes (16 x 125 mm) in 10 mL of Diamond's TYI-S-33 medium (TYI-See Appendix II) (pH 6.2) (Diamond *et al.*, 1978) supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, 100 µg/ml streptomycin (penicillin/streptomycin solution, Gibco-BRL) and 2.5 µg/mL amphotericin B (Fungizone, Gibco BRL). Tubes containing *T. vaginalis* cultures were incubated at 37°C with 5% CO₂, at a 45° angle. Cultures were passaged every two to three days. Viable organisms were enumerated on a Bright Line hemacytometer (VWR Canlab, Mississauga, ON), using trypan blue exclusion (1% Trypan Blue (Sigma, Sat. Louis, MO)) to distinguish between dead and healthy cells (Takahashi *et al.*, 1970).

***In vitro* Co-Incubation Experiments**

***T. vaginalis* and McCoy Cell Monolayers**

As described above, McCoy monolayers were allowed to grow to confluency in sterile chamber slides. Prior to the addition of *T. vaginalis*, monolayers were washed three times with sterile prewarmed PBS. *T. vaginalis* grown to mid-log phase was prepared in the following manner: *T. vaginalis* (isolate 202) growth culture was transferred to a centrifuge tube and the cells were spun at 900 x g for ten minutes. The pellet was washed three times and the final pellet was then suspended in two parts CMGA to one part TYI. The resuspended sample was counted with a hemacytometer and the concentration was adjusted accordingly. Depending on the desired concentration for each chamber (10^4 , 10^5 , or 10^6 *T. vaginalis* per mL), proportions of the prepared *T. vaginalis* and of the CMGA-TYI media were added to each of the prewashed McCoy monolayers, so that each chamber held 1 mL. One chamber on each slide was designated the control and was incubated with media only. The chamber slides were then placed in a 37°C incubator (with 5% CO₂) and were incubated for six hours. After the six hour incubation period, the pH of the culture supernatant was checked (pHydrion Vivid 6-8 (Fisher Scientific)), and removed from the chambers for transfer to pre-labeled cryovials for storage at -80°C.

Once the media was transferred, the chambers were removed from the glass slides, and were fixed for ten minutes in 1% paraformaldehyde (PFA-BDH supplied by VWR Canlab), in preparation for immunocytochemical staining.

For the time-course experiment, a portion of the slides was removed from the incubator at two hours and four hours. These slides were fixed in PFA and were then transferred to PBS (at 4°C) for temporary storage until all slides could be recovered from the incubator.

T. vaginalis Growth Media (Cell Free Filtrate) and McCoy Cell Monolayers

T. vaginalis cells were passaged into CMGA-TYI (2:1) at a concentration of 10^4 trichomonads per mL and were incubated for forty-eight hours, allowing the culture to reach mid-log phase. The sample was centrifuged at 900 x g for ten minutes. The supernatant was poured through a bottle top filter (0.22 μ m pore size (Corning-Fisher Scientific)) to remove any debris or cells still present after centrifugation. pH of the cell free filtrate was checked (pHydriion Vivid 6-8) and adjusted with 1N NaOH to a pH of 6.6 to 6.8.

McCoy cells were grown to confluency in chamber slides, as described above, and immediately before the addition of the cell free filtrate, were washed three times with sterile PBS. Cell free filtrate and fresh media were added in the appropriate proportions to each chamber, with a final volume of 1 mL in each. One chamber was again designated the control and to this chamber CMGA-TYI (2:1) media only was added. Monolayers were incubated at 37°C / 5% CO₂ for six hours.

Cytospins to Recover Detached Cells

Cells that had detached from the chamber slides during co-incubation were pelleted from the co-incubation media by centrifugation at 500 x g for ten minutes. The supernatant was then aspirated and the pellet was resuspended in 500 μ L 1% PFA for ten minutes to fix cells. The samples were then spun (Shandon cytospin centrifuge (Fisher Scientific)) onto glass slides at 1200 rpm for ten minutes.

Induction of Apoptosis in McCoy Cells Using Camptothecin (Positive Control)

McCoy cell monolayers were first allowed to grow to confluency as previously described. Monolayers were treated with camptothecin (concentrations of 5 μ M and 10 μ M) in CMGA

media for four hours; fresh CMGA was added to monolayer cells, which were then incubated for an additional forty-eight hours. After treatment, all chambers were removed and the slides were fixed for ten minutes in 1% PFA, in preparation for immunocytochemical staining.

Caspase Inhibition in McCoy Cells

McCoy monolayers were pre-treated with caspase inhibitors z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (z-IETD-fmk) (Enzyme Systems Products, Livermore, CA) and z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk) (Enzyme Systems Products) prior to co-incubation with *T. vaginalis*. McCoy monolayers were incubated with the inhibitors for twelve hours, and then with inhibitors and *T. vaginalis* for an additional six-hour period. Ten μM and 100 μM concentrations of z-VAD-fmk and z-IETD-fmk were used to treat different monolayers.

Chamber slides were incubated at 37°C in 5% CO₂. Once the incubation time had elapsed, the chambers were removed, and the slides were fixed for ten minutes in 1% PFA.

Immunocytochemical staining was then undertaken.

Jurkat Apoptosis Control Using Anti-CD95 Antibody

Healthy Jurkat cells were first transferred to chamber slides (3×10^5 cells per chamber). One-quarter of the chambers were then pre-treated with caspase inhibitor z-VAD-fmk and one-quarter with caspase inhibitor z-IETD-fmk (1 mL of RPMI media supplemented with inhibitor to give a concentration of 100 μM in each chamber) for ten hours.

After the ten hour pre-treatment with caspase inhibitors alone, CH11 (0.33 $\mu\text{g}/\text{mL}$) (anti-CD95 antibody clone IgM (Beckman Coulter-Immunotech, Burlington, ON)) was added to all chambers except one, which was untreated. Following fifteen hours of incubation, the contents of each chamber were transferred to microcentrifuge tubes and were spun at 300 x g for ten minutes. The

cells recovered in each pellet were then fixed in 500 μ L of 1% PFA for ten minutes. The fixed cell samples were then cytospun as described above and the resulting slides were stained.

Detection of Apoptosis Using the TUNEL Assay

The ApopTag[®] Plus Peroxidase *In Situ* Apoptosis Detection Kit (Intergen Company, Purchase, NY) was used in all TUNEL assays as suggested by manufacturer's protocol, except that counterstaining was carried out for twenty minutes with methyl green solution at a pH of 5.0.

The reagents provided in the kit are designed to label the free 3' OH DNA termini *in situ* with chemically labeled and unlabeled nucleotides. These nucleotides are enzymatically added to the DNA ends by terminal deoxynucleotidyl transferase (TdT) via a template-independent process. The incorporated nucleotides form an oligomer of digoxigenin nucleotide and unlabeled nucleotide in a random sequence. DNA fragments that have been labeled with the digoxigenin-nucleotide are then allowed to bind to an anti-digoxigenin antibody conjugated to peroxidase. The bound peroxidase antibody conjugate enzymatically generates a permanent, localized stain from chromogenic substrates, providing sensitive detection in tissues, or in individual cells (The Complete ApopTag[®] Manual, Intergen Company).

Detection of Apoptosis Using the Sandwich ELISA

As an alternate method of apoptosis detection, the Cell Death Detection ELISA^{Plus} Kit (Roche Molecular Biochemicals, Mannheim, Germany) was used as described by the manufacturer's instructions. This assay is used for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) formed as a result of induced cell death (From the Cell Death Detection ELISA^{Plus} Kit Instruction Manual, Roche Molecular Biochemicals Website <http://biochem.roche.com>).

The samples (in this case, co-culture supernatant) were placed in the wells of a streptavidin coated microtiter plate. To these wells, a mixture of anti-histone-biotin and anti-DNA-peroxidase (POD) was added. Once unbound components were washed out of the wells, nucleosomes in the samples were quantified by detecting the peroxidase still bound in the complexes. Peroxidase concentration was determined photometrically, five minutes after the addition of peroxidase substrate 2,2'-Azino-di- [3-ethylbenzthiazoline sulfonate] (ABTS) (from the Cell Death Detection ELISA^{Plus} Kit Instruction Manual, Roche Molecular Biochemicals Website <http://biochem.roche.com>).

Co-culture growth supernatants that had been frozen at -80°C after earlier TUNEL experiments were used as samples in these experiments. Samples were thawed and spun at 200 x g for ten minutes to remove cells and debris. Supernatant aliquots were then added to the appropriate wells of the microtiter plate and processed as per the manufacturer's instructions. Culture supernatant from camptothecin-treated McCoy cells was used as a positive control specific for these cells (See "Positive Apoptosis Control in McCoy Cells" above).

Cell Enumeration and Image Capture

A set of criteria was established, in order to gain consistency in the enumeration of cells. For each chamber or sample, at least five separate microscope fields were counted, with the minimum acceptable number of enumerated cells per chamber set at three hundred.

Fixed cells were observed under an Olympus BH-2 microscope fitted with a Sony Power HAD 3CCD Colour Video Camera. The video camera allowed for the capture of the desired still images, which were then transferred into Image Pro Plus software for digital preservation. All images were captured at a magnification of 400x.

RESULTS

Measurement of DNA Strand Breaks in McCoy Cells Using the TUNEL Assay

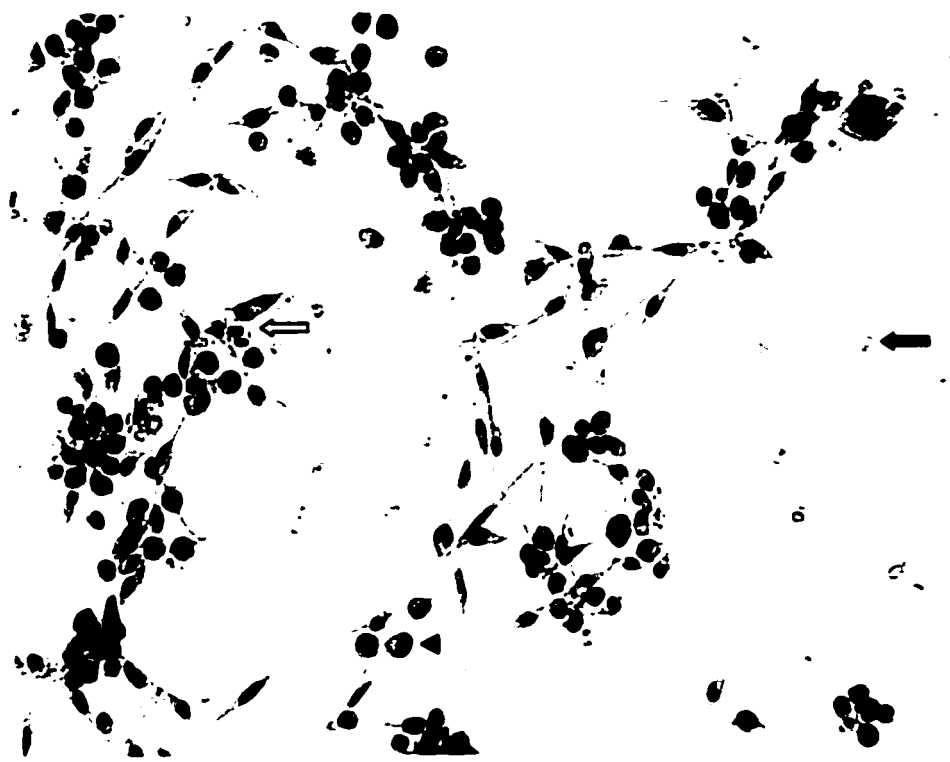
The TUNEL assay, which allows for the differentiation between apoptotic and non-apoptotic cells via a colourimetric reaction, was used to detect *T. vaginalis*-induced apoptosis in McCoy cells.

McCoy cell monolayers were allowed to grow to confluency and were incubated with fresh media (control) or media containing *T. vaginalis* in specific concentrations (ranging from 10^4 to 10^6 cells/mL). Following six hours of co-incubation in divided chamber slides, monolayers were rinsed with PBS and the remaining cells were fixed on the glass. Fixed slides were then treated with a TUNEL assay which employs a colourimetric reaction to distinguish between apoptotic (brown) cells and non-apoptotic (blue counterstained) cells.

Figure 4 shows the TUNEL-stained monolayer following six hours of co-culture *with T. vaginalis*. There were three distinct cell types present. The most prevalent cell type was blue and fibroblast-like in shape with a large nucleus in relation to the size of the whole cell (an example is indicated by the arrowhead in Figure 4). Based on staining observed in untreated control monolayers (not shown), these appeared to be non-apoptotic, adherent McCoy cells. The second cell type, indicated by the open arrow, was dark brown and had a large nucleus. These were relatively rare and appeared to be apoptotic McCoy cells. The third type of cell observed in the TUNEL experiments, indicated by the solid arrow, had not taken up any of the blue counterstain, but were very lightly stained a greyish/beige colour and were much smaller than the other cells observed. In addition, the nuclei seen in these cells were quite small and appeared to have a spindle or pinpoint shape.

Figure 4: Co-Culture Illustrating Three Cellular Morphologies

TUNEL assay on adherent cells remaining after co-culture. The first population consisted of fibroblast-shaped, blue cells with large dark-staining nuclei (arrowhead). The second type of cell, clearly apoptotic, also had a large nucleus, but it was stained dark brown (open arrow). The third cell type was smaller, did not counterstain well, but were light greyish/beige in colour (closed arrow). These cells also contained small pinpoint or spindle-shaped nuclei.



To address the possibility that the beige cells were trichomonads, *T. vaginalis*, trypsinized McCoy cells, and a mixture of both, were cytopun and stained with the TUNEL assay. This allowed for comparison of cellular morphology. The two cell types were found to be in the same approximate size range, although *T. vaginalis* showed more variability. With respect to staining, the McCoy cells counterstained well, while the *T. vaginalis* did not. For this reason, the trichomonads were more difficult to see. Non-specific brown staining of *T. vaginalis* was not observed, however. It was also discovered that the cytopun *T. vaginalis* did not resemble the lightly stained, small, beige cells found attached to the chamber slides following co-culture of *T. vaginalis* and McCoy cells (not shown).

It was speculated that perhaps the poorly-counterstained, beige cells were apoptotic McCoy cells. When these cells were compared with the positive control tissue (fixed rat mammary gland section (Strange *et al.*, 1995)) supplied with the TUNEL kit, however, the faint staining seen in the cells in question (solid arrow in Figure 4) was unlike the darker brown colour of the positive cells in the kit (solid arrows in Figure 5). This led to some doubt as to the accuracy of counting the small beige cells as apoptotic. Therefore, they were counted as separate, “possibly apoptotic”, cells in all subsequent experiments. Hence, there are three cell types in all tables: blue non-apoptotic cells, brown apoptotic cells, and beige cells which, at the time, were assessed as possibly apoptotic.

Initial TUNEL experiments showed that when incubated in media alone (i.e. no *T. vaginalis*), the McCoy monolayers remained largely intact and non-apoptotic McCoy cells made up 99.7% of the cells counted (Table 1). With the addition of trichomonads to the co-incubation, there were found to be fewer dark blue apoptosis-negative cells. As can be seen in Table 1, when the McCoy cells were incubated with 10^6 *T. vaginalis*, the majority of the cells counted after the six-hour co-incubation were the lightly positive greyish/beige cells (98.7%). Very few large blue McCoy

Figure 5: Rat Mammary Gland Stained with TUNEL Assay Reveals Apoptotic Cells

Panels (a) and (b) are two fields of TUNEL-stained sections of rat mammary gland that act as positive controls for the ApopTag® Plus Peroxidase *In situ* Apoptosis Detection Kit (Intergen Company). The dark brown apoptotic cells are easily detected (arrow).

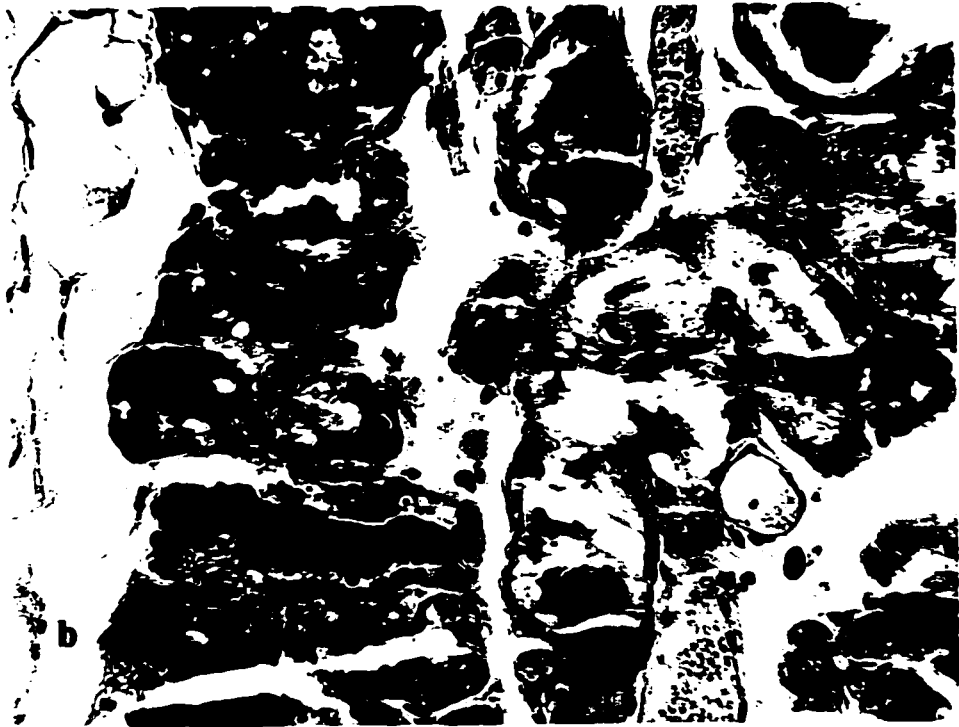


Table 1: Initial TUNEL Experiments

Early TUNEL experiments showed that when increasing numbers of trichomonads were added to the co-culture, fewer healthy McCoy cells resulted. After co-incubation, the majority of cells remaining were found to be stained a light beige colour, suggesting that apoptosis may have been occurring in McCoy monolayer cells as a result of the six hours of exposure to *T. vaginalis*.

Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number)	brown cells positive for apoptosis-% of total cells counted (raw number)	slightly beige cells which may be apoptotic-% of total cells counted (raw number)
McCoy cells alone	99.7% (1517)	0.3% (4)	0% (0)
McCoys and 10^4 <i>T. vaginalis</i>	97.0% (1189)	0% (0)	3.0% (37)
McCoys and 10^5 <i>T. vaginalis</i>	48.0% (547)	0% (0)	52.0% (593)
McCoys and 10^6 <i>T. vaginalis</i>	1.3% (27)	0% (0)	98.7% (2017)

cells remained (1.3% of cells counted).

A timecourse experiment (Table 2) showed that after four and six hours of co-incubation, almost all of the dark blue McCoy cells were replaced by the lightly staining beige cells. The faintly stained cells made up 99.7% of the cells counted on slides left to co-incubate for four hours with 10^6 *T. vaginalis* and made up 99.1% of cells counted on slides left to incubate for six hours with 10^6 *T. vaginalis*. A similar trend was seen at two hours, where 70.2% of the cells counted were light beige in colour. Throughout the TUNEL experiments, the percentage of cells that were dark brown and therefore definitely positive for apoptosis, remained uniformly low (generally less than 1%) (Tables 1 and 2).

