Purification and characterization of a lytic factor having phospholipase A□2 like activity in Trichomonas vaginalis

by Kirk James Lubick

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
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Abstract:
T vaginalis produces cytotoxic factors that have been reported to have the ability to damage target cells in vitro. It has been hypothesized that these factors may play a role in the pathogenesis of human trichomoniasis. Parasite extracts were subjected to octyl-Sepharose and gel filtration chromatography to purify a lytic factor (LF) from T. vaginalis, which was further subjected to biochemical tests to determine the molecular characteristics of LF. LF was cytotoxic to WEHI164 cells and bovine red blood cells and inactivation of the LF by treatment with trypsin (130 μg/ml), suggested cytotoxicity was due to a protein. Results of a fluorescence assay in which carboxyfluorescein (CF) labeled liposomes composed of phosphatidylcholine: cholesterol (1:1 ratio) showed that liposomes were hydrolyzed by LF suggesting that LF has phospholipase activity. LF hydrolyzed BODIPY-labeled phosphatidylcholine into components resolved by thin layer chromatography (TLC), providing further evidence of phospholipase A2-like (PLA2) activity. A monoclonal antibody (mAb), 11-5-14, was identified that was specific for an epitope on the 220 kDa protein, which hydrolyzed BODIPY labeled phosphatidylcholine into components resolved by TLC. Flow cytometry analysis using mAb 11-5-14 revealed LF is internally expressed in the parasite and is not expressed on the parasites surface. Flow cytometry and ELISA analysis further revealed increased expression of LF when T. vaginalis was stimulated with fixed WEHI164 cells. T vaginalis appears to store LF in cytoplasmic vesicles that are disrupted when parasites are treated with Brefeldin A (BFA) and BFA-treated T. vaginalis incubated on fixed WEHI164 cells showed a decrease in cytotoxicity when subsequently assayed on live WEHI164 cell targets. These results suggest that LF is a virulence factor of T. vaginalis that may be important in the destruction of host cells contributing to tissue damage and inflammation in trichomoniasis.

PURIFICATION AND CHARACTERIZATION OF A LYTIC FACTOR HAVING PHOSPHOLIPASE A_2 LIKE ACTIVITY IN *TRICHOMONAS VAGINALIS*

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Kirk James Lubick

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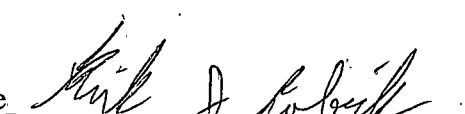
Signature 

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TABLE OF CONTENTS

1. *TRICHOMONAS VAGINALIS* 1

Introduction 1
Epidemiology 2
Morphology 3
Metabolism 4
Carbohydrate and Amino Acid Metabolism 4
Nucleotide Metabolism 5
Phospholipids and Glycoconjugate Metabolism 6
Hyodrogenosome 7
Treatment 8
Pathogenesis 8
Pathogenic Mechanisms and Factors 9
Adhesion Protein 10
Proteinases 12
Hemolysis 14
Cell-Detaching Factor 15
Contact-Independent Mechanisms 17
T. vaginalis Destruction of Target Cell Cytoskeletion 18
Virulence Dependence on Iron 19
Hypothesis 20
Project Goals 20

2. PURIFICATION AND ANALYSIS OF A LYTIC FACTOR IN *TRICHOMONAS VAGINALIS* 22

Introduction 22
Materials and Methods 24
Parasites 24
Parasite-Antigens 25
Hydrophobic Chromatography 25
Gel Filtration 26
SDS-Polyacrylamide Gel Electrophoresis 26
2-Dimensional Electrophoresis 26
Protein Digestion 27
MALDI-TOF 28
Periodate Sensitive Epitopes 28
Cytotoxicity Assays 29
Lipase Assay 30

TABLE OF CONTENTS-CONTINUDED

Protease Assay 30
Liposome Preparation 31
Phospholipid Hydrolysis 32
Statistical Analysis 32
Results 33
T. vaginalis CPE 33
Chromatographic Purification of LF 34
Enzyme Assays 37
LF Hydrolyses Phospholipase Substrates 40
Gel Filtration 42
2 Dimensional Electrophoresis and MALDI-TOF Analysis 44
Discussion 46

3. *TRICHOMONAS VAGINALIS*: SECRETED VIRULENCE FACTOR AND REGULATION 50

Introduction 50
Materials and Methods 51
Parasites 51
Parasite-Extract 52
SDS-Polyacrylamide Gel Electrophoresis 52
Phosopholipid Hydrolysis 52
Stimulation and Secretion Assays 53
ELISA 53
Flow Cytometry 54
Confocal Microscopy 55
Cytotoxicity Assays 55
Results 56
Phospholipase specific monoclonal antibody 58
LF is secreted and stimulated by external factors 58
LF is stored in vesicles 64
Discussion 65

4. CONCLUSIONS 68

LIST OF TABLES

Table Page

1. Proteomic analysis of LF proteins 46

LIST OF FIGURES

Figure Page

1. *T. vaginalis* cytotoxicity toward WEHI 164 cells is present with or without cell-to-cell contact 34

2. LF is cytotoxic to WEHI 164 cells 36

3. Cytotoxic activity of LF is due to protein activity 38

4. LF function is not due to detectable protease activity 39

5. LF hydrolyses phospholipase substrates 41

6. LF hydrolyzes BPC with PLA_2-like activity 42

7. LF activity corresponds to a 167 kDa protein according to gel filtration 43

8. LF activity corresponds to heterodimer of approximately a 220 kDa protein according to 10% SDS-PAGE 44

9. 2-D electrophoresis of LF 45

10. BFA treatment of *T. vaginalis* decreases cytotoxicity of *T. vaginalis* toward WEHI 164 cells 57

11. mAb 11-5-14 recognizes an epitope specific for a 220 kDa protein in the LF preparation 59

12. The mAb 11-5-14 recognizes LF, which hydrolyzes BPC 60

13. LF is localized internally in *T. vaginalis* 61

14. *T. vaginalis* expression of LF is stimulated by WEHI 164 cells 62

15. Anti-LF ELISA confirms increase in LF in *T. vaginalis* due to target cell exposure 63

16. LF localizes in cytoplasmic vesicles in *T. vaginalis* 64

Abstract

T. vaginalis produces cytotoxic factors that have been reported to have the ability to damage target cells *in vitro*. It has been hypothesized that these factors may play a role in the pathogenesis of human trichomoniasis. Parasite extracts were subjected to octyl-Sepharose and gel filtration chromatography to purify a lytic factor (LF) from *T. vaginalis,* which was further subjected to biochemical tests to determine the molecular characteristics of LF. LF was cytotoxic to WEHI 164 cells and bovine red blood cells and inactivation of the LF by treatment with trypsin (130 μg/ml), suggested cytotoxicity was due to a protein. Results of a fluorescence assay in which carboxyfluorescein (CF) labeled liposomes composed of phosphatidylcholine: cholesterol (1:1 ratio) showed that liposomes were hydrolyzed by LF suggesting that LF has phospholipase activity. LF hydrolyzed BODIPY-labeled phosphatidylcholine into components resolved by thin layer chromatography (TLC), providing further evidence of phospholipase A_2-like (PLA_2) activity. A monoclonal antibody (mAb), 11-5-14, was identified that was specific for an epitope on the 220 kDa protein, which hydrolyzed BODIPY labeled phosphatidylcholine into components resolved by TLC. Flow cytometry analysis using mAb 11-5-14 revealed LF is internally expressed in the parasite and is not expressed on the parasites surface. Flow cytometry and ELISA analysis further revealed increased expression of LF when *T. vaginalis* was stimulated with fixed WEHI 164 cells. *T. vaginalis* appears to store LF in cytoplasmic vesicles that are disrupted when parasites are treated with Brefeldin A (BFA) and BFA-treated *T. vaginalis* incubated on fixed WEHI 164 cells showed a decrease in cytotoxicity when subsequently assayed on live WEHI 164 cell targets. These results suggest that LF is a virulence factor of *T. vaginalis* that may be important in the destruction of host cells contributing to tissue damage and inflammation in trichomoniasis.

TRICHOMONAS VAGINALIS

Introduction

Trichomonas vaginalis is a eukaryotic flagellate parasitic protozoan of humans, and the cause of human trichomoniasis. *T. vaginalis* is a sexually transmitted parasite of the human urogenital track that can produce either asymptomatic or symptomatic infections, ranging from a mild infection to a severe state of inflammation (1). Trichomoniasis has been reported to be one of the most common non-viral sexually transmitted diseases (STD) estimated at 5 million new cases per year in the United States and 170 million new cases per year in the world (World Health Organization). *T. vaginalis* is classified in the class of Zoomastigophora and in the order of Trichomonadida (2).

The ability of *T. vaginalis* to cause disease and survive in the vaginal environment is remarkable due to cyclic changes during menstruation exposing the parasite to serum proteins, red blood cells, hormonal changes, and various macromolecules. In order for *T. vaginalis* to survive and be a pathogenic organism in this harsh and ever changing environment the parasite must possess appropriate molecular mechanisms for survival and to cause pathogenesis.

To date numerous investigators have reported possible molecular mechanisms that contribute to pathogenesis, host invasion, and survival in the host that give insight into the pathogenesis of trichomoniasis. Investigation of the pathogenesis of *T. vaginalis*

has uncovered numerous virulence factors, of which cysteine proteinases have been studied in depth, while the functions of other virulence factors and enzymatic activities have yet to be elucidated.

Epidemiology

In non-selected populations *T. vaginalis* infection in women is reported to range from 5-20%, yet most epidemiological reports are biased in that selected groups were studied; such as women attending STD clinics (3). The World Health Organization has reported that there are 5 million new cases in the United States and 170 million new cases worldwide (4). Thus, *T. vaginalis* is cosmopolitan in distribution and has been reported to be the world's most common non-viral sexually transmitted disease.

Numerous epidemiological reports have been published and have suggested certain trends occur within *T. vaginalis*-positive humans. One report showed that *T. vaginalis* infection in women correlated with educational status showing that 18% of women having a high school education were infected with *T. vaginalis* whereas 12% of women having a college degree were positive for *T. vaginalis* (5). It was also shown that *T. vaginalis* infection can be correlated to marital status, such as the following: married (13.6%) < never married (25.6%) < divorced or separated women (37%) (5). Another study showed women belonging to families having poverty level incomes were more likely to be infected with *T. vaginalis* (6).

Of the numerous epidemiological reports published on *T. vaginalis* and the few mentioned here it appears that the prevalence of *T. vaginalis* infection can be attributed to

the lifestyles of humans. Due to the nature of the infection of *T. vaginalis* and the underestimation of the prevalence of asymptomatic cases at the present time it is impossible to determine the exact number of infected individuals in non-selected populations.

Morphology

T. vaginalis is pyriform in shape, yet shape and size of these parasites vary depending on environmental conditions. *T. vaginalis* has only one stage, trophozoite, therefore lacking a true cyst stage. There have been reports of *T. vaginalis* present in a psuedocyst stage, which has been described as rounding of the parasite and internalization of the flagella. Formation of these pseudocysts is thought to be due to the growth of the parasites under unfavorable conditions, and therefore is an artifact of culture conditions (7). The trophozoite possesses an axostyle that protrudes through the posterior end of the cell and is thought to anchor the trophozoite to the epithelium in both the reproductive tract and the urethra tract in women and men, respectively (7). The parasite contains five flagella (locomotor organelles) four of which are anterior flagella and the fifth flagellum is a recurrent one that is incorporated into the undulating membrane. The four flagella originate in the anteriorly located basal granule complex called the kinetosomal complex. This fifth flagellum and the undulating membrane give the parasite a characteristic quivering motion (7). The undulating membrane and the costa originate from the kinetosomal complex posterior and dorsal to the anterior flagella. The undulating membrane is normally shorter than the body. The costa corresponds in

length to the undulating membrane and is usually situated between two paracostal granules, which have been identified as hydrogenosomes (discussed below). Hydrogenosomes are arranged in three rows between the posterior end of the nucleus (anterior end of the body) and the anterior point of the axostyle (7).

Metabolism

Carbohydrate and Amino Acid Metabolism

Carbohydrate metabolism is the primary energy source in this parasite, as it has a high maintenance expenditure requiring carbohydrates to maintain carbon (energy) sources. Carbon sources are critical for maintaining homeostasis due to the changing environment such as mensis, pH, hormones, and the nutrient supply. Maltose is the primary carbohydrate source and is converted to glucose by α–glucosidase, which is expressed on the cell surface of these parasites (8). Carbohydrate metabolism occurs in two places, in the cytoplasm and in the hydrogenosome (discussed below). Glucose metabolism occurs in the cytoplasm and is converted to phosphoenolpyruvate and then to pyruvate by the Embden-Meyerhoff-Parnas pathway (9). The pyruvate then can be transported to the hydrogenosome and metabolized further. Glycerol and lactate can also be produced in the cytoplasm by glycerol-3-phosphate dehydrogenase and lactate dehydrogenase, respectively (10,11).

Although carbohydrates, mainly maltose, are the primary source of energy amino acids can be a substitute for a source of nutrients in the absence of carbohydrates (12). Under carbohydrate depletion the parasite uses amino acids as energy generators,

especially arginine, alanine, threonine, and leucine. In a recent report there was a high concentration of intracellular alanine and arginine (13). In summary *T. vaginalis* primary energy resource is through carbohydrates, primarily maltose initially converted to glucose. When carbohydrates are in low supply amino acids such as alanine and arginine are used as substitutes.

Nucleotide Metabolism

T. vaginalis does not contain the enzymatic activity needed for the *de novo* pathways, and therefore is unable to synthesize purines and pyrimidines. Thus salvage pathways are employed to generate purines and pyrimidines (14, 15). This was revealed by experiments in which purine and pyrimidine precursors were unable to incorporate into nucleic acids. Salvage of pyrimidines occurs by phosphoribosyltransferases and nucleoside kinases, such as cytidine phosphotransferase (16). Uridine is made by cytidine deaminase, which converts cytidine to uridine (16). Purine salvage is mediated through nucleoside phosphorylases and kinases. It has recently been reported that adenosine is the primary precursor of purine nucleotides (17). Nucleotide precursors adenosine and guanosine are salvaged through adenosine and guanosine kinases (17). Salvage pathways are a critical requirement in the nucleotide metabolism of *T. vaginalis* and cytotoxicity *in vivo* may be a critical requirement in order to salvage both purines and pyrimidines from host cells.

Phospholipids and Glycoconjugate Metabolism

T. vaginalis appears not to have the molecular machinery required for lipid synthesis, yet parasites contain phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin as the major phospholipids (18). Incorporation of phospholipids must be from external sources such as the host epithelium and red blood cells. There have been reports hypothesizing glycophosphosphingolipid synthesis occurring in *T. vaginalis*. A novel glycosphingolipid containing inositol phosphoceramide was identified, supporting the concept that glycophosphosphingolipid synthesis does occur in *T. vaginalis* (19).

The surface membranes of these parasitic protozoans contain a variety of carbohydrate – rich molecules and these surface glycoconjugates most likely play key roles in the survival of the parasite in hostile environments and in confrontation with host immune responses (20). Along with glycophosphosphingolipids there is also the presence of lipophosphoglycan-like glycoconjugates (LPG) on the cell surface, which is made up of a branched complex of oligosaccharides linked to an inositol anchor. The presence of LPG on the cell surface of *T. vaginalis* has been demonstrated by surface labeling with galactose oxidase and $NaB[^3H]_4$ techniques(21). There are an estimated 2.7 X 10^6 of LPG per cell in *T. vaginalis* and this high copy number on the cell surface suggests that LPG plays an important role in the biology of *T. vaginalis* and the infection it causes (21). The abundant LPG on the surface membrane of *T. vaginalis* forms a glycocalyx over the surface membrane of parasites. These glycoconjugates may influence the physicochemical properties of the plasma membrane, may play major roles

in cell-cell interaction and cell antigenicity, and they may also protect the membrane from chemical or enzymatic degradation in the environments of host cells (22).

The LPG of *T. vaginalis* most likely exists on the parasite surface as a conjugate containing the LPG moiety and a peptide component that is at least partially composed of hydrophobic sequences. This is suggested by results obtained with *Leishmania* spp. and the closely related trichomonad *Tritrichomonas foetus* in which polypeptide components were demonstrated to co-purify with the LPG structure (20, 23).

