BLASTOCYSTIS: INVESTIGATIONS ON HOST-PATHOGEN INTERACTIONS USING *IN VITRO* MODEL SYSTEMS

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“*The universe is full of magical things, patiently waiting for our wits to grow sharper.*” Eden Phillpotts

DEDICATED WITH LOVE TO MY

PARENTS & FAMILY

---------- *Peace I leave with you; my peace I give to you* ----------

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Manoj Kumar Puthia
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LIST OF ABBREVIATIONS

°C	degree Celsius
ATCC	American Type Culture Collection
B. hominis	*Blastocystis hominis*
B. ratti	*Blastocystis ratti*
EDTA	ethylenediaminetetraacetic acid
E-64	trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
FBS	fetal bovine serum
Fig	figure
FITC	fluorescein isothiocyanate
H	hour
HRP	horse radish peroxidase
IA	iodoacetamide
IEC	intestinal epithelial cells
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
Igs	immunoglobulins
IMDM	Iscove's modified Dulbecco's medium
ml	milliliter
P	p value

PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pep A	pepstatin A
PI	propidium iodide
PMSF	phenylmethylsulphonyl fluoride
PS	phosphatidylserine
Rpm	revolutions per minute
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TER	transepithelial resistance
TUNEL	TdT-mediated dUTP-biotin nick-end labeling
Vs	versus
ZO-1	zonula occludens-1

SUMMARY

Blastocystis is a ubiquitous enteric protozoan found in the intestinal tract of humans and a wide range of animals. Accumulating evidence over the last decade suggests association of *Blastocystis* with gastrointestinal disorders involving diarrhea, abdominal pain, flatulence and vomiting. Despite new knowledge of *Blastocystis* cell biology, genetic diversity, and epidemiology, its pathogenic potential remains controversial. Numerous clinical and epidemiological studies either implicated or exonerate the parasite as a cause of intestinal disease. Clinical and experimental studies have associated *Blastocystis* with intestinal inflammation and it has been shown that *Blastocystis* has potential to modulate the host immune response. *Blastocystis* is also considered an opportunistic pathogen and high prevalence is reported in immunocompromised HIV patients. However, nothing is known about the parasitic virulence factors and early events following host-parasite interactions. Therefore, the aim of this study was to investigate the pathogenic potential of *Blastocystis*, by studying the interactions of *Blastocystis* with intestinal epithelial cell lines. This study reports that *B. ratti* WR1 induces apoptosis in IEC-6 cells in a contact-independent manner. Furthermore, it was found that *B. ratti* WR1 rearranges F-actin distribution, decreases transepithelial resistance, and increases epithelial permeability in IEC-6 cell monolayers. In addition, it was demonstrated that *Blastocystis* effects on transepithelial electrical resistance and epithelial permeability were significantly abrogated with metronidazole treatment, an antiprotozoal drug. Results suggest that *Blastocystis*-induced apoptosis in

host cells and altered epithelial barrier function might play an important role in its pathogenesis.

In the present study, the molecular mechanisms by which *Blastocystis* activates IL-8 gene expression in human colonic epithelial T84 cells were also investigated. This study demonstrates for the first time that cysteine proteases of *Blastocystis* can activate IL-8 gene expression in human colonic epithelial cells. Furthermore this study shows that NF-κB activation is involved in the production of IL-8. Findings show that the antiprotozoal drug metronidazole treatment can avert IL-8 production induced by *Blastocystis ratti* WR1. It was also shown for the first time that the central vacuole of *Blastocystis* may function as a reservoir for cysteine proteases that can degrade human secretory immunoglobulin A. These findings will certainly help to understand pathobiology of a poorly studied parasite whose public health importance is increasingly recognized.

PUBLICATIONS ARISING FROM THE THESIS

A. Internationally-Refereed Journals

1. **Puthia MK**, Lu J, Tan KS (2008) *Blastocystis ratti* contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-kappaB-dependent manner. *Eukaryotic Cell* 7:435-443

2. **Puthia MK**, Sio SW, Lu J, Tan KS (2006) *Blastocystis ratti* induces contact-independent apoptosis, F-actin rearrangement, and barrier function disruption in IEC-6 cells. *Infection & Immunity* 74:4114-4123

3. Sio SW, **Puthia MK***, Lee AS, Lu J, Tan KS (2006) Protease activity of *Blastocystis hominis*. *Parasitology Research* 99:126-130 (***co-first author)**

4. **Puthia MK**, Vaithilingam A, Lu J, Tan KSW (2005) Degradation of Human Secretory Immunoglobulin A by *Blastocystis*. *Parasitology Research* 97:386-389

B. Book Chapter

1. **Puthia MK**, Tan KSW (2008) Blastocystosis. edited by Stephen Palmer, Lawson Soulsby, Paul Torgerson and David Brown (In Zoonses: II edition) Invited by *Oxford University Press* (submitted)

C. Manuscript under preparation

1. **Puthia MK**, Joanne LMY, Tan KSW (2008) *Blastocystis* augments the effects of cholera toxin on intestinal barrier function through a bystander effect by proteolytic degradation of antigen-specific immunoglobulins

D. Conference Papers

1. **Puthia MK**, Tan MH, Lu J, Tan KSW. *Blastocystis* infection compromises epithelial barrier function and affects tight junctions in human colonic epithelial cells. 8th Military Medicine Conference 2007, Singapore

2. **Puthia MK**, Sio SW, Lu J, Tan KSW. *Blastocystis* infection displaces ZO-1 in tight junctions and decreases transepithelial electrical resistance of human colonic epithelial monolayer. 16th International Microscopy Congress 2006 (IMC16), Sapporo, Japan

3. Sio SW, **Puthia MK**, Lu J, Tan KSW. Apoptosis of host intestinal epithelial cells following infection with the enteric protozoan *Blastocystis*. The 16th International Microscopy Congress 2006, Sapporo Convention Centre, Sapporo, Japan

4. **Puthia MK**, Sio SW, Lu J, Tan KSW. F-actin Rearrangement and Decreased Transepithelial Electrical Resistance in Intestinal Epithelial Monolayers Following *Blastocystis* Infection. 6th National Symposium on Health Sciences, 6-7 June 2006, Palace of Golden Horses, Kuala Lumpur, Malaysia

5. Sio SW, **Puthia MK**, Lu J, Tan KSW.Caspase-3 dependent killing of host cells by the Intestinal Protozoan *Blastocystis*. 6th National Symposium on Health Sciences, 6-7 June 2006, Palace of Golden Horses, Kuala Lumpur, Malaysia.

6. Tan KS, **Puthia MK**, Nasirudeen AMA, Ng GC: Recent advances in *Blastocystis* research: Implications for protozoan programmed cell death and parasite survival. International conference on anaerobic protists 2005. Chiostro di San Francesco, Alghero, Italy

7. **Puthia MK**, Lu J,Tan KSW: *Blastocystis* influences the permeability of human intestinal epithelial monolayers. Abstract accepted for Molecular parasitology Meeting 2005, Woods Hole (MA) USA

8. **Puthia MK**, Vaithilingam A, Lu J,Tan KSW: Degradation of human secretory immunoglobulin A (SIgA) by the intestinal protozoan *Blastocystis*. Research work presented in Combined Scientific Meeting 2005, Singapore

CHAPTER 1:

INTRODUCTION

1.1 INTRODUCTION

Blastocystis is an enteric protozoan parasite of humans and many animals. It was first described in the medical literature in 1911 (Alexeieff 1911) and since then its pathogenic significance has always been uncertain. *Blastocystis* has been ignored as a pathogen due to its association with mild nature of gastrointestinal symptoms and also with many asymptomatic cases. In addition, lack of controlled experimental studies addressing the pathogenicity aspects underestimated its status as a gastrointestinal pathogen. Moreover, most conclusions were made from conflicting case reports which led to confusion and disagreements among researchers and clinicians.

Blastocystis is commonly identified in stool specimens and it is one of the most common parasites that reside in the human intestinal tract. The disease it causes is called blastocystosis but most publications refer it to as *Blastocystis* infections. Clinical symptoms attributed to *Blastocystis* infections include recurrent watery diarrhea, mucous diarrhea, vomiting, abdominal cramps and flatulence. *Blastocystis* can infect both children and adults and its geographical distribution appears to be global with prevalence ranging from 30 to 50% in developing countries (Stenzel and Boreham 1996).

At first, the name *B. enterocola* was proposed by Alexeieff (1911) and later it was isolated from human feces and the name *B. hominis* was coined (Brumpt 1912). Initially, it was described as harmless intestinal yeast. Its association with human disease was suggested by a number of reports and eventually work by Zierdt (1991) increased the

awareness of *Blastocystis* infections in humans. In spite of its description about a century ago, the exact pathogenesis mechanisms of *Blastocystis* infections are uncertain. A number of clinical and epidemiological studies implicate the parasite as a potential pathogen, while others exonerate it as an etiology of intestinal disease (Tan 2004; Leder et al. 2005). Significant progress has been achieved on descriptions of the morphology and genetic diversity of *Blastocystis* but most aspects of its life cycle, molecular biology, and pathogenicity remain unresolved (Stenzel and Boreham 1996; Tan 2004).

1.2 TAXONOMY

The taxonomic classification of *Blastocystis* is a controversial subject and there are many disagreements among researchers. *Blastocystis* was earlier described to be a yeast or a fungus (Alexeieff 1911; O'Connor 1919), a cyst of another protozoa (Bensen 1909), or a degenerating cell (Swellengrebel 1917). *Blastocystis* was described as a protist on the basis of morphological and physiological features (Zierdt et al. 1967). These protistan features included presence of one or more nuclei, smooth and rough endoplasmic reticulum, Golgi complex, mitochondria-like organelles, inabilty to grow on fungal medium, ineffectiveness of antifungal drugs, and susceptibility to some antiprotozoal drugs. Later, *Blastocystis* was classified as a sporozoan (Zierdt 1991) and finally reclassified as a sarcodine.

Molecular sequencing studies of *Blastocystis* partial small-subunit rRNA (ssrRNA) showed that *Blastocystis* is not monophyletic with the yeasts, fungi, sarcodines, or

sporozoans (Johnson et al. 1989) and it was concluded that *Blastocystis* is not related to yeasts. In another study, the complete *Blastocystis* ssrRNA gene was sequenced and phylogenetic analysis suggested that *Blastocystis* should be classified within the Stramenopiles (also known as Heterokonta) (Silberman et al. 1996). Molecular phylogenetic analysis showed that *Blastocystis* is closely related to the Stramenopile *Proteromonas lacerate* (Arisue et al. 2002). Another study involving molecular analysis of *Blastocystis* ssrRNA, cytosolic-type 70-kDa heat shock protein, translation elongation factor 2, and the non-catalytic 'B' subunit of vacuolar ATPase confirmed that *Blastocystis* is a Stramenopile (Arisue et al. 2002). Stramenopiles characteristically possess flagella with mastigonemes. Interestingly, since *Blastocystis* does not have flagella and is non-motile, it was therefore placed in a newly formed Class Blastocystea in the Subphylum Opalinata, Infrakingdom Heterokonta, Subkingdom Chromobiota, and Kingdom Chromista (Cavalier-Smith 1998). In addition, elongation factor- 1α (EF- 1α) sequencing for phylogenetic analysis also showed that *Blastocystis* is not a fungus and suggested that it diverged before *Trypanosoma*, *Euglena*, *Dictyostelium* and other eukaryotes. Most studies in the past named *Blastocystis* species according to host origin and this may have resulted in confusion regarding specificity, cell biology and pathogenicity of the parasite. Recently, a consensus report on the terminology for *Blastocystis* genotypes was published (Stensvold et al. 2007b). Based on this report humans can be host to *Blastocystis* from a variety of animals including mammals (subtype 1), primates (subtype 2), rodents (subtype 4), cattle and pigs (subtype 5), and birds (subtype 6 and 7) (Noel et al. 2005; Yan et al. 2007).

1.3 SPECIATION AND GENETIC DIVERSITY

Blastocystis has been isolated from an extensive range of hosts that includes primates, pigs, rodents, reptiles, insects and birds (Boreham and Stenzel 1993). Morphological differences among isolates are not significant and cannot be used for speciation, therefore other methods for instance karyotyping and molecular phylogenetic analysis have been used to differentiate *Blastocystis* from different hosts (Tan 2004). In the past, description of new species was based on host of origin and parasite ultrastructure (Belova 1992). Others used pulsed-field gel electrophoresis for karyotyping and speciated *Blastocystis* isolated from rats (Chen et al. 1997b), reptiles (Teow et al. 1991), tortoise and rhino iguana (Singh et al. 1996). However, diverse intra-species karyotypes were observed and it was realized that karyotyping might not be a good method for the speciation of *Blastocystis* (Yoshikawa et al. 2004b). Consequently, there are arguments against assigning different species names, other than *B. hominis*, based on presumed host specificity and morphology (Tan 2004).

Recently, analysis of ssrRNA sequencing of 16 *Blastocystis* isolates from humans and other animals showed that isolates can be divided phylogenetically into seven distinct groups that are morphologically similar but genetically different (Arisue et al. 2003). Concurrently, other studies reported the presence of these distinct genotypes in a variety of other animal hosts (Abe et al. 2003a; Abe et al. 2003b; Yoshikawa et al. 2004a, b; Noel et al. 2005). Altogether, these studies strongly suggested that *Blastocystis* is a zoonotic parasite. More recently, it was shown in an extensive ssrRNA sequence analysis

that most of the 78 isolates of *Blastocystis* can be clearly grouped into seven clades referred to as groups I to VII (Noël et al. 2005). More importantly, *Blastocystis* isolates from both humans and animals were present in six of the seven groups. It was suggested that group I (subtype 1) comprised of zoonotic isolates of mammalian origin, group II (subtype 2) comprised of isolates of primates origin, group III (subtype 3) comprised of isolates of human origin, group IV (subtype 4) represented zoonotic isolates of rodent origin, group V (subtype 5) comprised of isolates from pigs and cattle and group VI (subtype 6) and VII (subtype 7) possibly comprised of zoonotic isolates of avian origins (Yoshikawa et al. 2004b, Noel et al. 2005, Yan et al. 2007). Overall, these studies suggested that *Blastocystis* is a zoonotic parasite and animal-to-animal, animal-to-human, and human-to-animal transmission can occur.

Random amplified polymorphic DNA (RAPD) analysis of 16 *Blastocystis* isolates, comprising eight isolates from symptomatic and eight asymptomatic patients, suggested a possible link between genotype with pathogenicity (Tan et al. 2006). However, other studies failed to show any correlation between genotype and pathogenesis of *Blastocystis* (Böhm-Gloning et al. 1997, Yoshikawa et al. 2004b). In a more recent study, correlation between the genotype and symptoms was evaluated using PCR subtyping and a significant correlation between subtype 2 and the asymptomatic group was found among both pediatric and adult patients (Dogruman-Al et al. 2008).

1.4 THE CELL BIOLOGY OF *BLASTOCYSTIS*

Blastocystis is a highly polymorphic and pleomorphic protozoan and there are four major forms (vacuolar, granular, amoeboid and cyst) of the parasite reported from *in vitro* culture and fecal samples (Stenzel and Boreham 1996, Tan et al. 2002). There is little information on the transition of one form to another and available information is limited to the description of individual forms based mostly on microscopic studies. The extensive heterogeneity of various forms of *Blastocystis* has led to the misinterpretation of findings from different studies. *Blastocystis* contains typical organelles of eukaryotes and the most apparent structures in transmission electron microscopy are nuclei, Golgi apparatus and mitochondria-like organelles. It has been shown that *Blastocystis* nuclei are spherical to ovoid and a crescent-shaped chromatin mass is often observable at one end of the organelle (Tan et al. 2001). As *Blastocystis* is an anaerobe, the presence of mitochondria-like organelles needs to be elucidated and it was suggested that these may instead be hydrogenosomes (Boreham and Stenzel 1993, Tan et al. 2002, Stechmann et al. 2008) as a number of typical mitochondrial enzymes were not found in *Blastocystis*. Hydrogenosomes are anaerobic organelles related to mitochondria first described in trichomonads (Lindmark and Müller 1973). In a recent study Stechmann et al. (2008) reported that *Blastocystis* organelles have metabolic characteristics of both anaerobic and aerobic mitochondria and of hydrogenosomes. They suggested that *Blastocystis* mitochondria-like organelles are convergently similar to organelles in the unrelated ciliate *Nyctotherus ovalis*.

Vacuolar form

The vacuolar form is also known as the vacuolated or central body form and it is the most predominant form in axenized *in vitro* cultures, liquid cultures and stool samples (Fig. 1.1A). This form varies significantly in size, ranging from 2-200 μm in diameter with average diameters of cells usually being between 4-15 μm (Zierdt 1991). Vacuolar forms are spherical and contain a characteristic large vacuole surrounded by a thin rim of peripheral cytoplasm. Cellular organelles like nucleus, mitochondria-like organelles, Golgi are located within the cytoplasmic rim. Multiple nuclei can be seen in *Blastocystis* and an average of four nuclei is common (Zierdt 1973). The plasma membrane of *Blastocystis* has pits that appear to have a role in endocytosis (Stenzel et al. 1989).

The exact function of the central vacuole in the *Blastocystis* is currently unclear. It may act as storage organelle to participate in schizogony-like reproduction (Suresh et al. 1994; Singh et al. 1995) or for the deposition of apoptotic bodies during parasite programmed cell death (Tan and Nasirudeen 2005). It was also suggested that the central vacuole may act as a repository for carbohydrates and lipids required for cell growth (Yoshikawa and Hayakawa 1996).

A surface coat or fibrillar layer of varying thickness often surrounds the organism. This surface coat is thick in freshly isolated parasites from feces but it gradually becomes thinner with prolonged laboratory culture (Cassidy et al. 1994). The exact role of the surface coat is not understood but it has been suggested to play a role in trapping and

degrading bacteria for nutrition (Zaman et al. 1997; Zaman et al. 1999) and protecting against osmotic shock (Cassidy et al. 1994).

Granular form

The granular form of *Blastocystis* is morphologically identical to the vacuolar form except that granules are present in the cytoplasm or more commonly within the central vacuole (Fig 1.1B). The size of this form ranges from 3-80 μm in diameter. Granules in the central vacuole may differ considerably in appearance and described as myelin-like inclusions, small vesicles, crystalline granules and lipid droplets (Dunn et al. 1989). Bacterial remnants in lysosome-like compartments in the central vacuoles were also observed (unpublished observation). The granular form is commonly observed in non-axenized or older cultures (Tan 2004).

Amoeboid form

The amoeboid form (Fig. 1.1C) is rarely observed and there are conflicting reports about its description (McClure et al. 1980; Dunn et al. 1989). These forms have been observed in antibiotic treated cultures, old cultures or in fecal samples (Zierdt 1973). Amoeboid forms are smaller and its size ranges from 2.6-7.8 μm in diameter. Dunn et al. (1989) reported ameboid forms with extended pseudopodia but a central vacuole, Golgi and mitochondria were not seen. On the contrary, Tan et al. (2001) showed by transmission electron microscopy that this form possess a central vacuole, numerous Golgi bodies and mitochondria within the cytoplasmic extension of pseudopods suggesting that this is a highly active form. In contrast to amoebae, these pseudopodia do

not seem to be involved in locomotion. It was suggested that this form may be phagocytic in nature as ingested bacteria were found within the parasite in transmission electron microscopy analysis (Boreham and Stenzel 1993).

Cyst form

An environmentally resistant cyst form (Fig. 1.1D) is the most recently reported form of *Blastocystis* (Mehlhorn 1988; Stenzel and Boreham 1991; Zaman 1998) and it is considered significant for the fecal-oral transmission of infection (Yoshikawa et al. 2004c). This form is in general much smaller and its size ranges from 2-5 μm in diameter. It is protected by a multi-layered cyst wall which is sometimes covered with a loose surface coat (Moe et al. 1996). Unlike vacuolar and granular forms, this form has been shown to survive in water for up to 19 days at normal temperatures (Moe et al. 1996). Another study has shown that *Blastocystis* cysts could survive up to 1 month at 25^0C and 2 months at 4^0C (Yoshikawa et al. 2004c). Experimental infection studies in mice (Moe et al. 1997), rats (Yoshikawa et al. 2004c) and birds (Tanizaki et al. 2005) have shown that the cyst form is indeed the transmissible form of *Blastocystis*.

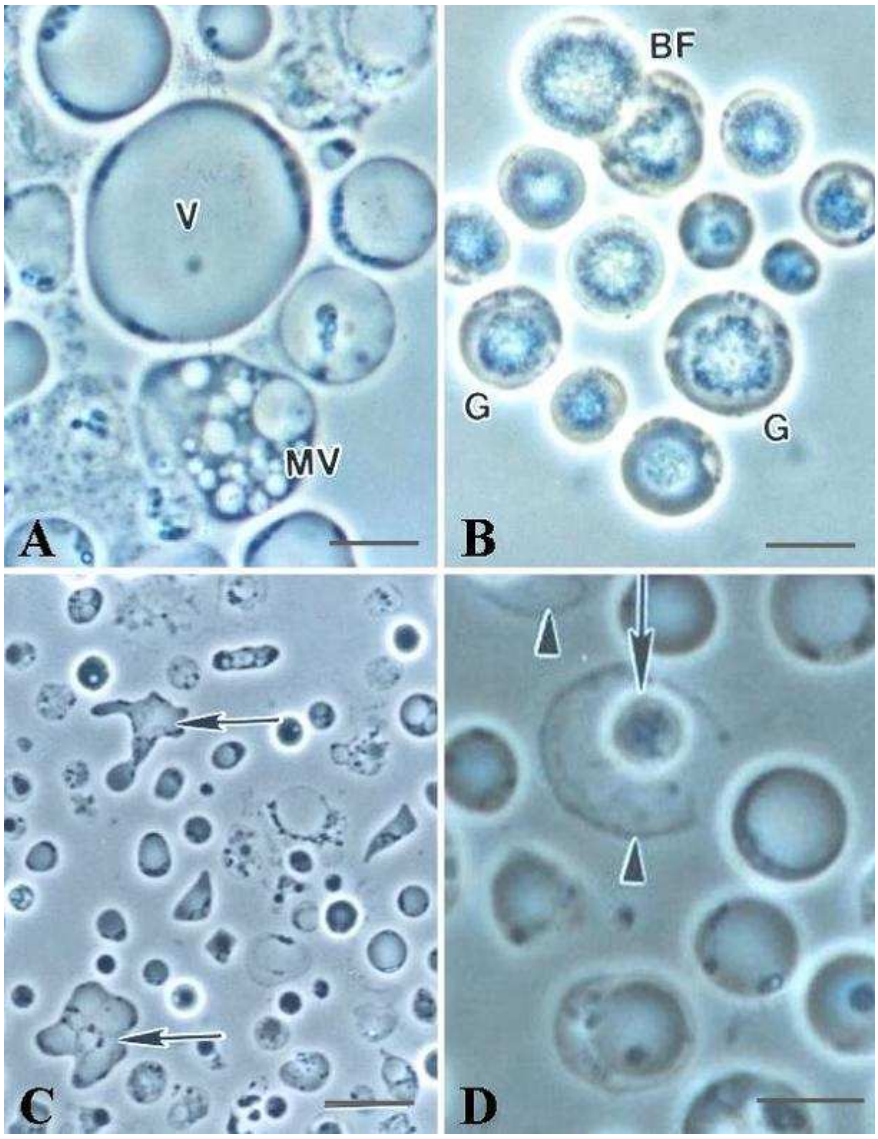

Fig. 1.1 Four morphological forms from axenic cultures of *Blastocystis* under phase-contrast microscopy.

(A) Vacuolar (*V*) and multivacuolar (*MV*) forms. Cells are showing extensive variations in their size. Bar = 10 μm.
(B) Granular forms (*G*). One of the cells appears to be dividing (*BF*). Bar = 10 μm.
(C) Amoeboid forms (arrow). Bar = 10 μm.
(D) Cyst form. Refractile cyst (arrow) with loose fibrillar layers (arrowhead). Bar = 5 μm.
Adapted from Chen (1999).

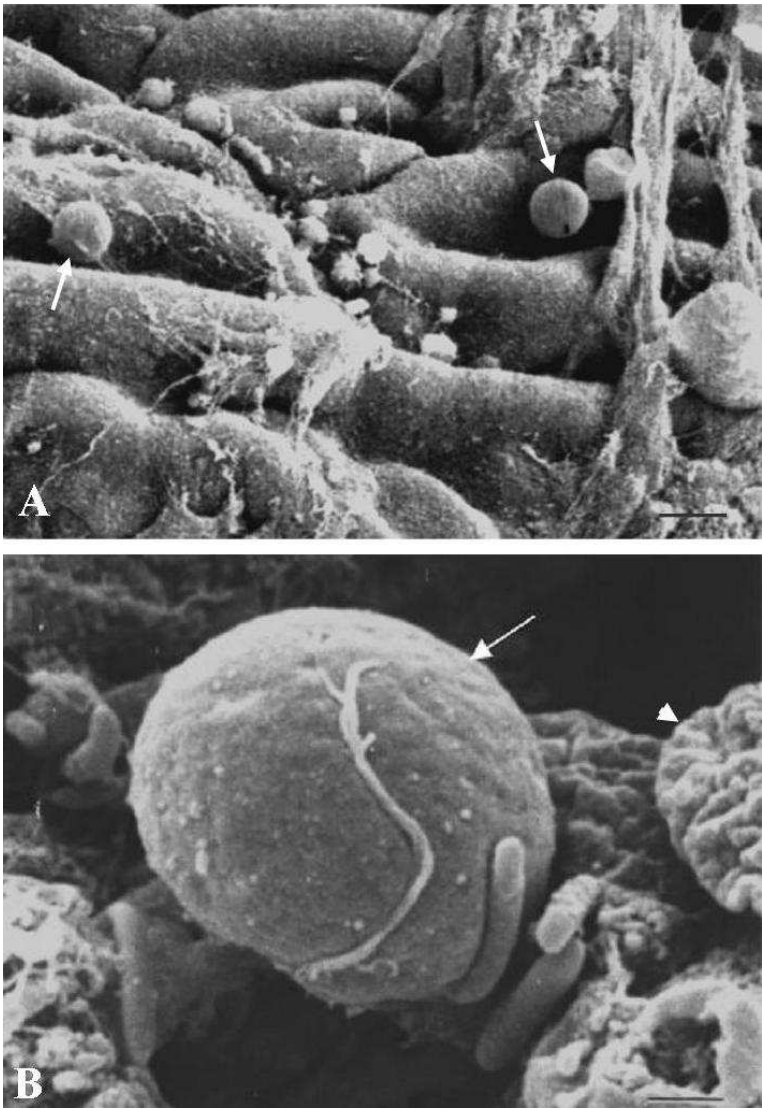

Fig 1.2 Scanning electron micrographs of *Blastocystis* rat isolate. (A) Sphere shaped *Blastocystis* (arrow) can be seen in folds of large intestine of wistar rat. Bar = 10 μm. (B) An enlarged view showing a well rounded healthy *Blastocystis* cell (arrow) and another that appears to be a dying *Blastocystis* cell (arrowhead). Bar = 1 μm. (Chen 1999).

1.5 LIFE CYCLE

Many life cycles have been proposed for *Blastocystis* (Alexeieff 1911; Boreham and Stenzel 1993; Singh et al. 1995; Stenzel and Boreham 1996; Tan 2004); owing to a lack of controlled experimental studies and the pleomorphic nature of the organism. The first life cycle was proposed by Alexeieff (1911) and it described the involvement of binary fission and autogamy. Some of the reports suggest modes of division like plasmotomy and schizogony (Zierdt 1973; Singh et al. 1995). Most of these observations were based on microscopic analysis. Although *Blastocystis* had been isolated from laboratory animals (Fig 1.2), the lack of a suitable animal model was considered to be a major reason for the disagreement on its life cycle (Tan 2004). Recent studies have shown successful experimental infection of *Blastocystis* in chickens (Iguchi et al. 2007) and rats (Yoshikawa et al. 2004c; Iguchi et al. 2007; Hussein et al. 2008). Rats appear to be good animal models for *Blastocystis* infection but reproducibility of animal infection needs to be ascertained.

A life cycle proposed by Tan (2004) states that infection is initiated when cysts of *Blastocystis* are orally ingested by humans or animals (Fig 1.3). Ingested cysts develop into vacuolar forms in the large intestine and later reproduce by binary fission. Some of the vacuolar forms encyst and are passed through the feces and the cycle is repeated. The role of the amoeboid and granular form in the life cycle of *Blastocystis* is not understood and remains to be elucidated. More recently, Tan (2008) revised the life cycle and included findings from molecular typing suggesting that *Blastocystis* isolated from

humans actually comprise human and zoonotic genotypes of varying host specificities. A modified life cycle of *Blastocystis* must take into consideration the large reservoir of this parasite in a range of animal populations with humans as potential hosts (Fig. 1.4).

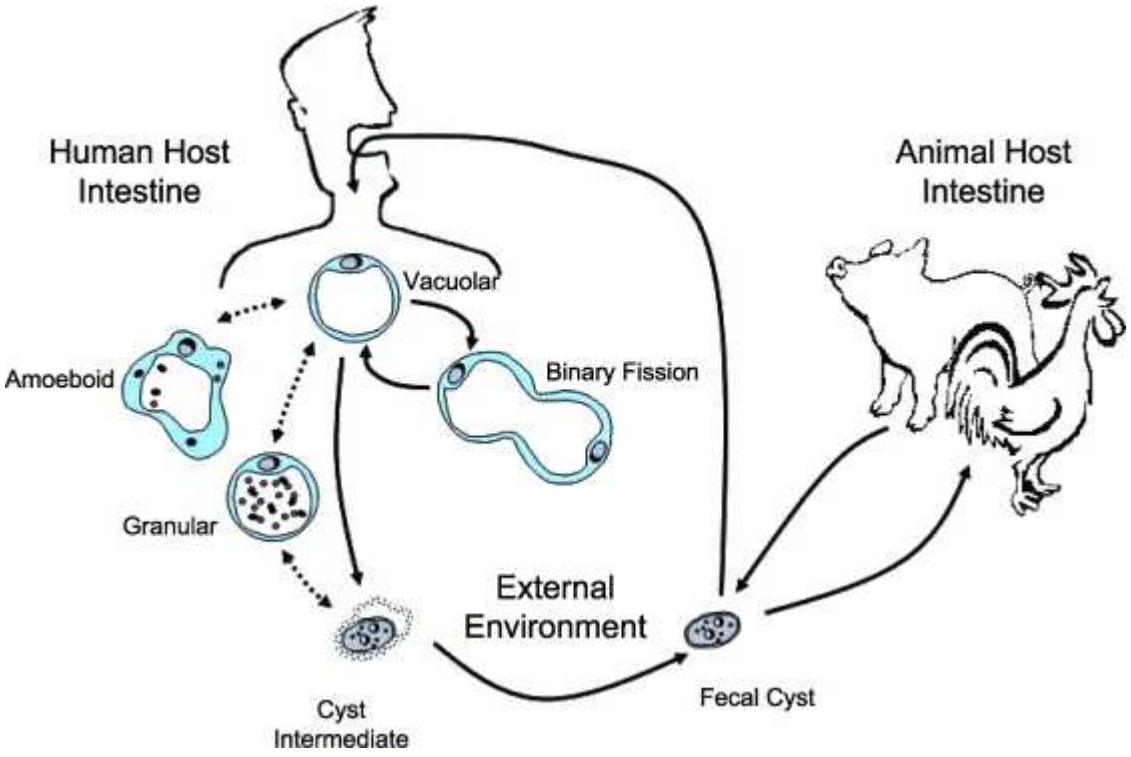

Fig. 1.3 Life cycle of *Blastocystis* as proposed by Tan (2004). Infection is initiated when cysts of *Blastocystis* are orally ingested by humans or animals. Ingested cysts develop into vacuolar forms in the large intestine and later reproduce by binary fission. Some of the vacuolar forms encyst and passed through the feces and cycle is repeated by fecal-oral route. The development of other forms is less well understood and is represented with dashed lines.

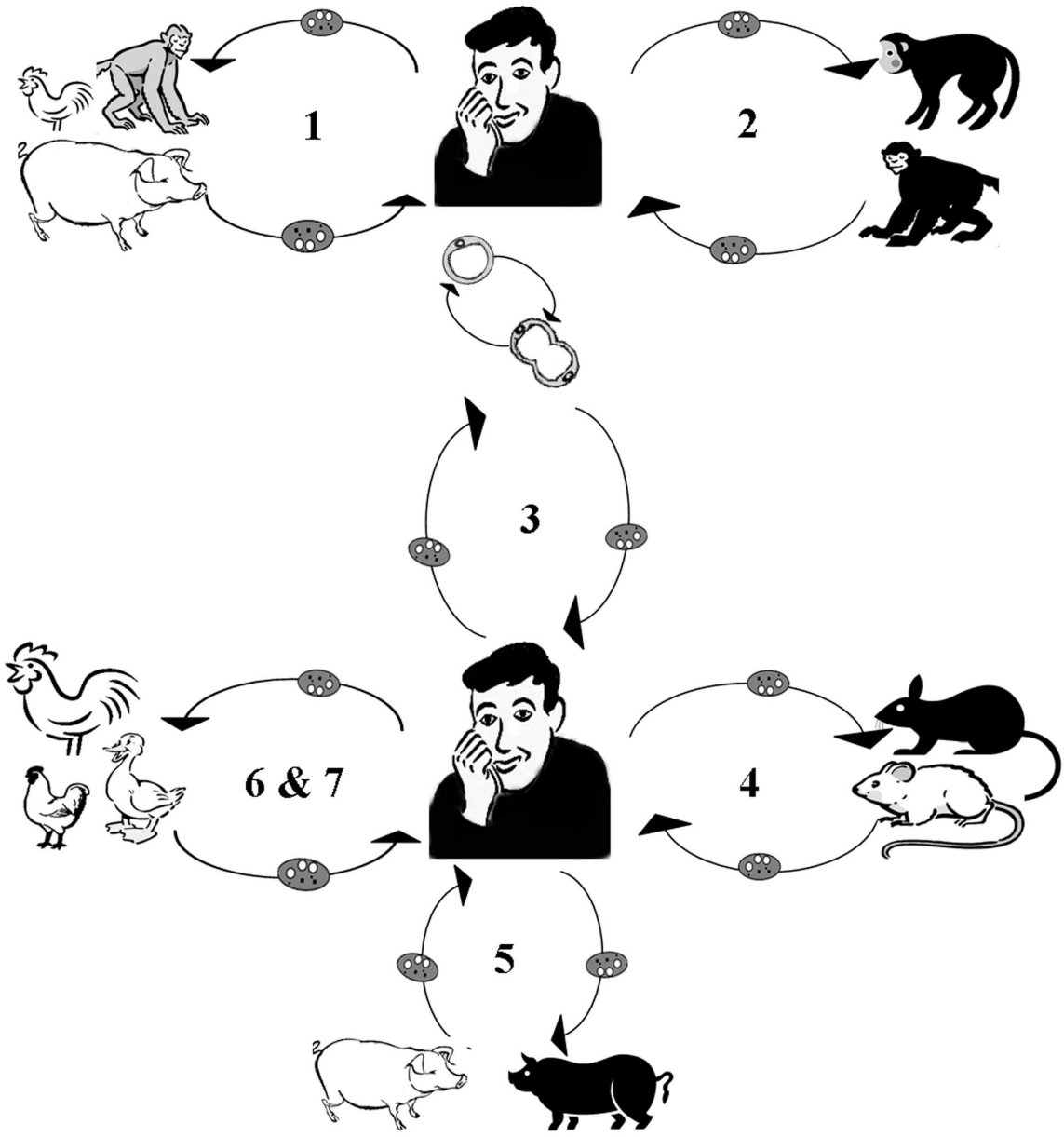

Fig. 1.4 Revised life cycle of *Blastocystis* as proposed by Tan (2008). This life cycle also suggests existence of zoonotic genotypes of *Blastocystis* (Subtypes 1-4, 6 and 7) with different host specificities. Fecal cysts of *Blastocystis* infect human and animal hosts and develop into vacuolar forms in the large intestine. Cross-infection can occur among mammalian and avian isolates of subtype 1. Subtype 2, 3 and 4 comprises primate, human and rodent isolates, respectively. Subtype 5 comprises isolates from pigs and cattle whereas subtype 6 and 7 comprise avian isolates. This proposal suggests that certain animals act as reservoirs of *Blastocystis* for human infections; and humans can be potentially infected by six or more species of *Blastocystis*. (Adapted from Tan 2008).

1.6 ZOONOSES

Over the last decade, *Blastocystis* is increasingly recognized as a cause of human gastrointestinal disease. Research interest on *Blastocystis* is on the rise and a Pubmed search indicates that in the last 5 years there is an increase in the number of articles on *Blastocystis* (from approximately 130 during 1998-2002 to approximately 220 during 2003-2007) (PubMed).

Recently, there have been reports that reveal many unexplored aspects of this pathogen's pathogenesis. *Blastocystis* is now considered to have zoonotic potential and it is believed that animals like pigs and chicken constitute large reservoirs of this protozoan for human infection via the fecal-oral route (Tan 2004). Many reports have shown strong phylogenetic evidences that designate *Blastocystis* as a zoonosis (Abe et al. 2003c; Yoshikawa et al. 2004a; Noel et al. 2005). In an extensive phylogenetic study, it was shown that *Blastocystis* could be classified in seven different clades with six main groups comprising of isolates from both humans and animals (Noel et al. 2005). It was suggested that animals represent a large potential reservoir for human infections. Numerous *Blastocystis* isolates from humans are believed to be potentially zoonotic because they have similar or fairly similar genotypes to isolates found in a variety of other animal and bird species. It has been reported that a number of genotypes from human isolates can infect chickens and rats (Iguchi et al. 2007; Hussein et al. 2008).

Blastocystis possesses a number of features that increase the chances of waterborne transmission and environmental contamination and thus demands zoonosis control. These include an extensive range of hosts and low host specificity, a transmissible cyst form that is resistant to adverse environmental conditions and a lack of knowledge of specific disinfection and treatment strategies.

Blastocystis is also very common among many animal species. It was suggested that humans are host for numerous *Blastocystis* genotypes isolated from animals (Noel et al. 2005). It has been reported in mammals, birds, reptiles, amphibians, annelids, and arthropods. In particular, some animals showing high prevalence include laboratory rats (60%; Chen et al. 1997a), pigs (70-95%; Abe et al. 2002), and birds (50-100%; Abe et al. 2002; Lee and Stenzel 1999). In Brisbane, Australia, *Blastocystis* has been detected in fecal samples from domestic dogs and cats (Duda et al. 1998). The prevalence was very high; with 70.8% dogs and 67.3% cats infected with *Blastocystis*.

In an extensive study, the prevalence of *Blastocystis* sp. was examined in fecal samples collected from cattle, pigs, and various zoo animals in Japan (Abe et al. 2002). A high prevalence of *Blastocystis* infection was reported in farm animals (95% in pigs; 71% in cattle), and in zoo animals (85% in primates; 80% in pheasants; 56% in ducks). In this study, *Blastocystis* isolates from various animals were morphologically indistinguishable from *Blastocystis* isolated from humans.

PCR-based characterization of *Blastocystis* isolates was reported from dogs and humans living in a localized endemic community in Thailand (Parkar et al. 2007). This phylogenetic study provided molecular-based evidence to support zoonotic transmission of *Blastocystis* infections from dogs, possums and primates in a community. It was reported that people working closely with animals were at significantly higher risk of *Blastocystis* infections suggesting that infection can be acquired from animals and work place safety is important for prevention of infection (Rajah Salim et al. 1999). It was found in this study that 41% of animal handlers were positive for *Blastocystis* in contrast to 17% of individuals who did not work with animals.

Human populations exposed to poor hygiene practices, contaminated food and water appeared to be at risk of *Blastocystis* infections (Tan 2008). Outbreaks of waterborne *Blastocystis* infections have been documented recently in some studies (Karanis et al. 2007). *Blastocystis* cysts have been detected in Scottish and Malaysian sewage treatment facilities; and viable cysts, found in the effluent, provided evidence that *Blastocystis* infections have potential for waterborne transmission (Suresh et al. 2005). Evidence is growing that contaminated water and food play an important role in the transmission of *Blastocystis* to humans. In a study (Cruz Licea et al. 2003), *Blastocystis* was detected from 41.7% of food vendors and risk analysis showed that it was associated with poor personal hygiene habits. This report suggested that customers were at risk of acquiring *Blastocystis* infection from food vendors.

1.7 SYMPTOMS AND SIGNS

Many epidemiological studies suggest that *Blastocystis* infection is associated with intestinal disorders (Barahona Rondon et al. 2003; Miller et al. 2003; Leelayoova et al. 2004; El-Shazly et al. 2005). On the contrary, a number of reports suggested that this parasite does not cause any disease (Rosenblatt 1990; Senay and MacPherson 1990; Udkow and Markell 1993).

The clinical symptoms and signs associated with *Blastocystis* infections are mainly diarrhea, abdominal pain, cramps, nausea as well as non-specific gastrointestinal signs that includes bloating, vomiting, anorexia, weight loss and flatulence (Qadri et al. 1989; Doyle et al. 1990; Nimri and Batchoun 1994; Stenzel and Boreham 1996). Although most cases of *Blastocystis* are mild and chronic, profuse watery diarrhoea (Logar et al. 1994) and fever (Gallagher and Venglarcik, 1985) has also been reported in acute cases. There are reports that suggest the association of *Blastocystis* with irritable bowel syndrome (Giacometti et al. 1999; Yakoob et al. 2004), a functional bowel disorder characterized by abdominal pain and changes in bowel habits.

Interestingly, some studies have also reported symptoms like itching (Garavelli and Scaglione 1990) and joint pain (Lee et al. 1990). Numerous case reports have suggested association of *Blastocystis* with cutaneous disorders like chronic urticaria (Armentia et al. 1993; Biedermann et al. 2002), angioedema (Micheloud et al. 2007) and palmoplantar pruritis (Kick et al. 2002). Recently in a case study of *Blastocystis* infection,

it was reported that acute urticaria was associated with amoeboid forms of *Blastocystis* subtype 3 (Katsarou-Katsari et al. 2008).

