Title
The role of Entamoeba histolytica Cysteine Proteinase 1 (EhCP1) in the pathogenesis of amebiasis

Permalink
https://escholarship.org/uc/item/49q6h9km

Author
Melendez-Lopez, Samuel G.

Publication Date
2007

Peer reviewed|Thesis/dissertation
The Role of *Entamoeba histolytica* Cysteine Proteinase 1 (EhCP1) in the Pathogenesis of Amebiasis

A Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular Pathology by Samuel G. Melendez-Lopez

Committee in charge:

Professor Sharon Reed, Chair
Professor Kim Barrett
Professor Laurence Brunton
Professor Joshua Fierer
Professor Frances Gillin
Professor Victor Nizet

2007
The Dissertation of Samuel G. Melendez-Lopez is approved, and it is acceptable in quality and form for publication on microfilm:

_____________________________________________________________________
_____________________________________________________________________
_____________________________________________________________________
_____________________________________________________________________
_____________________________________________________________________

Chair

University of California, San Diego

2007
DEDICATION

This dissertation is especially dedicated to my family: to my dear wife Lupita, and my sons Samuel and Miguel. I’m so blessed for their love and unyielding support during the past five and a half years.

I dedicate this dissertation to my mother Ana María (b.1937). I’m forever thankful for her love and support which allowed me to be the first in my family to get a college degree. As a single parent with three children, she showed me that if you have the will and determination you can overcome almost anything.

I also want to dedicate this work to the memory of my dear grandmother Guadalupe (Bela) (1915-2006), for her endless love and support for me and my family. And to the memory of my dear grandfather Guillermo (Bole) (1911-1985), a great man, a wonderful grandfather.

And finally, I dedicate this work to my extended family: My mother, father, sisters and brothers in law and other in-laws. Their continued support for me and my family has been instrumental for my success here at UCSD.
# TABLE OF CONTENTS

Signature Page ................................................................................................................. iii
Dedication ......................................................................................................................... iv
Table of Contents .............................................................................................................. v
List of Abbreviations .......................................................................................................... vi
List of Figures ................................................................................................................... ix
Acknowledgements .......................................................................................................... xi
Vita ...................................................................................................................................... xiii
Abstract ........................................................................................................................... xv

Chapter 1: Introduction: *Entamoeba histolytica* and Amebiasis ......................... 1
Chapter 2: The Virulence Factors of *Entamoeba histolytica* .............................. 13
Chapter 3: The Amebic Cysteine Proteinases ......................................................... 23
Chapter 4: Materials and Methods ............................................................................ 45
Chapter 5: Expression, Purification and Characterization of rEhCP1 ............... 63
Chapter 6: Release and Intracellular Localization of EhCP1 ............................... 77
Chapter 7: Inhibition of Released Amebic Proteinases Markedly Diminishes Amebic Invasion ......................................................... 83
Chapter 8: Discussion ................................................................................................. 94

References ....................................................................................................................... 102
LIST OF ABREVIATIONS

ELISA  Enzyme Linked Immunosorbent Assay
SCID  Severe Combined Immunodeficiency
Gal/GalNAc lectin  D-galactose/N-acetyl-D-galactosamine)-specific amebic lectin
EhCP1  Entamoeba histolytica Cysteine Proteinase 1
EhCP2  Entamoeba histolytica Cysteine Proteinase 2
EhCP3  Entamoeba histolytica Cysteine Proteinase 3
EhCP5  Entamoeba histolytica Cysteine Proteinase 5
Z-Arg-Arg-AMC(Z-RR-AMC)  Benzyloxy carbonyl-arginine-arginine-4-amino-7-methylcoumarin
Z-Phe-Arg-AMC(Z-FR-AMC)  Benzyloxy carbonyl-phenylalanine-arginine-4-amino-7-methylcoumarin
Z-Ala-Arg-Arg-AMC(Z-ARR-AMC)  Benzyloxy carbonyl-alanine-arginine-arginine-4-amino-7-methylcoumarin
Z-Phe-Ala-Arg-AMC(Z-FAR-AMC)  Benzyloxy carbonyl-phenylalanine-alanine-arginine-4-amino-7-methylcoumarin
E-64  L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane
ECM  Extracellular Matrix
IgA  Immunoglobulin A
sIgA  secretory Immunoglobulin A
IgG  Immunoglobulin G
IL-1  Interleukin 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>CCL13</td>
<td>chemokine (C-C motif) ligand 13</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>TSA</td>
<td>Thiol Specific Antigen</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Performing Liquid Chromatography</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>MES</td>
<td>2-Morpholinoethanesulfonic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative Fluorescence Unit</td>
</tr>
<tr>
<td>BME</td>
<td>Beta–mercaptoethanol</td>
</tr>
<tr>
<td>TBS-Tween</td>
<td>Tris Buffered Saline plus Tween 20</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>C3</td>
<td>Complement Component 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>pro-IL-18</td>
<td>Pro-Interleukin 18</td>
</tr>
<tr>
<td>K11777</td>
<td>N-methylpiperazine-urea-phenylalanyl-homophenylalanyl-vinylsulfone-benzene</td>
</tr>
<tr>
<td>HM-1: IMSS</td>
<td>Hospital de Maternidad-1 Instituto Mexicano del Seguro Social</td>
</tr>
<tr>
<td>WRR 483</td>
<td>N-methylpiperazine-urea-arginyl-homophenylalanyl-vinylsulfone-benzene</td>
</tr>
<tr>
<td>PBS-Cys++</td>
<td>Phosphate Buffered Saline plus Cysteine, Calcium and Magnesium</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
</tr>
<tr>
<td>LT</td>
<td>Lysotracker</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>7-AMC</td>
<td>7-Aminomethylcoumarine</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Medium</td>
</tr>
<tr>
<td>PPV</td>
<td>Pre-phagosomal Vacuoles</td>
</tr>
<tr>
<td>q-PCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 3.1 Model of the cysteine proteinases of *Entamoeba histolytica* ........... 27
Figure 5.1 Plasmid map of pBAD-Thio-pro-EhCP1 .................................. 65
Figure 5.2 Expression and purification of Thio-pro-EhCP1 ......................... 66
Figure 5.3 Purification of refolded active rEhCP1 .................................. 67
Figure 5.4 Cleavage of synthetic substrates by rEhCP1 .............................. 68
Figure 5.5 Substrate specificity determined by tetra-peptide libraries ............. 70
Figure 5.6 Cleavage of human C3 by rEhCP1 ........................................ 71
Figure 5.7 Cleavage of human IgG by rEhCP1 ........................................ 72
Figure 5.8 Degradation of human Pro-IL-18 by rEhCP1 ............................. 73
Figure 5.9 pH profile of rEhCP1 ......................................................... 74
Figure 5.10 Km for Z-RR-AMC .......................................................... 75
Figure 5.11 Km for Z-ARR-AMC .......................................................... 75
Figure 6.1 Quantification of released amebic proteinases ............................ 78
Figure 6.2 Localization of EhCP1 by IFA .............................................. 79
Figure 6.3 Localization of EhCP1 and EhCP3 by confocal microscopy .......... 80
Figure 6.4 Localization of EhCP1 and EhCP3 by immuno-EM .................... 81
Figure 6.5 EhCP1 localizes to acidic and non-acidic vesicles ...................... 82
Figure 7.1 The vinyl sulfone cysteine proteinase inhibitor K11777 ............... 84
Figure 7.2 IC50 of K11777 for released amebic proteinases ....................... 85
Figure 7.3 IC50 of K11777 for rEhCP1 ............................................... 86
<table>
<thead>
<tr>
<th>Figure 7.4</th>
<th>Pathology of human intestinal xenografts infected with amebic trophozoites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 7.5</td>
<td>Standard curve used for the quantification of <em>E. histolytica</em></td>
</tr>
<tr>
<td>Figure 7.6</td>
<td>Effect of K11777 on amebic invasion</td>
</tr>
<tr>
<td>Figure 7.7</td>
<td>Comparison of K11777 and WRR 483</td>
</tr>
<tr>
<td>Figure 7.8</td>
<td>IC50 of WRR483 for released proteinases</td>
</tr>
<tr>
<td>Figure 7.9</td>
<td>IC50 of WRR483 for rEhCP1</td>
</tr>
<tr>
<td>Figure 7.10</td>
<td>Effect of WRR 483 on amebic invasion</td>
</tr>
</tbody>
</table>
I would like to thank the members of my Thesis Committee:

Professor Sharon Reed
Professor Kim Barrett
Professor Laurence Brunton
Professor Joshua Fierer
Professor Frances Gillin
Professor Victor Nizet

For their time and help in getting my dissertation ready for defense

The Sharon Reed Laboratory
I would like to thank current and past members of the Reed lab, especially Ken Hirata and Scott Herdman for having the patience to share their expertise in culturing and handling *E. histolytica*, allowing me to successfully perform many crucial experiments. I would also like to thank Dr. Xuchu Que for important technical assistance, and Dr. Marco Antonio Ramos for many insightful discussions. I take this opportunity to specially thank my thesis adviser and mentor Dr. Sharon L. Reed, for allowing me the opportunity to conduct my thesis in her lab on this unique protozoan parasite. She is a wonderful adviser, who always encouraged and had confidence in me to write manuscripts, and to make public presentations of my work in national and international meetings.

I would like to thank our collaborators:
Dr. Lars Eckmann (Mucosal Immunity core, UCSD), Dr. Samuel Stanley Jr. and Dr. Xianjua Guo (Washington University, St Louis, MO), for help with the intestinal xenograft experiments.
Dr. James McKerrow, Dr. Conor Caffrey, Dr. Mojamed Sajid, and Elizabeth Hansell (UCSF), for providing us with key proteinase inhibitors and probes, and expert advise in enzyme-inhibitor kinetics.

Dr. Bibiana Chávez-Munguía (CINVESTAV, IPN, México) for expert help with electron microscopy.

Dr. Charles Craik and Dr. Youngchool Choe (UCSF) for their help with the peptide library experiments.

I would also like to thank: 
Dr. Colin Bloor and Dr. Mark Kamps former and current director of the Molecular Pathology Graduate Program, for their help and advise during my tenure here at UCSD.

I am greatly indebted and thankful to: 
Dr. Laurence Brunton and Dr. Francisco Villarreal, who were instrumental in my coming to UCSD for doctoral studies, and were always supportive with encouragement, thoughtful advice and financial aid during the five and a half years I have spent here at UCSD.