Hydrogenosome

This parasite differs from most other eukaryotic organisms in that it lacks mitochondria and instead has a hydrogenosome as its energy source (24).. The hydrogenosome is a redox organelle present in some anaerobic unicellular eukaryotes such as the genera *Metopusm, Neocallimastix, and Trichomonas.* The hydrogenosome present in *T. vaginalis,* and some other anaerobic protozoans, is enclosed by two membranes, differing from that of fungi, which is a single membrane organelle. It has been shown that the hydrogenosome of *T. vaginalis* has a NADH-ferredoxin oxidoreductase activity. The hydrogenosome is also where conversion of pyruvate to acetate, malate, CO_2 and H_2 occurs, with acetate being the major product (reviewed in 25).

Treatment

The current treatment of *T. vaginalis* is with metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), which is also used for the treatment of several anaerobic pathogens. Metronidazole enters the parasite through diffusion and is activated in the hydrogenosome (26,27). Electron transfer from ferredoxin to the 5-nitroimidazole group of metronidazole activates metronidazole. The crystal structure of the ferredoxin from *T. vaginalis* has been recently been determined and has a unique cavity compared to ferredoxins expressed in mitochondria and has been hypothesized to be responsible for metronidazole reduction (28). Once the 5-nitroimidazole group of the metronidazole compound is activated it breaks up the DNA of *T. vaginalis* (29). It is well known that *T. vaginalis* populations can become resistant to metronidazole. In one study it was shown that parasite populations resistant to metronidazole had reduced mRNA levels of ferredoxin (30). It has also been shown that metronidazole resistant parasites exhibit reduced expression levels of proteins such as pyruvate-ferredoxin oxidoreductase and hydrogenase (31). Suggesting that metronidazole has a primary effect on ferredoxin, yet affects a biological pathway.

Pathogenesis

The pathogenesis of trichomoniasis can range from a severe state of inflammation to an almost an asymptomatic, carrier state, however women are more likely to be symptomatic carriers of the disease than men. Signs of trichomoniasis in woman can

include vaginal discharge, leukorrhea, and dysuria. In men signs of trichomoniasis can include urethitis and urethal discharge, yet the majority of men infected with *T. vaginalis* are asymptomatic and usually thought to be infected due to having a sexual partner that is positive for *T. vaginalis*.

In pregnant women infected with *T. vaginalis* preterm rupture of membranes can lead to preterm birth (1). In some cases infected women may have vascular distention of blood vessels and hemorrhages, causing a strawberry appearance of the cervix (32). Infected women also may have characteristic vascularity of squamous epithelium, epithelial cells separating from each other, inflammation of squamous epithelial cells, and purulent exudate that covers the damaged epithelial surface. In some cases the purulent exudate will be replaced by superficial layers of epithelium, which leads to an influx of leukocytes into the remaining epithelium. In trichomoniasis damaged squamous epithelial cells can be surrounded by *T. vaginalis* (33).

Pathogenic Mechanisms and Factors

The specific cytopathic effects (CPE) of *T. vaginalis* on host cells, has been a subject of controversy. The controversy surrounds the molecular mechanism(s) of pathogenesis, and especially the issues dealing with contact dependent and contact independent CPE mechanisms (34, 35). Numerous reports have been published on contact dependent mechanisms (36,37,38,39,and 40) leading to CPE, yet contact independent mechanisms (41,42,43,44, and 45) have been more difficult to determine. Investigators have reported cytotoxic factors being secreted by *T. vaginalis*, yet specific

molecular characteristics of these, such as enzymatic activities, remain to be described. To date, the majority of literature on the CPE of *T. vaginalis* pertains to contact-dependent mechanisms, which include adhesion proteins, hemolyisis, and cysteine proteinases. The literature pertaining to contact independent mechanisms suggests little is known about the specific properties and functions (enzymatic activities) of secreted factors by *T. vaginalis* and their roles in pathogenesis and CPE on host cells.

Adhesion Proteins

The *T. vaginalis* contact-dependent mechanism of target cell killing has been well documented *in vitro*. The parasite's ability to adhere to the epithelial cells in the vagina also appears to be a critical step in the pathogenesis (46). Adhesion studies have shown that parasite adherence *in vitro* is greater to human epithelial cells compared nonhuman epithelial cells. *T. vaginalis* adherence to the host epithelial cells appears to be mediated by four adhesion proteins (AP), which are AP65, AP51, AP33, and AP23 (46). These APs are thought to bind to receptor ligands expressed on vaginal epithelial cells. Some studies indicate that parasites deficient in AP expression are not capable of adherence to vaginal epithelial cells (47). Alderete et al showed that p270, a major surface antigen, is expressed on the surface of *T. vaginalis* and is required for adhesion in addition to APs (48). APs are expressed in the presence of a glycoprotein P270 and APs are thought to alternate expression meaning only one AP is expressed at any given time. This alternating expression of APs has led to the hypothesis that this may be a mechanism whereby the parasite evades the host immune system. It has been shown that when *T.*

vaginalis is cultured in iron-replete medium that AP expression is increased relative to parasites cultured in medium devoid of iron (further review of Iron mediation on *T. vaginalis* virulence discussed below) (49).

Although these results suggest APs have a role in adherence in parasite-host cell pathogenesis, there are other data that contradict the exclusiveness of the four APs in controlling parasite adherence to host cells. Brugerolle et al (50) used monoclonal antibodies specific to APs to demonstrate that APs are not present on the surface yet are present in the hydrogenosome. Supporting this data is that AP65 and AP33 have been sequenced and have sequences resembling known hydrogenosomal proteins specifically, malic dehydrogenase and succinyl coenzyme A synthetase (51,52). These gene similarities of these APs to proteins found in the hydrogenosome has been postulated to be a form of molecular mimicry. In addition, the APs ability to recognize specific receptor ligands on the surface of epithelial cells has not been demonstrated and some studies have shown that APs recognize different cell types indiscriminately such as HeLa, CHO, Vero, erythrocytes, and *Mycroplasma hominis* in the absence of the cell wall (53). Although there seems to be controversy in the APs role in recognizing specific ligands on host cells, there is a general acceptance that these APs aid in the adherence of *T. vaginalis* to host cells.

T. vaginalis also expresses surface proteins and carbohydrates that interact with fibronectin and laminin (54), which are extracellular matrix-basement membrane components that help maintain the integrity of the vaginal epithelium. *T. vaginalis* binding to fibronectin seems to be increased in the presence of iron and calcium, yet *T.*

vaginalis binding to laminin does not seem to be influenced by the presence of either iron or calcium (55). A recent report showed that *T. vaginalis* binds to several domains of fibronectin such as the cell-binding domain, N-terminal domain, and the gelatin binding domain (56). Two fibronectin-like binding proteins have been identified in *T. vaginalis* and it has been hypothesized that these two proteins have an additive effect in the parasite binding to fibronectin thus leading to the colonization of mucosal surfaces (57).

Proteinases

Several proteinases have been well characterized in *T. vaginalis*, as a lot of work has been done to characterize the role proteinases have in virulence and immune evasion, and in the pathogenesis of trichomoniasis. Over 20 cysteine proteinases and a few metallo-proteinases have been identified in *T. vaginalis* (58,59,60). Coombs et al first reported the presence of cysteine proteinases in *T. vaginalis* and showed proteinase activity was dependent on DTT (57). Expressed cysteine proteinases were detected from 20-110 kDa, whereas metallo-proteinases were larger, 142 kDa and 220 kDa (58,60). The large number along with the range in protein size suggests cysteine proteinases may play a number of roles in the biology and pathogenesis of *T. vaginalis*.

Trichomonad proteinases, in particular cysteine proteinases, have been extensively studied in the last few years to determine their roles in immune evasion. Cysteine proteinases have been shown to degrade immunoglobin G (IgG) and IgA, which are present in serum and vaginal washes of infected hosts (61). It has been hypothesized that cysteine proteinases serve as a molecular mechanism in evading the host immune

system (61). Degradation of host complement system has also been examined. It was shown that the complement component C3 (alternate pathway) is degraded by the parasite and the parasite has increased susceptibility to complement when treated with proteinase inhibitors (62). In addition the immune system secretes a protease inhibitor termed secretory leukocyte protease inhibitor (SLPI), present in abundant amounts on mucosal surfaces and known to have antimicrobial properties, which is degraded by *T. vaginalis* cysteine proteinases (63), once again illustrating their role in immune evasion.

Cysteine proteinases are thought to play a role in the pathogenesis of trichomoniasis and have been implicated to play a role in the cytoadherence, with specific proteinases expressed on the surface of the parasites playing a major role. Two cysteine proteinases (termed CP65 and CP30) were shown to be expressed on the surface of *T. vaginalis,* and shown to bind to and play a role in the cytotoxicity effect of *T. vaginalis* on HeLa cells (36,37,38). Parasites with low levels of CP65 and CP30 have a low level of cytotoxicity on HeLa cells (36).

Secretion of hydrolytic enzymes by trichomonads, particularly small molecular weight proteins, is well known. Secreted cysteine proteinases have been linked to cell detachment and cell lysis *in vitro.* Scott et al reported the secretion of two cysteine proteinases having activity towards either benzyloxycarbonyl-arginyl-ariginine 4-nitroanilide (Z-RR-Nan) or *N*-benzyl-prolyl-phenylalanyl-arginine 4-nitroanilide (Bz-PFR-Nan), which are cysteine proteinase substrates (42). The secretion pathway of these two proteinases are thought to involve the release from lysosomes (42). This hypothesis of cysteine proteinases being released from lysosomes was due to the stimulated release

of proteinase activity by treatment with amines and monensin. The lysosomal released cysteine proteinases appeared to be present in their active form, rather than in the precursor form in the lysosomes due to the inability of pepsin to stimulate any activity. Specific host-derived substrates have not been identified, although once identified would lead to a greater understanding of the role of these enzymes in the pathogenesis of trichomoniasis. Of the potential virulence factors present in *T. vaginalis* proteinases, particularly cysteine proteinases, have been the most studied and appear to participate in several facets of the biology of *T. vaginalis* including immune evasion and cytotoxicity.

Hemolysis

It is hypothesized that the hemolytic activity on red blood cells by *T. vaginalis in vivo* is a molecular mechanism for the acquisition of lipids and iron. Hemolytic activity and acquisition of iron have been suggested to be responsible for the exacerbation of symptoms observed to occur during and following menstruation. Iron acquisition is required and is critical in the pathogenic mechanisms, specifically the expression of adhesions. Hemolytic activity was first reported in the early 1980's by Kreiger et al when he described a beta-hemolytic activity, which correlated with virulence of particular *T. vaginalis* strains (64). This beta-hemolytic activity is not host specific in that human, sheep, rabbit and chicken red blood cells were lysed. The specific enzymatic activity required for hemolytic activity is uncertain, yet Alderete et al have shown that hemolytic activity can be partially inhibited by the use of proteinase inhibitors, suggesting

proteinases may play a direct role in the observed hemolytic activity or processing the lytic molecule (65).

Hemolysis is sensitive to certain physiological conditions, including temperature, Ca^{2+}, pH, and concentration (34). *T. vaginalis* hemolytic ability is optimal at 37°C and is abrogated at 4°C. The hemolytic activity is released when the pH is lower then 6.5 and this pH requirement is also required for functional proteins required for hemolytic activity. Ca^{2+} depletion experiments showed that hemolytic activity is inhibited in Ca^{2+}-depleted media. Hemolytic activity seems to be concentration dependent in order to measure hemoglobin release.

Cell-Detaching Factor

T. vaginalis secretes a cell-detaching factor (CDF), which has been characterized as a 200 kDa glycoprotein that has cytopathic effects on mammalian cell cultures *in vitro* (43,65). In the presence of CDF anchored mammalian cells detach and then aggregate, yet remain viable. CDF is pH sensitive with an optimal activity at 7.2, although CDF activity can be detected in a pH range from 5.0 to 8.5 (43,65). The pH range in which CDF is active is important due to the fact that the vagina is normally at a pH 4.5, yet when infected with *T. vaginalis* the pH is 5.0. *T. vaginalis* raising the pH may be critical in having active CDF enzymatic activity possibly leading to sloughing of epithelial cells, characteristic in the vaginal mucosa during acute infections with this parasite.

Investigations on CDF activity showed it could be detected by stimulating parasites on mammalian cells, concentrating the cell free filtrates, and then adding the

concentrated cell-free filtrates to mammalian cells. This approach differs from the approach of some other investigators who studied soluble factors secreted from this parasite. Garber et al revealed that the concentration of CDF *in vitro* is dependent on three factors. The concentration of the *T. vaginalis* inoculum, growth of *T. vaginalis* prior to collection of cell free filtrate, and pH of the cell free filtrate (discussed in previous paragraph) (43). CDF secretion seems to be dependent on activation of parasites by the presence of mammalian cells, yet secretion of CDF does not seem to be cell specific.

Lushbaugh et al reported a secreted factor termed *T. vaginalis* culture factor (TVF), which altered the morphology of mammalian cells (41). The TVF morphological effects on mammalian cells were reversed in the absence of TVF medium, yet required media containing 40% fetal bovine serum. TVF is heat and pH labile and has an approximate size of 250 kDa according to gel filtration, yet size was reduced to approximately 50 kDa on SDS-PAGE. TVF appears to be cell specific affecting certain mammalian cell lines, such as baby hamster kidney cells (BHK-21) and Chinese hamster ovary cells (CHO-K1), where as no effect was detected on rabbit kidney cells (RK-13) and murine macrophage-like cells (WEHI-3). Lushbaugh et al failed to culture parasites in the presence of mammalian monolayers to determine if secretion increased due to the presence of the monolayers. Nevertheless a secreted factor was detected in the cell-free filtrate that altered the morphology of mammalian cells *in vitro*.

Reports of *T. vaginalis* secreting a cell-detaching factor support the idea of contact independent virulence factors in the pathogenesis of trichomoniasis. The *in vitro*

mechanisms leading to cell detachment may have valid implications in the pathogenesis of trichomoniasis in humans, in which characteristic sloughing of epithelial cells occurs in acute infections. Data suggest that CDF and TVF may possibly be due to the same type of enzymatic activity, yet further investigation of these cell-detaching factors needs to be pursued to elucidate the molecular identity of these parasite products and their specific mechanisms.

Contact-Independent Mechanisms

The determination of virulence factors secreted from *T. vaginalis* has been controversial. Using hanging drop cultures of human cells Hogue first observed the pathogenesis of *T. vaginalis in vitro* and hypothesized that the pathogenesis was a result of secreted factors rather than caused by host cell-parasite direct interactions (66). Several other reports on secreted factors have been published in the literature and one report showed that cell-free culture filtrates caused morphological changes to chick liver cultures and inhibited fibroblast cell division (67). Fiori et al were able to show that under "triggering" conditions pore-forming proteins were released in a contact-independent mechanism (68). It has also been recently reported that neither hemolytic nor cell lysis effects of *T. vaginalis* were contact dependent suggesting that soluble factors may play a role in the pathogenesis of trichomoniasis (44).

Reasons why secreted virulence factors have been a spot of controversy may be directly related to the nature of the experiments being performed. Currently most of the discoveries of virulence factors have been from *in vitro* studies. In order for proper

enzymatic activity and function there needs to be a wide array of environmental conditions met such as identification of optimal pH, ion concentration, stimulus, *etc.* This may be why some investigators were able to detect contact-independent mechanisms (41, 43, 65), yet others were not (36,37,38,39,40). For example, CDF was detected by stimulating the parasites prior to collecting the cell free filtrate (43). Stimulation was also used for the optimal secretion of proteases (42). The idea of detection of secreted virulence factors after stimulation makes sense due to the fact that *T. vaginalis*, like all life forms, is likely conservative of its energy resources and virulence factors are not going to be secreted unless the parasite comes into contact with a stimulus such a host cells. Ion concentration and pH play a major role in preserving enzymatic activity and these parameters must be maintained. For example hemolytic activity requires Ca^{2+} and is only active in a specific pH range. Many of the investigators that failed to observe cytotoxic activity in cell free filtrates may have failed to maintain the physiological conditions that may be critical in detecting secreted virulence factors in cell-free filtrates.

T. vaginalis Destruction of Target Cell Cytoskeletions

A hallmark of *T. vaginalis* cytotoxicity towards nucleated cells is the loss of fibroblast like morphology. This loss of fibroblast like morphology is followed by cell rounding and detachment *in vitro*. It has been shown that *T. vaginalis* directly targets cell cytoskeletons, which was first shown on red blood cells (68). Initial host cell cytoskeleton studies employed red blood cells due to the biconcave shape of these cells. Studies have showed red blood cells in contact with *T. vaginalis* result in disappearance

of spectrin expression, which is a major component of red blood cell membranes (68). Investigators have shown that contact is required between viable parasites and red blood cells (34). Disappearance of spectrin occurs prior to red blood cell lysis (34). Loss of cell spectrin is not exclusive to just red blood cells, as reports have shown that epithelial cells also lose expression in membranes during contact with *T. vaginalis* (34). Further analysis into spectrin disappearance revealed an active proteinase specific for spectrin termed spectrinase (34). Spectrinase activity was only detected in parasite lysates and was absent in cell free filtrates indicating spectrinase activity is probably due to an intracellular enzyme. The current hypothesis is that spectrinase activity is secreted in order to degrade an internal target. Furtado et al. suggested that spectrinase may be secreted once the membrane fusion occurs between parasite-human epithelial cells (69).