Numerous reports suggested that HIV and immunocompromised patients are more likely to acquire *Blastocystis*-associated intestinal disease and suggested that this parasite is opportunistic (Brites et al. 1997; Ok et al. 1997; Florez et al. 2003; Rao et al. 2003; Hailemariam et al. 2004). Diarrhoea and other gastrointestinal symptoms were reported in these *Blastocystis* infected immunocompromised patients. *Blastocystis* was observed to be the most common parasite isolated from patients undergoing chemotherapy for hematological malignancies and it was suggested to be associated with diarrhoea, abdominal pain and flatulence (Tasova et al. 2000).

Many cases of *Blastocystis* infections appear to be asymptomatic and it is common to detect large number of *Blastocystis* in stool samples from patients who do not show any sign of disease. It appears that absence of clinical symptoms might be due to infections with nonpathogenic genotypes of *Blastocystis*, however, whether this disease is genotype linked or not is still an unresolved issue. In a study involving PCR-RFLP ribotyping of *Blastocystis* isolates, it was observed that subtypes 1, 2 and 4 were associated with symptoms whereas subtype 3 was associated with asymptomatic infections (Kaneda et al. 2001). In an another genotyping study, subtype 1 was found in patients with gastrointestinal symptoms, subtype 7 was found in asymptomatic and subtypes 3 and 6 were found in both groups of patients (Hussein et al. 2008). On the contrary, some studies have indicated that there is no association between symptoms and

Blastocystis genotypes (Böhm-Gloning et al. 1997; Yoshikawa et al. 2004b). In summary, studies suggest that subtype 1 might be associated with disease and subtype 3 may be non-pathogenic; however there is a need for more studies with larger sample sizes to resolve this issue. In addition, it is evident that *Blastocystis* infections can cause a variety of symptoms, not necessarily confined to the intestinal tract.

1.8 CLINICAL FEATURES

The clinical significance of *Blastocystis* is presently ambiguous. There are many reports that either implicate or exonerate this organism as a cause of gastrointestinal disease. This parasite can be found in the intestinal tract of both healthy individuals and patients exhibiting gastrointestinal symptoms (Stenzel and Boreham 1996).

Many case reports have suggested the association of *Blastocystis* with a variety of clinical features including terminal ileitis (Tsang et al. 1989), colitis (Russo et al. 1988), ulcerative colitis (Jeddy and Farrington 1991), and infective arthritis (Lakhanpal et al. 1991). Interestingly, various reports associate *Blastocystis* infections with cutaneous lesions particularly urticaria (Giacometti et al. 2003; Cassano et al. 2005; Gupta and Parsi 2006) suggesting host allergic response to some unknown parasitic factors.

Fecal leukocytes (Cohen 1985; Diaczok and Rival 1987) and rectal bleeding has been reported in *Blastocystis* infections (Al-Tawil et al. 1994). Enlargement of liver and spleen was also reported in a study (Garavelli and Scaglione 1990). Blood analysis of a number of patients showed eosinophilia (Garavelli and Scaglione 1990; Lambert et al. 1992). There are reports that associated *Blastocystis* with intestinal disorders in HIV or immunocompromised patients (Ok et al. 1997; Cirioni et al. 1999; Florez et al. 2003; Rao et al. 2003; Hailemariam et al. 2004) suggesting that *Blastocystis* is an opportunistic organism.

Inflammation and edema of intestinal mucosa has been reported in *Blastocystis* infections (Russo et al. 1988; Garavelli et al. 1991; Zuckerman et al. 1994). In *Blastocystis* infected patients, endoscopy results showed that there was no invasion of the colonic mucosa (Kain et al. 1987; Dawes et al. 1990; Zuckerman et al. 1990). On the other hand, Al-Tawil et al. (1994) reported the presence of colonic ulceration and infiltration of superficial lamina propria by *Blastocystis*. Intestinal permeability was reported to be significantly increased in *Blastocystis* patients and it was suggested that *Blastocystis* infections may damage the intestinal wall (Dagci et al. 2002).

1.9 PATHOGENESIS

Very few *in vivo* and *in vitro* studies have addressed the pathogenicity aspects of *Blastocystis* infections. Live *Blastocystis* cells and parasitic lysates isolated from symptomatic and asymptomatic individuals caused significant cytopathic effects on Chinese Hamster Ovary (CHO) cells (Walderich et al. 1998). Another study showed that *Blastocystis* reduced *Escherichia coli* or LPS-induced secretion of IL-8 and it was proposed that *Blastocystis* is capable of modulating host immune responses at initial stages of infection (Long et al. 2001). This suggested that *Blastocystis* may down-regulate host immune responses in order to improve survival in the gut.

Other isolated clinical reports described intestinal inflammation and edema in patients infected with *Blastocystis* (Kain et al. 1987; Russo et al. 1988; Garavelli et al. 1992). One study reported significantly increased intestinal permeability after *Blastocystis* infections (Dagci et al. 2002). A number of studies have described experimental infections involving rats, mice, guinea pigs and chickens (Phillips and Zierdt 1976; Pakandl 1992; Moe et al. 1997; Abou El Naga and Negm 2001; Yoshikawa et al. 2004c; Tanizaki et al. 2005; Iguchi et al. 2007; Hussein et al. 2008). However, reproducibility of these animal models needs to be ascertained. In experimentally infected mice, histological examination of the cecum and colon revealed intense inflammatory cell infiltration, edematous lamina propria, and mucosal sloughing (Moe et al. 1997). Lesions on the ileocecal mucosa were reported in mice experimentally infected with *Blastocystis* (Zhang et al. 2006). Experimentally, it has been shown that *Blastocystis* infection can be

established with oral inoculation of cysts in mice (Moe et al. 1997) and rats (Yoshikawa et al. 2004c). Wistar rats were successfully infected with oral inoculation of *Blastocystis* cysts recovered from feces of another infected rat and it was demonstrated that an oral dose of as low as ten cysts was sufficient to establish infection. These studies indicated that the fecal cyst form was responsible for fecal-oral transmission of the parasite. Clinical reports and experimental studies indicate that *Blastocystis* is not invasive but it is capable of causing gastrointestinal pathogenesis. Experimentally, it was shown that germ free guinea pigs can be infected by oral or intracecal inoculations and heavy infections caused diarrhoea and cecal hyperemia (Phillips and Zierdt 1976). Recently, *Blastocystis* isolates from symptomatic and asymptomatic humans were experimentally tested for their infectivity in rats (Hussein et al. 2008). It was reported that *Blastocystis* isolates from symptomatic patients induced moderate to severe pathological changes in infected rats but isolates from asymptomatic individuals caused mild pathological changes. Authors suggested that subtype 1 was pathogenic, while subtypes 3 and 4 consisted of both pathogenic and non-pathogenic variants.

Interestingly, accumulating reports suggest an association between *Blastocystis* infections and cutaneous lesions. The mechanism is possibly that of typical cutaneous allergic hypersensitivity where parasitic antigens induce the activation of specific clones of Th2 lymphocytes (Pasqui et al. 2004). Eventually, cytokines release and the consequent IgE production may lead to an allergic reaction. Moreover, it was also suggested that some *Blastocystis* virulence factors may activate the complement pathway with the production of anaphylotoxins (Valsecchi et al. 2004). These anaphylotoxins,

upon interaction with basophils and mast cells induce histamine production which may result in cutaneous disorders.

A number of earlier reports suggested that *Blastocystis* disease is dependent on parasite load however recent studies suggest that clinical outcome of *Blastocystis* infection is multifactorial and depends on parasite genotype, parasitic load, and host immune status (Tan 2008). In a number of cases, *Blastocystis* infections appear to be self-limiting and spontaneous elimination of infection has been reported (Sun et al. 1989).

1.10 DIAGNOSIS

Because of its uncertain pathogenesis, reasonable clinical significance is seldom given to *Blastocystis* infections. Generally, diagnosis and other important aspects of *Blastocystis* infections are not included in the curriculum of medical studies and thus diagnosis of *Blastocystis* remains a challenging task for a diagnostic laboratory. Although an experienced laboratory technician can perform diagnosis in direct fecal smears, most diagnostic laboratories do not have expertise on identification of this parasite and there is a need for training to enable identification of all forms of *Blastocystis* in fecal samples. Identification of *Blastocystis* in direct fecal smears is relatively difficult as the parasite can be confused with yeast, *cyclospora*, or fat globules.

In the past, laboratory diagnosis of *Blastocystis* was based on the identification of vacuolar and granular forms in direct fecal specimens (Katz and Taylor 2001). Direct microscopy of fecal specimens is performed by wet mounts with Lugol's iodine or permanent fixed smears with Giemsa, acid-fast, trichrome and Field's staining. Rather than the characteristic vacuolar form, the cyst form may predominate fecal samples. Cyst forms might be difficult to identify by direct microscopy because of their small size (3-5 μm) but these can be effectively concentrated by density-gradient methods (Zaman 1996). Diagnostic labs should therefore include the fecal cyst form as an indicator of *Blastocystis* infection.

Many researchers suggest that when all other known bacterial, viral or parasitic causes of symptoms are absent and *Blastocystis* is present in large numbers it should be treated as a pathogen. More than five organisms per high power field (×400 magnification) should be considered as a heavy infection. For confirmative diagnosis in stool samples, *in vitro* culture in Jones' medium is a method of choice (Suresh and Smith 2004). It was reported that *in vitro* culture of fecal samples was six times and twice more sensitive than direct fecal smears and trichrome staining methods respectively (Termmathurapoj et al. 2004). However, it was also reported in this study that the *in vitro* culture method failed to detect some parasites suggesting that not all *Blastocystis* isolates can be readily cultured in laboratory. *Blastocystis* can be cultured in various mediums including Jone's medium, Boeck and Drbohlav's inspissated medium or diphasic agar slant medium with Jone's as a medium of choice for patient samples. Diphasic agar slant medium was reported to be good for the culture of *Blastocystis* from pigs, cattle and chickens (Abe et al. 2003b; Abe 2004). In axenized cultures, cell densities of up to 2.5 $\times 10^7$ can be achieved (Ho et al. 1993) and doubling time may vary from 6 to 23 h, depending on type of medium and isolate (Boreham and Stenzel 1993). Colony growth of *Blastocystis* has been shown on solid medium and cultures were viable for up to 2 weeks (Tan et al. 2000).

Molecular approaches, particularly PCR-based diagnosis have been described for *Blastocystis* (Stensvold et al. 2007a). PCR amplification using subtype specific primers is suggested to be useful for identifying and genotyping *Blastocystis* from patient samples. Knowledge of the genotype can be extremely valuable if certain *Blastocystis* genotypes

are found to be more virulent than others. A recent study has demonstrated that PCR-based detection of *Blastocystis* from fecal specimens is more sensitive than *in vitro* propagation (Parkar et al. 2007). Recently, a sensitive and specific real-time light cycler PCR assay was developed to detect a 152 bp sequence in an uncharacterized region of the *Blastocystis* genome and 11 strains of *Blastocystis* from subtypes 1, 3, and 4 were with this method (Jones et al. 2008). Using this method, *Blastocystis* was detected in stool samples that were found *Blastocystis* negative during microscopy and conventional PCR. In addition, this method showed no cross-reactivity with other common gastrointestinal pathogens.

Other methods like enzyme-linked immunosorbent assay (ELISA) and immunofluorescence detection have not been comprehensively investigated for *Blastocystis*. Although development of monoclonal antibodies against *Blastocystis* has been reported (Tan et al. 1996), antigenic diversity of *Blastocystis* seems to be a limiting factor in the use of immunological methods. *Blastocystis* infections have been reported to induce IgG and IgA responses in patients and detected by indirect fluorescent antibody test (IFA) and ELISA (Zierdt and Nagy 1993; Zierdt et al. 1995; Hussain et al. 1997; Kaneda et al. 2000; Mahmoud and Saleh 2003). ELISA titers ranged from 1:50-1:1,600 (Zierdt et al. 1995) and it was observed that high titers were associated with symptomatic infections of *Blastocystis* (Zierdt and Nagy 1993; Zierdt et al. 1995; Hussain et al. 1997; Mahmoud and Saleh 2003). In a recent study using ELISA, secretory IgA, serum IgA and serum IgG levels were detected in *Blastocystis* infected patients with and without clinical symptoms (Mahmoud and Saleh 2003). It was found that serum from only symptomatic

patients had significantly higher antibody levels. On the other hand, Kaneda et al. (2000) reported asymptomatic patients with serum antibodies to *Blastocystis* and high levels were observed in chronic cases. Overall, it may be desirable to develop specific monoclonal antibodies against different genotypes and evaluate different serological assays for the diagnosis of *Blastocystis* infections.

Diagnosis of blastocystosis has been reported with the help of invasive diagnostic techniques like endoscopy but it has not been evaluated. *Blastocystis* colonization in the lower ileum and cecum of a patient was detected in the microscopic examinations of the lumen fluids aspirated during endoscopy (Matsumoto et al. 1987). As *Blastocystis* can be detected in feces and no characteristic intestinal lesions are associated with infection, invasive diagnostic techniques are not recommended for routine examinations.

In brief, a number of methods have been described for the diagnosis of *Blastocystis*. Direct microscopy of stained fecal smears is useful and it should be supplemented with numbers of parasites observed per high power field to help clinicians ascertain parasitic load. For confirmatory diagnosis, microscopic examination should be supplemented by *in vitro* culture and/or PCR-based methods.

1.11 TREATMENT AND PROGNOSIS

Due to ambiguity surrounding the pathogenesis of *Blastocystis* and the non-specific nature of symptoms, the need to treat *Blastocystis* infections has been disputed. In most cases, treatment with antiprotozoal drugs, in particular metronidazole, is warranted if no other cause of symptoms is observable (Nigro et al. 2003; Cassano et al. 2005; Moghaddam et al. 2005). Metronidazole is a nitroimidazole drug used primarily for infections caused by obligate anaerobes. After uptake by anaerobic organisms, it is reduced by the pyruvate:ferredoxin oxidoreductase system in the mitochondria. Reduced intermediate products interact with intracellular targets and disrupt DNA helical structure and inhibit nucleic acid synthesis. Whether the mode of action of metronidazole on *Blastocystis* is similar to that described for other protozoan parasites is currently unknown. It has been shown that metronidazole induces programmed cell death in *Blastocystis* with a number of features similar to apoptosis in higher eukaryotes (Nasirudeen et al. 2004). In *Blastocystis* infections, various dosage of metronidazole has been recommended in different studies (Waili 1987; Qadri et al. 1989; Garavelli and Libanore 1990; Nassir et al. 2004; Guirges and Al- Moghaddam et al. 2005). In summary, treatment regime ranges from 250-750 mg three times a day for 5 to 10 days, 200 mg four times per day for 7 days or 2 gm/day for 5 days. There have been reports where metronidazole has been used successfully in combination with other drugs particularly paromomycin (Pasqui et al. 2004) and co-trimoxazole (Andiran et al. 2006). Some studies also reported that metronidazole was not efficacious in eliminating *Blastocystis* infection from patients (Cohen 1985; Schwartz and Houston 1992). *In vitro* studies have

reported that different *Blastocystis* isolates show variable sensitivities to metronidazole (Haresh et al. 1999). In particular, cysts of *Blastocystis* have been shown to be resistant to high concentrations (up to 5 mg/ml) of metronidazole (Zaman and Zaki 1996). Overall, treatment failures may occur due to extensive genetic heterogeneity and differences in the susceptibility of different forms of *Blastocystis* to drugs.

In addition to metronidazole, many other drugs have been used to treat *Blastocystis* infections with high or reasonable efficacy. Among these are furazolidone, quinacrine, ornidazole, tinidazole, ketoconazole, and trimethoprim-sulfamethoxazole (co-trimoxazole) (Reviewed in Stenzel and Boreham 1996). Recently, a number of studies reported that the broad spectrum antiparasitic drug nitazoxanide is effective for use in *Blastocystis* infections (Cimerman et al. 2003; Rossignol et al. 2005).

There is a lack of controlled studies involving large numbers of patients and extensive studies are needed to verify the efficacy of different drugs on *Blastocystis* infections. Thus far, metronidazole seems to be drug of choice for blastocystosis, even though there are some evidences of treatment failure. In such cases, other drugs should be employed empirically. Chemotherapy should be employed when symptoms are persistent and no other pathogen than *Blastocystis* is detected. In mild cases of *Blastocystis* infections, intervention may not be required as infection is usually self-limiting (Babb and Wagener 1989; Doyle et al. 1990; Markell 1995).

1.12 EPIDEMIOLOGY, PEVENTION AND CONTROL

Blastocystis is reported to be one of the most common protozoans found in fecal samples of both symptomatic patients and asymptomatic individuals (Cirioni et al. 1999; Taamasri et al. 2000; Windsor et al. 2002). Recently, there is a significant increase in prevalence reports which has helped us to better understand the distribution of genotypes, mode of transmission and pathogenicity aspects. *Blastocystis* has a worldwide distribution and findings of many surveys reported it to be most frequently isolated protozoan parasite (Pegelow et al. 1997; Taamasri et al. 2000; Florez et al. 2003; Baldo et al. 2004). Prevalence of *Blastocystis* infection is higher in developing countries than in developed countries (Stenzel and Boreham 1996; Tan et al. 2002) and occurrence as high as 60% were reported from some developing countries (Pegelow et al. 1997). Occurrence of *Blastocystis* varies from country to country. A low prevalence of 0.5% has been reported among asymptomatic healthy individuals in Japan (Horiki et al. 1997). A moderate prevalence of 14-21% and 23% was reported in Thailand (Yaicharoen et al. 2005) and United States (Amin 2002) respectively. A high prevalence of 40.7% and 60% was reported in Philippines (Baldo et al., 2004) and Indonesia (Pegelow et al. 1997) respectively. High incidences (36.9-44%) of *Blastocystis* were also observed in Thai military personnel (Taamasri et al. 2002; Leelayoova et al. 2004). Prevalence of *Blastocystis* may vary widely within various geographical regions of the same country. In Thailand, a prevalence of 0.8% and 45.2% was reported from Nan province (Waikagul et al. 2002) and Pathum Thani province (Saksirisampant et al. 2003) respectively. Variations in the same geographical region may represent true differences between

communities or living conditions. Nevertheless, these reported variations might be due to lack of a standardized diagnostic methodology and difficulty in identifying parasitic forms other than the common vacuolar form. Recent studies have used PCR-based approaches to further elucidate genotype information which has shed light on the distribution of *Blastocystis* genotypes in humans and animals. Studies have found that *Blastocystis* subtype 3 was the most common subtype among isolates from countries including Turkey (Ozyurt et al. 2008), Greece (Menounos et al. 2008), Singapore (Wong et al. 2008), Japan, Pakistan, Bangladesh, and Germany (Yoshikawa et al. 2004b). In summary, studies suggest that there is no association between specific genotype and geographic origin; and due to its predominance in urbanized countries, subtype 3 is probably the subtype of human origin.

It has been observed that humans with compromised health and poor hygiene are more susceptible to *Blastocystis* infections. *Blastocystis* infections are also of special clinical interest to developed countries as millions of travelers going to developing countries are at risk of acquiring infection (Sohail and Fischer 2005). *Blastocystis* infections are more common during hot weather and during the pre-monsoonal months (Stenzel and Boreham 1996).

Based on current knowledge, it is generally accepted that *Blastocystis* is transmitted by the fecal-oral route. This assumption is strengthened by animal infection studies (Yoshikawa et al. 2004c; Tanizaki et al. 2005) and reports showing high prevalence of *Blastocystis* in population living in poor hygiene (Nimri 1993; Cruz Licea

et al. 2003). Therefore, control measures should consist of good hygiene practices and community sanitary facilities.

Because *Blastocystis* is generally regarded as a zoonotic parasite, animals and their fecal material represent a risk for human infection. Contamination of food, water, and environment by animal fecal material should be prevented. High prevalence of *Blastocystis* has been shown in pets particularly dogs and cats and it was suggested that these domestic animals could be an important source of infection to humans (Duda et al. 1998). Routine antiparasitic treatment practice for pet animals may be useful to eliminate the parasite. Animal handlers must take additional precautions for their personal hygiene and may go for stool examination especially if experiencing any gastrointestinal symptoms. In unhygienic and high *Blastocystis* prevalence areas, sterilization of water is recommended. Currently, the best sterilization method is to boil water as chemical methods of water sterilization have not been extensively studied for *Blastocystis*. Travelers to high prevalence areas should ensure that they consume clean water and cooked food. *Blastocystis* has been found in sewage (Suresh et al. 2005) and there is growing evidence for waterborne transmissions (Leelayoova et al. 2004; Hakim et al. 2007; Karanis et al. 2007) which makes it necessary to develop preventive measures to ensure water sanitation.

1.13 OBJECTIVES OF THE PRESENT STUDY

The pathogenesis of *Blastocystis* is, to date, controversial and inconclusive. Whether it is a pathogenic or a commensal intestinal parasite; or it has potential to be a pathogen in particular circumstances is currently debatable. Several epidemiological and clinical case reports have associated *Blastocystis* with a variety of gastrointestinal symptoms but only a small number of controlled experimental studies have been carried out to investigate the pathogenesis of this organism. Even though, there is no suitable animal model available, there is scarcity of *in vitro* studies that have addressed this issue.

In vitro models are used extensively in biomedical research and they have contributed tremendously to the understanding of basic cellular processes and interactions. The study of host-pathogen interactions in intestine presents significant methodological problems and *in vitro* model systems were developed in an attempt to circumvent these limitations. *In vitro* intestinal cell culture models simulate the human physiology of intestinal tract and represent a highly reproducible experimental system. White (2001) stated that *in vitro* models mimic *in vivo* drug clearance profiles and represent a significant tool for carrying out pharmacodynamic studies in a more cost-effective, timely, and easily controlled manner. Intestinal *in vitro* cell culture methods were suggested to be easy and elegant ways to study cell interactions, cellular behavior, and cell signaling pathways (Simon-Assmann et al. 2007). Many intestinal epithelial cell lines including Caco-2, T84, HT-29 and HCT-8 have been extensively used in various *in vitro* models for studies involving host pathogen interactions, infection, immunology,

pharmacodynamics of drugs and cell biology. Cell lines (transformed cells) have the advantage over *in vivo* models as they allow the study of cell types in isolation which helps in understanding the responses of a particular cell type. Cell lines are easy to maintain and can be passaged many times without loss in their characteristics. As a result of their differentiation characteristics, many of these cell lines have been exploited extensively as substitutes for normal intestinal cells. Even though all variables seen *in vivo* can not be incorporated in *in vitro* experiments, they do provide valuable information (White 2001). The key benefit involving *in vitro* models is that single variable experiments can be performed under highly controlled conditions.

The authors of the most extensive review on *Blastocystis* (Stenzel and Boreham 1996) stated in their conclusion "Our current knowledge of *Blastocystis* hominis and the putative disease it causes is insufficient to determine the significance of the parasite in humans". Because of the limited information on *Blastocystis* virulence factors, it was investigated in the current work if *Blastocystis* possesses any protease activity as proteases are considered to be virulence factors in many protozoan parasites. Many of these proteases in particular cysteine type are likely to play crucial roles in host–parasite interactions (Sajid and McKerrow 2002; Mottram et al. 2004). Interactions of *Blastocystis* proteases with human secretory immunoglobulin A and intestinal epithelial cell lines were further investigated. *Blastocystis* infections are associated with gastrointestinal symptoms but nothing is known about its pathophysiology. An association between disruption of intestinal barrier function and diarrhea has been described in other diseases (Madara 1988; Madara 1990) and thus in present study; the

effects of the *Blastocystis* exposure on intestinal epithelial monolayer permeability and transepithelial resistance were studied.

In this study, using *in vitro* model systems, aim was to investigate various aspects of *Blastocystis* pathogenicity with particular focus on interactions with the intestinal epithelium. For most experiments, parasitic lysates were used. To ensure that damaging effects on intestinal epithelial cells are *Blastocystis* specific, live *Blastocystis* cells were also used in all experiments.

Specific objectives of this study were:-

1. To study the protease activity of *Blastocystis*.
2. To investigate if *Blastocystis* can degrade human secretory immunoglobulin A (S-IgA).
3. To investigate if *Blastocystis* can induce apoptosis in intestinal epithelial cells.
4. To study the effects of *Blastocystis* on intestinal epithelial tight junctions and barrier function.
5. To investigate if *Blastocystis* is capable of inducing proinflammatory cytokine interleukin-8 response from intestinal epithelial cells and to study parasitic factors and molecular events involved in this phenomenon.

CHAPTER 2:

PROTEASE ACTIVITY OF *BLASTOCYSTIS*

2.1 INTRODUCTION

In recent years, there has been an increased interest in the proteases of pathogens. Proteases or peptide hydrolases catalyze the cleavage of peptide bonds in the proteins or peptides. Proteases from many protozoan and other pathogens have been described and their importance in host-pathogen interactions is well understood in a number of diseases (McKerrow et al. 1993; Sajid and McKerrow 2002). In protozoan parasites, proteases have been reported to have important roles in host cell invasion and egress, encystation, excystation, catabolism of host proteins, differentiation, cell cycle progression, cytoadherence, and both stimulation and evasion of host immune responses (Reviewed in Klemba and Goldberg 2002). Serine and cysteine proteases depend on the nucleophilic character of the serine and cysteine side chains respectively, and directly attack the peptide bond to form a transient covalent enzyme-substrate intermediate (Klemba and Goldberg 2002). Aspartic proteases and metalloproteases use a water molecule for nucleophilic attack and do not form a covalent intermediate. Proteases, in particular cysteine (thiol or sulfhydryl proteases), are known to have cytopathic effects and they were reported to function as virulence factors of many protozoan parasites (Sajid and McKerrow 2002).

High protease activity was reported in *Trichomonas vaginalis* and it was found that this activity is mainly due to parasitic cysteine proteases (Coombs and North 1983). Protease activity of *T. vaginalis* was suggested to contribute to its adherence to host cells and protease inhibitors were shown to prevent this interaction (Arroyo and Alderete

1989). It was also reported that these proteases in *T. vaginalis* are located in lysosomes (Lockwood et al. 1988). Parasitic proteases differ in their pH dependence and sensitivities to different protease inhibitors. Knowledge of pH dependence of proteases can aid us in understanding their actual activity in *in vivo* physiological and pathological conditions.

Numerous other protozoans were reported to have cysteine proteases as their major proteases. *Entamoeba histolytica*, the cause of amoebiasis, has cysteine proteases that can damage host tissue and helps in parasite invasion and cytotoxicity was prevented with the use of protease inhibitors (McGowan et al. 1982; Gadasi and Kobiler 1983). High protease activity, in particular of the cysteine type, was also reported in *G. lamblia* and Lindmark (1988) showed that the proteases are localized in lysosome-like subcellular structures. In summary, numerous pathogenic protozoan parasites exhibit significant protease activity and these proteases may have important roles in parasite survival, multiplication and pathogenicity.

Blastocystis is a protozoan parasite found in intestinal tract of humans and many other animals. Pathogenicity aspects of *Blastocystis* are thus far ambiguous and there are many conflicting reports that either implicate or exonerate the parasite as a cause of gastro-intestinal disease. Numerous studies have also suggested zoonotic potential of *Blastocystis* (Yoshikawa et al. 2004a; Noel et al. 2005). Despite considerable progress in understanding of the morphology and epidemiology of *Blastocystis*, virulence factors of this parasite remain unidentified.

In this chapter, the protease activity and specificity of *Blastocystis* were investigated. For the first time, the protease activity of *Blastocystis ratti* WR1 and *Blastocystis hominis* B is reported and it was observed that this parasite possesses high protease activity, in particular of the cysteine protease type. It is also demonstrated that central vacuole of *Blastocystis*, an organelle of unknown function, contains cysteine proteases.

2.2 MATERIALS AND METHODS

2.2.1 Parasite culture

For this study, two isolates of *Blastocystis*; *Blastocystis ratti* WR1 and *Blastocystis hominis* B were used. Parasites were cultured in pre-reduced Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% inactivated horse serum and incubated anaerobically at 37^0C in an ANAEROJAR (Oxoid, UK) as described previously (Ho et al. 1993). After washing and dilution in PBS, counting of the parasites was performed using a haemocytometer. Viability of 5 days old parasites was microscopically determined and healthy cultures with >95% viability were used for all experiments.

2.2.2 Preparation of lysate

For the analysis of protease activity by azocasein assay, parasites were washed two times in PBS (pH 7.4) and lysates were prepared by 3 freeze-thaw cycles in liquid nitrogen and 37^0C water bath respectively. Lysates of 4×10^6 parasites were used for each sample, except in studies with concentration dependent activity where different amounts of parasites were used.

2.2.3 Azocasein assay for the measurement of protease activity of *Blastocystis*

Protease activity of *Blastocystis* was measured by the azocasein assay as previously described (Scholze and Tannich 1994) with a few minor modifications. To activate proteases, parasitic lysates were preincubated with 2mM dithriothreitol (DTT) (Sigma) at 37^0C for 10 min. 100µl of 5mg/ml azocasein (Sigma) solution (in PBS, pH 7.4) and incubated with 100µl of lysate at 37^0C for 1h. To stop reaction, 300µl of 10 % trichloroacetic acid was added and samples were incubated for 30 min on ice. To remove undigested azocasein, samples were centrifuged at (5000×g, 5 min). The resulting supernatant was transferred to a new clean tube containing 500µl of NaOH (525mM). Finally, absorbance of the sample was measured at 442nm on a spectrophotometer (Tecan Magellan). For some controls, to see if azocasein digestion is due to *Blastocystis* proteases, parasitic lysates were boiled at 90^0C for 15 min to inactivate proteases. For experimental positive controls, 100 µl trypsin (2.5mg/ml) was used.

2.2.4 Protease inhibition

To determine the types of proteases that are present in both WR1 and B isolates of *Blastocystis*, effects of protease inhibitors on the protease activity of *Blastocystis* were studied by adding one of the following inhibitors during 1h incubation of parasitic lysate with azocasein: E-64, iodoacetamide, EDTA, PMSF (all 1mM); and pepstatin A (100µg/ml). All inhibitors were purchased from Sigma except pepstatin A (CHEMICON), iodoacetamide (M P Biomedicals, LLC) and PMSF (BDH) (Table 2.1).

Protease inhibitor	Specificity	Concentration used
E-64	Cysteine protease inhibitor	1mM
Iodoacetamide	Cysteine protease inhibitor	1mM
EDTA	Metallo-protease inhibitor	1mM
PMSF	Serine protease inhibitor	1mM
Pepstatin A	Aspartic protease inhibitor	100µg/ml

Table 2.1 Different protease inhibitors used in this study, their specificities and concentrations.

2.2.5 Determination of optimum pH for protease activity

To determine the pH dependence of the protease activity of both isolates of *Blastocystis* in azocasein assays, 1 h incubation with parasitic lysate was performed in buffers of different pH containing 1mM DTT. The pH of these buffers ranged from 3.0 to 8.5. The following buffers were used: 0.1M Glycine/HCl (for pH 3.0), 0.1M sodium acetate/acetic acid (for pH 4.0 and 5.5), 0.1M sodium phosphate (for pH 7.0 and 7.6) and 0.1M Tris-HCl (for pH 8.5).

2.2.6 Secretory product extraction

To extract secretory components of *Blastocystis*, 5 day old parasites were washed twice in PBS (pH 7.4) and re-suspended in incubation buffer (PBS with 10 mM HEPES, and 0.05% L-ascorbic acid, pH 6.2) and incubated at 37°C for 3h, as reported previously for *Trichomona*s (Sommer et al. 2005). After incubation, parasites were examined microscopically for their viability and found to be viable and healthy. After incubation, the suspension was centrifuged at a low speed (2,000rpm for 10min, 4^0C) so that no leakage of parasitic products occurs. To remove any debris, supernatant was again centrifuged at high speed (6,000rpm for 10min, 4^0C) and resulting supernatant was filtered using 0.22µm filters and kept at -80°C for further analysis.

2.2.7 Cellular localization of cysteine proteases

After discovering that *Blastocystis* possesses high cysteine protease activity, cellular localization of cysteine proteases of living *Blastocystis* was determined as described previously (Scholze and Tannich 1994). Briefly, cells were incubated at room temperature in phosphate-buffered saline (pH 7.0) containing 5mM of the substrate Arg-Arg-4-methoxy-2-naphtylamide and 2.5mM 5-nitro-2-salicylaldehyde. After 2h, cells were spun down and washed 4 times in PBS at 2500rpm. Smears were made on a glass slide and viewed under a fluorescence microscope using 360-430nm excitation and 550-600nm emission. For inhibition of cysteine proteases, parasites were preincubated for 1h in PBS containing iodoacetamide (50µM).

2.3 RESULTS

2.3.1 Protease activity of *Blastocystis*

Azocasein assay was employed for the determination of parasitic protease activity. Azocasein consists of casein conjugated to an azo-dye and serves as a substrate for proteolytic enzymes. Degradation of casein liberates free azo-dye into the supernatant that can be quantitatively analyzed. Fig. 2.1 shows the protease activity of *B. ratti* WR1 represented in azocasein units. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD units per h. Lysates of *B. ratti* WR1 showed significant protease activity (35.3 ± 3.2; $P < 0.01$) in comparison to control (0.8 ± 0.1). A significant inhibition of protease activity of WR1 lysate was observed with the use of cysteine protease inhibitors iodoacetamide (4.1 ± 0.8; $P < 0.01$) and E-64 (9.8 ± 2.9; $P < 0.01$) (Fig. 2.1). Partial inhibition was noticed with aspartic protease inhibitor pepstatin A (25.1 ± 1.3; $P = 0.058$) and insignificant inhibition was seen with metallo-protease inhibitor EDTA and serine inhibitor PMSF. These findings suggested that protease activity of *B. ratti* WR1 is mainly due to cysteine proteases of the parasite.

Figure 2.2 represents protease activity of *B. hominis* B and it shows that lysate has significantly high protease activity (45.7 ± 2.6; $P < 0.01$) in comparison to control (0.7 ± 0.2). A significant inhibition of protease activity of *B. hominis* B lysate was observed with the use of cysteine protease inhibitors iodoacetamide (9.1 ± 2.0; $P < 0.01$) and E-64 (13.1 ± 1.3; $P < 0.01$) (Fig. 2.2). Serine protease inhibitor PMSF also inhibited protease

activity (37.8 ± 2.2; *P* = 0.03 versus control). Metallo-inhibitor EDTA and aspartic inhibitor pepstatin A showed no inhibition. The results suggest that *B. hominis* B proteases mainly consist of cysteine and serine proteases.

The effect of parasite concentration on the rate of azocasein hydrolysis was also measured. As expected, results for both isolates show that azocasein degradation is proportional to parasite concentration (Fig. 2.3 and 2.4).

2.3.2 Optimum pH for *Blastocystis* protease activity

To determine optimum pH for protease activity in azocasein assay, incubation with parasitic lysates was performed in 0.1M Glycine/HCl (pH 3.0), 0.1M sodium acetate/acetic acid (pH 4.0 and 5.5), 0.1M sodium phosphate (pH 7.0) and 0.1M Tris-HCl (pH 8.5). Protease activity of both isolates *B. ratti* WR1 and *B. hominis* B showed pH dependence and it was highest at pH 7 and lowest protease activity was observed at acidic 3 pH (Fig. 2.5 A and 2.5 B).

2.3.3 Protease activity of *Blastocystis* secretory products

To investigate if *Blastocystis* secretory products have similar protease activity as lysates, *Blastocystis* secretory products were extracted by incubating live parasites in incubation buffer. Secretory products of *B. ratti* WR1 showed significant protease activity (17.1 ± 1.8; $P < 0.01$) in comparison to control (1.3 ± 0.4) (Fig. 2.6). Secretory

products of *B. hominis* B also showed significantly high protease activity (27.2 ± 2.9; $P < 0.01$) in comparison to control (1.7 ± 0.7) (Fig. 2.6).

2.3.4 Cysteine proteases are confined to the central vacuole

To determine cellular localization of cysteine proteases, live parasites were incubated in PBS containing substrate Arg-Arg-4-methoxy-2-naphtylamide and 5-nitro-2-salicylaldehyde. 5-nitro-2-salicylaldehyde forms an insoluble adduct with the proteolytically released napthylamine derivative. Fluorescent microscopy results for *B. ratti* WR1 (Fig. 2.7) showed that fluorescence is clearly localized within the central vacuole of the parasite. The central vacuole localization of cysteine proteases is evident in the exceptionally large *Blastocystis* cells which are surrounded by smaller cells (Fig 2.7, second column). *Blastocystis* is a pleomorphic protozoan and sizes can significantly vary (up to 50-100 times larger parasites can be observed in the same culture). In figure 2.8, fluorescence is also limited to central vacuole of *B. hominis* B suggesting that cysteine proteases are mainly contained in the central vacuole of the parasite. For both isolates, almost no fluorescence was observed when parasites were pretreated with specific cysteine protease inhibitor iodoacetamide.

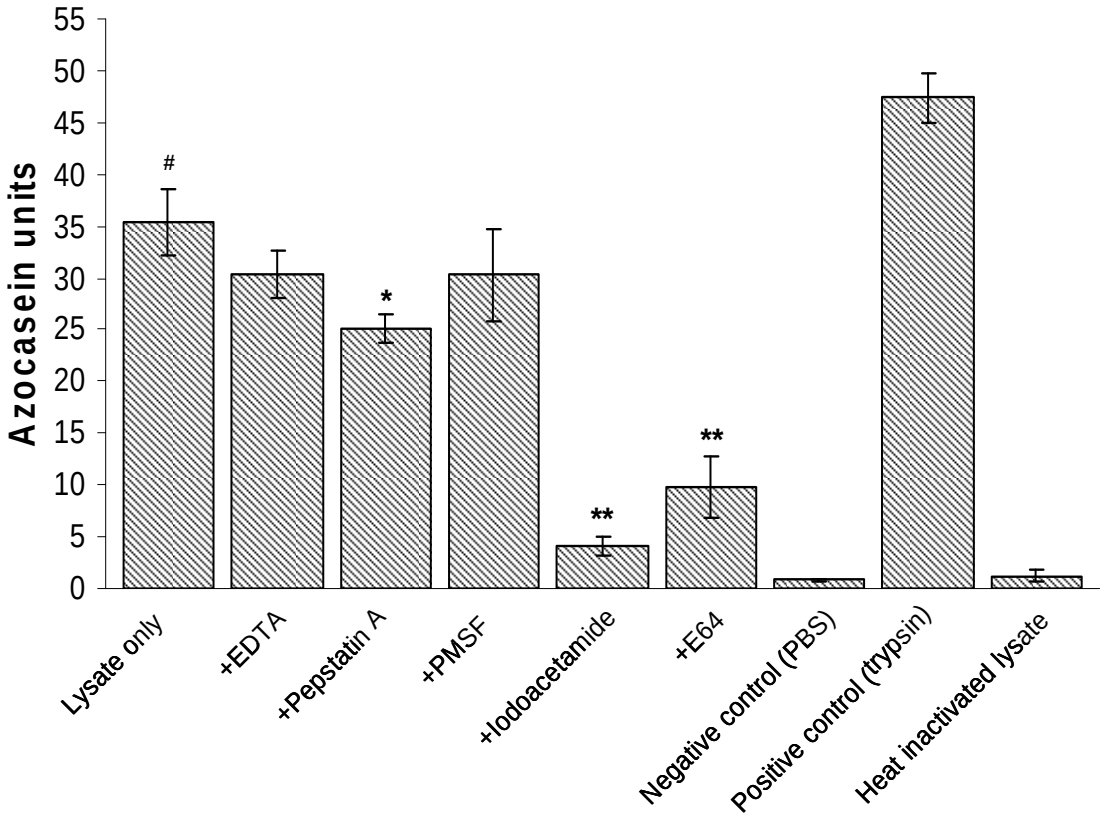

Fig. 2.1 Protease activity of *B. ratti* WR1 and effect of inhibitors. Protease activity was determined with azocasein as a substrate. Lysates from 4×10^6 parasites were used for each sample. The assay was performed with/without protease inhibitors as described in Material and Methods. A significant inhibition of protease activity can be noticed with cysteine protease inhibitors (iodoacetamide and E-64) and less significant inhibition is seen with aspartic inhibitor pepstatin A. Metallo-protease inhibitor EDTA and serine inhibitor PMSF show no inhibition. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD units/h. Values are means ± SD (n = 3). $^{\#}P <$ 0.01 versus negative control, * $P = 0.058$ versus lysate, ** $P < 0.01$ versus lysate.