Role of Contact Between *T. vaginalis* and McCoy Cells in Monolayer Destruction and Induction of McCoy Cell Apoptosis

Based on the hypothesis that apoptosis was taking place in the McCoy cells, the mechanism of its induction was assessed. It has been well established that *T. vaginalis* exerts a negative effect on cell monolayers, but there is some debate as to the need for contact in this process. Thus, McCoy cell monolayers were grown to confluency, but rather than being incubated with whole trichomonads, *T. vaginalis* culture supernatant was added to the monolayers. Culture supernatant was prepared by growing *T. vaginalis* to mid-log phase and removing the cells by centrifugation. The *T. vaginalis* supernatant was filtered and the pH was adjusted prior to co-incubation. Control monolayers incubated with fresh media showed no signs of damage after six hours (100% blue McCoy cells-Table 3). Monolayer cells incubated with the *T. vaginalis* culture supernatant, however, had to be recovered with a cytopspin technique because the monolayer almost completely detached from the glass slide. Once these cells were collected, spun onto fresh glass slides, and stained with the TUNEL assay, it was observed that 100% of them were negative for

Table 2: Timecourse Experiment

Co-cultures were conducted as before, but this time cells were left for two, four and six hours. Similar to initial TUNEL experiments, the timecourse showed an inverse relationship between the number of trichomonads and the number of healthy McCoy monolayer cells remaining after four and six hours of co-culture. (The same trend was seen, to a lesser extent, after two hours of co-culture). This experiment also showed that with increasing co-incubation times, there were fewer healthy blue McCoy cells and more lightly stained beige (and possibly apoptotic) cells.

Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number) after 2, 4, and 6 h			brown cells positive for apoptosis-% of total cells counted (raw number) after 2, 4, and 6 h			slightly beige cells which may be apoptotic-% of total cells counted (raw number) after 2, 4, and 6 h		
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
McCoy cells alone	99.8% (1316)	99.7% (997)	99.0% (900)	0.2% (3)	0.3% (3)	1.0% (9)	0% (0)	0% (0)	0% (0)
McCoy's and 10^4 <i>T. vaginalis</i>	99.8% (1370)	98.0% (904)	95.6% (986)	0% (0)	0.4% (4)	0.1% (1)	0.2% (3)	1.5% (14)	4.3% (44)
McCoy's and 10^5 <i>T. vaginalis</i>	87.2% (1072)	80.4% (701)	64.7% (738)	0.2% (3)	0.2% (2)	0.2% (2)	12.6% (155)	19.4% (169)	35.1% (400)
McCoy's and 10^6 <i>T. vaginalis</i>	29.8% (248)	0.3% (2)	0.9% (9)	0% (0)	0% (0)	0% (0)	70.2% (584)	99.7% (763)	99.1% (996)

Table 3: Examining the Role of Contact in *T. vaginalis* Pathogenesis

McCoy cell monolayers were incubated with *T. vaginalis* supernatant for six hours. Monolayer cells detached from the glass slide after co-culture with the growth media but once recovered by cytopsin, were found to be negative for apoptosis.

Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number)	brown cells positive for apoptosis-% of total cells counted (raw number)	slightly beige cells which may be apoptotic-% of total cells counted (raw number)
McCoys attached to chamber slide following incubation in fresh media only	100% (839)	0% (0)	0% (0)
McCoys recovered via cytopsin following incubation with 1:4 supernatant dilution	99.9% (1003)	0.1% (1)	0% (0)
McCoys recovered via cytopsin following incubation with 1:2 supernatant dilution	100% (1000)	0% (0)	0% (0)
McCoys recovered via cytopsin following incubation with full strength supernatant	100% (1000)	0% (0)	0% (0)

apoptosis (Table 3 and Figure 6). Similarly, 1:4 and 1:2 dilutions of the *T. vaginalis* conditioned media yielded no apoptotic cells after the TUNEL assay.

Role of Caspases in *T. vaginalis*-Induced McCoy Cell Apoptosis

Co-incubation experiments were repeated in the presence of caspase inhibitors in order to explore the mechanism of apoptosis induction in the McCoy cells. Caspases play a central role in the majority of apoptotic cascades, therefore it was postulated that caspase inhibitors would prevent the induction of McCoy cell apoptosis by *T. vaginalis*. This would yield important information about the mechanism of apoptosis occurring in these cells and would also act as an alternate way of confirming that the McCoy cells were indeed dying by apoptosis.

As expected from previous experiments, McCoy cells incubated with media alone were healthy and 99.9% were stained blue after the TUNEL assay (Figure 7a and Table 4). Also following the pattern observed earlier, non-apoptotic blue cells made up only 84.3% and 0% of cells counted, when incubated with 10^5 (Figure 7b) and 10^6 *T. vaginalis*/mL respectively. The lightly staining beige cells (solid arrow) made up 15.7% and 100% of cells counted when the co-incubation included 10^5 (Figure 7b) and 10^6 *T. vaginalis*/mL respectively.

The lightly staining beige cells were also observed in similar numbers in chambers treated with the caspase inhibitors. As illustrated in Table 4, co-incubations of McCoy cells and 10^6 *T. vaginalis* in the presence of 10 μ M and 100 μ M z-IETD-fmk (a caspase-8 inhibitor) resulted in a uniform population (100% of cells counted) of the lightly staining beige cells. When McCoy cells were grown with 10^5 *T. vaginalis* in the presence of 10 μ M and 100 μ M z-IETD-fmk, it was observed that 41% and 49.6% of cells, respectively, stained light beige (solid arrows in Figures 7c and 7d) (Table 4). After co-incubation of McCoy cells and 10^6 *T. vaginalis* in the presence of 10

Figure 6: Addressing the Need For Contact in *T. vaginalis* Pathology

Incubation of McCoy cell monolayers with *T. vaginalis* supernatant was found to cause widespread detachment of the McCoy cells from the glass substrate. Once the detached McCoy cells were recovered via a cytospin, and stained with the TUNEL assay, it was discovered that they were rounded but not apoptotic.

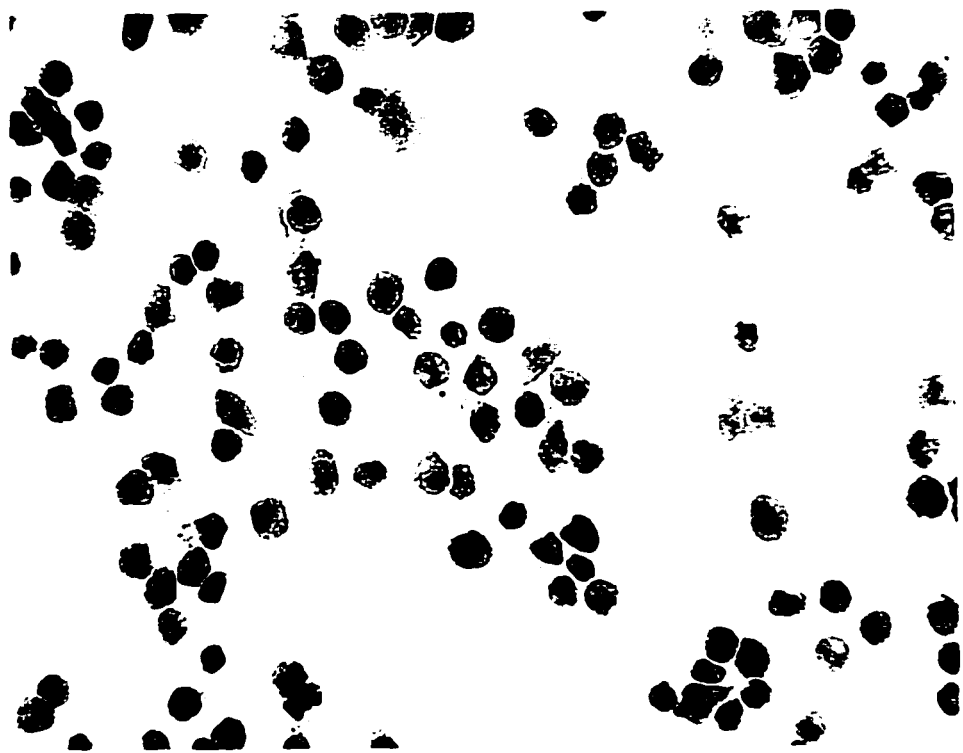


Figure 7: Effect of Caspase Inhibitors on McCoy Cells

The co-culture experiments were repeated in the presence of two different caspase inhibitors - z-IETD-fmk (a caspase-8 inhibitor) and z-VAD-fmk (a general caspase inhibitor). (a) McCoy cells incubated in media alone (control) and then stained with TUNEL assay were found to be non-apoptotic. (b) McCoy cells co-cultured with 10^5 *T. vaginalis*; some of the cells were stained light beige (arrow). The lightly stained beige cells (arrow) were also observed when McCoy cells were co-cultured with 10^5 *T. vaginalis* in the presence of 10 μ M (c) or 100 μ M (d) concentrations of z-IETD-fmk. (e) The same phenomenon was observed when the McCoy cells were co-cultured with 10^5 *T. vaginalis* in the presence of 10 μ M z-VAD-fmk.

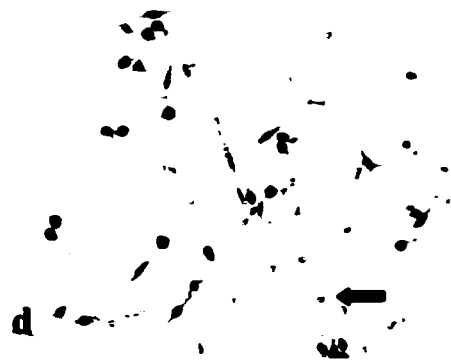
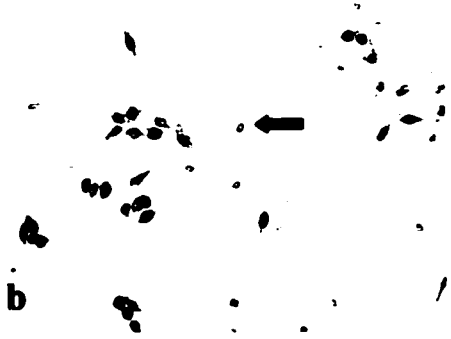


Table 4: Investigating the Involvement of Caspases

Co-culture experiments were repeated in the presence of caspase inhibitors (z-IETD-fmk and z-VAD-fmk). Caspase inhibitors did not have any effect on the numbers of slightly beige (and possibly apoptotic) cells present.

Caspase Inhibitor	Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number)	brown cells positive for apoptosis-% of total cells counted (raw number)	slightly beige cells which may be apoptotic-% of total cells counted (raw number)
Control- No drug	McCoys in media alone	99.9% (2855)	0.1% (3)	0% (0)
	McCoys and 10 ⁵ <i>T. vaginalis</i>	84.3% (307)	0% (0)	15.7% (57)
	McCoys and 10 ⁶ <i>T. vaginalis</i>	0% (0)	0% (0)	100% (300)
10 μM z-IETD-fmk	McCoys with drug but no <i>T. vaginalis</i>	100% (650)	0% (0)	0% (0)
	McCoys with drug and 10 ⁵ <i>T. vaginalis</i>	59.0% (453)	0% (0)	41.0% (315)
	McCoys with drug and 10 ⁶ <i>T. vaginalis</i>	0% (0)	0% (0)	100% (314)
100 μM z-IETD-fmk	McCoys with drug but no <i>T. vaginalis</i>	100% (557)	0% (0)	0% (0)
	McCoys with drug and 10 ⁵ <i>T. vaginalis</i>	50.4% (227)	0% (0)	49.6% (223)
	McCoys with drug and 10 ⁶ <i>T. vaginalis</i>	0% (0)	0% (0)	100% (472)
10 μM z-VAD-fmk	McCoys with drug but no <i>T. vaginalis</i>	100% (583)	0% (0)	0% (0)
	McCoys with drug and 10 ⁵ <i>T. vaginalis</i>	48.5% (236)	0% (0)	51.5% (251)
	McCoys with drug and 10 ⁶ <i>T. vaginalis</i>	0.2% (1)	0% (0)	99.8% (568)

μM z-VAD-fmk (a general caspase inhibitor), 99.8% of the cells enumerated were found to be light beige. A co-culture of McCoy cells and 10^5 *T. vaginalis* under the same conditions showed that 51.5% of those cells enumerated were light beige in colour (solid arrow in Figure 7e) (Table 4). It should be noted that in this experiment, higher concentrations of z-VAD-fmk were used in some chambers, but it was found that these concentrations exerted direct negative effects on the McCoy cells, resulting in disruption of the monolayer in control as well as test chambers.

Given that the caspase inhibitors did not block what we had postulated to be apoptosis in the McCoy cells, it was important that the inhibitors be tested in a cell system where apoptosis was confirmed. A Jurkat control, in which apoptosis was induced and inhibited, was established to demonstrate the ability of the caspase inhibitors to block caspase-8 (z-IETD-fmk) and caspases in general (z-VAD-fmk). The Jurkat cells were cultured and were then pretreated with the caspase inhibitors (z-IETD-fmk and z-VAD-fmk) for ten hours. After the pretreatment, an anti-CD95 antibody (CH11) was added to all chambers and the cells were left for a fifteen-hour incubation period. Controls were also included in which Jurkat cells were cultured in media with no CH11 or inhibitor and in media with CH11 only (no inhibitor). When Jurkat cells were incubated in media alone (with no treatment), 100% of the cells were blue and non-apoptotic (Figure 8a), while those cells treated with CH11 yielded two populations following the TUNEL assay. 24.9% of the cells counted were dark brown and definitely apoptotic (closed arrow in Figure 8b), while 75.1% were found to be blue and non-apoptotic (Table 5). The numbers of non-apoptotic cells returned to control levels when the caspase inhibitors were added to the incubation chambers. 99.7% of Jurkat cells treated with CH11 and z-IETD-fmk were non-apoptotic (Figure 8c) while 100% of those treated with CH11 and z-VAD-fmk were found to be non-apoptotic (Figure 8d and Table 5). This suggested that the caspase inhibitors were working properly and that the findings in the McCoy/*T. vaginalis* caspase inhibition experiment could not be explained by inactivity on the part of the inhibitors.

Figure 8: Control Experiment to Verify Efficacy of Caspase Inhibitors

Jurkat cells were incubated with an anti-CD95 antibody (CH11). (a) Jurkat cells incubated in media alone, cytospun, and stained with the TUNEL assay. (b) Jurkat cells, a number of which appear to be apoptotic (arrow), following incubation with the anti-CD95 antibody. (c) and (d) Non-apoptotic Jurkat cells were the result when the culture media contained both the anti-CD95 antibody and a caspase inhibitor (z-IETD-fmk (c) or z-VAD-fmk (d)).



Table 5: Caspase Inhibitor Control

Anti-CD95 induced apoptosis was inhibited in Jurkat cells via the use of caspase inhibitors (z-IETD-fmk and z-VAD-fmk) in the culture media.

Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number)	brown cells positive for apoptosis-% of total cells counted (raw number)	slightly beige cells which may be apoptotic-% of total cells counted (raw number)
Jurkats recovered via cytopsin following incubation with media only	100% (600)	0% (0)	0% (0)
Jurkats recovered via cytopsin following incubation with CH11	75.1% (426)	24.9% (141)	0% (0)
Jurkats recovered via cytopsin following incubation with CH11 and z-IETD-fmk (100 μM)	99.7% (685)	0.3% (2)	0% (0)
Jurkats recovered via cytopsin following incubation with CH11 and z-VAD-fmk (100 μM)	100% (765)	0% (0)	0% (0)

Adherence of *T. vaginalis* to Monolayer Following Detachment of McCoy Cells

During the course of one of the six-hour *T. vaginalis*/McCoy co-incubation experiments, prior to fixation, the co-culture was examined under the inverted microscope. It was noted that many of the cells stuck to the monolayer possessed the distinct corkscrew movement patterns of trichomonads. It was hypothesized that *T. vaginalis* was inducing apoptosis in the McCoy cells but that these compromised cells were detaching and allowing for the attachment of the *T. vaginalis*. It was estimated that by collecting detached cells after the usual six-hour co-incubation of McCoy cells and *T. vaginalis*, this possibility could be addressed. The detached cells were cytopspun onto fresh glass slides and these slides were stained with TUNEL reagents.

When McCoy cells were incubated with fresh media only, 99.9% of the attached cells counted were blue and non-apoptotic (Table 6). The co-incubation media from the control chambers were not cytopspun because of a lack of cellular detachment in these chambers. (The monolayers were fully intact). At a *T. vaginalis* concentration of 10^4 trichomonads/mL, the large majority of cells counted were blue, non-apoptotic McCoy cells (98.7% and 99.1% of attached and detached cells counted respectively) (Table 6). When the monolayers were incubated with 10^5 *T. vaginalis*/mL, 70.5% of the attached cells were assessed to be blue and non-apoptotic, while 84.9% of the detached cells were non-apoptotic. None of the detached cells from the 10^4 *T. vaginalis*/mL or 10^5 *T. vaginalis*/mL co-incubations were found to be dark brown (apoptotic). Following the co-incubation of McCoy cells and 10^5 *T. vaginalis*/mL, 29.5% of the attached cells were found to have been lightly stained and beige in colour, while 15.1% of the detached cells were lightly stained. Figure 9 illustrates the cells captured via cytopspin following the co-incubation of a McCoy cell monolayer and 10^5 *T. vaginalis*. Because of the massive destruction of the monolayers when McCoy cells were incubated with 10^6 *T. vaginalis*, the cytopspin yielded too many cells to count properly. It was noted, however, that none of the cells in these cytopspins was

Table 6: Assessing Detached Monolayer Cells

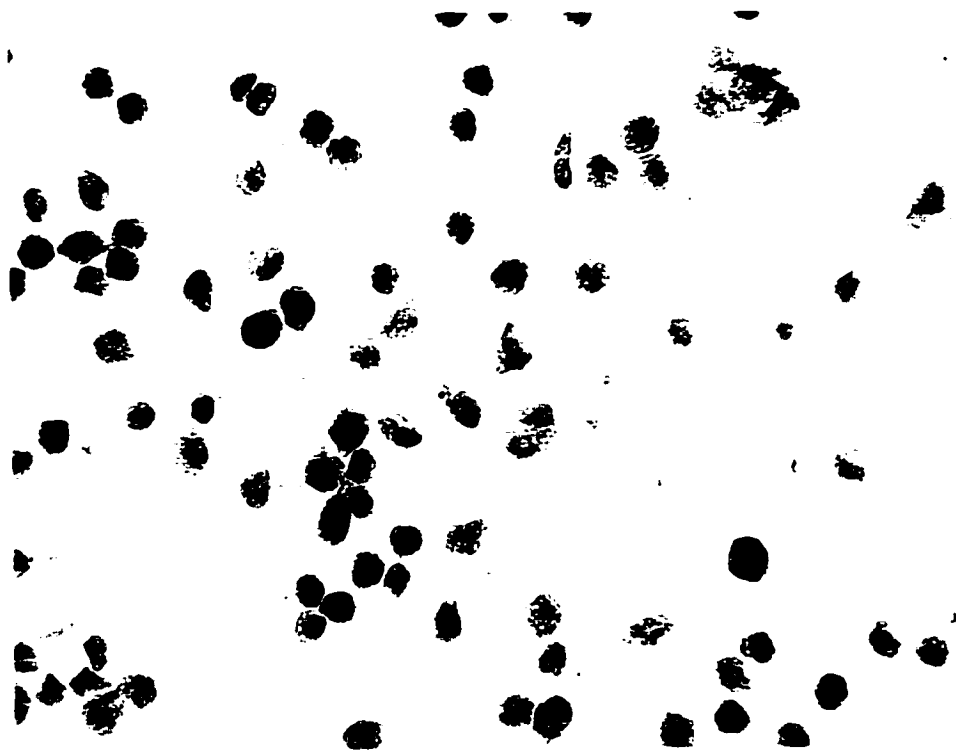
Co-culture experiments were conducted as per the standard protocol. Following the six hour co-incubation, TUNEL staining was done on both attached cells and detached cells recovered by cytopsin from the co-culture media. Increasing concentrations of *T. vaginalis* led to augmented detachment of monolayer cells. None of the detached cells were found to stain dark brown.