Virulence Dependence on Iron

Iron is an important part of the host environment and thus has been hypothesized that trichomoniasis is thought to be greatly dependent on the presence of iron. Ryu et al reported that the pathogenesis of *T. vaginalis* is influenced by the presence of iron (70). It has been reported that there is an increase in AP expression on *T. vaginalis* when grown in iron replete media (49). Further evidence of AP expression on iron was the characterization of iron-responsive promoter in the *AP65-1* gene (71). Iron also seems to be critical in the evasion of the host immune system, in which in the presence of iron *T. vaginalis* is resistant to complement (62). This is performed by the increase of expression of a C3 specific proteinase. In another report *T. vaginalis* was shown to have

increased binding to fibronectin in the presence of both iron and calcium (fibronectin-*T. vaginalis* interaction discussed above) (55) supporting the idea that appropriate iron availability may facilitate adhesion function leading to higher cytotoxic capacity.

Hypothesis

Multiple virulence factors of *T. vaginalis* likely play a role in the pathogenesis toward host cells and identification and characterization of these virulence factors should give insight into the pathogenesis of trichomoniasis. We hypothesize that *T. vaginalis* produces virulence factors both secreted and contact-dependent that are important in the pathogeneses of trichomoniasis. Molecular characterization of parasite molecules, that mediate target cell lysis will elucidate specific mechanisms of host cell destruction and will identify (at the molecular level) virulence molecules of this parasite.

Project Goals

To test this hypothesis, we set a number of goals to answer this hypothesis. A primary goal was to identify and characterize novel virulence factors isolated from *T. vaginalis* that may play a role in trichomoniasis. Once we identified and characterize a novel virulence factor we set a goal of determining if the virulence factor is secreted, how it is regulated, and if it is stored in vesicles. We isolated and characterized a virulence factor, termed lytic factor (LF), produced by *T. vaginalis* with CPE to mammalian cells *in vitro*. Studies are described that show that inactivation of LF by treatment with trypsin

(125 ng/ml) suggesting cytotoxicity was due to a protein. LF hydrolyzed phospholipase substrates but lacked any activity toward protein substrates, thus indicating LF enzymatic activity was likely due to a phospholipase. Gel filtration (Sephacryl-200) and SDS-PAGE analysis revealed LF components of 167.9 kDa and 220 kDa (non-reducing conditions), respectively. Further experiments showed that *T. vaginalis* upregulated expression of LF when stimulated with mammalian cells *in vitro*. The results also indicate *T. vaginalis* appears to store LF in cytoplasmic vesicles as parasites treated with Brefeldin A were unable to store LF in these cytoplasmic vesicles. We suggest that molecular characterization of such virulence factors should lead to a greater understanding in the molecular mechanisms of the pathogenesis of trichomoniasis and, ultimately, to new approaches to alleviating this disease.

PURIFICATION AND ANALYSIS OF A LYTIC FACTOR PRESENT IN *TRICHOMONAS VAGINALIS*

Introduction

Human trichomoniasis a sexually transmitted disease caused by *Trichomonas vaginalis,* is a disease ranging from a mild or unapparent infection to a more severe infection with a substantial level of inflammation in women and urethritis in men (1). *T. vaginalis,* an extracellular, flagellate, protozoan parasite, is one of the most common causes of vaginitis in women and accounts for 20-30% of all vaginitis cases worldwide (2). The mechanisms of pathogenesis of the trichomonad parasite in the urogenital tract and avoidance of the host immune system response are not well understood. Virulence factors of *T. vaginalis* likely play a role in pathogenesis by causing cytopathic effects (CPE) on host cells so that it is important to identify such virulence factors and to determine and understand their role in pathogenesis.

The CPE of *T. vaginalis* on mammalian cells *in vitro* has been documented, though the particular molecular mechanisms have not been fully elucidated. The CPE has been proposed to be due to both contact-dependent and contact-independent mechanisms. Four adhesion proteins (AP), AP65, AP51, AP33, and AP23, have been described that may mediate adherence to the vaginal epithelial cells *in vivo* (46) an event that leads to efficient CPE (47). Contact-dependent CPE has been well studied and numerous potentially pathogenic molecules such as cysteine proteinases, hemolytic

activity, and pore forming proteins have been reported (36,37,38,39,68) that may be involved in contact-dependent CPE. Two known cysteine proteinases are expressed on the surface of these parasites (36,37,38). In contrast the role of secreted proteins by *T. vaginalis* in CPE and their specific functions in pathogenesis has not been elucidated and their molecular mechanisms remaining unclear.

Sixty years have past since Hogue observed the pathogenesis of *T. vaginalis* on hanging drop cultures of human cells and hypothesized that the pathogenesis observed *in vitro* was directly do to secreted toxins from *T. vaginalis* (66). Since then investigators have published contradicting reports on the CPE of cell-free, parasite-derived filtrates on cultured cells (36,37,38,39,39,42,43, 65). Several reports of secreted soluble factors, termed cell-detaching factor (CDF) and *T. vaginalis* factor (TVF), have suggested these parasite products to cause a variety of morphological changes to certain mammalian cells *in vitro* (42,43,65). Many of these morphological effects were reversible when the mammalian cells were subsequently cultured in media without CDF or TVF (42,43,65) but no data on the biochemical alterations on the target cells were reported. Other than the cysteine proteinases the parasite enzymatic activities involved in the CPE on host cells or the pathogenesis of trichomoniasis, including target cell lysis, remain unknown. We hypothesized that lipolytic activity may play a role in the pathogenesis of *T. vaginalis* towards host cells since lytic activity has been described in almost all pathogenic organisms, including *T. vaginalis*. Because several microbial lytic molecules (eg., bacterial hemolysins) have been identified as either a phospholipase or a lipase, we

further hypothesized that a lipase/phospholipase could account for lytic activity and be an important virulence factor of *T. vaginalis.*

Lipolytic activity has been described in all most all organisms analyzed for its presence. In this study we addressed the hypothesis that lipolytic activity may play a role in the CPE of *T. vaginalis* towards host cells. In the analysis of *T. vaginalis* for the presence of lipolytic activity we partially purified and analyzed a lytic factor (LF) that hydrolyzes lipids and phospholipids. This LF lyses nucleated mammalian cells, red blood cells, and hydrolyses liposomes *in vitro,* suggesting the presence of enzymatic activity consistent with lipase or phospholipase activity. Further investigation revealed that LF hydrolyzes phosphatidylcholine similar to phospholipase A_2 (PLA_2) suggesting the major LF component responsible for host cell destruction is likely to be a PLA_2.

Materials and Methods

Parasites

Trichomonas vaginalis Strain UAB 5-1 (provided by Dr. J. Swebke, University Alabama Medical Center, Birmingham, AL) was grown in modified Diamond's medium pH 6.0 (72) containing 10% heat inactivated Fetal Bovine Serum (Atlanta Biological, Atlanta, Ga) at 37°C in 500 ml screw-capped serum bottles. Parasites were counted with a hemocytometer and collected by centrifugation (400xg @ RT, 5 min) and washed twice in PBS pH 7.2 (10 mM sodium phosphate, 150 mM NaCl).

Parasite-Antigens

Whole parasite antigen was extracted by adding $1x10^8$ parasites/ml to extraction buffer (50 mM Tris pH 8, 100 mM NaCl, 5 mM ethylenediaminetetracetic acid (EDTA), 1% Trition-X 100, 10 μM leupeptin, and 10 μM trans-epoxysuccinyl-L-luecylamido-(4-guanidine) butane(E-64) (Sigma, St. Louis, Mo) followed by incubation on ice for 30 minutes with gentle mixing every 5 minutes. Cellular debris was pelleted (1000x g @ 10°C, 30 min.) and the supernatant was stored at -20°C. The BCA protein assay (Pierce Chemicals, Rockford, IL) was used to determine protein yields.

Hydrophobic Chromatography

LF was purified by octyl-Sepharose (Amersham-Pharmacia, Arlington Heights, IL) chromatography (73, 74). Briefly, octyl-Sepharose beads were washed twice with two volumes of 5% N-propanol in 0.1 M ammonium acetate pH 7.0 and parasite antigen (2 mg/ml of beads) or BSA (500 μl/ml of beads); (negative control) was added and the mixture was rotated end-over-end overnight at 4°C. The supernatant was decanted and the beads were washed with 20 volumes of 5% N-propanol in 0.1 M ammonium acetate pH 7.0. The column was eluted with a ladder gradient of 10-60% N-propanol (10% N-propanol increments-30 ml/increment) in 0.1 M ammonium acetate. The presence of fractions containing LF or BSA was monitored by periodic acid-Schiff (Sigma, St. Louis, MO) (PAS) on nitrocellulose paper and by BCA protein assay (Pierce Chemicals, Rockford, IL). Fractions containing carbohydrate material or protein (BSA) were

concentrated under vacuum (Speedvac, BioRad), re-suspended in Hanks balanced salts solution, pH 7.2, (HBSS) and pooled into groups of four fractions for further analysis.

Gel Filtration

Octyl-Sepharose LF preparations were further fractionated by gel filtration chromatography. A Sephacryl 200 column (Amersham Pharmacia, Arlington Heights, IL) was packed in a glass column with 1 cm by 25 cm dimensions in 0.1 M ammonium acetate, pH 7.0 (buffer). The packed column was calibrated with a sample containing four known molecular weight standards (myosin, BSA, ovalbumin, and β-lactoglobulin) all at 1 mg/ml. One ml fractions were collected at a flow rate of 450 μl/min for both the standards and the LF preparations and 500 μl of LF preparations were typically added to the column for each chromatogram. Fractions collected from the column were subjected to the BCA protein assay (Pierce Chemicals, Rocford, IL) to detect fractions containing protein and retained at 4°C for functional assays.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to Laemmli (75) using 10% T gels that were either developed with silver staining (Novex) or electroblotted (76) onto nitrocellulose membrane and analyzed by Western blot.

2-Dimensional Electrophoresis

2-D electrophoresis was performed according to O'Farrell (77). 3-10 isoelectric focusing (IEF) strips (Bio-Rad, Hercules, CA) were rehydrated with 100 μl of protein

solution (100 mg) and 350 µl redydration buffer ((67 mM DTT, 9 M Urea (Fisher Scientific, Denver, CO), 2% Triton X-100 (Sigma, St. Louis, MO), 1.5 % 3/10 Ampholyte (Bio-Rad, Hercules, CA)) for 19 hours at RT. Strips were then rinsed three times with water followed by electrophoresis for 24 hours at a constant voltage of 800V. After first dimension electrophoresis strips were equilibrated in equilibration buffer I (0.1 M Tris-HCL pH 6.9, 6 M urea, 20% SDS, 26 mM DTT, 30% glycerol) for 15 min followed by equilibration buffer II (0.1 M Tris-HCL pH6.9, 6 M urea, 20% SDS, 26 mM iodoacetamide (Sigma, St. Louis, MO), 30% glycerol)) for 10 min. After strips were equilibrated the strips were subjected to SDS-PAGE on a 10% T gel.

Protein Digestion

Protein spots were removed from 2-D gels and digested with trypsin. Briefly, gel pieces were incubated in 10 µl of acetonitrile (Sigma, St. Louis, MO) for 10 min at RT and dried in a vacuum centrifuge (Speedvac, BioRad). 10 mM DTT (Sigma, St. Louis, MO) in 100 mM ammonium hydrogenocarbonate (NH_4HCO_3) was added and incubated for one hour at 56°C, liquid was removed and 20 µl of 55 mM iodoacetamide (Sigma, St. Louis, MO) in 100 mM NH_4HCO_3 was added and incubated for 45 min at RT in the dark. Liquid was removed and gel pieces were washed in 20 µl of 100 mM NH_4HCO_3 for 10 min, liquid was than removed, and gel pieces were incubated in 20 µl of acetonitrile for 10 min, gel pieces were washed with 20 µl of 100 mM NH_4HCO_3 for 10 min and liquid was than removed (step was repeated). 20 µl of acetonitrile was added and incubated for time min, liquid was removed and dried in a vacuum centrifuge. Gel pieces were

digested with 20 µl of 12.5 ng/µl trypsin (Worthington biochemical, Lakewood, NJ) in in trypsin buffer (50 mM NH_4HCO_3 and 5 mM $CaCl_2$) for 3 hours on ice. 10 µl of trypsin buffer was added and incubated at 37°C overnight, supernatant was removed, 20 µl of 100 mM NH_4HCO_3 was added and incubated at RT for 15 min, and supernatant (step was repeated). Supernatant was pooled and dried in a vacuum centrifuge and resuspend in water, Samples were purified with a $ZipTip_{C18}$ (Millipore, Bedford, MA) and eluted with 50% acetonitrile. Samples were then added to saturated α-Cyno-4-hydroxycinnamic acid (Sigma, St. Louis, MO)at a 1:1mixture.

MALDI-TOF

Positive-ion MALDI-TOF MS analysis was performed using model ReflexIII delayed-extraction MALDI-TOF mass spectrometer (Bruker Daltonics, Billarica, MA) equipped with a 337 nm nitrogen laser; alpha-cyano-4-hydroxycinnamic acid (MALDI-Quality, Hewlett-Packard) was used as the matrix. Prospector was used to analyze peptide fragments and identify possible homologues.

Periodate Sensitive Epitopes

Western blots were probed with antibodies or treated with periodate and then probed with antibodies. Briefly, strips were washed with 50 mM sodium acetate buffer, pH 7.0, for 5 min. Strips were then either soaked in 20 mM periodate in 50 mM sodium acetate buffer, pH 4.5, or in 50 mM sodium acetate, pH 4.5, in the dark for one hour. Strips were washed 3X 10 min each in 50 mM sodium acetate. Strips were then soaked in 1% glycine in 150 mM NaCl for 30 min and washed in 5% blotto (5% skim milk in

phosphate buffer saline pH 7.2) for 30 min. Goat anti-*T. vaginalis* serum was added and incubated overnight at 4°C. Strips were washed 3X with 5% blotto followed by peroxidase-conjugated rabbit anti-goat IgG (ICN/Cappel, Costa Mesa, CA) and incubated for an hour. Strips were washed 3X times in PBS and then developed with TMB membrane peroxidase substrate (KPL, Gaithersburg, MD).

Cytotoxicity Assays

Cytotoxicity of *T. vaginalis* parasites and purified LF was measured on WEHI 164 cells (murine fibroblast cell line; Ruddle *et al*; ATCC, McClean, VA). WEHI 164 cells were plated in 96 or 24 well plates at 2.0 X 10^5/well or 2.0 X10^6/well, respectively, depending on experimental design, in complete DMEM (4 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), 25 μg/ml gentimicin sulfate). Plates were incubated at 37°C/ 5% CO_2 humidified incubator for 4 to 6 hours to allow the WEHI 164 cells to adhere to culture plates. Serial dilutions of the parasite material (viable parasites, extract, LF, etc) or LF treated with trypsin (20 μl of 130 μg/ml) or E-64 (20 μl of 100 mM) (Sigma, St. Louis, MO) were added to the WEHI 164 cells. Viable parasites were incubated on WEHI 164 cells or added to a transwell filter (Corning Incorporated, Corning, NY). Negative controls were prepared with serial dilutions of BSA fractions that eluted from the octyl-Sepharose column and a positive control of TNF-α standard (NIH) were used for cytotoxicity assays. After incubation at 37°C/ 5% CO_2 humidified incubator overnight medium was decanted and monolayers were washed with warm HBBS. The plate was then fixed with 4% formalin for 10 min and stained

with crystal violet solution (25% ethanol, 0.55% crystal violet, 0.145 M NaCl) for 10 min. Wells were then washed 3X with HBSS and allowed to dry over night. Plates were solublized with an acidic acid solution (0.5 %SDS and 0.5 M acetic acid) and absorbance was measured at 550 nm on a spectrophotometer (Thermomax Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated with the following equation:

$$\% \text{ Cytotoxicity} = \frac{(\text{Control}_{\text{O.D. 550nm}} - \text{Unknown}_{\text{O.D. 550nm}})}{(\text{Control}_{\text{O.D 550nm}})} \times 100$$

Lipase Assay

Lipase activity was measured with polyoxyethylene sorbitan substrates (Tween 20 and Tween 80) as described by Tirunarayanan (78). The reaction mixture which consisted of 0.01 ml of 10% Tween in 50 mM Tris hydrochloride pH 7.6 (Tris buffer), 0.01 ml of 1M $CaCl_2$ in Tris buffer, 0.23 ml of 50mM Tris hydrochloride pH 7.6 buffer was mixed with 0.05 ml of fraction material or phospholipase D. Reagent blanks were prepared with 0.05 ml of water. Duplicates of each mixture were prepared and incubated at 37°C for two hours. Turbidity of each mixture was measured at 405 nm on a spectrophotometer (Thermomax Molecular Devices, Sunnyvale, CA).

