CHAPTER 1

INTRODUCTION
1.1 History
The protozoan parasite *Entamoeba histolytica* was first identified by Fedor Losch in 1875 while working with human gut-infecting organisms (Losch, 1875) but a formal classification of this organism was proposed by Fritz Schaudin (Schaudin et al., 1903). Clifford Dobell also classified this organism into several species based on the number of nuclei present in the cysts (Dobell, 1919). In 1925, Emile Brumpt proposed that there were in fact two species within what was being called *E. histolytica*. One of these was infective to humans while the other was not. He named the latter as *Entamoeba dispar* (Brumpt, 1925). However as the two species were morphologically indistinguishable, Brumpt's hypothesis was largely ignored for 50 years. The evidence for two distinct sub-groups within *E. histolytica* came from several biochemical, immunological, and genetic studies which showed differing lectin agglutination properties, different isoenzyme patterns and nucleotide sequences of highly conserved genes of isolates obtained from different patients with invasive disease and asymptomatic carriers (Sergeaunt et al., 1987; Strachan et al., 1988; Tannich et al., 1989). Finally, after around 70 years, *E. dispar* was formally separated from *E. histolytica* and this classification was formally adopted in the field of amoebiasis research (Diamond and Clark, 1993).

1.2 Epidemiology
*E. histolytica*, the causative agent of the enteric human disease amoebiasis, infects large number of individuals worldwide and is responsible for around 100,000 deaths per annum. This figure places it third in terms of mortality caused due to protozoan parasites (WHO, 1997). Amoebiasis spreads through contaminated food and water. The parasite usually thrives in the intestine, but during the invasive form of the disease it penetrates the intestinal mucosa and enters the blood stream from where it goes to the vital organs, mainly the liver and sometimes the brain causing abscesses which are fatal if not diagnosed timely.

1.3 Life Cycle
*E. histolytica* exists in two morphologically distinguishable forms – the infective, non-motile, dormant form known as the cyst and the non-infective, motile and dividing
form known as the trophozoite. Infection in humans as shown in Fig. 1, starts with the ingestion of cysts present in food and water contaminated with fecal matter. The cysts pass through the gastro-intestinal tract into the ileo-cecal region, where under favourable conditions, they excyst to produce eight trophozoites per cyst. The trophozoites migrate to the colon and continue to divide further and colonize the host tissues, causing dysentry. They are however incapable of causing fresh infection in another individual; they are only responsible for pathogenesis. Under certain conditions of infection (which are not understood) the trophozoites can penetrate the intestinal mucosa and give rise to typical flask shaped ulcers. They can further move on to the blood stream, from where they travel to various organs like the liver and brain colonising them and causing abscesses which are fatal. Some of the trophozoites in the colon excyst to produce quadrinucleated cysts which are released with the faeces. The cysts have a hard outer wall and can survive in the outside environment in a dormant state.

*Entamoeba* species have a simple life cycle consisting of an infective cyst stage and a multiplying trophozoite stage. Within the genus *Entamoeba*, different species produce cysts with one, four or eight nuclei. There is no cyst stage in a few species, such as *E. gingivalis*, which implies that direct transmission must be taking place through saliva (Clark et al., 2000). In *E. histolytica* the mature cyst contains four nuclei. The process of excystation has been studied in vitro (Marinetts et al., 1997) and in monkeys with essentially identical results (Espinosa-Cantellano and Martinez-Palomo, 1991). The first stage involves movement of the amoeba within the cyst wall. This is followed by the thinning of the wall at one position through which the trophozoite emerges, usually after numerous extensions and retractions of pseudopodia, leaving behind an empty cyst wall. The multi-nucleated trophozoite may start feeding even before fully emerging from the cyst. The four-nucleated amoeba undergoes three rounds of cytokinesis and one round of nuclear division to give rise to eight daughter amoebae (Marinetts et al., 1997). Trophozoites ingest bacteria and multiply by binary fission in the colon where, in response to unknown stimuli, they encyst. Cysts are shed periodically in the stool, thus completing the cycle.
Life Cycle of *Entamoeba histolytica* in Man