Co-Culture	blue cells negative for apoptosis-% of total cells counted (raw number)		brown cells positive for apoptosis-% of total cells counted (raw number)		slightly beige cells which may be apoptotic-% of total cells counted (raw number)	
	attached to chamber slide following incubation	recovered via cytopsin following incubation	attached to chamber slide following incubation	recovered via cytopsin following incubation	attached to chamber slide following incubation	recovered via cytopsin following incubation
McCoy cells alone	99.9% (1250)	n/a	0.1% (1)	n/a	0% (0)	n/a
McCoys and 10 ⁴ <i>T. vaginalis</i>	98.7% (1072)	99.1% (883)	0.2% (2)	0% (0)	1.1% (12)	0.9% (8)
McCoys and 10 ⁵ <i>T. vaginalis</i>	70.5% (659)	84.9% (1095)	0% (0)	0% (0)	29.5% (276)	15.1% (194)
McCoys and 10 ⁶ <i>T. vaginalis</i>	0.1% (1)	(>1200)*	0% (0)	(0)	99.9% (949)	(>1200)*

Note: * = too many cells cytopsun onto slide to count

Figure 9: Lack of Apoptosis in Detached McCoy Cells

Detached McCoy cells recovered, after co-incubation with 10^5 *T. vaginalis*, by cytopspin, and stained with TUNEL assay.



dark brown in colour (Figure 9). When the *T. vaginalis* and McCoy cells contained in the co-incubation media were cytopun, it became quite difficult to count the *T. vaginalis*. The trichomonads did not counterstain well and tended to flatten onto the slide (Note the faint, lightly stained cells in the background of Figure 9).

Induction of Apoptosis in McCoy Cells Using Camptothecin (Positive Apoptosis Control)

In order to determine whether the lightly stained beige cells were apoptotic McCoy cells or adherent *T. vaginalis*, a positive apoptosis control in McCoy cells was required. This was achieved by incubating McCoy cells with camptothecin (CAM), which is used to induce apoptosis in a number of different cell types. McCoy cell monolayers were incubated in media supplemented with CAM (5 μ M or 10 μ M concentrations) for four hours and were then left for forty-eight hours in regular growth media. At this point, slides were stained with TUNEL and cells were counted.

McCoy cells incubated in media only (no CAM) were largely blue and non-apoptotic (94.3%) (Table 7), with a slightly higher than normal background level of apoptotic cells (5.7%) likely due to natural deterioration of the monolayer after the long post-confluency incubation period. 17.6% of the McCoy cells treated with 5 μ M CAM (solid arrow in Figure 10 and Table 7) and 23.3% of the McCoy cells treated with 10 μ M CAM were found to be dark brown and apoptosis positive. It should be noted that no lightly staining beige cells with spindle nuclei were seen in the culture. The colour of the positive cells in this experiment was comparable to the brown staining seen in the kit control tissues (solid arrows in Figures 5 and 10) and was easily distinguished from the blue counterstaining seen in the non-apoptotic McCoy cells (open arrow in Figure 10).

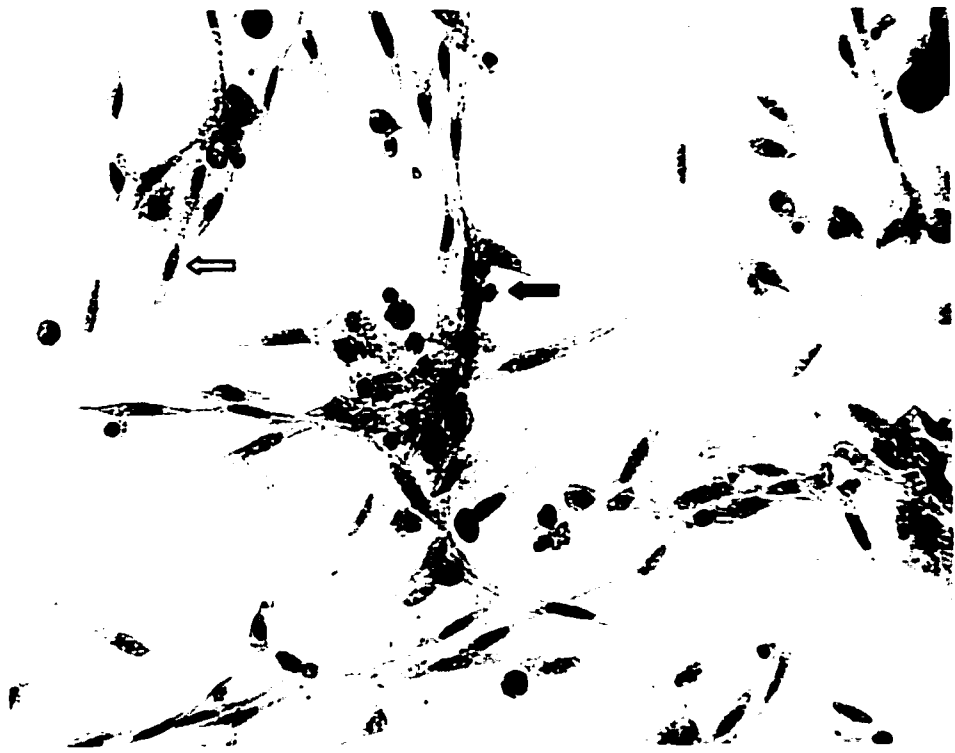
Table 7: Apoptosis Control in McCoy Cells

McCoy cells were incubated with camptothecin (CAM) for four hours before being stained with the TUNEL assay. More apoptotic cells were detected in those monolayers treated with CAM than in those incubated in media alone. Examination of the treated monolayer cells revealed none of the lightly stained beige cells that had been observed in previous experiments.

Co- Culture	blue cells negative for apoptosis-% of total cells counted (raw number)	brown cells positive for apoptosis-% of total cells counted (raw number)	slightly beige cells -% of total cells counted (raw number)
McCoy cells in media only	94.3% (741)	5.7% (45)	0% (0)
McCoys in media with 5 μ M CAM	82.4% (575)	17.6% (123)	0% (0)
McCoys in media with 10 μ M CAM	76.7% (490)	23.3% (149)	0% (0)

Figure 10: Apoptosis of McCoy Cells Induced by CAM

McCoy cells were incubated with camptothecin (CAM) (5 μ M concentration) for four hours. After a further forty-eight hours, cells were fixed and stained with the TUNEL assay. Resulting apoptotic cells (closed arrow) were dark brown and were easily distinguished from the blue, non-apoptotic McCoy cells (open arrow).



Measurement of DNA/Histone Fragments Resulting from Apoptosis by ELISA Assay (An Alternate Means of Assessing Apoptosis)

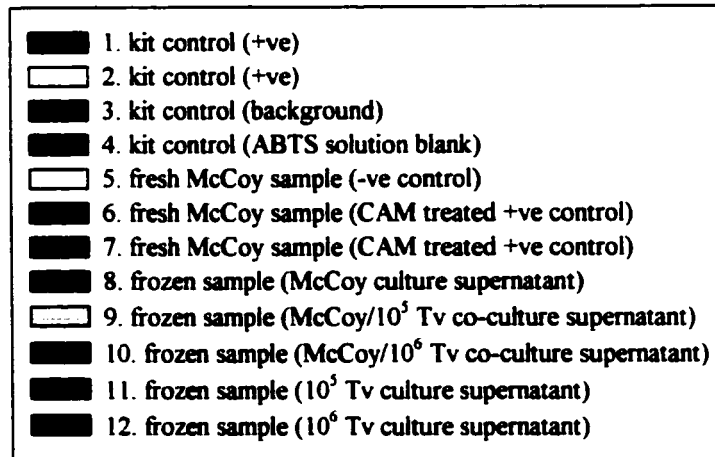
The Cell Death Detection ELISA^{Plus} Kit was employed as an alternative method for identifying apoptosis in the co-cultures of McCoy cells and *T. vaginalis*. If the trichomonads were inducing apoptosis in the McCoy cells during co-culture, the resulting DNA/histone fragments in the culture supernatant would be detected via the sandwich ELISA.

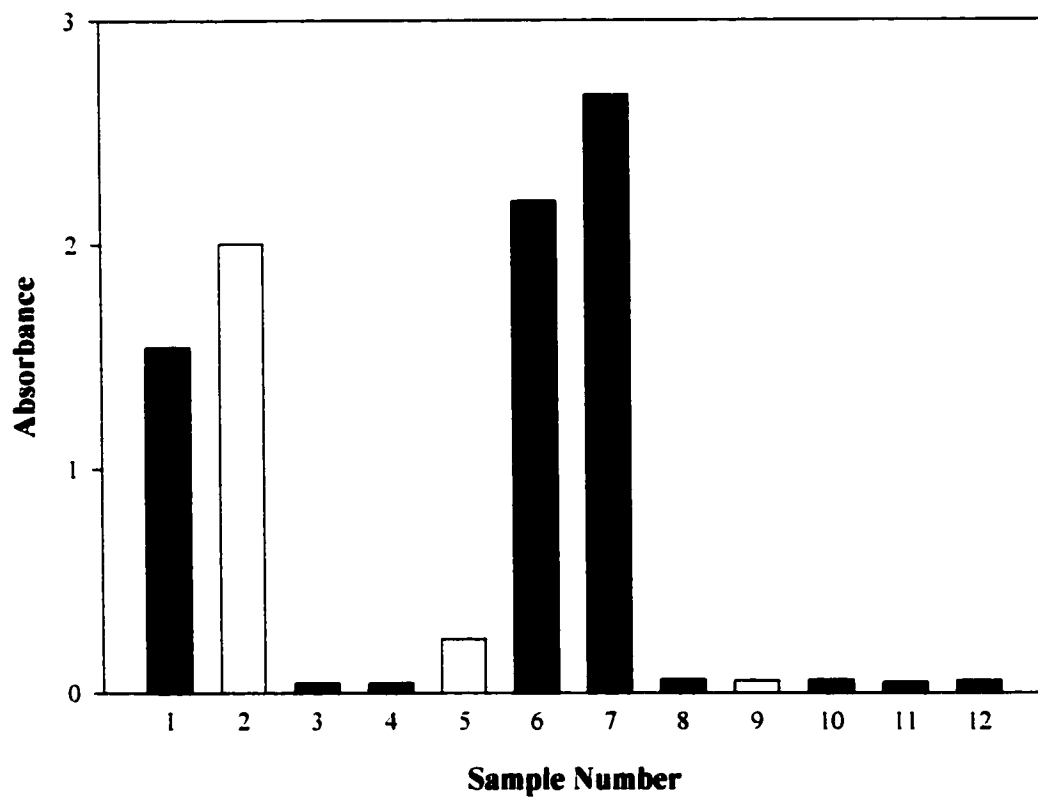
As expected, the negative controls included in the Cell Death Detection ELISA^{Plus} Kit gave very low absorbance values (bars 3 and 4 in Figure 11). Conversely, positive controls provided with the kit gave significantly higher absorbance values when tested with the ELISA protocol (bars 1 and 2 in Figure 11). Culture supernatants from McCoy monolayers were also incorporated, in a control capacity, into the ELISA experiment. As outlined in the Materials and Methods, monolayers incubated with the apoptosis-inducing compound CAM served as a positive control. Figure 11 illustrates the large difference in absorbance values between the co-culture supernatant taken from a monolayer grown in media only (bar 5) and those incubated instead with CAM-containing media (bars 6 and 7). This confirmed that CAM induces DNA fragmentation in McCoy cells.

The last group of co-culture supernatant samples used in the ELISA experiment came from frozen aliquots preserved from earlier TUNEL experiments. If DNA fragmentation was occurring in the McCoy cell/*T. vaginalis* co-incubations, the ELISA assay should detect the resulting DNA/histone fragments released into the culture supernatant. As can be seen in Figure 11, control supernatants (McCoy cells grown in media alone (bar 8) and *T. vaginalis* cultured alone (bars 11 and 12)) showed very low absorbance levels, indicating a lack of DNA/histone fragments. Very low (approaching background) levels of absorbance for supernatants from the

Figure 11: Cell Death Detection ELISA

The Cell Death Detection ELISA^{PLUS} Kit (Roche Molecular Biochemicals) was used to test for apoptosis in McCoy cells co-cultured with *T. vaginalis* (Tv). Negative kit controls gave very low absorbance values (bars 3 and 4). Positive kit controls gave comparatively high absorbance levels (bars 1 and 2). McCoy cells incubated with media alone (bar 5) gave an absorbance comparable to the background level, while those that had been cultured with CAM (to induce apoptosis) (bars 6 and 7) provided absorbance values greater than the kit positive controls. This indicated that CAM-induced McCoy cell apoptosis was being detected by the sandwich ELISA assay. Supernatants from six-hour co-cultures of McCoy cells with *T. vaginalis* were also assayed for DNA/histone fragments; the resulting low absorbance values (bars 9 and 10), suggested that apoptosis did not occur in the McCoy cells cultured with *T. vaginalis*. As expected, no DNA/histone fragments were detected in supernatant from McCoy cell growth alone in media (bar 8) or from *T. vaginalis* cultured alone in media (bars 11 and 12).





McCoy/10⁵ *T. vaginalis* (bar 9 in Figure 11) and the McCoy/10⁶ *T. vaginalis* (bar 10 in Figure 11) co-incubations suggested that DNA/histone fragments were not present and therefore that apoptosis was not taking place. The ELISA findings further supported the data from the TUNEL assays and indicated that the lightly staining cells seen in previous histological analyses of co-cultures were not apoptotic McCoy cells.

Assessment of Adherent *T. vaginalis* via the TUNEL Assay

All signs pointed to the beige cells seen in the co-incubation experiments being *T. vaginalis*, but this could not be concluded until a *T. vaginalis* control was developed. To do this, healthy *T. vaginalis* (at varying concentrations) was transferred to separate chamber slides and allowed to incubate for six hours. All conditions were comparable to the usual six-hour co-incubation, except for the omission of the McCoy cells. Once the slides were stained with TUNEL, it was discovered that the *T. vaginalis* did stick quite well to the glass substrate of the chamber slides (Figure 12a). Their morphology there was also noted to be quite different from cytopun *T. vaginalis*. In fact, it was observed that the *T. vaginalis* allowed to attach to the glass during a six hour incubation period (Figure 12a) looked almost identical to the lightly staining beige cells with the spindle nuclei which had previously been identified as apoptotic McCoy cells (open arrow in Figure 12b). McCoy cells grown in the absence of *T. vaginalis* (Figure 12c) have a distinctly different morphology from the trichomonads.

Figure 12: Morphology of *T. vaginalis* Adherent to Glass Substrate (Alone and in Co-Culture with McCoy Cells)

(a) Healthy *T. vaginalis* was added to chamber slides and was then allowed to incubate for six hours. After fixation and staining with TUNEL, it became apparent that the parasite did indeed adhere well to the glass substrate. It was also noted that the trichomonads did not counterstain and took on a faint beige hue. **(b)** A co-culture of *T. vaginalis* and McCoy cells. The large blue cells are non-apoptotic McCoy cells (closed arrow), and by comparison with **(a)**, it is apparent that the second cell type in **(b)** is adherent *T. vaginalis* (open arrow). **(c)** McCoy cells incubated on their own display the expected morphology and staining following treatment with the TUNEL assay. No apoptosis is apparent and no lightly-stained beige cells can be found.



DISCUSSION

The experiments outlined here suggest that *T. vaginalis*, while displaying a cytopathic effect on the target McCoy cells, does not induce apoptosis. This finding is in contrast with our original hypothesis that an apoptotic cascade would be initiated in the McCoy cells as a result of exposure to *T. vaginalis*.

Detection of Apoptosis

There are a number of different ways to detect apoptosis in cells (McGahon *et al.*, 1995). Some of these rely on the detection of morphological changes. For instance, membrane budding, chromatin condensation, cell shrinkage, and the formation of apoptotic bodies can all be seen to a certain extent with appropriate staining and the use of electron microscopy, phase-contrast microscopy or confocal laser scanning microscopy (Sgonc and Wick, 1994). The disadvantage of these techniques is that they are qualitative, not quantitative, and it is also possible that some subtle cellular changes occurring in early apoptosis may go unnoticed. Changes in size and granularity can also be detected by flow cytometry. This kind of analysis is very rapid and eliminates the subjectivity of microscopy, but the hallmark changes that give rise to variations in side and forward scatter are not present in all apoptotic cells (Sgonc and Wick, 1994).

Translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which occurs early in apoptosis, can be detected by flow cytometric analysis. This technique relies on the high affinity binding of Annexin-V for phosphatidylserine (Allen *et al.*, 1997). This has proven to be useful quantitative measure of early apoptosis, but may not be as applicable to adherent cells.

A second way to detect apoptosis is through the analysis of DNA degradation, a biochemical

feature common to all apoptotic cells. This can be done by agarose gel electrophoresis analysis, in which the uniformly fragmented DNA forms a “ladder” configuration. This is a well-established method of identifying apoptotic cells, but some drawbacks should be noted. These include the requirement for large numbers of cells, and the fact that it reveals no indication as to the percentage of apoptotic cells in a population, and does not recognize apoptotic cells in a heterogeneous cell population (Sgonc and Wick, 1994). Facchinetti *et al.* (1991) outlined a method that improves on the sensitivity of the gel technique, by incorporating a transfer to a Southern blot and hybridization with radiolabeled total cellular DNA probe. DNA fragmentation and subsequent loss can also be measured by flow cytometry. DNA content can be determined by the measurement of DNA using intercalating dyes like propidium iodide (PI), ethidium bromide, and acridine orange, or with dyes that bind externally to the DNA such as Hoechst 33342 and mithramycin. DNA strand breaks resulting from endonuclease activity can also be visualized by labeling the cells *in situ* (Wijsman *et al.*, 1993; Migheli *et al.*, 1995). This method can be applied to individual fixed, permeabilized cells or in tissue sections by *in situ* nick translation (ISNT) (Gold *et al.*, 1993) or by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (Negoescu *et al.*, 1996; Smith and Cartwright, 1997; Labat-Moleur *et al.*, 1998). In these methods, the 3'-hydroxyl ends of the DNA fragments are labeled either with biotin-dUTP, digoxigenin-dUTP, or FITC-dUTP. Apoptotic cells can be detected after labeling via flow cytometric methods, or by microscopy. *In situ* labeling systems have the advantage of enabling the detection of early DNA breaks during apoptosis, which allows for the quantification of the percentage of apoptotic cells, and can also detect small numbers of apoptotic cells in a heterogeneous population (Sgonc and Wick, 1994; Gorzyca *et al.*, 1995). DNA fragmentation can also be detected through the use of a cell death detection ELISA, where DNA/histone fragments are detected in growth media or in cellular cytoplasm (Bonfoco *et al.*, 1995; Terui *et al.*, 1995; Aragane *et al.*, 1998). This technique is advantageous because is quantitative, simple, and can be very sensitive.

McCoy Monolayer/*T. vaginalis* Co-Cultures: TUNEL and *T. vaginalis* Staining

Initial McCoy monolayer/*T. vaginalis* co-culture experiments yielded fields of cells with three distinct morphologies. There were found to be blue, non-apoptotic McCoy cells with a recognizable fibroblast shape. In addition, there were similarly-sized, rounded cells with dark-brown stained nuclei. These were determined to be apoptosis positive McCoy cells with a degree of staining comparable to that seen in the control slides processed along with experimental slides. The third cell type found was smaller than the first two and appeared to be much more lightly stained. In fact, the whole cell showed a light beige hue, with no counterstaining apparent. These cells also had smaller, spindle-shaped nuclei, which were quite different from the nuclei found in the McCoy cells determined to be positive for apoptosis. Garber and Bowie (1990) previously observed the morphologically variant nuclei of McCoy cells and *T. vaginalis*.