Protease Assay

Protease activity was measured with Azocoll as described by Hageman (79). Briefly, 11 mg/ml Azocoll (Sigma, St. Louis, MO) in 0.1 M potassium phosphate buffer, pH 7.8, was pre-equilibrated at 42°C for 20 minutes in 1.0 ml aliquots. Reaction was

initiated by the addition of 0.1 ml of enzyme to the Azocoll solution. 0.25 ml of the sample was removed at 5-min intervals and added to 1 ml 5% of trichloroacetic acid to stop reaction and absorbance was measured at 550 nm on a spectrophotometer (Thermomax Molecular Devices, Sunnyvale, CA).

Liposome Preparation

Liposomes were prepared as described by Gregoriadis (80). Briefly, 0.25 ml of phosphatidylcholine (Sigma, St. Louis, MO) in chloroform (100 mg/ml) was mixed with 0.63 ml cholesterol (Sigma, St. Louis, MO) in chloroform (20 mg/ml) and the lipid solution (1:1 molar) was dried by rotary evaporation in a 50 ml round bottom flask. Two ml of aqueous 20 mM 5-(and-6)-carboxyfluorescein (CF) (Molecular Probes, Eugene, OR), warmed to 40°C was added to the lipid film with shaking to dislodge lipid film followed by sonication to reduce the mutlilaminer vesicles to small unilamellar vesicles (liposomes). Sonication was performed in a 40°C water bath with 1 minute bursts (60% output) using the 50 Sonic Dismembrator (Fisher, Denver, CO) followed by 30 second cooling periods (Total sonication time 10 minutes). Large vesicles together with liberated probe tip fragments were pelleted (3000x g, @ RT, 7 min). Unincorporated CF was removed from the liposomes by gel filtration (CL-4B) and centrifugation (1000x g @ 10C, 30min.). CF incorporated liposome fractions were stored at 4°C. Liposomes were incubated with LF, BSA, or buffer (PBS) at 37°C for two hours. Treatment groups were then read on a fluorometer at an excitation wavelength of 435 nm and emission

wavelength of 538 nm. The extent of lysis was expressed as the percent release, calculated with the following equation:

$$\% \text{ CF release} = \frac{(\text{Control}_{\text{O.D. 538nm}} - \text{Unknown}_{\text{O.D. 538nm}})}{(\text{Control}_{\text{O.D 538nm}})} \quad X\ 100$$

Phospholipid Hydrolysis

10 µl of 1 mM BIODIPY labeled phosphatidylcholine (BPC) (Molecular Probes, Eugene, OR) was dried under a slow stream of nitrogen. Once dried BPC was dissolved in 50 µl of solution I (0.5 mM octylglucoside, 0.4 mM NaCl, and 60 mM HEPES, pH 7.0) and briefly sonicated. 50 µl of solution II (5 mM EGTA, 2 mM EDTA, and 1 mM DTT) or HBSS, pH 7.0, was added to the BPC solution. A volume of 5 µl of LF, phospholipase D (PLD) (Sigma, St. Louis, MO) phospholipase A_2 (PLA_2) (Sigma, St. Louis, MO), or blank was added to 12.5 µl of BPC and treatment groups were incubated for 2 hour at 37°C. 5 µl was then plated on a K6 Silca Gel 60 A thin layer chromatography plate (Whatman, Maidstone, UK) and allowed to dry. Samples were resolved in the chloroform:methanol:acetic acid solvent (45:45:10) and hydrolysis was detected by inspection under a far UV (365 nm).

Statistical Analysis

Data were analyzed by a one-way ANOVA followed by Tukey's multiple comparison test was used to detect significant differences at $P<0.05$ among treatment groups.

Results

T. vaginalis CPE

CPE of *T. vaginalis* on mammalian cells *in vitro* has been reported to be due to cell-to-cell contact (36,37,38,39,40) and secreted proteins (42,43,65). The basic pathogenic effects of *T. vaginalis* on WEHI 164 cells were examined in the presence or absence of transwell filters to measure the cytotoxic effect of *T. vaginalis*-WEHI 164 cells contact versus secreted proteins on the WEHI 164 cells (Fig. 2.1). *T. vaginalis* added directly to the WEHI 164 cells (cell-to-cell contact) produced a significant cytotoxic effect ($P < 0.05$) in the presence or absence of FBS (Fig. 2.1). At all parasite to WEHI 164 cell ratios tested (10:1, 5:1, and 1:1) the parasite cytotoxicity on the WEHI 164 cells was greater then 80%.

T. vaginalis in FBS-free medium added to transwell filters had cytotoxic levels of 35% and 85%, respectively, when 5:1 and 10:1 WEHI 164 cells: *T. vaginalis* ratios were used. *T. vaginalis* in media containing FBS in transwell filters produced cytotoxicity levels of 13% and 50% at concentrations of 5:1 and 10:1, respectively. 1:1 ratio using the transwell filters did not produce a significant level of cytotoxicity in the presence or absence of FBS. *T. vaginalis* added directly to the WEHI 164 cells and to the transwell filters maintained higher then 95% viability 24 hours after being added to the monolayers (data not shown).

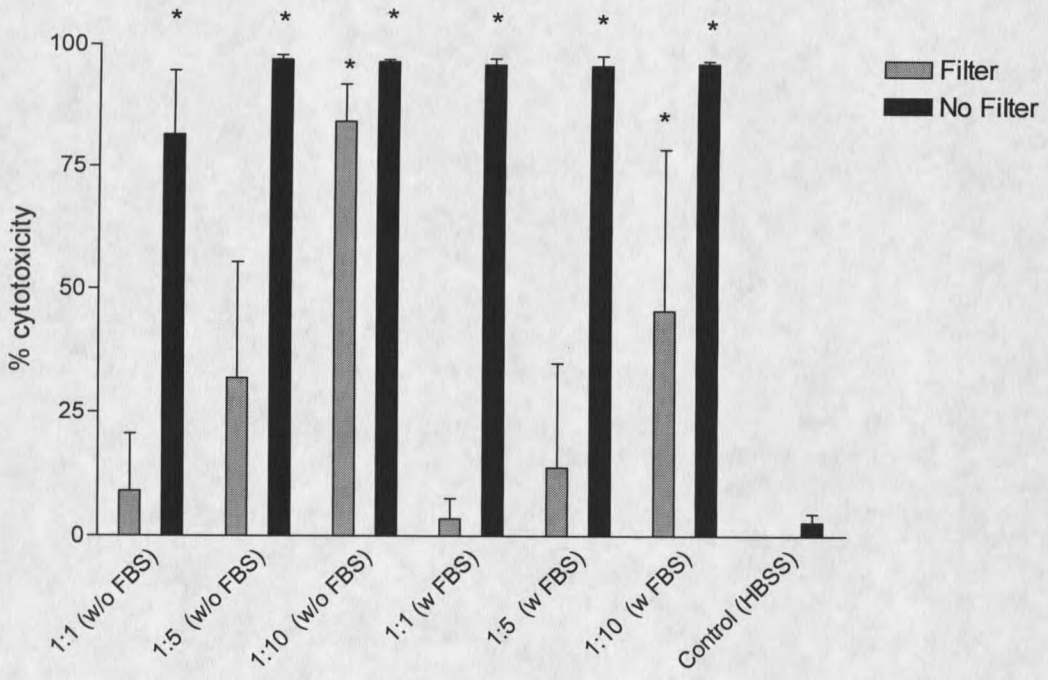


Figure 2.1: *T. vaginalis* cytotoxicity toward WEHI 164 cells is present with or without cell-to-cell contact. *T. vaginalis* were cultured on WEHI 164 cells in the presence or absence of transwell filters with or without FBS medium. *T. vaginalis*:WEHI 164 cells were incubated for 24 hours and then cytotoxicity was measured. Data pooled from three individual experiments. Significant differences of treatment groups compared to negative control (HBSS) indicated by * is $P<0.05$.

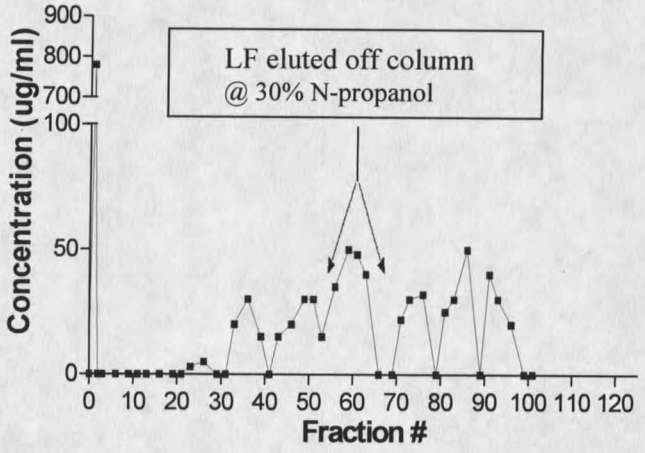
Chromatographic Purification of LF

Since we observed cytotoxic activity of soluble parasite material on WEHI 164 cells and it is well known that several microorganisms express lypolytic activity that can play a role in their pathogenesis, including host cell membrane destruction (81) we wanted to see if *T. vaginalis* expressed any lipolytic activity. Octyl-Sepharose chromatography was used to resolve molecules in *T. vaginalis* extract according to

species hydrophobicity (Fig 2.2A) since we reasoned that lipolytic enzymes are predominately hydrophobic species and are capable of association and disassociation from hydrophobic matrices (82). Fractions that eluted at 30% N-propanol contained cytotoxic material when added to WEHI 164 cells (Fig 2.2B) and we termed this material lytic factor (LF). When LF was tested for cytotoxic activity on WEHI 164 cells it exhibited a cytotoxic effect with the kinetics of cell lysis ranging from 4 to 24 hours and depended on the concentration of LF in the preparation (data not shown). The addition of 10 µl of LF to 5.0 X 10^5 WEHI 164 cells had a cytotoxic effect of greater than 90% on the WEHI 164 cells ($P<0.05$). LF when added at 5 µl and 2.5 µl had cytotoxic effect on WEHI 164 cells of 50%, 25%, respectively, and LF lysis was also observed with bovine red blood cell targets (data not shown). When 10 µl of BSA material (negative control) was added to WEHI 164 cells there was no significant cytotoxic effect (Fig 2.2B), which eluted off the column at 40% N-propanol in 0.1 M ammonium acetate (data not shown). LF positive fractions also contained carbohydrate material detected by PAS staining of fractions spotted onto nitrocellulose paper (data not shown).

Next the LF was digested with trypsin and assayed to determine if LF depended on protein activity. Treatment of LF (10 µl) with trypsin inhibited the cytotoxic activity on the WEHI 164 cells by 90% (Fig 2.3A). As a control for the effects of trypsin on WEHI 164 cells BSA treated with serial dilutions of trypsin ranging from 1:2 out to 1:256 (confirmed by SDS-PAGE) and the dilutions that hydrolyzed BSA were added to BSA and the mixture then applied to WEHI 164 cells. 130 µg/ml of trypsin, which hydrolyzed BSA, produced no detectable lysis of the WEHI 164 cells (data not shown).

A



B

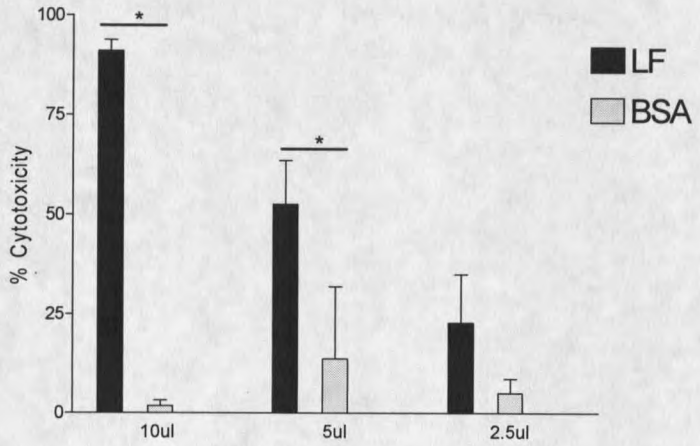


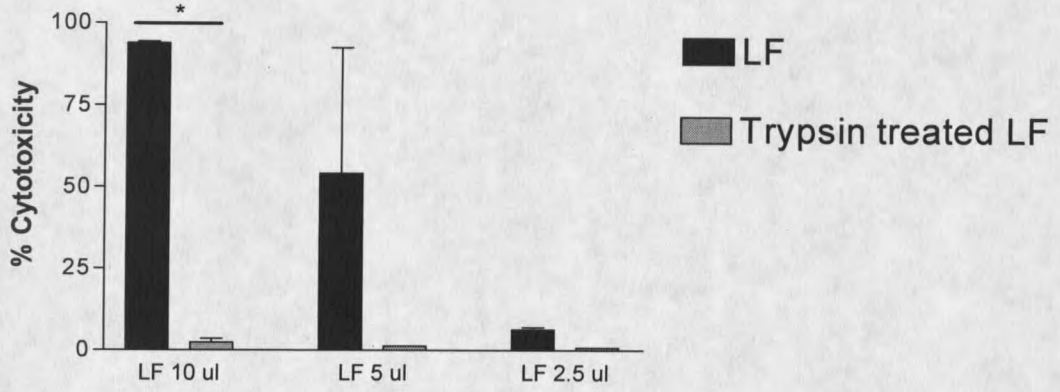
Figure 2-2: LF is cytotoxic to WEHI 164 cells. *T. vaginalis* extract or BSA was subjected to octyl-Sepharose chromatography and separated by elution of the column with increasing amounts of N-propanol in 0.1 M ammonium acetate. Fractions were tested for the presence of protein (BCA) and carbohydrates (periodic acid-Schiff) and fractions positive for both protein and carbohydrates were lypholized. Lypholized fractions were reconstituted in HBSS, incubated on WEHI 164 cells, and cytotoxicity was measured. A) Octyl-Sepharose chromatogram of *T. vaginalis* extract. B) WEHI 164 cell cytotoxicity assay. Data pooled from three individual experiments. Significant differences among treatment group indicated by * is $P<0.05$.

Trypsin treated LF, which inhibited cytotoxicity to WEHI 164 cells, was electrophoresed onto nitrocellulose and probed with goat anti-*T. vaginalis* polyvalent serum. Untreated LF probed with goat anti- *T. vaginalis* polyvalent serum revealed two major, distinguishable bands (Fig 2.3B) at approximately 60 and 57 kDa. When trypsin treated LF was probed with goat anti-*T.vaginals* polyvalent serum there was only one distinguishable band at 57 kDa. Interestingly, when LF was treated with periodate the goat anti-*T. vaginalis* polyvalent serum was unable to recognize the epitopes of LF on the 60 and 57 kDa bands (Fig 2.3B) indicating that these epitopes detected in the two bands are glycosylated.

Enzyme Assays

T. vaginalis has many proteases, most of which are cysteine proteases and at least 23 cysteine proteases have been identified in *T. vaginalis* (58). Since proteases have been implicated in target cell damage we examined LF function when treated with a cysteine protease inhibitor. Treatment of LF with E-64 (cysteine protease inhibiter) did not inhibit cytotoxicity of LF on WEHI 164 cells and BSA treated with E-64 showed no signs of cytotoxicity on WEHI 164 cells (Fig 2.4A). LF material and trypsin (positive control) were also compared for protease activity using Azocoll. No protease activity was detected in LF using the Azocoll assay (Fig 2.4B), compared to an equal protein concentration of trypsin, which hydrolyzed Azocoll as early as 25 min of treatment.