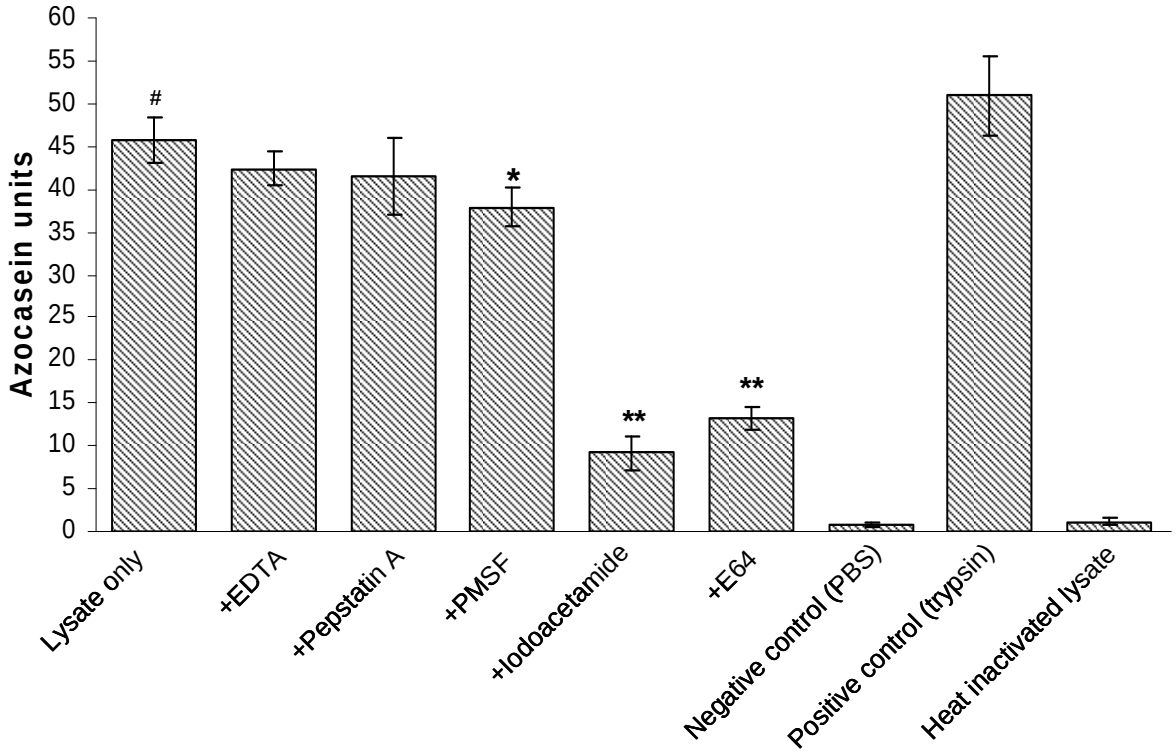

Fig. 2.2 Protease activity of *B. hominis* B and effect of inhibitors. Protease activity was determined with azocasein as a substrate. Lysates from 4×10^6 parasites were used for each sample. The assay was performed with/without protease inhibitors as described in Material and Methods. A significant inhibition of protease activity can be noticed with cysteine protease inhibitors (iodoacetamide and E-64). Serine protease inhibitor PMSF also inhibited protease activity. Metallo-inhibitor EDTA and aspartic inhibitor pepstatin A show no inhibition. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD units/h. Values are means ± SD (n = 3). $^{\#}P < 0.01$ versus negative control, * $P = 0.03$ versus lysate, ** $P < 0.01$ versus lysate.

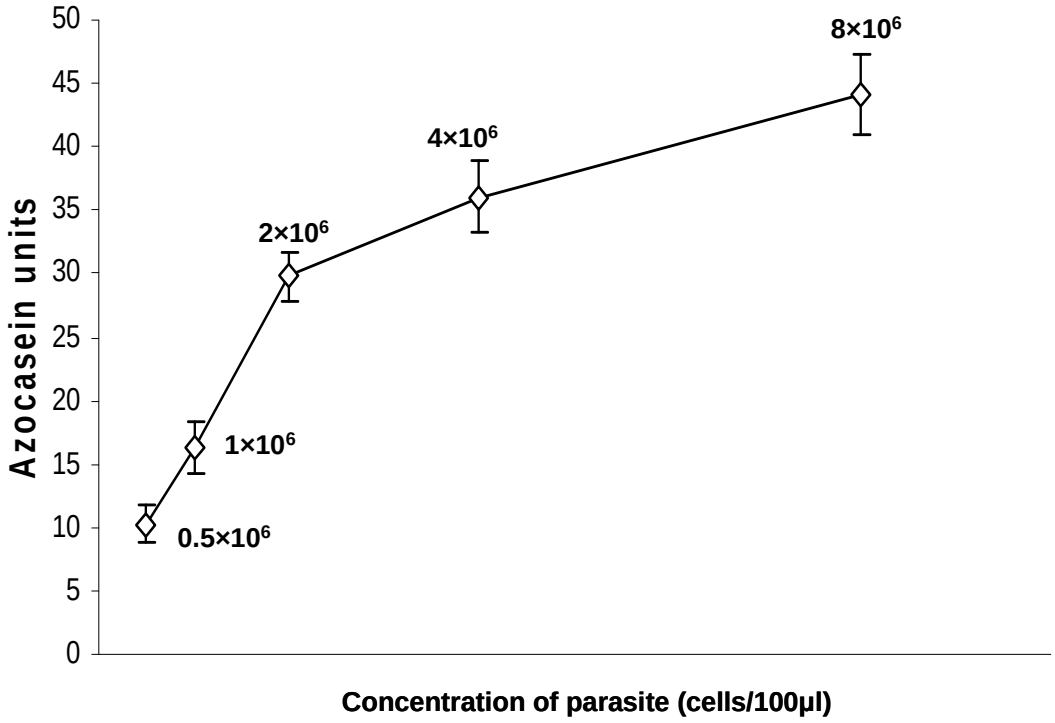

Fig. 2.3 Histogram showing the effect of parasite concentration on the rate of azocasein hydrolysis by *B. ratti* WR1. The azocasein assay was performed as described in Material and Methods. Lysates from varying concentration of *Blastocystis ratti* WR1 were used for azocasein assay. A dose dependent increase in protease activity can be observed. Values are averages from three independent experiments with duplicate samples in each. Standard deviations are indicated with error bars.

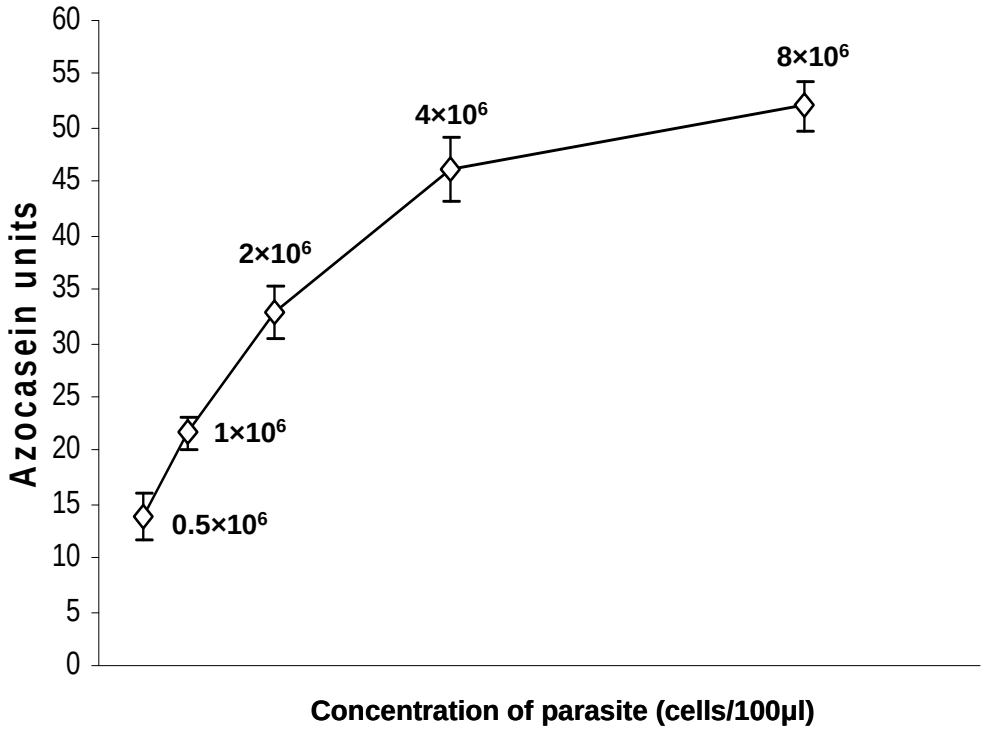

Fig. 2.4 Histogram showing the effect of parasite concentration on the rate of azocasein hydrolysis by *B. hominis* B. The azocasein assay was performed as described in Material and Methods. Lysates from varying concentration of *Blastocystis hominis* B were used for azocasein assay. A dose dependent increase in protease activity can be observed. Values are averages from three independent experiments with duplicate samples in each. Standard deviations are indicated with error bars.

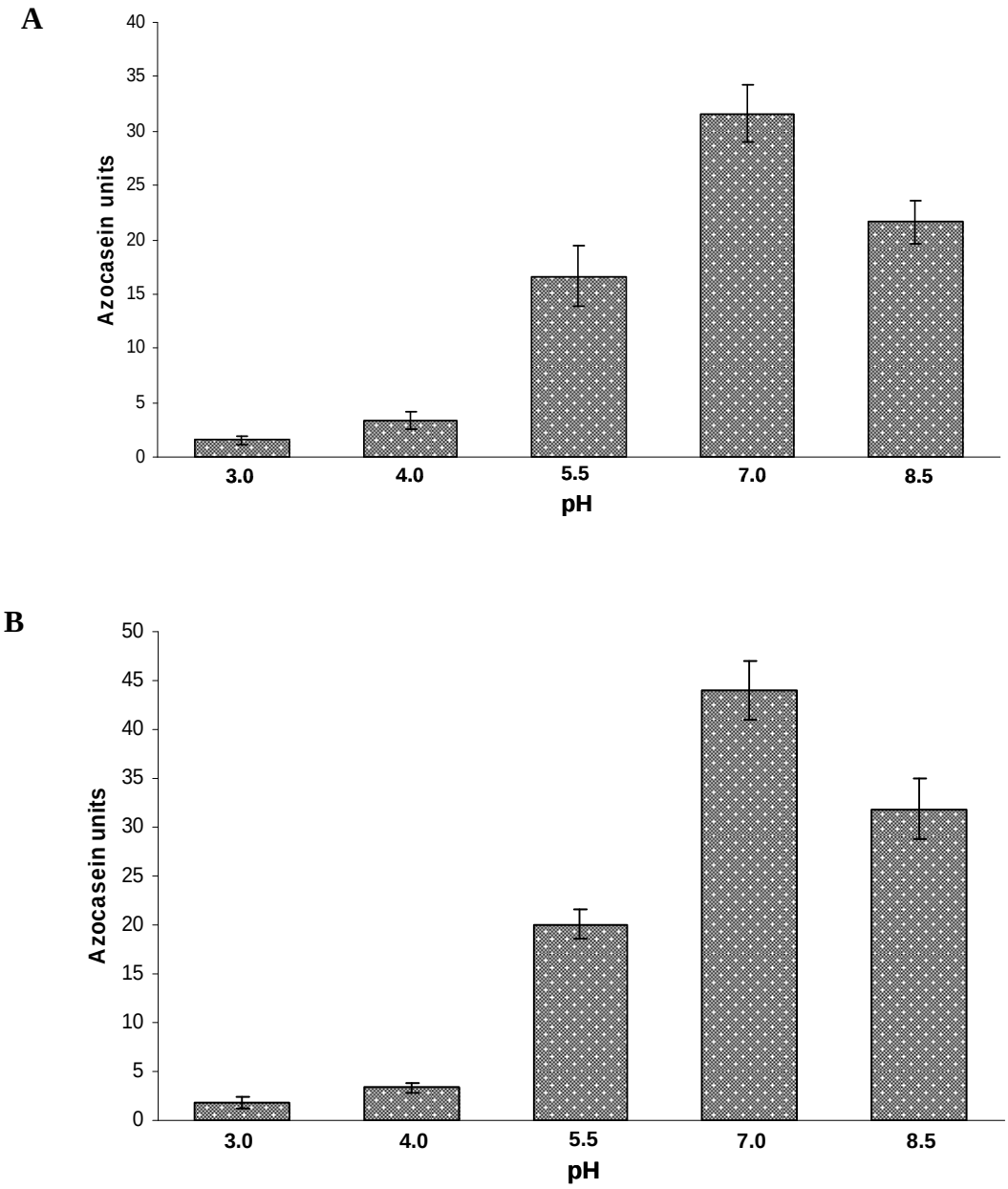

Fig. 2.5 Protease activity of *B. ratti* WR1 (A) and *B. hominis* B (B) at different pH. Azocasein was used as a substrate and incubation with parasitic lysates was performed in 0.1M Glycine/HCl (pH 3.0), 0.1M sodium acetate/acetic acid (pH 4.0 and 5.5), 0.1M sodium phosphate (pH 7.0) and 0.1M Tris-HCl (pH 8.5). For both isolates, highest protease activity was observed at 7 pH whereas lowest activity was at acidic 3 pH. Values are averages from three independent experiments with duplicate samples in each. Standard deviations are indicated with error bars. All values are significantly different ($P < 0.05$) when compared with each other.

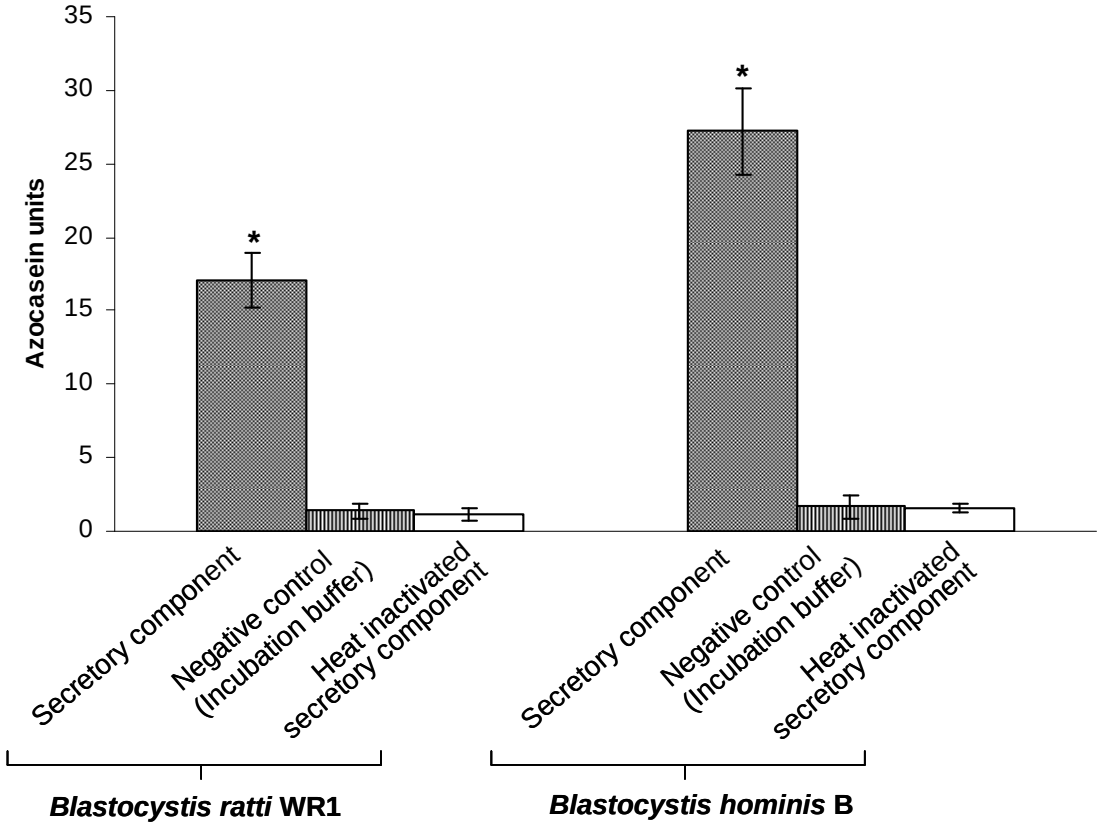

Fig. 2.6 Protease activity of *B. ratti* WR1 and *B. hominis* B secretory products. Parasites were incubated for 3 hours in incubation buffer to extract secretory fraction as described in Materials and Methods. Protease activity of secretory product was determined with azocasein as a substrate. Secretory products of both isolates show significant protease activity. Secretory fraction of *B. hominis* B shows comparatively higher protease activity than that of *B. ratti* WR1. Values are means ± SD (n = 3). $^{*}P <$ 0.01 versus negative control (i.e. incubation buffer) .

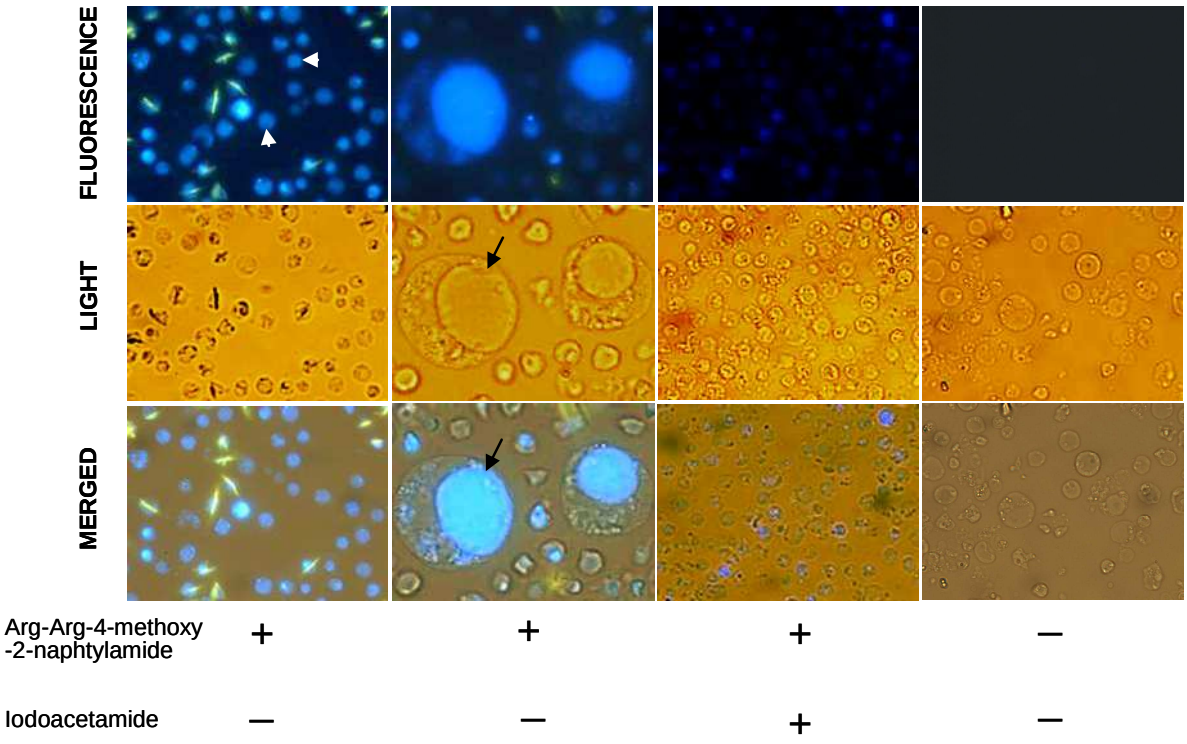

Fig. 2.7 Representative fluorescence, light, and merged micrographs show activity and localization of cysteine proteases in live parasites of *B. ratti* WR1. Live parasites were incubated in PBS containing substrate Arg-Arg-4-methoxy-2-naphtylamide and 5-nitro-2-salicylaldehyde. 5-nitro-2-salicylaldehyde forms an insoluble adduct with the proteolytically released napthylamine derivative. Blue color fluorescence is an indication of *in situ* protease activity (arrowhead, first column). Higher magnification (400×) picture in second column shows unusually large *Blastocystis* cells surrounded by smaller cells. It can be noticed that fluorescence is limited only to the central vacuole (arrow) of the parasite. Treatment of parasites with cysteine inhibitor iodoacetamide resulted in diminished fluorescence (third column). Control parasites without cysteine protease substrate showed no fluorescence (fourth column). (Magnification in first, third and fourth column is 100×).

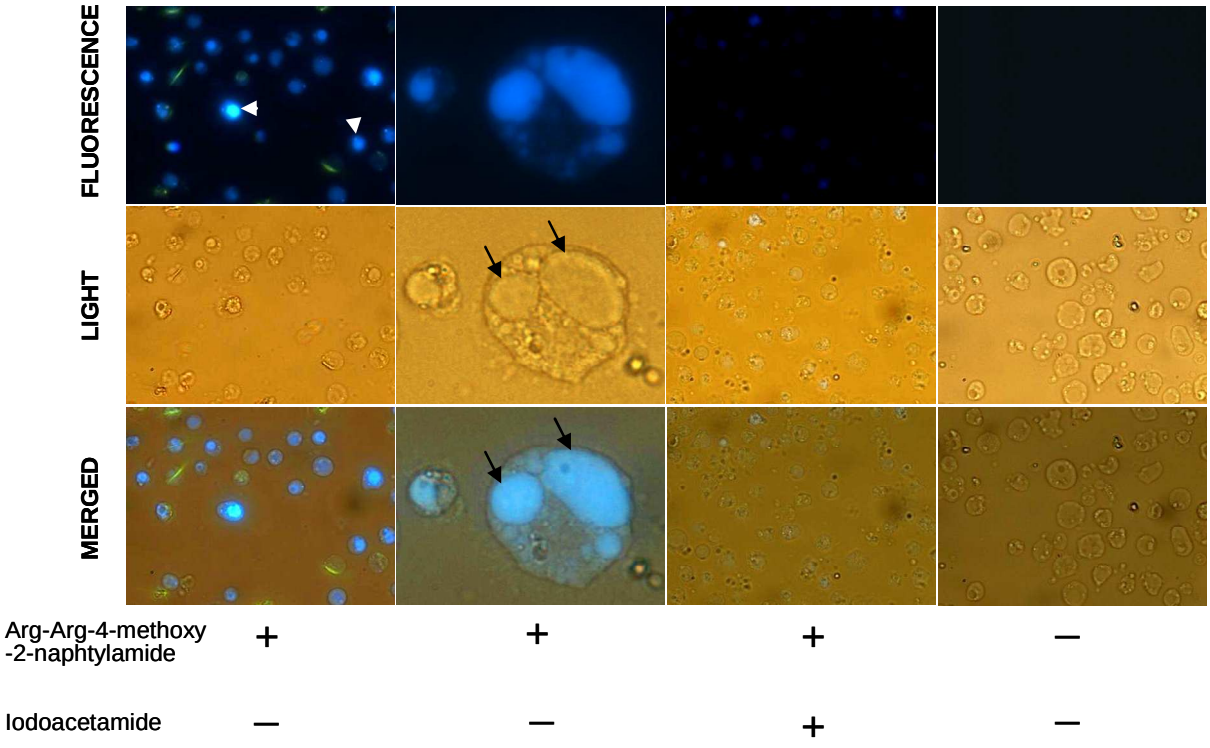

Fig. 2.8 Representative fluorescence, light, and merged micrographs show activity and localization of cysteine proteases in live parasites of *B. hominis* B. Live parasites were incubated in PBS containing substrate Arg-Arg-4-methoxy-2-naphtylamide and 5-nitro-2-salicylaldehyde. 5-nitro-2-salicylaldehyde forms an insoluble adduct with the proteolytically released napthylamine derivative. Blue color fluorescence is an indication of *in situ* protease activity (arrowhead, first column). Higher magnification (400×) picture in second column shows unusually large *Blastocystis* cell. It can be noticed that fluorescence is limited only to the central vacuoles (arrow) of the parasite. Treatment of parasites with cysteine inhibitor iodoacetamide resulted in diminished fluorescence (third column). Control parasites without cysteine protease substrate showed no fluorescence (fourth column). (Magnification in first, third and fourth column is 100×).

2.4 DISCUSSION

Results from the protease profile study showed that *B. ratti* WR1 and *B. hominis* B possess high protease activity. Inhibition of protease activity of *Blastocystis* showed that these proteases consist mainly of cysteine proteases. The active-site thiol group of cysteine proteases can function only under reduced conditions (McKerrow et al. 1993) and because *Blastocystis* is an anaerobic enteric protozoan, these cysteine proteases may have important roles in its life cycle. Most other pathogenic protozoans including *Entamoeba*, *Giardia*, *Trichochmonas*, *Leismania*, *Trypanosoma*, and *Plasmodium* are known to possess cysteine proteases (reviewed in North 1982; McKerrow et al. 1993) that have been shown to be important for the development, differentiation, and pathogenicity of these parasites. These cysteine proteases can be promising chemotherapeutic or vaccine targets for many parasites (Sajid and McKerrow 2002). Based on protease activity, it was observed that *B. ratti* WR1 showed less cysteine protease activity than that for *B. hominis* B. High protease activity has been reported in amebic clones of high virulence (Espinosa-Cantellano and Martinez-Palomo 2000) and it would be important to study if high protease activity is associated with virulence in *Blastocystis*.

Furthermore, in a parallel investigation, gelatin-SDS-PAGE analysis was performed and it was found that majority of *Blastocystis* proteases were of cysteine type, with molecular weight ranging between 20 to 33 kDa (Sio et al. 2006). Interestingly, *Entamoeba histolytica* is also reported to have cysteine proteases within this range (22-29

kDa) and these have been reported to be responsible for the hydrolysis of extracellular matrix proteins (Keene et al. 1986).

Intestinal permeability was found to be increased in patients with *Blastocystis hominis* infections and it was suggested that *Blastocystis* can damage the intestinal wall (Dagci 2002). Other reports describe adherence of *Blastocystis* and colonic ulcerations on the intestinal mucosa (Al-Tawil et al. 1994). Findings suggest that cysteine proteases activity of *Blastocystis* may possibly contribute to its pathogenicity. Proteolytic activity of *B. ratti* WR1 and *B. hominis* B proteases was found to be highest at neutral pH. This is in agreement with the pH of human large intestine where *Blastocystis* colonizes. This finding suggests that *Blastocystis* proteases can have maximum *in vivo* activity at neutral pH in the large intestine of humans. Significantly, it was shown that *Blastocystis* secretory products also possess protease activity. Proteases can be found inside the protozoan cell, on the plasma membrane or in the parasitic secretions (Que and Reed 2000). *Entamoeba histolytica* has been reported to have cysteine proteases that were present in parasitic secretions and suggested to play role in its pathogenesis (Ocadiz et al. 2005). *Blastocystis* is suggested to be a non-invasive organism and finding that it has secretory proteases has relevance to its prospective *in vivo* pathogenesis.

Inhibitors of cysteine proteases have already been shown to be effective against numerous protozoan parasites *in vitro* (North 1994) suggesting that proteases might be potential targets for specific antiprotozoal drugs. As cysteine proteases are found to be

essential for the life cycle and pathogenicity of many parasites, they might be potential chemotherapeutic or vaccine targets (Sajid and McKerrow 2002).

Blastocystis is a polymorphic organism and its recognized forms are vacuolar, granular, amoeboid, and cyst forms. The vacuolar form, also referred to as vacuolated or central vacuole form, is the most common form observed in axenized cultures. It has a characteristic large central vacuole and a thin rim of peripheral cytoplasm (Tan 2004). Vacuolar forms are spherical and most of the parasite's volume is occupied by the central vacuole. The exact role of central vacuole in *Blastocystis* is not clear to date but it has been suggested that it may act as a storage organelle for the deposition of apoptotic bodies during programmed cell death of the parasite (Nasirudeen et al. 2001). Other reports suggest that the central vacuole may have a role in schizogony-like reproduction (Dunn et al. 1989; Suresh et al. 1994). Results from this study suggest for the first time that central vacuole may also function as a reservoir for cysteine proteases. A number of studies on protozoans including *T. cruzi* have shown that the major protease activity is located in the lysosomes (De Souza 1984; Souto-Padrón et al. 1990). Findings suggest that central vacuole might be an unusually big lysosome in *Blastocystis*. Preliminary results from our laboratory using Lyso tracker staining are in agreement with this suggestion. It was observed that *Blastocystis* central vacuole containing cysteine proteases are located in close proximity with the plasma membrane of parasite. It will be interesting to know how the extracellular trafficking of these proteases occurs.

Interestingly, in adverse conditions like insufficient nutrition or in old cultures, many granular forms are seen which is characterized by granules in central vacuole. There is a need to further elucidate if cysteine proteases have a role in the formation of granules in central vacuoles.

In this chapter, it was demonstrated for the first time, the protease activity of *Blastocystis ratti* WR1 and *Blastocystis hominis* B and found that this parasite possesses high protease activity, in particular of the cysteine protease type. It was also showed that the central vacuole of *Blastocystis* contains cysteine proteases. The pathogenicity of *Blastocystis* is until now unknown and further characterization of *Blastocystis* proteases will lead to a better understanding of the parasite life cycle and its interactions with the host.

CHAPTER 3:

EFFECTS OF *BLASTOCYSTIS* ON HUMAN SECRETORY IMMUNOGLOBULIN A

3.1 INTRODUCTION

Adherence of pathogens to the host surface, for example gastrointestinal mucosal surface, is a significant step in host pathogen interactions. Once the pathogen adheres to the host surface, it is able to initiate certain biological processes like proliferation, toxin secretion and cell invasion that can result in disease.

At the mucosal surfaces of gastrointestinal tract, immunoglobulin A (IgA) is the predominant immune defense against ingested pathogens and their toxins. IgA is vital for limiting mucosal adhesion and colonization by microorganisms and neutralizing a range of microbial toxins (Renegar and Small 1999; Russel et al. 1999; Chintalacharuvu et al. 2003). In the body, IgA is produced in more amounts than all other immunoglobulin classes combined and is mainly found in mucosal secretions in the form of secretory IgA (S-IgA). S-IgA is made of IgA dimer or tetramer covalently joined to the secretory component which is an epithelium-derived polyimmunoglobulin receptor.

Two subclasses of IgA, IgA1 and IgA2, are found in humans. They are different in their structure as IgA2 lacks 16 proline-rich amino acid sequence in its hinge region which makes it more resistant to proteases. The distribution of the two IgA subclasses may vary in different secretions and it was reported that 70% IgA1 and 30% IgA2 is found in the gut (Olbricht et al. 1986). However, some reports mentioned that IgA2 may predominate in the distal parts of gastrointestinal tract and it can be as high as 60% (Kilian et al. 1996).

IgA in secretions has been reported to have many functions including inhibition of adherence and colonization of pathogens by agglutination, trapping antigen in the mucus layer, inhibition of antigen penetration, neutralization of viruses, and neutralization of bacterial toxins and enzymes (Kilian et al. 1996). In general, IgA is quite resistant to most proteolytic enzymes of host and pathogen origin. However, numerous pathogens can evade the IgA immune response and adhere to the host cell surface by secreting IgA proteases. These IgA proteases cleave IgA in the hinge region and produce intact Fab and Fc fragments and thus interfere with IgA mediated inhibition of attachment (Plaut 1983). Although cleaved Fab fragments can still bind to the pathogen surface, loss of Fc fragment prevents most antigen-elimination mechanisms (Kilian et al. 1996).

IgA proteases have been described in protozoan parasites (Kelsall and Ravdin 1993; Provenzano and Alderete 1995), bacteria (Kilian et al. 1980; Plaut 1983) and they are known to mostly cleave secretory IgA at defined site in proline-rich sequence of hinge region. On the other hand, many other parasitic and bacterial proteases do not show similar unique substrate specificity and they may cause extensive degradation of the entire IgA molecule (Kilian et al. 1996) as observed for *Entamoeba histolytica* (Kelsall and Ravidin 1993) IgA proteases have been implicated as virulence factors in the pathogenesis of mucosal infections (Kilian et al. 1996).

Blastocystis is a zoonotic enteric protozoan (Yoshikawa et al. 2004a; Noel et al. 2005) that colonizes the large intestine of humans and many other animals. There are many reports associating *Blastocystis* with gastrointestinal and other clinical symptoms

including recurrent watery diarrhea, mucous diarrhea, constipation, nausea, vomiting, and bloating (Qadri et al. 1989; Doyle et al. 1990; Nimri and Batchoun 1994). Despite its description about a century ago (Alexeiff 1911), little is known about its colonization strategies and association with clinical symptoms.

Findings in previous chapter that *Blastocystis* possesses high levels of protease activity prompted us to investigate if it does possess IgA degrading proteases. These proteases, if present, may shed new light on *Blastocystis* host-pathogen interactions. In this chapter, it is demonstrated for the first time that *Blastocystis* has proteases that can degrade both subclasses of IgA.

3.2 MATERIALS AND METHODS

3.2.1 Culture of *Blastocystis*

Two axenic isolates of *Blastocystis*, *B. ratti* WR1 and *B. hominis* B were used in this study. Parasites were cultured in pre-reduced Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% inactivated horse serum and incubated anaerobically at 37^0C in an ANAEROJAR (Oxoid, UK) as previously described in chapter 2. Parasite viability was microscopically determined and cultures with >95% viable parasites were used for this study. Counting of parasites was performed using a haemocytometer.

T. vaginalis was cultured in Hollander's medium containing 10% foetal-calf serum and used as a positive control.

3.2.2 Preparation of conditioned medium and cell lysates

For the IgA study, four-to five-day old *Blastocystis* cultures were harvested by centrifugation at 400g and the clear culture supernatant was designated as the conditioned medium. The conditioned medium was centrifuged at 2000rpm (10 minutes, 4^0C) to remove any suspended cells. The cell pellet was washed with PBS (pH 7.4), resuspended in 1ml PBS and cell lysates were obtained by repeated freeze-thaw action in liquid nitrogen and 37^0C water bath respectively. The protein content of the cell lysates and

conditioned medium was estimated using a commercially available Bradford protein assay kit (Bio-Rad). Cell lysates of *T. vaginalis* were also prepared as for *Blastocystis*.

3.2.3 Assay of IgA degradation by *Blastocystis*

For IgA degradation assays, 30µg of parasite protein (cell lysate) and conditioned medium were each incubated with 1µg of secretory-IgA (US Biological) for two hours at 37^0C. This secretory-IgA is a natural mixture of both IgA subtypes (IgA1 and IgA2). After incubation, the reaction was stopped by addition of SDS sample buffer containing 2-β-mercaptoethanol and the mixture was boiled for 15 minutes. Approximately 30µg of various protein preparations were electrophoresed in 12% polyacrylamide gels (Laemmli 1970). After SDS-PAGE, the proteins were electroblotted onto a nitrocellulose membrane (Amersham). The membrane was blocked overnight at 4^0C in 5% milk-PBST and detected with 1:50,000 dilution of goat polyclonal anti-IgA heavy chain antibody conjugated to horseradish peroxidase (CHEMICON) for 1h at room temperature. This anti-IgA antibody was human α chain specific and can react with IgA1 and IgA2. Blots were developed on Hyperfilm ECL (Amersham) using chemi-luminescent ECL Plus Detection Reagent (Amersham).

In other experiments, *B. hominis* B was used to determine the effects on subtypes of IgA, 30µg of parasitic lysate or conditioned medium were incubated with IgA1 (2µg) or IgA2 (2µg), for 2h at 37°C. Western blot was performed as described for IgA except

that membrane was probed with 1:25,000 dilutions of a HRP-conjugated goat polyclonal antibody cocktail (mixture of anti-IgA, IgM, IgG; US Biological).

In some experiments, IgA breakdown was quantified by determining Immunoblots using a gel documentation and analysis system (Gel Doc XR, Bio Rad). The % of heavy chain remaining intact after exposure to *Blastocystis* samples is calculated as follows: % remaining heavy chain = (optical density of remaining heavy chain/ optical density of total heavy chain) × 100. In experiments with proteinase inhibitors, degree of inhibition was calculated as follows: % inhibition = [(% remaining heavy chain with inhibitor - % remaining chain without inhibitor)/ (100- % remaining heavy chain without inhibitor)] × 100.

3.2.4 Inhibition of IgA proteinase activity

The proteinase inhibitors used consisted of 25mM EDTA (BDH), 2 mM phenylmethylsulfonyl fluoride (PMSF) (BDH), 2mM idoacetamide (IA) (M P Biomedicals, LLC), and 0.8mM pepstatin A (CHEMICON). Parasite preparations were incubated with the inhibitors for 1h at room temperature before IgA, IgA1 or IgA2 was added and incubated for another 2h. The reaction was stopped with SDS sample buffer containing 2-β-mercaptoethanol. The samples were analyzed by immunoblotting as described before.

3.2.5 Immunoglobulin substrate SDS-PAGE assay

To further study the *Blastocystis* immunoglobulin degrading proteases and their molecular weights, immunoglobulin substrate SDS-PAGE assay was performed. This SDS-PAGE method requires the inclusion of substrate (IgA1 or IgA2) in an acrylamide gel. IgA1 (100 µg/3ml) or IgA2 (100 µg/3ml) were copolymerized into 16% polyacrylamide gel. 2 µg lysate proteins were incubated with sample buffer (without mercaptoethanol) at room temperature for 20mins before loading. Samples were not boiled so that protease activity is retained. Electrophoresis was performed at a constant current of 30mA/gel for 1.5 h. After electrophoresis, each lane of gel was cut into strips for further treatment. To renature proteases, gels were incubated in reducing buffer (12.5% Triton-X, 100mM sodium acetate, 1mM DTT) for 2 hours without inhibitors and with inhibitors EDTA (2mM), Iodoacetamide (30µM), pepstatin A (30µM) and PMSF (2mM) (37 °C, shaking). The gel was stained in 0.025% commassie blue for 2 hours with shaking. Destaining was done in destaining solution I (40% methanol, 7% acetic acid) for 30 minutes and in solution II (10% methanol, 7% acetic acid) for one h.

3.3 RESULTS

3.3.1 *Blastocystis* lysates and conditioned medium degrade secretory IgA

Lysates of *Blastocystis* isolates WR1 and B (Fig. 3.1) were able to degrade human secretory IgA over 2 h at 37^0C. A significant loss of intact heavy chain and increase of IgA fragments was noticed. After exposure to WR1 and B lysate, only 31% and 39% IgA heavy chain remained intact respectively (Table 3.1). Positive control *T. vaginalis* lysate (Fig. 3.1, lane 7) showed degradation of secretory IgA (Provenzano and Alderete 1995). IgA degradation activity for conditioned medium of WR1 (Fig. 3.1, lane 2) was also noticed with 47% intact heavy chain remaining after treatment. Conditioned medium from *B. hominis* isolate B also degraded IgA with 64% heavy chain remaining (Table 3.1). Condition medium shows less extensive IgA degradation pattern than that of lysate which might be due to some proteases that are not secreted but are present only in the lysate.

3.3.2 Effect of protease inhibitors on degradation of secretory IgA

Degradation of secretory IgA by WR1 lysate was inhibited significantly (99%) by aspartic-specific inhibitor pepstatin A (Fig. 3.2, lane 6) and also by cysteine-specific inhibitor IA (57%) (Fig. 3.2, lane 5). With WR1 lysate, negligible inhibition (10%) was noticed by serine-specific inhibitor PMSF and an unexpected increase in degradation of IgA was observed with metallo-specific inhibitor EDTA. In contrast, degradation of IgA by B isolate was inhibited most by IA (79%) (Fig 3.2, lane 10) followed by aspartic-

specific inhibition by pepstatin A (52%) and less significant inhibition was noticed by serine (10%) and metallo-specific inhibitors (23%). The effects of inhibitors on *Blastocystis* proteinase activity are summarized in Table 3.2.

3.3.3 Degradation of IgA1 and IgA2

Human secretory immunoglobulin A has two subtypes, IgA1 and IgA2. IgA1 and IgA2 differ in their structure and distribution in the gut. The effects of *Blastocystis hominis* B exposure on subtypes IgA1 and IgA2 were studied. As showed in the fig. 3.3, exposure of *Blastocystis hominis* B lysate to immunoglobulins resulted in the degradation of the heavy chain of IgA1. In IgA1 exposed to lysates, a big band (~50 kDa) and a small band (~42 kDa) cleaved from heavy chain can be observed. The 42 kDa band is faint which might be due to small amounts of degraded heavy chain fragments of this molecular weight. Exposure of *Blastocystis hominis* B lysate to IgA2 resulted in significant loss of heavy chain and a small cleaved band (~45 kDa) is observed (Fig 3.4). Additionally, the conditioned medium showed proteolytic activity for both subtypes of IgA as well and loss of heavy chain can be seen. No degradation was observed in controls exposed to PBS only. In addition to heavy chain, in results (Fig. 3.3 to Fig. 3.8), light chain of IgA1 and IgA2 (~23 kDa) can be observed. This is because the antibody used for these experiments can also detect light chains of IgA1 and IgA2. Differences in the band intensity of light chains was also observed which may be due to some cleaved fragments of the heavy chain the might have stacked at the same region of the light chain (Fig. 3.3 to Fig. 3.8).

A concentration-dependent assay was performed to study the effect of varying amount of *Blastocystis* lysate on IgA1 and IgA2 degradation. As the dose of lysate increased, degradation of IgA1 and IgA2 gradually increased as shown by loss of band intensity of the heavy chain (Fig. 3.5 and 3.6). Exposure to 50 µg of *Blastocystis* lysate resulted in complete loss of heavy chain in both IgA1 and IgA2.

3.3.4 Effect of protease inhibitors on degradation of IgA1 and IgA2

Various protease inhibitors were used to investigate types of specific proteases that are responsible for degradation of IgA1 and IgA2. It was observed that cysteine proteinase inhibitor iodoacetamide completely inhibited the degradation of both IgA1 and IgA2 and no cleaved fragment of heavy chain can be seen (Fig. 3.7 and 3.8). Some inhibition effect was also observed with aspartic-specific inhibitor pepstatin A. However, the inhibition effect was not as pronounced as Iodoacetamide, as small cleaved fragments of heavy chain can still be seen. Metallo-specific inhibitor EDTA and serine-specific inhibitor PMSF did not show any significant inhibition, as loss of heavy chain and cleaved fragments were of the same pattern as of lysate. A slight increase in degradation of IgA2 heavy chain is observed with EDTA (Fig. 3.8) which is consistent with observation previously (Fig. 3.2).

3.3.5 Immunoglobulin substrate SDS PAGE assay

An immunoglobulin substrate SDS-PAGE assay was performed to further study *Blastocystis* immunoglobulin degrading proteases and their molecular weights. This SDS-PAGE method requires the inclusion of substrate (IgA1 or IgA2) in an acrylamide gel. Immunoglobulin substrate is digested by proteases after electrophoresis and clear bands can be observed against a dark background after Coomassie Brilliant blue staining.