And finally, I would like to acknowledge: 
The Autonomous University of Baja California (UABC), for allowing me to take an extended leave of absence from my academic duties, and for four years (2001-2005) of continued financial support (PROMEP-UABC Scholarship: UABC-123, Clave P/PROMEP, 2000-12-01; Ministry of Public Education, Mexico).
VITA

1987 B.Sc., National Autonomous University of México
1990 M. Sc., National Autonomous University of México
2007 Ph.D., University of California, San Diego

PUBLICATIONS


ABSTRACTS


ABSTRACT OF THE DISSERTATION

The Role of *Entamoeba histolytica* Cysteine Proteinase 1 (EhCP1) in the Pathogenesis of Amebiasis

by

Samuel G. Melendez-Lopez

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2007

Professor Sharon L. Reed, Chair

Amebiasis, defined as invasive intestinal or extra-intestinal infection with the protozoan parasite *Entamoeba histolytica*, is a major cause of morbidity in developing countries and the second leading cause of death from parasitic disease worldwide. More than 500 million people worldwide are infected with *Entamoeba*, causing about 50 million cases of invasive amebiasis and more than 50,000 deaths every year. Based upon biochemical, immunological, and genetic studies, *E. histolytica* has been reclassified into two morphologically identical, but genetically distinct species: *E. histolytica*, which is potentially invasive, and *E. dispar*, which is not. Cysteine proteinases are considered key virulence factors of the protozoan parasite, *E. histolytica*, and play a central role in tissue invasion and disruption of host
defenses. Of all the reported virulence factors found in *E. histolytica* to date, only cysteine proteinases are encoded by unique genes, with at least two of the cysteine proteinase genes absent from *E. dispar* strains (EhCP1 and EhCP5).

The main focus of this dissertation tested the hypothesis that, EhCP1, which is unique to *E. histolytica*, plays a crucial role in amebic invasion: **Specific Aim 1:**

**Tested the hypothesis that *E. histolytica* Cysteine Proteinase 1 (EhCP1) differs from the other cysteine proteinases in its specificity for substrates.** In these studies I: a) Expressed active recombinant EhCP1 and purified it to homogeneity. Recombinant pro-EhCP1 was expressed in *E. coli* and an optimized refolding protocol yielded active enzyme. b) Characterized EhCP1 by its pH optimum, Km for synthetic substrates, and active site mapping. The purified recombinant enzyme had a Km of approximately 2 μM with the fluorogenic peptidyl substrate Z-Arg-Arg-AMC. Its pH optimum was 6.0-6.5, but retained activity >50% between pH 6.0 and 9.0. We mapped the specificity of the P1-P4 subsites of the active site cleft using a positional-scanning synthetic tetra-peptide combinatorial library. Arginine was strongly preferred at the P2 position, an unusual specificity among Clan CA proteinases. c) Determined the specificity of EhCP1 for synthetic and physiological (biological) substrates. Recombinant EhCP1 cleaved C3, human IgG and pro-IL-18 in a time- and dose-dependent manner similar to purified native proteinases

**Aim 2:** **Tested the hypothesis that EhCP1 differs in its release or localization from other cysteine proteinases by:** a) Identifying and quantifying the major released cysteine proteinases by quantitative ELISA. EhCP1 is one of the major
released cysteine proteinases as shown with a sensitive ELISA using monoclonal or polyclonal antibody to recombinant forms of EhCP1, EhCP2, EhCP3, and EhCP5 to quantify released proteinases. EhCP2 was the major released cysteine proteinase followed by EhCP5>EhCP1>EhCP3. 

b) Determining the intracellular localization of EhCP1 by fluorescence, confocal and electron microscopy. EhCP1 localized to large cytoplasmic vesicles, separate from those containing EhCP3.

**Aim 3: Tested the hypothesis that inhibition of EhCP1 blocked or significantly diminished invasion.** In these studies I compared the effect of specific cysteine proteinase inhibitors on *in vivo* invasion in the human intestinal xenograft model in SCID mice. Both pathology and quantification of the number of invading ameba by a sensitive PCR assay demonstrated that the specific irreversible vinyl sulfone cysteine proteinase inhibitors, K11777 and WRR 483, almost completely blocked amebic invasion in the human intestinal xenograft model. Thus, EhCP1 is a major released cysteine proteinase of invasive *E. histolytica*, which is important in both invasion and disruption of the host response.
Chapter 1

Introduction: *Entamoeba histolytica* and Amebiasis.

HISTORICAL BACKGROUND

Protozoa were not recognized until the invention of the microscope by Antonie van Leeuwenhoek at the end of the 17th century. The study of parasitic protozoa only really began two centuries later, following the discovery of bacteria and the postulation of the germ theory by Pasteur and his colleagues at the end of the 19th century [1].

AMEBA AND AMEBIASIS

Humans can harbor nine species of intestinal ameba, of which only one, *Entamoeba histolytica*, is a pathogen. The life cycle is simple. The amebas live and multiply in the gut and form cysts that are passed out in the feces and infect new individuals when they are consumed in contaminated water or food. Most infections are asymptomatic, but some strains of *E. histolytica* can invade the gut wall, causing severe ulceration and amebic dysentery characterized by bloody stools. If the parasites gain access to damaged blood vessels, they may be carried to extra intestinal sites anywhere in the body, the most important of which is the liver, where the ameba cause amebic liver abscess. Evidence that both the intestinal and hepatic forms of the disease were recognized from the earliest times is circumstantial because there are so many
causes of both the bloody dysentery characteristic of amebiasis and the symptoms of amebic liver abscess that many of these records are open to other interpretations [2]. With these reservations in mind, the earliest record is possibly that from the Sanskrit document *Brigu-samhita*, written about 1000 BC, which refers to bloody, mucose diarrhea [3]. Assyrian and Babylonian texts from the Library of King Ashurbanipal refer to blood in the feces, suggesting the presence of amebiasis in the Tigris-Euphrates basin before the sixth century BC [2,4], and it is possible that the hepatic and perianal abscesses described in both *Epidemics* and *Aphorisms* in the *Corpus Hippocratorum* refer to amebiasis [5]. In the second century AD, Galen and Celsus both described liver abscesses that were probably amebic, and the works of Aretaeus, Archigenes, Aurelanus, and Avicenna toward the end of the first millennium give good accounts of both dysentery and hepatic involvement [6]. As amebiasis became widespread in the developed world, there were numerous records of "bloody flux" in Europe, Asia, Persia, and Greece in the Middle Ages [7]. The disease appears to have been introduced into the New World by Europeans sometime in the 16th century [8] and with the later development of European colonies and increased world trade; there are numerous clear descriptions of both the intestinal and hepatic forms of amebiasis. In the 19th century, several books mainly concerned with diseases in India, including *Researches into the Causes, Nature and Treatment of the More Prevalent Diseases of India and of Warm Climates Generally* by James Annersley, clearly refer to both intestinal and hepatic amebiasis [9], and it is now generally agreed that this book contains the first accurate descriptions of both forms of the disease. The connection
between amebic dysentery and liver abscesses was described by William Budd, the English physician who discovered the method of transmission of typhoid [10].

*Entamoeba histolytica* was first linked to amebic colitis and liver abscess by Lösch in 1875, and was named by Schaudinn in 1903 for its ability to destroy host tissues. In 1925, Emil Brumpt proposed the existence of a second, non-pathogenic *Entamoeba* species, *Entamoeba dispar*, which is morphologically indistinguishable from *E. histolytica* to explain why only a minority of individuals infected with *Entamoeba* develop invasive disease. Although Brumpt’s hypothesis was not accepted during his lifetime, it is now clear that he was correct [11]. In 1993, based on cumulative clinical, biochemical, immunologic, and genetic data, *E. histolytica* (Schaudinn, 1903) was redefined to include two morphologically indistinguishable, but genetically distinct species: *E. histolytica*, the cause of invasive amebiasis, and *E. dispar*, a nonpathogenic intestinal commensal parasite [11].

**EPIDEMIOLOGY**

*Entamoeba histolytica* is a parasite of global distribution, but the preponderance of the morbidity and mortality due to amebiasis occurs in Central and South America, Africa, and the Indian subcontinent [12]. Fortunately, the majority of the 500 million individuals worldwide previously believed to be asymptomatic *E. histolytica* cyst passers are actually infected with *E. dispar*, which has not been shown to cause human disease. The best current estimate is that *E. histolytica* causes 34 to 50 million symptomatic infections annually worldwide, resulting in between 40,000 and
100,000 deaths each year [13,14]. In Dhaka, Bangladesh, where diarrheal diseases are the leading cause of childhood death, approximately 50% of children have serologic evidence of exposure to *E. histolytica* at 5 years of age [15].

*E. histolytica* has a simple, two-stage life cycle consisting of an infectious cyst and a motile trophozoite. The cyst form measures 5 to 20 μm in diameter and contains four nuclei. The amoeboid trophozoite, which is responsible for tissue invasion, measures 10 to 60 μm, and contains a single nucleus with a central karyosome. The cysts are relatively resistant to chlorination and desiccation, and survive in a moist environment for several weeks. Infection occurs after the cysts are ingested in fecally contaminated food or water. In the lumen of the distal small intestine, the quadrinucleate cyst undergoes nuclear and cytoplasmic division, giving rise to eight trophozoites [16]. Approximately 90% of infected individuals become asymptomatic cyst passers, completing the organism’s life cycle. In the remaining 10%, trophozoites invade the colonic epithelium and cause colitis [11]. Trophozoites that are able to gain access to the bloodstream may spread hematogenously to establish infection in other organs, most commonly the liver.

**PATHOGENESIS, PATHOLOGY AND IMMUNOLOGY**

After infection with *E. histolytica*, microscopic studies suggest a well-defined sequence of events: adherence, tissue invasion, cytolysis, and inflammation leading to disease [17-20]. After excystation within the lumen of the small intestine, trophozoites first adhere to colonic mucins and epithelial cells via the amebic galactose/N-acetyl-D-
galactosamine inhibitable surface lectin [21-24]. Secreted cysteine proteinases then facilitate tissue invasion by degrading extracellular matrix proteins, thereby disrupting the colonic mucous and the epithelial barrier [25-28]. During tissue invasion, trophozoites kill epithelial and immune cells by a contact-dependent mechanism that requires amebic adherence to host cells via the galactose-inhibitable lectin [21]. Finally, damaged and killed epithelial cells release pro-interleukin-1-β, which is processed by the amebic cysteine proteinases to its active form, resulting in the tissue inflammation and edema seen during early disease [29].