A



B

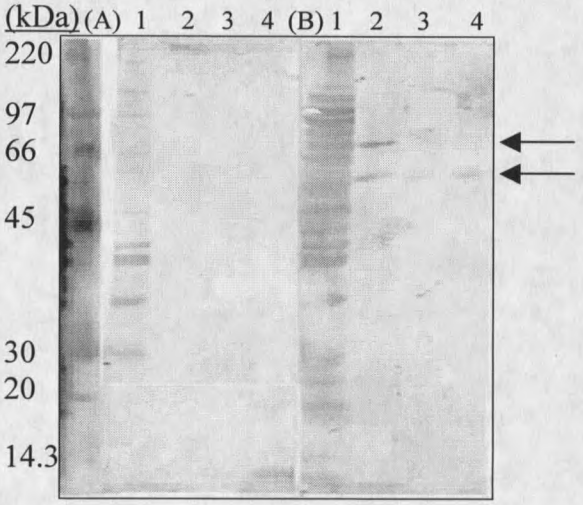
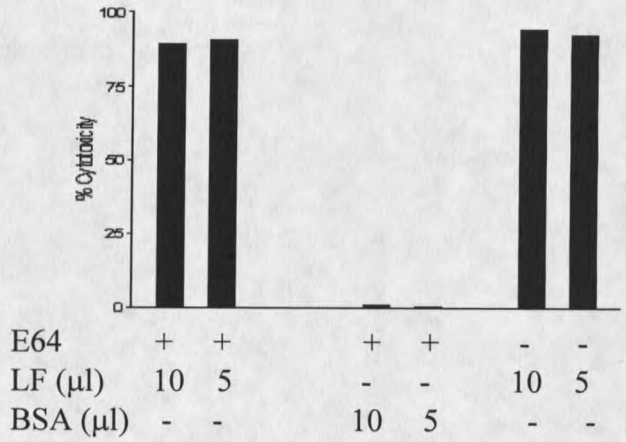


Figure 2-3: Cytotoxic activity of LF is due to protein activity. LF or BSA was treated with trypsin for two hours added to WEHI 164 cells or subjected to SDS-PAGE. (A) WEHI 164 cell cytotoxicity assay. Data pooled from two individual experiments. Significant differences among treatment group indicated by * is $P<0.05$. B) 10% SDS-PAGE was electroblotted on to a nitrocellulose membrane and treated with the following; A) Periodate treated, B) Probed with goat anti-*T. vaginalis*. Lane 1) *T. vaginalis* extract; Lane 2) LF; Lane 3) LF (1:4 dilution) treated with trypsin; Lane 4) LF (1:8 dilution) treated with trypsin. Western blot representative of three individual experiments.

A



B

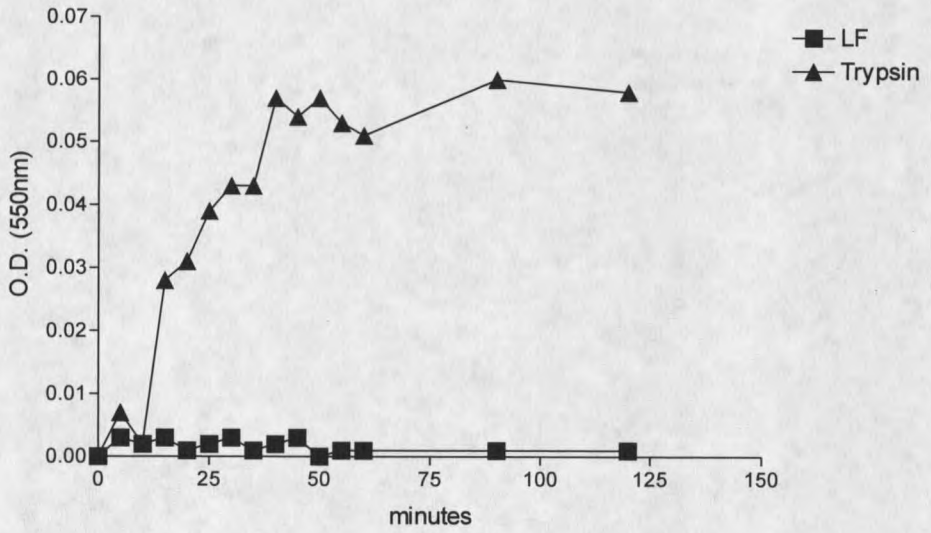


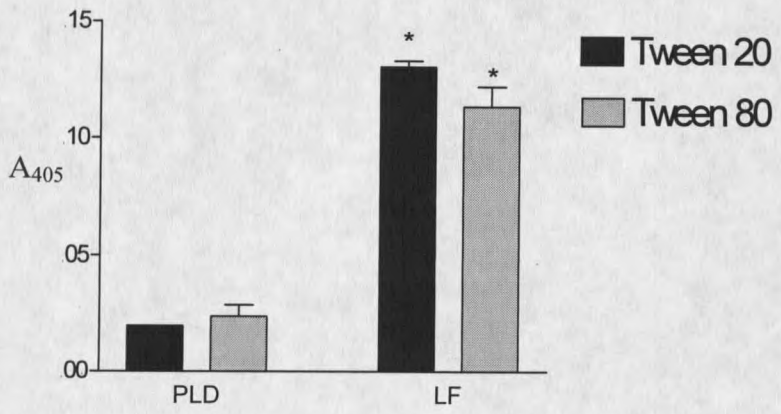
Figure 2.4: LF function is not due to detectable protease activity. A) LF treated with E-64, BSA treated with E-64, or untreated LF was incubated on WEHI cells overnight and cytotoxicity was measured by staining WEHI cells with crystal violet. B) LF and trypsin were incubated with Azocall to observe if any proteases were present in samples. LF showed no signs of absorbance at 550 nm. Trypsin showed signs of absorbance at 550 nm after approximately 25 minutes of incubation at 37°C. Experiments representative of three individual experiments.

LF Hydrolyses Phospholipase Substrates

LF was examined for lipase activity using the lipase substrates Tween 20 and Tween 80. Enzymes possessing lipase or phospholipase activity will hydrolyze these substrates and produce an insoluble product that can be measured by its turbidity at 405 nm. LF incubated with both Tween substrates produced detectable activity. LF produced a higher level (6.5 fold with Tween 20 and 4.7 fold with Tween 80) of turbidity compared to the turbidity after treatment with phospholipase D (PLD), which was used as a positive control (Fig 2.5A).

Liposome assays were performed next since the variation and sensitivity were superior to Tween assays and liposomes composed of precise phospholipase target molecules (phosphatidylcholine) could be used. Liposomes that consisted of phosphatidylcholine:cholesterol (1:1 ratio) were prepared to determine if LF would hydrolyze phospholipids that are substrates for all four subclasses of phospholipases (PLA, PLB, PLC, PLD). Carboxyfluorescein (CF) labeled liposomes incubated at 37°C with LF for 2 hours released up to 90% of the maximum releasable CF by treatment with 1% Triton-X 100 (Fig 2.5B). Decreasing concentrations of LF added to the liposomes resulted in decreasing release of CF demonstrating lysis due to LF occurred in a dose dependent manner. Control fractions that eluted from the octyl-Sepharose column at 60% N-propanol (fraction #161), distinct from the fractions containing LF, were also tested for CF release after lyophilization and reconstituted in the same manner as LF. When 50 μl and 25 μl of fraction #161 were incubated with liposomes only 25% and 12% CF release, respectively, was observed.

A



B

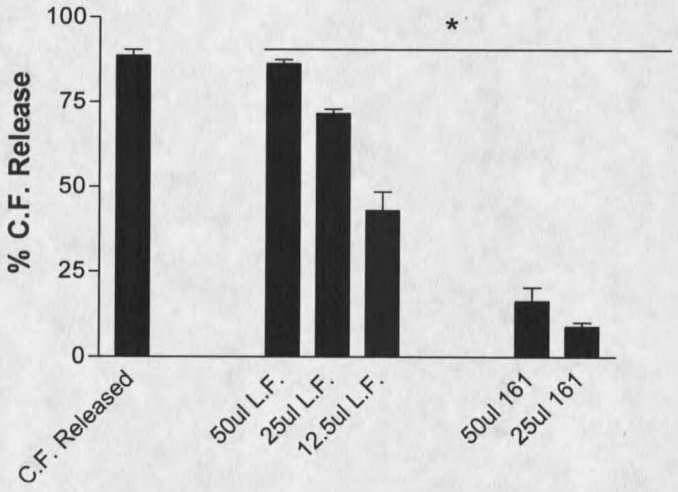


Figure 2.5: LF hydrolyses phospholipase substrates. A) LF and PLD (positive control) were incubated with Tween 20 or Tween 80 for two hours at 37°C and turbidity was recorded at 405 nm. LF hydrolyzed both Tween 20 and Tween 80. Date pooled from two individual experiments. Significant differences among treatment group indicated by * is $P<0.05$. B) LF, fraction #161 (negative control), or 1% Triton X-100 (maximum release) were incubated with CF entrapped liposomes composed of phosphatidylcholine:cholesterol (1:1 ratio) for two hours at 37°C. Various amounts of LF incubated with liposomes containing CF hydrolyzed the liposomes indicated by CF release detected at an excitation wavelength of 435 nm and emission wavelength of 538 nm. Data pooled from three individual experiments. Significant differences among treatment group indicated by * is $P<0.05$.

Since we had evidence that LF had phospholipase like activity we next wanted to determine what type of phospholipase activity was present in LF. BOIDIPY labeled phosphatidylcholine (BPC) was hydrolyzed in the presence of LF and the products of treatment with LF and PLA_2 had similar migration patterns when separated by TLC methodology (Fig 2.6). LF only hydrolyzed BPC when 2X LF was added to BPC, whereas 1X LF did not hydrolyze BPC. This migration pattern differed from the negative control not hydrolyzed BPC (lane 5) and PLD hydrolyzed BPC (Lanes 6,7) (Fig 2.6).

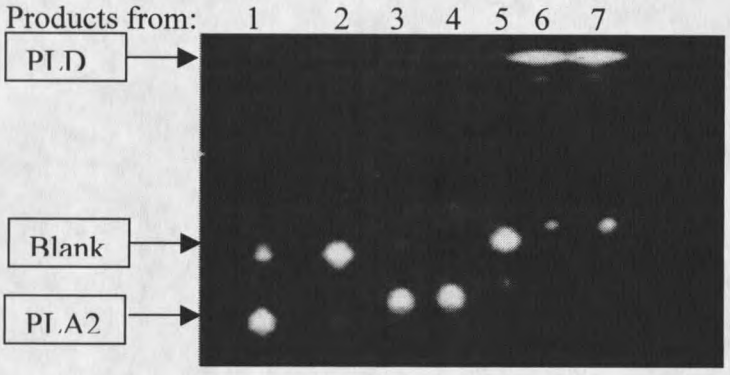


Figure 2.6: LF hydrolyzes BPC with PLA_2-like activity. LF, PLA_2, Blank (HBSS), or PLD were incubated with BPC for 2 hours at 37°C and products were resolved by TLC in chloroform:methanol:acetic acid solvent (45:45:10) and products were detected by inspection under far UV (365 nm). Lane 1) 2X LF; Lane 2) 1X LF; Lane 3) 1X PLA_2; Lane 4) 2X PLA_2; Lane 5) Blank (HBSS); Lane 6) 2X PLD; and Lane 7) 1X PLD. Experiment representative of three individual experiments.

Gel Filtration

Sephacryl 200 was next used to fractionate LF material from the octyl-Sepharose column by size. Material in the LF preparation eluted off the Sephacryl 200 column in

two major regions of the chromatogram: one in fractions 1-5 and the other in fractions 11-15 (Fig 2.7A). 1-5 and 11-15 correlated to 167 kDa and 144 kDa, respectively, based on the calibration of the gel filtration column (data not shown). The first twenty-two fractions were tested for the ability to hydrolyze liposomes and a detectable level of CF release was observed with fractions 1-5 (Fig 2.7B). SDS-PAGE analysis of fractions 1-5 under reducing and non-reducing conditions, revealed 60 kDa and 57 kDa bands under reducing conditions and a 220 kDa band under non-reducing conditions (Fig 2.8).

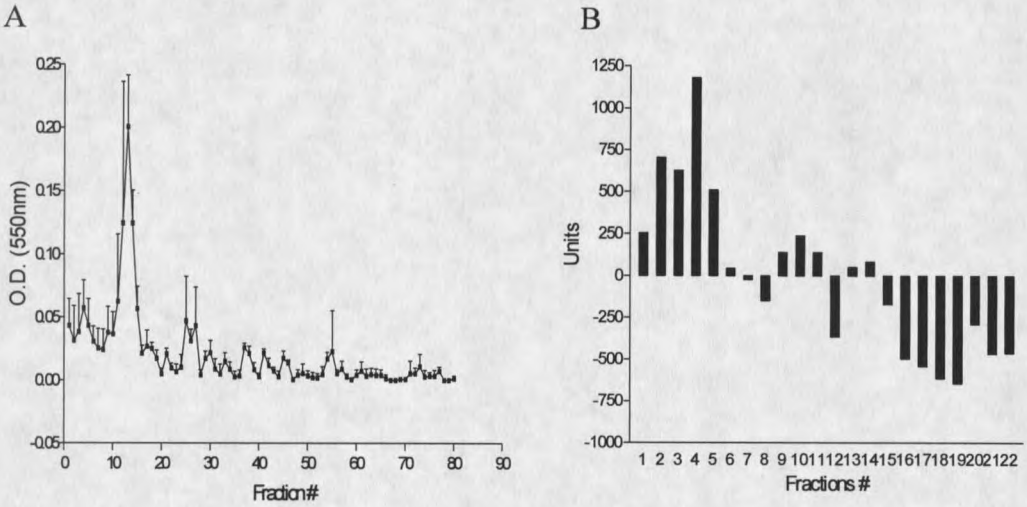


Figure 2.7: LF activity corresponds to a 167 kDa protein according to gel filtration. A) Octyl-Sepharose LF material was added to the gel filtration (Sephacryl 200) column and eluted with 0.1 M ammonium acetate buffer, pH 7.0. Protein was detected with the BCA assay at 550 nm. B) Gel filtration fractions were incubated with CF entrapped liposomes composed of phosphatidylcholine:cholesterol (1:1 ratio) for two hours at 37°C and CF release was detected at an excitation wavelength of 435 nm and emission wavelength of 538 nm. Phospholipase activity was detected in the first five fractions as indicated by CF release from liposomes. Experiments representative of three individual experiments.

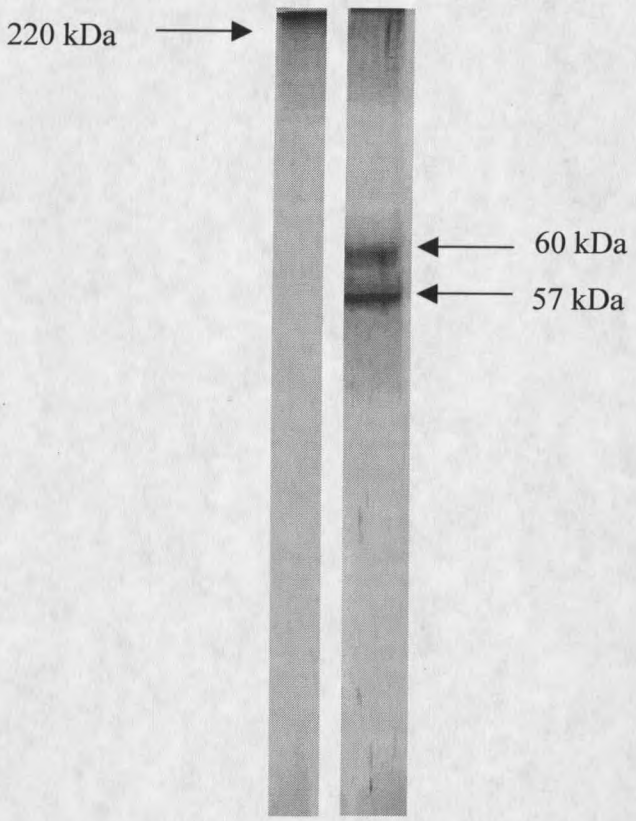


Figure 2.8: LF activity corresponds to heterodimer of approximately a 220 kDa protein according to 10% SDS-PAGE. Fractions containing liposome hydrolytic activity (fractions 1-5) were pooled and electrophoresed under non-reducing conditions (1) or reducing conditions (2) on a 10% SDS-PAGE. Experiment representative of three individual experiments.