Three protease bands were similarly observed in both IgA1 and IgA2 gels, one higher molecular weight band (~70kDa) and two lower molecular weight band (~17kDa and 19kDa) (Fig. 3.9 and 3.10). Both 17kDa and 19kDa proteases were completely inhibited by cysteine proteinase inhibitor iodoacetamide. The 19kDa band was partially blocked by aspartic-specific inhibitor pepstatin A. The 70kDa protease was inhibited by serine specific inhibitor PMSF in IgA1 and IgA2 gels. In addition, two protease bands (~27kDa and 30kDa) were also observed in IgA1 gel only (Fig. 3.9). These 27kDa and 30kDa proteases were completely inhibited by cysteine proteinase inhibitor iodoacetamide and aspartic-specific inhibitor pepstatin A. A smear of lower bands (below 17kDa) was observed that was completely blocked by pepstatin A and iodoacetamide.

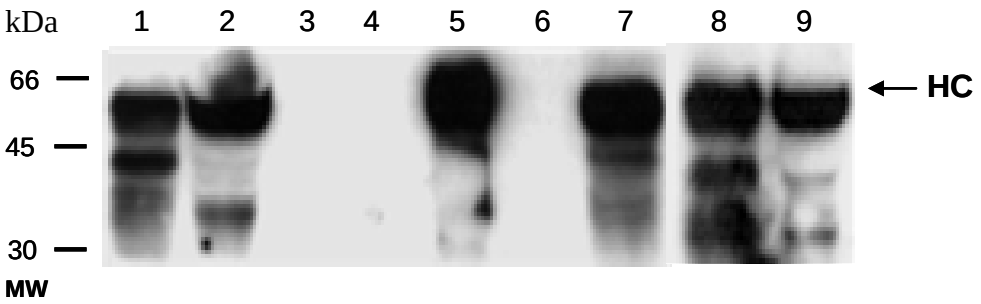

Fig. 3.1 Degradation of human secretory IgA by cell lysate and conditioned medium of *B. ratti* WR1 and *B. hominis* B. 30µg of parasitic protein or conditioned medium were incubated with S-IgA (1µg) for 2h at 37^0C, and subjected to SDS-PAGE and immunoblotting with antibody to human IgA heavy chain. Lane 1, IgA with WR1 lysate showing loss of intact IgA heavy chain into lower molecular-weight immunoreactive fragments. Lane 2, IgA with WR1 conditioned medium showing degradation of IgA. Lane 3, WR1 lysate only (negative control). Lane 4, conditioned medium only (negative control). Lane 5, IgA only showing intact heavy chain. Lane 6, *T. vaginalis* lysate only. Lane 7, IgA with *T. vaginalis* lysate (positive control) showing degradation of IgA. Lane 8, IgA with B lysate. Lane 9, IgA with B conditioned medium. (MW, molecular weight; HC, heavy chain).

Blastocystis isolates	**% of secretory IgA intact heavy chain remaining after incubating with parasitic lysate and conditioned medium**	
	Parasitic lysate	Conditioned medium
B. ratti WR1	31	39
B. hominis B	47	64

Table 3.1. Percentage of secretory IgA intact heavy chain remaining after incubation with the lysates and conditioned medium of *Blastocystis* (Representative results from 3 sets of experiments)

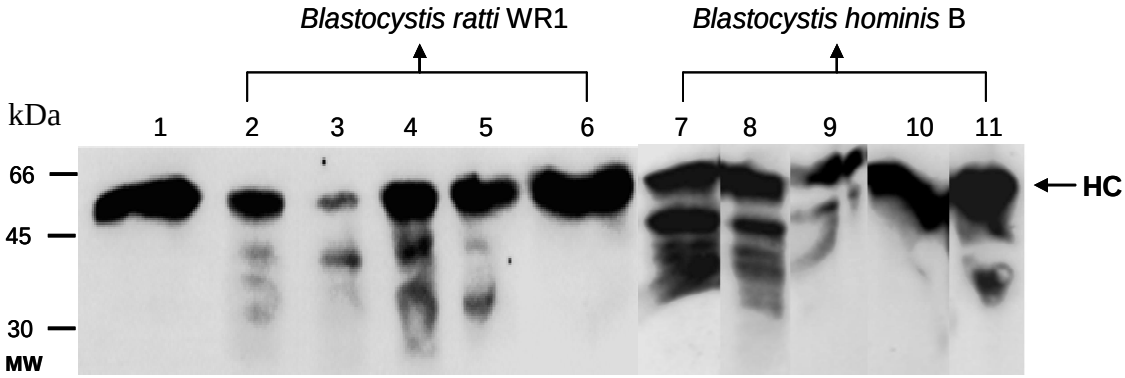

Fig. 3.2 Effect of proteinase inhibitors on IgA degradation by lysates from *B.ratti* isolate WR1 (lane 2-6) **and *B. hominis* isolate B** (lane 7-11). Parasitic lysates were incubated with inhibitors for 1h at 37^0C before IgA was added. Lane 1, IgA alone. Lane 2 and 7, IgA with lysates of *B.ratti* isolate WR1 and *B. hominis* isolate B respectively showing degradation of IgA. 25 mM EDTA (lane 3 & 8), 2 mM PMSF (lane 4 & 9), 2 mM Iodoacetamide (lane 5 & 10), and 0.8 mM pepstatin A (lane 6 & 11) treatment showing varying degrees of inhibition of IgA degradation. (MW, molecular weight; HC, heavy chain).

Blastocystis isolates	**% inhibition with different proteinase inhibitors**			
	EDTA	PMSF	IA	Pepstatin A
B. ratti WR1	-9	10	57	99
B. hominis B	23	10	79	52

Table 3.2 Degrees of inhibition by different proteinase inhibitors on IgA degradation by lysates of *Blastocystis*. (Representative results from 3 sets of experiments)

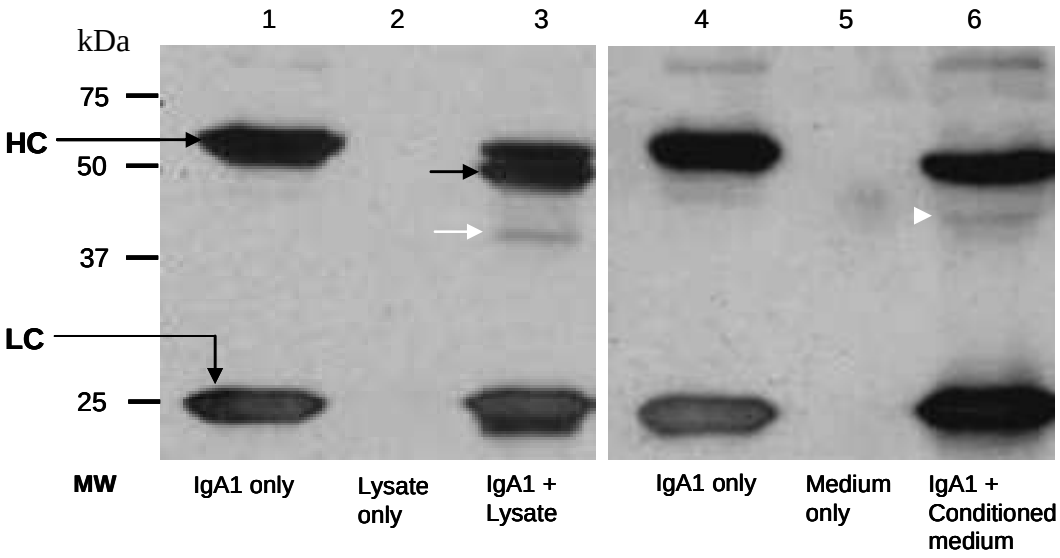

Fig. 3.3 Representative results showing degradation of human IgA1 by *B. hominis* B parasitic lysates and conditioned medium. 30µg of parasitic protein or conditioned medium were incubated with IgA1 (2µg) for 2h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Incubation with parasitic lysate resulted in the degradation of the heavy chain of IgA1. In IgA1 exposed to lysates significant loss of heavy chain, a big band (~50kDa, black arrow) and a small band (~42kDa, white arrow) cleaved from heavy chain can be observed. Lane 1 and 4 show intact heavy chain of IgA1. Conditioned medium also shows proteolytic activity as loss of heavy chain and a small cleaved band (arrowhead) from heavy chain can be seen. Lane 2 and 5 are the controls to show that only lysate and medium respectively do not cross react with antibody. (MW, molecular weight; HC, heavy chain; LC, light chain).

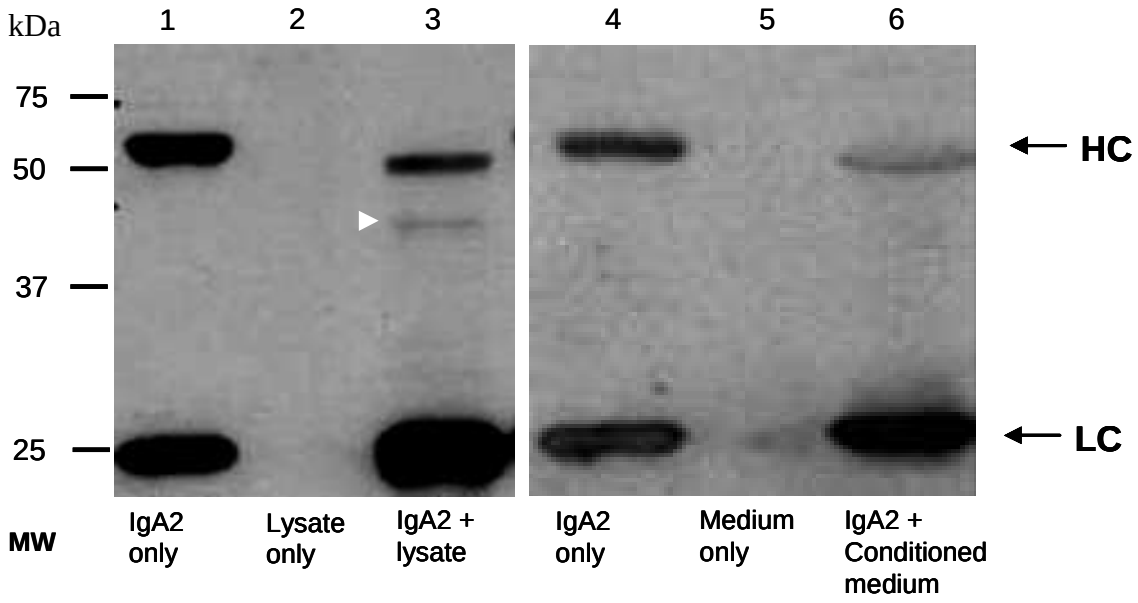

Fig. 3.4 Representative results showing degradation of human IgA2 by *B. hominis* B parasitic lysates and conditioned medium. 30µg of parasitic protein or conditioned medium were incubated with IgA2 (2µg) for 2 h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Incubation with parasitic lysate resulted in the degradation of the heavy chain of IgA2. In lane 3, IgA2 exposed to lysate shows significant loss of heavy chain, a small band (~45kDa, arrow head) cleaved from heavy chain can be observed. Lane 1 and 4 show intact heavy chain of IgA2. Conditioned medium also shows proteolytic activity as significant loss of heavy chain. Lane 2 and 5 are the controls to show that only lysate and medium respectively do not cross react with antibody. (MW, molecular weight; HC, heavy chain; LC, light chain).

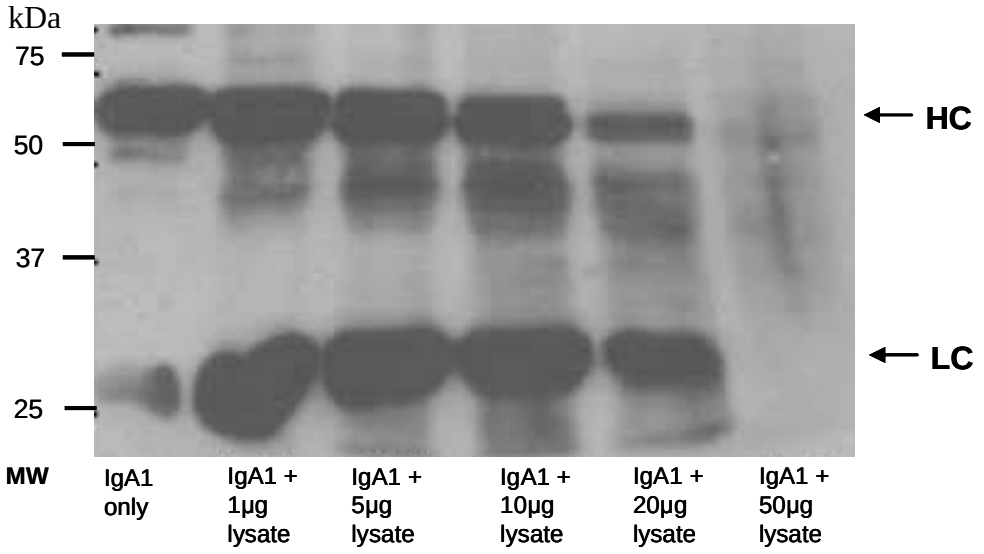

Fig. 3.5 Representative results showing concentration-dependent degradation of human IgA1 by *B. hominis* B parasitic lysates. Varying amount of *Blastocystis hominis* B lysate (from 1µg to 50µg) were incubated with IgA1 (2µg) for 2h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Degradation of heavy chain gradually increased as shown by loss of band intensity. Exposure to 50µg of *Blastocystis* lysate resulted in complete loss of heavy chain of IgA1. (MW, molecular weight; HC, heavy chain; LC, light chain).

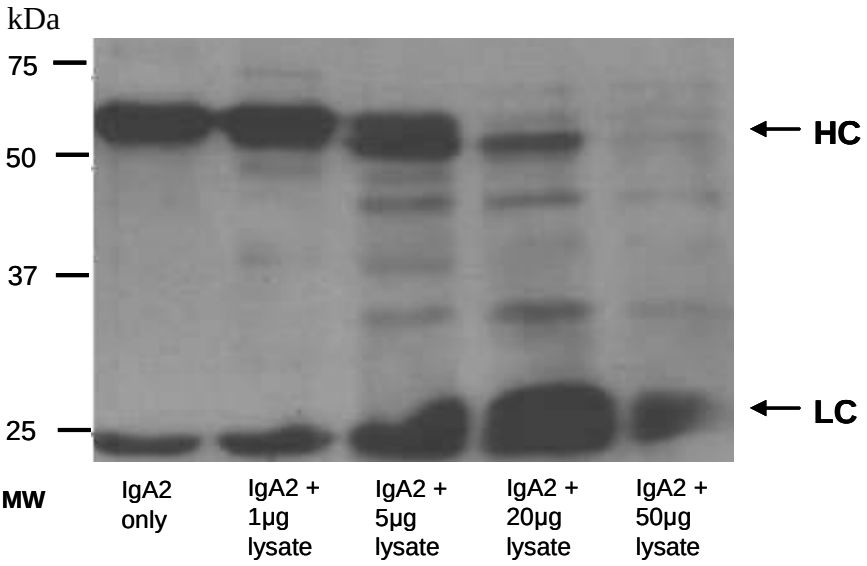

Fig. 3.6 Representative results showing concentration-dependent degradation of human IgA2 by *B. hominis* B parasitic lysates. Varying amount of *Blastocystis hominis* B lysate (from 1µg to 50µg) were incubated with IgA2 (2µg) for 2h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Degradation of heavy chain gradually increased as shown by loss of band intensity. Exposure to 50µg of *Blastocystis* lysate resulted in complete loss of heavy chain of IgA2. (MW, molecular weight; HC, heavy chain; LC, light chain).

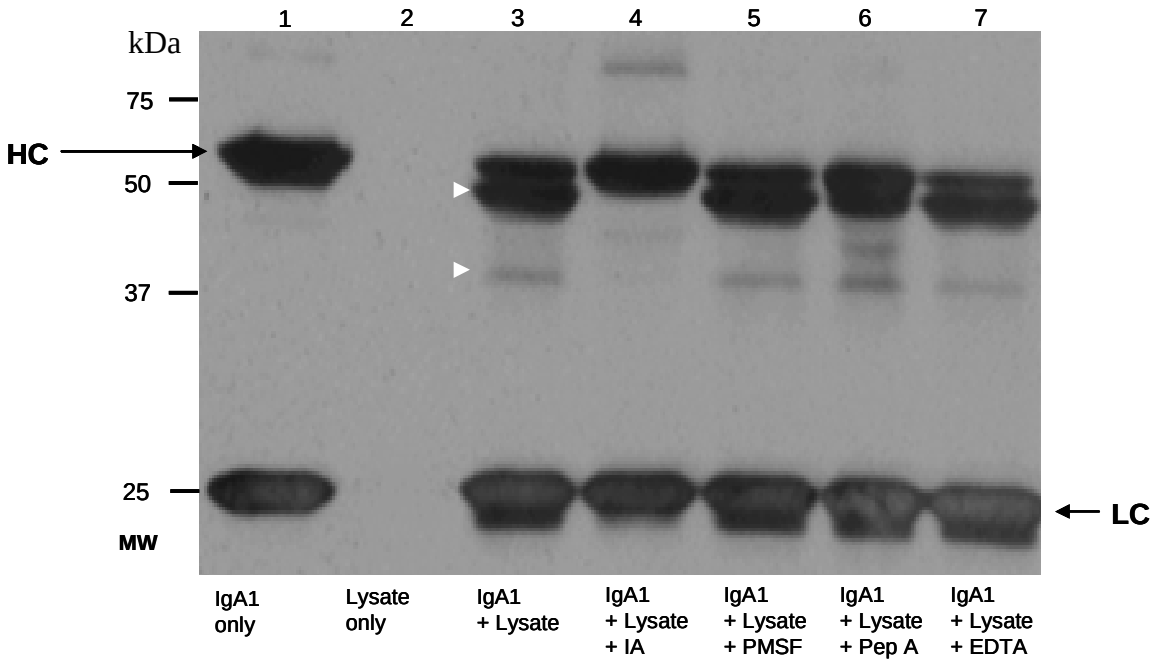

Fig. 3.7 Effect of proteinase inhibitors on IgA1 degradation by lysates from *B. hominis* B. Parasitic lysates were incubated with inhibitors for 1h at 37^0C before IgA1 was added and incubated for 2h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Lane 1 shows intact heavy chain of IgA1. Exposure to parasitic lysate resulted in degradation of heavy chain and cleaved fragments can be seen (arrowhead, lane 3). Cysteine proteinase inhibitor iodoacetamide completely inhibited the degradation of IgA1 and no degradation of heavy chain can be seen (Lane 4). Some inhibition is seen with aspartic-specific inhibitor pepstatin A (Lane 6). Metallo-specific inhibitor EDTA and serine-specific inhibitor PMSF do not show any significant inhibition, as loss of heavy chain and cleaved fragments similar to lysate can be seen. (MW, molecular weight; HC, heavy chain; LC, light chain).

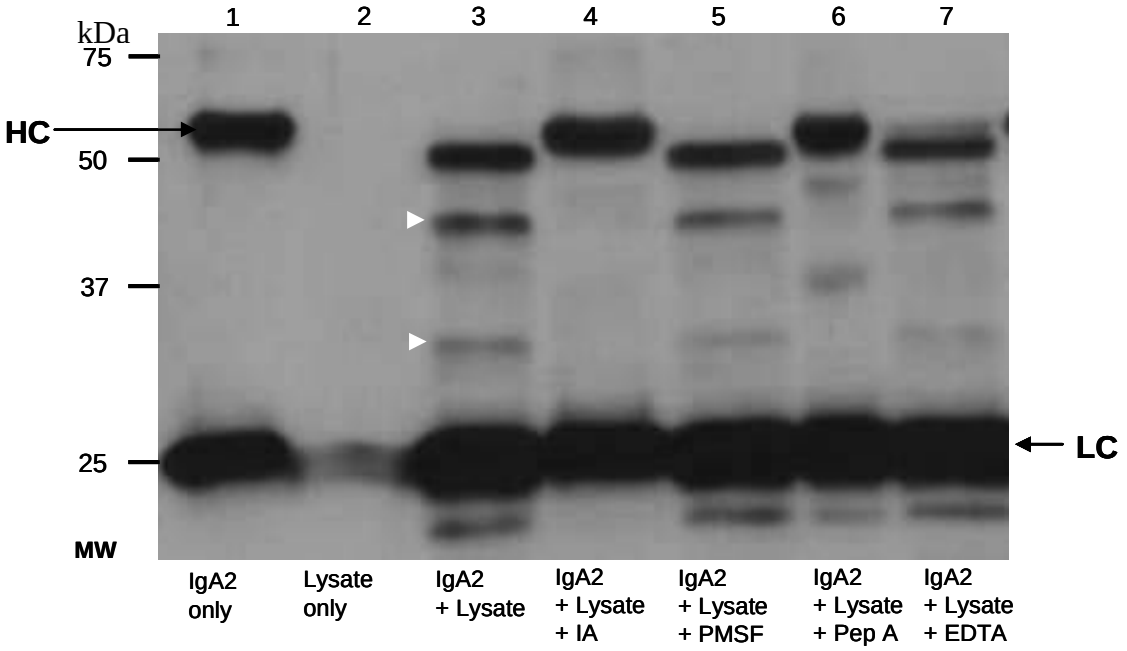

Fig. 3.8 Effect of proteinase inhibitors on IgA2 degradation by lysates from *B. hominis* B. Parasitic lysates were incubated with inhibitors for 1h at 37^0C before IgA2 was added and incubated for 2h at 37^0C, and subjected to SDS-PAGE in 12% acrylamide gel and immunoblotting with antibody to anti-IgA antibody as described in Materials and Methods. Lane 1 shows intact heavy chain of IgA2. Exposure to parasitic lysate resulted in degradation of heavy chain and cleaved fragments can be seen (arrowhead, lane 3). Cysteine proteinase inhibitor iodoacetamide completely inhibited the degradation of IgA1 and no fragments of heavy chain can be seen (Lane 4). Some inhibition is seen with aspartic-specific inhibitor pepstatin A (Lane 6). Serine-specific inhibitor PMSF do not show any significant inhibition, as loss of heavy chain and cleaved fragments similar to lysate can be seen. An unexpected increase in heavy chain degradation is observed with metallo-specific inhibitor EDTA (Lane 7). (MW, molecular weight; HC, heavy chain; LC, light chain).

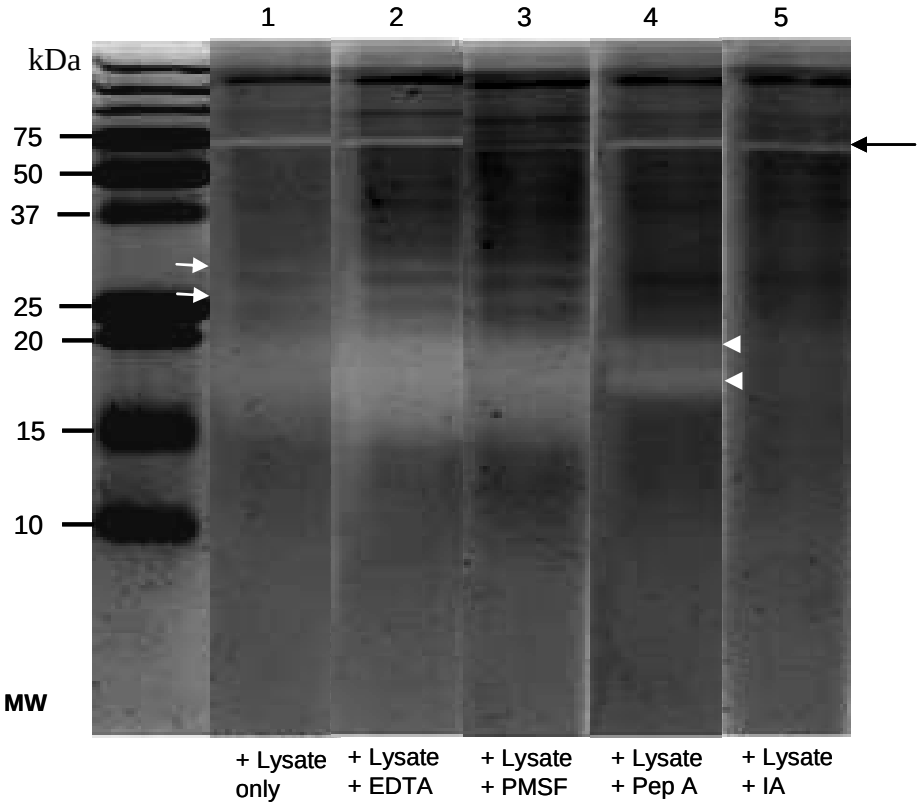

Fig. 3.9 Analysis of *B. hominis* B proteases by IgA1 substrate SDS-PAGE. This SDS-PAGE method requires the inclusion of substrate (IgA1) in an acrylamide gel. Immunoglobulin substrate is digested by proteases after electrophoresis and clear protease bands can be observed against a dark background after Coomassie Brilliant Blue staining. Protease bands can be seen as clear bands against a dark background. One higher molecular weight band (~70kDa, black arrow) and two lower molecular weight band (~17kDa and 19kDa, arrowhead) can be seen. The 17kDa and 19kDa proteases are completely inhibited by cysteine-inhibitor iodoacetamide (Lane 5). The 70kDa protease was inhibited by serine specific inhibitor PMSF (Lane 3). In addition, two faint protease bands (~27kDa and 30kDa, white arrow) visible which are completely inhibited by aspartic specific inhibitor pep A and cysteine-inhibitor iodoacetamide. A smear of low molecular weight proteases (below 17kDa; lane 1, 2 and 3) can be seen which is also completely inhibited by aspartic specific inhibitor pep A and cysteine-inhibitor iodoacetamide. (MW, molecular weight)

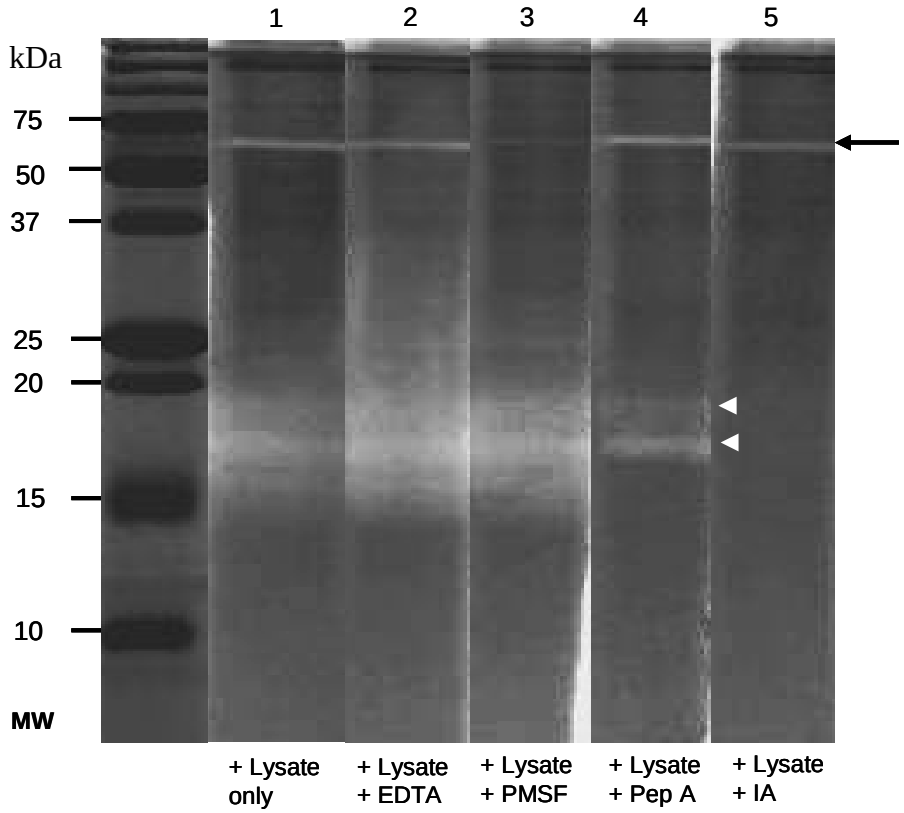

Fig. 3.10 Analysis of *B. hominis* B proteases by IgA2 substrate SDS-PAGE. This SDS-PAGE method requires the inclusion of substrate (IgA2) in an acrylamide gel. Immunoglobulin substrate is digested by proteases after electrophoresis and clear protease bands can be observed against a dark background after Coomassie Brilliant blue staining. One higher molecular weight band (~70kDa, black arrow) and two lower molecular weight band (~17kDa and 19kDa, arrowhead) can be seen. The 17kDa and 19kDa proteases are completely inhibited by cysteine-inhibitor iodoacetamide (Lane 5). The 70kDa protease was inhibited by serine specific inhibitor PMSF (Lane 3). A smear of low molecular weight proteases (below 17kDa; lane 1, 2 and 3) can be seen which is completely inhibited by aspartic specific inhibitor pep A and cysteine-inhibitor iodoacetamide. (MW, molecular weight)

3.4 DISCUSSION

Proteases have not been previously studied in *Blastocystis*, even though such enzymes are known to be virulence factors in many protozoal and bacterial infections (Plaut 1983; Que and Reed 2000). Evidence of human IgA degradation by protozoan proteases has previously been reported for *E. histolytica* (Quezada-Calvillo and Lopez-Revilla 1986; Kelsall and Ravdin 1993) and *T. vaginalis* (Provenzano and Alderete 1995). It was determined that various *Blastocystis* proteases contribute to the breakdown of IgA suggesting that this is a mechanism by which the parasite can persist in gut. Conditioned medium also showed the ability to cleave IgA suggesting active secretion of parasitic IgA proteases. Degradation of IgA by *Blastocystis* lysate and condition medium was inhibited by cysteine and aspartic-specific inhibitors indicate that *Blastocystis* cysteine and aspartic proteinases were mainly accountable for IgA degradation in B and WR1 isolates respectively. The contribution of metallo and serine proteinases to immunoglobulin A degradation was not significant.

Altogether, results from degradation experiments established that *Blastocystis* can significantly degrade both subtypes of immunoglobulin A, IgA1 and IgA2. This finding differs from most pathogenic bacterial IgA protease, which can not cleave IgA2 due to the absence of proline-rich vulnerable sequence in hinge region (Plaut 1983). This suggests that *Blastocystis* proteases do not specifically target the hinge region of IgA; and other sites in the heavy chain may be the target of these proteases. In dose dependent experiments, complete loss of heavy chain was observed suggesting that the heavy chain

has been degraded into fragments too small to be detected by antibody. The conditioned medium also showed IgA1 and IgA2 degradation activity, indicating that these proteases can be secreted by the parasite.

Findings from the protease inhibition experiments showed that iodoacetamide was most significant inhibitor for both IgA1 and IgA2 degradation by *B. hominis* B. Iodoacetamide can covalently bind to cysteine residues and is used as a cysteine protease inhibitor. The results show that most of the IgA proteases are cysteine proteases. To a lesser extent, pepstatin A also inhibited degradation of IgA1 and IgA2. Pepstatin A is a very specific aspartic protease inhibitor and probably there are few aspartic proteases that are involved in the degradation of immunoglobulins. The significant activity of cysteine protease and aspartic protease was also confirmed in the immunoglobulin substrate SDS PAGE assay. Inhibition with serine-inhibitor PMSF and metallo-inhibitor EDTA did not result in any significant inhibition. Interestingly, the presence of EDTA has an effect in increasing the degradation of IgA2. Possibly, this is because of an upstream regulator protein, which can inactivate the immunoglobulin degrading proteases in the presence of metal ions. As soon as the metal ions are chelated by EDTA the regulator protein is inactivated and activity of proteases is enhanced.

The results show that *Blastocystis hominis* B contains at least five IgA1 degrading proteases (~17kDa, 19kDa, 27kDa, 30kDa and 70kDa proteases) and three IgA2 degrading proteases (~17kDa, 19kDa, and 70kDa proteases). The 17kDa and 19kDa proteases can be blocked completely by iodoacetamide, which suggests that it is a

cysteine protease. The 27kDa and 30kDa proteases can be blocked by both pepstatin A and iodoacetamide. Since pepstatin A is a very specific inhibitor, these 27kDa and 30kDa proteases seems to be aspartic proteases that probably also contain an essential cysteine residue. The 70kDa protease seems to be a serine protease as it was blocked by serine-inhibitor PMSF in IgA2 substrate gel. The smear of lower bands (below 17kDa) was completely blocked by pepstatin A and iodoacetamide, but the resolution of bands was not high enough to draw any conclusion.

Blastocystis seems to have similar IgA protease profiles to *Trichomonas vaginalis*. *T. vaginalis*, the causative agent for trichomoniasis, was reported to have IgA degrading proteases (Provenzano and Alderete 1995). These *T. vaginalis* proteases were mainly cysteine proteases and suggested to be a parasitic virulence factor. *Blastocystis* proteases degrade both IgA1 and IgA2 and this may suppress the host intestinal immune response. This might create an immune defective milieu that can indirectly help other pathogens to establish infections or may enhance the chances of their survival. In summary, the present study demonstrates for the first time that *Blastocystis* possesses proteases that can degrade both subclasses of IgA, IgA1 and IgA2.

CHAPTER 4:

BLASTOCYSTIS-INDUCED INTESTINAL EPITHELIAL CELL APOPTOSIS

4.1 INTRODUCTION

The primary site for *Blastocystis* colonization is the intestine and in the previous chapter, it was suggested that proteases produced by *Blastocystis* contribute to its colonization and pathogenesis. Recently, a variety of intestinal pathogens were found to induce apoptosis in intestinal epithelial cells and their association with pathogenicity was suggested (Fiorentini et al. 1998; Kim et al 1998; Crane et al. 1999; Valenti et al. 1999; Huston et al. 2000; McCole et al 2000; Chin et al. 2002). Studies have reported that pathogens can modulate host cell apoptosis process and this can play important roles in the pathogenesis of various diseases (Gao et al. 2000). A complex interaction of parasitic virulence factors and host cell surface receptors is required for the induction of host cell apoptosis (Gavrilescu and Denkers 2003).

Apoptosis (programmed cell death) is a form of cell death distinguishable from necrosis and involves a series of biochemical events (mainly caspase activation) that lead to a range of morphological changes, including blebbing, changes in plasma membrane, loss of cellular attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation. Most of the time, unlike necrosis, apoptosis occurs without any accompanying inflammation. Caspases are cysteine protease and their activation in the host cell is considered essential for apoptosis to occur (Riedl and Shi 2004). Towards the end of apoptosis, the cell fragments into membrane bound vesicles that are efficiently ingested by neighboring immune cells (Wyllie et al. 1980). Apoptosis is considered a tightly regulated event that removes unwanted cells and it is prevalent in all multicellular

organisms. Gastrointestinal epithelial cells have a high turnover and apoptosis plays an important role in maintaining the balance between cellular proliferation and death; however, enhanced apoptosis in intestinal epithelial cells may lead to disturbed gut homeostasis and gastrointestinal symptoms (Ramachandran et al. 2000).

Induction of apoptosis by pathogens may play an important role in the commencement of infection, survival of pathogens or in escape from the host immune system; and in some infections, induction of apoptosis might be a host response to reduce microbial replication (Ojcius et al. 1998). Overall, induction of host cell apoptosis can be both advantageous and disadvantageous for the pathogen. Pathogens can benefit by modulation of apoptosis which can result in 1) substantial host cell deletion 2) initiation of inflammation and 3) inhibition of apoptosis (Zychlinsky et al. 1997). Massive depletion of host cells by apoptosis may assist in the invasion and replication of pathogen. *Salmonella* and *Shigella* cause gastrointestinal disease and they are known to induce apoptosis in macrophages (Siebers and Finlay 1995, Rathman et al. 1997). Moreover, depletion of host cell (e.g. intestinal epithelial cells) by apoptosis may provide other bystander pathogens a more conducive environment for invasion or multiplication. On the contrary, numerous pathogens including *Toxoplasma gondii* and *Leishmania donovani* can inhibit host cell apoptosis to ensure their own survival in the infected cells (Heussler et al. 2001). Apoptosis of macrophages by *Shigella* is accompanied by secretion of proinflammatory cytokine IL-1β leading to inflammation (Zychlinsky et al. 1997). This inflammatory response results in tissue destruction and therefore provides

opportunities for further tissue invasion. Hence, knowledge of host cell apoptosis modulation helps us to better understand host-pathogen interactions.

In vitro studies have demonstrated that *Blastocystis* is capable of causing significant cytopathic effects on Chinese Hamster Ovary cells (Walderich et al. 1998) but the exact nature of cell death mechanisms are not understood. This study was aimed to investigate the interactions of *Blastocystis ratti* WR1, an isolate of zoonotic potential, with a non-transformed rat intestinal epithelial cell line, IEC-6. Here, it is reported for the first time that *B. ratti* WR1 induces apoptosis in IEC-6 cells in a contact-independent manner.

4.2 MATERIALS AND METHODS

4.2.1 Intestinal epithelial cell culture

To investigate if *Blastocystis* can induce apoptosis of intestinal epithelial cells, a non-transformed rat intestinal epithelial cell line, IEC-6 (ATCC) was used. Cell stocks of IEC-6 were maintained in T-75 flasks in a humidified 37^0C incubator with 5% CO_2. For this study, cell cultures within 5-15 passages were used. The cell culture medium consisted of DMEM (Sigma) with 10% heat inactivated fetal bovine serum (Hyclone), 1% sodium pyruvate (Gibco), and 0.1% bovine insulin (Sigma). The culture medium was changed every 2-3 days. Trypan Blue assay was used to assess the viability of cells and cell cultures with >95% viability were used for all experiments. To remove cells from flask or culture plates, trypsinization was performed with 0.25% trypsin-EDTA (Gibco). A seeding density of 2×10^5 cells/ml was used for the experiments. Confluent IEC-6 cell monolayers were grown on 12-well tissue culture plates (Costar), or to poly-L-lysine treated 12 mm glass coverslips. Poly-L-lysine coating of coverslips is described in appendix III.

Additionally, human colonic carcinoma epithelial cells T84 (ATCC) were also used. T84 cells growth medium consisted 1:1 mixture of Dulbecco modified Eagle medium and Ham's F-12 (DMEM/F-12) (Sigma) supplemented with 5 % heat inactivated fetal bovine serum (Gibco).

4.2.2 Parasite culture and lysate preparation

Blastocystis ratti WR1 was used for this study. Parasites were cultured in pre-reduced Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% inactivated horse serum (Gibco) and incubated anaerobically at 37^0C in an ANAEROJAR (Oxoid, UK) as previously described (Ho et al. 1993). Parasites (5 days old) were harvested and washed 2 times in ice cold IEC-6 complete media at 500 × g for 10 min at 4^0C. Resulting pellet was resuspended in IEC-6 cell complete media. The parasites were counted with a hemocytometer and concentration was adjusted to 1×10^7 parasites/ml. Parasites were examined microscopically for their viability in IEC-6 complete media and found to be viable for >48 h in IEC-6 growth conditions. To prepare parasitic lysates, three freeze-thaw cycles in liquid nitrogen and 37^0C water bath were respectively performed. Lysates were microscopically checked for the complete cell lysis.

4.2.3 Experimental planning and inoculation protocol

For all experiments, a density of 1×10^7 parasites/ml was used. A cell density of 1×10^7 *Blastocystis* cells/ml was used for all experiments. Studies for other protozoan parasites have used similar or even higher concentration of parasites (up to 2×10^7 cells/ml for *Giardia intestinalis*) (Chin et al. 2002). Although titers of naturally occurring infections are not known for *Blastocystis*, a concentration of 1×10^7 *Blastocystis* cells/ml was used based on previous studies on protozoan parasites like *Giardia* (Teoh et al. 2000). Either live parasites or equivalent parasitic lysate were added to IEC-6 monolayers grown in 12 well

plates (2ml inoculum), 24 well plates (1 ml inoculum). For experiments, 2-3 days old confluent monolayers of IEC-6 were used and culture medium was changed 24 h before the experiment. In some experiments, to inhibit caspases, cell monolayers were pretreated for 2 h with either general caspase inhibitor Z-VAD-fmk (50µM) or caspase-3 inhibitor Z-DEVD-fmk (120µM) prior to addition of parasites or lysate. To investigate if apoptosis of IEC-6 cells requires direct contact with parasites, cells were grown in 12 well plates and kept separate from parasite by porous filters (Millicell-HA filter) during coincubation (Fig. 4.1).

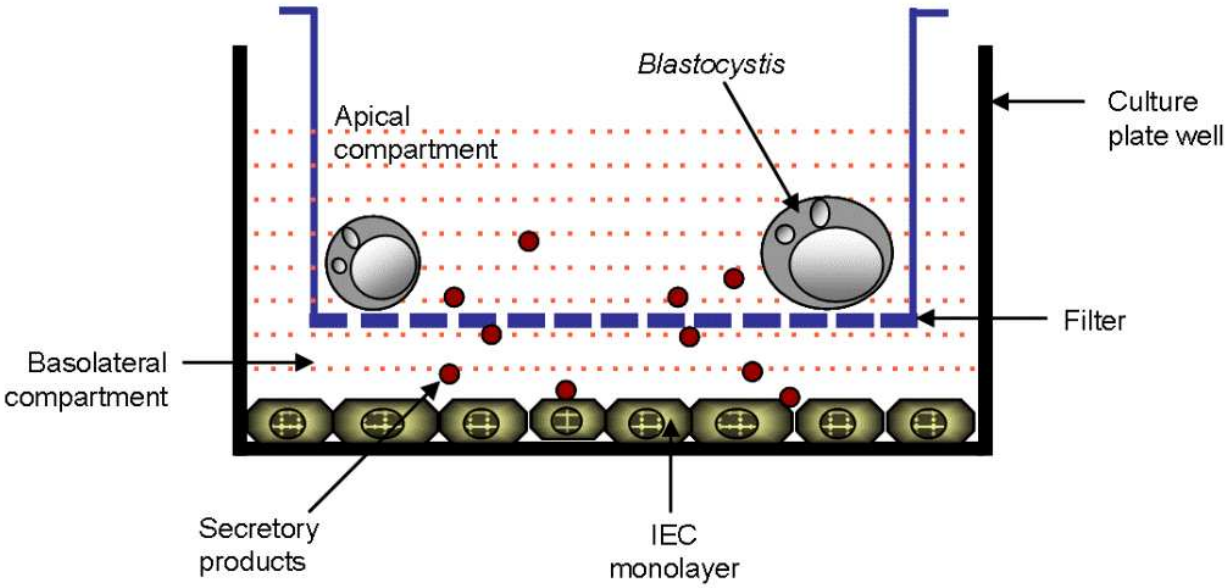

Fig. 4.1 Simplified diagrammatic representation of Millicell-HA membrane inserts used for the parasite-host cell contact-independent experiments. IEC-6 cells were grown on the culture plate well base, and live *Blastocystis ratti* WR1 parasites were added to the apical compartment (filter insert) to study contact-independent effects on host cells. The small pore size (0.4 µm) does not allow *Blastocystis* to pass through filter but secretory components can pass and interact with IEC monolayer grown on the base.