The cecum and ascending colon are the most commonly affected regions of the large bowel, although in severe disease the entire colon may be involved. Gross pathology may range from mucosal thickening to multiple punctuate ulcers with normal intervening tissue, to frank necrosis. The downward invasion of amebic trophozoites is often halted at the level of the muscularis mucosa. Subsequent lateral spread of ameba undermines the overlying epithelium resulting in the clean-based, flask-shaped ulcers that characterize amebic colitis [30,31]. Early in infection, an influx of neutrophils is typical, but, in well-established ulcers, few inflammatory cells are seen [20][30-32]. Organisms may be seen ingesting red blood cells (erythrophagocytosis). At distant sites of infection (e.g., liver abscess), similar pathologic characteristics include central liquefaction of tissue surrounded by a minimal mononuclear cell infiltrate [31-33].

The evidence for acquired immunity to E. histolytica infection is limited. In a retrospective study of 1021 Mexican patients cured of amebic liver abscess between
1963 and 1968, only three patients were readmitted to the study hospital with recurrent liver abscess [34]. Although this number is substantially fewer than expected, no control population was included in the study. Better evidence of immunity comes from the natural history of asymptomatic infection. Greater than 90% of individuals colonized with *E. histolytica* spontaneously clear the infection within 1 year [35].

More recently, epidemiological studies performed by Haque’s and Petri’s groups in Bangladeshi children have shown that a lower incidence of infection and disease by *Entamoeba histolytica* is linked to the absence of serum anti-trophozoite IgG and the presence of intestinal IgA against the carbohydrate recognition domain of the Gal/GalNAc lectin [36,37].

The contributions of humoral and cellular immunity to protection from amebiasis remain unclear. Nearly everyone with invasive amebiasis develops a systemic and a mucosal humoral immune response [38,39,41]. Antibodies alone are unable to clear established infection, since asymptomatic cyst passers remain infected for months after anti-amebic antibodies develop [35,36]. In addition, Bangladeshi children who had a high IgG serum response were more likely to develop invasive disease [36].

Passive immunization experiments in a severe combined immunodeficient (SCID) mouse model of liver abscess, however, demonstrate an important role for preexisting humoral immunity in protection from infection [42]. Reports that individuals receiving corticosteroids may be at increased risk of severe amebic colitis suggest that cellular immunity also plays an important role in control of *E. histolytica*
infection [33]. To date, however, no increase in the severity of disease in patients with the acquired immunodeficiency syndrome (AIDS) has been observed.

CLINICAL PRESENTATION

Infection with *E. histolytica* results in one of three outcomes. Approximately 90% of infected individuals remain asymptomatic. The other 10% of infections result in invasive amebiasis characterized by dysentery (amebic colitis) or, in a minority of cases, extra intestinal disease (most commonly amebic liver abscess) [10,35].

The major diagnostic challenge for the clinician seeing a patient with amebic colitis is to distinguish the illness from other causes of bloody diarrhea. The *differential diagnosis* includes causes of bacterial dysentery (eg, *Shigella*, *Salmonella*, and *Campylobacter* species and enteroinvasive or enterohemorrhagic *Escherichia coli*), and noninfectious diseases, including inflammatory bowel disease, and ischemic colitis [12,43]. In contrast to bacterial dysentery, which typically begins abruptly, amebic colitis begins gradually over 1 to several weeks. Although greater than 90% of patients with amebic colitis present with diarrhea, abdominal pain without diarrhea may occur. The presence or absence of abdominal pain, tenesmus, and fever is highly variable. Weight loss is common because of the chronicity of the illness, and only microscopic blood is present in the stool of a majority of patients [12,43,44].

The most lethal manifestation of amebic dysentery is acute necrotizing colitis with toxic megacolon, which occurs in 0.5% of cases. This complication manifests as
an acute dilatation of the colon, and 40% of patients die from sepsis unless it is promptly recognized and treated surgically [45,46]. Other unusual complications of amebic colitis include the formation of enterocutaneous, rectovaginal, and enterovesicular fistulas and amebomas. Ameboma, due to intraluminal granulation tissue, can cause bowel obstruction and mimic carcinoma of the colon [12,43].

**Amebic liver abscess.** Although a history of dysentery is a common finding, the majority of patients do not have coexistent symptomatic dysentery [47-49]. Other extra-intestinal sites of infection rarely occur, and typically result either from direct extension of liver abscesses (e.g., amebic pericarditis or lung abscess) or hematogenous spread (e.g., brain abscess) [12,43,50].

**DIAGNOSIS**

Microscopic examination of stool samples to diagnose amebiasis, which for decades has been used as the initial diagnostic test of choice for amebic colitis, has low sensitivity (30%–60%). [51,52]. More importantly, light microscopy cannot distinguish *E. histolytica* infection from infection with the intestinal commensal parasite *E. dispar*, which appears to be approximately ten times more common [11,14]. The presence of erythrophagocytic trophozoites in stool samples suggests *E. histolytica* infection, but these are rarely seen [53]. Confusion between *E. histolytica*, other non-pathogenic ameba (e.g, *Entamoeba coli*), and white blood cells also contributes to the over diagnosis of amebiasis [51]. Thus, stool examinations for cysts
and/or trophozoites should no longer be relied on as the sole test to diagnose amebiasis [14].

Because serum anti-amebic antibodies do not develop in patients infected with *E. dispar*, serologic tests for amebiasis accurately distinguish *E. histolytica* and *E. dispar* infection. Seventy-five percent to 85% of patients with acute amebic colitis have detectable anti-amebic antibodies on presentation, and convalescent titers develop in greater than 90% of patients [37,38,60]. For amebic liver abscess, 70% to 80% of patients have detectable antibody titers on presentation, and convalescent titers develop in greater than 90% of patients. In the setting of recent travel to an endemic region and a positive antibody titer, diagnosis is confirmed by an appropriate response to anti-amebic treatment.

The most specific clinically available test for diagnosis of amebiasis is an ELISA to detect *E. histolytica* adherence lectin antigen in fecal specimens (*E. histolytica* II test, TechLab, Blacksburg, VA). Of the many ELISA tests developed thus far, this is the only commercially available test capable of accurately distinguishing *E. histolytica* from *E. dispar* [52][61-67]. The sensitivity of this method for detection of amebic antigen in the stool of patients with colitis is greater than 85%, and its specificity when compared to the “gold standard” of stool culture followed by isoenzyme analysis is greater than 90% [64]. Prior to initiation of treatment, amebic lectin antigen can also be detected in the serum of greater than 90% of patients with amebic liver abscess [68].
Colonoscopy should be performed when noninfectious causes of bloody diarrhea are strong considerations in the differential diagnosis (eg, ulcerative colitis). Since the cecum and ascending colon are most frequently affected, colonoscopy is preferred to sigmoidoscopy. Classically, multiple punctuate ulcers measuring 2 to 10 mm are seen with essentially normal intervening tissue; however, the colonic epithelium may simply appear indurated with no visible ulcerations, and, in severe cases where the ulcers have coalesced, the epithelium may appear necrotic. Histological examination of a biopsy specimen of the edge of an ulcer reveals amebic trophozoites and a variable inflammatory infiltrate [31]. The identification of ameba can be aided by periodic acid-Schiff staining of biopsy tissue, which stains trophozoites magenta.

*Entamoeba histolytica* can be differentiated accurately from *E. dispar* infection using culture with isoenzyme analysis, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) against the amebic lectin antigen. Culture may require more than a week, and isoenzyme requires special laboratory facilities, making it unsuitable for use in all but research settings. Moreover, culture specimens must be inoculated rapidly since delays in stool sample processing dramatically reduce sensitivity [54]. Numerous investigators have developed PCR-based tests with sensitivities and specificities greater than 90% [55-59]. Currently, these tests are most suitable for use in research settings where their ability to differentiate between strains of *E. histolytica* will make them very useful. In the clinical setting, anti-amebic
antibody titers and an ELISA test to detect amebic adherence lectin antigen in stool and serum samples are the most useful available tests.

TREATMENT

The agents for treatment of amebiasis can be categorized as luminal or tissue amebicides on the basis of the location of their anti-amebic activity. The luminal amebicides include iodoquinol, diloxanide furoate, and paromomycin [71,72]. Of these, paromomycin, a non-absorbable aminoglycoside, is preferred because of its safety and the short duration of required treatment. Its major side effect is diarrhea [43]. The tissue amebicides include metronidazole and tinidazole [14,64]. Of these, metronidazole is the drug of choice, with cure rates greater than 90% [72].

Because an estimated 10% of asymptomatic cyst passers will develop invasive disease, *E. histolytica* carriers should be treated [11,14]. For noninvasive disease, treatment with a luminal agent alone is adequate [70]. Patients with amebic colitis should be treated with oral metronidazole (followed by a luminal agent such as paromomycin to prevent recurrent disease [70,72,73]. Most patients with colitis respond promptly to metronidazole with resolution of diarrhea in 2 to 5 days [12]. Metronidazole (750 mg three times a day for 10 days) followed by a luminal agent is also the treatment of choice for amebic liver abscess [70,73].
CONTROL AND PREVENTION

Prevention and control of *E. histolytica* infection depends on interruption of fecal-oral transmission. Water can be made safe for drinking and food preparation by boiling (for 1 minute), chlorination, iodination, or filtration [16]. In the United States and Europe, modern water treatment facilities effectively remove *E. histolytica*. The importance of safe drinking water is highlighted by a recent outbreak of amebiasis in Tbilissi, Republic of Georgia, where there was a waterborne epidemic due to decay of water treatment facilities following the demise of the Soviet Union [74]. More importantly, in the vast majority of the developing world, no modern water treatment facilities exist and none are likely to be constructed in the foreseeable future. This has led many investigators to focus on developing a vaccine for amebiasis. Because humans and some higher non-human primates are the only known hosts for *E. histolytica*, a vaccine that successfully prevents colonization might enable eradication of the disease [75,76].
Chapter 2

The Virulence Factors of *Entamoeba histolytica*

**VIRULENCE FACTORS ASSOCIATED WITH INVASION**

Several virulence factors of *E. histolytica* thought to be involved in trophozoite invasion have been identified and characterized. These virulence factors include: the galactose-inhibitable lectin, which is required for trophozoite attachment [77], a pore-forming peptide called the amebapore [78], and the cysteine proteinases. Although the genome project has identified genetic differences between *E. histolytica* and *E. dispar* [79]. Equivalent genes for the Gal-lectin and amebapore are also present and expressed in the non-pathogenic species *E. dispar*. Cysteine proteinases are the only putative virulence factors to date for which *E. histolytica* has unique genes (i.e. absent from *E. dispar* strains) [80,81].