2 Dimensional Electrophoresis and MALDI-TOF Analysis

To determine the molecular complexity of LF 2-dimensional electrophoresis was performed on LF material collected from the octyl-Sepharose column. 2-dimensional electrophoresis of LF material resolved 8 major spots (Fig 2.9). All resolved spots were in a pI range of approximately 7-9 with two major spots of approximately 57 kDa and 60

kDa (Fig 2.9). Spot 4 had a spot of approximately 60 kDa and spot 7 had a spot of approximately 57 kDa. Spots were arbitrarily numbered (1-8) and spots 4,5, and 7 were subjected to MALDI-TOF mass spectrometry. MALDI-TOF data of the trypsinized spot 4 has mass fit homology similar to a cytotoxin homolog, myotoxin, and neurotoxin among others (Table 2.1). Spot 5 had homolog similarity to a nonspecific lipid-transfer protein precursor among others (Table 2.1).

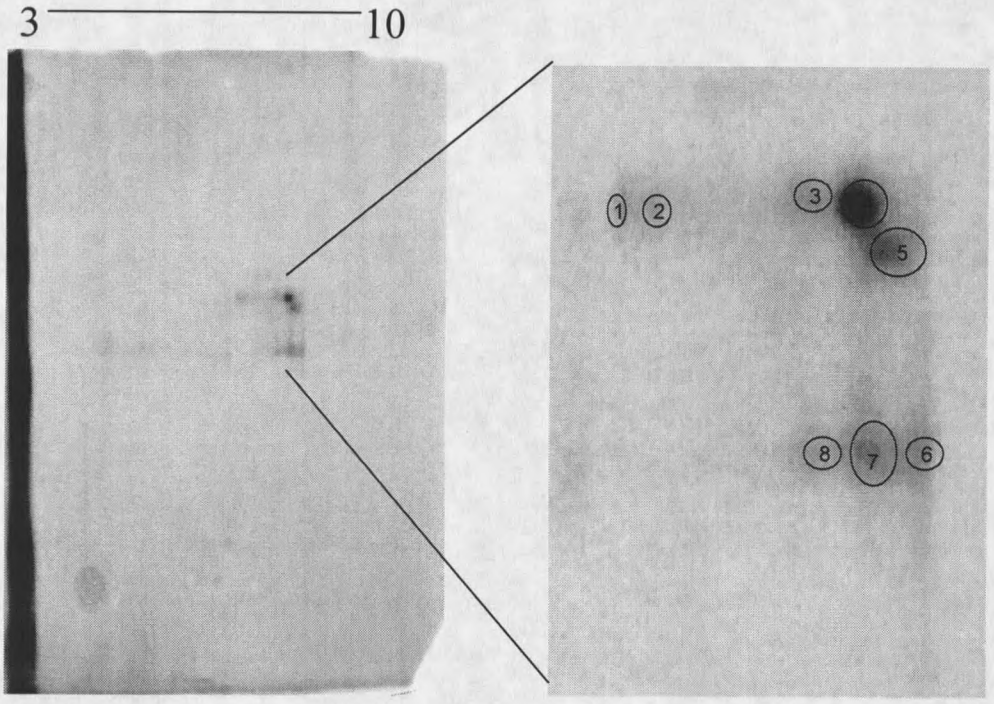


Figure 2.9: 2-D electrophoresis of LF. LF octyl-Sepharose preparation was electrophoresed on 3-10 IEF strip, electrophoresed on a 10% SDS-PAGE, and silver stained. Spots 4, 5, and 7 were cut out and subjected to MALDI-TOF mass spectrometry. Experiment representative of three individual experiments.

Spot number	pI	MW	% Cov	Protein (homology)	Species	pI Exp	MW Exp
4	8	60000	66	60S ribosomal protein L13a	*Spinacia aleracea*	8.7	1489
-	-	-	69	ATP synthase e chain, mitochondrial	Saccharomyces cerevisiae (83)	5.8	10876
-	-	-	73	NONSPECIFIC LIPID-TRANSFER PROTEIN PRECURSOR	Striga hermonthica (84)	9.6	12719
5	8	59000	95	Myotoxin	*Crotalus adamanteus* (85)	9.7	5202
-	-	-	90	Cytotoxin homolog	Naja kaouthia (86)	9.4	6995
-	-	-	84	Neurotoxin III	*Buthus occitanus tunetanus* (87)	8.2	7272

Table 2.1: Proteomic analysis LF proteins. 2-D protein spots were subjected to a in gel trypsin digest. Peptide masses were subjected to Prospector to identify possible homolog candidates. MW and pI were determined from 2-D gel. % cov is the matched peptides cover % of the protein. Exp of the pI and MW are the expected pI/MW of the protein homolog in Prospector.

Discussion

The molecular mechanisms of the CPE towards host cell and the pathogenesis of trichomoniasis in humans are not well defined. In the current literature there are two main schools of thought on the molecular mechanisms of the CPE: 1) contact dependent (36,37,38,39,40) and 2) contact independent (42,43,65). We show that *T. vaginalis* can have cytotoxic effects on mammalian cells through both contact and contact independent pathways (Fig 2.1). However, since *T. vaginalis* probably secretes multiple virulence factors a direct correlation between *T. vaginalis* supernatant and purified LF cannot be

stated. It has previously been clearly shown that *T. vaginalis* produces cytotoxic factors that play a role in the CPE on target cells (36,37,38,39,40,42,43,65). In this study we identified a novel cytotoxic factor, termed lytic factor (LF) that has PLA_2-like activity.

To date numerous virulence factors with a variety of enzymatic activities have been recorded in various pathogens. In some of the most virulent pathogens, enzymes can be attributed to a specific type of catalytic activity, the hydrolysis of lipids and phospholipids. Several pathogens that have been well studied for their destructive effects on biological tissues secrete phospholipases that have been implicated in their pathogenesis (81,82). Extracellular phospholipases secreted by pathogens often function as toxins and are known to cause damage to host cell membranes, which allows tissue invasion, particularly in bacterial pathogens. A secreted phospholipase of interest is the α–toxin secreted by *Staphylococcus aureus*, which has been shown to cause membrane damage to an array of mammalian cells and is active on artificial membranes (i.e. liposomes)(88). *Candida albicans* an opportunistic fungal pathogen has been reported to have three types of phospholipase activities, which include lysophospholipase, lysophospholipase transacylase, and phospholipase B (89,90,91). Our goal in this study was to determine if the characteristics of the LF *T. vaginalis* expresses and if a phospholipase (LF) is involved in the CPE mechanisms.

It is well known that in general phospholipases are hydrophobic. Therefore, in order to determine if a phospholipase is expressed in *T. vaginalis* we decided to use hydrophobic chromatography (octyl-Sepharose), a method that resolves molecules according to the species hydrophobicity. The results indicate that LF activity eluted off

the octyl-Sepharose column at 30% N-propanol (Fig 2.2A) demonstrating that LF is hydrophobic and suggesting LF may be a phospholipase or lipase.

Fractions containing LF activity were shown to contain carbohydrate material. Western blot analysis showed two distinct bands around 60 kDa and 57 kDa when electrophoresed under reducing conditions. Western blots on LF fractions revealed periodate-sensitive epitopes, demonstrating fraction material was partially composed of carbohydrate material. This can be stated since the primary antibody used to probe the Western blots was a polyvalent serum containing epitopes for both carbohydrates and peptide sequences. When fraction material was treated with trypsin the 60 kDa band was hydrolyzed indicating that this band is composed of both peptide and carbohydrate sequences, yet the 57 kDa band was not hydrolyzed by the treatment of trypsin. More importantly, when the LF material was treated with trypsin there was no cytotoxic activity toward mammalian cells indicating that the LF activity was due to a protein.

LF activity hydrolyzed phospholipase substrates indicating that LF had phospholipase activity. The phospholipid phosphatidylcholine, which is a substrate of phospholipases (PLA, PLB, PLC, and PLD), was hydrolyzed by LF (Fig 2.5B). Further analysis revealed that LF hydrolyzed phosphatidylcholine in a manner characteristic of PLA_2 suggesting the phospholipase activity in LF may be primarily a PLA_2 (Fig 2.6). Mass spectrometry analysis of three major spots in LF resolved by 2D gel electrophoresis revealed that spot 4 had homology similar to toxins from snake venom. Toxins from snake venom contain some of the best-characterized PLA_2 enzymes and purified several mammalian PLA_2 enzymes have been shown to have a high degree of sequence

homology with venom (PLA_2) (92). Although the majority of PLA_2 enzymes are between 13-15 kDa, there are other examples of higher molecular weight PLA_2 (92). For example a PLA_2 purified from sheep platelets is a dimer and is a 58 kDa protein on SDS-PAGE (93), which is similar to the composition observed here in LF from *T. vaginalis* (Fig 2.8). Other examples of PLA_2 enzymes found as dimers are those in the outer membrane in many gram-negative bacteria (94).

The major symptoms of trichomoniasis seem largely due to the inflammatory response *T. vaginalis* causes in the host. Of particular importance to this process is the activity of PLA_2 which can release the fatty acid arachidonic acid, which is then converted to prostanoids and leukotrienes by cyclooxygenases (COX) and lipoxygenases (LOX) (95,96,97). Arachidonic acid is a critical substrate to initiate the pro-inflammatory response. We have shown that *T. foetus,* which is similar to *T. vaginalis,* stimulates a macrophage cell line (J774) to produce COX-2 (98), which could act synergistically with released arachidonic acid thereby enhancing the inflammatory potential in trichomoniasis.

In summary we demonstrated the LF of *T. vaginalis* has PLA_2 like activity, destroys mammalian cells without the requirement of parasite-target cell contact. Since LF is cytotoxic to both nucleated and non-nucleated mammalian cells and hydrolyzes phospholipase substrates we conclude LF contains PLA_2 like activity, as a major component constituting the primary enzymatic mechanism of LF from *T. vaginalis.* These findings suggest the *T. vaginalis* LF could play an important role in the pathogenesis of human trichomoniasis.

TRICHOMONAS VAGINALIS: SECRETED VIRULENCE FACTOR AND REGULATION

Introduction

Trichomonas vaginalis is a flagellate parasitic protozoan that is the causative agent of trichomoniasis in humans and is the world's most common non-viral sexually transmitted disease. The World Health Organization estimates that 3-5 million women in the United States and 170 million worldwide are infected every year. *T. vaginalis* reportedly causes 20-30% of all vaginitis cases reported in women (2). Trichomoniasis can range from a severe state of inflammation of the urogenital tract to an asymptomatic carrier state in both men and women (1). A percentage of pregnant women infected with *T. vaginalis* can have premature births, which can cause perinatal morbidity leading to substantial health care costs (3).

The pathogenic mechanisms of *T. vaginalis* in the urogenital tract that are the basis of trichomoniasis are not well understood. Virulence factors of *T. vaginalis* likely play a role in the pathogenesis toward host cells and tissues (eg, vaginal epithelial cells, placenta) therefore it is important to identify such virulence factors and to determine their role in pathogenesis. For example virulence factors expressed by *T. vaginalis* that are cytotoxic to mammialian cells *in vitro* may play a role in the pathogenesis of trichomoniasis. Other parasite products such as cysteine proteinases are well-characterized virulence factor candidates in *T. vaginalis* with over 20 reported (58). *T. vaginalis* expresses cysteine proteinases on the cell surface (36,37,38) and secretes

proteolytic enzymes from lysosomes similar to the process in cytotoxic T cells (42). Other virulence factors detected in *T. vaginalis* that may be enzymatic such as hemolytic activity and a cell detaching factor (CDF) remain only partially investigated (43). We have identified a virulence factor, termed lytic factor (LF), that has phospholipase A_2-like activity (PLA_2) and is cytotoxic to both nucleated and nonnucleated mammalian cells (Chapter 2).

In this study we wanted to determine the extent of LF secretion or expression on the parasites surface and whether LF is up regulated by *T. vaginalis* under certain conditions. We also identified a monoclonal antibody (mAb) specific for the PLA_2. The results indicate that LF is stored in cytoplasmic vesicles and is upregulated when the parasites are stimulated by contact with target cell monolayers suggesting a signaling event from the targets induce expression of increased PLA_2.

Materials and Methods

Parasites

Trichomonas vaginalis Strain UAB 5-1 (Provided by Dr. J. Swebke, University Alabama Medical Center) was grown in modified Diamond's medium pH 6.0 (72), containing 10% heat inactivated fetal bovine serum (Atlanta Biological, Atlanta, Ca) at 37°C. Parasites were counted with a hemocytometer and collected by centrifugation and washed twice in PBS pH 7.2 (10 mM sodium phosphate, 150 mM NaCl).

Parasite-Extract

Whole parasite extracts were prepared by treatment of $1x10^8$ parasites/ml with extraction buffer (50 mM Tris pH 8, 100 mM NaCl, 5 mM ethylenediaminetetracetic acid (EDTA), 1% Trition-X 100 (Sigma, St. Louis, MO), 10 μM leupeptin (Sigma, St. Louis, MO), and 10 μM trans-epoxysuccinyl-L-luecylamido-(4-guanidine) butane(E-64) (Sigma, St. Louis, MO) by incubation on ice for 30 minutes with gentle mixing every 5 minutes. Cellular debris was pelleted (1000x g @ 10°C, 30 min.) and the supernatant was stored at -20°C. The BCA protein assay (Pierce Chemicals, Rockford, IL) was used to determine protein concentrations.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to Laemmli (73) using 10% T gels that were developed with silver staining (Novex) or electroblotted onto nitrocellulose membrane and analyzed by Western blot with anti-*T. vaginalis* monoclonal antibody 11-5-14 (mAb).

Phosopholipid Hydrolysis

10 μl of 1 mM BIODIPY labeled phosphatidylcholine (BPC) (Molecular Probes Eugene, OR) was dried under a slow stream of nitrogen. Once dried BPC was resuspended in 50 μl of solution I (0.5 mM octylglucoside, 0.4 mM NaCl, and 60 mM hepes, pH 7.0) and briefly sonicated. 50 μl of solution II (5 mM EGTA, 2 mM EDTA, and 1 mM DTT) or HBSS, pH 7.0, was added to the BPC solution. A volume of 5 μl of LF, phospholipase D (PLD), or HBSS (blank) was added to 12.5 μl of BPC and treatment

groups were incubated for 2 hour at 37°C. 5 µl was then plated on a K6 Silca Gel 60 thin layer chromatography plate (Whatman, Maidstone, UK) and allowed to dry. Samples were resolved in a chloroform:methanol:acetic acid solvent (45:45:10) and hydrolysis was detected by inspection under far UV illumination (365 nm).

Stimulation and Secretion Assays

Parasites were washed twice with DPBS and depending on experimental design parasites were labeled with 3 µl of 1 mM vibrant DiI (Molecular Probes Eugene, OR) for every 1 X 10^6 parasites/ml for 15 min at 37°C in DPBS. Parasites were then washed twice with DPBS and resuspended in complete Diamond's medium, pH 6.0. In some treatment groups parasites were treated with 10 mg/ml of brefeldin A (BFA). Confluent WEHI 164 cell monolayers, murine fibroblast cell line, were fixed with 4% formalin for 30 min and were then washed 8 times with DBPS. Parasites were then added to 6 or 48 well plates or T-75 flasks (depending on experimental design) in the presence or absence of fixed monolayers of the WEHI 164 cells. Treatment groups (parasites) were incubated at 37°C for 24 hours and then subjected to ELISA, flow cytometry, microscopy, or cytotoxicity assays, as appropriate.

ELISA

T. vaginalis treatment groups (as described in Stimulation and secretion assays) were extracted with protein extraction buffer as described above and protein concentration was determined with the BCA assay. Protein from each treatment group was adjusted to 1 mg/ml and 96 well, flat bottomed, high affinity ELISA plates were

coated with protein and allowed to dry overnight. The wells were then blocked with PBS/1% heat inactivated donor calf serum (DCS) for 30 min and triplicate wells of each treatment were incubated with either mAb 11-5-14 (anti-*T. vaginalis*) or mAb RB6.8C5 (anti-mouse) overnight at 4°C. The following day the wells were washed 3X in PBS/ 5 X 10^{-4} % Tween 20 and the secondary antibody, HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove. PA), at 1:1000 dilution in ELISA buffer (0.005% DCS, 5 X 10^{-4} Tween 20 in PBS), was added and incubated for 2 hours. The wells were washed 3X in PBS/5 X 10^{-4} % Tween 20, developed with ABTS peroxidase substrate (KPL, Gaithersburg, MD) for 20 min and 80 µl of the developed supernatant was transferred to a new plate and the absorbance was read at 450 nm.