After, Smyth, 1994

Amoebae may be carried to liver

Portal vein

Excystation in small intestine

Metacystic amoeba

Non-virulent form feeding on bacteria and debris in gut lumen

Virulent form invading gut

Abcess bursts

Reinvasion

Invasion of mucosa and submucosa; formation of abcess

Pre-cystic amoeba

Encystation

Quadrinucleate cyst

Survival of cyst 2-5 weeks at room temperature

Faeces

**PATHOGENIC**

**NON-PATHOGENIC**

Figure 1. Human infection by *E. histolytica*. Showing multinucleated cysts; infection begins with ingestion of cysts via contaminated food or water; cysts undergo excystment in the intestine giving rise to trophozoites; invasion of intestinal mucosa by trophozoites results in ulceration; and upon encystment, trophozoites convert back into cysts which are excreted in the faeces.
Trophozoites are short-lived outside the body and do not survive passage through the upper gastrointestinal tract. In contrast, cysts may remain viable in a humid environment and stay infective for several days. The invasive form of the parasite is the trophozoite which can penetrate the intestinal mucosa and disseminate to other organs.

1.4 Pathogenesis
One of the properties of \textit{E. histolytica} that plays a crucial role in pathogenesis is its ability to destroy human tissues. Due to lack of a satisfactory animal model which can best mimic this disease in an experimental system, our understanding of the molecular basis of pathogenesis is limited. Certain animal models like hamsters and gerbils have been used. On directly injecting actively dividing trophozoites into the liver of these organisms lesions are produced. The mechanism of pathogenesis can be elucidated to some extent using this model. Adherence can be scored by using Chinese Hamster Ovary (CHO) cell lines. Phagocytosis can be measured by counting the number of erythrocytes ingested by each trophozoite. None of these actually mimic the human disease and are used due to the lack of a suitable animal model. The three major processes which are involved in pathogenesis are:

1) \textbf{Colonization and interaction with the intestinal flora}
Colonization of the intestinal gut by \textit{E. histolytica} trophozoites is the first step in invasive amoebiasis. The trophozoites constantly interact with the intestinal flora, undergoing changes as a result of this interaction. Axenically growing avirulent trophozoites can regain virulence when associated with bacteria e.g. \textit{Escherichia coli}, \textit{Salmonella typhosa} or \textit{S. paratyphi}. It has been shown that direct association of \textit{E. histolytica} with viable bacteria is required for virulence (Wittner and Rosenbaum, 1970). Heat killed or glutaraldehyde fixed bacteria do not increase virulence. However, association with bacteria is not an absolute requirement for invasion by \textit{E. histolytica}.

2) \textbf{Adherence of trophozoite to target cell}
Trophozoites adhere to the target cells and this contact is required for cytotoxic activity. When amoeba interacts with CHO cells on a glass cover slip, the cells which are in direct contact with amoeba display membrane blebbing and are released from
the cover slip. Cells not in contact with amoeba remain viable. It has been shown that dextran prevents adherence of trophozoites to target cells (Ravdin and Guerrant, 1981). Adherence to CHO cells at 37°C is also inhibited by cytochalasins B and D. This may be due to the fact that intact amoebic microfilament is needed for the process.

There are reports of two surface molecules which are responsible for adherence. One of them is inhibited by galactose or N-acetyl-D galactosamine (GalNAc) (Bracha and Mirelman 1983; Petri et al., 1987; Ravdin and Guerrant, 1981). The other one is inhibited by N-acetyl-D-glucosamine (GlcNAc) polymers (Kobiler and Mirelman, 1981). The Gal/GalNAc inhibitable lectin has been characterized in detail (reviewed by McCoy, 1994). The following data leads to the conclusion that this molecule plays an essential role in amoebic adherence to target cells—(1) binding of trophozoites to CHO cells was inhibited 90-95% by 50 mM galactose and GalNAc while other sugars had no effect (Chadee et al., 1987, 1988; Ravdin and Guerrant, 1981; Ravdin et al., 1985; Salata and Ravdin, 1986); (ii) a mutant of CHO cells defective in production of N- and O-linked terminal galactose containing oligosaccharides was almost completely resistant to adherence, and (iii) complex branched polysaccharides containing terminal galactose groups were 1000-fold more effective by weight than galactose, in inhibiting adherence to CHO cells (Petri et al., 1987). The lectin which is a transmembrane protein, has a molecular weight of 260 kDa and dissociates into heavy (170 kDa) and light (35-31 kDa) subunits under denaturing conditions (Petri et al., 1989). Three genes (hgl 1-3) which code for 170 kDa subunit, have been identified and characterized (Mann et al., 1991; Purdy et al., 1993; Tannich et al., 1991a; Alder et al., 1995; Purdy et al., 1996). Two light subunit genes (lgl 1-2) have also been identified and characterized (McCoy et al., 1993a, b; Tannich et al., 1992).