The true identity of the lightly-staining cells with the spindle nuclei was obscured by the fact that they exhibited a beige colour, which prompted the initial erroneous conclusion that they were McCoy cells at a different stage of apoptosis. Subsequent experiments indicated that these cells were *T. vaginalis* attaching to the substrate in place of the lifting McCoy cells.

Why the trichomonads took on a beige colour after TUNEL staining is somewhat of a mystery. One hypothesis could be that *T. vaginalis* possesses some kind of peroxidase activity that acts upon the peroxidase substrate provided in the ApopTag kit. The peroxidase substrate is meant to interact with the peroxidase conjugated to the anti-digoxigenin antibody, which in turn attaches to the digoxigenin-nucleotides added to the 3'-OH DNA ends resulting from the apoptotic cascade. The ApopTag kit manual does state, however, that on occasion, certain tissues or cells may give excessive background or positive staining.

There appears to be some debate in the literature as to whether *T. vaginalis* possesses peroxidase activity. Some groups (Ellis, *et al.*, 1994; Brown *et al.*, 1995) have investigated the possibility that *T. vaginalis* may use peroxidase enzymes to aid in the defense against oxidant stress encountered in its environment. While some other protozoa did demonstrate such an oxygen detoxification system, these researchers could not find peroxidase activity in the trichomonads examined.

Dierickx *et al.* (1990) looked at glutathione transferase (GST) activity in a number of anaerobic and aerobic protozoa. They stated that GST enzymes have “peroxidase activity for organic hydroperoxides and therefore, the potential to detoxify lipid and DNA hydroperoxides arising from radical damage in the presence of oxygen” (Dierickx *et al.*, 1990). They found that the anaerobic flagellates studied (including *T. vaginalis* grown anaerobically) did not demonstrate any trace of GST activity. On the other hand, while the levels were variable, GST activity could be found in the aerobic protozoa studied, suggesting that the peroxidase activity of the GST enzymes might be important for aerobic organisms. Since GST enzyme function can be found in some aerobic protozoa, it is possible that *T. vaginalis* grown in aerobic conditions (as were the trichomonads used in our research) may display such enzymatic properties.

Two more recent studies have suggested that *T. vaginalis* does indeed display peroxidase activity (Page-Sharp, *et al.*, 1996; Wenlie *et al.*, 1996). Page-Sharp *et al.* (1996) found that *T. vaginalis* contains ascorbate peroxidase, an enzyme that they suggest is employed as a scavenger at low environmental hydrogen peroxide concentrations. Wenlie *et al.*, (1996) investigated ultrastructural cytochemistry in *T. vaginalis* and showed that peroxidase activity was present in the secondary lysosomes of the parasite. The authors postulate that the lysosomal system may play a role in the pathogenic capabilities of the trichomonad. They propose that toxic residues such as hydrolases may be released from the parasite lysosomal system, causing pathology in the

surrounding host tissues. Their findings may also point to a possible interaction between endogenous trichomonal peroxidase and 3,3' Diaminobenzidine (DAB), the peroxidase substrate provided in the ApopTag kit. It should be noted that the TUNEL protocol does include a step to counter the possibility that the tissues or cells being stained contain endogenous peroxidase; slides are quenched in 3.0% hydrogen peroxide for five minutes. It is not known how this would affect the trichomonads adherent to the slides, or if all endogenous peroxidase would be quenched under these conditions. Unfortunately, in no other studies has *T. vaginalis* been treated with the TUNEL protocol. While the presence of endogenous peroxidase could cause *T. vaginalis* to pick up some brown colour after TUNEL staining, there is also the possibility that the colour is as a result of some unidentified non-specific binding.

It is apparent that *T. vaginalis* does not counterstain well. After the application of peroxidase substrate, and prior to addition of counterstain, the chamber slides (background and cells) take on an overall faint beige hue. For some reason, the trichomonads do not take up the methyl green counterstain; perhaps they simply maintain this pale beige hue. It is suggested in the Complete ApopTag Manual that if staining of both nuclei and cytoplasm is detected (which is the case with the beige *T. vaginalis*), the “nonspecific component can often be mitigated by reducing the development time in substrate”. It is possible that the beige colour seen in the trichomonads could be eliminated in this way.

Investigating Involvement of Caspases in the McCoy Cell Death Process

At the time, potential caspase involvement seemed like an important avenue to explore in the cell death process we thought we had observed in early co-culture experiments. It has been suggested that many different stimuli “may result in the same set of terminal proteolytic events, accounting for the remarkable stereotypy of apoptosis, regardless of the reason for its initiation.” (Wyllie,

1997) Current evidence suggests that caspases are vital players in the proteolytic cascade that is initiated in response to a death stimulus, possessing roles in both the direction and implementation portions of the apoptotic event (Thornberry, 1997). Workers in all areas of apoptosis research have reported that z-IETD-fmk is a potent inhibitor of caspase-8 (Kobayashi *et al.*, 1999; Tafani *et al.*, 2000; Schempp *et al.*, 2001; Seol *et al.*, 2001) and that z-VAD-fmk effectively blocks caspases in general (Bortner and Cidlowski, 1999; Fadeel *et al.*, 1999; Gamen *et al.*, 2000; Gao *et al.*, 2001). Of course, the caspase inhibitors did not block what was initially identified as apoptosis due to the fact that the cells in question turned out to be adherent trichomonads displaying a faint beige colour.

A Positive Apoptosis Control in McCoy Cells

It has been well established that camptothecin (a mammalian DNA topoisomerase I poison) is an effective inducer of both DNA strand breaks and protein-DNA crosslinks (Hsiang *et al.*, 1989; Liu, 1989; Del Bino *et al.*, 1990; Zhang *et al.*, 1990; Bertrand *et al.*, 1991; Roberts *et al.*, 1999). It interferes with the “breakage reunion reaction of mammalian DNA topoisomerase I by trapping the key covalent reaction intermediate, the cleavable complex.” (Liu, 1989) “This stabilized intermediate-an unusual ternary complex of drug, DNA, and topoisomerase-may then collide with the DNA replication machinery and thus trigger cell death.” (Zhang *et al.*, 1990)

Camptothecin was used in our experiments in order to see how the McCoy cells would look when they were known to be undergoing DNA fragmentation. Liu (1989) has suggested that cell killing by topoisomerase poisons might, in fact, share some common steps with apoptosis. This made it an appropriate apoptosis control in McCoy cells, and allowed for comparison with the beige cells, which had originally been identified as apoptotic. TUNEL staining of the CAM-treated McCoy cells revealed a population of dark brown cells that were very unlike the beige

cells with spindle nuclei, but instead resembled the apoptotic cells in the rat mammary gland tissue employed as a control in the TUNEL assays.

ELISA Experiments Support a Lack of Apoptosis in McCoy Cells

The ELISA experiments were an important verification that there was no apoptosis occurring in the McCoy cells co-cultured with *T. vaginalis*. All the controls (both positive and negative) worked well, lending more weight to the experimental sample results. Media from trichomonad/McCoy cell co-incubations gave very low (approaching background) levels of absorbance, suggesting that there were very few DNA/histone fragments present. This indicated a lack of apoptosis in these co-cultures, supporting the conclusion that the beige cells seen in the co-cultures were adherent *T. vaginalis*. We were confident that the Cell Death Detection ELISA kit provided reliable and accurate results; there are many references to the kit's effectiveness in the apoptosis literature (Bonfoco *et al.*, 1995; Terui *et al.*, 1995; Aragane *et al.*, 1998).

McCoy Monolayer/*T. vaginalis* Co-Culture: Pathology

While McCoy cell apoptosis was not induced by *T. vaginalis*, there were a number of other pathogenic effects observed following co-culture. The most obvious consequence of adding *T. vaginalis* to the McCoy monolayer was the rapid detachment of the McCoy cells from the glass substrate. An increase in the concentration of *T. vaginalis* led to a monolayer with fewer healthy McCoy cells. After six hours of co-culture, 10^4 *T. vaginalis* in 1 mL (parasite to target cell (P:C) ratio of 0.04:1) caused little damage to the monolayer. 10^5 *T. vaginalis* in 1 mL (P:C ratio of 0.4:1) caused much more damage, leaving less than half (48%) of the original McCoy monolayer intact. Conversely, 52% of the cells counted turned out to be adherent trichomonads. Even more extensive (almost total) destruction of the monolayer was detected after a six hour co-culture of the monolayer with 10^6 *T. vaginalis* in 1 mL (P:C ratio of 4:1). A scant 1.3% of the remaining cells were identified as McCoy cells; the large majority was adherent trichomonads.

Stages of Monolayer Destruction Following Exposure to *T. vaginalis*

A number of researchers have looked closely at the progression of monolayer destruction over time in response to co-culture with *T. vaginalis* (Hogue, 1943; Christian *et al.*, 1963; Farris and Honigberg, 1970; Heath, 1981; Alderete and Pearlman, 1984; Alderete and Garza, 1985; Silva Filho and de Souza, 1988). Since the current investigation was more focused on the endpoint condition of the McCoy cells (i.e. Are they apoptotic after co-culture with *T. vaginalis*?), the parasite/monolayer cell interaction throughout the co-culture was not closely monitored or photographed. In early experiments, however, periodic inspections of the co-culture revealed basic stages of monolayer destruction very similar to those described by Christian *et al.* (1963). Christian's group studied *T. vaginalis* infected human HeLa cell cultures and found that the parasite/cell relationship could be divided into four basic stages. The first stage gets underway when a monolayer of HeLa cells is exposed to an active culture of trichomonads. As was seen upon inoculation of our McCoy cell monolayers, the *T. vaginalis* spread freely over the HeLa monolayer but appear to have no negative effects. Stage Two takes place once the trichomonads settle onto the monolayer and when the chamber is observed from a side view, no parasites can be seen up in the culture media. Christian *et al.* (1963) reported that there was no way to tell the difference between the HeLa cells and the trichomonads, but in our experiment, the McCoy cells were longer (fibroblast shaped) and larger than the *T. vaginalis*.

The third stage of the parasite/monolayer cell interaction begins with the formation of small holes or "plaques" in the monolayer sheet where cells are detaching. Christian's group described these plaques as being lined with trichomonads, which were attached to the HeLa cells via the opposite side of the undulating membrane. Alderete and Garza (1985) later reported the same orientation when *T. vaginalis* was allowed to attach to HeLa cells in a monolayer. The parasites attached to the McCoy monolayers in our experiments also appeared to have this orientation because

distinctive *T. vaginalis* flagella motility could be noted even when the trichomonads could not be lifted from the monolayer by shaking the chamber slide.

Christian *et al.* (1963) went on to report that during Stage Three, the trichomonads infiltrated the area around the plaques by moving between, below and over the HeLa cells. They surmised that this sort of activity helped to dislodge the monolayer cells and would thus contribute to the widening of the plaques. It cannot be concluded that this happened in the McCoy co-cultures because we had more of a “bird’s eye view” through the inverted light microscope, which provided much less magnification, but could indicate more general movement and trends with respect to cellular detachment. Hence, we were able to see the formation of plaques in the McCoy monolayer.

According to Christian’s report, Stage Four begins when the plaques that began to form in Stage Three become more confluent. We also found that the plaques joined together and with higher inocula of *T. vaginalis*, virtually the entire monolayer lifted by the end of the co-culture. In Christian’s HeLa cell experiment, he found that after destruction of the monolayer, some of the trichomonads attached themselves, with pseudopods, to the glass substrate. We too found that with increased inocula of *T. vaginalis*, there were elevated numbers of trichomonads stuck to the glass substrate following co-culture. Other researchers have noted adherence of *T. vaginalis* to non-biological substrates such as glass (Cappuccinelli and Varesio, 1975; Cappuccinelli *et al.*, 1975; Heath, 1981; Bonilha *et al.*, 1992; and Crouch and Alderete, 1999). It has been suggested that the adhesiveness of *T. vaginalis* is likely dependent on parasite cell membrane properties and most importantly, to the presence of glycoprotein on the cell surface (Cappuccinelli *et al.*, 1975).

Appearance of *T. vaginalis* On Glass Substrate

Another confounding factor in the early co-culture experiments proved to be the fact that *T. vaginalis* has a flexible morphology; cellular appearance can be quite different depending on how the cells become attached to a surface. When trichomonads were allowed to settle onto the glass substrate, they took on the small, pear-shaped appearance that was first seen in the early co-incubation experiments. When *T. vaginalis* and McCoy cells were cultured separately and then combined to be cytopun onto a glass slide, however, the trichomonads flattened onto the glass and looked much larger than when they attached on their own.

Timing of Monolayer Destruction and Parasite/Cell Ratios

While the basic stages in the destruction of McCoy monolayers followed the same general path as those published in the literature, the timing of these steps was not always in accordance with those found in previous reports. The timecourse experiment conducted with two, four and six hour co-cultures of McCoy cells and *T. vaginalis* demonstrated how rapidly the monolayers were destroyed by the trichomonads. After only two hours of co-culture with 10^6 trichomonads (P:C ratio of 4:1), a count showed that less than 30% of the remaining cells came from the original monolayer. The rest were adherent *T. vaginalis*. After a four hour incubation, virtually all the monolayer cells had detached and were replaced by trichomonads. When cultured with fewer parasites, the monolayer destruction was less striking, but the trend remained the same.

A study conducted by Juliano *et al.* (1987) involving the co-incubation of *T. vaginalis* and mouse CL1D fibroblast cultures yielded results comparable to what was observed in our experiments. These researchers found that with the highest concentrations of trichomonads (10^6 parasites per well), the cytopathic effect was rapid and severe, with massive fibroblast detachment from the support in less than one hour. Subsequent experiments conducted with 2×10^5 *T. vaginalis* per

well showed adherence of the parasites to the monolayers after thirty minutes. By six hours, a large number of the fibroblasts had lifted from the slide surface, giving rise to the formation of “acellular, irregularly shaped areas” (Juliano *et al.*, 1987).

Most other researchers have reported that monolayer destruction by *T. vaginalis* happened via a slower progression of similar events. Kulda (1967) looked at the effect of various trichomonads on monkey kidney cell cultures. Similar to our observations, Kulda reported that *T. vaginalis* showed a distinct affinity for the monolayer soon after inoculation, but went on to say that most of the trichomonads had adhered to the monolayer only after four to eight hours of co-culture. Host cell changes were observed as the co-incubation progressed, and intercellular spaces were noted. Complete destruction of the monolayer was finally reported to have occurred after twenty-eight to thirty-two hours, an incubation time much longer than was necessary to see monolayer destruction in our experiments.

Similar to Kulda’s observations, Farris and Honigberg (1970) reported that a relatively pathogenic strain of *T. vaginalis* caused the formation of cell free areas or lesions in chick liver cultures (consisting of macrophages, epithelial cells, and fibroblasts) within two hours. The progression to complete destruction of the chick liver monolayer was more gradual, however, since this was not achieved until twenty-four hours after the start of co-culture. When the same experiment was done with a relatively mild, less adherent strain of *T. vaginalis*, at no time were the cultures riddled with lesions, and even after twenty-four hours of co-culture, the monolayers were still largely intact.

Heath (1981) followed the destruction of rabbit kidney tubule epithelial cell cultures by *T. vaginalis* (4×10^5 trichomonads per mL). The first signs of injury to the monolayer were apparent after two to three hours, but after six to ten hours, only 10% of the area of the monolayer

was destroyed. Lesion enlargement continued until about thirty-six hours after inoculation, when most of the cell monolayer was destroyed.

Rasmussen *et al.* (1986) showed that trichomonads settled onto and attached to human vaginal epithelial cell monolayers after one hour, but that monolayer cell destruction was apparent only after twenty-four hours of co-culture. Interestingly, this cytopathology was observed to a much greater degree when the researchers used a fresh isolate of *T. vaginalis* in their co-incubation with the vaginal epithelial cells. With the fresh parasites, they showed a distinct cytotoxic effect at a concentration of 10^3 trichomonads per well. When they employed a strain of *T. vaginalis* which had been cryopreserved, the parasites failed to cause much of a cytotoxic effect or to even attach to the host cell monolayer at concentrations of less than 10^5 to 10^6 trichomonads per well.

It is difficult to draw conclusions when comparing our findings with those of other researchers because of the strain differences with respect to virulence or cellular pathogenicity of *T. vaginalis* (Farris and Honigberg, 1970). In addition, *T. vaginalis* has been studied in co-culture with many different host cell types. This also makes direct comparisons among studies more complicated, because each host cell type is affected in a different manner and to a different degree (Pindak *et al.*, 1986). In a study by Alderete and Garza (1985), it was stated that parasite adherence was greater with HeLa and Hep-2 epithelial cells than with fibroblast cells, and that "cytotoxicity with fibroblast cells never exceeded 20% of the level of cell killing observed for epithelial cells". Lastly, variation in experimental design, particularly in the parasite to host cell (P:C) ratio employed, may have a large impact on the observed outcome. The number of cells making up a confluent monolayer in a given well or chamber is not always given, making it hard to compare ratios. In studies where this information was provided, it appeared that previous researchers generally needed a greater P:C ratio to achieve pathology comparable to what was seen in our experiments. In a study of the relationship between *T. vaginalis* and Marine-Darby canine kidney

epithelial cells (Silva Filho and de Souza, 1988), the researchers used a P:C ratio of 5:1 since ratios lower than 5:1 did not induce complete monolayer destruction until five days after the initiation of co-culture. As in previous examples, workers found that the initial process of epithelium destruction began rapidly (one hour after the start of co-incubation), but reported detachment of “many epithelial cells or whole fragments of the monolayers” from the substrate only after one whole day of co-culture (Silva Filho and de Souza, 1988). In a study of the effects of *T. vaginalis* on HeLa cell monolayers (Alderete and Pearlman, 1984), it was noted that co-cultures with P:C ratios of 4:1, 6:1 and 8:1 produced maximum cytotoxicity within eight hours, which is comparable to our results. Lower ratios were also tested. With a 1:1 P:C ratio, maximum cytotoxicity was not achieved until eighteen hours after the initiation of co-incubation.

Ultimate Fate of Detached McCoy Cells Following Co-Culture With *T. vaginalis*

In early co-culture experiments, the viability of the McCoy cells that had detached from the monolayer was not investigated because, at that time, it was not known that they were lifting from the substrate. Once this realization was made, however, an experiment was designed to determine the fate of the detaching cells. The goal was to determine if the six hour co-incubation of McCoy cells and 10^6 *T. vaginalis* truly destroyed the host cells or merely caused them to lift. After co-culture, the media was treated with metronidazole to eliminate trichomonads. The detached McCoy cells were then transferred to fresh media and were allowed to re-settle in clean chamber slides. Chamber slides were monitored for signs of reattachment. It was found that many of the McCoy cells (approximately 50%) were able to reattach once the trichomonads were removed. A portion of the cells (50%) remained detached in the media and these were presumed dead. It should be noted, however, that in control chambers where the cells were removed from the substrate by trypsinization and then allowed to reattach, the same phenomenon was observed; approximately 75% of the trypsinized cells reattached, while 25% remained detached. This

experiment indicated that while some cells were irreparably damaged by contact with the trichomonads, others remained viable. Our investigation into the fate of the co-cultured cells was not exhaustive, however, and there exists the possibility that the McCoy cells may have been damaged by the metronidazole treatment, washes and spins employed in this experiment.

A number of published studies on *T. vaginalis* pathogenesis have looked at the ultimate fate of detached host cells following co-culture with *T. vaginalis* (Hogue, 1943; Heath, 1981; Alderete and Pearlman, 1984; Krieger *et al.*, 1985). These researchers have generally arrived at the conclusion that when direct contact between the cultured cell line and the trichomonads was allowed, the result was death and not just detachment of the host cells. Hogue (1943) was one of the first investigators to look at the pathogenic mechanisms of *T. vaginalis*. While her report does not include extensive details on methods, Hogue does conclude that tissue culture cells in co-culture with trichomonads died before they were able to take up neutral red stain (a cationic, vital dye that is selectively taken up and concentrated by proliferating mitotic cells (VanderWerf *et al.*, 1997)). This was in contrast to tissue culture cells, on their own, which gave a “beautiful red stain and lived for days”.