Flow Cytometry

T. vaginalis from stimulation and secretion assays were washed twice in DBPS resuspended in DPBS/4% DCS at 1.0 X 10^6 parasites/ml and incubated on ice for 30 min. Parasites were incubated in PBS-S (0.1 % Saponin (Sigma, St. Louis, Mo) 1 mM Calcuim Chloride, 0.6 mM Magnesium Sulfate, 4% DCS, 10 mM HEPES, pH 7.2) or PBS/4% DCS for 30 minutes on ice. Parasites were incubated with either mouse anti-*T. vaginalis* mAb (11-5-14) or mouse-anti PMN mAb (RB6.8C5) for 30 min. Parasites were then washed 3X in PBS/4% DCS and the secondary antibody, FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), was added and incubated for 30 min. Parasites were washed twice with DPBS and flow cytometry

analysis was performed on FACScan or FACScaliber (Beckton-Dickenson, Mountain View, CA). In each treatment group data were collected on a total of 10,000 events.

Confocal Microscopy

T. vaginalis from selected stimulation and secretion assays were placed on glass cover slips, washed twice with DPBS and fixed with 4% paraformaldhyde for 10 min. Fixed parasites were washed twice with DPBS and then incubated in DPBS/1% DCS for 30 min followed by incubation with either mAb 11-5-14 or mAb RB6.8C5 overnight at 4°C. Coverslips were then washed 3X with DPBS/1% DCS and incubated with FITC conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) for two hours. Coverslips were washed 3X with DPBS, mounted onto glass slides, and microscopy was performed with a confocal microscope (Zeiss LSM 510).

Cytotoxicity Assays

Cytotoxicity of *T. vaginalis* in stimulation and secretion assays was measured on WEHI 164 cells as previously described (Chapter 2). Briefly, 1.0 $X10^6$ *T. vaginalis* were added to WEHI 164 cells in complete DMEM (4 mM L-glutamine, 1 mM Sodium pyruvate, 10% Fetal bovine serum, 25 μg/ml gentimicin sulfate), triplicate wells for each treatment. Additional wells containing WEHI 164 cells without parasites served as controls. Treatment groups were incubated at 37°C for the desired time and WEHI 164 cells were washed with HBSS, fixed with 4% formalin, stained with crystal violet, washed 3X with water, and allowed to dry overnight. Crystal violet stained monolayers

were solubilized (0.05 M acetic acid and 0.5% SDS) and the absorbance was read at 550 nm. Percent cytotoxicity was calculated with the following equation:

$$\% \text{ Cytotoxicity} = \frac{(\text{Control}_{\text{O.D. 550nm}} - \text{Unknown}_{\text{O.D. 550nm}})}{(\text{Control}_{\text{O.D 550nm}})} \quad X\ 100$$

Results

Since we previously were able to detect LF secreted by *T. vaginalis* that was cytotoxic toward mammalian cells (chapter 2) we wanted to determine the effects of secretion inhibition on *T. vaginalis* cytotoxicity toward WEHI 164 cells. *T. vaginalis* were treated with BFA in the presence or absence of WEHI 164 cells for 24 hours and then the cytotoxicity of parasites on the WEHI 164 cells was measured (Fig. 3.1). A significant decrease of *T. vaginalis* cytotoxicity toward WEHI 164 cells was observed when parasites were treated with BFA compared to the cytotoxicity due to untreated parasites ($P<0.05$) (Fig. 3.1A). *T. vaginalis* were also cultured in the absence of WEHI 164 cells to observe BFAs effect on the parasite population and the results indicated BFA reduced the numbers of viable parasites slightly by the 24-hour time point but not by a significant amount (data not shown). *T. vaginalis* is known to be "sticky" and adhere to culture plates so parasites were cultured in parallel wells and stained with crystal violet to control for the possibility of increased staining due to staining of the adhered parasites. The results indicated that significant numbers of parasites do not seem to be sticking to

the wells and therefore is not the basis of increased staining by the crystal violet of groups with BFA-treated *T. vaginalis* (compare 3.1B, rows 1,3,4).

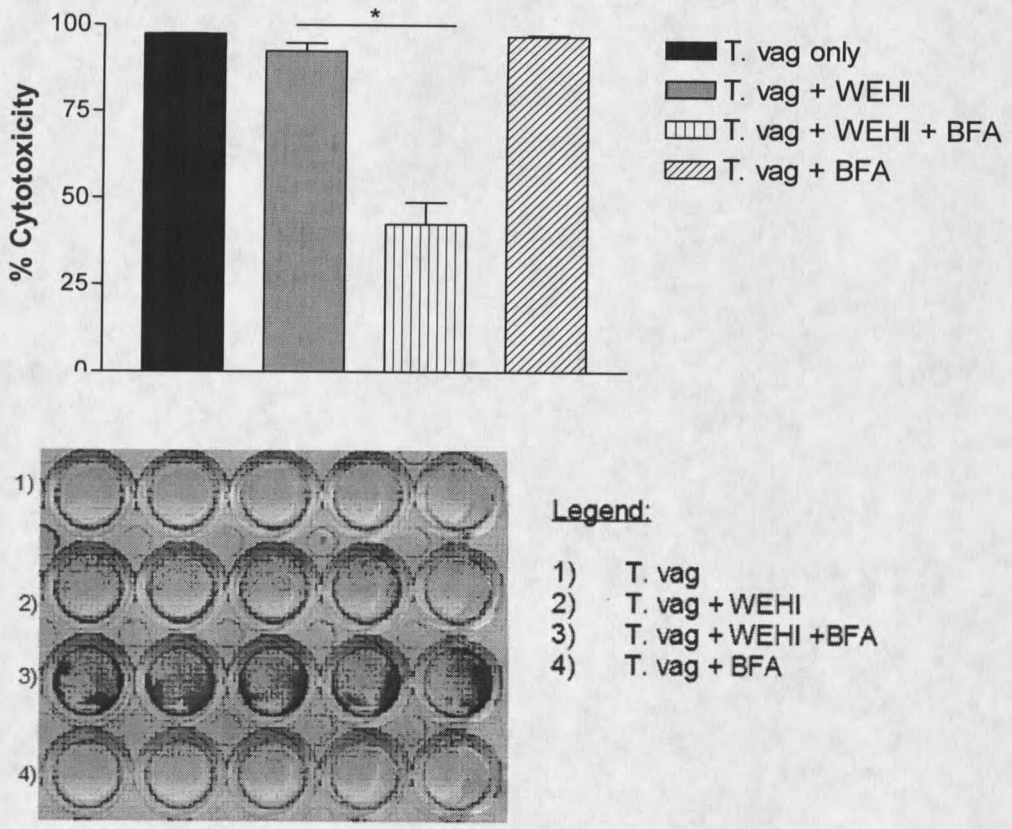


Figure 3.1: BFA treatment of *T. vaginalis* decreases cytotoxicity of *T. vaginalis* toward WEHI 164 cells. A) *T. vaginalis* were incubated in the presence or absence of WEHI 164 cells with BFA or without for 24 hours. WEHI 164 cells were stained with crystal violet, cytotoxicity was measured, and percent cytotoxicity was calculated. Data pooled from three individual experiments. Significant differences among treatment group indicated by * is $P<0.05$. B) *T. vaginalis* were incubated in the presence or absence of WEHI 164 cells with BFA or without for 24 hours. WEHI 164 cells were stained with crystal violet. Row 1) *T. vaginalis* only. Row 2) *T. vaginalis* + WEHI 164 cells. Row 3) BFA treated *T. vaginalis* + WEHI 164 cells. Row 4) BFA treated *T.vaginalis.* Experiment representative of three individual experiments.

Phospholipase specific monoclonal antibody

We have identified a PLA_2-like activity in LF present in *T. vaginalis* that has cytotoxic activity on mammalian cells (Chapter 2). To further explore the issue of the secretion of LF we identified a mAb, 11-5-14, that recognizes an epitope on the protein of approximately 220 kDa protein in the LF preparation (Fig. 3.2). Gel analysis of the octyl-Sepharose LF preparation showed three distinct bands of approximately 220, 55, and 30 kDa when silver stained (Fig. 3.2). The 11-5-14 antibody recognizes the 220 kDa protein, which coincides with the LF activity as we have already reported (Chapter 2). To further document that 11-5-14 recognizes an epitope on LF a functional assay was developed in which the 11-5-14 mAb or an irrelevant mAb, RB6.8C5, were dotted on nitrocellulose paper and incubated with an LF preparation. Treatment groups were then assayed for LF activity by incubating the nitrocellulose paper with the phospholipase substrate BIODIPY labeled phospotidilycholine (BPC) and the treatment products were subjected to TLC. LF incubated with the 11-5-14 mAb migrated slower than the negative control (blank nitrocellulose paper) further indicating that mAb 11-5-14 specifically bound an epitope on the PLA_2 present in LF (Fig. 3.3). In contrast, control nitrocellulose paper, with RB6.8C5 incubated with the LF, did not have detectable activity on the BPC.

LF is secreted and stimulated by external factors

We next wanted to determine if LF was localized internally or on the cell surface of *T. vaginalis.* Flow cytometry together with specific antibodies was employed to

determine LF localization in *T. vaginalis*. Either viable or sapponin-treated parasites were analyzed for the presence of LF with mAb 11-5-14. When viable parasites were gated to ensure a true viable population and then analyzed for the presence of LF on the cell membrane, 3.92% of viable parasites bound the mAb (Fig. 3.4A). However, sapponin-treated parasites analyzed by flow cytometry after treatment with 11-5-14 had 22.98% positive, a substantial increase in LF detection compared to viable parasites (Fig. 3.4B). This data indicates that LF is primarly localized internally and is not expressed on the surface of the parasite.

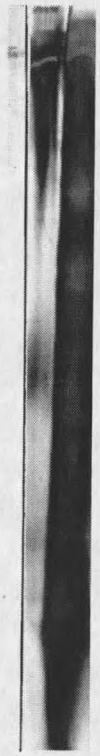


Figure 3.2: mAb 11-5-14 recognizes an epitope specific for a 220 kDa protein in the LF preparation. LF or parasite extract were run on a 10% SDS-PAGE and then either silver stained or a Western blot was performed. Lane 1) Western blot of LF probed with mAb 11-5-14. Lane 2) LF silver stained. Lane 3) *T. vaginalis* silver stained. Experiment representative of three individual experiments.

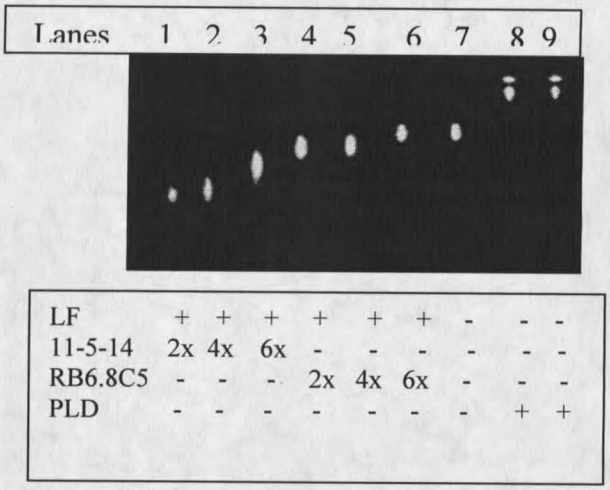


Figure 3.3: The mAb 11-5-14 recognizes LF, which hydrolyzes BPC. Either 11-5-14 or RB6.8C5 at various concentrations (2x, 4x, or 6x) were allowed to bind to the nitrocellulose paper and LF was added to the nitrocellulose (NC) and incubated overnight at 4°C (Lanes 1-6). Treatment groups were then added to the BPC reaction mixture, incubated at 37°C for two hours, and then separated on a TLC plate. Lane 1) LF + 11-5-14 (2X); Lane 2) LF + 11-5-14 (4X); Lane 3) LF + 11-5-14 (6X); Lane 4) LF + RB6.8C5 (2X); Lane 5) LF + RB6.8C5 (4X); Lane 6) LF + RB6.8C5 (6X); Lane 7) HBSS (negative control); Lane 8) PLD (2X); and Lane 9) PLD (4X). Experiment representative of three individual experiments.

We next wanted to examine the relationship of parasites secretion and the effects on target cells. Parasites were treated with or without BFA in the presence or absence of stimulation by WEHI 164 cells for 24 hours. Parasites were labeled with the lipophillic dye DiI, fixed and treated with sapponin to allow internal staining with mAb 11-5-14. Treatment with BFA, which inhibits secretion by blocking Golgi apparatus function, resulted in a significant increase ($P<0.05$) in accumulation of LF in *T. vaginalis*

compared to *T. vaginalis* only (control) (Fig 3.5A). Stimulation of the parasites by WEHI 164 cells also resulted in a significant increase in accumulation of LF in *T. vaginalis* compared to control (Fig 3.5A). Parasites in the *T. vaginalis* + WEHI 164 cell group were 60.93% positive with mAb 11-5-14 while parasites in the *T. vaginalis* + WEHI 164 cell + BFA group were 69.29% positive (Fig. 3.5B). BFA treated parasites had 63.88% while control had 53.01% positive (Fig. 3.5B)

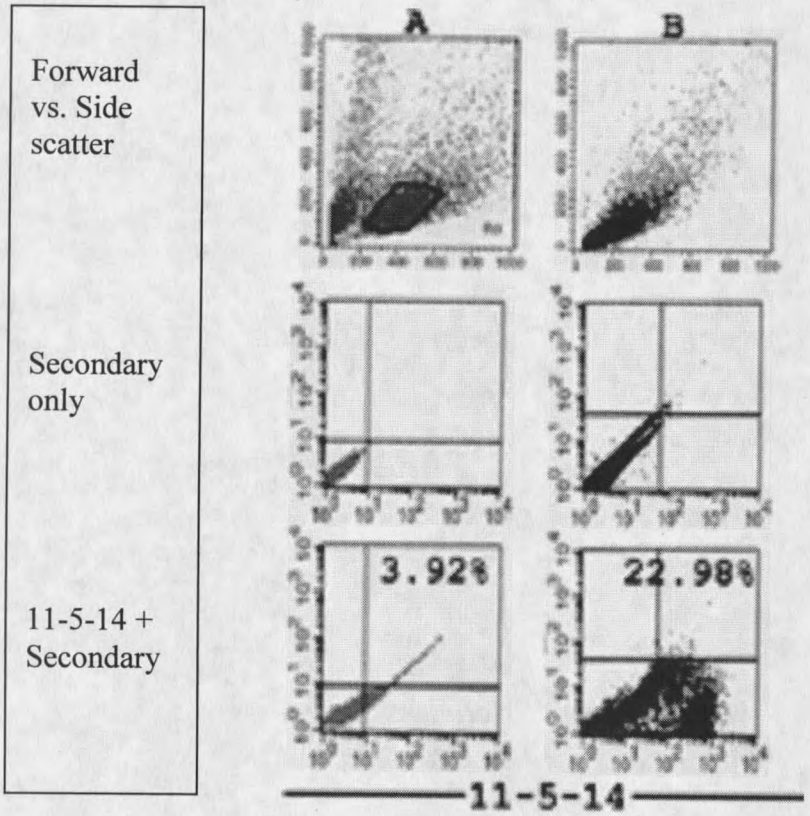
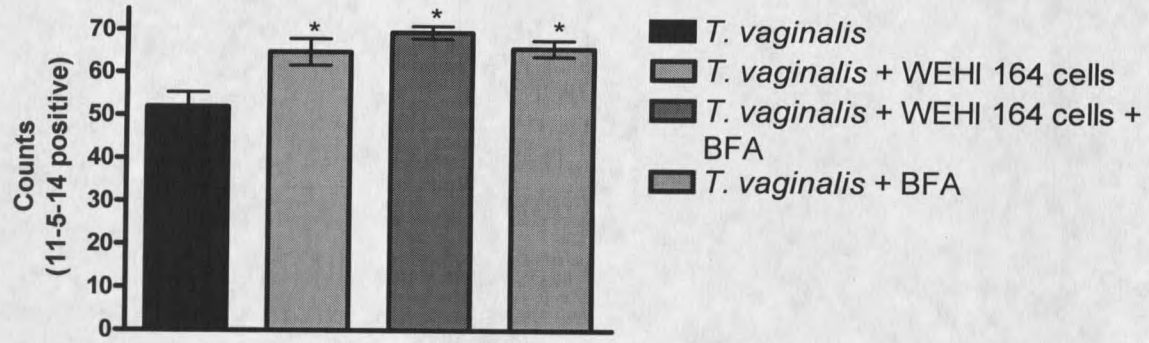


Figure 3.4: LF is localized internally in *T. vaginalis*. Parasites were incubated with or without sapponin and then stained with 11-5-14 + secondary or secondary only. Flow cytometry was performed and viable parasites were gated to obtain a true population of intact parasites. A) Viable parasites and B) sapponin treated parasites. Experiment representative of three individual experiments.