**A) Gal-GalNAc lectin.** Adherence of amebic trophozoites to the colonic mucus and the intestinal epithelium occurs mainly through a surface Gal-GalNAc(D-galactose/N-acetyl-D-galactosamine)-specific amebic lectin which binds to exposed terminal Gal-GalNAc residues of target cell glycoproteins [23,24,82]. The lectin is a 260 kDa heterodimeric glycoprotein composed of a 170 kDa heavy subunit linked covalently to a 31–35 kDa light subunit by disulfide bonds [82]. Evidence for the involvement of the Gal-lectin molecule in the process of adhesion of the parasite has
been demonstrated by reduced amebic adherence to human erythrocytes, neutrophils, colonic mucins, epithelia and to certain bacteria when the lectin is inhibited by galactose [23,83-85]. Complete inhibition of *E. histolytica* adherence to target cells or colonic mucins is not observed, even when the lectin is blocked with high (100 to 500 mM) concentrations of galactose or GalNAc monomers [24,86,87]. It has been suggested that the spacing of multiple GalNAc residues on the surface of target cells is important for optimal lectin binding [88]. In addition, several other proteins that might participate in adherence have also been identified. These include a 150-kDa intermediate-weight lectin that co-purifies with the Gal/GalNAc lectin, the serine-rich *E. histolytica* protein (SREHP) and the 112-kDa surface protein [89-92]. Lectin-mediated adherence is also required for cytolysis of host cells by ameba, since mammalian cells lacking N-terminal Gal and GalNAc protein modifications are resistant to amebic killing [93,94]. Contact-dependent target cell lysis is also reduced in the presence of galactose, and a monoclonal antibody against the heavy subunit is capable of partially inhibiting cytolysis without blocking adherence [95]. Considering that the purified lectin has no cytotoxic effect even at high concentrations, it has been proposed that the adhesin is involved in the signaling of cytolysis [95,96]. Furthermore, the adhesin binds to purified C8 and C9 components of complement and blocks the assembly of the complement membrane attack complex on the amebic plasma membrane, suggesting a role in mediating amebic resistance to complement lysis through components C5b through C9 [97]. The heavy subunit of the Gal-GalNAc adhesin is encoded by five genes in *E. histolytica* strain HM1: IMSS [98-101]. Analysis of the recently completed *E. histolytica* genome project identified an
additional gene coding for the heavy subunit of the galactose lectin [102]. The derived amino acid sequences suggest that the heavy subunit is an integral membrane protein with a small cytoplasmic tail and a large extracellular N-terminal domain containing a cysteine-rich region. The cytoplasmic tail has sequence identity with β₂-integrin cytoplasmic tails and seems to be involved in the inside-out signaling that controls the extracellular adhesive activity of the amebic lectin [103]. The cysteine-rich region seems to be essential for several activities described for the lectin, including adherence and complement resistance [104]. Moreover, the carbohydrate recognition domain is found within the cysteine-rich region of the heavy subunit [77]. The light subunit shows two 35- and 31-kDa isoforms and has a glycosylphosphatidylinositol anchor. This subunit is also encoded by a gene family that has 79 to 85% nucleotide sequence identity [105]. Comparison between E. histolytica and E. dispar reveals very similar Gal-GalNAc adhesins that efficiently bind to target cells and colonic mucins [84,86,106]. Derived amino acid sequences encoded by homologous genes show that E. dispar heavy- and light-subunit molecules are 86 and 79% identical in their primary structures, respectively, to their E. histolytica counterparts [86,106]. In vitro, E. dispar exhibits adherence and cytotoxicity to target cells that is mediated by the Gal-GalNAc lectin [86], although to a lesser extent than E. histolytica [86,107].

Leroy’s group, in studies of the initial interactions of E. histolytica with polarized intestinal epithelial cells, found that ameba disrupt the enterocyte epithelial tight junctions early, while ameba sonicates had no effect [108]. They show that inhibition of the Gal/GalNAc lectin blocked tight junction disruption, demonstrating the requirement for contact of the intact parasite with the epithelium. The tight
junction proteins ZO-1 and ZO-2 were proteolytically degraded during the interaction and ZO-2 was also dephosphorylated. Tight junction disruption of the enterocytes required transfer of the Gal/GalNAc from trophozoites. This lectin transfer occurs by an unknown mechanism that requires viable trophozoites. The transferred lectin localizes to the basolateral surface of polarized Caco2 cells upon transfer. It is not clear what portions or subunits of the lectin are transferred, as not all anti-lectin monoclonal antibodies recognize the transferred lectin. There is, thus, the question of processing or conformational changes in the lectin during the transfer process. [109].

B) Amebapores. Following contact of *E. histolytica* trophozoites with mammalian cells *in vitro*, rapid cytolysis takes place, resulting in swelling, surface blebbing, and lysis of the target cell, including lymphocytes, polymorphonuclear leukocytes, and macrophages, leaving the parasite unharmed. The similarity of this event to the perforin-mediated lysis of target cells by cytotoxic T lymphocytes [110], initially suggested the participation of a channel-forming protein called the amebapore, whose activity had been identified in *E. histolytica* lysates [111-113]. The amebapore of *E. histolytica* is a channel-forming peptide of 77 amino acid residues, which has now been purified; the protein has been sequenced, and the respective genes have been cloned [114,115]. Three isoforms, amebapores A, B, and C, are present at a ratio of 35:10:1, respectively, with the genes showing 35 to 57% deduced amino acid sequence identity. The molecules share six cysteine residues at identical positions and a histidine residue near the C terminus. Structural modeling suggests a compact tertiary structure
composed of four α-helical structures stabilized by three disulfide bonds [116]. Thus, amebapores are different from the much larger (65- to 70-kDa) perforins that contain three amphipathic segments, two α-helices and one β-sheet [110]. However, similarities at the structural and functional levels have been found between amebapores and NK-lysin, a polypeptide present in natural killer (NK) cells and cytotoxic T lymphocytes of pigs [117,118]. Like other pore-forming peptides, amebapores are readily soluble but are capable of rapidly changing into a membrane-inserted stage [118]. Early observations suggested that the molecule forms multi-state channels with similar properties to those found in the barrel-stave aggregates of toxins such as alamethacin [119]. With the elucidation of the primary and secondary structures, it is now believed that amebapores aggregate through the arrangement of their amphipathic α-helices. In a model proposed by Andrä and Leippe [120], amebapores bind to negatively charged phospholipids via protonated lysine residues; this is followed by the insertion of the peptide into the lipid bilayer driven by the negative membrane potential of the target membrane. Oligomerization of the peptide occurs with the participation of a key histidine residue (His\textsuperscript{75}), that may either interact with another monomer through the formation of hydrogen bonds or stabilize the predicted fourth α-helix. The oligomer forms a channel through the plasma membrane, allowing the passage of water, ions, and other small molecules, resulting in lysis of the target cell. In vitro, amebapores exert cytolytic activity against several human cultured cell lines. Amebapore C seems to be the most effective, while amebapore A is not efficient in lysing erythrocytes. In addition, the peptides show potent antibacterial activity against
gram-positive bacteria by damaging their surface membranes. Damage to the outer membrane-shielded gram-negative bacteria requires high concentrations of amebapore or removal of the wall with lysozyme [121-123]. With the use of synthetic peptides, of the four α-helices present in the molecule, helix 3 was found to be responsible for the membrane penetration, displaying the highest antibacterial activity. Interestingly, helix 3 is also the most highly conserved domain in the three isoforms [122]. The peptide derived from isoform C was the most active of several synthetic peptides studied, reaching a magnitude of activity in the range of the whole molecule [121]. Amebapores are localized in cytoplasmic vesicles, as evidenced by positive immunofluorescence staining and by the presence of typical signal peptides of intracellular transport in its primary translation products. The peptides show maximum activity at acidic pH, which is consistent with previous observations that lysis of target cells by E. histolytica required a pH of 5.0 within amebic vesicles [115]. A peptide homologous to E. histolytica amebapore A has been identified in E. dispar [124]. The molecules have common structural and functional properties, such as insertion into negatively charged liposomes, highest activity at low pH, localization in cytoplasmic vesicles, 95% identity of primary structures, and a high degree of similarity of secondary-structure predictions. In spite of these similarities, the specific activity of the E. dispar amebapore is 60% lower than that of the one in E. histolytica. This may be related to a shortened amphipathic helix in the former [124].

Surprisingly, in spite of all the advances in the biochemistry and molecular biology of amebapores, their participation in the cytolytic event produced by
*E. histolytica* has not yet been demonstrated. Amebapores are not spontaneously secreted from viable trophozoites [125]. Whether the molecule is able to insert into target cell membranes upon adherence *in vivo* remains to be established. The presence of pore-forming activity in the noninvasive *Entamoeba dispar* suggests that the primary function of amebapores is to destroy phagocytosed bacteria, the main intestinal source of nutrients of amebas; thus, they have a similar function to defensins found in mammalian phagocytes that kill bacteria and fungi to prevent intracellular microbial growth within digestive vacuoles [118,126]. The anaerobic environment found in the colon may favor oxygen-independent mechanisms as a means of destroying bacteria, rather than using oxygen metabolites and nitric oxide. Amebapores and other proteins, like the recently characterized ameba lysozyme that co-localizes to the same cytoplasmic granules of amebapores [127,128] could synergistically enhance the antibacterial activity.

*Entamoeba histolytica* trophozoites with reduced cytolytic activity *in vitro* are less able to cause liver abscess in animal models, providing the best evidence for the role of amebic host cell killing in the pathogenesis of invasive disease [103,129]. The mechanism of cell killing has been the subject of intense investigation and remains controversial. Gal/GalNAc-mediated contact with host cells is required [93,94]. The ability of purified amebapore to lyse cells at micromolar concentrations suggests a necrotic mechanism of host cell death [130]. However, simple cell lysis (necrosis) does not fully explain the phenotype of dying cells, suggesting a more complicated mechanism of cell killing. Ameba-induced nuclear chromatin condensation and
internucleosomal DNA fragmentation consistent with apoptotic cell death have been observed both in vitro and in animal models of colitis and liver abscess [131-133]. Furthermore, an irreversible rise in intracellular calcium concentration and activation of caspase 3, a distal effector molecule in the apoptotic pathway, occur rapidly in the host cell following amebic contact and are required for cell death to proceed in vitro [133,134]. The importance of an apoptotic mechanism of host cell killing is emphasized by the ability of the non-specific caspase inhibitor z-VAD-fmk to block amebic liver abscess formation in mice [135]. In actuality, the relative contributions of apoptotic versus necrotic cell death and indeed where the phenotype of any given dying cell falls on the spectrum from pure necrosis to pure apoptosis probably depends on the type of host cell, as well as other host and parasite variables that at present remain unknown [136].