A later study by Heath (1981) demonstrated rabbit kidney tubule epithelial cell death via contact with *T. vaginalis* by ten hours post-inoculation. No staining was done to assess epithelial cell integrity, but lysis was instead determined by electron microscope images, which showed a trichomonad lying “beneath a collection of cytoplasmic constituents...released by the rupture of the plasma membrane of an epithelial cell”. The parasite to cell ratio (P:C) is unclear, but appears to be within the same approximate range as was used in our work. The results reported by Heath do not necessarily disagree with what was seen in our experiments. While some of the detached McCoy cells did survive, it was apparent that others may have been irreparably damaged by contact with the trichomonads. Perhaps an electron micrograph of these cells would show lysis

like that shown in Heath's report. Similarly, had Heath transferred the detached epithelial cells to fresh media without the influence of *T. vaginalis*, some may have reattached.

A subsequent study by Alderete and Pearlman (1984), however, suggested that contact with *T. vaginalis* caused extensive cytotoxicity. Monolayers were co-incubated with varying concentrations of parasites in this study (P:C ratios of 1:4, 1:2, 1:1, and 2:1). Maximum cytotoxicity was found at the P:C ratio of 1:1, and resulted in a complete loss of HeLa cells after eighteen hours of co-culture. Shorter co-cultures were also done with larger P:C ratios (P:C of 4:1, 6:1, and 8:1 over an eight hour period). They concluded that initiation of monolayer disruption seemed to be a function of trichomonad densities in the incubation mixture. They went on to report that re-culture of detached HeLa cells was not even attempted because trypan blue uptake by all liberated monolayer cells was indicative of widespread irreversible host cell death (Trypan blue results were not shown, but were mentioned in the results section.). It is unknown if this general statement applies to all the liberated cells in all the co-cultures, or merely to the co-cultures with high P:C ratios where maximal cytotoxicity was demonstrated. The authors stated that the cytotoxicity was a function of trichomonad density, but also seemed to imply that HeLa cell death was an all or nothing phenomenon. This is in contrast to what was observed in our research, where varying degrees of pathology could be observed in the McCoy cells (i.e. some detached cells were viable while others were dead). Such a gradation in the pathology of host cells exposed to *T. vaginalis* was more evident in the tritium release assays reported in the same paper by Alderete and Pearlman (1984). The authors looked at ³H release from HeLa cells after co-culture with several densities of *T. vaginalis*. They found that a greater level of tritium was released from HeLa cells when they were incubated with a 10:1 ratio of *T. vaginalis* than when they were incubated alone. After six hours, they observed that approximately 30 to 35% of the original tritium contained in the HeLa cells was released into the culture medium. It should be noted, though, that in the control culture (with no trichomonads), the six-hour incubation time

resulted in the release of approximately 10 to 15% of the tritium in the HeLa cells. This suggests that while some HeLa cell death occurred as a result of co-culture with *T. vaginalis*, not all cells suffered the same fate.

Cell Free Filtrate Experiments-Cellular Detachment Without Trichomonad Contact

T. vaginalis culture supernatant (containing no trichomonads and adjusted to an appropriate pH) caused complete detachment of the McCoy cell monolayers with which it was incubated. The complete lifting of the monolayers was very rapid; there were few traces of the McCoy cells after only six hours of co-culture with the filtrate. 1:2 and 1:4 dilutions of the growth media did not lessen the ability of the filtrate to induce lifting of the monolayers from the substrate. The detached McCoy cells were recovered by cytopspin and were then assessed via the TUNEL staining technique. The detached McCoy cells remained whole and counterstained well. They did not stain brown at all, indicating a lack of DNA fragmentation (and hence no apoptosis) as a result of co-culture with *T. vaginalis* culture supernatant. This finding is in keeping with a study done by Garber *et al.* (1989) wherein filtrates obtained from twenty-two-hour growth of *T. vaginalis* totally disrupted McCoy and other monolayers within six hours. The cell-free supernatants were found to cause widespread detachment, but not McCoy cell death, when the pH was controlled. (This was determined by Trypan blue exclusion.) Garber *et al.* (1989) kept the pH at 6.5, while, in our study, pH was adjusted to a similar value of 6.6-6.8. Garber's group describes the same sloughing phenomenon, where monolayer cells round up and focal areas of detachment develop over the course of the co-culture experiment. The component of the filtrate thought to cause the detachment of monolayer cells *in vitro* (and likely vaginal epithelial cells *in vivo*), has been characterized and termed cell detaching factor (CDF). The cellular detachment observed is quite comparable to what has been reported when McCoy cells are co-cultured with whole, live *T. vaginalis*. Garber *et al.* (1989) report, however, that the main difference between

the contact-dependent and independent pathologies is the ultimate fate of the monolayer cells. With *T. vaginalis* contact, the McCoy cells do not merely detach, but perish.

A number of other authors have reported varying degrees of contact-independent pathology in cultured cell lines exposed to *T. vaginalis* (Hogue, 1943; Honigberg and Ewalt, 1963; Farris and Honigberg, 1970; Nielson and Nielson, 1975; Gentry *et al.*, 1985; Pindak *et al.*, 1986; Silva Filho and de Souza, 1988; Garber and Bowie, 1990; Pindak *et al.*, 1993). These studies employed both direct and indirect methods to prove that contact-independent pathology is a part of the *T. vaginalis* pathogenesis repertoire. Silva Filho and de Souza (1988) showed that trichomonad growth media filtrates caused destruction of epithelial cell monolayers in a manner similar to that seen with whole parasites. This idea was supported, indirectly, by their conclusion that treatment with trypsin alters the ability of *T. vaginalis* to adhere to monolayer cells, but does not affect pathology. Thus, even when contact is prevented by an external factor (trypsin), the trichomonads are able to cause pathology in the monolayer cells.

Pindak *et al.* (1986) published a report which further supports the idea that *T. vaginalis* employs contact-independent mechanisms. The authors concentrated CDF from growth filtrates and found that, when applied to monolayers, it caused cellular detachment. Furthermore, the same isolated factor prevented the attachment of healthy monolayer cells to substrate, leaving them viable but free-floating.

More indirect evidence to support contact-independent pathology has come from Nielson and Nielson (1975) who looked, via electron microscopy, at the interaction of *T. vaginalis* and the vaginal epithelium in disease. In some cases, their electron micrographs show gaps between trichomonads and damaged host cells in biopsies, suggesting that the pathological effects induced by the parasites may occur over a distance.

Farris and Honigberg (1970) suggested that the degree of pathology seen in response to a given supernatant differs depending on the isolate from which the supernatant was derived. Their experiments employ two different isolates-one described as relatively pathogenic and one designated to be comparably mild. These assessments were made based on clinical and pathological data from patient histories and on the subcutaneous mouse assay introduced by Honigberg *et al.* (1966). Farris and Honigberg (1970) found that the filtrates from growth of the more pathogenic strain had more destructive effects on chick liver cell cultures than did the filtrates from the growth of the less pathogenic strain.

In our experiments, an isolate designated "202" was used. Garber and Lemchuk-Favel (1990), attempted to correlate *in vitro* production of CDF with pathogenicity according to Honigberg's subcutaneous mouse assay, and *in vivo* clinical disease. In their study, isolate 202 was designated to be moderate with respect to clinical disease and they further concluded that CDF production and activity correlated significantly with this clinical disease assessment. This would seem to be in agreement with our findings that *T. vaginalis* 202 causes virtually complete detachment of the McCoy monolayer within six hours. Garber and Bowie (1990) used an isolate designated as severe (with respect to clinical disease severity and the mouse assay) and observed total destruction of the McCoy monolayer within two to four hours.

In contrast to our findings, a number of researchers have reported a lack of contact-independent pathology in *T. vaginalis* (Kotcher and Hoogasian, 1957; Christian *et al.*, 1963; Kulda, 1967; Alderete and Pearlman, 1984; Krieger *et al.*, 1985; and Rasmussen *et al.*, 1986). Gentry *et al.* (1985) proposed that contact-independent pathology may have gone undetected in some previous studies due to binding of the toxic agent to serum proteins. Gentry *et al.* found a decrease in

cytopathic effect in response to *T. vaginalis* lysates when the incubation was conducted in the presence of serum.

Christian *et al.* (1963), Alderete and Pearlman (1984) and Krieger *et al.* (1985) all concluded that if a soluble toxic factor is produced by *T. vaginalis*, it is likely present in small quantities or is a labile substance. Subsequent studies have shown that CDF is heat and acid labile, may account for some of the difficulty in detecting it. Garber *et al.* (1989) studied the effect of pH on McCoy cell monolayers. They found that exposure of McCoy cells to pH values of less than 6.0 resulted in rapid cell death, but had no effect on monolayer confluence. (This further supports the notion that contact-independent pathology is a phenomenon caused by a trichomonad-expressed factor, and is not a by-product of pH). With respect to the effects of temperature and pH on CDF, Garber *et al.* (1989) found that CDF activity could be abrogated by heating *T. vaginalis* growth filtrate to 59°C for thirty minutes, or by exposing it to a low pH (3.5-4.5). CDF activity was found to be present between pH values of 5.0 and 8.5, with a peak at a pH of 6.5. Garber and Bowie (1990) went on to study the involvement of culture pH in *T. vaginalis* pathology. This was done using a Millicell system, wherein the parasites and McCoy cells could be cultured in the same media, but were physically prevented from making direct contact. It was found that monolayer disruption occurred both in the presence and absence of parasite/McCoy cell contact. The rate of McCoy cell death was significantly lowered, however, when pH was rigidly controlled. This may indicate, then, that some of the cell death previously reported to be exclusively contact-dependent was in fact due in part to the decrease in pH level that occurs when *T. vaginalis* is grown *in vitro*. The other side of the coin, of course, is that when the pH is not controlled, the CDF activity is diminished due to the acid lability of this factor. This would help to explain the apparent discrepancy between what was found in our experiments and what was reported by those authors who did not observe pathology in response to co-culture with *T. vaginalis* supernatants. In our contact experiments, pH was not allowed to dip below 5.0, hence

we saw that many of the detached McCoy cells survived to reattach to fresh substrate. In our filtrate experiments, we controlled the pH and found that monolayer detachment (and therefore, CDF activity) was striking. The idea that contact-dependent cytotoxicity may be due, in part, to acid production in the co-culture media is supported by a study by Pindak *et al.* (1993). They concluded that production of acid metabolites by the parasite, whether in direct contact with the target (erythrocyte) cells or not, can lead to haemolysis or death of the cultured cells. It is noteworthy that very few of the studies that point to exclusively contact-dependent pathology document endpoint pH values. While it is very likely that contact between the parasite and the host cell does play an important part in the pathological process, contact-independent factors like CDF and acid metabolites (each of which exerts very different effects on host cells) likely also play a role in pathogenesis.

Conclusions

Contrary to the original hypothesis, *T. vaginalis* does not appear to induce levels of apoptosis in McCoy cell monolayers above those seen in control monolayers without the parasite. It was observed that the numbers of truly apoptotic cells remained at a low and constant level irrespective of the concentration (or even the presence) of *T. vaginalis*. Given that it has been established that the beige cells on these slides are not apoptotic McCoy cells, but adherent *T. vaginalis*, it must be concluded that the true low-level McCoy cell apoptosis observed does not result from co-culture with the parasite. The ELISA assays and caspase inhibition experiments all support this conclusion. It is difficult to link these *in vitro* findings with what may happen in the *in vivo* setting. Within the given parameters, we did not detect apoptosis in the McCoy target cells cultured with *T. vaginalis*, but apoptosis may still be involved in the observed clinical pathology. Perhaps *T. vaginalis* might induce host cell apoptosis in a more biologically relevant cell line like freshly isolated vaginal epithelial cells, or in a comparable cell system like the

human amnion membrane (which more closely mimics the cellular layers of the urogenital region). The McCoy cell monolayer is an accepted system for studying *T. vaginalis* growth and cytopathology, and numerous studies have shown that McCoy monolayers suffer marked pathology when co-cultured with trichomonads. However, it has been demonstrated that fibroblast cells, like McCoy cells, are generally more resistant to the negative effects of *T. vaginalis in vitro*. As with any *in vitro* study, care must be taken in concluding clinical relevance from laboratory findings. It might be interesting to assess the apoptotic status of vaginal epithelial cells as they are sloughed from the vaginal walls of trichomoniasis-sufferers.

While the pathological assault of *T. vaginalis* may not have been shown to incorporate the induction of apoptosis, it definitely included the destruction of the McCoy cell monolayer. In keeping with what has been shown by many previous researchers, we found that cell monolayers co-cultured with *T. vaginalis* and with *T. vaginalis* culture supernatants were rapidly disrupted and dissolved. A follow up experiment suggested that a portion of the cells cultured in contact with *T. vaginalis* may have been irreparably damaged. Given what has been shown by a number of different laboratories, *T. vaginalis* likely caused the lifting of the monolayers via the production of metabolites like CDF. This would make sense since the detachment was observed with and without trichomonad/McCoy cell contact. The irreparable damage seen in a portion of the McCoy cells cultured in contact with *T. vaginalis* may have been induced by a multi-step process that is slowly being characterized by pathogenesis researchers. It has been shown that this process relies, in part, on the production and employment of various adhesins, cysteine proteinases, and perforins. Clinical symptoms of trichomoniasis, including cell sloughing, and the development of lesions, are likely *in vivo* reflections of the *in vitro* experimental pathology observed in co-culture experiments like ours.

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APPENDIX I:

The Intravaginal Mouse Model of *Trichomonas vaginalis* Infection Revisited

Introduction

The second portion of the *T. vaginalis* research focused on the intravaginal mouse model of infection and the immunization of mice against the parasite.

***T. vaginalis* Vaccine Potential**

Research into the development of a vaccine against *T. vaginalis* has shown some promise in recent years. *T. vaginalis* strains possess unique as well as shared or common epitopes (Garber *et al.*, 1986; Wos and Watt, 1986 and Mason and Gwanzura, 1988), suggesting that an immune response mounted against antigens on an immunizing strain of *T. vaginalis* could potentially cross react with shared antigens found on an infecting strain of the parasite.

There have been two separate vaccine candidates used in human trials to combat *T. vaginalis* infection. The first was reported in the 1960s and involved one hundred women with refractory cases of trichomoniasis. The patients were intravaginally inoculated with increasing numbers of heat-killed *T. vaginalis*; all showed clinical improvement, but the controversial procedure has not been repeated since (Aburel *et al.*, 1963).

A second vaccine was introduced in the 1970s. This vaccine, known commercially as SolcoTrichovac or Gynatren, was manufactured from inactivated “abnormal strains of lactobacilli” isolated from vaginal secretions of patients suffering from *T. vaginalis* infection (Pavic and Stojkovic, 1983). A number of mechanisms were suggested by Pavic and Stojkovic (1983) to account for the proposed action of SolcoTrichovac on *T. vaginalis*, but research subsequent to the initial studies has shown that the fundamental idea behind the vaccine itself was

flawed (Gombosova *et al.*, 1986; Alderete, 1988; Spiegel, 1990). In future, it seems reasonable that vaccine development for *T. vaginalis* infection will make use of the trichomonad itself as a means of eliciting a specific and protective immune response.

***In vivo* Models of *T. vaginalis* Infection**

Trichomonad antibiotic resistance and refractory cases of trichomoniasis are becoming an increasing problem. Researchers hope that new drug and vaccine development may not only provide improved treatment, but may offer protective immunity and prophylaxis. Unfortunately, this research has been limited by a lack of understanding of the pathogenesis of the disease, and the role of the host immune response to *T. vaginalis* infection. A reliable and reproducible animal model of infection is required for proper standardized research into the pathogenesis, host immune response, and treatment of *T. vaginalis* infection.

A wide variety of animal models for *T. vaginalis* infection have been attempted (Jirovec and Petru, 1968; Honigberg, 1978; Kulda, 1990). The intraperitoneal models of Teras and Roigas (1966) and Cavier *et al.* (1972) and the subcutaneous model of Honigberg *et al.* (1966) have been used extensively for pathogenesis and drug studies. The intraperitoneal injection of *T. vaginalis* results in visceral lesions that can ultimately lead to death, while subcutaneous introduction of trichomonads produces a local abscess. The extent and severity of the host reactions to these manipulations was then used to estimate the degree of virulence in the experimental isolate used, but it has been suggested that the findings, with respect to virulence, did not correlate well with clinical disease (Garber and Lemchuk-Favel, 1990). A genital infection model would be preferable as it would mimic more closely the disease state in humans.

Vaginal infection of the squirrel monkey was found to produce symptomatic disease for up to three months, and horizontal transmission was achieved (Street *et al.*, 1983; Gardner *et al.*, 1987). The resulting immune response was weak, however, and the presence of indigenous trichomonads in the animals was problematic (Hollander and Gonder, 1985). The studies using this model have been small and infrequent, leaving many questions as to the suitability of the monkey as a test subject. The associated cost and upkeep of such a large and complex host animal is also prohibitive.

Establishing an intravaginal infection in the guinea pig has been shown to be relatively simple, requires no supportive estrogen therapy, and results in a symptomatic infection similar to that seen in humans. From this model, a series of reports examining the pathology of trichomoniasis was published (Soska *et al.*, 1962; Kazanowska *et al.*, 1983; Honigberg, 1978). Surprisingly, the guinea pig model did not progress beyond the scope of the original research. The model has however been used in some drug studies with the bovine trichomonad pathogen *Tritrichomonas foetus* (Maestrone and Semar, 1967; Michaels, 1968).

The intravaginal infection of the hamster, also achieved without estrogen treatments, persists up to several months, but has proven asymptomatic. The hamster model has been used mainly in *T. foetus* (Ryley and Stacey, 1963) and *T. vaginalis* (Michaels, 1968; Wildfeuer, 1974; Kulda, 1990) drug assays. As with the squirrel monkey, the hamster harbours indigenous trichomonads, rendering the model and results obtained from it, potentially unreliable (Kulda, 1990).

The rat is a popular laboratory animal whose physiological and immunological profile has been thoroughly researched, so it made sense that researchers attempted to use this animal in *T. vaginalis* infection studies. Intravaginal infection of the rat can be accomplished, but only after the surgical removal of the ovaries and subsequent treatment with estrogen (Cavier and Mossion,

1956; Meingassner *et al.*, 1981), thus making it a less than ideal choice for an infection model.

Intravaginal infection in the rat lasts a few weeks, and is generally asymptomatic. The rat model has been used in some drug studies.

The Intravaginal Mouse Model of Infection

With the resurgence of interest in finding novel antitrichomonal therapies, the mouse has gained attention as an appropriate animal for use in models of trichomonad infection. Mice have the potential to be good animal model candidates for many reasons. They are readily available, have been thoroughly researched with respect to immunological and physiological parameters, they are relatively inexpensive and their husbandry is simple. Furthermore, *T. vaginalis* infection can be achieved in the mice without preparatory surgery, and they are free of the indigenous trichomonads that have confounded many other animal models (Meysick and Garber, 1992; McGrory and Garber, 1992)

Mouse intraperitoneal and subcutaneous *T. vaginalis* infections have provided much useful information regarding drug pharmacology and parasite pathogenesis, however, the intravaginal infection model has the most relevance to human vaginal infection. Early attempts to colonize the mouse with trichomonads via the intravaginal route were met with limited success due to technical difficulties. Research was impeded by the fact that the animal models employed did not closely replicate the milieu found in the human vagina, where the epithelium is exposed to estrogen through most of the menstrual cycle (Larsen, 1993).