4.2.4 DAPI staining for nuclear fragmentation and condensation

IEC-6 cells were grown on poly L-lysine coated 12 mm glass coverslips and coincubated with *B. ratti* WR1 live parasites or parasitic lysate. After 24 h of incubation, monolayers were washed with PBS and fixed with 2 % (w/v) paraformaldehyde in PBS (pH 7.4). After fixation, cells were washed twice with PBS and stained with 0.5µg/ml of DNA binding dye DAPI (4', 6-diamidino-2-phenylindole; Sigma) for 5 min in the dark (at room temperature). After staining with DAPI, cells were washed in PBS and mounted on a glass slide with fluorescent mounting medium (VECTASHIELD). Stained cells were viewed under a fluorescence microscope (Olympus BX60, Japan) for changes in nuclear morphology and counted.

4.2.5 Annexin V binding assay for expression of phosphatidylserine molecules on cell surface

As an early marker of apoptosis, annexin V binding assay was performed. IEC-6 cells were grown in 12 well culture plates. Grown monolayers were then coincubated with *B. ratti* WR1 live parasites or parasitic lysate for 5 h. Expression of phosphatidylserine molecules on plasma membrane was analyzed by using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Pharmingen) following the manufacturer's instructions. Within the kit, propidium iodide (PI) was used for the exclusion of dead cells from apoptotic cells. After annexin V staining, samples were analyzed by flow cytometry (Dako Cytomation Cyan LX) using 488 nm excitation and a

515 nm bandpass filter for FITC detection and a 600 nm filter for PI detection. In the dot plot, a region was defined that represents the apoptotic cell population showing annexin $^{+}$ and PI $^{-}$ staining (lower right quadrant).

4.2.6 Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL)

IEC-6 cells were cultured on poly L-lysine coated 12 mm glass coverslips. Grown cell monolayers were coincubated with *B. ratti* WR1 live parasites or lysate for 12 h. To detect *in situ* DNA fragmentation, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) was performed using the *in situ* cell death detection kit (Roche) following the manufacture's instructions. The samples were analyzed by a fluorescence microscope and apoptotic cells were counted.

4.2.7 Caspase-3 activity

To analyze caspase-3 activity, the ApoAlert^{TM} Caspase Fluorescent Assay Kit (BD Pharmingen) was used. IEC-6 cells were grown in 12 well culture plates and coincubated with *B. ratti* WR1 live parasites or parasitic lysate for 6, 12, 24 h. After coincubation, cells were washed twice with ice cold PBS and harvested using a cell scraper. After washing, cells were resuspended in 50µl of chilled cell lysis buffer and incubated on ice for 10 min. Cell homogenates were centrifuged (13000 rpm) for 10 min to precipitate cellular debris. Resulting supernatants were transferred to new tubes. After this, 50µl of 10mM DTT in

1ml of 2× reaction buffer mix followed by 5µl of 1mM caspase-3 substrate (DEVD-AFC; 50µM final concentration) was added to each sample and incubated in a water bath (1 h at 37^0C). Samples were transferred to a 96 well plate and analysis was done by measuring absorbance in a fluorometer (SpectraMax) with a 400 nm excitation filter and 505 nm emission filter. Caspase-3 activity was expressed as relative fluorescence units (RFUs).

4.2.8 Positive control

In some of the abovementioned apoptosis assays, 0.25 µM staurosporine (Sigma) was used as a positive control to induce apoptosis.

4.3 RESULTS

4.3.1 *Blastocystis* induces apoptosis in IEC-6 cells

To investigate whether *Blastocystis* has potential to induce apoptosis in intestinal epithelial cells, range of experiments was performed. These experiments involved (a) DAPI staining to detect chromatin condensation and nuclear fragmentation, (b) Annexin V binding assay to analyze externalization of phosphatidylserine molecules during the early phase of apoptosis, (c) TUNEL for the analysis of internucleosomal nicks in DNA during the late stages of apoptosis and (d) Caspase-3 assay to analyze the presence of active caspase-3. In addition, caspase inhibitors were used to confirm findings. Results from all experiments consistently revealed that exposure of IEC-6 cells to *B. ratti* WR1 live parasite and parasitic lysates induces significant levels of apoptosis in IEC-6 cells. Moreover, the results showed that the secretory products of *Blastocystis* can induce apoptosis and parasite-host cell contact is not essential.

4.3.1.1 Cellular detachment

For proper growth, function and survival, most of the cells require attachment to each other and subsequent proliferation on the extracellular matrix (ECM). This dependence is known as ‘anchorage dependence’ of cells. Without attachment, cells often undergo apoptosis, a process known as ‘anoikis’ (Frisch and Ruoslahti 1997). Anoikis is a well documented form of programmed cell death in freshly isolated human intestinal

epithelial cells (Grossmann et al. 1998). IEC-6 cell monolayers were coincubated with *B. ratti* WR1 live parasites and parasitic lysates for 6 h and viewed by inverted phase-contrast microscopy. Results show that cellular detachment is significantly higher in monolayers exposed to live parasite and parasitic lysate compared to normal looking cell monolayers in control monolayer (Fig. 4.2). Monolayers treated with positive control (staurosporine) were disrupted and extensive cell detachment was observed. It has been reported that cellular detachment occurs due to caspases which can degrade cytoskeletal proteins and some proteins in ECM, resulting in the detachment of cells (Huppertz et al. 1999, Ramesh et al. 2000) and suggests that the induction of caspase activity is an early event in the apoptotic pathway.

4.3.1.2 Changes in nuclear morphology

Distinguishing nuclear morphological changes in the cells undergoing programmed cell death were considered as important indicator of apoptosis (Kerr et al. 1972). Staining of cells with nuclear binding dye DAPI detects changes in the nuclear morphology. IEC-6 cell monolayers exposed to *Blastocystis* showed characteristic apoptotic nuclear condensation and fragmentation (Fig. 4.3). IEC-6 cells exposed to *B. ratti* WR1 live parasites and parasitic lysates for 24 hours, showed highly condensed and fragmented nuclei in apoptotic cells. Monolayers of control IEC-6 cells showed healthy intact nuclei with normal chromatin distribution. Cells coincubated with live parasites (7.6% ±1.4, P <0.05 versus control) and parasitic lysate (13.5% ±2.4, P <0.05 versus control) showed about 4 and 7 fold respective significant increase in percentage of apoptotic cells compared

with the control (2.0% ±0.5) (Fig 4.4). Cells in contact-independent assay also showed a significant increase in apoptotic cells (5.9% ±0.7, P <0.05 versus control) suggesting that parasitic secretory products have potential to induce apoptosis. In addition, to provide direct evidence that apoptosis of IEC-6 cells by *Blastocystis* involved caspase-3, pretreatment of cells with a caspase-3 inhibitor Z-DEVD-fmk before exposure to parasitic lysate significantly inhibited apoptosis (5.3% ±1.1, P <0.05 versus parasitic lysate treatment).

4.3.1.3 Externalization of phosphatidylserine molecules on cell surface

Externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane is one of the main features of apoptosis and it is generally assumed that PS exposure is a result of caspase activation, another hallmark of apoptosis (Ferraro-Peyret et al. 2002). Annexin V, a 35-36 kDa protein, in the presence of calcium ions, binds with high affinity to PS molecules (Raynal and Pollard 1994). Annexin V-FITC and propidium iodide double staining can effectively detect viable, apoptotic and necrotic cells in a mixed population (Vermes et al. 1995).

In this method, annexin^{+} and PI^{-} stained cells represent apoptotic cell population in flow cytometry dot plots (lower right quadrants, Fig. 4.5). IEC-6 cells exposed to *Blastocystis* for 5 hours showed a marked increase in percentage of apoptotic cells. Cells coincubated with live parasites (19.3 %, Fig. 4.5B) and with parasitic lysate (34.2 %, Fig. 4.5C) relative to the control (5.8 %, Fig. 4.5A) in the lower right quadrants. The population

dots in B, C, E and F compared to A in figure 4.5, show an overall shift in the population of cells from the lower left quadrant towards the lower right quadrant. This shift indicates the increase in the percentage of apoptotic cells with preservation of plasma membrane integrity. Pretreatment with general caspase inhibitor Z-VAD-fmk prior to coincubation with parasitic lysate significantly prevented the apoptosis of IEC-6 cells (8.4 %, Fig. 4.5D). Moreover, a significant increase in the number of apoptotic cells induced by live parasites in a contact-independent manner was observed (14.6%, Fig. 4.5E). This observation suggests that some secretory products of *B. ratti* WR1 may induce apoptosis of IEC-6 cells without the need for direct contact with the host cells.

4.3.1.4 TUNEL

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) was performed as a late marker for the detection of endonuclease activity and analysis of apoptosis in IEC-6 cells exposed to *Blastocystis*. The low molecular weight DNA fragments and single strand breaks (also called as 'nicks') in high molecular weight DNA can be detected by labeling free 3'-OH end with modified nucleotides in an enzymatic reaction. TUNEL is reported to be a good assay for detecting DNA fragmentation when used in combination with other techniques (Huppertz et al. 1999).

Results from fluorescent microscopy revealed labeling of distinctly fragmented DNA (Fig. 4.6). IEC-6 cells exposed to *B. ratti* WR1 live parasites and parasitic lysate

showed distinct increase in fluorescence in comparison to the cells of negative control. Flow cytometry was performed for the quantification and results showed significantly higher percentages of TUNEL labeled cells exposed to live parasites (16.7% ±2.6, *P* <0.05 versus control) and parasitic lysate (21.8% ±4.9, *P* <0.05 versus control) as compared to the negative control (2.0% ±0.5). Overall, TUNEL and in DAPI staining results clearly suggest that *Blastocystis* causes DNA fragmentation in IEC-6 cells.

4.3.1.5 Increase in caspases-3 activity

Caspases are cysteine proteases that are essential for the execution of apoptosis (Riedl and Shi 2004) and they control the dismantling and clearance of dying cells. Caspase-3 (also called as CPP32, apopain, YAMA) has been identified as being a key mediator of apoptosis in mammalian cells (Kothakota et al. 1997), during which cells undergo morphological changes particularly DNA fragmentation, chromatin condensation, and apoptotic body formation. There was a significant increase in caspase-3 activity in IEC-6 cells exposed to *B. ratti* WR1 live parasites and parasitic lysates (Fig. 4.7). Caspase-3 activity was observed to peak at 12 h after incubation. After 12 h, activity of caspase-3 decreased up to 24 h. Importantly, a significantly high caspase-3 activity was seen in IEC-6 cells coincubated with live parasites in a contact-independent manner, using Millicell-HA filters. This finding was consistent with DAPI staining and annexin V binding results (Fig. 4.4 and 4.5) providing evidence that *B. ratti* WR1 can induce apoptosis of IEC-6 cells in a contact-independent manner. Additionally, to prove that *Blastocystis* induced apoptosis of IEC-6 cells involved caspase-3, IEC-6 monlayers were

pretreated with caspase-3 specific inhibitor, Z-DEVD-fmk, before coincuabation with parasitic lysate. Caspase-3 activity was found to be completely inhibited with the use of Z-DEVD-fmk (Fig. 4.7). Overall findings suggest that caspases-3 is actively involved in *Blastocystis* induced apoptosis of IEC-6 cells.

4.3.2 *Blastocystis* induces apoptosis in T84 cells

In addition to the non-transformed rat intestinal epithelial cell line IEC-6, it was investigated if *Blastocystis* could induce apoptosis in human colonic carcinoma epithelial cells (T84). T84 Cells were grown on poly-L-lysine coated glass coverslips and incubated for 24 h with *B. ratti* WR1 live parasite or parasitic lysate. Cells coincubated with live parasites and parasitic lysate show nuclear condensation and fragmentation (Fig. 4.8). A significant increase in percentage of apoptotic cells in T84 monolayers exposed to live parasite (8.3% ±1.6) and parasitic lysate (14.6% ±2.7) was noticed in comparison to the negative control (2.5% ±0.5) (For both $P < 0.05$ in comparison to control).

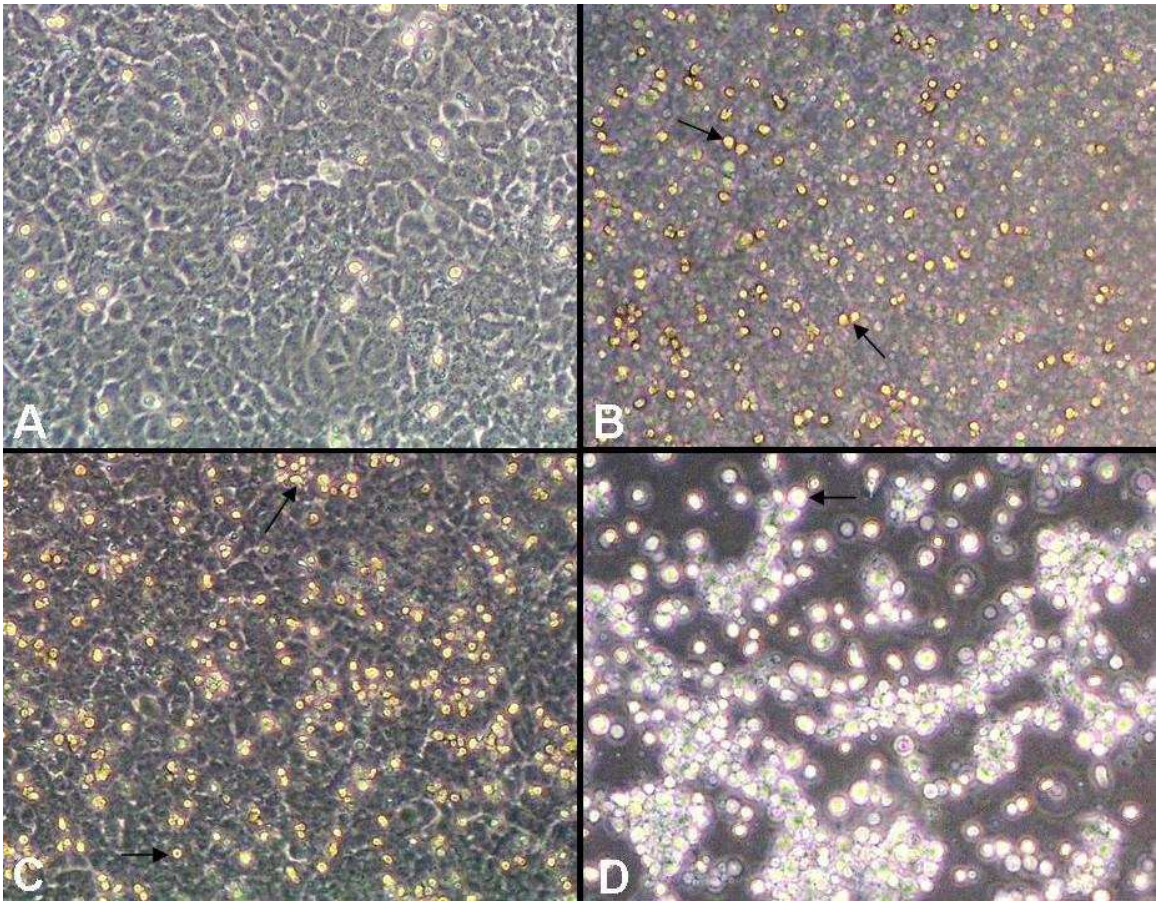

Fig. 4.2 Representative light microscopy pictures of apoptotic cells showing cellular detachment from stratum. Confluent IEC-6 cell monolayers were grown on 12-well tissue culture plates and coincubated with *B. ratti* WR1 live parasites and parasitic lysates for 6 h and viewed by inverted phase-contrast microscopy. Overall observation shows that cellular detachment (shiny spots) is much higher in monolayers exposed to live parasite (B) and parasitic lysate (C) compared to a normal looking cells in control monolayer (A). In the positive control (D), the monolayer integrity was disrupted and extensive cell detachment can be seen. (100× magnification).

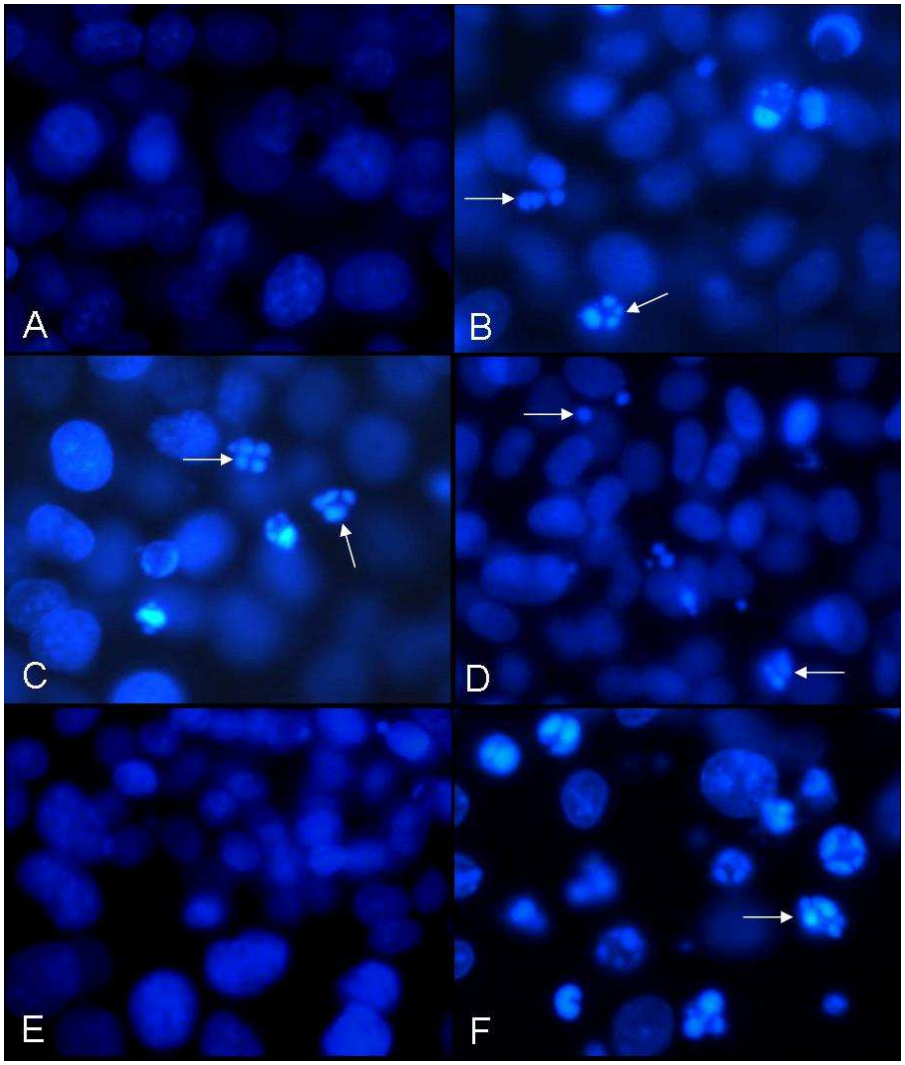

Fig. 4.3 Fluorescence photomicrographs after DAPI staining shows apoptosis of IEC-6 cells. Cells were grown on glass coverslips and incubated for 24 h with either culture media (A), *B. ratti* WR1 live parasite (B), parasitic lysate (C), and 0.25 µM staurosporin as positive control (F). Cells coincubated with live parasites, parasitic lysate and staurosporin show nuclear condensation and fragmentation (arrow). Parasite-host cell contact independent assay also shows apoptosis of cells (D). Pretreatment with a caspase-3 inhibitor Z-DEVD-fmk rescued cells from apoptosis (E). (400× magnification).

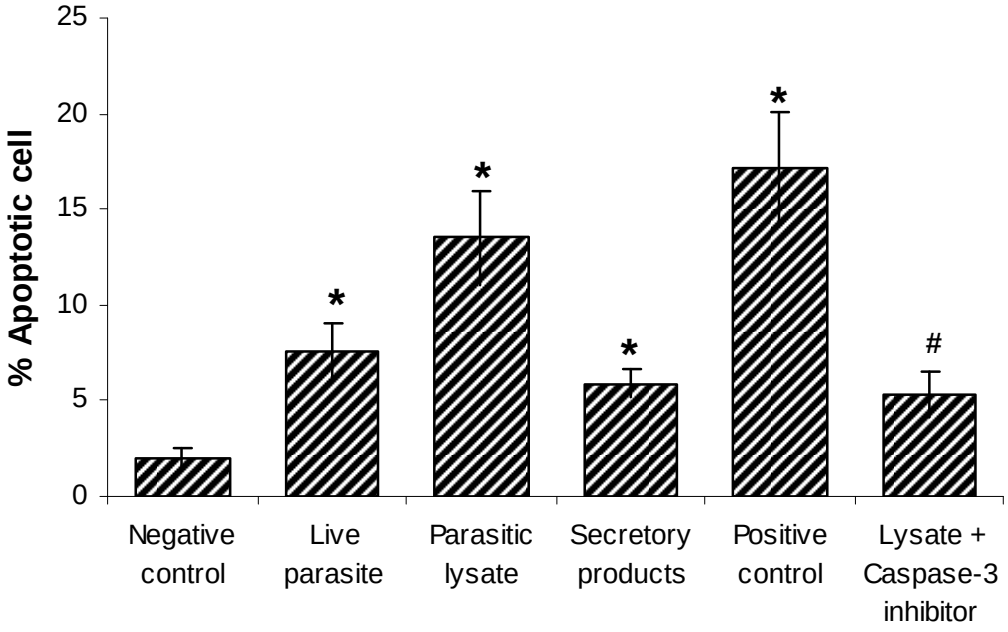

Fig. 4.4 Histograms showing percentage of apoptotic cells after DAPI fluorescence assay. IEC-6 monolayers were grown on glass coverslips and incubated for 24 h with either culture medium, *B. ratti* WR1 live parasite, parasitic lysate, or 0.25 µM staurosporin. Cells coincubated with live parasites and parasitic lysate show a significant increase in percentage of apoptotic cells in comparison to the negative control. Contact independent assay also shows significant increase in apoptotic cells suggesting that secretory products of *Blastocystis* has potential to induce apoptosis. Pretreatment of cells with a caspase-3 inhibitor Z-DEVD-fmk significantly inhibited the apoptosis. For each sample, 1000 cells were counted at 400× magnification. Values are means ± standard deviations (n = 3 per group). *, $P < 0.05$ versus negative control; $^{\#}$, $P < 0.05$ versus parasitic lysate treatment.

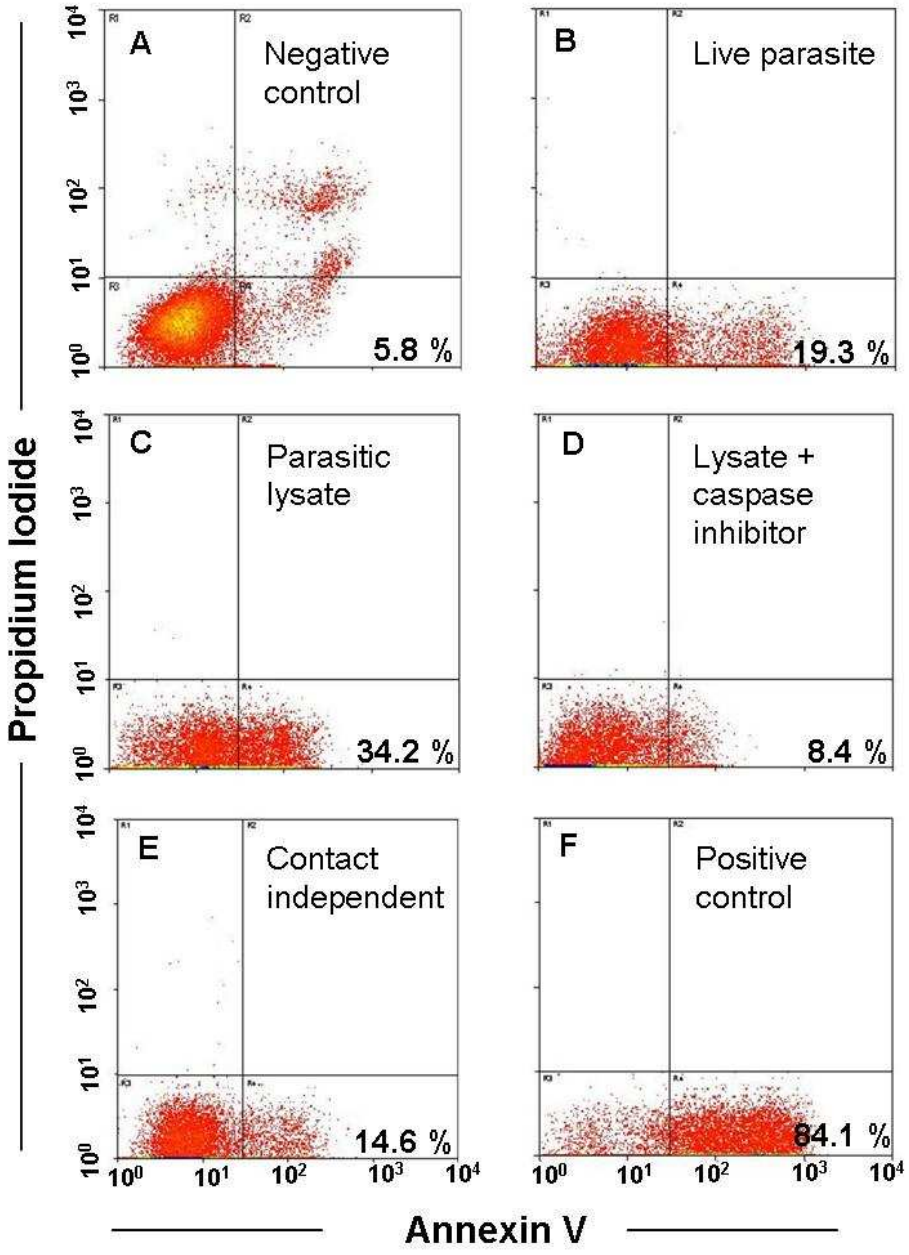

Fig. 4.5 Flow cytometry analysis of annexin V-FITC and propidium iodide staining. Representative dot plots of controlcells (A), cells incubated for 5 hr with *B. ratti* WR1 live parasites (B), with parasitic lysate (C), with lysate after pretreatment with caspase inhibitor Z-VAD-fmk (D), with live parasite on Millicell-HA filter for contact-independent assay (E), and with 0.25µM staurosporin as a positive control (F). B, C, E and F show a significant increase in annexinV^+ and PI^- apoptotic cell population (lower right quadrant). 2×10^4 cells from each sample were analyzed.

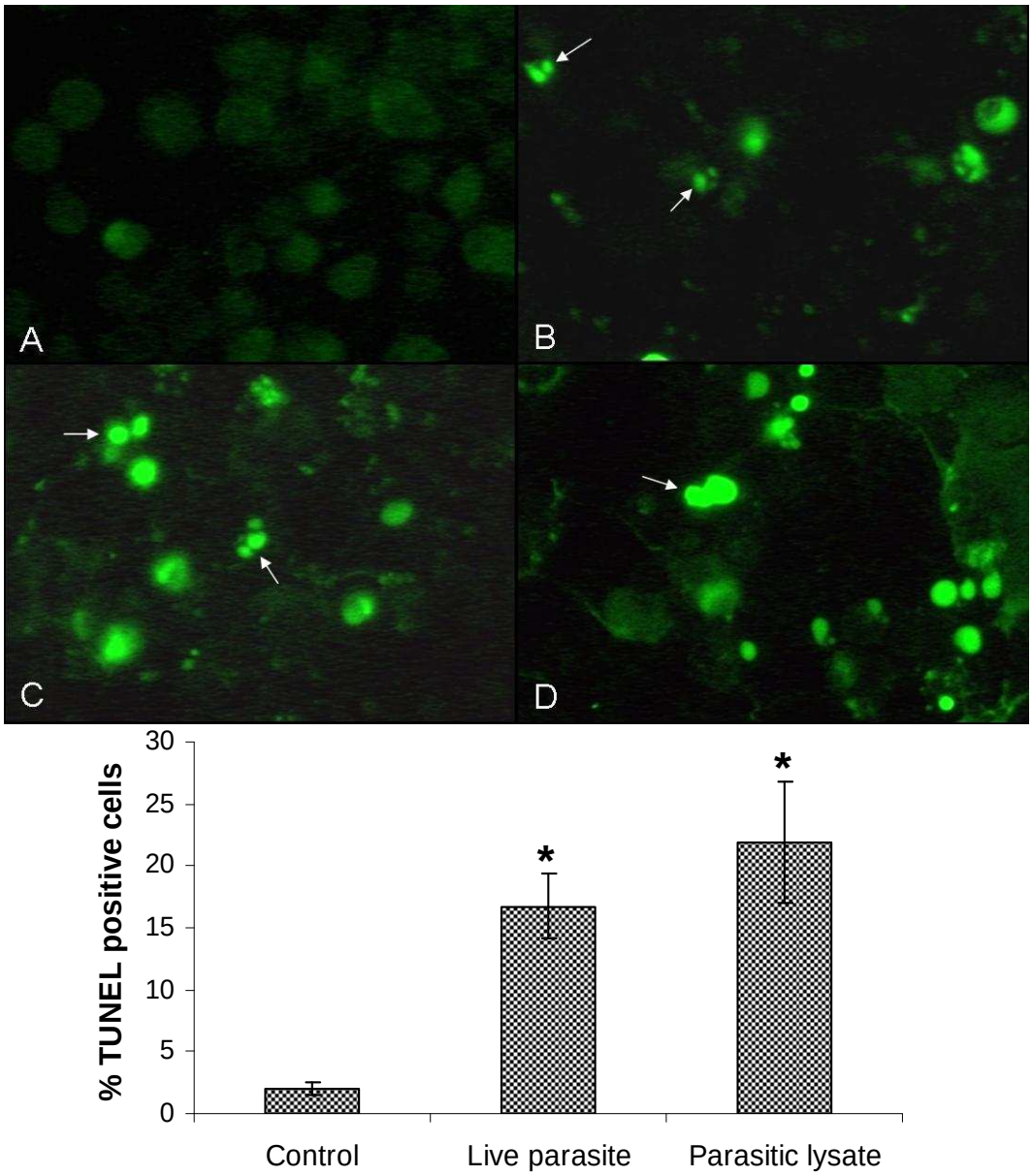

Fig. 4.6 TUNEL for the detection of *in situ* DNA fragmentation. IEC-6 cells were cultured on poly L-lysine coated 12 mm glass coverslips. To detect *in situ* DNA fragmentation, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) was performed. Fluorescence micrographs of cells grown on glass coverslips and coincubated for 12 h with culture medium (A), *B. ratti* WR1 live parasite (B), parasitic lysate (C) and staurosporin (D). A significant number of TUNEL positive cells (green fluorescence) can be seen in B, C and D. Histogram shows TUNEL positive cell population determined by flow cytometry shows a significant increase in TUNEL positive IEC-6 cells after coincubation with live parasite and parasitic lysates. Values are means ± standard deviations (n = 3 per group). *, $P < 0.05$ versus control. (400× magnification).

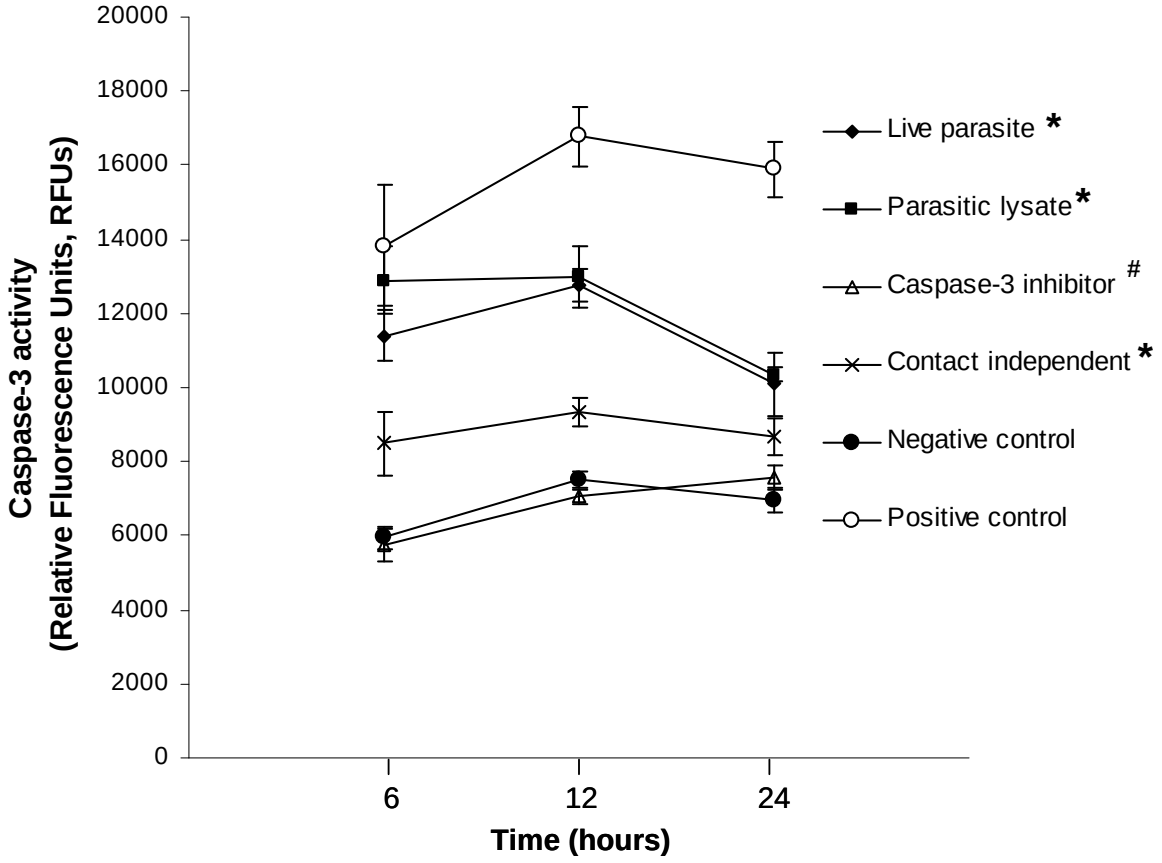

Fig. 4.7 Line chart showing caspase-3 activity of IEC-6 cells. IEC-6 cells were grown in 12 well culture plates and coincubated with *B. ratti* WR1 live parasites or parasitic lysate. Caspase-3 activity was measured at 6, 12 and 24 h time points as using a Caspase-3 Fluorescent Assay Kit. A significant increase in caspase-3 activity was observed in cells treated with live parasite, parasitic lysates and positive control (0.25 µM staurosporin) after 6 and 12 h. Caspase-3 activity was gradually reduced at 24h. Pretreatment of cells with caspase-3 inhibitor Z-DEVD-fmk significantly reduced the caspase-3 activity. Values are means ± standard deviations (n = 3 per group). *, $P < 0.05$ versus negative control for all time points; $^{\#}$, $P < 0.05$ versus parasitic lysate for all time points.

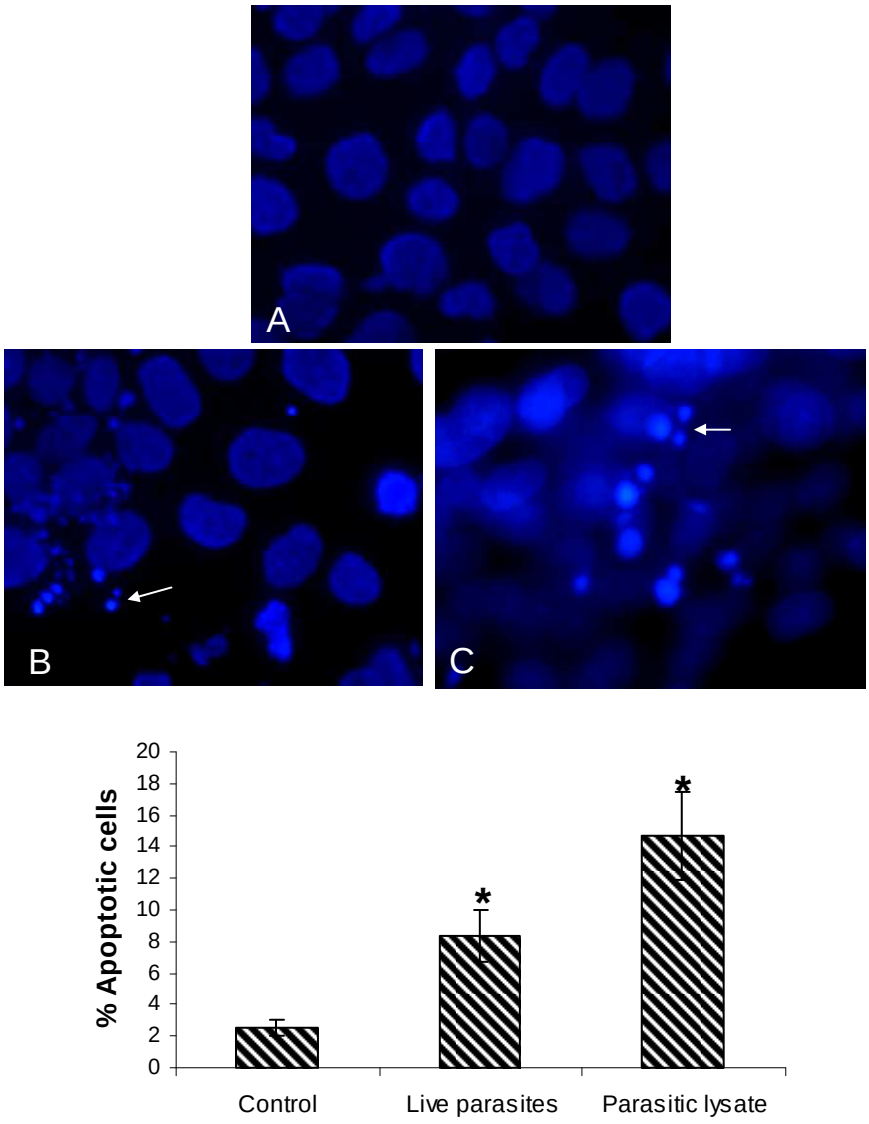

Fig. 4.8 Fluorescence photomicrographs and histogram showing apoptosis of human colonic carcinoma epithelial cells (T84) after DAPI staining. Cells were grown on glass coverslips and incubated for 24 h with either culture media (A), *B. ratti* WR1 live parasite (B), or parasitic lysate (C). Cells coincubated with live parasites and parasitic lysate show nuclear condensation and fragmentation (arrow). From the histogram, a significant increase in percentage of apoptotic cells in T84 monolayers exposed to live parasite and lysate can be noticed in comparison to the negative control. For each sample, 1000 cells were counted. Values are means ± standard deviations (n = 3 per group). * $P < 0.05$ versus negative control. (400× magnification).

4.4 DISCUSSION

Here, it was demonstrated for the first time that *B. ratti* WR1 can induce apoptosis in intestinal epithelial cells. The apoptosis of IEC-6 cells also occurred in a contact-independent manner and suggests that *Blastocystis* may be secreting certain virulent factors that can initiate the apoptotic pathway in IEC-6 cells.

Apoptosis can occur in response to infection with numerous invasive and non-invasive pathogens (McCole et al. 2000). It was reported recently that many protozoan parasites including *G. lamblia, C. parvum, E. histolytica* and other pathogens particulary *L. monocytogenes, E. coli, C. difficile* (Fiorentini et al. 1998; Kim et al. 1998; Crane et al. 1999; Valenti et al. 1999; Huston et al. 2000; McCole et al. 2000; Chin et al. 2002) can induce apoptosis of intestinal epithelial cells. The cytopathic effects of *Blastocystis* were reported earlier and it was shown to be able to kill host cells *in vitro* (Walderich et al. 1998), but the mechanism of cell death was not investigated. Findings from this study demonstrate that *B. ratti* WR1 can cause apoptosis of intestinal epithelial cells and parasite-enterocyte contact is not required. These results suggest that *Blastocystis* has the potential to cause *in vivo* pathogenic effects without adhering to the gut mucosa. On the other hand, studies have shown that intestinal pathogens in particular *E. histolytica* and *C. parvum*, and enteropathogencic *E. coli* usually establishes contact with the host cells to cause apoptosis (Crane et al. 1999; Huston et al. 2000; McCole et al. 2000).

Although the ability of *Blastocystis* to adhere to the intestinal epithelium has not been established, the majority of studies suggest *Blastocystis* to be a non-invasive protozoan parasite (Stenzel and Boreham 1996; Tan 2004). Hence, finding that *Blastocystis* has the ability to cause apoptosis without adhering to the gut mucosa is important for its pathogenicity. Numerous intestinal protozoans are reported to secrete parasitic factors, for example proteases, that are accountable for their virulence (Rosenthal 1999). It was also shown in previous chapter that *Blastocystis*-secreted proteases can degrade human secretory immunoglobulin A and they were suggested to be parasitic virulence factors. Invasion does not necessarily lead to host cell apoptosis and there are pathogens that can invade but do not induce apoptosis (Crane et al. 1999). Further investigations and characterization of *Blastocystis* secretory products can subsequently shed more light on its pathogenesis.