**Lysis of target cells**

A prerequisite to amoebic invasion is the parasite's ability to colonize and invade through the colonic mucins overlying the intestinal epithelium. Intracellular calcium levels in target cells rise approximately 20-fold within seconds of direct contact with an amoebic trophozoite and is associated with membrane blebbing. Cell death occurs 5-15 min. after the lethal hit is delivered. Other than Ca²⁺ flux, amoebic
microfilament movement and phospholipase A are thought to be required for the cytolysis of target cells. It is believed that thiol-proteases, which can be activated by thiols may be involved in pathogenesis (McKerrow, 1993). Patients with invasive disease produce antibodies against this enzyme whereas those with non-invasive disease do not (Reed et al., 1989).

Another candidate for cytolysis may be the pore-forming peptide. Various amoebic pore forming proteins (30, 14 and 5 kDa proteins) have been described (Dodson and Petri, 1994). Of these, a purified 5-kDa amoebapore and a synthetic peptide based on the sequence of its third amphipathic α-helix have been shown to have cytolytic activity for nucleated cells at high concentrations (10-100 μM) (Leippe et al., 1991, 1995, 1997). Proteolytic activities, such as collagenase is also believed to be involved in the lysis of cells and the extracellular matrix of the host.

Leukocytes have the potential to lyse *E. histolytica* trophozoites and vice-versa (McCoy et al., 1994). Only virulent amoeba can lyse polymorphonuclear leukocytes (PMNs) and lysis is blocked by GalNAc (Petri, 1996). *E. histolytica* can kill macrophages and T lymphocytes in vitro.

1.5 Biochemistry

*E. histolytica* is essentially a microaerophilic anaerobe requiring small amount of oxygen for survival. Two known *E. histolytica* enzymes can generate hydrogen peroxide: the NADPH-flavin oxidoreductase (Lo and Reeves, 1980; Bruchhaus et al., 1997) and the iron containing superoxide dismutase (SOD) (Bruchhaus and Tannich, 1994). Catalase is not known to be present (Tanaka et al., 1997); neither is glutathione or its related enzymes (Fahey et al., 1984). Hydrogen peroxide is thought to be degraded by a thiol-dependent peroxidase, previously known as the 29kDa cysteine-rich antigen (Bruchhaus et al., 1997; Poole et al., 1997). There is also evidence to suggest that cysteine is synthesized in *E. histolytica* as genes encoding cysteine synthase have been identified (Nozaki et al., 1998).