C) Cysteine Proteinases. Cysteine proteinases appear to have multiple roles in amebic invasion. Clinical E. histolytica isolates release 10–1000-fold more cysteine proteinase activity into culture supernatants than E. dispar isolates [137]. Cysteine proteinases purified from axenic cultures of E. histolytica cleave collagen, elastin, fibrinogen, and laminin, components of the extracellular matrix that trophozoites must penetrate to cause invasive disease [26,27]. Cysteine proteinases are directly involved in the detachment of tissue culture monolayers, the most widely used assay for amebic toxins. The observed cytopathic effect on fibroblast monolayers that results from contact of supernatants from clinical E. histolytica strains is completely inhibited by specific irreversible cysteine proteinase inhibitors, which are non-toxic to host cells
Amebic secreted cysteine proteinases (EhCPs) disrupt the polymeric structure of MUC2; the major component of human colonic mucus, and degraded mucus is less efficient at blocking amebic adherence to host cells *in vitro* [138]. Therefore, it appears likely that mucus degradation *in vivo* enables subsequent contact-dependent cytolysis of epithelial cells. More than forty genes coding *E. histolytica* cysteine proteinases have been identified [92,139,140]. *E. dispar* lacks genes for two of the cysteine proteinases, EhCP1 and the membrane-bound proteinase EhCP5 [139,141]. These data suggest that qualitative differences in cysteine proteinase expression might be more important than the level of expression. Consistent with this, overexpression of EhCP2 in *E. dispar* increased tissue monolayer destruction *in vitro*, but had no effect on liver abscess formation in gerbils [142]. The substrate specificity of the amebic proteinases is typical for that of cathepsin B-like proteases in the papain family and *in vitro* degradation of relevant extracellular matrix proteins including type I collagen, fibronectin and laminin has been demonstrated [26,143]. Recent data indicate that the cysteine proteinases may also mediate proteolysis of enteric cell villin and cause effacement of microvilli [144]. Pro-interleukin-1β (IL-1β) released from lysed epithelial cells is cleaved and activated by the cysteine proteinases, and a direct role for cysteine proteinases in the development of inflammation during early colonic invasion has been demonstrated by antisense knockout studies in severe combined immunodeficient (SCID)–human mice [29].

Additionally, cysteine proteinases interfere with the function of the host’s immune system. A cysteine proteinase purified from *E. histolytica* can specifically
cleave C3 by a unique mechanism that enables *E. histolytica* to activate complement in the fluid phase [145]. This proteinase also degrades immunoglobulin A (IgA), and the anaphylatoxins C3a and C5a, which may explain the relative paucity of neutrophils noted in amebic liver abscesses [146]. The proteinases must be released during the course of invasive amebiasis, because more than 80% of infected patients make antibodies to cysteine proteinases [137]. Studies in animal models by the groups of Mirelman and Stanley have demonstrated that inhibition of cysteine proteinase activity with chemical inhibitors or antisense constructs to EhCP5 significantly decrease liver abscess formation in SCID mice [147] and hamsters [148]. All together, the data mentioned above, clearly supports the notion that amebic cysteine proteinases play a key role in virulence. [149].
MULTIPLE FORMS OF AMEBIC CYSTEINE PROTEINASES

Cysteine proteinases are the most abundant proteinases in *Entamoeba histolytica*. Cell fractionation studies indicate that although present in the cytosol, they are enriched in the plasma and internal membranes. Also, *E. histolytica* releases extracellularly high amounts of cysteine proteinases. Several cysteine proteases purified directly from *Entamoeba histolytica* cultured trophozoites have been described: a 16-kDa cathepsin-B-like molecule [28], a 56-kDa neutral cysteine protease [26], a 22/27-kDa protease named amoebapain [150], a 26/29-kDa histolysain [27], a 30-kDa cysteine protease (EhCP$_3$) [141] and a cysteine protease associated directly with the 112-kDa adhesin (EhCP112) [92].

Prior to the completion of the *Entamoeba histolytica* genome project seven cysteine proteinase genes were identified: Reed *et al.* [80] isolated three genes from the HM-1 strain designated as *acp1*, *acp2* and *acp3*. Tannich *et al.* [81][151] identified six different cysteine proteinase genes (*ehcp1* to *ehcp6*). Some are identical to those described previously by Reed *et al.* [80]: *ehcp1*, *ehcp2* and *ehcp3* correspond to *acp3*, *acp2* and *acp1*, respectively. Orozco’s group isolated the cysteine proteinase gene *ehcp112*, which codes for a cysteine proteinase tightly associated with a surface adhesin [92]. Two of the previously mentioned genes are absent in *E. dispar* (*ehcp1*, *ehcp5*), the non-invasive *Entamoeba*. In contrast, genes analogous to four of the six
genes identified in *E. histolytica* were found to be present also in *E. dispar* (*ehcp2, ehcp3, ehcp4, ehcp6*) [81,152]. All of the cysteine proteinase genes described so far encode for the pre-proforms of the proteinases. The enzymes encoded by three of these genes have been purified and characterized, namely, EhCP1 (ACP3; previously known as amebapain) [153,154] EhCP2 (ACP2; earlier reported as histolysin) [27,151], and EhCP5, a membrane-bound proteinase [141]. These three enzymes, all with a molecular mass of ~30 kDa, account for ~90% of cysteine proteinase transcripts and for virtually all of the cysteine proteinase activity found in *E. histolytica* lysates [81].

More recently, analysis of the just completed *E. histolytica* Genome Project (www.tigr.org) has resulted in the identification of more than 40 genes coding for cysteine proteinases [102,139,155]. It is surprising that only a small number of the cysteine proteinase genes identified, so far, are actually expressed in cultured trophozoites, Tannich’s group confirmed previous observations that *ehcp1, ehcp2,* and *ehcp5* are indeed strongly expressed in *E. histolytica*, and a medium level of expression was found for *ehcp3, ehcp8,* and *ehcp9* [139].
SUBSTRATE SPECIFICITY

The substrate specificity of *E. histolytica* proteinases is typical of cathepsin B-like proteases of the papain family [26,27]. All of the amebic cysteine proteinases (native and recombinant), tested so far, are very active against the synthetic peptide substrate, Z-Arg-Arg-AMC (benzyloxycarbonyl-arginine-arginine-4-amino-7-methylcoumarin), with arginine at the P1 and P2 positions. The amebic cysteine proteinases (native and recombinant) show very low affinity for the cathepsin L substrate, Z-Phe-Arg-AMC, and the cathepsin H substrate, Z-Arg-AMC, although the molecular analysis of the cysteine proteinase genes has identified only cathepsin L-like sequences. Even though the pH optima of *E. histolytica* cysteine proteinases is broad, ranging from about pH 6.0 to 8.5, most of these enzymes are active in slightly acidic and neutral pH environments [156].

INHIBITOR SPECIFICITY

Amebic cysteine proteinases are classified as cysteine (thiol) proteinases because their activity is inhibited by the cysteine proteinase-specific inhibitor L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (E-64) and not by the serine proteinase-specific inhibitor phenylmethylsulfonfyl fluoride. Amebic cysteine proteinases are also inhibited by sulphydryl reagents (*p*-chloromercuribenzoate [PCMB]) and activated by dithiothreitol (DTT) and 2-mercaptoethanol.
Inhibition of proteinase activities is a new approach to anti-infectious therapy, which has been revolutionized by the therapeutic efficacy of synthetic protease inhibitors specifically active against the aspartyl proteinase of human immunodeficiency virus. New generations of cysteine proteinase-specific inhibitors, including diazomethanes, vinyl sulfones, and synthetic peptide inhibitors, have been active in the micromolar to nanomolar range against *E. histolytica* [80], as well as against other protozoan parasites [157,158]. A specific vinyl sulfone cysteine proteinase inhibitor prevented lethal *Trypanosoma cruzi* infection in mice [159], supporting at least potentially the use of cysteine proteinase inhibitors as novel anti-parasitic therapy.

**STRUCTURAL ASPECTS OF AMEBIC CYSTEINE PROTEINASE GENES**

**Structure and Processing of Amebic Cysteine Proteinases**

Amebic cysteine proteinases are synthesized as precursor proteins with a 12- to 14-amino-acid hydrophobic pre-domain signal peptide, a 78- to 82-amino-acid pro-domain, a 216- to 225-amino-acid catalytic domain, and no C-terminal extension. The pre-pro-enzymes are subsequently processed to the mature enzymes. The signal sequences consist of a N-terminal charged region (n-region), a central hydrophobic region (h-region) and a polar C-terminal region (c-region). A putative processing site between the signal peptide and pro-peptide region is predicted by the -3, -1 rule of von Heijne [160]. The pro-domains contain a stretch of sequence containing the ERFNIN
motif \([\text{Glu-X}_3-\text{Arg-X}_2-(\text{Val/Ile})-\text{Phe-X}_2-\text{Asn-X}_3-\text{Ile-X}_3-\text{Asn}]\) near residue -50 of all amebic cysteine proteinases. This motif has been identified at a similar position in all cathepsin H- or L-like proteinases but not in cathepsin B-like enzymes [161]. See figure 3.1.

The pro-domain of eukaryotic cathepsins has at least two well-defined functions: 1) to maintain the enzyme in an inactive form (zymogen) until it reaches an appropriate site for protease function and 2) to act as a chaperone like structure to ensure proper folding during translation [162]. EhCP5 contains an Asn-X-(Ser/Thr) recognition sequences within the prosequence, which may be post-translationally modified by glycosylation [141]. The mature regions of the amebic cysteine proteinases are homologous to cathepsin L-like cysteine proteinases and contain all conserved cysteine residues implicated in the maintenance of the three-dimensional structure.

![Figure 3.1: Model of the cysteine proteinases of Entamoeba histolytica](image)

The relative sizes of the pre-enzyme, pro-enzyme, and mature enzymes are depicted. The ERFIN motif of cathepsin L in the prosequence is shown, along with the three residues of the active site, cysteine (C), histidine (H), and asparagine (N). aa, amino acids. From Que, X. and Reed, S.L. Clin Microbiol Rev. 2000 April; 13(2): 196–206[149].
ROLES OF CYSTEINE PROTEINASES IN HOST-PARASITE INTERACTIONS

Nutrient Acquisition and Developmental Cycle

Cysteine proteinases play numerous and indispensable roles in the biology of parasitic organisms. Studies of other protozoan parasites suggest that the functions of cysteine proteinases are diverse [157,163,164]. Aside from previously known general catabolic functions and protein processing, cysteine proteases may be crucial to parasite immunoevasion, excystment/encystment, exsheathing and cell and tissue invasion. Parasite cysteine proteinases are highly immunogenic and have been used as serodiagnostic markers and vaccine targets [164].