Clostridium difficile, a cause of diarrhea and colitis in human, produces toxin B that can cause apoptosis in intestinal cells with nuclear fragmentation and chromatin condensation (Fiorentini et al. 1998). It was reported that *H. pylori* colonization causes increased apoptosis of gastric cells and it was prevented by the removal of organism (Moss et al. 1996). Purified lipopolysaccharide of *H. pylori* has also been reported to induce apoptosis of epithelial cells *in vitro* (Wagner et al. 1997).

Findings suggest that *Blastocystis* induces moderate levels of apoptosis in intestinal epithelial cells; and this may limit the host inflammatory response, which might be detrimental to the survival of the parasite. Ideally, it seems that induction of apoptosis

of host intestinal cells would not be beneficial to a non-invasive parasite like *Blastocystis* as it may possibly result in the loss of colonization sites for the parasite. This unintentional initiation of host cell apoptosis might be a host response against some parasitic factors like proteases which are essential for parasite's own growth and life-cycle. Extension of these findings in suitable animal model would greatly help to elucidate this issue.

Apoptosis or necrosis of intestinal epithelial cells can lead to altered intestinal barrier function (Bojarski et al. 2000). Findings showed that the inhibition of caspases in IEC-6 cells with Z-VAD-fmk has apparently no significant effect on TER and permeability changes induced by *Blastocystis*. *Giardia lamblia* parasitic factors have been reported to increase epithelial permeability by inducing enterocyte apoptosis (Chin et al. 2002). This difference in observation may be due to low percentage of apoptosis induced by *Blastocystis*. In addition, *Blastocystis* may have induced non-apoptotic cell death, such as necrosis, autophagy or paraptosis (Broker et al. 2005) that may have resulted into increased epithelial permeability.

In summary, results from the present study report for the first time that *Blastocystis* induces apoptosis in enterocytes and there is active involvement of caspase-3. In addition, it was demonstrated that this apoptosis may occur in a contact-independent manner and suggested that *Blastocystis* may secrete virulent factors that can initiate the apoptotic pathway in IEC-6 cells.

CHAPTER 5:

EFFECTS OF *BLASTOCYSTIS* ON EPITHELIAL TIGHT JUNCTIONS AND BARRIER FUNCTION

5.1 INTRODUCTION

Intestinal permeability was reported to be considerably increased in *B. hominis* patients and it was suggested that *Blastocystis* infections may damage the intestinal wall (Dagci et al. 2002). Many gastrointestinal disorders particularly bacterial enteritis, celiac disease and inflammatory bowel disease are reported to be associated with a breakdown of epithelial barrier function (Bjarnason et al. 1995; Chin et al. 2002). The main function of intestinal mucosa is to provide a barrier that keeps luminal contents separate from the interstitium. This barrier function can be compromised in many diseases and may result in a leaky barrier and gastrointestinal symptoms including diarrhea.

Recently it was known that cytoskeleton and tight junctions play important roles in regulating the mucosal barrier function (Clayburgh et al. 2004). The cytoskeleton, in particular the actin microfilaments, is key in maintaining cell shape and regulating tight junction permeability. The organization of actin filaments is crucial for the assembly of intestinal tight junctions (Madara et al. 1986) and its disorganization may lead to increased epithelial permeability. Intestinal barrier function is critically regulated by tight junctions which consist of a number of transmembrane proteins, particularly claudins and occludin, and cytoplasmic peripheral membrane proteins, including zonula-occludens-1 (ZO-1), 2 and 3 and cingulin (Clayburgh et al. 2004). Many tight junctions proteins interact with F-actin and these interconnections are considered to stabilize the tight junction. Intestinal pathogens for example *Giardia lamblia* and *Escherichia coli* were reported to rearrange F-actin

distribution in intestinal epithelial cells and compromise barrier function (Gerhard et al. 1998).

ZO-1 is a 220-kDa peripheral membrane protein that interacts with tight junctional occludin at its N terminus and with cytoskeletal F-actin at its C terminus. ZO-1 plays an intermediary role between the cytoskeleton and the tight junction and thus has importance in regulating paracellular permeability (Fig 5.1) (Chin et al. 2002). ZO enterotoxins (Zot) released by some pathogens bind to Zot receptors expressed on mature intestinal epithelial cells and in turn cause the displacement of tight junctional proteins including ZO-1 (Fasano et al. 1991). Studies have shown that disruption of ZO-1 integrity can increase epithelial permeability and decrease transepithelial electrical resistance (TER) (Youakim and Ahdieh 1999).

TER is the measurement of electrical physical resistance provided by a cell monolayer. To measure TER, an electrical pulse of known amplitude is passed across the epithelium and the corresponding transepithelial voltage deflection is measured. Ohm's law is used to calculate electrical resistance and it is corrected for system and fluid resistance. Transepithelial electrical resistance is thought to be well correlated with the change in paracellular permeability of the cell monolayer (Madara 1988). Generally, transepithelial electrical resistance decreases during many gastro-intestinal infections and it is inversely proportional to the permeability of epithelial monolayer. In conclusion, understanding the pathogen’s effects on regulation of barrier function may yield new insights into the pathogenesis of disease that it causes.

In chapter 4, it was shown that *Blastocystis* can cause apoptosis of IECs and it has been suggested that epithelial cell apoptosis caused by the protozoan *G. lamblia* can contribute to the disorders of intestinal permeability (Chin et al. 2002). In following experiments, aim was to characterize the effects of *Blastocystis* on tight junctions and the cytoskeleton of intestinal epithelial cells. In addition, it was investigated if *Blastocystis* can modulate the permeability of the intestinal epithelium.

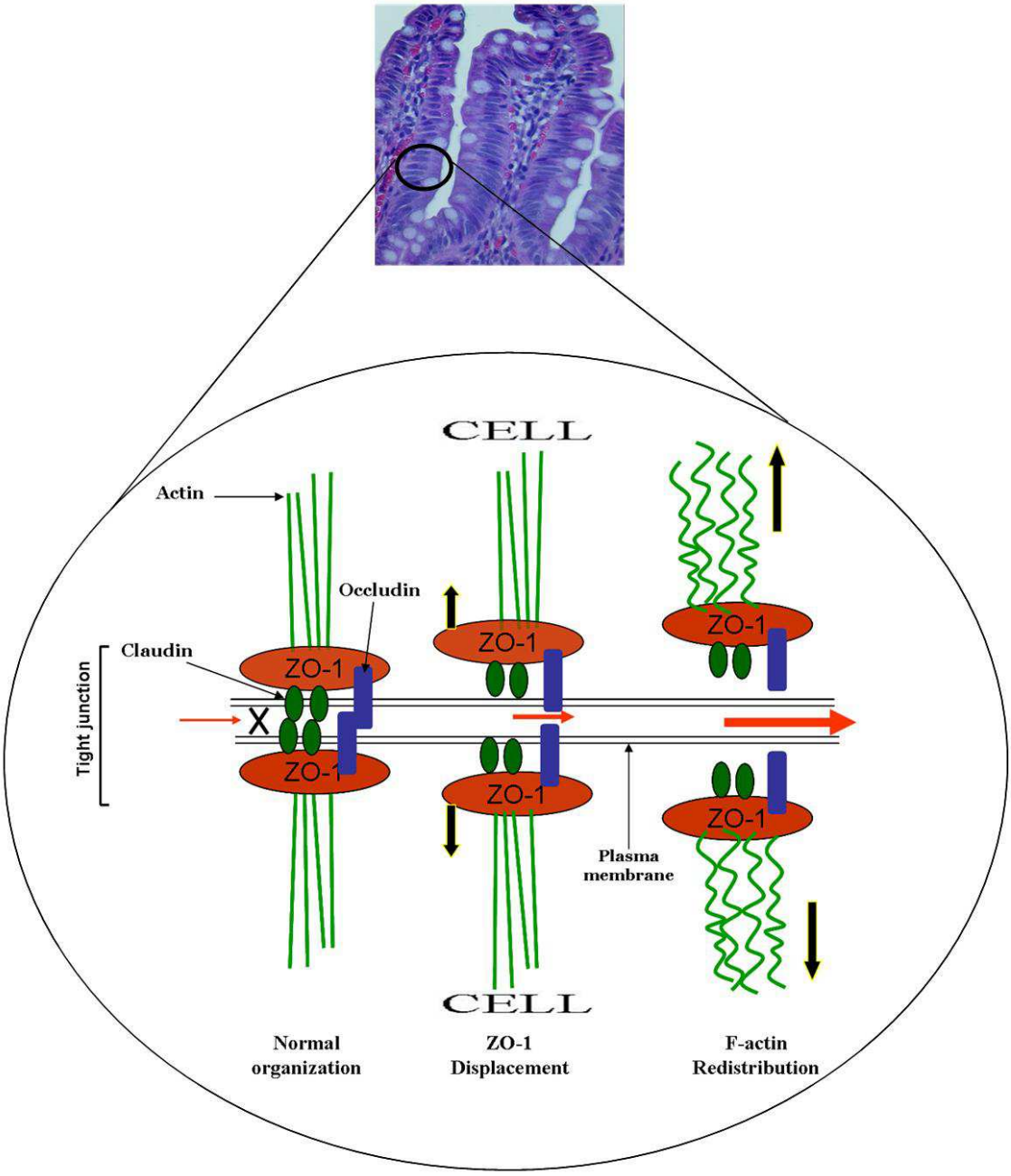

Fig 5.1 A simplified schematic of tight junctions. Tight junction protein ZO-1 interacts with actin filaments and the occludin-claudin complex. Displacement of ZO-1 or abnormalities in the distribution of F-actin may affect tight junction integrity and thus barrier function.

5.2 MATERIALS AND METHODS

5.2.1 Culture of non-transformed rat intestinal cell line

All experiments were performed with a non-transformed rat intestinal epithelial cell line, IEC-6 (ATCC). IEC-6 cell were cultured and maintained as described in chapter 4. The culture medium was replenished every 2-3 days. Cell viability was analyzed by Trypan Blue assay and only cell cultures with >95% viability were used. Cells were trypsinized with 0.25% trypsin-EDTA (Gibco) and 2×10^5 cells/ml seeding density was used. Cells were grown to confluency on poly-L-lysine treated 12 mm glass coverslips. For transepithelial resistance (TER) and monolayer permeability assays, cells were grown on 0.6 cm^2 Millicell-HA filters (Millipore) using 24 well tissue culture plates (costar) (Fig 5.2A).

For the ZO-1 study, HCT8 (ATCC) human ileocecal epithelial cells were grown in RPMI 1640 medium containing 10% FBS, 1 mM sodium pyruvate, and 2 mM l-glutamine (HCT8 medium). HCT8 cells were grown at 37°C in a 5% CO_2 environment. Problems of IEC-6 detachment from coverslips during harsh immunostaining procedure were experienced and thus HCT8 cells were used for ZO-1 study. HCT8 cells also exhibit tight junctions and have been used to study ZO-1 (Jensen-Jarolim et al. 1998).

5.2.2 Parasite culture and preparation of lysate

Blastocystis ratti isolate WR1 was cultured as described in chapter 4. Five days old parasites at log phase were washed 2 times in ice cold IEC-6 complete media at 500 × g for 10 min at 4^0C. The pellet was resuspended in IEC-6 cell complete media, parasites were counted with a hemocytometer and concentration was adjusted to 1×10^7 parasites/ml. Parasites were examined microscopically for their viability in IEC-6 and HCT8 complete medium and found to be viable for >48 h in IEC-6/HCT8 growth conditions. Parasitic lysates were prepared by 3 freeze-thaw cycles in liquid nitrogen and 37^0C water bath respectively.

5.2.3 Inoculation protocol and experimental planning

A density of 1×10^7 parasites/ml was used for inoculation. Either live parasites or parasitic lysate were added to the apical side of confluent monolayers grown on 0.6 cm^2 Millicell-HA filters (400 µl). For phalloidin-FITC staining, 2-3 days old confluent monolayers were used. For TER and epithelial permeability experiments, monolayers grown on Millicell-HA filters were used 5-6 days after seeding when they reached peak electrical resistance of ~30 Ω/cm^2. In some TER and permeability assays, antiprotozoal drug metronidazole (10 µg/ml, Sigma) was added in medium prior to addition of live parasites. Monolayers were washed twice with cold Hanks Balanced Salt Solution (HBSS, Gibco) before addition of parasite or lysate suspension.

5.2.4 Phalloidin-FITC staining of F-actin

IEC-6 cells were grown on poly L-lysine coated glass coverslips and confluent monolayers were coincubated with live parasites, parasitic lysate or 5 µg/ ml cholera toxin as positive control for 24 and 48 hrs. After incubation, cells were washed with PBS and fixed in 3.7% paraformaldehyde in PBS for 5 min. Cells were washed with PBS and dehydrated with acetone, permeabilized with 0.1% TritonX-100 in PBS and washed again in PBS. Cells were stained with 50 µg/ml phalloidin-FITC conjugate solution in PBS for 40 min at room temperature and then washed 5-6 times with PBS to remove unbound phalloidin conjugate. Coverslips were mounted on glass slides with fluorescent mounting medium (Vectashield) and viewed by confocal microscopy (Olympus Fluoview FV500).

5.2.5 Tight junctional ZO-1 immunostaining

HCT-8 cells were grown on LabTek chamber slides (NUNC). 4-5 days old confluent monolayers were used to determine the effects of *Blastocystis ratti* WR1 on tight junctional ZO-1 integrity. Cells were coincubated with live parasite or parasitic lysates for 24 h. Monolayers were washed and fixed with fresh 2% paraformaldehyde in PBS (2 h, RT) and washed three times with PBS (5 min, RT). Cells were permeabilized with 0.4% Triton X-100 (15 min, room temperature) and washed three times with PBS (5 min, RT). Nonspecific binding was blocked with pure FBS (Gibco) (10 min, RT) and washed three times with PBS (5 min, RT). After this, the monolayers were incubated (37°C, 1 h, humidified chamber) with rabbit polyclonal anti-ZO-1 antibody (1:200 in PBS; Zymed)

and washed three times with PBS (5 min, RT). Next, the monolayers were incubated (37°C, 1 h, dark humidified chamber) with Goat anti-rabbit IgG conjugated to FITC (1:200 in PBS; Sigma) and washed three times with PBS (5 min, RT). Lastly, monolayers were mounted with fluorescent mounting medium (Vectashield) and visualized by confocal microscopy (Olympus Fluoview FV500).

5.2.6 Measurement of transepithelial resistance

Transepithelial resistance was determined with a Millipore Electrical Resistance system (Millipore-ERS) (Fig 5.2B). IEC-6 monolayers were grown on 12mm Millicell-HA filters and TER was tested on alternate days until it reached peak TER (~30Ω/cm^2) by day 6 (Fig. 5.2). Parasites or lysate were added to the apical side of confluent monolayers grown on Millicell-HA filters and coincubated for 12, 24 and 48 hr. After coincubation, monolayers were washed 2 times with HBSS to ensure that parasites would not affect TER measurements. 400µl warm (37^{0}C) complete media was added to the apical compartment and TER was measured with Millipore-ERS. To avoid fluctuations in temperature, TER was measured at 37^{0}C with the aid of a heating plate. Epithelial resistance was expressed in Ω/ cm^2. The complete procedure for measuring TER is explained in the appendix V.

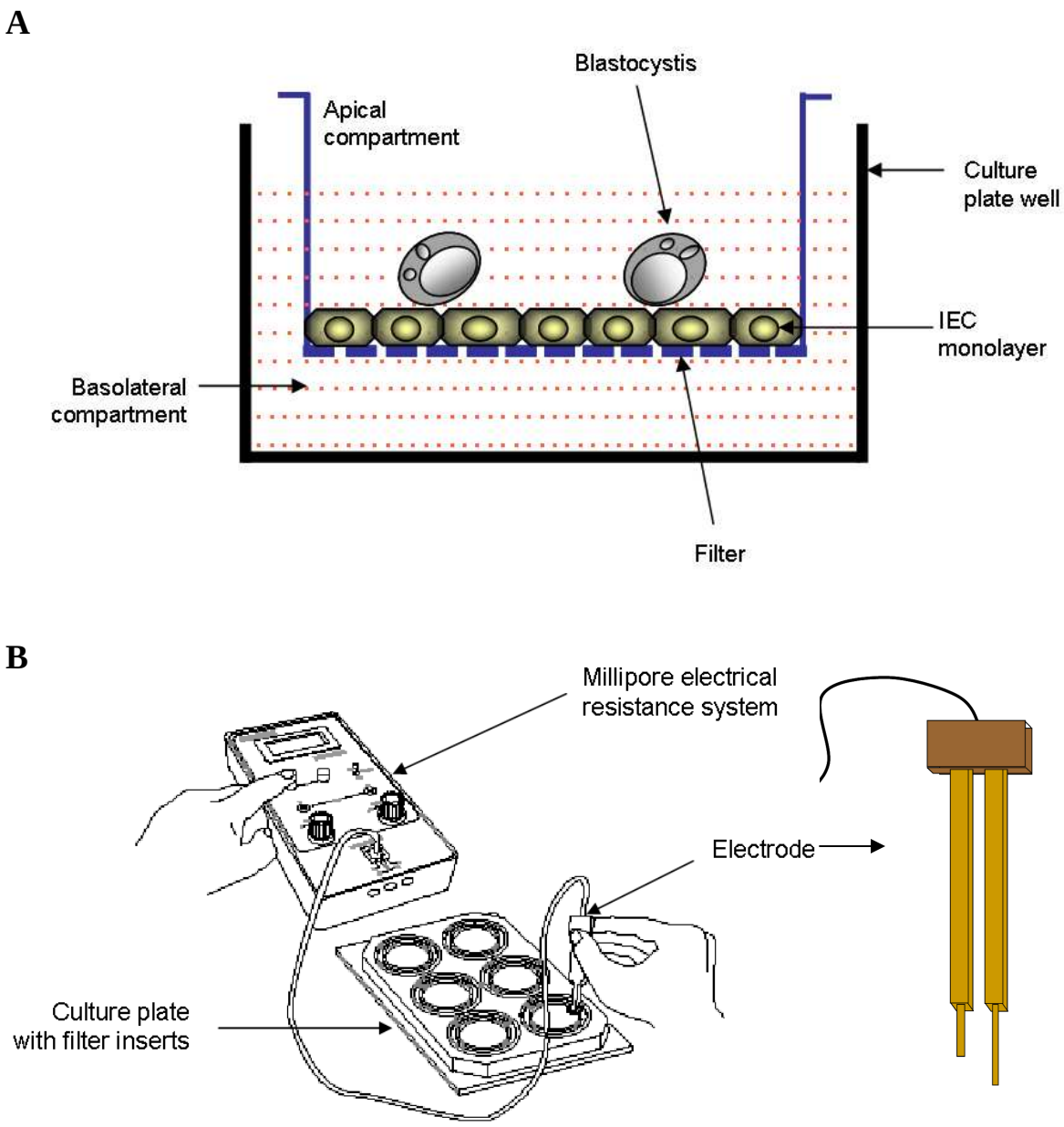

Fig. 5.2 Simplified diagrammatic representation of Millicell-HA filter inserts and Millipore electrical resistance system. (A) IEC-6 cells were grown on the filter insert, and live *Blastocystis ratti* WR1 or parasitic lysates were added to the apical compartment to study transepithelial resistance and permeability. (B) Experimental setup for the measurement of transepithelial resistance showing Millipore electrical resistance system and an electrode with a short and a long leg. To measure TER, electrodes are immersed in a way that the shorter electrode is in the apical compartment of the culture plate insert and the longer electrode is in the outer well. The shorter electrode should not contact cells growing on the membrane. (Picture of Millipore electrical resistance system is adapted from www.millipore.com)

5.2.7 Determination of epithelial permeability by Lucifer yellow

To confirm confluence of cell monolayers grown on Millicell-HA filters, transepithelial resistance was measured with Millipore-ERS (Fig. 5.2). After reaching peak transepithelial resistance (~30Ω/cm^2), the effect of *Blastocystis* on epithelial barrier function was assessed with Lucifer yellow (482 MW, Molecular Probes). In brief, after 24 h of coincubation, apical and basolateral compartments were washed gently 3 times with HBSS. 300µl of Lucifer yellow (100µg/ml in HBSS) was added to apical compartment and 500µl of HBSS was added to the basolateral compartment. After 3 h of incubation at 37^{0}C in a humidified incubator with 5% CO_2, 250µl of sample was taken from the basolateral compartment. Fluorescence absorbance was determined using wavelengths of 485nm excitation and 535nm emission. Values were expressed as percent Lucifer yellow passage across the cell monolayer.

5.3 RESULTS

5.3.1 Rearrangement of F-actin

The cytoskeleton, in particular the actin microfilaments, is key in maintaining cell shape and regulating tight junction permeability. The organization of actin filaments is crucial for the assembly of intestinal tight junctions (Madara et al. 1986). Phalloidin, a fungal toxin, binds to F-actin with high affinity and stabilizes it. Phalloidin-FITC conjugate was used to detect changes in F-actin distribution. When IEC-6 monolayers were coincubated with *B. ratti* WR1 live parasites, elongation of cortical actin filaments, actin disruption, and formation of stress fibers in the cytoplasmic zone was noticed (Fig. 5.3). A more pronounced formation of stress fibers and actin condensation was noticed in the monolayers incubated with parasitic lysate and cholera toxin (positive control) (Fig. 5.3 B and C). IEC-6 monolayers in controls showed normal actin distribution in the cortex zone of cells with negligible stress fiber formation in the cytoplasmic area (Fig. 5.3 D). IEC-6 cells incubated with live parasites and parasitic lysate for 24 h showed significantly high percentage of cells showing stress fibers formation (34.3% and 36.4%) in comparison to controls (5.7%) (Table 5.1). After 48 h coincubation, the percentage of cells showing stress fiber formation was slightly higher than at 24 h (Table 5.1).

5.3.2 ZO-1 displacement from tight junctions

In tight junctions, ZO-1 acts as an intermediary junction between the cytoskeleton (F-actin) and the tight junctional proteins (occludin) thus have importance in regulating paracellular permeability. HCT8 monolayers were exposed to the *Blastocystis ratti* WR1 live parasites and parasitic lysates and performed ZO-1 immunostaining. Results (Fig. 5.4) show that ZO-1 protein from tight junctions is displaced in cells exposed to live parasite and parasitic lysates whereas control monolyers showed continuous pericellular organization of ZO-1 typical of a healthy cell. Significantly high percentage of cells showed ZO-1 displacement after exposure to live parasite (45.7 ± 4.8) and parasitic lysate (65.0 ± 7.0) in comparison to control cells (9.9 ± 2.1) (for both, $P < 0.05$ Vs control; Table 5.2). In *Blastocystis ratti* WR1-exposed cells, migration of ZO-1 towards the nuclei was observed.

5.3.3 Decrease in transepithelial resistance

F-actin rearrangement has been associated with reduced TER in a number of studies (Hecht et al. 1988; Teoh et al. 2000). Hence, experiments were conducted to investigate if rearrangement of F-actin induced by *Blastocystis* is associated with changes in TER. IEC-6 cell monolayers achieved a peak average resistance of ~30Ω/cm^2 after about 6 days from seeding. *B. ratti* WR1 caused a significant decrease in transepithelial resistance of IEC-6 monolayers in a time dependent manner (Fig. 5.5). Epithelial resistance of monolayers coincubated with *B. ratti* WR1 live parasites dropped from 32.2 ±1.1 to 27.2 ±1.1 Ω/cm^2 by

12 h, from 32.8 ±1.5 to 24.2 ±2.6 Ω/cm^2 by 24 h and dropped further from 32.6 ±1.7 to 22.4 ±2.6 Ω/cm^2 by 48 h (for all, P <0.05 in comparison to control). A similar significant decrease in TER was observed with parasitic lysate which suggested that parasitic soluble and non-soluble products are capable of inducing a decrease in TER. To show that the effect on transepithelial resistance was due to live parasites, antiprotozoal drug metronidazole (10µg/ml) was added in the incubation medium as the drug has been reported to induce *Blastocystis* cell death (Nasirudeen et al. 2004). Addition of metronidazole to monolayers coincubated with live parasites, significantly prevented a reduction in TER after 24 h, bringing the levels of TER close to control values (P <0.05 in comparison to monolayers incubated with live parasites only). In contrast, a significant reduction in TER was noted in monolayers coincubated with live parasites only (Fig. 5.6).

To test if *Blastocystis*-induced apoptosis of IEC-6 cells plays an important role in inducing a reduction in TER, monolayers were pretreated with general caspase inhibitor Z-VAD-fmk before coincubation with parasites. Results show that the inhibition of caspases in IEC-6 cells did not significantly prevent reduction of TER (P =0.07 in comparison with monolayers incubated with parasites only, Fig. 5.6).

5.3.4 Increase in epithelial permeability

The effects of *B. ratti* WR1 live parasites and parasitic lysate on permeability of IEC-6 monolayers were analyzed by measuring apical to basolateral flux of Lucifer yellow. IEC-6 cells exposed to *B. ratti* WR1 live parasites or parasitic lysates disrupted the monolayer

barrier function as evidenced by an increased flux of Lucifer yellow across the monolayer (Fig. 5.7). There was a significant increase in the flux of Lucifer yellow in monolayers coincubated for 24 h with live parasites (22.4% ±1.2, P <0.05), and with parasitic lysates (22.1% ±1.2, P <0.05) in comparison to control monolayers (17.2% ±0.6). Pretreatment of IEC-6 monolayers with general caspase inhibitor Z-VAD-fmk prior to coincubation with live parasites, did not significantly decrease Lucifer yellow flux (20.6% ±0.3, P =0.2 in comparison to live parasite treated monolayers) across monolayers as compared to parasite free control (17.2% ±0.6). This suggests that *Blastocystis*-induced apoptosis of intestinal epithelial cells might not play a major role in disrupting epithelial permeability but there are other factors responsible for this effect.

To prove that changes in permeability of IEC-6 cell monolayer was due to the effect of live parasites of *Blastocystis*, antiprotozoal drug metronidazole was added during coincubation and it was found that it significantly abrogated the increased flux of Lucifer yellow across IEC-6 cell monolayer (18.5% ±0.4, P <0.05 in comparison to live parasite treated monolayers).

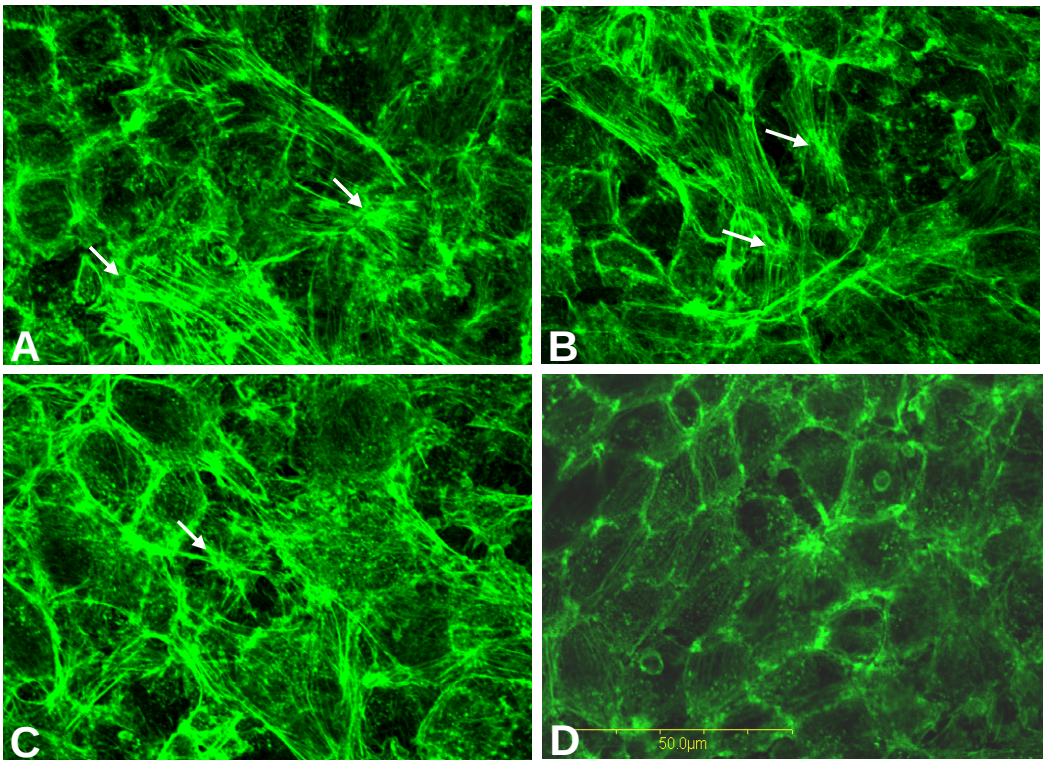

Fig. 5.3 Effect of *B. ratti* WR1 exposure on actin cytoskeleton. IEC-6 cells were grown to confluency on poly-L-lysine treated 12 mm glass coverslips, stained with fluorescein-phalloidin and analyzed by confocal microscopy. Prominent formation of stress fibers (arrow) can be noticed in monolayers coincubated with *B. ratti* WR1 live parasite (A), parasitic lysate (B), and 5 µg/ ml cholera toxin (C) as positive control. Negative control (D) showing normal distribution of F-actin in the cortex zone of cells. Bar = 50µm

Period of incubation	Cells coincubated with WR1 live parasite (%)	Cells coincubated with parasitic lysate (%)	Negative control (%)	Cells coincubated with 5µg/ml cholera toxin (positive control) (%)
24 hrs incubation	34.3	36.4	5.7	42.8
48 hrs incubation	37.2	45.7	7.8	60.7

Table 5.1. Percentage of IEC-6 cells showing stress fiber formation in response to *Blastocystis ratti* WR1 infection (500 cells were counted for each sample).

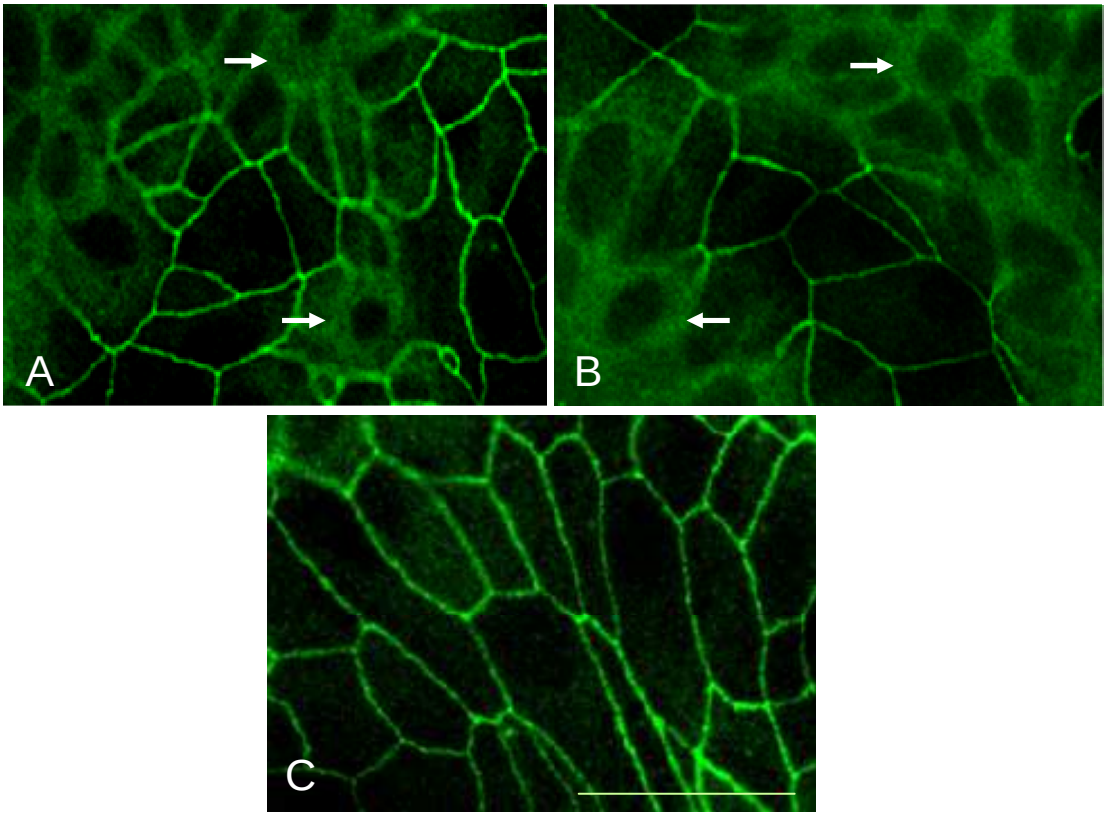

Fig. 5.4 Representative confocal scanning laser micrographs illustrating ZO-1 integrity in human HCT-8 epithelial monolayer. HCT-8 cells were grown on LabTek chamber slides. Monolayers were fixed, permealized and immunostained with rabbit polyclonal anti-ZO-1 antibody. Control monolayer (C) exhibits typical ZO-1 pericellular organization at the periphery of enterocytes. Monolayers incubated with whole parasite (A) or parasitic lysate (B) exhibit focal disruption of ZO-1 along pericellular junctions (arrows). Bar = 50µm

Cells coincubated with WR1 live parasite	Cells coincubated with parasitic lysate	Negative control
45.7 ± 4.8	65.0 ± 7.0	9.9 ± 2.1

Table 5.2 Percentage of HCT-8 cells showing displacement of ZO-1 at pericellular junctions. The data present the mean ±SD from 2 different experiments.

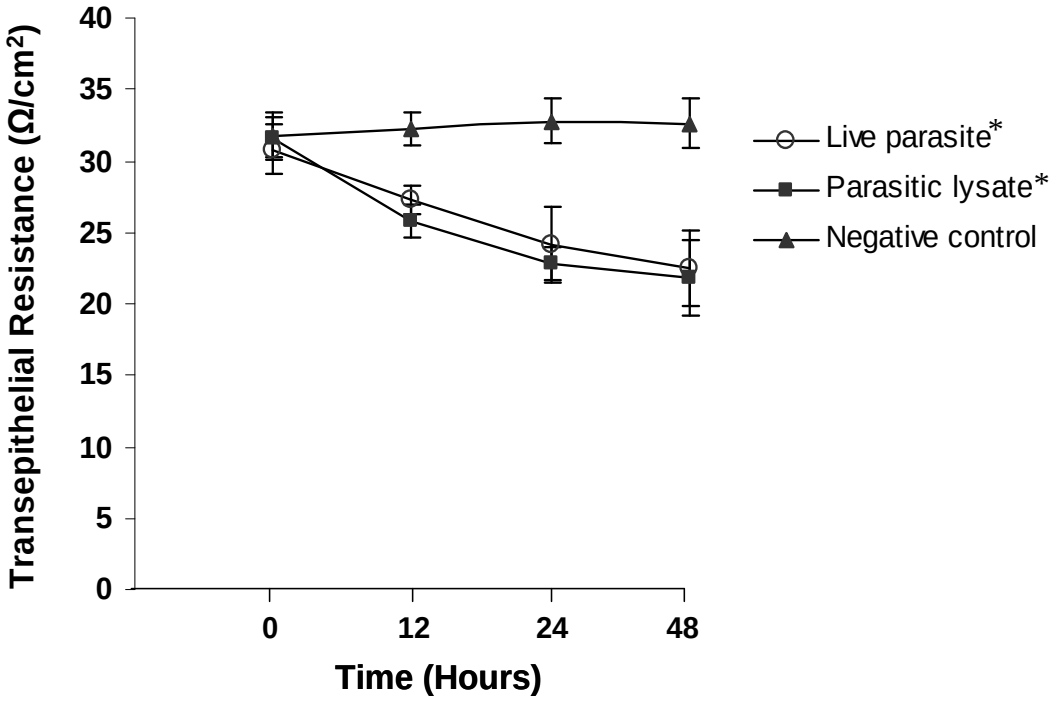

Fig. 5.5 Effect of *B. ratti* WR1 on transepithelial resistance of IEC-6 cell monolayers. Confluent monolayers of IEC-6 cells were grown on Millicell-HA filters and coincubated for indicated times with live parasite, parasitic lysate or growth media (negative control). Thereafter, transepithelial resistance (TER) was measured using Millipore electrical resistance system. Live parasite and lysate-treated monolayers showed a significant drop in TER after 12, 24 and 48h. Values are means ± standard deviations (n = 3 per group). *P < 0.05 versus negative control for all time points.

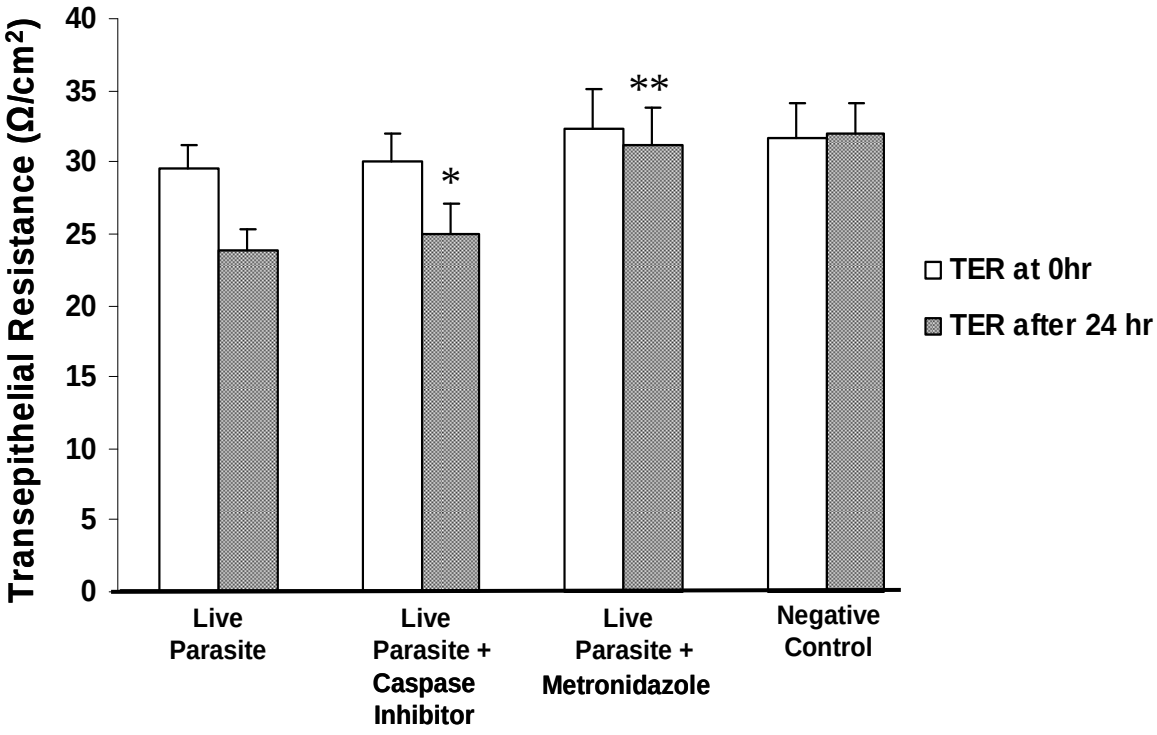

Fig. 5.6 Effect of caspase inhibition and metronidazole on *B. ratti* WR1-induced decrease in transepithelial resistance of IEC-6 monolayers. Confluent monolayers of IEC-6 cells were grown on Millicell-HA filters and incubated for 24h with live parasite, live parasites after pretreatment of cells with broad spectrum caspase inhibitor Z-VAD-fmk, or live parasites along with antiprotozoal drug metronidazole. Monolayers incubated with growth media served as negative control. Thereafter, transepithelial resistance (TER) was measured using Millipore electrical resistance system. Treatment with caspase inhibitior does not considerably abolish *Blastocystis*-induced effect but metronidazole treatment abolished the effect significantly. Values are means ± standard deviations (n = 3 per group). *, P = 0.07; **, $P < 0.05$ (both versus live parasite treated samples).

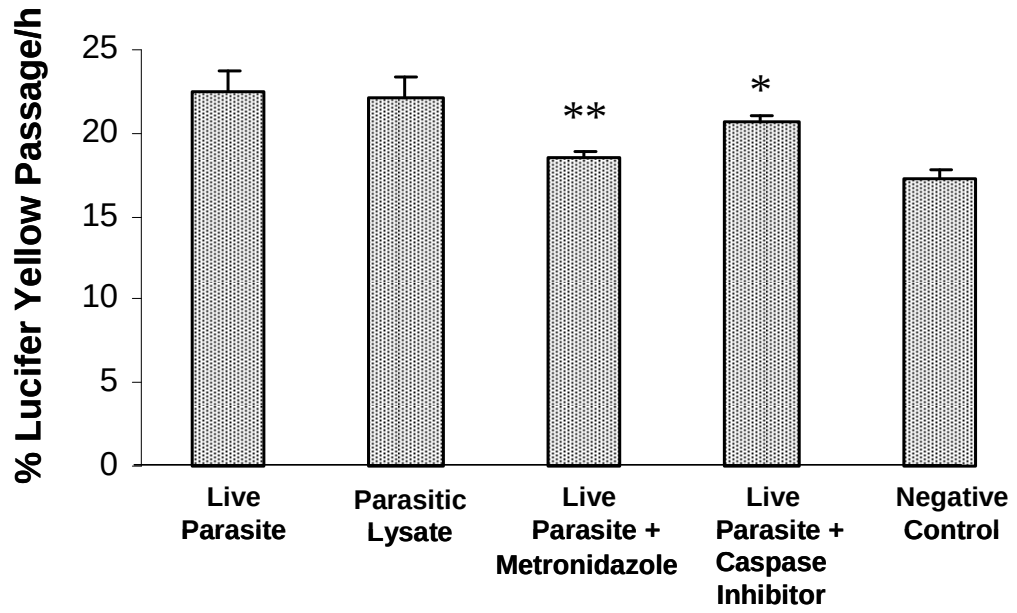

Fig. 5.7 Flux measurement with Lucifer yellow. Confluent monolayers of IEC-6 cells were grown on Millicell-HA filters and incubated for 24h with live parasite, live parasites after pretreatment of cells with broad spectrum caspase inhibitor Z-VAD-fmk, or live parasites along with antiprotozoal drug metronidazole. Monolayers incubated with only growth media served as negative control. Permeability was determined by measurement of Lucifer yellow fluxes across the monolayer. Mean values of two experiments ±SD are shown. Significant increase in epithelial permeability can be noticed after incubation with live parasite and parasitic lysate in comparison to control monolayer. Caspase inhibition does not considerably abolish the *Blastocystis*-induced effect on permeability but metronidazole treatment abolished the effect significantly. Values are means ± standard deviations (n = 3 per group). *, P = 0.2; **, $P < 0.05$ (both versus live parasite treated samples).