A



B

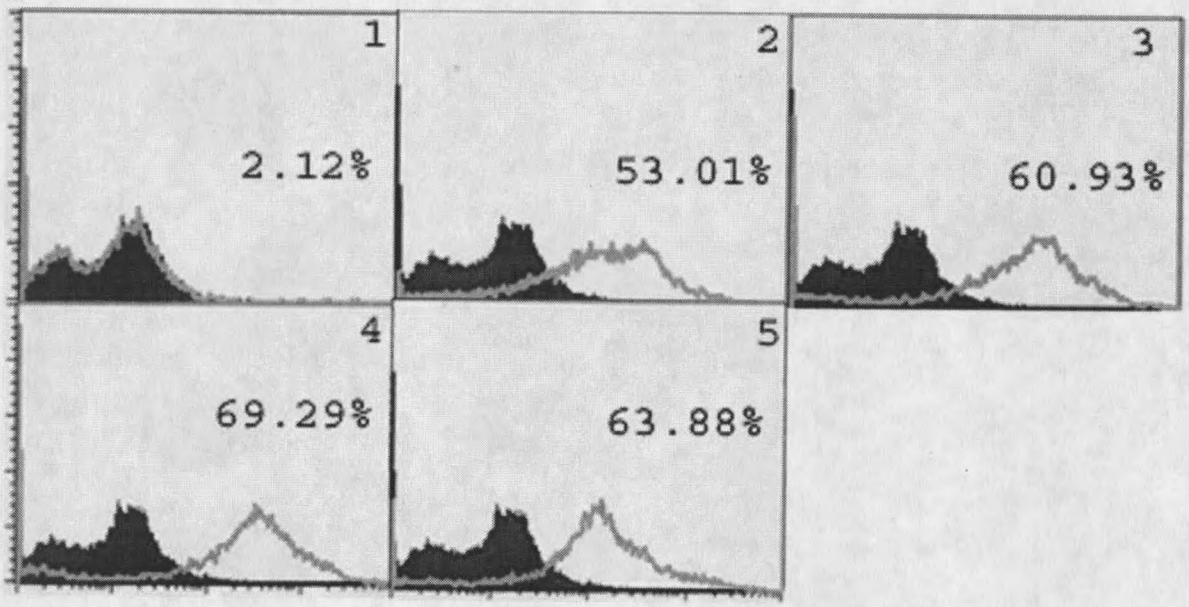


Figure 3.5: *T. vaginalis* expression of LF is stimulated by WEHI 164 cells. *T. vaginalis* parasites were cultured overnight in the presence or absence of WEHI 164 cells and either treated with BFA or left untreated. Treatment groups were either analyzed by flow cytometry. A) *T. vaginalis*; *T. vaginalis* + WEHI 164 cells; *T. vaginalis* + WEHI 164 cells +BFA; and *T. vaginalis* + BFA. Data pooled from three individual experiments. Significant differences among treatment group indicated by * is $P<0.05$. B) 1) *T. vaginalis* + 2° only; 2) *T. vaginalis*; 3) *T. vaginalis* + WEHI 164 cells; 4) *T. vaginalis* + WEHI 164 cells + BFA; and 5) *T. vaginalis* + BFA. Experiment representative of three individual experiments.

In order to verify these BFA effects an ELISA assay using the mAb 11-5-14 and the same experimental groups as is in figure 3.5 was performed on these treatment groups. The ELISA was set up using parasite extracts prepared with Triton-X 100. Parasites stimulated by WEHI 164 cells showed a significant upregulation in LF production compared to parasites not stimulated with WEHI monolayers (Fig 3.6). Parasites treated with BFA displayed either increased or significant difference in LF production compared to parasites that were not treated with BFA, although there was a small increase in LF compared to parasites not treated with BFA (Fig. 3.6).

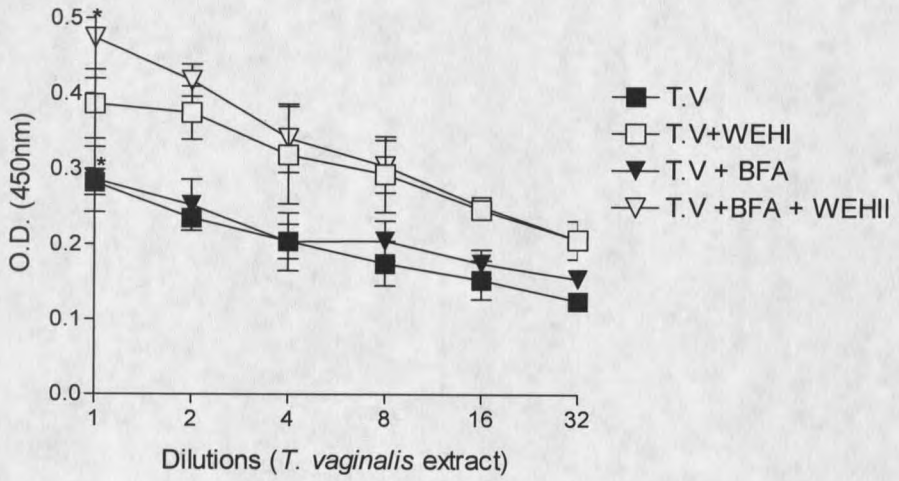


Figure 3.6: Anti-LF ELISA confirms increase in LF in *T. vaginalis* due to target cell exposure. Treatment groups were extracted with Triton X-100, brought up to 1mg/ml for each treatment group, added to the ELISA plate at the indicated dilutions, and ELISA was performed. ( ) *T. vaginalis* only; ( )*T. vaginalis* + WEHI 164 cells; ( )BFA treated *T. vaginalis*; and ( ) BFA treated *T. vaginalis* + WEHI 164 cells. Data pooled from two individual experiments. Significant differences among treatment groups indicated by * is $P<0.05$.

LF is stored in vesicles

In order to visually confirm LF localization parasites were examined by confocal microscopy. The results indicated that LF in *T. vaginalis* is localized in discrete cytoplasmic vesicles appearing to be concentrated at the periphery of the cytoplasm (Fig 3.7A). In contrast, BFA-treatment of parasites disrupted this pattern of concentrated vesicles resulting in a dispersed pattern of smaller vesicles containing LF (Fig 3.7B). However, in both cases the LF still accumulated in the periphery of the cytoplasm, indicating continued synthesis of LF by the parasite. Parasites treated with BFA had increased overall accumulation of LF compared to parasites not treated with BFA (compare Figs 3.7C & D). Parasites stimulated with WEHI 164 cells accumulated LF on the periphery of the parasite as observed in Fig 3.7 A & B. In contrast, parasites stimulated with WEHI 164 cells and treated with BFA seem to be accumulating the LF throughout the parasite cytoplasm (Fig 3.7 D) suggesting disruption of normal cytoplasmic transport/secretion of LF.

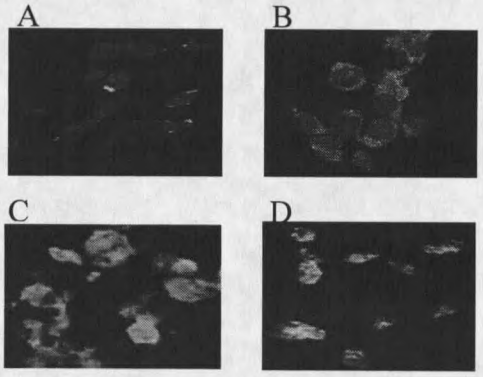


Figure 3.7: LF localizes in cytoplasmic vesicles in *T. vaginalis*. *T. vaginalis* were labeled with a red membrane dye (DiI) and probed with a mAb (11-5-14) green. A) *T. vaginalis*. B) BFA treated *T. vaginalis* C) *T. vaginalis* + WEHI 164 cells. D) BFA treated *T. vaginalis* + WEHI 164 cells.

Discussion

To date numerous virulence factors both secreted and contact-dependent have been identified and suggested to play a role in the pathogenesis of trichomoniasis (36,37,38,39,68). In this study we assessed *T. vaginalis* localization of LF its secretion and whether external stimuli would result in upregulation of LF.

LF does not appear to be expressed on the surface of the plasma membrane of *T. vaginalis*, rather it localizes in cytoplasmic vesicles near the plasma membrane. BFA, which destroys the ability of the Golgi to function properly so that proteins cannot be transported or secreted (99), partially inhibited cytotoxicity of *T. vaginalis* toward WEHI 164 cells (Fig. 3.1). These data suggest that LF is being secreted, and that this secreted virulence factor is important in the CPE of *T. vaginalis* toward host cells. Furthermore, BFA treatment of *T. vaginalis* exposed to fixed WEHI 164 cells increased the intracellular accumulation of LF compared to identically treated parasites except without BFA (Fig. 3.5B). *T. vaginalis* has a well a defined Golgi (7) and these treatment effects suggest BFA inhibits transport of LF molecules to the proper cellular location and secretion of LF, leading to a cytoplasmic build up of LF. BFA treated *T. vaginalis* are not capable of proper cytoplasmic location of LF relative to LF localization in untreated *T. vaginalis*.

LF production is at least partially dependent on external stimuli, which was evident from the increased LF produced when *T. vaginalis* was stimulated with fixed WEHI 164 cells. Like other organisms *T. vaginalis* has been shown to upregulate

proteins in response to certain stimuli (41,42,43,49,65). For example adhesion proteins in *T. vaginalis,* thought to aid in the binding to host cells, are upregulated in the presence of iron (49). A cell-detaching factor thought to be a virulence factor, is also upregulated when *T. vaginalis* is stimulated with various types of mammialian cells (43,65). In addition, *T. vaginalis* is known to store proteinases in cytoplasmic vesicles, which are thought to be lysosomes, and when activated by treatment with amines or monensin the parasite releases these protolytic enzymes (42). LF appears to be stored in cytoplasmic vesicles similar to that of lysosomes, although further work needs to be done to determine the precise type of cytoplasmic vesicle(s) involved. Lysosomes are usually thought to be dead end vesicles in eukaryotic cells, yet there are some examples of lysosomes being able to release their contents, such as those present in cytotoxic T cells (100). Lysosomal contents are secreted when the cytotoxic T cell comes into close contact with its target (100). It is tempting to speculate that *T. vaginalis* can respond to the presence of target cells by up regulating production and secretion of LF to facilitate lysis of targets and reliance for nutrients essential to parasites (e.g. nucleosides, lipids).

It has been hypothesized that contact dependent mechanisms are required for *T. vaginalis* to have a cytotoxic effect on host cells in which the four APs are thought to play a role through facilitating firm adhesion to the target (49). However prior to this report there is no evidence of the parasites secreting a cytotoxic molecule as a result of contact between the parasite and the target cell. In this paper we addressed the issue of whether LF is upregulated upon stimulation by target cell contact and the effects of this contact on release of LF. The results showed that parasite contact with target cells

increased expression of LF (Fig 3.5A, B). In addition LF is stored in cytoplasmic vesicles that may be similar to lysosomes.

In the current literature there is evidence of *T. vaginalis* secreting virulence factors that are cytotoxic to host cells (41,43,65). However, the molecular nature of these factors remains largely unknown, as does their mechanism of action. Previous work on LF from *T. vaginalis* indicated it contained potent PLA_2-like activity (chapter 2). Results presented here indicate the molecule recognized by anti-*T. vaginalis* mAb,11-5-14, binds to the phospholipase component of LF (Fig. 3.3). Results of microscopic and flow cytometry analysis of LF in *T. vaginalis* with mAb 11-5-14 further demonstrated that LF localizes in cytoplasmic vesicles (Fig 3.6) and that production is stimulated by parasite contact with WEHI 164 cells (Fig 3.5).

In summary, the results presented here suggest LF is released after *T. vaginalis* contacts target cells through an active secretion pathway and the mechanisms by which LF damages target cells involves PLA_2-like activity. Determination how *T. vaginalis* regulates LF production and release provides insight into the role of LF in host cell damage and the role it may play in the pathogenesis of trichomoniasis.

CONCLUSIONS

T. vaginalis is the causative agent of trichomoniasis in humans and identification of virulence factors that play a role in the pathogeneses will lead to a greater understanding of trichomoniasis. *T. vaginalis* is known to produce many virulence factors that may play a role in the pathogenesis of trichomoniasis. Known virulence factors of *T. vaginalis* have been studied *in vitro* thus their contribution to the pathogenesis of trichomoniasis is unknown, yet understanding their cytopathic effects (CPE) on host cells will lead to a greater understanding of the possible molecular mechanisms that may play a role in trichomoniasis.

We successfully identified a novel virulence factor, termed lytic factor (LF) that is cytotoxic to host cells and may play a role in the pathogenesis of *T. vaginalis*. LF lysed nucleated mammalian cells and red blood cells suggesting that LF has a CPE on host cells *in vitro* and may play a role in the pathogenesis trichomoniasis. LF has a CPE on the nucleated mammalian cells specifically WEHI 164 cells and HeLa cells, which are murine fibroblast cells and human epithelial cervix derived cells, respectively. Critical experiments that need to be performed are to test the CPE of LF on human primary vaginal epithelial cells and observe the effect of LF *in vivo* using a mouse model to assess the role of LF in the pathogenesis of trichomoniasis. These experiments will give insight into LF's effect and role on specific host cells.

LF has phospholipase A_2 (PLA_2)-like activity, which was suggested by LF having the ability to hydrolyze phospholipase substrates. LF hydrolyzed Tween 20 and Tween

80, which can be hydrolyzed by phospholipases among other enzyme classes. To further suggest that LF has phospholipase activity we tested LF activity on liposomes composed of phosphatidylcholine:cholesterol (1:1 ratio), which provided a more stringent assay for detecting phospholipase activity. LF constantly hydrolyzed liposomes suggesting LF contains phospholipase activity. LF hydrolyzed BIODIPY-labeled phosphatidylcholine similar to PLA_2 activity, suggesting LF has PLA_2-like activity. Suggesting that LF has PLA_2-like activity can be stated due to the nature of BIODIPY-labeled phosphatidylcholine can be hydrolyzed by all four classes of phospholipases producing products that can be differentiated by further analysis. More evidence of LF having PLA_2-like activity came be MALDI-TOF mass spectrometry data providing possible protein homologues. LF had homology toward snake venom toxins, specifically myotoxin, cytotoxin homolog, and neurotoxin. It is well known that snake venom toxins contain PLA_2 activity among others and myotoxin is one of the snake toxins that have PLA_2 activity.

It is very likely that LF has PLA_2-like activity, yet it would be advantageous to perform more experiments that would further validate LF having PLA_2-like activity. Obtaining the sequence of LF would greatly enhance the future experiments that could be performed. If mutant LF constructs that disrupted the activity could be made and demonstrated that LF activity is abrogated toward phospholipase substrates and host cells this would be valuable evidence that LF activity is of the PLA_2 class. These mutant constructs along with wild type constructs could also be utilized an *in vivo* mouse model to ascertain the role LF may play in the pathogenesis of trichomoniasis.

Another project goal was to assess *T. vaginalis* localization of LF, to determine if LF is secreted, and whether external stimuli would result in upregulation of LF. LF does not appear to be expressed on the surface of the plasma membrane of *T. vaginalis*, rather it localizes in cytoplasmic vesicles near the plasma membrane. Flow cytometry data showed that LF was not expressed on the surface of *T. vaginalis*, yet rather expressed in the cytoplasm of the parasite. This observation of expression in the cytoplasm was further confirmed by confocal microscopy showing that *T. vaginalis* stores LF in cytoplasmic vesicles on the periphery of the parasites. When parasites were treated with Brefeldin A the localization of LF in cytoplasmic vesicles was not apparent. The next logical experiment to be performed would be to determine what type of vesicle LF is stored in.

LF is upregulated when *T. vaginalis* is stimulated with external stimuli such as WEHI 164 cells. This was confirmed by ELISA and flow cytometry showing that when BFA treated *T. vaginalis* stimulated with WEHI 164 cells had a higher level of LF when compared to *T. vaginalis* only and BFA treated *T. vaginalis.* The conformation of LF upregulation can be further investigated at the mRNA level if sequence information is provided. If this can be achieved then determination of regulation can be further investigated to determine if regulation is at the translational or transcriptional level.

The data in this thesis project supports the hypothesis that *T. vaginalis* produces virulence factors both secreted and contact-dependent that are important in the pathogeneses of trichomoniasis. Molecular characterization of parasite molecules, that

mediate target cell lysis will elucidate specific mechanisms of host cell destruction and will identify (at the molecular level) virulence molecules of this parasite.

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