Susceptibility of laboratory animals to intravaginal infection is related to their hormonal status and varies with changing phases of the sexual cycle (Kulda, 1990). The estrous cycle of mice includes four separate phases: proestrus, estrus, metestrus and diestrus (Jacoby and Fox, 1984).

Optimal conditions for intravaginal growth of *T. vaginalis* can be found during proestrus and estrus, when an augmented level of estrogen is present, and neutrophils and bacteria are at a minimum (Corbeil *et al.*, 1985). Jirovec and Petru (1968) went further to suggest that *T. vaginalis* can grow only in an environment that includes estrogen. To keep the animals in protracted estrus, researchers have adopted a supportive treatment with estrogen, thus preventing the animals from cycling into the less desirable stages of metestrus and diestrus (Kulda, 1990).

The first attempt at murine intravaginal inoculation was made by Patten *et al.* (1963), when they repeatedly introduced *T. vaginalis* into the mouse vagina over a number of weeks. They examined the pathology in response to continuous introduction of the organisms, but they did not verify the duration of infection, nor did they monitor for the presence of trichomonads between inoculations. Subsequent researchers, however, were more interested in the establishment of an infection after an initial introduction of the parasite. Cappuccinelli's group (1974) was the first to establish successfully an intravaginal *T. vaginalis* infection in the mouse with a preliminary introduction of the organism, and exogenously administered estrogen. This group suggested that the estrogen therapy was necessary for induction of infection, but not for maintenance. A number of ensuing studies have supported the need for estrogenization (Coombs *et al.*, 1986; McGrory and Garber, 1992; Meysick and Garber, 1992; Abraham *et al.*, 1996). There have been inconsistencies, however, in the duration of infection in estrogenized mice.

The factors directly responsible for the enhancement of growth of trichomonads in estrogenized animals are unknown and the exact role of this hormone is still under debate (Van Andel *et al.*, 1996). There may be a possible association between increased susceptibility to infection and accumulation of glycogen, a substrate that may be used as a nutrient source by *T. vaginalis* (Kulda, 1990). The relationship is likely more complex and may involve factors such as the

modification of vaginal epithelial cells, changes in the normal vaginal flora, and the number of protective neutrophils in the vagina (Corbeil *et al.*, 1985).

While it is generally accepted that estrogen levels and stage of estrous are important in the establishment of an intravaginal *T. vaginalis* infection, sustained estrogenization alone is insufficient to support long-term genital *T. vaginalis* infection in a murine model (Van Andel *et al.*, 1996). Investigators have therefore made an attempt to alter the mouse vaginal environment so that it more closely reflects that human vaginal milieu. Meingassner (1977) co-inoculated mice with *Candida albicans* in order to secure a more reliable model for use in drug testing. Vaginal flora and pH are markedly dissimilar in mice and humans; high numbers of lactobacilli and low pH are hallmarks of the human vagina (Paavonen, 1983; Hanna *et al.*, 1985; Larsen, 1993), while only a small percentage of mice harbour lactobacilli and mouse vaginal pH is neutral (Meysick and Garber, 1992). With this fact in mind, McGrory and Garber (1992) introduced *Lactobacillus acidophilus* into the vaginas of mice prior to inoculation with *T. vaginalis* and observed a significantly more consistent and sustained infection. Of mice preinoculated with *L. acidophilus* prior to introduction of *T. vaginalis*, sixty-nine percent were still infected (with trichomonads) after twenty-four days; only eleven percent of the control group (no *L. acidophilus*) maintained the infection. They also noted that the addition of *L. acidophilus* did not otherwise significantly alter the resident vaginal flora found in the mice.

The precise nature of the relationship between *L. acidophilus* preinfection and greater duration of *T. vaginalis* colonization is not clear. Lactobacilli may become a nutrient source for the *T. vaginalis*, when the invading trichomonads phagocytose the bacteria. The prolonged infection seen in the presence of *L. acidophilus* may also be attributed to direct or indirect actions of the lactobacilli on the vaginal environment, providing conditions which help facilitate the establishment of a long-term *T. vaginalis* infection (McGrory and Garber, 1992).

Murine strains are not equally susceptible to infection with *T. vaginalis*. Experiments conducted by Landolfo *et al.* (1981) support the use of the BALB/c mouse strain for such studies. While investigating the genetic control of resistance or susceptibility to *T. vaginalis* infection in various strains of mice, this group found that female BALB/c and DBA/2 mice are highly susceptible to intraperitoneal, subcutaneous, and intravaginal infection with the parasite. Common laboratory strains C57BL/6 and C3H female mice display intermediate levels of resistance following intraperitoneal or subcutaneous inoculation, whereas they show high levels of resistance to intravaginal infection (Landolfo *et al.*, 1981), and would thus be inappropriate for use in the intravaginal infection model. The age of the mice also plays an important role in the establishment of infection. Landolfo *et al.* (1981) found that susceptibility of BALB/c mice to *T. vaginalis* decreases with age; susceptibility is maximal at three to four weeks and minimal at forty to forty-two weeks of age.

The Intravaginal Mouse Model of Infection and the Anti-*T. vaginalis* Vaccine

The intravaginal mouse model can be employed in the search for a suitable *T. vaginalis* vaccine. With the potential for drug intolerance in some patients, and the emergence of increasing numbers of metronidazole-resistant strains of *T. vaginalis*, such a strategy may prove to be very important. While the research has been relatively limited, promising data have been published (Abraham *et al.*, 1996) that demonstrate the efficacy of a whole-cell vaccine preparation against *T. vaginalis*.

The published vaccine protocol works around the timeline of the vaginal infection protocol, and begins fifty-six days prior to the intravaginal inoculation of the mice with *T. vaginalis*. On Day -56, log-phase *T. vaginalis* is harvested, suspended in Freund's complete adjuvant, and is then injected subcutaneously into the mice. A similar booster immunization, with the trichomonads

suspended in Freund's incomplete adjuvant, is given at Day -28. Once the *T. vaginalis* is suspended in the adjuvant, the cells are rendered immobile and cease to grow (Abraham *et al.*, 1996).

Abraham *et al.* (1996) showed that mice that were vaginally infected, treated with metronidazole, and reinfected vaginally, did not develop protective immunity or an increased ability to clear infection. This mirrors the human condition, where women suffer numerous reinfections without the development of protective immunity. However, mice which had received subcutaneous immunization and boosting with the *T. vaginalis* vaccine prior to intravaginal challenge, were significantly less likely to develop infection. Only twenty-five percent of immunized mice were infected after seven days, while ninety-one percent of sham-immunized and naïve mice were infected at this time point. The mice also demonstrated a significantly increased rate of trichomonad clearance when compared with control animals. While only four percent of vaccinated mice still harboured the organism after twenty-eight days, fifty-two percent of those not immunized with *T. vaginalis* vaccine were still infected. In addition, a measurable serum and vaginal antibody response was observed in mice immunized with *T. vaginalis* prior to intravaginal challenge (Abraham *et al.*, 1996).

Thus, it appears that the method of antigen presentation to the immune system (systemic versus vaginal) may be important in inducing a measurable immune response. While there is still much work to be done, this study supports the notion that a vaccine could be an effective anti-trichomonal therapy.

Hypothesis and Objectives

Hypothesis

It was predicted that by pre-treating mice with *L. acidophilus* and estrogen, there would be a sustained *T. vaginalis* infection established in the mice, similar to what had been published previously. The hypothesis was that in cross-protection studies, immunization with one isolate of *T. vaginalis* would protect the mice against a second isolate of *T. vaginalis*.

Objectives

The initial objective was to reestablish the intravaginal mouse model of *T. vaginalis* infection in order to continue studies designed to further our knowledge of the immunological relationship between host and parasite. Specifically, the intention was to conduct cross-protection studies with different immunizing and challenging isolates of *T. vaginalis*.

Materials and Methods

Microorganisms

T. vaginalis

T. vaginalis culture (100 mL) was grown to mid log phase in preparation for inoculation into the mice. On inoculation days, the *T. vaginalis* culture was enumerated by hemacytometer and the total number of organisms was then calculated. The cells were harvested by centrifugation (Omnifuge RT) for ten minutes at 900 x g (at 4°C), and were then washed three times in pre-cooled, sterile PBS (pH 7.2). The final pellet was then resuspended in pre-warmed (37°C) media (TYI-S-33 supplemented with 10% FBS and 0.32% agar (QueBact, Quelab Laboratories, Montreal, Canada)) to yield a final concentration of 2.5×10^7 cells per mL (McGrory and Garber, 1992; Meysick and Garber, 1992; Abraham *et al.*, 1996). Table 8 outlines the timing of the basic elements of the intravaginal infection protocol.

Lactobacillus acidophilus

L. acidophilus was obtained from the American Type Culture Collection (ATCC), (Rockville, MD; strain #4356) (McGrory and Garber, 1992; Abraham *et al.*, 1996). A start-up culture was streaked on Bacto Lactobacilli MRS agar plates (Difco Laboratories, Detroit, MI), and was then subcultured into 10 mL Bacto Lactobacilli MRS broth (Difco Laboratories) in glass screw-capped tubes. Cultures were then incubated at 37°C in 5% CO₂, and were passaged every two to three days.

On the day before inoculation of *L. acidophilus* into the mice, a 1 L volume of MRS broth was seeded with 1 mL of pure *L. acidophilus* subculture (grown to log phase). The culture was then left to grow overnight at 37°C in 5% CO₂. On inoculation days, the culture was quantified using

Table 8: Intravaginal Mouse Infection Protocol

This outlines the manipulations carried out in preliminary experiments. In subsequent experiments, it was necessary to deviate from the basic protocol in order to investigate various factors more closely.

Day of Protocol	Procedure
Day -9	<ul style="list-style-type: none"> ▪ subcutaneous injection of Delestrogen
Day -7	<ul style="list-style-type: none"> ▪ vaginal smear and intravaginal inoculation with <i>L. acidophilus</i>
Day -6	<ul style="list-style-type: none"> ▪ intravaginal inoculation with <i>L. acidophilus</i>
Day -2	<ul style="list-style-type: none"> ▪ subcutaneous injection of Delestrogen
Day -1	<ul style="list-style-type: none"> ▪ vaginal wash to check for <i>L. acidophilus</i>
Days 0 and 1	<ul style="list-style-type: none"> ▪ intravaginal inoculation with <i>T. vaginalis</i>
Days 7, 14, 21	<ul style="list-style-type: none"> ▪ vaginal washes for <i>T. vaginalis</i> (washes are cultured and monitored for presence of live trichomonads)

a spectrophotometer to measure absorbance at 650 nm (Beckman DU-88). Organisms were harvested by centrifugation for ten minutes at 5000 x g at 4°C (Beckman Centrifuge, Model J2-21M). The pellet was then washed with pre-cooled sterile PBS (pH 7.2) three times and resuspended in MRS broth to yield a final concentration of 10¹⁰ *L. acidophilus*/mL. In order to verify the concentration of *L. acidophilus* inoculated into the mice, serial ten-fold dilutions in PBS were made from the original inoculum. The exact number of *L. acidophilus* colony forming units (cfu) was determined by spreading 100 µL of each dilution on to MRS plates. The colony forming units were then counted after a forty-eight hour incubation period at 37°C (5% CO₂) (McGrory and Garber, 1992; Abraham *et al.*, 1996).

Animal Care

Female BALB/c mice (Charles River Co., Montreal, Canada) were approximately six to eight weeks of age or 22 to 24 g in weight at the beginning of each experiment. Mice were housed in plastic cages and were supplied with food and water *ad libitum*. They were provided with twelve hours of artificial illumination each day. All aspects of housing and husbandry were performed (under Protocol #OGH-58) within the regulations set forth by the University of Ottawa Animal Care Committee and the Canadian Council on Animal Care (Olfert *et al.*, 1993). In order to provide environmental enrichment, mice were also provided with nesting material and polyvinyl chloride (PVC) tubes. Mice were also weighed regularly and inspected to assess health status.

Pretreatment of Mice with Estrogen

Mice were pretreated with estrogen in one of two ways-injection or pellet implantation. Injections were subcutaneous and each delivered 0.05 mL of Delestrogen (estradiol valerate 10 mg/mL; Squibb Canada, Montreal, Canada) (Cappuccinelli *et al.*, 1974; McGrory and Garber, 1992; Meysick and Garber, 1992; Abraham *et al.*, 1996).

Estradiol valerate pellets (1.5 mg twenty-one day release pellets) (Innovative Research of America, Inc., Sarasota, FLA) were implanted surgically. Animals receiving pellets were first given an analgesic injection and were then anaesthetized with halothane/O₂ prior to and during surgery. BNP ointment was put in each eye to prevent drying, and 1 mL of saline was injected subcutaneously into the hind end of each mouse to prevent dehydration. A small area on the back of the mouse was then shaved and disinfected with a savlon/alcohol solution. The pellet was inserted subcutaneously through a small hole in the skin on the back of the mouse. The hole was then pinched together with tweezers, and the halothane was shut off. (In some mice, the hole was clipped shut with a small suture, which was removed two days later). Once the pellet was in place, the mouse was then placed in a warm incubator for a short recovery period. Animals were monitored closely after surgery to ensure a healthy resumption of activity.

Determination of Mouse Estrous Stage

The estrous stage of each mouse was assessed by vaginal smear. A swab, constructed by wrapping a small amount of cotton around the tip of a round toothpick, was moistened with saline, and then gently rolled in the vagina. The swab was then removed and the collected cells were rolled onto a clean glass slide. The cell smear was fixed onto the slide with CytoPrep (Fisher Scientific, Nepean, Canada). The smear was then stained with the Diff-Quick Stain Set (Dade Diagnostics, Aguada, PR), and examined to determine stage of estrous.

Vaginal Inoculation of Mice

The inocula were introduced into the mouse vagina with an Eppendorf pipette (Brinkmann Instruments, Westbury, NY). The inoculum (20 µL of *T. vaginalis* or *L. acidophilus* suspension) was delivered by carefully inserting the tip of the pipette into the vagina and dispelling the contents of the pipette tip.

Vaginal Washes in Mice

The wash technique was similar to the inoculation procedure. The pipette tip was inserted into the vagina and 50 μ L of wash media (TYI or MRS media depending on whether *T. vaginalis* or *L. acidophilus* was to be recovered) was injected and drawn back into the pipette several times until it was turbid (McGrory and Garber, 1992; Meysick and Garber, 1992; Abraham *et al.*, 1996).

One to two days before *T. vaginalis* inoculation, mice were washed with pre-warmed (37°C) MRS broth in order to check for *L. acidophilus* infection. The wash material recovered from each mouse was incubated separately in glass screw-capped tubes containing MRS supplemented with 5 μ g/mL ciprofloxacin (Cipro I.V., Bayer, Etobicoke, Canada) and 20 μ g/mL metronidazole, which would inhibit growth of other vaginal bacteria that might multiply and confound results. The tubes were incubated at 37°C in 5% CO₂ for twenty-four to forty-eight hours.

To detect *T. vaginalis* infection, washing was done with 50 μ L of pre-warmed TYI medium supplemented with 10 % FBS. The recovered wash material was transferred into glass tubes containing pre-warmed TYI supplemented with 300 U/mL penicillin, 300 μ g/mL streptomycin, 2.5 μ g/mL amphotericin B, and 10 μ g/mL gentamicin (Gentamicin Reagent Solution, Gibco BRL). As before, the antibiotics were incorporated to prevent unwanted bacterial contamination. The tubes were incubated at 37°C (5 % CO₂) at a 45° angle, and were examined daily with an inverted microscope; the presence of motile *T. vaginalis* was noted (McGrory and Garber, 1992; Meysick and Garber, 1992; Abraham *et al.*, 1996). To avoid false negatives, lack of infection was concluded only after two consecutive negative washes (Coombs *et al.*, 1986; Abraham *et al.*, 1996).

RESULTS

By reestablishing the intravaginal mouse model that had been published in the literature, it would be possible to continue to further the study of the immunological relationship between the mouse host and the *T. vaginalis* parasite. Specifically, once the infection model was reestablished, it could then be used to look at cross protection among isolates of *T. vaginalis*.

In these experiments, mice were pretreated with estrogen and *L. acidophilus* as outlined in published accounts of the model (McGrory and Garber, 1992; Abraham *et al.*, 1996). A short-term *T. vaginalis* colonization was achieved, but a marked decrease in the numbers of “infected” mice by seven days post-inoculation was noted. In order to investigate the effectiveness of a vaccine against *T. vaginalis* and to do more in depth cross-protection studies, it would be necessary to initiate and maintain a more long-term infection in these mice. It was for this reason, then, that the protocol was repeated in various forms, the aim being the identification of the factors preventing the establishment of a reliable long-term infection in the mice.

Table 8 outlines the basic elements of the mouse intravaginal infection protocol. The most preliminary experiments (done with an isolate named DG) followed this outline and the results showed a lack of *T. vaginalis* infection in all mice at Days 7, 14 and 21 (data not shown).

The Role of the *T. vaginalis* Isolate in the Mouse Infection Model

The first aspect of the intravaginal mouse model to be addressed was the actual isolate of *T. vaginalis* used to infect the mice. Well-characterized isolate 263 was employed as an alternate to isolate DG, which had been used in preliminary experiments. These *T. vaginalis* isolates, originally harvested from infected patients, have been used extensively in the laboratory for the

last ten years (Garber and Lemchuk-Favel, 1990; Meysick and Garber, 1992; McGrory and Garber, 1992; Abraham *et al.*, 1996).

Table 9 shows that while live trichomonads could be recovered from the mice one day after inoculation, these numbers dropped off by seven and fourteen days post-inoculation. The Day 1 wash was not in the original protocol, but was conducted just to be sure that the trichomonads were surviving the initial inoculation into the mice. It was not known if a vaginal wash at this early timepoint would have a detrimental effect on the establishment and maintenance of an infection, so only a representative portion of each group (half) was washed. Neither isolate led to a long-term infection in the mice. In response to concern that the spins and washes used to prepare the trichomonads may have had some detrimental effect on the viability of the *T. vaginalis*, an aliquot of the prepared *T. vaginalis* sample used to vaginally infect the mice was passed into fresh media. The passaged *T. vaginalis* grew well and this proved that the isolation protocol did not harm the *T. vaginalis*; they were in fact still viable when introduced into the mouse. It should also be noted that in this experiment, none of the washes done on Day-1 showed any evidence of live *L. acidophilus*. Later experiments would investigate the *L. acidophilus* portion of the protocol in further detail.

The Importance of Estrous Stage in Establishment of the Intravaginal Infection

Early experiments suggested that not all the mice sampled were in the desired stage of estrous (i.e. proestrus or estrus when fewer potentially inhibitory neutrophils and bacteria are present in the mouse vagina). In order to investigate this area of the protocol, daily vaginal smears were incorporated such that the stage of estrous could be assessed throughout the given timeline. To help illustrate the different populations of cells seen at each stage of the estrous cycle, representative vaginal smears have been included here (Figure 13a-d).

Table 9: Importance of the *T. vaginalis* Isolate in the Mouse Model

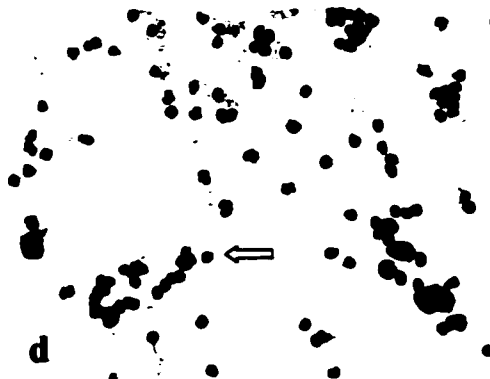
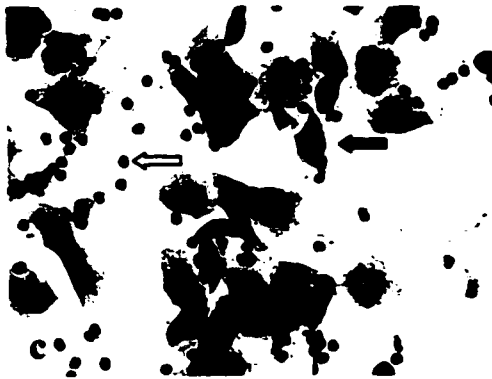
The intravaginal mouse infection protocol was conducted with two different *T. vaginalis* isolates. Live trichomonad recovery dropped off by seven and fourteen days post-inoculation. Isolate did not appear to influence intravaginal survival of *T. vaginalis*.