5.4 DISCUSSION

In this study, it was demonstrated that *B. ratti* WR1 rearranges F-actin distribution, displaces ZO-1, decreases transepithelial electrical resistance, and increases epithelial permeability across the cell monolayer. Furthermore, results showed that the antiprotozoal drug metronidazole abolishes the effects of *B. ratti* WR1 on epithelial barrier function. Together, these findings suggest for the first time that *Blastocystis*-induced disruption of the epithelial barrier function may lead to the pathogenesis of *Blastocystis*-related gastrointestinal symptoms.

A variety of intestinal pathogens have been shown to cause changes in F-actin arrangement in intestinal epithelial cells (Teoh et al. 2000) and it was reported that dynamic rearrangements of F-actin contribute to an increase in epithelial permeability (Hecht et al. 1988) and affects regulation of epithelial salt and water transport (Matthews et al. 1994) which may consequently lead to diarrhea. *Blastocystis* has been shown to cause cytopathic effects in Chinese Hamster Ovary cells (Walderich et al. 1998) but its effects on intestinal epithelial cytoskeleton and barrier functions were not reported. The present study demonstrates for the first time that *Blastocystis* adversely affects the cytoskeletal proteins in intestinal epithelial cells, causing a rearrangement of F-actin distribution. IEC-6 cells exposed to live *B. ratti* WR1 and parasitic lysate showed enhanced formation of condensed actin filaments in cells. A significant number of IEC-6 cells exhibited the formation of stress fibers in cytoplasmic zone of cells.

Findings that *B. ratti* WR1 causes apoptosis of intestinal epithelial cells (previous chapter) and rearrangement of F-actin and ZO-1 distribution, prompted us to assess the effect of *Blastocystis* on transepithelial electrical resistance and permeability. An *in vitro* model involving confluent monolayers of IEC-6 cells grown on filters were used and findings demonstrated that on interactions with apical surface of the epithelial cells, *B. ratti* WR1 causes significant decrease in epithelial resistance and increase in permeability across monolayers. The intestinal mucosa forms a barrier that separates luminal contents from the interstitium and the intestinal barrier can become compromised in parasitic infections, bacterial infections, and in many intestinal diseases in particular inflammatory bowel disease (Clayburgh et al. 2004). Intestinal barrier function is mainly regulated by tight junctions and the function of tight junctions can be measured as a decrease in transepithelial electrical resistance and an increase in paracellular flux of macromolecules (Berkes et al. 2003).

Studies have suggested that *Blastocystis* may interact with epithelial cells of the gastrointestinal tract to modulate their transport characteristics and increase intestinal permeability (Dagci et al. 2002). *In vitro* findings from this study show for the first time that *Blastocystis* has the ability to disrupt barrier function of the intestinal epithelium. Results consistently showed an increase in transepithelial flux of Lucifer yellow with decrease in TER in the IEC-6 monolayers exposed to live *B. ratti* WR1 and parasitic lysates. Results from experiments suggest that *Blastocystis*-induced F-actin and ZO-1 rearrangement is associated with reduced TER and altered permeability. The cause of *Blastocystis*-associated diarrhea is so far unidentified, but an association between increase

in epithelial permeability and diarrhea has been described for other diseases (Madara 1988; Madara 1990). It is possible that *Blastocystis* induced changes in permeability may also occur *in vivo* and lead to electrolyte imbalance and diarrhea.

It was observed that monolayers coincubated with *B. ratti* WR1 live parasite in the presence of metronidazole, did not show any change in barrier function and retained their normal TER and epithelial permeability. This finding suggests that the effect on monolayer barrier function was due to live parasite only and dead parasites cannot induce such effects on epithelial TER and permeability. Metronidazole is known to induce programmed cell death in *Blastocystis* and plasma membrane integrity of parasite remains preserved (Nasirudeen et al. 2004), therefore there is no leakage of intracellular parasitic proteases and other products that can induce an effect on TER and permeability. However, a change in TER and permeability was noticed in the monolayers coincubated with parasitic lysates as it contains all parasitic soluble or non-soluble products which would have resulted in the observed effect. *G. lamblia* live parasite and parasitic lysates were shown to exhibit similar effects on intestinal epithelial cytoskeleton and TER (Teoh et al. 2000). In this study, it was also found that *B. ratti* WR1 live parasite and parasitic lysates have similar effects on F-actin distribution and epithelial barrier function.

Previously, it has been reported that *E. histolytica* trophozoite can decrease TER of enteric monolayers (Leroy et al. 2000). In contrast to this study, coincubation of *E. histolytica* parasitic lysate with human colonic epithelial monolayer failed to lower epithelial resistance indicating that the increase in permeability was not due to the release

of proteinases or other cytotoxic molecules (Leroy et al. 2000). It appears that *E. histolytica* induces increase in permeability by invasion and thus directly damaging the epithelium.

The need to treat *Blastocystis* infections is controversial because of its uncertain pathogenicity. There is a lack of standardized treatment for *Blastocystis* infections and studies have shown that metronidazole inhibits the growth of *Blastocystis* (Moghaddam et al. 2005). In clinical cases where *Blastocystis* is implicated for gastrointestinal symptoms, metronidazole is a drug of choice (Tan 2004). Results show that metronidazole can avert the adverse effects of *Blastocystis* on intestinal epithelial barrier function and suggest that metronidazole is a therapeutic drug for *Blastocystis* infections.

In summary, results from the present study show for the first time that *Blastocystis* was able to rearrange F-actin and ZO-1 distribution and disrupt epithelial barrier function; and metronidazole could abrogate these effects. In addition, the current findings show that IEC-6 monolayers grown on permeable filters provide a useful *in vitro* model for studying the interactions of *Blastocystis* with intestinal epithelial cells.

CHAPTER 6:

HOST CELL INTERLEUKIN-8 RESPONSE AGAINST *BLASTOCYSTIS*

6.1 INTRODUCTION

In previous experiments, it was reported that the *Blastocystis ratti* WR1, an isolate of zoonotic potential (Yoshikawa et al. 2004a; Noël et al. 2005), can induce contact-independent apoptosis and disrupt barrier function in IEC-6 cells. It was also showed that it possesses proteases a fraction of which could degrade human secretory immunoglobulin A (sIgA). Reports suggested that this parasite can cause acute and chronic gastroenteritis (Nimri and Batchoun 1994); and inflammation of intestinal mucosa was associated with *Blastocystis* infections (Kain et al. 1987; Russo et al. 1988; Garavelli et al. 1992; Zuckerman et al. 1994). Intense infiltration of inflammatory cells in the colon was shown after *Blastocystis* infection in mice (Moe et al. 1997) and it was reported that *Blastocystis* modulates interleukin-8 (IL-8) response in intestinal epithelial cells (Long et al. 2001), however nothing is known about the parasitic virulence factors and the early events occurring in host cells following *Blastocystis*-host interactions. Proteases from protozoan parasites are known to play significant roles in pathogenesis (McKerrow et al. 1993; Sajid and McKerrow 2002) and proteases from some pathogens have been reported to induce the production of proinflammatory cytokines from host cells (Borger et al. 1999).

IL-8 (CXCL8) is a CXC chemokine which attracts polymorphonuclear leukocytes to the site of inflammation, activates monocytes and is considered to play an important role in the pathogenesis of inflammatory diseases (Oppenheim et al. 1991; Charo and Ransohoff 2006). Expression of the IL-8 gene is regulated by a number of pathways and its promoter region has binding sequences for a range of transcription factors including NF-κB, NF-IL-6,

and AP-1 (Mukaida et al. 1994). In most cell types, activation of NF-κB is the most important step for IL-8 gene transcription (Mori et al. 1999). In unstimulated cells, NF-κB exists in an inactive form in the cytoplasm, bound to inhibitory proteins called IκBs. Stimulation by various inducers may activate a signaling cascade that culminates in the phosphorylation of IκBs, resulting in the degradation of IκB proteins (DiDonato et al. 1997) and the released NF-κB translocates to the nucleus to activate target genes. Numerous pathogens including *Helicobactor pylori* (Beswick et al. 2006), *Escherichia coli* (Dahan et al. 2002), and *Bacteroides fragilis* (Kim et al. 2002) have been shown to induce IL-8 production from host cells via NF-κB activation.

In this chapter, aim was to investigate if *B. ratti* WR1 is capable of inducing IL-8 response from intestinal epithelial cells. Moreover, parasitic factors and molecular events responsible for this IL-8 induction were further investigated. It is demonstrated for the first time, that *B. ratti* WR1 cysteine proteases can activate IL-8 gene expression in human colonic epithelial cells. Furthermore, it is shown that NF-κB activation is involved in the production of IL-8. In addition, findings show that the antiprotozoal drug metronidazole treatment can decrease IL-8 production induced by *B. ratti* WR1.

6.2 MATERIALS AND METHODS

6.2.1 Parasite culture and preparation of lysate

B. ratti WR1, an axenized zoonotic isolate was used in this study and cultured as described in previous chapters. In brief, parasites were cultured in pre-reduced Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% inactivated horse serum (Gibco) and incubated anaerobically at 37^0C in an ANAEROJAR (Oxoid, UK). 4-5 days old parasites at log phase were washed 2 times in ice cold T84 cell complete medium at 500 × g for 10 min at 4^0C. The pellet was resuspended in T84 cell medium supplemented with only 0.5% fetal bovine serum, parasites were counted with a hemocytometer and concentration was adjusted to 1×10^7 parasites/ml. Parasites were examined microscopically for their viability in this T84 cell medium and found to be viable for >48 h in T84 growth conditions. Parasitic lysates were prepared by 3 freeze-thaw cycles in liquid nitrogen and 37^0C water bath respectively.

6.2.2 Colonic cell culture, inoculation protocol and experimental planning

T84 human colonic carcinoma epithelial cells were obtained from American Type Culture Collection. T84 cells are considered a good *in vitro* model to study IL-8 (Yu and Chadee 1997) and these cell lines have been widely used in other studies involving host cytokine responses (Yu and Chadee 1997; Zhou et al. 2004; Bandyopadhaya et al. 2007). T84 cell stock was maintained in T-75 flasks in a humidified 37^0C incubator with 5% CO_2

and passages 20-25 were used for all experiments. The complete growth medium consisted 1:1 mixture of Dulbecco modified Eagle medium and Ham's F-12 (DMEM/F-12) (Sigma) supplemented with 5 % heat inactivated fetal bovine serum (Gibco). Cell viability was analyzed by Trypan Blue assay and cell cultures with >95% viability were used for the experiments. Cells were trypsinized with 0.25% trypsin-EDTA (Gibco) and seeded in 6 well plates (Costar) and grown to 75-80 % confluency for all experiments. In some assays, cells were grown to confluency on collagen (BD) coated 12 mm glass coverslips in 12 well tissue culture plates (Costar). As described previously, a concentration of 1×10^7 parasites or equal parasitic lysate per 1 ml medium was used for all experiments. Either live parasites or parasitic lysate were added to T84 cells grown in 6 well or 12 well plates and coincubated for indicated time points in humidified 37^0C incubator with 5% CO_2. Culture medium was changed one day before experiments and fresh complete medium supplemented with only 0.5 % heat inactivated fetal bovine serum was used during incubation with parasite or parasitic lysate.

In protease inhibition experiments, parasitic lysates were preincubated for 1 hr in T84 complete medium containing one of the following inhibitors: EDTA, E-64, Pepstatin A (all 10μM); PMSF (1 μM), and iodoacetamide (50 μM). For antiprotozoal drug treatment, live parasites were pretreated in complete medium with 10 μg/ml metronidazole (Sigma) prior to experiments. In some experiments, for NF-κB inhibition, T84 cells were pretreated for 3 hr in complete medium containing 30 μM BAY11-7082 (Sigma). Cholera toxin (5 μg/ml) was added in the medium in some experiments as a positive control for the production of IL-8.

In addition to T84 cells, just to study reproducibility in another cell line, human colon adenocarcinoma cells HT29 (ATCC) were used in NFκB nuclear translocation experiment. HT29 culture medium consisted of Dulbecco modified Eagle medium (DMEM, Sigma) and 10 % heat inactivated fetal bovine serum (Gibco).

6.2.3 ELISA & real-time reverse transcription-polymerase chain reaction (RT-PCR) for interleukin-8

An IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R & D) was used to measure IL-8 in the supernatants of T84 cell cultures co-incubated with *B. ratti* WR1 live parasite or parasitic lysates for various time points. For real-time PCR, total cellular RNA was extracted by single-step method using Trizol reagent (Invitrogen). First strand cDNA was synthesized with 1 μg RNA using SuperScript II RT (Invitrogen) following manufacturer's instructions. The quantitative real-time PCR was performed with an Applied Biosystems 7500 instrument (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). The reaction conditions were designed as follows: initial denaturation at 95^0 C for 10 min, followed by 40 cycles of 15 s at 95^0 C, 1 min at 60^0 C, and finally 15 s at 95^0 C, 1 min at 60^0 C and 15 s at 95^0 C. Relative quantitation of IL-8 mRNA was calculated by $\Delta\Delta C_T$ method and the amount of the target relative to the β-actin mRNA was expressed as $2^{-(\Delta\Delta CT)}$. The primers used were: IL-8 sense (5' ATGACT TCCAAGCTGGCCGTGGCT 3') and antisense (5' TCTCAGCCCTCTTCAAAAAC

TTCTC 3'); β-actin sense (TGACGGGGTCACCCACACTGTGCCCATCTA 3') and antisense (5' CTAGAAGCATTGCGGTGGACGATGGAGGG 3').

6.2.4 Western blot for IκB-α

T84 cells grown on 6 well plates were exposed to *B. ratti* WR1 live parasite and parasitic lysate for 6 h were first lysed in cell lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM EDTA, 1% Triton X-100, 0.5% SDS and protease inhibitor cocktail). Equal amounts of proteins were loaded on 10% polyacrylamide gel for SDS-PAGE in the Mini-PROTEAN II system (Bio-Rad) and blotted onto nitrocellulose membrane (Amersham). After blocking with 5% nonfat milk in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.1% Tween-20), the membrane was probed first with rabbit anti-IκB-α antibody (Santa Cruz) and then with HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz). Rabbit anti-actin antibody (Sigma) was used as a loading control. Blots were developed on Hyperfilm ECL (Amersham) using chemi-luminescent ECL Plus Detection Reagent (Amersham). Density of bands was quantified by a gel documentation and analysis system (Gel Doc XR, Bio-Rad).

6.2.5 EMSA and measurement of NF-κB activation by ELISA

Nuclear extracts were prepared using a NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce) and electrophoretic mobility shift assay was performed according to manufacturer's protocol using LightShift chemiluminescent EMSA kit

(Pierce). Target DNA sequences were used as described previously (Sun et al. 2006). Oligonucleotide duplexes containing either wild-type NF-κB binding site (sense strand, 5' GATCCGTGGAATTTCCTCTG 3') present in the IL-8 promoter or a mutant NF-κB binding site (sense strand, 5' GATCCGTTAACTTTCCTCTG 3') were biotinylated at 3' end (synthesized by 1st Base, Singapore).

In a separate experiment, the NF-κB activity was measured with a commercial ELISA kit (TransAM NF-κB p65 Assay Kit; Active Motif) as described by manufacturer. In brief, this assay uses a 96 well plate coated with an oligonucleotide containing the NF-κB consensus binding site (5'-GGGACTTTCC-3'). The active form of NF-κB in the nuclear extracts binds to the consensus site and detected by a primary antibody specific for the activated p65 of NF-κB. A HRP-conjugated secondary antibody is then used for the colorimetric quatification by spectrophotometry at 450nm. Results are expressed as fold increase over the control group.

6.2.6 Immunostaining for NFκB nuclear translocation

T84 cells grown on collagen coated glass coverslips were exposed to *B. ratti* WR1 parasitic lysate for 6 h and fixed in 2 % paraformaldehyde for 30 min. All procedures were carried out at room temperature. Monolayers were permeabilized with 0.25 % Triton-X 100 for 15 minutes and washed 3 times with PBS (1 minute each). Monolayers were blocked with blocking solution (Protein Block, DAKO) for 10 minutes and solution was decanted. Monolayers were incubated for 1 h with 1:100 dilutions of rabbit anti-NF-

κB p50 antibody (Santa Cruz) diluted in 0.2 % bovine serum albumin and were subsequently washed 3 times with PBS (5 minutes each). Cells were incubated for 30 minutes in the dark with 1:200 dilutions of a Cy3-conjugated sheep anti-rabbit secondary antibody (Sigma) diluted in 0.2 % bovine serum albumin. Monolayers were washed 4 times with PBS (5 minutes each) and mounted with mounting medium (Vectashield) with 4', 6-diamidino-2-phenylindole (DAPI) to counterstain the nucleus. Monolayers were visualized by fluorescent microscopy (Olympus) and images were captured using the Image Pro software.

6.3 RESULTS

6.3.1 Cysteine proteases of *B. ratti* WR1 induce IL-8 production

To assay for IL-8 protein in culture supernatants, T84 cells were incubated with *B. ratti* WR1 live cells or lysates for various time points. As shown in Fig. 6.1, significant production of IL-8 protein occurred 12, 24 and 48 h after stimulation with *B. ratti* WR1 live parasite (166 ± 22, 482 ± 58, and 620 ± 60 pg/ml respectively; $P < 0.05$) and with parasitic lysates (260 ± 25, 654 ± 64, and 802 ± 87 pg/ml respectively). *B. ratti* WR1 induced-increase in IL-8 was significantly inhibited with the use of protease inhibitor cocktail for 12, 24, and 48 hrs (149 ± 16, 395 ± 27, 492 ± 34 pg/ml respectively; $P < 0.05$ in comparison to lysate). These observations suggest that *B. ratti* WR1 proteases are involved in the induction of IL-8 from T84 cells. Pretreatment of live *B. ratti* WR1 parasites with the antiprotozoal drug metronidazole significantly retarded the ability of parasites to induce increase in IL-8 production at 12, 24, and 48 h (111 ± 8, 289 ± 26, 429 ± 15 pg/ml respectively; $P < 0.05$).

To study the type of particular protease involved, different protease inhibitors were used and it was found that cysteine protease inhibitors iodoacetamide (243 ± 9; $P = 0.002$), and E-64 (265 ± 23 pg/ml; $P = 0.007$) significantly inhibited IL-8 increase induced by parasitic lysates at 24 h (Fig. 6.2). A less significant reduction in IL-8 production is also seen with aspartic protease inhibitor pepstatin A (426 ± 57; $P = 0.048$). Insignificant inhibition is noticed with serine and metallo protease inhibitors, PMSF and

EDTA respectively. These findings suggest that *B. ratti* WR1 cysteine proteases are mainly responsible for the induction of increased IL-8 from T84 cells and aspartic proteases also contribute to some extent to IL-8 production.

6.3.2 *B. ratti* WR1 cysteine proteases increase IL-8 mRNA levels in human colonic cells

To determine whether *B. ratti* WR1 or its cysteine proteases can increase IL-8 mRNA expression, T84 cells were co-cultured with *B. ratti* WR1 live parasite or lysate for 12 h. Total RNA extracted from the cells was used for real-time reverse-transcriptase (RT)-PCR and β-actin was used as a housekeeping gene. As shown in Fig. 6.3, there was a marked increase in the expression of IL-8 mRNA in T84 cells after co-incubation with *B. ratti* WR1 lysate (~4 fold) and live parasite (~3 fold). WR1 lysate induced IL-8 mRNA expression levels were significantly inhibited when a cysteine protease inhibitor iodoacetamide was used (~1.4 fold). To find out if the NF-κB pathway is activated upon exposure to *B. ratti* WR1, T84 cells were pretreated with a NF-κB inhibitor BAY11-7082 and co-incubated with parasitic lysate. BAY11-7082 markedly decreased *B. ratti* WR1-induced increase in mRNA levels (~1.7 fold). Altogether, these findings indicated that *B. ratti* WR1 cysteine proteases are capable of increasing IL-8 mRNA in human colonic T84 cells with involvement of the NF-κB pathway.

6.3.3 *B. ratti* WR1 degrades IκB-α and activates NF-Kb

One of the key pathways for NF-κB activation involves the phosphorylation of IκBs, which is followed by degradation of IκBs and subsequent translocation of NF-κB dimers from the cytoplasm to the nucleus. Hence, kinetics of IκB-α degradation were examined by western blot analysis in T84 cells co-cultured with *B. ratti* WR1 live parasite or lysate for 5 h. As shown in Fig. 6.4, cells incubated with parasitic lysate and live parasite show significant degradation of IκB-α (74.7% and 55.8% degradation respectively). Use of cysteine protease inhibitor iodoacetamide decreased the *B. ratti* WR1 lysate induced degradation of IκB-α (43.5%). Similarly, pretreatment of T84 cells with NF-κB inhibitor BAY11-7082 also decreased the *B. ratti* WR1 lysate induced-degradation of IκB-α (34.7 %).

To determine NF-κB binding activity with the IL-8 promoter, oligonucleotide duplexes containing wild-type NF-κB binding site present in the IL-8 promoter were used in EMSA. Stimulation of T84 cells with *B. ratti* WR1 lysate for 6 h caused NF-κB/DNA binding activity in nuclear extracts of T84 cells (Fig. 6.5). EMSA with a mutant NF-κB oligo-duplex did not show any NF-κB/DNA binding. A decreased NF-κB/DNA binding activity was noticed when T84 cells were pretreated with NF-κB inhibitor BAY11-7082. To measure the NF-κB p65 activity, nuclear extracts were subjected to a specific ELISA. As shown in Fig. 6.6, coincubation of T84 cells with *B. ratti* WR1 lysate caused a significant increase in NF-κB p65 activity ($P < 0.05$). Pretreatment of T84 cells with NF-

κB inhibitor BAY11-7082 significantly inhibited the *B. ratti* WR1 lysate induced increase in NF-κB p65 activity.

6.3.4 Nuclear translocation of NF-κB

The most commonly found form of NF-κB is a hetero-dimer composed of the p50 and p65 subunits. In unstimulated cells, NF-κB resides in the cytosol in the inactive form bound to inhibitory IκB proteins. Various stimuli initiate a series of signaling events that culminate in the phosphorylation and degradation of IκBs. Activated free NF-κB translocates to the nucleus and stimulates transcription by binding to correlated κB sites in the promoter regions of various target genes. To visualize the nuclear translocation of NF-κB, immnohistochemistry was performed on T84 cell monolayers stimulated for 6 h with *B. ratti* WR1 lysates (Fig. 6.7). Monolayers were probed with rabbit anti-NF-κB p50 antibody and a Cy3-conjugated sheep anti-rabbit secondary antibody was used for visualization. Nuclei were counterstained with DAPI to determine the cellular localizationof p50. T84 cells exposed to *B. ratti* WR1 lysate show nuclear translocation of p50 subunit (Fig. 6.7, image C). Control cells showed a normal cytosolic localization of p50 (Fig. 6.7, image F). A significant increase in the percentage of cells showing nuclear translocation of NF-κB p50 was observed after exposure to *B. ratti* WR1 lysate ($P < 0.05$) (Histogram, Fig. 6.7).

In addition to the T84 cells, it was investigated if *Blastocystis* can induce NF-κB activity in HT29 (human colon adenocarcinoma) cells. Fig. 6.8 shows the nuclear

translocation of NF-κB p50 in HT29. A significant increase in the percentage of cells showing nuclear translocation of NF-κB p50 was observed after exposure to *B. ratti* WR1 lysate ($P < 0.05$ versus control) (Histogram, Fig. 6.8).

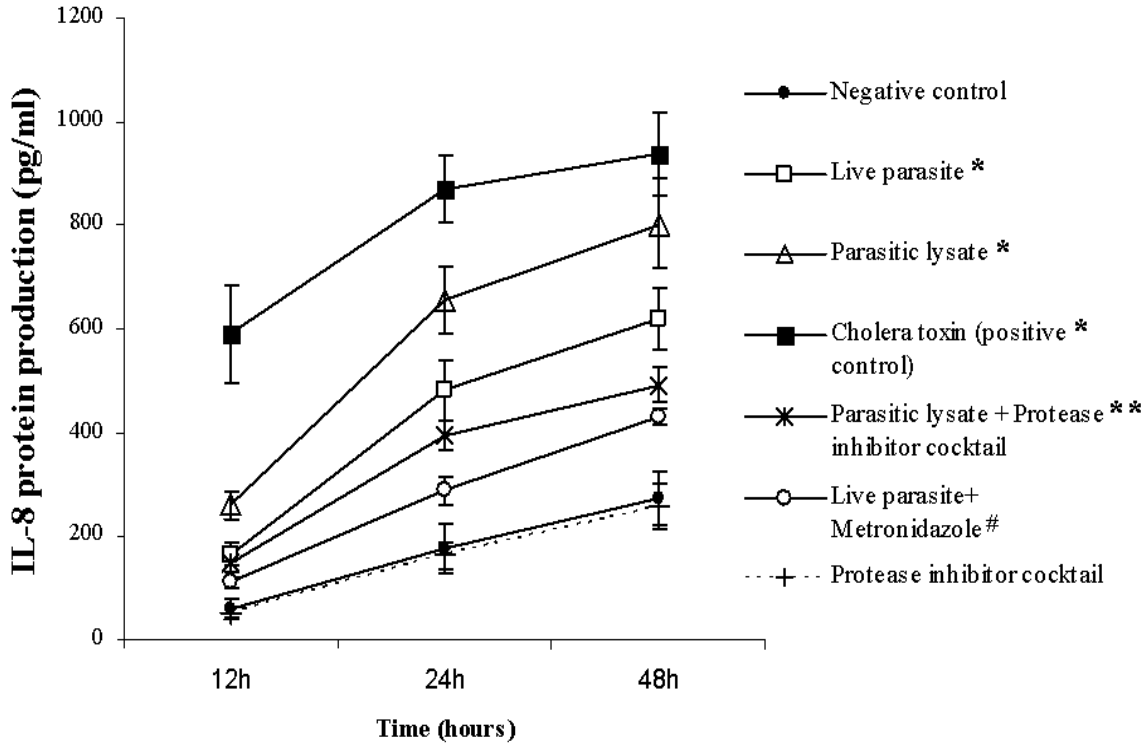

Fig. 6.1 Induction of IL-8 production in human intestinal epithelial T84 cells by *Blastocystis ratti* WR1. Cells were grown to confluency in 12 well tissue culture plates. T84 cells were co-incubated with *Blastocystis ratti* WR1 for 12, 24, and 48 h. The IL-8 concentration in supernatants was measured by ELISA. WR1 live parasite and lysate induce significant increase in IL-8 production in a time-dependent manner (* $P < 0.05$ versus negative control for each time point). Use of protease inhibitor cocktail reduced the WR1 lysate-induced IL-8 production significantly (** $P < 0.05$ versus WR1 lysate). Pretreatment of live parasites with antiprotozoal drug metronidazole significantly reduced the IL-8 production induced by WR1 live parasite ($^{\#}P < 0.05$ versus live WR1 parasite). Treatment of cells with only protease inhibitor cocktail did not show any significant effect on baseline production of IL-8. To know if experiment is working, purified cholera toxin was used as a positive control for IL-8 induction. Values are mean ± SD ($n = 3$).

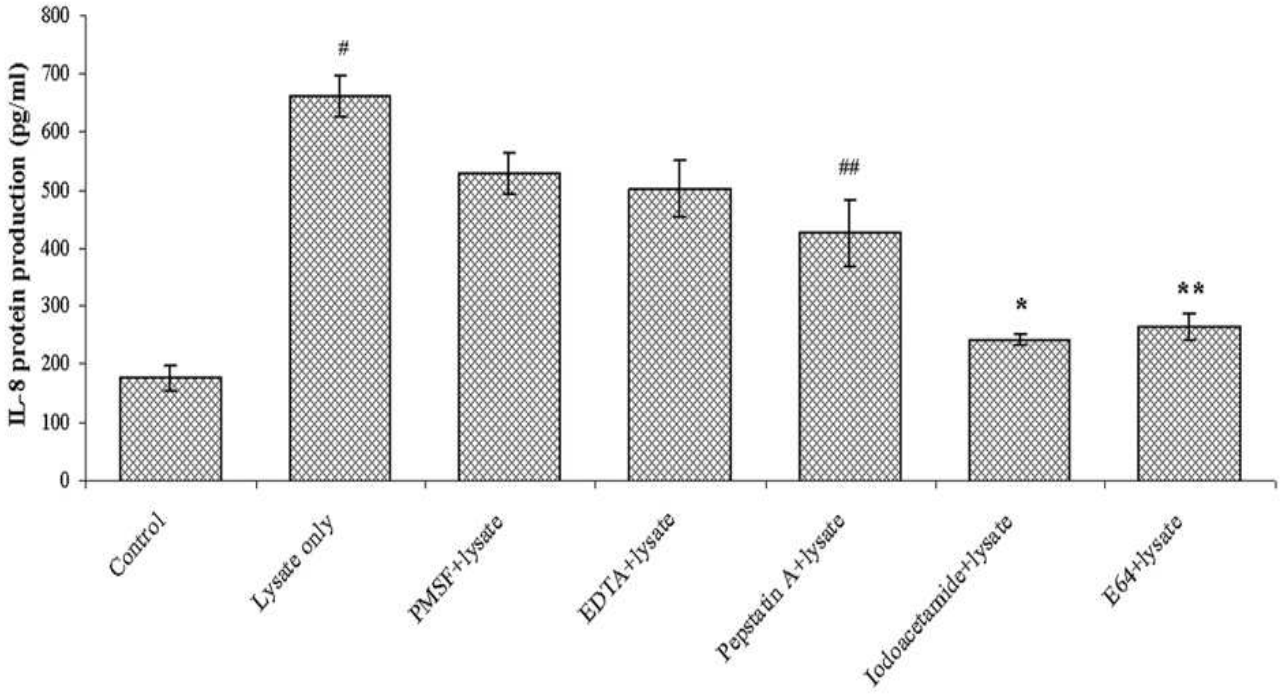

Fig. 6.2 Effect of protease inhibitors on IL-8 production from T84 cells induced by *Blastocystis ratti* WR1. Cells were grown to confluency in 12 well tissue culture plates. T84 cells were co-incubated for 24 h with WR1 lysate pretreated with one of various protease inhibitors. The IL-8 concentration in supernatants was measured by ELISA. Cysteine protease inhibitors, iodoacetamide and E-64, most significantly reduce the WR1 induced IL-8 production from T84 cells ($^{*}P = 0.002$, $^{**}P = 0.007$; both versus WR1 lysate). Considerable reduction in IL-8 production is seen with aspartic inhibitor pepstatin A ($^{\#\#}P = 0.04$ versus WR1 lysate). Minimal inhibition is observed with serine and metallo protease inhibitors, PMSF and EDTA respectively. Values are means ± SD ($n = 3$). $^{\#}P < 0.02$ versus control.

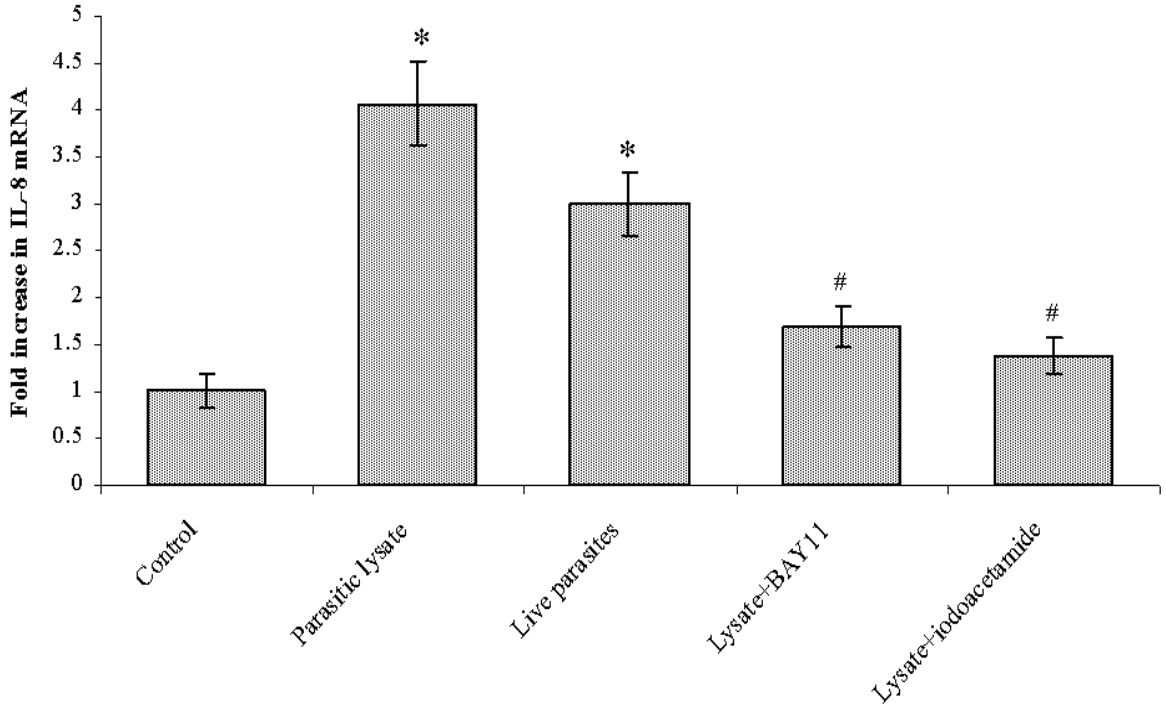

Fig. 6.3 *Blastocystis ratti* WR1 induces up-regulation of IL-8 mRNA levels in T84 cells. T84 cells were co-incubated with WR1 live parasite or parasitic lysate for 12 h. Total cellular RNA was isolated and IL-8 and β-actin mRNA levels were measured by quantitative real-time RT-PCR as described in 'Materials and Methods'. WR1 lysate causes significant increase in IL-8 mRNA levels. Significant decrease in IL-8 mRNA levels is noticed after treatment with NF-κB inhibitor BAY11-7082 and cysteine protease inhibitor iodoacetamide. The fold increase in IL-8 mRNA relative to untreated T84 cells was calculated for each experiment based on internal β-actin controls. Values are means ± SD (n = 3). * $P < 0.05$ versus control; $^{\#}$ $P < 0.05$ versus WR1 lysate.

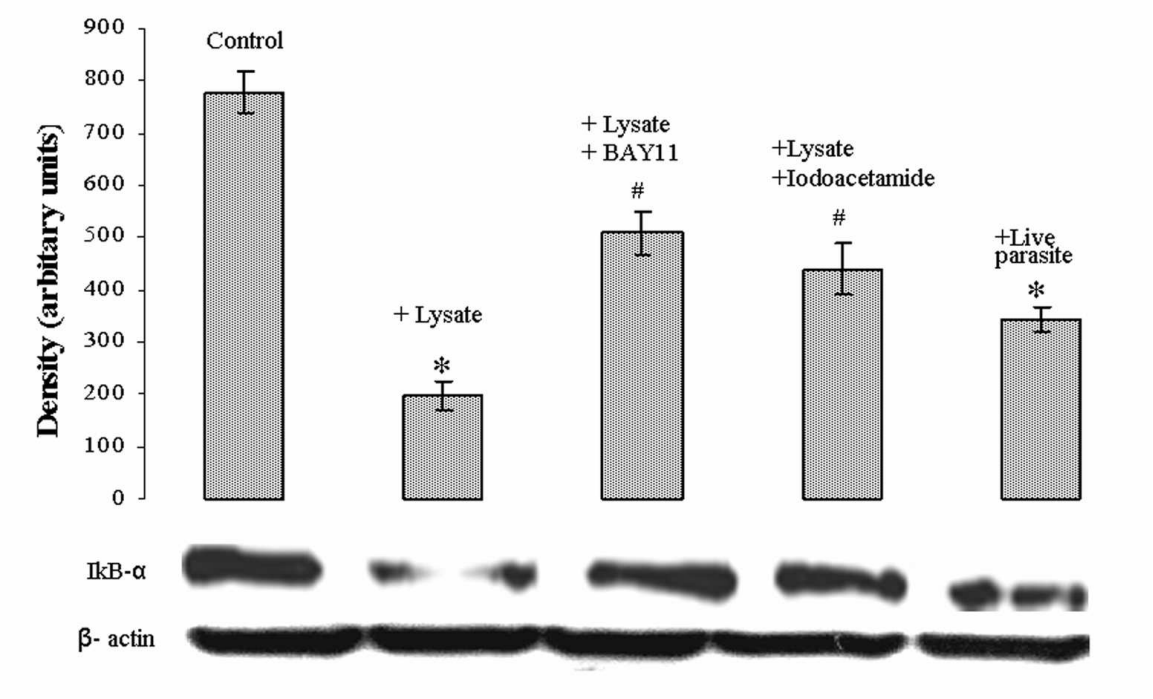

Fig. 6.4 *Blastocystis ratti* WR1 exposure to intestinal epithelial cells causes IκB-α degradation. WR1 live parasite or lysate co-incubation with T84 cells resulted in IκB-α degradation at 5 h shown in a representative result with a β-actin loading conrol. The cell lysates were analyzed by Western blot using an anti-IκB-α antibody. Pretreatment of cells with NF-κB inhibitor BAY11-7082 resulted in decreased IκB-α degradation. Iodoacetamide, a cysteine protease inhibitor, decreased WR1 lysate-induced degradation of IκB-α. Histogram represents densitometry values expressed as arbitary units

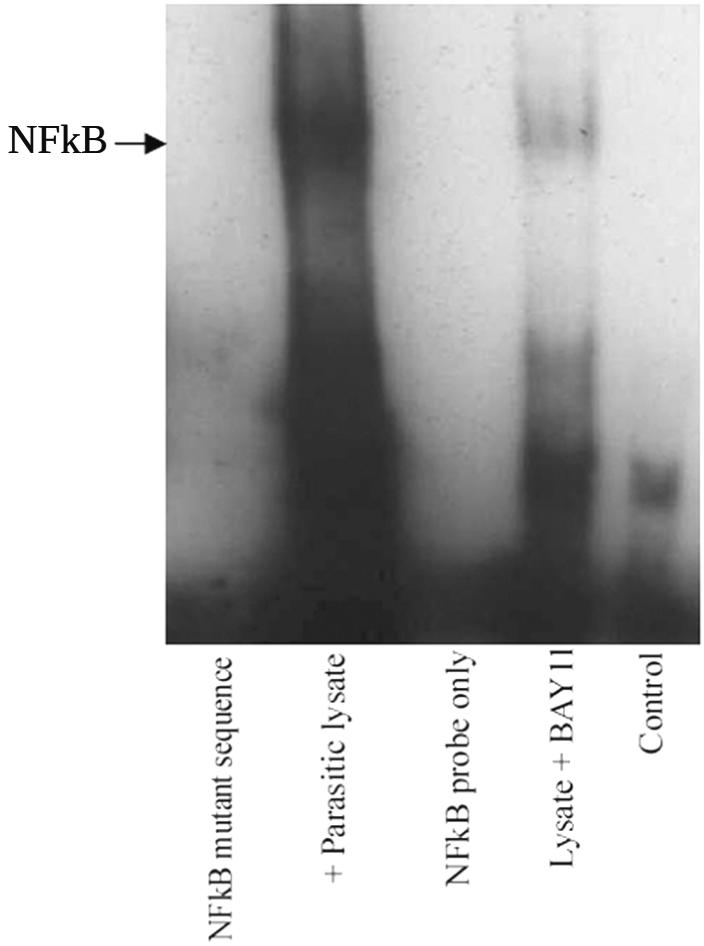

Fig. 6.5 Representative electrophoretic mobility shift assay (EMSA) shows NF-κB/IL-8 promoter binding activity in nuclear extracts. Cells were grown to confluency in 12 well tissue culture plates. Nuclear extracts were prepared using a NE-PER nuclear and cytoplasmic extraction reagent kit and electrophoretic mobility shift assay was performed using LightShift chemiluminescent EMSA kit. T84 cells co-incubated with WR1 lysate for 6 h. No NF-κB/DNA binding is observed in mutant NF-κB oligo-duplex incubated with nuclear extract, wild-type NF-κB oligo duplex without nuclear extract, and control. Pretreatment of T84 cells with NF-κB inhibitor BAY11-7082 resulted in decreased WR1 induced NF-κB/DNA binding.