Parasitic cysteine proteinases often have unique structural and biochemical properties. These functional and structural differences of the parasite cysteine proteinases compared to their counterparts in the host are now opening the way for the development of novel chemotherapy to treat parasitic infections [165]. Parasite cysteine proteinases play fundamental roles in the acquisition of nutrients and as chief participants in their developmental cycles [164,166]. The *Plasmodium* cysteine proteinases hydrolyze globin to free amino acids for the growth of the intra-erythrocytic parasites [166,167], and the proteinase inhibitors that block hemoglobin degradation also block the development of cultured malaria parasites [168]. In a more recent finding, a new family of cystene proteinase inhibitors called peptidyl vinyl sulfones, was shown to inhibit *P. falciparum* development at low nanomolar concentrations, and blocked the development of *P. falciparum* in culture, and exerted
antimalarial effects in vivo. [169]. Cysteine proteinase inhibitors also block the intracellular development of *T. cruzi* [170] and *Leishmania* [171]. Although *E. histolytica* is not an intracellular parasite, when its trophozoites were incubated with a diazopeptidyl inhibitor, growth was decreased by 50% [172] suggesting that cysteine proteinases are important for the acquisition of nutrients, even from a liquid medium.

Cysteine proteinases also play a role in the development of many parasites. Specific inhibitors blocked the transformation from epimastigotes to trypomastigotes [173] and from amastigotes to trypomastigotes in *Trypanosoma cruzi* [174]. In *Trypanosoma brucei*, cysteine proteinase activity increased during differentiation from long slender to short stumpy forms [175]. Furthermore, cysteine proteinases have been shown to be critical for excystation of *Giardia* [176]. In *Entamoeba invadens*, which has served as a model for the study of encystation/excystation of *E. histolytica*, specific cysteine proteinase inhibitors significantly decreased the efficiency of encystation [177]. Further studies are required to clarify whether cysteine proteinases play a direct role in the initiation of encystation or whether the effect of inhibition is secondary through decreased trophozoite multiplication.
The Role of Cysteine Proteinases in Host Invasion

Cysteine proteinases play a crucial role in host invasion for a number of parasites. Specific inhibitors of cysteine proteinases block invasion in Trypanosoma cruzi [173], Plasmodium falciparum [178], Cryptosporidium parvum [179], and Toxoplasma gondii [180]. The role of cysteine proteinases in tissue invasion has been very well documented in Entamoeba histolytica. Purified proteinases from Entamoeba histolytica degrade components of the extracellular matrix, including fibronectin, laminin, and collagen, as well as an extracellular matrix from vascular smooth muscle [26]. Cysteine proteinases are primarily responsible for the cytopathic effect, an in vitro assay of Entamoeba histolytica virulence that measures detachment of cells from monolayers [62,80]. The observed cytopathic effect correlates with the amount of cysteine proteinase activity that is released into the medium by clinical isolates of E. histolytica [137] and can be inhibited by specific peptide inhibitors [80]. Mutants of the Entamoeba histolytica strain HM-1 that are deficient in both proteinase expression and cytopathic effect have been identified [181]. The in vitro cytopathic effect correlates well with the early pathology of invasion in animal models in which the intestinal epithelial cells separate before making direct contact with trophozoites, presumably from disruption of the extracellular matrix [182]. In summary, E. histolytica possesses the necessary machinery to degrade the ECM components it encounters during invasion.
The Roles of Cysteine Proteinases in Immune System Evasion and Inflammation

Degradation of Human IgA

IgA is the main antibody defense at mucosal surfaces [183,184]. Although not all of the biological functions of IgA are understood, some of the known functions of IgA include the immobilization and prevention of adherence of microorganisms, the binding of toxins, and inhibition of antigen absorption [183-185]. Most B lymphocytes found at the external mucosa are dedicated to the production of IgA, which is in turn released into the bowel as a dimer linked by disulfide bonds and a carbohydrate-rich secretory component, which is necessary for transepithelial secretion [184]. It has been suggested that IgA may play an indirect role in the antibody-mediated cytotoxicity through Fcε receptors and potentiation of the action of non-specific antibacterial factors such as lactoferrin, lactoperoxidase, and lysozyme [185]. The presence of immune secretory IgA (sIgA) has been demonstrated in patients with invasive amebiasis by measurement of fecal [186,187], colostral [188], and salivary [41] antibodies. Ximenez’s group showed that carriers of E. dispar also developed a salivary sIgA response [189]. Both human salivary sIgA [190] and anti-amebic monoclonal IgA [191] blocked the adherence of Entamoeba histolytica trophozoites to epithelial or colonic cell monolayers. Recent epidemiological studies performed by Haque’s and Petri’s groups in Bangladeshi children have shown that a lower incidence of infection and disease by Entamoeba histolytica is linked to the presence of intestinal IgA against the carbohydrate recognition domain of the Gal/GalNAc lectin [36,37].
Pathogenic bacteria capable of invading mucosal surfaces, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, produce IgA1 proteases [183,192-195]. All IgA1 proteinases, characterized so far, are extracellular, neutral endopeptidases that cleave specifically the hinge region of IgA1 to produce Fab and Fc fragments [183,192,196]. Cleavage of IgA1 might result in reduction of its affinity for antigen; interfere with antigen disposal or masking of immunogenic determinants by coating them with the resulting Fab fragments [185]. Similarly in *Entamoeba histolytica*, Quezada-Calvillo et al. [197] demonstrated limited proteolytic cleavage of human IgA by whole ameba. And, Kelsall and Ravdin [198] found that the degradation of human IgA appeared to be mediated predominantly by secreted amebic cysteine proteinases.

**Disruption of Immune IgG**

A number of parasites release proteinases that cleave IgG. Trypomastigotes of *Trypanosoma cruzi* bind IgG through the Fab fragment and then cleave the Fc fraction with cruzain, its major cysteine proteinase [199]. *Schistosoma mansoni* binds immunoglobulins via their Fc receptor and then degrades the Fab portion of IgG [200]. Cysteine proteinases of *Tritrichomonas foetus* [201], *Trichomonas vaginalis* [202], and *Fasciola hepatica* [203] also degrade IgG.

A systemic IgG response develops in patients who are colonized or have invasive infection with *Entamoeba histolytica*, in contrast to those colonized with *E. dispar* [204]. Any protective role for IgG in amebic disease has been difficult to establish, and
IgG antibody levels correlate with the length of disease, not with the clinical response to infection [204]. In animal models, a serum antibody response did not protect hamsters, but SCID mice were passively protected by rabbit polyclonal immune serum [42]. Studies done in our laboratory found that both intact trophozoites and the purified extracellular cysteine proteinases cleaved the heavy chain of IgG [205]. When a monoclonal antibody to the surface thiol-specific antioxidant was cleaved by purified proteinase, binding to trophozoites was decreased by more than 80% [206]. In a recent epidemiological study of amebiasis in Bangladeshi children, Haque et al [37] found that individuals who developed mainly a high serum IgG titer of anti-amebic antibodies did not seem to be protected from re-infection and recurrence of disease. These results suggest that cleavage of IgG by the extracellular cysteine proteinase may limit the effectiveness of the host humoral response.

**Resistance to Complement-Mediated Lysis**

To invade successfully, trophozoites must be able to evade multiple local and systemic host defenses, including the action of activated complement proteins. Trophozoites have a carbohydrate-rich surface and were shown to consume components of both the classical and alternative pathways of the complement cascade [206,207]. The substrate specificity of the amebic cysteine proteinases for positively charged amino acids is very similar to that of the proteinases generated during complement activation. Our research group has shown that *Entamoeba histolytica* trophozoites activate complement by a unique mechanism, cleavage of the α-chain of
C3, generating a product, which acts as functionally active C3b [137]. The activated terminal complement components are also generated, which lyse *Entamoeba dispar* but not *E. histolytica* by reactive lysis [145]. *Entamoeba histolytica* resists lysis by the membrane attack complex because the galactose-inhibitable lectin has antigenic cross-reactivity with CD59, a membrane inhibitor of C5b-9 in human blood cells [97]. The former may be one mechanism by which *Entamoeba dispar* gets confined to the lumen of the bowel by complement-mediated killing in tissues or the bloodstream. Other groups have reported that *Entamoeba dispar* strains are resistant to complement-mediated lysis while *Entamoeba histolytica* strains are susceptible [208]. It is not clear whether the conflicting results are due to different culture conditions, but from a clinical standpoint, only *Entamoeba histolytica* can cause invasive disease.

**Degradation of Anaphylatoxins C3a and C5a**

The anaphylatoxins, C3a and C5a, are very potent stimulators of the host inflammatory response. C3a and C5a are generated by cleavage of the α-chains of C3 and C5 by their correspondent convertases. The small remaining portion of each molecule, C3b and C5b, participate in the activation of the late-acting complement components, which lead to the formation of the membrane attack complex or MAC [209]. C3a exerts multiple physiologic effects, including increasing vascular permeability and smooth muscle contraction, suppressing T-cell proliferation, and releasing histamine from mast cells and interleukin-1 (IL-1) from macrophages [76].
C5a induces chemotaxis of neutrophils, activates macrophages, and stimulates the release of IL-1, IL-6, and IL-8 [210]. The same extracellular cysteine proteinase(s) from *Entamoeba histolytica* that activates complement to produce active C3b [211] also degrades the C3a and C5a, which were subsequently formed in a dose-dependent fashion [146]. The same extracellular cysteine proteinase(s) of *Entamoeba histolytica*, which activate complement, may also aid in the evasion of the normal host immune response by inactivating the anaphylatoxins, C3a and C5a.