Carbohydrates are the main source of energy for this parasite. The pathway involved in the fermentation of glucose includes some unusual enzymes that use pyrophosphate (PPI) rather than a nucleoside triphosphate as the phosphate donor.
(Tanaka et al., 1997). Both D-glucose and D-galactose are transported and actively metabolized by *E. histolytica* (Tanaka et al., 1997). A cDNA encoding the enzyme galactokinase, an enzyme in the galactose utilization pathway, has been detected in a library prepared from glucose-grown *E. histolytica*, suggesting that this enzyme may be expressed constitutively. This is consistent with earlier biochemical observations (Tanaka et al., 1997). Galactose enters the glycolytic pathway via glycogen which is present at high levels in axenic *E. histolytica* (Bakker-Grunwald et al., 1995). Identification of genes encoding glycogen phosphorylase (Tanaka et al., 1997) and phosphoglucomutase (Ortner et al., 1997) and the detection of the necessary uridyl transferase and epimerase activities (Tanaka et al., 1997) suggests that *E. histolytica* may have a pathway for glycogen metabolism similar to that of mammalian systems. The presence of both α and β-amylase activities (Werries and Muller, 1986) and the usually compact structure of *E. histolytica* glycogen have been documented (Bakker-Grunwald et al., 1995). In the glycolytic pathway, genes encoding glucokinase (hexokinase) (Ortner et al., 1997), two phospho fructokinases (Ppi dependent) (Huang et al., 1995; Bruchhaus et al., 1996; Deng et al., 1997), triosephosphate isomerase (Landa et al., 1997), glyceraldehyde-3-phosphate dehydrogenase (Azam et al., 1996; Nozaki et al., 1998), enolase (Beanan and Bailey, 1995; Hidalgo et al., 1997), pyruvate phosphate dikinase (Bruchhaus and Tannich, 1993; Saavedra-Lira and Perez-Montfort, 1994), ferredoxin and pyruvate:ferredoxin oxidoreductase (Tannich et al., 1991b) have been identified. From acetyl-CoA, ethanol is produced by action of acetate thiokinase (Bruchhaus et al., 1997), a bifunctional acetaldehyde/alcohol dehydrogenase (Poole et al., 1997; Nozaki et al., 1998) and a NADP dependent alcohol dehydrogenase (Diamond and Cunnick, 1991; Rosenthal et al., 1997).

The presence of ubiquinone in *E. histolytica* has been demonstrated (Bakker-Grunwald et al., 1995) and this may well accept electrons from Ferredoxin, but the subsequent pathway to oxygen, if indeed oxygen is the terminal acceptor, is unknown. While the terminal acceptor may be oxygen when oxygen is present, the terminal acceptor under anaerobic conditions is completely unknown. Given that *E. histolytica* has no detectable haem iron (Weinbach et al., 1976), cytochromes are not involved. The relative levels of NAD, NADP, NADH, NADPH and ATP molecules
are likely to be very important in *E. histolytica* due to lack of a mitochondrial electron transport chain and tricarboxylic acid cycle. The gene encoding adenylate kinase has been reported (Azam et al., Tanaka et al., 1997). Adenylate kinase maintains ATP balance within the cell by interconversion of AMP, ATP and ADP. It can generate energy not only from the high energy γ-phosphate but also the β-phosphate which may be significant for this organism. The balance among the different forms of pyridine nucleotides may be maintained in part by the enzyme pyridine nucleotide transhydrogenase which catalyzes hydrogen exchange between the two nucleotides (NAD and NADP) (Harrow et al., 1976; Clark and Roger, 1995).

*E. histolytica* is a purine auxotroph but can apparently synthesize pyrimidines (Tanaka et al., 1997). Little is known about lipid metabolism. At least three phospholipase activities A1, A2 and L1 have been detected (Vargas-Villarreal et al., 1995). *E. histolytica* appears to be able to synthesize at least some of its lipids (for example ceramide aminoethyl phosphonate, which is presumably absent from the medium) but details are scarce. Some results indicate an ability to synthesize cholesterol and isoprenoids (Lujan and Diamond, 1997). The only detectable polyamine present is putrescine, at 9.5 mM (Bakker-Grunwald et al., 1995). As it is present only in trace amounts in the growth medium it must therefore be synthesized by the amoeba, and perhaps acts as an osmolyte.

Calcium is also an important molecule in cellular signal transduction and can be released from internal stores by a mechanism based on inositol trisphosphate (IP3) receptor activation (Stanley et al., 1992). Although biochemical evidence for the presence of a Ca$^{2+}$/calmodulin pathway has been reported (Munoz et al., 1992) no calmodulin gene has yet been identified. In addition *E. histolytica* has a novel calcium signal transduction system, as the gene for a calcium binding protein of the calmodulin family, but with no specific homology to any known protein, has been discovered in this species (Espinosa-Cantellano and Martinez-Palomo, 1991). This protein is biochemically different from calmodulin. A novel calcium-binding protein (*EhCaBP*) has been identified and characterized from *E. histolytica*. It is different from calmodulin in the gene sequence and unlike calmodulin it does not stimulate cAMP-phosphodiesterase activity (Yadava et al., 1997).