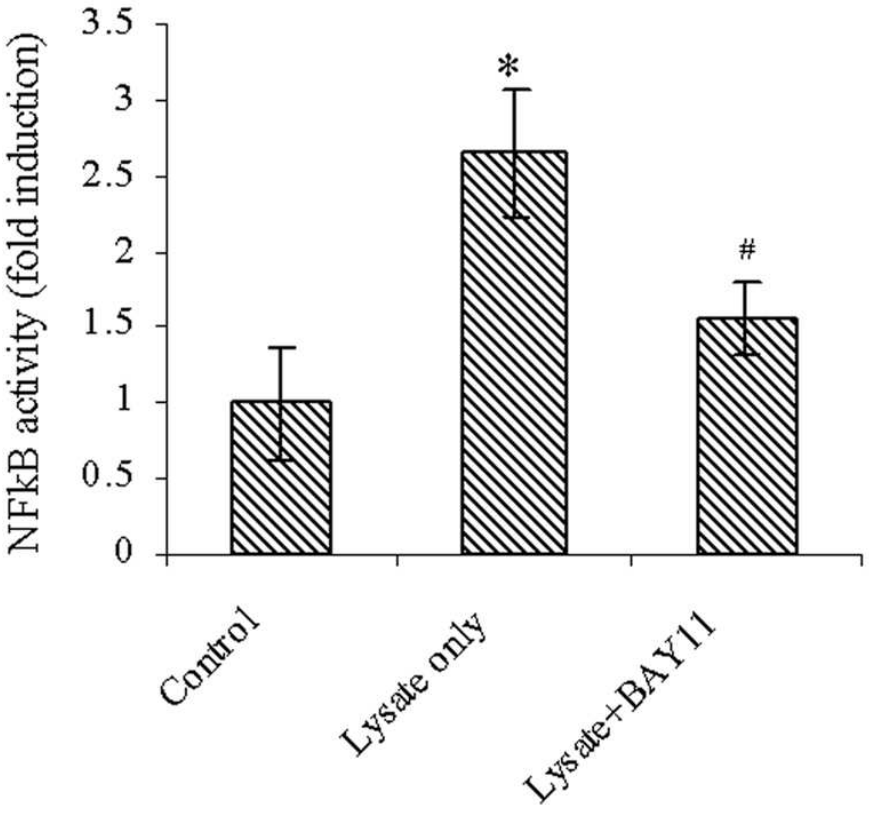

Fig. 6.6 Histogram showing fold increase in NF-κB activity in nuclear extracts of T84 cells. Cells were grown to confluency in 12 well tissue culture plates. Cells were co-incubated with WR1 lysate for 6 h and NF-κB activity in the nuclear extracts was measured by specific ELISA. Exposure to parasitic lysates significantly increased the NF-κB activity in T84 cells. Pretreatment of cells with NF-κB inhibitor BAY11-7082 resulted in decreased NF-κB activity. Values are means ± SD (n = 3). * $P < 0.05$ versus control; $^{\#}$ $P < 0.05$ versus WR1 lysate.

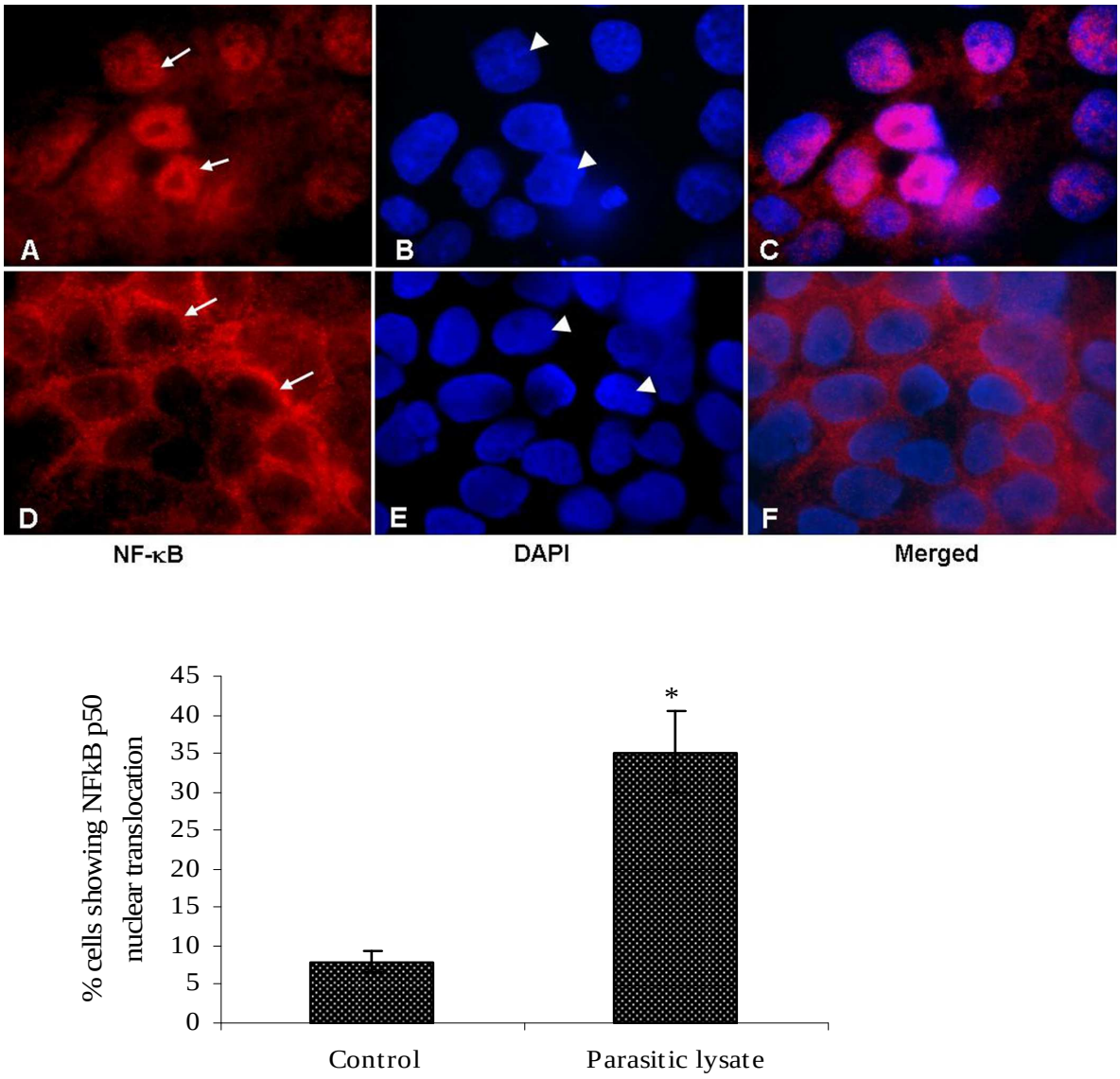

Fig. 6.7 Representative micrographs showing nuclear translocation of NF-κB in intestinal epithelial T84 cells following exposure to *Blastocystis ratti* WR1. T84 cell monolayers were grown on collagen coated glass coverslips and co-incubated with WR1 lysate for 6h. Monolayers were fixed and first incubated with rabbit anti-NF-κB p50 antibody and then with Cy3 conjugated sheep anti-rabbit secondary antibody. Monolayers were mounted using mounting medium containing DAPI to counterstain nucleus and visualized by fluorescent microscopy. T84 cells exposed to WR1 lysate show nuclear translocation of the p50 subunit (A-C). Control cells exhibit normal perinuclear localization of p50 (D-F). All micrographs were obtained at a magnification of ×400. (Arrow: p50 localization; arrowhead: nucleus). From the histogram, a significant increase in the percentage of cells showing nuclear translocation of NF-κB p50 can be noticed in cells exposed to WR1 lysate. The percentage of cells with NF-κB p50 nuclear translocation was calculated from observation of 200 cells in three independent experiments. Values are means ± SD. * $P < 0.05$ versus control.

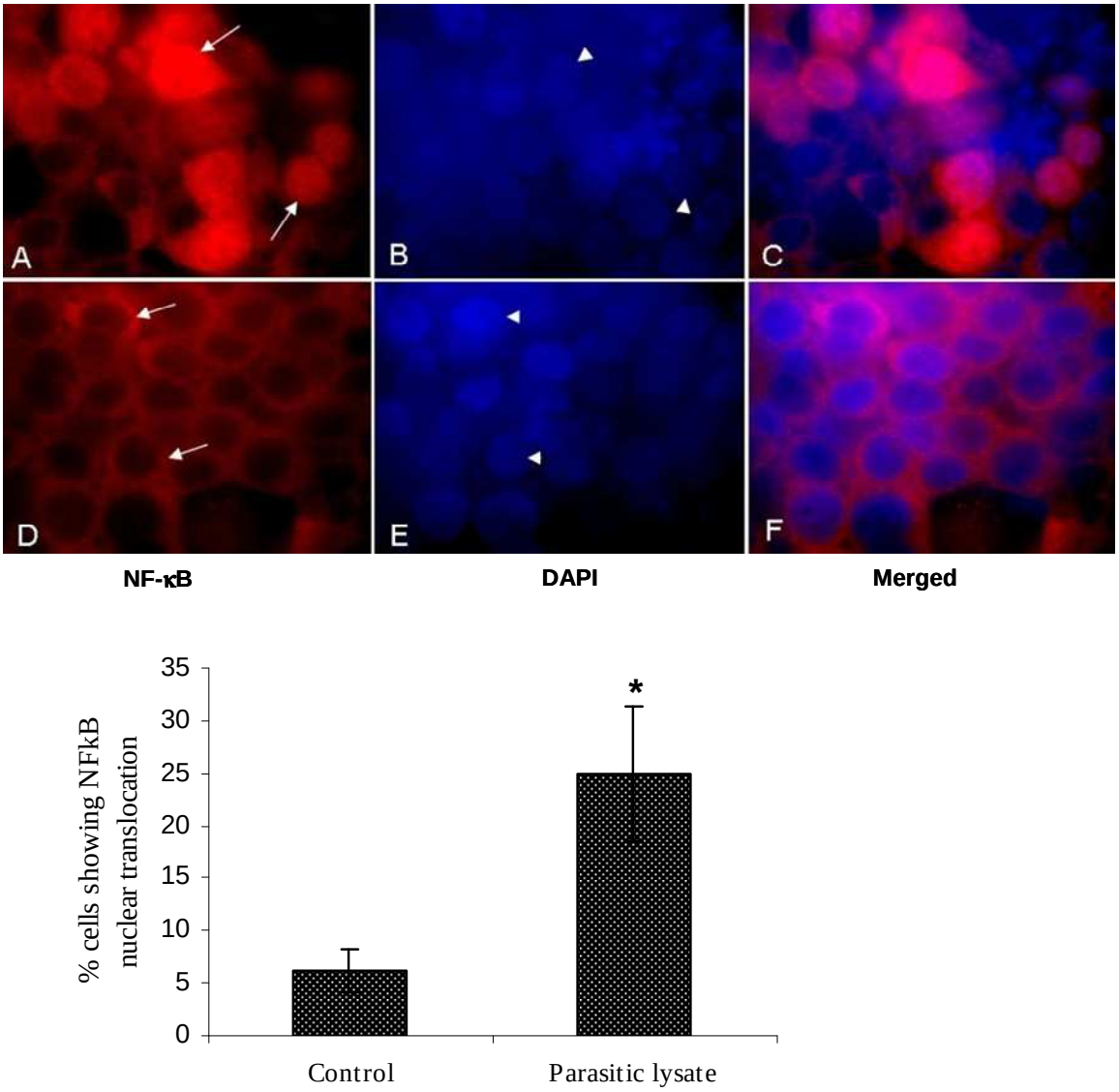

Fig. 6.8 Representative micrographs showing nuclear translocation of NF-κB in intestinal epithelial HT29 cells following exposure to *Blastocystis ratti* WR1. HT29 monolayers were grown on collagen coated glass coverslips and co-incubated with parasitic lysate for 6h. Monolayers were fixed and first incubated with rabbit anti-NF-κB p50 antibody and then with Cy3 conjugated sheep anti-rabbit secondary antibody. Monolayers were mounted using mounting medium containing DAPI to counterstain nucleus and visualized by fluorescent microscopy. HT29 cells exposed to WR1 lysate show nuclear translocation of the p50 subunit (A-C). Control cells exhibit normal perinuclear localization of p50 (D-F). All micrographs were obtained at a magnification of ×400. (Arrow: p50 localization; arrowhead: nucleus). Histogram shows a significant increase in the percentage of cells showing nuclear translocation of NF-κB p50 in cells exposed to WR1 lysate. The percentage of cells with NF-κB p50 nuclear translocation was calculated from observation of 200 cells in three independent experiments. Values are means ± SD. * $P < 0.05$ versus control.

6.4 DISCUSSION

In previous chapters, the protease activity of *B. ratti* WR1 and *B. hominis* B was reported; and it was also shown that *B. hominis* B and *B. ratti* WR1 proteases can degrade human secretory immunoglobulin A. It was also demonstrated that *B. ratti* WR1 induces contact-independent apoptosis, F-actin rearrangement, and barrier function disruption in IEC-6 cells. These studies suggested that *B. ratti* WR1 secretory products exert detrimental effects on host cells and warrant further investigations. Phylogenetic data suggest that *Blastocystis* is a zoonotic parasite and animal to human transmissions are common (Yoshikawa et al. 2004a; Noel et al. 2005). Intestinal inflammation and edema was reported in patients infected with *Blastocystis* (Kain et al. 1987; Russo et al. 1988; Garavelli et al. 1992; Zuckerman et al. 1994). In a murine model, intense infiltration of inflammatory cells in colon and inflammation with edematous lamina propria in the cecum and colon was reported following *Blastocystis* infection (Moe et al. 1997). One *in vitro* study showed that *Blastocystis* may modulate IL-8 response in intestinal epithelial cells (Long et al. 2001). Taken together, these observations suggest that *Blastocystis*-induced intestinal inflammation is mediated by IL-8 recruitment of inflammatory cells and some secretory parasitic factors are responsible for this.

It has been reported that *E. histolytica* cysteine proteases are involved in the production of IL-8 from intestinal epithelial cells and these proteases also induce gut inflammation (Zhang et al. 2000). Results demonstrate that *B. ratti* WR1 induces IL-8 production from colonic epithelial T84 cells in a time-dependent manner. Use of protease

inhibitors showed that cysteine proteases of *B. ratti* WR1 were mainly responsible for the induction of IL-8 production. Moreover, increased cytokine production was paralleled by increase gene transcription. Numerous pathogenic gastrointestinal pathogens like *E. histolytica* (Eckmann et al. 1995), *C. parvum* (Seydel et al. 1998), *Salmonella*, *E. coli* (Eckmann et al. 1993), and *Vibrio cholerae* (Rodriguez et al. 2001) are known to induce IL-8 production from the intestinal epithelium. Intestinal epithelial cell production of IL-8 causes influx of inflammatory cells into the intestinal mucosa with resultant tissue damage and gastrointestinal disturbances. It was reported that invasion of intestinal epithelium by pathogens is not necessary for the induction of inflammation (Berkes et al. 2003) and since *Blastocystis* is non-invasive parasite, secreted products from the parasite might initiate the inflammatory process by activating cell surface receptors. Although, *E. histolytica* is an invasive protozoan, it can also induce IL-8 secretion from human colonic epithelial cells without parasite-enterocyte contact (Yu and Chadee 1997). Zhang et al. (2000) showed that the expression of cysteine proteinases by *E. histolytica* is crucial to cause intestinal inflammation and tissue damage in amoebic colitis. In a human intestinal xenograft model, they demonstrated that *E. histolytica* trophozoites deficient in cysteine proteinase production failed to induce IL-1B, IL-8 and subsequent gut inflammation. They showed that amoebic cysteine proteinases have IL-1 converting enzyme (ICE) activity which might stimulate the production of IL-8 in adjacent intestinal cells through induction of NF-kB and cause intestinal inflammation. Similarly, *Blastocystis* proteases, in a contact-independent manner, may initiate IL-8 mediated inflammatory processes that ultimately lead to gastrointestinal symptoms.

It was observed that metronidazole treatment of *B. ratti* WR1 live parasites markedly reduced the induction of IL-8 production from T84 cells. Metronidazole induces programmed cell death in *Blastocystis* and plasma membrane integrity of parasite remains preserved (Nasirudeen et al. 2004), therefore there is no leakage of intracellular parasitic proteases and other products that can induce high IL-8 response. As metronidazole inhibits nucleic acid synthesis in parasite, it will in turn inhibit the parasites ability to produce proteases. However, a pronounced IL-8 response was observed when T84 cells were exposed to parasitic lysates as it contains all parasitic soluble and non-soluble products. It was previously shown in chapter 5 that metronidazole can avert the adverse effects of *B. ratti* WR1 on intestinal epithelial barrier function. Altogether, these findings suggest the therapeutic potential of metronidazole in *Blastocystis* infections.

This study investigated the molecular mechanisms by which IL-8 gene expression is regulated in colonic epithelial cells after exposure to *B. ratti* WR1. Firstly, it is demonstrated that *B. ratti* WR1 can degrade IκB-α and the use of a cysteine protease inhibitor markedly inhibited the degradation of IκB-α suggesting the involvement of a parasite-derived cysteine protease. Secondly, results from EMSA demonstrated that NF-κB is involved in the induction of IL-8 promoter activity in colonic epithelial cells. NF-κB plays a pivotal role in intestinal inflammation and both invasive and non-invasive enteric pathogens can trigger the inflammatory cascade through activation of NF-κB (Berkes et al. 2003). In most cells, including intestinal epithelial cells, activation of NF-κB is critical for inducible expression of the proinflammatory response genes. NF-κB-

mediated induction of IL-8 has been reported in numerous enteric pathogens like *H. pylori* (Beswick et al. 2006), *E. coli* (Dahan et al. 2002), and *B. fragilis* (Kim et al. 2002). Protozoan parasites like *E. histolytica* (Zhang et al. 2000) and *C. parvum* (Chen et al. 2001) are also capable of activating NF-κB in host cells. Results from immunohistochemistry also support these findings and show nuclear translocation of NF-κB. The finding that *B. ratti* WR1 has the ability to activate NF-κB has important implications since other NF-κB responsive genes like IL-1 and IL-6 may also be affected in *Blastocystis* infections, which may lead to altered intestinal homeostasis.

In summary, present study demonstrates for the first time that *B. ratti* WR1 cysteine proteases induce IL-8 production in colonic epithelial T84 cells and an NF-κB-dependent transcriptional process is involved. In addition, findings show that metronidazole treatment can markedly inhibit the ability of *B. ratti* WR1 to induce IL-8 production. These findings will contribute to an understanding of the pathobiology of a poorly studied parasite whose public health importance is increasingly recognized.

CHAPTER 7:

GENERAL DISCUSSION

AND

CONCLUSIONS

7.1 GENERAL DISCUSSION

Blastocystis infections have a worldwide distribution and incidences of up to 60 % have been reported in tropical, subtropical and developing countries. The clinical symptoms of *Blastocystis* infections are mainly diarrhea and abdominal pain as well as nonspecific gastrointestinal symptoms particularly nausea, vomiting, bloating and anorexia (Doyle et al. 1990; Nimri and Batchoun 1994, Sohail et al. 2005). Intriguingly, even though it was first described almost a century ago (Alexeieff 1911; Brumpt 1912), the pathogenic mechanisms of this enteric protozoan still remain elusive. In recent years, many reports have shown that *Blastocystis* is associated with intestinal disorders (Barahona-Rondon et al 2003; Leelayoova et al. 2004; El-Shazly et al. 2005). Patients with HIV infections and immunosuppressive therapy were reported to be more susceptible to *Blastocystis*-associated diarrhea (Florez et al. 2003; Rao et al. 2003; Hailemariam et al. 2004). Similar to giardiasis, most of the people infected with *Blastocystis* are asymptomatic and infection frequently resolves spontaneously (Stenzel and Boreham 1996).

So far, many epidemiological and clinical case reports have implicated *Blastocystis* as a cause of disease but only a small number of controlled experimental studies have been carried out to investigate its pathogenicity. Although lack of a suitable animal model remained a major impediment, there is scarcity of *in vitro* studies that have addressed this issue. In this study, using *in vitro* model systems, various aspects of *Blastocystis* pathogenicity were investigated. Different intestinal cell lines and zoonotic

isolates of *Blastocystis* were employed*;* and results from this study consistently suggested that this parasite has pathogenic potential.

Parasite-derived proteases are important to the parasite life cycle and the pathogenesis of disease they produce. Proteases of *Blastocystis* were studied and it was demonstrated for the first time that *Blastocystis ratti* WR1 and *Blastocystis hominis* B possess high levels of protease activity, in particular of the cysteine protease. Protease activity has been well documented in many parasitic protozoa and a number of roles were assigned for protease function in host-parasite relationships (North 1982). Some of the roles of parasite proteases include immune evasion or modulation by degradation of host immune molecules, invasion of the host facilitated by catalyzing degradation of connective tissues, and metabolism within the host by taking advantage of specific host proteins.

Cysteine proteases from *E. histolytica* were reported to degrade extracellular matrix proteins (Keene et al. 1986; Zhang et al. 2000) and their expression is necessary for intestinal inflammation and tissue damage in amoebic colitis. It would be interesting to investigate if *Blastocystis* proteases similarly degrade extracellular matrix proteins. The function of proteases in such infections is suggested to be hydrolysis of proteins of connective tissue in particular collagens and keratins, which then increases the invasiveness of organism. Intestinal permeability was found to be significantly increased in *B. hominis* patients and it was suggested that *Blastocystis* infections can cause damage to the intestinal wall (Dagci et al. 2002). Further studies on *Blastocystis* should include

the role of proteases in intestinal tissue damage. Additionally, characterization of *Blastocystis* proteases will lead to a better understanding of the parasite life cycle and its interactions with host. Parasite cysteine proteases are immunogenic and have been exploited as vaccine targets and diagnostic markers in serology. Because of the complex diversity of *Blastocystis* genotypes, proteases may be a good choice as diagnostic markers. Parasitic proteases are suggested to be promising targets for the development of new anti-parasitic chemotherapy (Sajid et al. 2002). It has also been shown that cysteine protease inhibitors are effective against numerous protozoan parasites (North 1994).

There has been controversy over the role of the *Blastocystis* central vacuole. It was shown for the first time that central vacuole of *Blastocystis* contains cysteine proteases and this finding will certainly help to further elucidate the functional importance of *Blastocystis* organelles with respect to their localizations. As the central vacuole is quite a large organelle, there is a need to further investigate if it functions only as storage organelle or it also plays an important role in the synthesis of proteases.

It was further investigated if *Blastocystis* proteases can degrade secretory immunoglobulin A (S-IgA). Pathogen-produced immunoglobulin A (IgA) proteases cleave human secretory IgA which compromises IgA function. Directly, this may help the pathogen in mucosal adhesion or this might indirectly help other pathogens in adhesion and invasion. To study if the enteric protozoan *Blastocystis* degrades human secretory IgA, parasitic lysate and conditioned medium were exposed to secretory immunoglobulin A. Secretory IgA was degraded by both cell lysate and conditioned

medium with mainly cysteine proteinase activity in *B. hominis* B isolate and aspartic proteinase activity in *B. ratii* WR1 isolate. These findings suggest that, by degrading S-IgA, *Blastocystis* proteases may play a role in parasite survival *in vivo* as S-IgA prevents the pathogens from colonizing in gut.

Many infections with *G. intestinalis*, *E. histolytica* and other protozoans are subclinical but these may exhibit clinical symptoms if a particular isolate or sympathetic host immune milieu is involved. *Blastocystis* was found to be associated with clinical symptoms in many cases of HIV infection (Garavelli et al. 1990; Cirioni et al. 1999). The high prevalence of *Blastocystis* in AIDS patients may be attributable to the defective immune response (Rodgers and Kagnoff 1988) which leads to lower secretory IgA concentrations in mucosal secretions (Challacombe and Sweet 1997) of these patients and possibly provides a conducive environment for parasitic colonization. Numerous studies have demonstrated strong correlations between titers of specific SIgA in secretions and resistance to infections (Russel et al. 1999).

Finding that *Blastocystis* degrades secretory immunoglobulin A has another significant implication in the use of specific pathogen free (SPF) laboratory animals. At present, SPF laboratory animals are not screened for the *Blastocystis* infections by animal suppliers (Charles River Laboratories). Up to 60 % SPF rats were found infected with *Blastocystis* in well maintained animal facilities (Chen et al. 1997). In *Blastocystis* infected SPF laboratory animals, degradation of secretory immunoglobulin A can interfere with other experimental gastrointestinal infection studies. This interference

could be in the form of a changed intestinal milieu due to reduced secretory immunoglobulin A levels in gut. Furthermore, *Blastocystis* proteases may modulate intestinal homeostasis and may directly affect the pathogen of study, ultimately generating misleading results.

One of the most important functions of intestinal epithelium is to provide a barrier between the intestinal lumen and the underlying compartments. The host intestine houses a wide variety of microorganisms and a single layer of epithelial cells that lines the intestinal mucosa is the first site of interaction with pathogens. These epithelial cells work as a first line of defense against pathogens and elicit a variety of protection strategies through widespread communication with the innate and adaptive immune components. Any breach or insult of the epithelial integrity may expose underlying tissues to an array of antigens and might lead to a pathological host response. In this work, *in vitro* interactions of *Blastocystis* with the intestinal epithelium were studied and it was found that it induces apoptosis of intestinal epithelial cells, and disrupts the epithelial barrier function possibly by causing filamentous-actin rearrangements and ZO-1 disruption. Enteric pathogens are known to adopt several strategies to disrupt the tight junctions of intestinal epithelial cells. They can either alter the cytoskeleton of cell or they can affect particular tight junction proteins. Tight junctions can be regulated via the cytoskeleton indirectly through changes in the perijunctional actomyosin ring or directly through changes in specific tight junction proteins (Berkes et al. 2003). These changes may lead to disturbances in epithelial functions particularly changes in paracellular permeability. The function of tight junctions can be studied as a change in transepithelial

electrical resistance or in the paracellular flux of macromolecules. *Bacteroides fragilis* is known to cause diarrhea and it can disrupt tight junctions by proteolytic degradation of tight junction proteins resulting in decrease in transepithelial electrical resistance and increase in permeability (Berkes et al. 2003). This study has shown that *Blastocystis* can displace ZO-1 proteins from tight junctions. Further investigations are needed to see if *Blastocystis* proteases can directly degrade ZO-1 or other tight junction proteins.

It was found that *Blastocystis* can decrease transepithelial resistance and increase permeability of intestinal epithelial monolayer. This study supports the finding from other clinical reports where significantly increased intestinal permeability was reported after *Blastocystis* infections (Dagci et al. 2002). Some of these cytopathic effects are comparable to that of other pathogenic protozoans like *G. lamblia* and warrant further elucidation.

Importantly, this study has shown that *Blastocystis* secretory products can induce apoptosis in intestinal epithelial cells. This suggest that contact with host cells is not required for *Blastocystis* to cause any cytopathic effects and being a non-invasive organism, this finding has significant implications for its pathogenicity. However, the role of pathogen-induced apoptosis in gut epithelial cells is not well understood. It's not clear whether the host or pathogen benefits by this phenomenon. Pathogen-induced apoptosis can be significant in maintaining homeostasis by controlling inflammation and immunological silent removal of cells. At the same time, this response can benefit some extracellular pathogens but some pathogens may lose their colonization sites. It appears that only extensive dysregulated apoptosis may lead to pathology due to cytokine or Fas-

FasL overexpression as in *Shigella* and *Salmonella* infections where apoptosis becomes pro-inflammatory and damage of host cells occurs.

When exposed to certain toxins, the intestinal epithelium can be induced to undergo cell death via apoptosis or necrosis, which can in turn affect intestinal barrier function, resulting in decreased transepithelial permeability and increased permeability (Bojarski et al. 2000). In experiments, it was tested if *Blastocystis* induced apoptosis of IEC-6 cells has an effect on epithelial permeability by pretreating cell monolayers with general caspase inhibitor Z-VAD-fmk before coincubation with parasites. Results show that the inhibition of caspases in IEC-6 cells did not prevent reduction of TER and did not significantly decrease Lucifer yellow flux across cell monolayer. Therefore, in the case of *Blastocystis* infection, it appears that activation of host cell apoptosis in a contact-independent manner does not play a major role in increasing epithelial permeability but there might be other factors responsible for increased permeability. In contrast, studies on *G. lamblia* have established that while *Giardia* is a non-invasive pathogen, it releases parasitic factors which may induce enterocyte apoptosis and increase epithelial permeability (Chin et al. 2002). The apparent discrepancy in observation may be due to two factors. Firstly, the contribution of apoptosis to increased permeability may be minor because of the low percentage of apoptotic cells, evidenced by DAPI staining, as compared to cells with F-actin rearrangement. Secondly, *Blastocystis* may have induced non-apoptotic cell death, for example necrosis, autophagy or paraptosis (Broker et al. 2005) that may have contributed to the impaired barrier function.

Gastrointestinal symptoms along with inflammation of gastrointestinal tract were associated with *Blastocystis* infections. However it was not clear how *Blastocystis* triggers the inflammatory response. Results show for the first time that *Blastocystis* cysteine proteases induce IL-8 production in colonic epithelial cells and an NF-κB-dependent transcriptional process is involved. Production of IL-8 causes influx of inflammatory cells into the intestinal mucosa which causes tissue damage and gastrointestinal disturbances. *E. histolytica* cysteine proteases can induce the production of IL-8 from intestinal epithelial cells and these proteases also induce gut inflammation (Zhang et al. 2000). Protozoan parasites like *E. histolytica* (Eckmann et al. 1995) and *C. parvum* (Seydel et al. 1998) are capable of inducing IL-8 production from intestinal epithelial cells. *E. histolytica* can induce IL-8 from intestinal cells in a contact-independent manner and it was reported that invasion of intestinal epithelium by pathogens is not necessary for the induction of inflammation (Berkes et al. 2003). As *Blastocystis* is non-invasive parasite, secreted products from parasite appears to initiate the inflammatory process by activating cell surface receptors. It would be interesting to investigate if proteinase activated receptors (PARs) of intestinal epithelial cells play a role in *B. ratti* WR1 cysteine protease-mediated induction of IL-8. PARs are seven transmembrane domain receptors that are activated by specific proteolytic cleavage of their N terminal extracellular domain (Vergnolle et al. 2005). It is known that IL-1 may act as an intermediary factor for the induction of IL-8 secretion. Since T84 cells were used and they lack type-1 IL-1 receptors and IL-1β does not have significant role in T84 cells (Yu and Chadee 1997), more studies need to be performed to ascertain if *Blastocystis*-induced IL-8 secretion involves IL-1.

Results show that treatment of *Blastocystis* with metronidazole can avert the adverse effects of *Blastocystis* on intestinal epithelial barrier function. Metronidazole treatment also resulted in reduced interleukin-8 activity from intestinal epithelial cells. This is controversial if there is a need to treat *Blastocystis* infections because of its doubtful pathogenicity. Treatment for *Blastocystis* infections is not yet standardized in clinics. Previous studies have shown that metronidazole inhibits the *in vitro* growth of *Blastocystis* (Moghaddam et al. 2005). Reports show that metronidazole is a drug of choice in cases where *Blastocystis* is suspected for gastrointestinal symptoms (Tan 2004). Taken together, this suggests that metronidazole has a therapeutic potential for *Blastocystis* infections and treatment with anitprotozoal drugs, in particular metronidazole, is warranted if no other cause of disease is observable.

Accumulating evidence over the last decade suggests association of *Blastocystis* with gastrointestinal disorders (Sohail et al. 2005). Mostly inconclusive clinical and epidemiological studies implicated or exonerated *Blastocystis* as an etiology of disease. Unlike most other enteric pathogens, *Blastocystis* is essentially considered non-invasive and does not cause any consistent inflammatory response. However, it was observed that *Blastocystis* infections may cause gastrointestinal symptoms similar to irritable bowel syndrome (IBS) involving diarrhea, abdominal pain, constipation, nausea and fatigue (Tungtrongchitr et al. 2004; Boorom 2007). Approximately 4 billion diarrhea episodes occur worldwide each year which account for 4% of all deaths and 5% of days lost to disability (Sazawal et al. 2006). The pathophysiological basis of diarrhea in *Blastocystis*

infections can be multifactorial and may involve at least in part intestinal epithelial damage, compromised barrier function, as well as induction proinflammatory cytokine production. In figure 7.1, a model is proposed for the pathogenic potential of *Blastocystis* that shows a relationship between *in vitro* experimental findings of this study and their patho-physiological correlation to gastrointestinal symptoms.

Although discovered almost a century ago, scientific knowledge on this parasite has increased significantly only in last few years. It is now known that humans can be infected by numerous *Blastocystis* genotypes and many of these genotypes can be zoonotic. All mammalian and avian isolates are now classified in nine subtypes (Stensvold et al. 2007b). Previously conflicting observations on *Blastocystis* pathogenesis are possibly due to the existence of pathogenic and non-pathogenic genotypes. Laboratory rats appear to be reasonably good animal models for infection studies and fecal-oral route has been established as the mode of transmission. There is a need to further identify and characterize *Blastocystis* virulence factors and associated genes. Most importantly, major efforts should be directed to elucidate the subtype linked differences in pathogenesis of this interesting parasite.

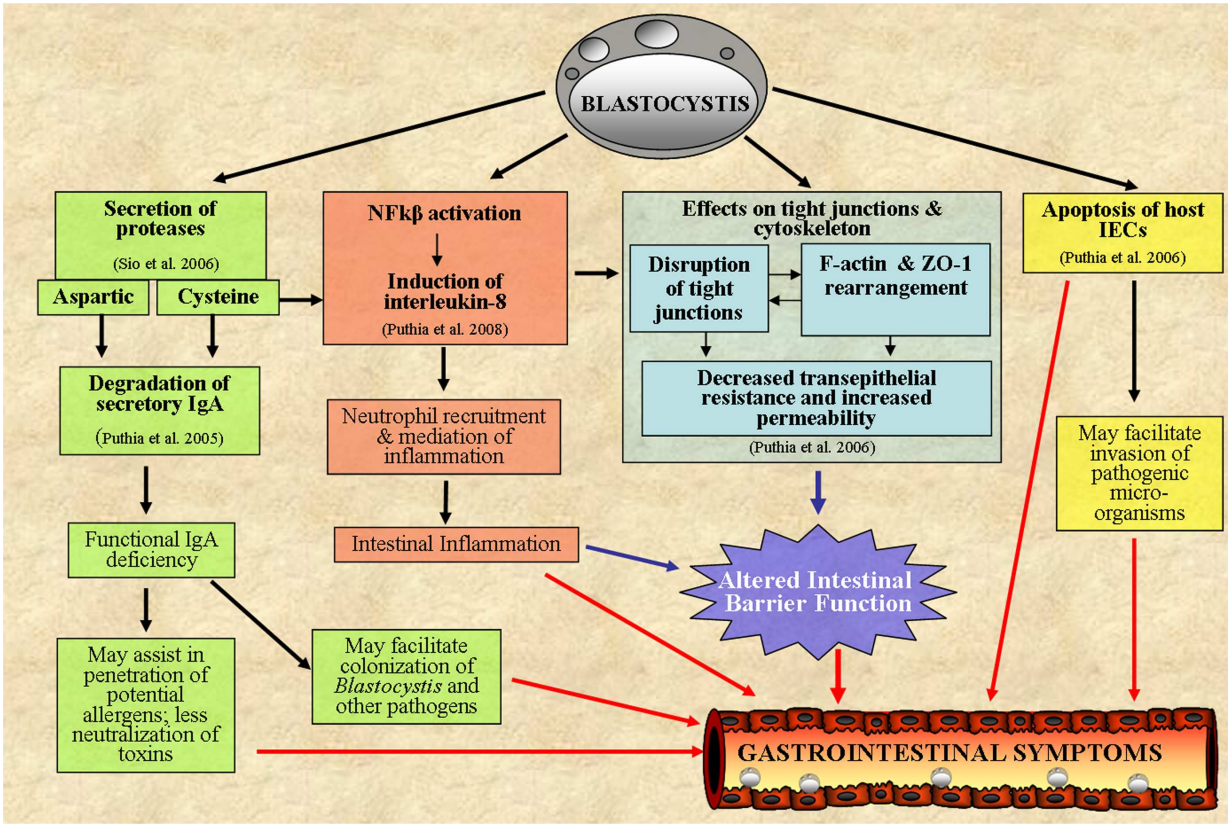

Fig 7.1 Proposed model for the pathogenic potential of *Blastocystis*. This model correlates *in vitro* findings from this study and their patho-physiological relevance to gastrointestinal symptoms. Results from this study show that *Blastocystis* secretes protease that can degrade S-IgA that may facilitate colonization of *Blastocystis* or may assist other pathogens in invasion. *Blastocystis* proteases also induce interleukin-8 production and may lead to neutrophil recruitment and intestinal inflammation. In addition, *Blastocystis* can also alter the intestinal barrier function by disturbing tight junctions and cytoskeleton. In addition, *Blastocystis* can induce apoptosis in intestinal epithelial cells which may directly affect intestinal homeostasis or indirectly facilitate invasion of other pathogens. Altogether, these events may eventually lead to gastrointestinal symptoms.

7.2 CONCLUSIONS

1. This study has showed for the first time, that *Blastocystis* possesses high levels of protease activity, in particular of the cysteine protease type. It was also demonstrated that central vacuole of *Blastocystis* contains cysteine proteases (Sio et al. 2006; Puthia et al. 2008).

2. Present study showed for the first time that *Blastocystis* has proteases that can degrade secretory immunoglobulin A (Puthia et al. 2005). The degradation effect is shown in both subtypes of IgA, IgA1 and IgA2.

3. This study reported for the first time that *Blastocystis* induces apoptosis in enterocytes and there is active involvement of caspase-3. In addition, this apoptosis occurred in a contact-independent manner (Puthia et al. 2006).

4. This study demonstrated for the first time that *Blastocystis* can rearrange F-actin and ZO-1 distribution and disrupt epithelial barrier function; and metronidazole can abrogate these effects. In addition, findings showed that IEC-6 monolayers grown on permeable filters provide a useful *in vitro* model for studying the interactions of *Blastocystis* with intestinal epithelial cells (Puthia et al. 2006).

5. This study showed for the first time that *Blastocystis* cysteine proteases induce interleukin-8 production in colonic epithelial cells and an NF-κB-dependent transcriptional process is involved. In addition, it was demonstrated that metronidazole treatment can markedly inhibit the ability of *Blastocystis* to induce IL-8 production (Puthia et al. 2008).

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APPENDICES

Appendix I **Medium for culturing *Blastocystis***

Appendix II **Medium for culturing *Trichomonas vaginalis***

Appendix III **Poly-L-lysine coating of glass coverslips**

Appendix IV **Coomassie (Bradford) Protein Assay**

Appendix V **Measuring cell monolayer resistance by Millicell-ERS**

Appendix I

Medium for culturing *Blastocystis*

For culturing *Blastocystis*, each 15 ml culture test-tube contained the following:

a) Iscove's Modified Dulbecco's Media (IMDM) (Gibco) 9ml

b) Inactivated horse serum (Gibco) 1ml

Loose capped tubes with medium were kept for 24 h in anaerobic jar with gas pack inside to remove oxygen (prereduction of medium). Prereduced medium was inoculated with a *Blastocystis* cells, they were placed in an anaerobic jar and incubated at 37ºC for optimal growth.

Appendix II

Medium for culturing *Trichomonas vaginalis*

For culturing *Trichomonas vaginalis*, each screw-capped test-tube contained the following:

a) Hollander's Medium	9ml
b) Inactivated foetal-calf serum	1ml

Once the test-tubes containing the medium were inoculated with *Trichomonas vaginalis*, they were placed in a test-tube holder and incubated at 37ºC. Sub-culturing was performed every three to five days by inoculating 0.5ml of the turbid suspension into a fresh test-tube containing medium.

Preparation of Hollander's Medium

Trypticase peptone:	20.0g
Yeast extract:	10.0g
Maltose:	5.0g
Ascorbic acid:	1.0g
KCl:	1.0g
KHC03:	1.0g
Kh2PO4:	1.0g
FeSO4:	0.1g
Purified agar:	0.5g

1) Dissolve in 900ml of water
2) Autoclave at 15lb, 15 mins
3) Store at 4 degrees

Appendix III

Poly-L-lysine coating of glass coverslips

1. Acid wash glass coverslips
    a. Soak coverslips in hydrochloric acid overnight (keep in fume hood)
    b. Rinse under tap water for 15-30 minutes
    c. Rinse in sterile water, autoclave and dry
    d. Coverslips are ready for coating
2. Add 50 ml of sterile tissue culture grade water to 5 mg of poly-L-lysine.
3. Aseptically coat culture surface of coverslips with poly-L-lysine solution enough to cover surface
4. Rock gently to ensure even coating of the culture surface
5. After 5 minutes, remove solution by aspiration and thoroughly rinse surface with sterile tissue culture grade water.
6. Allow to dry at least two hours before introducing cells and medium.

Appendix IV

Coomassie (Bradford) Protein Assay (Pierce)

Micro Microplate Protocol (Working Range = 1-25 μg/ml)

1. Pipette 150 μl of each standard or unknown sample into the appropriate microplate wells.
2. Add 150 μl of the Coomassie Reagent to each well and mix with plate shaker for 30 seconds.
3. Remove plate from shaker and incubate plate for 10 minutes at room temperature for consistent results.
4. Measure the absorbance at 595 nm on a plate reader.
5. Subtract the average 595 nm measurements for the Blank replicates from the 595 nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average blank corrected 595 nm measurement for each BSA standard vs. its concentration in μg/ml.
7. Using the standard curve, determine the protein concentration estimate for each unknown sample.

Appendix V

Measuring cell monolayer resistance by Millicell-ERS

(http://www.millipore.com)

Set up the laminar flow hood with a:

- Millicell-ERS
- Millicell culture plate insert without cells
- Millicell culture plate insert with cells
- 70% ethanol solution

Procedure:

To sterilize electrodes, immerse the electrodes in 70% ethanol for 15 minutes. Allow them to air dry for 15 seconds.

1. Rinse the electrodes in sterile cell culture medium or in 0.1–0.15 M KCl or NaCl.
2. For resistance measurements, the electrode is now ready to use.
3. Switch the MODE switch to “R.”
4. Turn the POWER switch to “ON.”
5. Immerse the electrodes so that the shorter electrode is in the Millicell culture plate insert and the longer electrode is in the outer well. The shorter electrode should not contact cells growing on the membrane.

6. Do not push the “R” button while the electrodes are outside solution or the meter could be damaged. Completely immerse the metal sheath at the electrode tips in solution to obtain accurate resistance measurements.
7. Press the MEASURE button. The meter should indicate a stable resistance reading of the solution.
8. Record the resistance.
9. The blank resistance must be measured and then substracted from the resistance reading across monolayer in order to obtain the true tissue resistance.
10. The unit area resistance is obtained by multiplying the meter readings by the effective surface area of the filter membrane.
11. Resistance of a unit area = Resistance (W) x Effective Membrane Area (cm^2).