**Amebic Cysteine Proteinases in the Induction of an Inflammatory Response in the Human Colon**

Cysteine proteinases released by *Entamoeba histolytica* trophozoites also seem to play an important role in colonic invasion and inflammation. *Entamoeba histolytica* trophozoites secrete 100 to 1,000-fold more cysteine proteinases than the non-invasive ameba *Entamoeba dispar* [137]. Most of the proteinase activity seen in *Entamoeba histolytica* lysates and secretions can be attributed to just four proteinases: EhCP1, EhCP2, EhCP3 and EhCP5 [81,80,139]. When *Entamoeba histolytica* trophozoites with reduced levels of proteinase activity were generated by the episomal expression of an antisense message to the *ehcp5* gene [212], the total cysteine proteinase activity in these trophozoites was reduced by about 90% in comparison to levels in control trophozoites. These proteinase-deficient trophozoites were less virulent in a hamster model of amebic liver abscess, their phagocytic capability was considerably reduced [148,212] and they showed markedly reduced virulence when used to infect human
intestinal xenografts [29]. Infected xenografts had less intestinal inflammation attributed to reduced levels of IL-1 and IL-8 and decreased neutrophil influx [29]. The reduced virulence of cysteine proteinase-deficient *Entamoeba histolytica* trophozoites may be explained by multiple factors. The inability to cleave extracellular matrix proteins could reduce trophozoite invasiveness, and the still unexplained phagocytosis defect in protease-deficient ameba could have physiological effects as well [212]. Cysteine proteinases may have important functions within the parasite (digestion of ingested bacteria and red blood cells, activation of other enzymes, etc.), and inhibition of these functions may impact virulence in subtle ways. *Entamoeba histolytica* lysates or purified amebic cysteine proteinases are capable of mimicking the activity of IL-1-converting enzyme (ICE or caspase 1) and can cleave pro-IL-1β to form the active mature cytokine [29]. Intestinal epithelial cells lysed by *Entamoeba histolytica* trophozoites may release pro-IL-1β, which could then be activated by extracellular amebic cysteine proteinases and further amplify the inflammatory process in amebic colitis. Que et al [213] showed that secreted amebic proteinases and recombinant EhCP5 cleaved pro and mature IL-18 to biologically inactive fragments, thus potentially blocking the host’s inflammatory response. Pertuz-Bellosa *et al* [214] found that purified EhCP2 is capable of cleaving *in vitro* the chemokines CCL2, CCL13 and CXCL8, and that this specific proteolytic cleavage by EhCP2 leads to reduced amounts of biologically active chemokines. Proteolytic cleavage of CCL2, CCL13 and CXCL8 impair the chemotactic responses by monocytes and granulocytes.
This could be one of the putative regulatory mechanisms used by this pathogen to decrease leucocyte recruitment and activation.

ANIMAL MODELS FOR THE STUDY OF AMEBIC CYSTEINE PROTEINASES

Intestinal and Hepatic Amebiasis in Gerbils and Hamsters

One of the main problems in experimental amebiasis has been the absence of an animal model that closely mimics human disease, in which liver abscesses develop after intestinal infection. Early stages of amebic invasion were observed after the injection of trophozoites into the cecum of gerbils [32,215] or guinea pigs [18]. Two of the most widely used models of amebic liver abscess utilize direct inoculation of trophozoites into the livers of baby gerbils or hamsters [216,217]. The size of liver abscesses can be readily quantified, and these models have been very useful for studying potential vaccine candidates, including the galactose-inhibitable lectin [218], the serine-rich Entamoeba histolytica protein [219], and the 29-kDa thiol-specific antioxidant (TSA) [220,221]. In vivo models have also been useful to confirm that Entamoeba dispar could not form amebic liver abscesses, even when injected directly into the liver [222]. A cysteine proteinase-deficient and phagocytosis-deficient mutant of Entamoeba histolytica strain HM-1 also did not form liver abscesses [223].
Liver Abscess in SCID Mice

Stanley's research group showed that liver abscesses could be formed after direct injection of *Entamoeba histolytica* trophozoites into the livers of SCID mice [42]. The size of amebic liver abscesses was significantly decreased by pre-incubating *Entamoeba histolytica* trophozoites with laminin, a molecule which binds to and blocks the activity of amebic cysteine proteinases [143]. They showed that an affinity-purified antibody against a recombinant cysteine proteinase of *Entamoeba histolytica* localized the proteinase in amebic trophozoites and extracellularly in amebic liver abscesses of infected SCID mice [147]. Pre-treatment of *Entamoeba histolytica* trophozoites with the specific cysteine proteinase inhibitor E-64 blocked or greatly decreased the size of liver abscesses after 48h of infection. [148]. These studies suggest that cysteine proteinases have a crucial role in amebic liver abscess formation.

Human Intestinal Xenografts in SCID mice

*Entamoeba histolytica* infects only humans and non-human primates, and there has been limited success at establishing reproducible animal models of intestinal amebiasis. Intestinal xenografts provide a sterile and biologically relevant animal model system for studying host-parasite interactions, particularly the first stages of invasion in the human intestine. Human fetal intestinal tissue is engrafted into the subcutaneous space on the backs of SCID mice [224]. After 8 weeks, a functional human intestine develops. Amebiasis in this model closely mimicked the pathological
findings reported in cases of human amebic colitis, with an early phase of mucosal damage and subsequent invasion of ameba into submucosal tissues with the formation of amebic ulcers [224]. An early neutrophil response was detected, confirming previous findings in other animal models [32,215]. Stanley’s group also demonstrated that human intestinal epithelial cells can produce inflammatory cytokines in vivo, including upregulation of IL-1β and IL-8, in response to amebic infection [224]. The previous results suggest that signals produced by the intestinal epithelial cells may play an important role in inducing the early host inflammatory response to infection. The human xenograft model is a useful tool to study the role of cysteine proteinases in the initial stages of bowel invasion.

**CYSTEINE PROTEINASE INHIBITORS**

**Natural Cysteine Proteinase Inhibitors**

Both endogenous and exogenous inhibitors affect parasitic proteinases. Papain-like inhibitors have been detected in a variety of parasitic protozoa, such as *Leishmania, Trichomonas*, and *Trypanosoma*, suggesting that cystatin-like molecules may be widely distributed among protozoan parasites [225]. A gene encoding a cystatin-like molecule has been found in *Schistosoma mansoni* [226]. It is very likely that cystatins that occur within the parasites may have as their main role protecting the parasite from its own cysteine proteinases, and the determination of the structure of
these inhibitors should provide important information on the binding requirements of the parasite enzymes. Recently, two novel natural cysteine proteinase inhibitors have been identified in *Entamoeba histolytica* [227-230]. They are both expressed in cultured trophozoites and localize to different sub-cellular compartments [229,230]. EhICP1 (*Entamoeba histolytica* inhibitor of cysteine proteinase 1) is highly homologous to Chagasain, the endogenous inhibitor of cruzain the major cysteine proteinase of *Trypanosoma cruzi*[228].

The finding that pro-regions of cysteine proteinases can inhibit the corresponding mature enzyme may also be relevant to parasite cysteine proteinases [162]. Analysis of the structures of the pro-regions of the parasite cysteine proteinases could also provide valuable information on the inhibitor specificity of the enzymes themselves and aid in the design of specific inhibitors. In order to protect cells from uncontrolled degradation, almost all proteinases are synthesized as inactive precursors (proenzymes). The N-terminal pro-peptide extension of these precursors have the tasks of facilitating folding of the mature enzyme by acting as an intramolecular chaperone, maintaining proteinase stability during trafficking of the enzymes through the secretory pathway, and acting as a very specific intrinsic inhibitor of the enzyme. The mature and active enzyme is formed after the release of the pro-region by autoproteolytic cleavage under acidic conditions. A number of studies of mammalian and plant cysteine proteinases have demonstrated that free pro-peptides are potent and highly selective inhibitors for their corresponding mature enzymes [231,232]. Interestingly, the pro-peptides exhibited the highest inhibition selectivity for the
enzyme from which they originated; for example, cathepsin L pro-peptide was more selective for cathepsin L than for related members of the superfamily, such as cathepsin S, and showed no inhibitory activity against cathepsin B \[162,233\] Roche et al. have investigated the specificity of the pro-peptide of Fasciola hepatica cathepsin L proteinase for its mature cognate enzyme \[231\]. The recombinant pro-peptide of Fasciola hepatica cathepsin L1 (CL1), expressed in Escherichia coli, was purified and shown to be a potent inhibitor of the mature cathepsin L1 enzyme and, to a lesser extent, of Fasciola hepatica cathepsin L2 (CL2) \[231\]. Taylor et al. also showed that the specificity of inhibition of plant cysteine proteinases of the papain superfamily by papain pro-peptide correlated with their sequence identity \[232\]. Phil Rosenthal’s group has shown that the folded pro-domain of falcipain-2 one of the major cysteine proteinases of the malarial parasite Plasmodium falciparum acts as a potent, competitive, and reversible inhibitor of mature falcipain-2. \[234\]. Determination of the molecular mechanism of inhibition of cysteine proteinases by their pro-peptides will be one avenue that should allow for the design of more selective and potent synthetic anti-parasitic drugs.

**E-64 and novel Synthetic Peptide Inhibitors of Cysteine Proteinases**

E-64 \[L\text{-}trans\text{-}epoxysuccinyl\text{-}leucylamido \text{(4-guanidino) butane}\], which was isolated from Aspergillus japonicum, is a broad inhibitor of cysteine proteinases \[235\]. It has been an invaluable tool in experiments to block the activity of cysteine
proteinases but is limited by an inability to penetrate cells. E-64 completely blocks the cysteine proteinase activity of cultured *Entamoeba histolytica* trophozoites and inhibits the destruction of mammalian cell monolayers [137,172]. In studies with infected SCID mice, Stanley’s group found that preincubation of *Entamoeba histolytica* trophozoites with E-64 markedly reduced liver abscess formation [224].

Efforts have been made to design and synthesize highly specific non-peptide proteinase inhibitors or peptidomimetic inhibitors with specificity for parasite enzymes by computer-aided secondary-structure analysis and modeling techniques. A number of peptidyl chloromethyl ketone, diazomethyl ketone, and fluoromethyl ketone inhibitors block parasite proteinase activity when present at micromolar concentrations [157]. Studies using biotinylated derivatives of these inhibitors have verified that cysteine proteinases are likely targets [178]. Specific peptide inhibitors against *Trypanosoma cruzi* are not concentrated in mammalian lysosomes, and no morphologic changes could be detected by electron microscopy of host cells [236]. The vinyl sulfone cysteine proteinase inhibitor, K11777 (N-methylpiperazine-phenylalanyl-homophenylalanyl-vinylsulfone-benzene), has been found to be effective in the treatment of Chagas’ disease in two different animal models (mouse and dog) [159,237]. Additionally, several toxicology studies [238] have shown that K11777 is much safer than other families of currently used cysteine proteinase inhibitors. Therefore, K11777 was first chosen for its in vivo efficacy in intestinal xenografts in these studies. Subsequently, Dr. Bill Roush from Scripps Research Institute, Florida, synthesized a newer derivative of K11777, WRR483, based on the specificity we
identified for the amebic enzymes. The application of future and currently available cysteine proteinase inhibitors in the study of the pathogenesis of amebiasis should allow for the discovery of promising new drugs with anti-amebic activity.