Estrogen Delivery	Infection Protocol	<i>L. acidophilus</i> Recovery	Live <i>T. vaginalis</i> Recovery		
		Day -1	Day 1	Day 7	Day 14
Days -9/-2	<i>L. acidophilus</i> : Days -7/-6 <i>T. vaginalis</i> (DG): Days 0/1	0/4	2/2*	2/4	0/4
Days -9/-2	<i>L. acidophilus</i> : Days -7/-6 <i>T. vaginalis</i> (263): Days 0/1	0/6	3/3*	2/6	0/6

*Note: Half the mice were washed on Day 1.

Figure 13: Mouse Vaginal Smears to Determine Stage of Estrous

Cells in vaginal smears were fixed onto glass slides and then stained with Diff-Quick Stain Set. **(a)** Panel shows cells representative of a mouse in proestrus. Note the predominance of round nucleated cells (closed arrow) and a lack of leukocytes. **(b)** Smear shows cells commonly found during estrus. Anucleated cornified epithelial cells prevail (closed arrow) while leukocytes are scarce. **(c)** The hallmark of metestrus, the presence of high numbers of leukocytes (open arrow) together with scattered cornified cells (closed arrow), can be seen here. **(d)** A preponderance of leukocytes (open arrow) is found during diestrus.



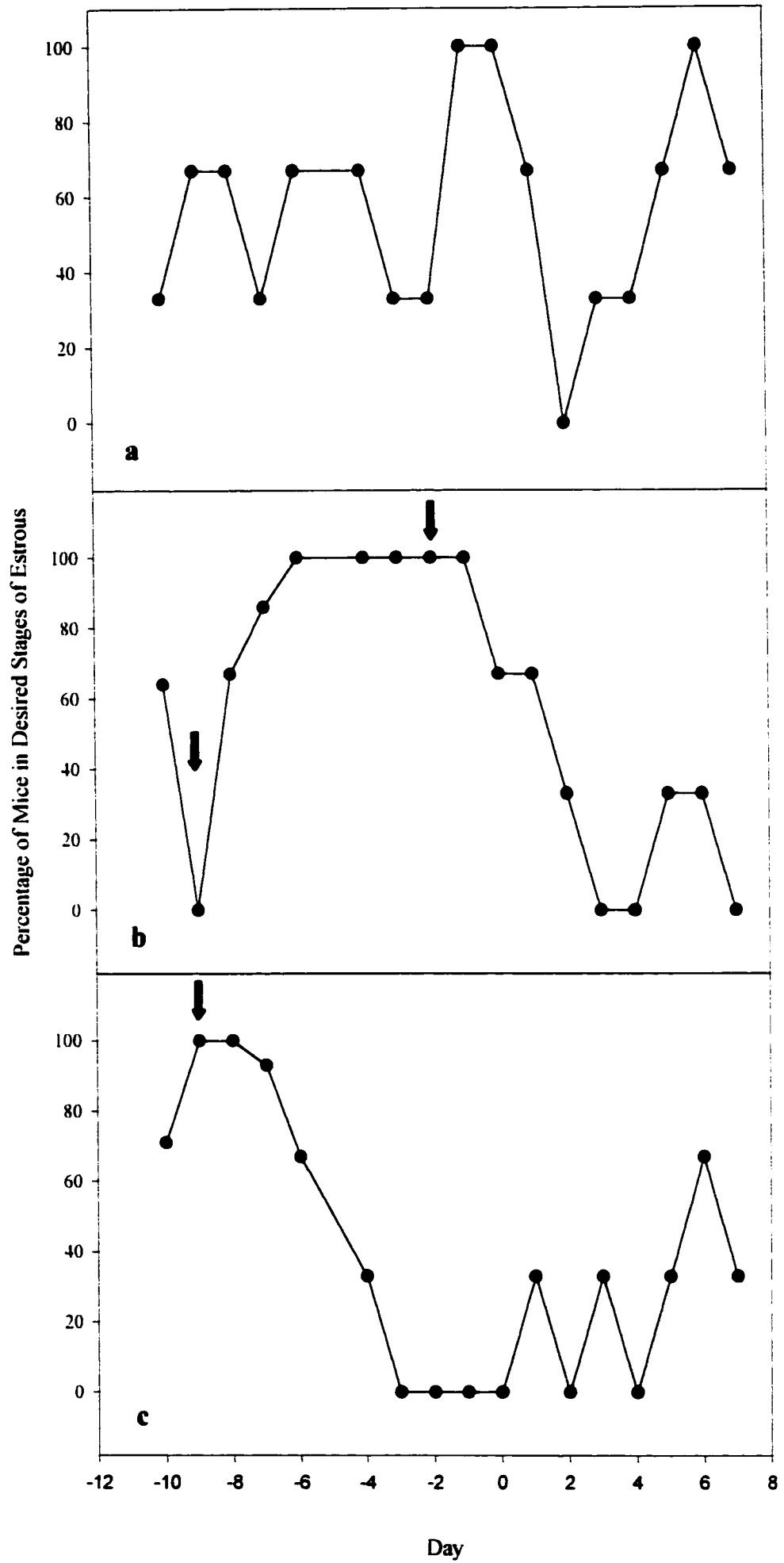
Stage of estrous was estimated by comparison with established criteria (Fox and Laird, 1970; Jacoby and Fox, 1984). The exfoliative cytology at proestrus is distinguished by a lack of leukocytes, and a predominance of round nucleated cells (closed arrow in Figure 13a). Estrus is distinguished by the presence of anucleated cornified epithelial cells (closed arrow in Figure 13b), and a scarcity of leukocytes. In metestrus, high numbers of leukocytes (open arrow in Figure 13c) can be found together with scattered cornified cells (closed arrow in Figure 13c) and nucleated squamous cells. Diestrus is identified by a predominance of leukocytes (open arrow in Figure 13d) (Kulda, 1990).

This experiment also incorporated a new technique for delivery of estrogen to the mice. Estrogen pellets, designed to deliver a specific amount of hormone over a set period of time, were implanted into a portion of the mice, with the hope that this alternate system of estrogen delivery would result in more consistency with respect to estrous stage.

As can be seen in Figure 14, the pellet system did not lead to maintenance of proestrus/estrus in the majority of mice. Instead, there appeared to be an initial spike in the percentage of mice in estrus after the implantation of the pellet (arrow in Figure 14c), with a gradual decline throughout the rest of the sampling period. When estrogen was delivered via an injection, the percentage in estrus peaked a few days after the first injection (first arrow in Figure 14b). This peak was maintained for about five or six days and then the numbers of mice in estrus started to decline gradually around the Day 0 mark of the protocol. It seemed that the second estrogen injection (given at Day -2) (second arrow in Figure 14b) had a much more diminished effect on the estrous cycles of the mice when compared with the initial (Day -9) injection. A pattern is not readily apparent in the untreated control mice, which cycled in and out of the desired stages of estrous rather rapidly.

Figure 14: Effect of Estrogen Delivery Method on Estrous Stage

(a) Control mice, given no external hormone, cycled in and out of proestrus/estrus. **(b)** In those animals given estrogen by injection (closed arrows), the percentage in the desired stage of estrous peaked initially, but started to decline around Day 0 of the protocol. **(c)** Implantation of the estrogen pellet (closed arrow) in the animals led to an initial peak in the percentage in proestrus/estrus. This effect was short-lived, however, and the percentage dropped rather rapidly giving way to the same cycling in and out of the desired stage that had been observed in the control group.



The pellets did not lead to greater rates of infectivity in the estrogenized mice (Table 10). In fact, the best rates of infection were found in those animals that had been estrogenized by injection as per the usual protocol. Therefore, it was concluded that while the pellets were designed to deliver a set amount of hormone over a specific period of time, this did not afford greater consistency with respect to estrous stage, nor did it lead to improved rates of infectivity.

The next experiment to address the question of estrous stage involved doubling the frequency of estrogen injections to see if this would lead to better maintenance of the desired stage of estrous in the mice. One group of mice was given the usual dosage of estrogen at Days -9 and -2, while animals in a second group were given two extra doses of the hormone. These individuals received the normal dosage of estrogen at Days -9, -3, 2 and 7, resulting in an overall doubling of the estrogen given. Figure 15 illustrates the results found when the mice were sampled for estrous stage. The percentage of mice in the desired stage of estrous varied in the control group (similar to what was observed in the previous experiment) (Figure 15a). In the group receiving the normal dosage of estrogen, the percentage in proestrus/estrus peaked two days after the administration of the first estrogen dose (first arrow in Figure 15b), but dropped off fairly rapidly (by Day -4). By Days 0 and 1 (vaginal *T. vaginalis* inoculation), the majority of the mice were not, in fact, in the desired stage of estrous. A similar peak and drop-off, after the initial estrogen dose (first arrow in Figure 15c), was seen in the third group of mice (those given augmented estrogen treatments). The percentage of mice in the desired stage of estrous remained low even after the subsequent doses of estrogen were delivered at Days -3, 2 and 7 (Figure 15c). As in the last experiment, the first dosage of estrogen appeared to have had a much greater impact on estrous stage when compared with the subsequent treatments.

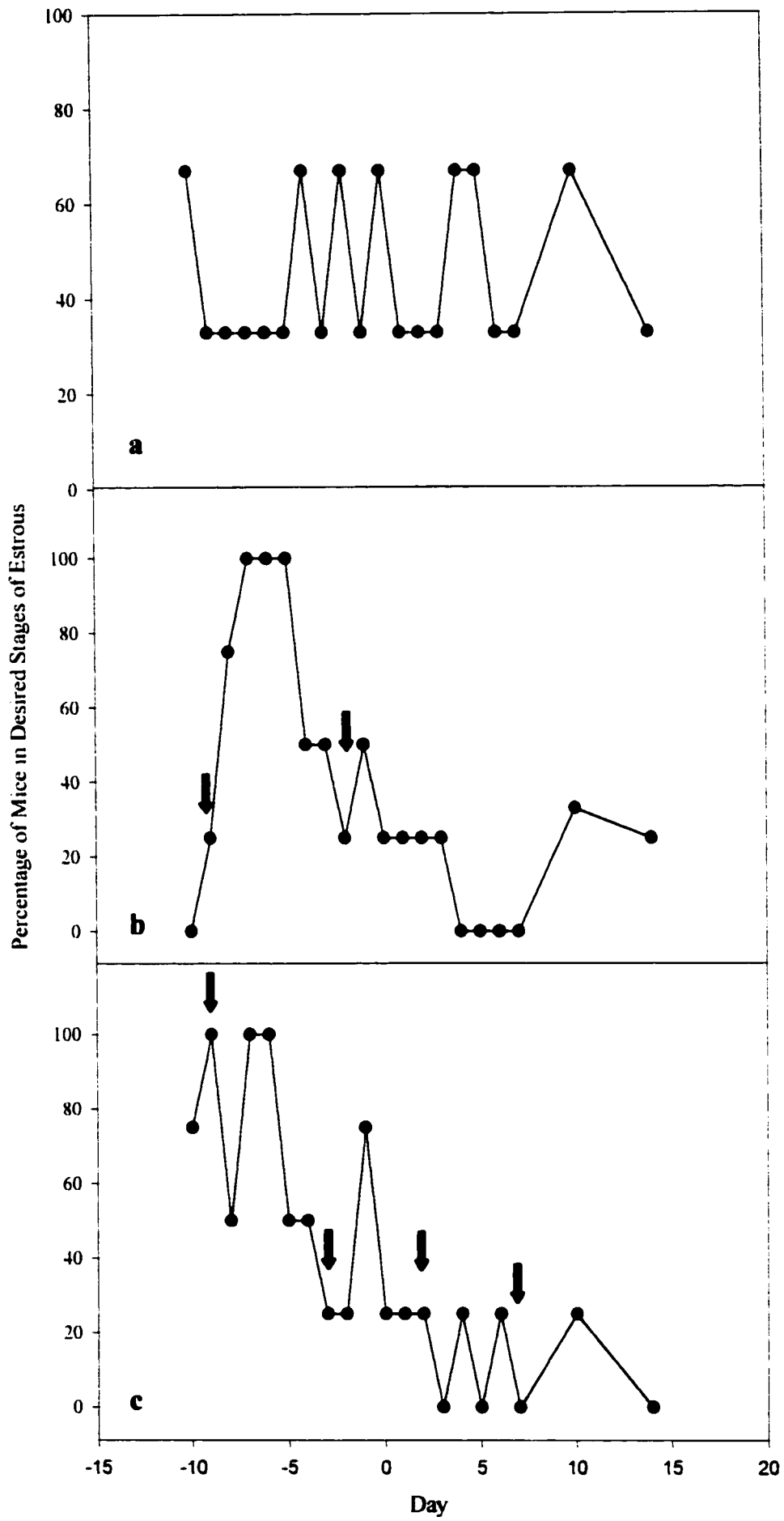
Table 10: Estrogen Delivery Method and Establishment of Intravaginal Infection

The intravaginal mouse infection protocol was altered with respect to delivery of estrogen. In half of the mice, estrogen was given via slow release pellets that had been implanted subcutaneously. The pellets did not lead to increased recovery of *L. acidophilus* or *T. vaginalis* from mice.

Estrogen Delivery	Infection Protocol	<i>L. acidophilus</i> Recovery at Day -1	Live <i>T. vaginalis</i> Recovery		
			Day 3	Day 7	Day 14
Estrogen by injection (Days -9 & -2)	<i>L. acidophilus</i> : Days -7 & -6 <i>T. vaginalis</i> : Days 0 & 1	4/4	10/11	7/11	3/11
Estrogen pellet (Day -9)	<i>L. acidophilus</i> : Days -7 & -6 <i>T. vaginalis</i> : Days 0 & 1	1/3	4/11	2/11	1/11

Figure 15: Effect of Extra Estrogen Injections on Estrous Stage

(a) Control mice, given no external hormone, cycled in and out of the desired stages of estrous (proestrus/estrus) rapidly. (b) In the group given the usual dosage of estrogen (injections indicated by closed arrows), an initial peak in the percentage of mice in the proper stage of estrous was observed. This was followed by a rather rapid decline by Day -4. (c) Mice in the last group received double the number of injections (closed arrows), but there was observed the same decline in the percentage of mice in the desired stages of estrous after an initial peak.



Doubling the number of doses of estrogen did not have any effect on infection rates in the mice (Table 11). Mice given extra estrogen did not harbour trichomonads for a greater period of time and the number of infected mice dropped dramatically after Day 7.

It appeared then, that despite much variation in the delivery of estrogen to the mice, there remained inconsistency with respect to maintenance of the desired stage of estrous. The role that this inconsistency may have played in infection (or lack thereof) in the mice remains to be seen.

***L. acidophilus* and its Role in the Maintenance of a Long-Term Infection in the Mice**

The next aspect of the intravaginal mouse infection protocol to be addressed was the pre-treatment with *L. acidophilus*. Earlier experiments had shown inconsistency with respect to *L. acidophilus* recovery from representative portions of the mice. In this experiment, the objective was to investigate more closely the establishment of *L. acidophilus* colonization in the mice. It was proposed that perhaps better infection rates would be observed with *L. acidophilus* and subsequently with *T. vaginalis*, if the concentration of *L. acidophilus* initially inoculated into the mice was increased.

Half of the mice received the usual dose of *L. acidophilus* (10^{10} cfu/mL), while the rest received a ten-fold higher amount of the bacteria (10^{11} cfu/mL). A portion of the mice in each of these groups was washed for *L. acidophilus* at an earlier time in the protocol (Day -4) as well as the usual time (Day -1) and were not inoculated with *T. vaginalis*. The rest of the mice were given *T. vaginalis* as usual at Days 0 and 1 but were not washed for *L. acidophilus*. There was some concern that by washing out some of the *L. acidophilus*, this would eliminate the vaginal *L. acidophilus* infection. This in turn would likely have a subsequent effect on the establishment of a *T. vaginalis* infection.

Table 11: Estrogen Dosage and Establishment of Intravaginal Infection

The frequency of estrogen injections was doubled in half of the mice. Recovery of live *T. vaginalis* was not enhanced by the addition of extra estrogen injections to the protocol.

Estrogen Treatment	Infection Protocol	<i>L. acidophilus</i> Recovery at Day -1	Live <i>T. vaginalis</i> Recovery		
			Day 3	Day 7	Day 14
Estrogen at Days -9 & -2	<i>L. acidophilus</i> : Days -7 & -6 <i>T. vaginalis</i> : Days 0 & 1	0/3	5/6	4/6	0/6
Estrogen at Days -9, -3, 2 & 7	<i>L. acidophilus</i> : Days -7 & -6 <i>T. vaginalis</i> : Days 0 & 1	2/3	5/6	2/6	1/5

The results of this experiment indicated that at Day -4 all mice harboured *L. acidophilus*, but that this number dropped slightly by Day -1 (Table 12). There seemed to be no difference when comparing the group given the usual dosage with the group given a ten-fold higher number of *L. acidophilus*. With respect to live *T. vaginalis* recovery, the results looked promising at Day 7, with washes from five of seven mice showing *T. vaginalis* growth. By Day 14, however, these numbers had dropped and only two of seven mice were still infected at this timepoint. Contrary to the hypothesis in this experiment, the mice pretreated with the higher concentration of bacteria did not show better *T. vaginalis* infection rates. In fact, as Table 12 shows, none of these mice was infected with *T. vaginalis* at Day 14.

Influence of Timing of Various Elements of the Infection Protocol

The last issue that was addressed, in an effort to understand a frustrating lack of a long-term infection in the mice, was the timing of the various elements in the overall infection protocol. The previous experiment showed that the number of mice infected with *L. acidophilus* was greater at Day -4 than at Day -1 (Table 12). Based on the assumption that prior colonization with *L. acidophilus* is very important in the establishment of a long-term *T. vaginalis* infection, it was decided to try inoculating with *T. vaginalis* at an earlier timepoint (Days -4 and -3).

While investigating the importance of estrous stage in earlier experiments, it was found that most of the mice were not in the desired stages of estrous at Day 0 (the usual time for *T. vaginalis* inoculation). In those given the normal 50 μ L dose of estrogen at Days -7 and -6, there appeared to be an initial peak in the percentage of mice in the desired stages of estrous, with a gradual decline by Day 0 (Figure 14).

Table 12: *L. acidophilus* Concentration and Establishment of Intravaginal Infection

While the basic timeline of the protocol was followed, half of the mice were pre-treated with a dose of *L. acidophilus* ten times the usual concentration. The augmented dosage of bacteria did not lead to enhanced recovery of *T. vaginalis*.

Estrogen Treatment	Infection Protocol	<i>L. acidophilus</i> Recovery		Live <i>T. vaginalis</i> Recovery	
		Day -4	Day -1	Day 7	Day 14
Days -9/-2	<i>L. acidophilus</i> : 10 ¹⁰ cfu/mL on Days -7 and -6 <i>T. vaginalis</i> : none	4/4	2/3	n/a	n/a
Days -9/-2	<i>L. acidophilus</i> : 10 ¹⁰ cfu/mL on Days -7 and -6 <i>T. vaginalis</i> : Days 0 and 1	n/a	n/a	2/3	2/3
Days -9/-2	<i>L. acidophilus</i> : 10 ¹¹ cfu/mL on Days -7 and -6 <i>T. vaginalis</i> : none	4/4	3/4	n/a	n/a
Days -9/-2	<i>L. acidophilus</i> : 10 ¹¹ cfu/mL on Days -7 and -6 <i>T. vaginalis</i> : Days 0 and 1	n/a	n/a	3/4	0/4

Inoculating with *T. vaginalis* at an earlier timepoint (Days -4 and -3 rather than Days 0 and 1) looked like a viable option. It was estimated that enhanced *T. vaginalis* infection rates might be observed due to the fact that, at this earlier timepoint, a greater proportion of mice would be infected with *L. acidophilus* and in proestrus/estrus.

The results of the experiment showed that neither increasing the concentration of the *L. acidophilus* inoculum nor inoculating at an earlier timepoint with *T. vaginalis*, yielded better rates of infection in the mice. Similar to the last experiment, mice given 10^{11} cfu/mL *L. acidophilus* rather than 10^{10} cfu/mL did not demonstrate more consistency with respect to *L. acidophilus* infection nor *T. vaginalis* infection (Table 13). Numbers of mice infected with *L. acidophilus* were uniformly low throughout the wash dates. Initially, most mice were found to harbour *T. vaginalis*, but as seen in earlier studies, the number of infected mice dropped off by Days 7 and 14.

It should be noted that at the Day -7 sampling point, 100% of these mice were in the desired stage of estrous (proestrus/estrus), but introduction of *T. vaginalis* into the mice at the earlier timepoint did not lead to increased rates of infection. As in the mice given *T. vaginalis* at Days 0 and 1, those inoculated at Days -4 and -3 had good initial rates of infection (Day 7), but the numbers dwindled by Day 14, when only one mouse out of eight was still infected with *T. vaginalis*.

Table 13: Timing of *T. vaginalis* Inoculation and Establishment of Intravaginal Infection

Half of the mice used in this experiment were inoculated with *T. vaginalis* at an earlier timepoint in the protocol. It was discovered that neither an increase in *L. acidophilus*, nor earlier inoculation of *T. vaginalis* led to improved infection rates.

Estrogen Delivery	Infection Protocol	<i>L. acidophilus</i> Recovery				<i>T. vaginalis</i> Recovery			
		Day 0	Day 3	Day 7	Day 14	Day 0	Day 3	Day 7	Day 14
Days -9/-2	<i>L. acidophilus</i> : 10 ¹⁰ cfu/mL on Days -7/-6 <i>T. vaginalis</i> : none	n/a	1/5	1/5	1/5	n/a	n/a	n/a	n/a
Days -9/-2	<i>L. acidophilus</i> : 10 ¹⁰ cfu/mL on Days -7/-6 <i>T. vaginalis</i> : Days 0/1	n/a	1/4	0/4	1/4	n/a	4/4	2/4	1/4
Days -9/-2	<i>L. acidophilus</i> : 10 ¹¹ cfu/mL on Days -7/-6 <i>T. vaginalis</i> : Days 0/1	n/a	1/4	1/4	0/4	n/a	3/4	2/4	1/4
Days -9/-2	<i>L. acidophilus</i> : 10 ¹⁰ cfu/mL on Days -7/-6 <i>T. vaginalis</i> : Days -4/-3	n/a	n/a	n/a	n/a	4/4	n/a	3/4	0/4
Days -9/-2	<i>L. acidophilus</i> : 10 ¹¹ cfu/mL on Days -7/-6 <i>T. vaginalis</i> : Days -4/-3	n/a	n/a	n/a	n/a	2/4	n/a	2/4	1/4

DISCUSSION

Development of a Mouse Model for Vaccine Studies in Our Lab

The intravaginal mouse model of infection has been employed in our lab over the last decade. Preliminary development of the basic protocol design incorporated data generated by previous workers in the field (See Kulda, 1990 for review). Meysick and Garber (1992) investigated the effects, on the endemic flora and pH, of the introduction of *T. vaginalis* into the mouse vaginal ecosystem. It was hoped that this research would lead to a reliable model with applications in immunological and virulence studies. These early experiments compared the number and identity of bacterial species isolated from control (uninfected, non-estrogenized) mice with those animals which had been given hormone treatment and those which had been treated with estrogen and *T. vaginalis*. Mice in these groups were also monitored for vaginal pH and estrous stage. This study was prompted by the knowledge that, in human trichomonal infection, there tends to be a reduction in the lactobacillus population (Jirovec and Petru, 1968), a concomitant rise in anaerobic flora (Spiegel, 1990) and an increase in vaginal pH levels (Rein and Chapel, 1975; Hanna *et al.*, 1985; Rein, 1990). Meysick and Garber's basic model (1992), with pre-estrogenization of BALB/c mice prior to the introduction of *T. vaginalis*, demonstrated that estrogenization does not appear to significantly alter the frequency of isolation nor the type of bacterial species isolated from the mice. pH levels appeared to be slightly lower in those mice that had been estrogenized. Those mice vaginally inoculated with *T. vaginalis* were found to harbour vaginal flora similar to those left uninoculated. Vaginal pH was noted to be slightly higher in infected mice, compared with baseline estrogenized mice. Meysick and Garber (1992) noted variable results with respect to infection rates three days post inoculation, but saw a widespread decrease in the percentage of infected animals seven days post inoculation.

The same year, McGrory and Garber (1992) published a paper outlining their attempts to gain a more long-term infection in the mice. They continued directly from Meysick and Garber's work by building on the model protocol set out earlier that year. Modifications to this protocol came about when it was realized that mice harbour very low numbers of vaginal lactobacilli, which is in direct contrast to the predominance of lactobacilli found in the human vaginal compartment (Meysick and Garber, 1992). Given that a major aim of any animal model is to mimic, as closely as possible, the human disease, McGrory and Garber (1992) decided to pre-treat the mice with *L. acidophilus* prior to the introduction of *T. vaginalis*. Initial rates of infection were comparable between those pre-treated with *L. acidophilus* and those given *T. vaginalis* alone. Those mice pre-treated with *L. acidophilus* did, however, show an increase in duration of infection over those not pre-treated. The sharp decrease in infection rates that had been seen seven days post-inoculation, was not apparent in the *Lactobacillus*-treated group.

The application to immunological studies of *T. vaginalis* of the mouse model came to fruition in the form of vaccine research. Abraham *et al.* (1996) took the mouse model of intravaginal infection developed by previous researchers and used it to study the protective effect of subcutaneous immunization with a whole-cell *T. vaginalis* vaccine. Vaccine efficacy was monitored by infection rates and the measurement of vaginal and serum immunoglobulin levels. Immunized mice were found to have significantly fewer intravaginal infections and displayed increased antibody responses when compared with those of control mice.

These results were exciting and prompted us to further investigate the immunological parameters of the vaccine model. To this end, it was decided that current research should focus on cross protection among isolates of *T. vaginalis* and on the employment of possible alternative adjuvants, which would elicit a desirable anti-*T. vaginalis* immune response, but would be

acceptable for use in humans. In order to do these things, it would be necessary to first repeat what had been done previously.

Problems Encountered in Reestablishment of the Infection Model

Early attempts to reestablish the intravaginal mouse model of *T. vaginalis* infection proved largely unsuccessful. Given that the animals were pre-treated with estrogen and *Lactobacillus*, results comparable to those reported by McGrory and Garber (1992) were expected. In their experiment, 79% of inoculated mice were found to harbour live trichomonads four days post-inoculation, while 69% were found to be infected twenty-four days post-inoculation. In our preliminary experiments using the same protocol, culture of vaginal wash material yielded no live trichomonads (indicating a lack of infection) at Days 7, 14 and 21 post-inoculation. In order to begin more in-depth studies of the immunological response to parenteral vaccination with *T. vaginalis*, there needed to be total confidence that the basic infection part of the protocol was reproducible. Various aspects of the model, which may have been preventing long term establishment of infection, were therefore studied more closely.

Assessment of the Importance of the *T. vaginalis* Isolate in Establishment of an Infection

The earliest experiments conducted in an attempt to reestablish the intravaginal infection model utilized the isolate "DG", a cryopreserved isolate originally cultured from a woman with trichomoniasis. A number of researchers have demonstrated that there exists much variation among trichomonad isolates with respect to pathogenicity (Farris and Honigberg, 1970; Bremner *et al.*, 1986; Coombs *et al.*, 1987; Hook *et al.*, 1995). Therefore, when preliminary experiments showed a lack of infection at Days 7, 14 and 21, the protocol was repeated with an alternate isolate, with the hope that this might lead to a more dependable infection in the mice. The alternate isolate employed was a well-characterized one termed "263". This isolate was obtained

from the vaginal secretions of a woman with “moderate” symptoms and was also found to have moderate CDF activity and pathology (as determined by the mouse subcutaneous assay) (Garber and Lemchuk-Favel, 1990). Isolate 263 also seemed to be a good choice due to the fact that it had been used by previous researchers who had demonstrated success with the model (Meysick and Garber, 1992; McGrory and Garber, 1992; Abraham *et al.*, 1996). Isolate 263 afforded no greater success, however, with respect to maintenance of an infection in the mice past Day 1. Recovery rates at Day 1 suggested that the trichomonads were surviving the preparation process but the lack of *T. vaginalis* at Days 7 and 14 meant that the parasites were not flourishing.

Rasmussen *et al.* (1986) suggested that *T. vaginalis* that had been cryopreserved demonstrated a diminished cytopathic effect in *in vitro* studies with human vaginal epithelial cells. They showed that their axenic laboratory strains did not adhere to epithelial cell monolayers to the same degree that freshly isolated trichomonads did. Similarly, Bremner *et al.* (1986) indicated that prolonged *in vitro* passaging of trichomonads led to an “attenuation of their virulence”. While these *in vitro* studies do raise questions about the pathogenicity of isolates preserved in the lab for long periods of time, this theory would not explain the lack of infection encountered in our preliminary research. A number of workers in our lab have used the axenic isolate 263 in the mouse model (Meysick and Garber, 1992; McGrory and Garber, 1992; Abraham *et al.*, 1996) and have been able to successfully establish long-term intravaginal infections in BALB/c mice.

Estrogenization and Estrous Stage and their Part in the Infection Protocol

The next aspect of the intravaginal infection process that we explored in greater detail was the estrogenization and resulting mouse vaginal pathology. Preliminary sampling of the mice suggested that despite being given estrogen injections prior to inoculation with *L. acidophilus* and *T. vaginalis*, a majority of the animals were not in the desired stage of estrous (i.e. proestrus or

early estrus). The most optimal conditions for intravaginal growth of trichomonads occur during the stages of proestrus and preovulatory estrus when increased levels of estrogens are present (Kulda, 1990) and when fewer neutrophils and bacteria are found (Corbeil *et al.*, 1985; Coombs *et al.*, 1986). Kulda (1990) showed that a swing into the phase of metestrus and diestrus could terminate an infection or induce latency. It is for this reason, then, that supportive estrogen therapy is indicated for the successful achievement of an intravaginal infection (Cappuccinelli *et al.*, 1974; Wildfeuer, 1974; Meingassner, 1977; Coombs *et al.*, 1986; Meysick and Garber, 1992; McGrory and Garber, 1992; Abraham *et al.*, 1996).

Meysick and Garber (1992) found that when they sampled their pre-estrogenized mice before inoculating with *T. vaginalis*, a surprisingly high 53% were in the undesirable stage of metestrus. They hypothesized that this may have had something to do with the drop in the number of infected mice at seven days post-inoculation. They proposed that perhaps the extensive normal flora and neutrophils present at metestrus may have provided some protection against a sustained infection with the introduced trichomonads. Through sampling of the vaginal flora of mice with and without estrogenization, Meysick and Garber (1992) found that enhanced infectivity with estrogen treatment was not a result of marked floral changes, but was linked instead to the stage of estrous induced. These conclusions raised questions about what was happening in our mice, and prompted the use of alternate hormone delivery system which, it was hoped, would not only give the mice an initial dose of estrogen but would deliver the hormone over a longer period of time. It was predicted that this system would give rise to mice that would stay in the desired stage throughout the protocol and thus the infection would not be compromised by a shift into metestrus and/or diestrus. In contrast to what had been done previously, daily vaginal sampling of the mice was initiated. It was hoped that this system would give us a better idea of how the mice were or were not cycling and whether or not this was influenced by the pellet delivery system.

The pellet system did not lead to greater consistency with respect to maintenance of proestrus/estrus. An initial peak was followed by a steady decline in the number of mice in the desired stage. In those given estrogen by injection (as per the usual protocol), there was an initial peak in the percentage of mice in the desired stage, but numbers dropped off after Day 0 (the time at which mice were inoculated with *T. vaginalis*). The infection rates were not boosted by the implementation of the pellet system. This was not surprising given the fact that the pellets did not give rise to longer-term maintenance of estrous stage. As in preliminary experiments, the number of infected mice at Day 3 was quite high when the mice were given estrogen by injection, but the infection was cleared by the majority of the animals at Day 14.

It was decided, then, to try expanding on the estrogen delivery by injection. A portion of the mice in this experiment was given double the usual dosage of estrogen via extra injections. As in the previous experiment, the percentage of mice in the desired stage peaked within about forty-eight hours of estrogen delivery (normal estrogen regimen) and there was seen to be a gradual decline from that point on in the protocol. A minority of the mice was in the desired stage at the time of *T. vaginalis* inoculation. In the group of mice given the augmented estrogen, the percentage of mice in the desired stage peaked after the first two doses but this was followed by a steady decline similar to that seen in the mice given the normal hormone regimen. In both these groups, it appeared that the initial dosage of estrogen had a much more pronounced effect on the mice than did subsequent treatments. Following an initial peak, there was found to be a definite downward trend in the number of mice in the desired stage of estrous. This serves to highlight the fact that few of the mice were in proestrus/estrus at the time of inoculation with trichomonads. What effect this may have had on the maintenance of an intravaginal infection is unknown but the literature would indicate that the hormone state and estrous stage is very important. Our vaginal smear results are comparable to those reported by Meysick and Garber (1992) who found that only a small portion of their mice were in the desired stage of estrous at the time of trichomonad

introduction. It is difficult to compare results beyond that one timepoint, however, because Meysick and Garber (1992) and others did not do extensive daily sampling.

While still concerned about the dwindling numbers of mice in proestrus/estrus at the time of inoculation, it was determined that other avenues must also be investigated. It appeared that none of the variations we tried would give improved maintenance of estrous stage or intravaginal trichomonad infection.

Importance of *L. acidophilus* in Establishment of Infection

Our results, with respect to estrous stage and infection rate drop off at Day 7 post inoculation, tended to mimic those of Meysick and Garber (1992), but there was a major difference in the protocols. This was the inclusion in our protocol of the *Lactobacillus* pre-treatment prior to *T. vaginalis* inoculation that had been documented by McGrory and Garber (1992). In their research, McGrory and Garber (1992) found that the inclusion of the *Lactobacillus* pre-treatment step potentiated more long-term infections in the mice. In fact, they felt that pre-inoculation with *L. acidophilus* had a more demonstrable effect on the ability of *T. vaginalis* to establish a longer-lasting infection in the mice than did the stage of estrous. They postulated that the addition of the bacteria helped to create a mouse vaginal milieu more reminiscent of the human environment. Given that our results resembled those seen by McGrory and Garber (1992) in the control mice who did not receive *L. acidophilus* before *T. vaginalis*, we decided to look more closely at this step. This, coupled with the fact that recovery of *L. acidophilus* from vaginal washes had been inconsistent in previous experiments, suggested that perhaps bacterial colonization of the mice was not taking place.

In order to try to ensure a more consistent infection with *L. acidophilus*, a portion of the mice was given ten-fold higher doses of the bacteria intravaginally. The percentage of mice positive for *Lactobacillus* decreased slightly from Day -4 to Day -1, but the majority still harboured the bacteria. No distinction could be made with respect to bacterial culture positivity between the mice given the usual dosage and those given the ten-fold higher doses of *Lactobacillus*. Despite respectable rates of bacterial recovery, however, only a small percentage of those sampled after Day 7 were infected with *T. vaginalis*, indicating that the increase in *L. acidophilus* did not lead to a trichomonad infection of increased duration. It is not known why the mice in our experiments did not demonstrate the same type of prolonged *T. vaginalis* infection seen by other researchers (McGrory and Garber, 1992). All elements of the protocols appeared to be the same, but despite *Lactobacillus* pre-treatment, our results continued to mirror those seen by previous researchers when the bacterial pre-inoculation step was excluded.

Overall Timing of Various Elements in Protocol

The final attempt at troubleshooting was to address the issue of timing of the various elements of the protocol. It was hypothesized that inoculating with *T. vaginalis* at an earlier timepoint (Days -4/-3 rather than Days 0/1) might take better advantage of augmented *Lactobacillus* infection rates and higher percentages of mice in proestrus/estrus. (These phenomena had been observed in previous experiments.) While all mice were found to be in the desired stage of estrous at the time of bacterial pre-treatment, *Lactobacillus* recovery rates were poor throughout the timeline, and earlier introduction of trichomonads did not lead to improved *T. vaginalis* recovery after Day 7.

Conclusions

The fact that great difficulty was encountered in re-establishing the mouse intravaginal infection model prevented pursuit of the immunological studies that were to be the main focus in the first

place. Why the model has worked well for others in the past and has been so inconsistent recently is still a mystery. All attempts have been made to mimic the various elements of the model used in past research (Meysick and Garber, 1992; McGrory and Garber, 1992; Abraham *et al.*, 1996). The keys to the intravaginal mouse model of infection appear to be estrogenization and pre-treatment with *Lactobacillus*. With respect to the estrogen stage, we found that the majority was not in the desired stage throughout the protocol, but all attempts to alter this were met with limited success. Furthermore, due to the fact that previous studies incorporated estrous stage sampling at only one timepoint in the timeline, it is impossible to say whether or not the estrous cycling which we observed was occurring in past. Recovery of *Lactobacillus* has been very sporadic for unknown reasons. Lack of good bacterial infection could very well have played a role in the observed drop-off in trichomonad infection.

While it seems unlikely, it is not known if the BALB/c mice employed in current experiments are somehow different from those used by previous researchers. There is the possibility that the animals used by past workers were infected with parasites and/or pathogens that have since been eradicated by Animal Care. The veterinary staff in the Animal Care facility has reported that prior to 1994, there existed in the animal population a baseline of pinworms, mouse hepatitis virus, external fermites and enteric protozoa (including trichomonads and entamoeba). It is difficult to say whether these factors could have had any immune system effects on the mice used in past research, or even if these particular animals were infected with any or all of these pathogens, but the possibility should be considered given the paucity of other explanations.

APPENDIX II

Recipe for Diamond's TYI-S 33 Medium

Combine in a 2L flask:

783 mL distilled water

18.0 g casitone (casein digest peptone)

9.0 g yeast extract

9.0 g dextrose

1.8 g sodium chloride

0.54 g potassium phosphate (monobasic)

0.9 g potassium phosphate (dibasic anhydrous)

0.9 g L-cysteine hydrochloride

0.18 g L-ascorbic acid

20.5 mg ferric ammonium citrate

- ❖ Adjust pH to 6.5 with 1N NaOH.
- ❖ Autoclave for forty-five minutes to sterilize.
- ❖ Once cool, add 27 mL Medium NCTC-109 1x (supplemented with 0.1% Tween 80) (Gibco-BRL).

Recipe for Phosphate Buffered Saline (PBS) 10x Stock Solution

In a 2 L flask, combine:

800 mL distilled water

80 g sodium chloride (NaCl)

2 g potassium chloride (KCl)

11.5 g sodium monohydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)

2 g potassium dihydrogen phosphate (KH_2PO_4)

- ❖ Stir to dissolve all ingredients.
- ❖ Bring solution up to 1 L with distilled water.
- ❖ Confirm pH of 7.2.
- ❖ Dilute stock solution in distilled water to make 1x PBS solution.
- ❖ Autoclave forty-five minutes to sterilize.