**ANTISENSE INHIBITION OF CYSTEINE PROTEINASES**

Gene knockouts have not been successful in *Entamoeba* so the best genetic approach for blocking the expression of a specific cysteine proteinase gene is through the use of antisense RNA. Mirelman's group was able to generate a transfectant of *Entamoeba histolytica* strain HM-1 with an episomally replicating plasmid in which the transcribed *ehcp5* antisense RNA strongly reduced the expression of *ehcp5*. Because of the high homology of the antisense *ehcp5* transcript to other cysteine proteinases, the possibility of cross-reaction with transcripts from the other cysteine proteinases was very likely [212]. The total cysteine proteinase activity from lysates of the transfectant was approximately 90% less than the level of cysteine proteinase activity in the controls. The transfected trophozoites had significantly lower erythrophagocytic activity than did the parental strain, but they were not impaired in their ability to destroy tissue culture monolayers (cytopathic activity) [212]. The absense of an effect of antisense inhibition on cytopathology is surprising, since many laboratories had previously found that cysteine proteinase activity and cytopathic effect correlated quite well [26, 80, 137, 141]. The finding that monolayer destruction by the antisense transfectants could still be blocked by E-64 and that destruction was
decreased with amebic lysates but not with intact trophozoites highlights the need for further studies looking at the importance of surface versus released cysteine proteinases. The effect of antisense inhibition on the production of liver abscesses was dramatic, since no abscesses were detected in hamsters injected with transfected trophozoites in contrast to 100% of untreated controls [148].

Based on our current understanding of the biology of the amebic cysteine proteinases, we can describe their multiple roles in invasion as follows: a) they help to achieve trophozoite attachment to the intestinal mucosa by degrading the protective barrier of colonic mucus b) they facilitate the penetration of colonic tissue by digesting the extracellular matrix, c) they prevent immunological clearance of invading trophozoites by degrading innate and specific immune host proteins such as anaphilotoxins C3a, C5a and immune IgG and IgA, d) they manipulate the host’s local inflammatory response to invasion by proteolytic activation/inactivation of cytokines/chemokines released by the damaged colonic epithelial cells, and e) they aid the dissemination of trophozoites to extra-intestinal locations, particularly the liver.
Chapter 4

Materials and Methods

Cloning and expression of Thio-Pro-EhCP1 in E. coli. The sequence coding the pro-mature domains of the Entamoeba histolytica cysteine proteinase 1 gene (ehcp1) was amplified from Entamoeba histolytica genomic DNA by PCR (HotStarTaq Master Mix, Qiagen, Valencia, CA). The forward primer (pBAD/Thio-Pro-EhCP1-5’: 5’-ATT GAT TTC AAT ACA TGG GTT-3’) encoded the first seven amino acids at the amino-terminus of the pro-region of the EhCP1 gene. The reverse primer (pBAD/Thio-Pro-EhCP1-3’: 5’-GAG ATA TTC AAC ACC AGT TGG-3’) encoded the last seven amino acids (excluding the stop codon) at the carboxy-terminus of the EhCP1 mature proteinase region. The pBAD/Thio-Pro-EhCP1 plasmid was constructed by inserting the gel purified pro-mature EhCP1 gene sequence into the TOPO cloning site of the pBAD/Thio-TOPO plasmid (Invitrogen, Carlsbad, California), following the manufacturer’s instructions. The pBAD/Thio-TOPO plasmid allows the expression of the recombinant protein as a thioredoxin fusion (at the amino-terminus) with a six-residue histidine tail (at the carboxy-terminus). Chemically competent TOP 10 E. coli cells (Invitrogen, Carlsbad, California, USA) were transformed with the recombinant plasmid pBAD/Thio-pro-EhCP1 (2-4 µl of ligation reaction). To express Thio-pro-EhCP1, cells were grown in SOB medium (with Ca^{++} and Mg^{++}) plus ampicillin (100 µg/ml) at 37°C until the OD values of the culture reached 0.6-0.8 (at a wavelength of 600 nm); expression of the recombinant protein was induced by the addition of arabinose to a final concentration of 0.2%. 
Cells were harvested by centrifugation after 4h of induction, and the resulting cells pellets were frozen at -70°C, until used.

**Large scale purification of rEhCP1 from* E. coli.*** The equivalent of six liters of *E. coli* pellets were thawed at room temperature. Bacterial cells were lysed in a buffer containing 20 mM Tris, 20 mM Na phosphate, 0.5 M NaCl, pH 8.0, by sonication (ten, 15 sec pulses, at 70% intensity, with a Brownwill Biosonik IV sonicator), freeze-thawing (4 times from a methanol-dry-ice bath to room temperature water: -120°C to 25°C), and by passing cells through different gauge needles (18 to 25 gauge, at least 3 times with each needle). The resulting lysate was treated with DNAse for 2 h at 4°C. Urea was added to the lysate to a final concentration of 6M and rocked gently for another 4-6 hours or overnight. The urea lysate was cleared by centrifugation at 15,000 rpm for 30 min. The cleared lysate was further diluted in binding buffer (6M urea, 20 mM Tris, 20 mM Na phosphate, 0.5 M NaCl, pH 8.0) and filtered through a 0.45 µm membrane. The filtered lysate was loaded twice onto a Hi-Trap-Ni-charged-chelating column (5 ml column, Amersham Biosciences, UK) previously equilibrated with binding buffer with a peristaltic pump. The loaded column was connected to an FPLC system (Pharmacia, Uppsala Sweden) and washed with 5 column volumes of wash buffer: 6M Urea, 20 mM Tris, 20 mM Na Phosphate, 0.5 M NaCl, 20 mM imidazole, pH 8.0. Finally, the column was eluted with 10 column volumes of a 20-500 mM imidazole linear gradient (dissolved in 6M urea, 20mM Tris, 20 mM Na Phosphate, 0.5 M NaCl, pH 8.0). Selected elution fractions
were analyzed by SDS-PAGE (12% gels); fractions containing the recombinant protein Thio-Pro-EhCP1 were pooled and concentrated using Centricon ultrafiltration devices (Millipore, Boston, Massachusetts, USA). The total protein concentration of the pooled and concentrated fractions was determined by the method of Bradford [239].

Refolding of Thio-pro-EhCP1. Initially we used our modification [240] of a previously reported protein refolding screen [241] to refold denatured Thio-pro-EhCP1 by dilution with 16 different refolding buffers containing some or all of the following components: 50 mM MES (pH 6.0) or 50mM Tris, pH 8.5), 0.3/0.03 M NaCl, 0.1% PEG, 10mM MgCl₂, 10mM CaCl₂, 10mM EDTA, 25% Glycerol, 0.75 M L-arginine, 0.3mM n-dodecyl-B-D-Maltoside, 1mM DTT, 5mM GSH, 0.5mM GSSG. Denatured Thio-pro-EhCP1 was added drop wise and slowly to each refolding buffer (2 mL volume) to a final protein concentration of 1 µM. Refolding of Thio-pro-EhCP1 in each of the refolding buffers proceeded for 48 h at 4°C with gentle stirring or rocking. Each refolded recombinant protein (Thio-pro-EhCP1) solution was immediately dialyzed against TBS (Tris Buffered Saline) and concentrated at least 10-fold using Centricon ultrafiltration devices (Millipore Corporation, USA). The refolding of Thio-pro-EhCP1 with: 50mM MES, pH 6.0, 30 mM NaCl, 0.1% PEG 4000, 10mM EDTA, 750 mM Arginine, 5mM GSH, 0.5 mM GSSG (Condition 16 buffer), yielded the highest amount of active rEhCP1 as determined by the cleavage of
the synthetic peptide substrate Z-Arg-Arg-AMC, in a Fluoroskan-Ascent fluorometer (Labsystems, USA).

**Purification of refolded active rEhCP1 by ion-exchange chromatography.**

Concentrated, refolded active rEhCP1 was exchanged from TBS into Q-Binding Buffer (2mM EDTA, 25mM Tris, pH 8.0) by desalting (using a PD-10 desalting column, Amersham Biosciences, UK) or dialysis. Exchanged or dialyzed refolded active EhCP1 was loaded onto a 1ml Hi-Trap-Q column (Anion exchanger, Amersham Biosciences, UK), which had been previously connected to a FPLC system (Pharmacia-Amersham Biosciences, UK). The Hi-Trap-Q column was washed with 20 column volumes of Binding Buffer (2mM EDTA, 25mM Tris, pH 8.0), and then eluted with 30 column volumes of a linear (0-500 mM) NaCl gradient (in Binding Buffer: 2mM EDTA, 25 mM Tris, pH 8.0). All of the fractions (2 mL) collected were screened for proteinase activity by assaying aliquots from each fraction with the synthetic peptide substrate Z-Arg-Arg-AMC, in a Fluoroskan-Ascent Fluorometer (Labsystems, USA).

**Activity assay for refolded, active rEhCP1.** Refolded and active rEhCP1 was assayed by using synthetic peptide substrates commonly cleaved by amebic and other cysteine proteinases: Z-Arg-Arg-AMC, Z-Ala-Arg-Arg-AMC, Z-Phe-Ala-Arg-AMC, and Z-Phe-Arg-AMC. Five to 20 µl aliquots of refolded rEhCP1, were pre-incubated in 96 well plates) for 30 min in a total volume of 100 µl of a buffer containing 25 mM
Tris (pH 7.5) or 25 mM NaH$_2$PO$_4$(pH 6.5), 2mM EDTA, 5mM DTT or 5mM Cys. The cleavage of the fluorescent AMC leaving group was assayed kinetically by adding another 100 µl of substrate buffer containing 20 µM of the synthetic peptide substrate, in a Fluoroskan-Ascent fluorometer (Labsystems, USA) at 25°C for 5.0 min. Enzyme activity, slope curves and relative fluorescence units (RFU, the amount of proteinase activity needed for the liberation of 1 pmole of Aminomethylcoumarin = AMC, per minute from the fluorescent peptide substrate) were calculated with the Ascent software.

**Antibodies to amebic cysteine proteinases.** To raise antibodies against the main amebic proteinases. EhCP1, EhCP2, EhCP3, and EhCP5 were all expressed as recombinant proteins. Polyclonal antibodies were raised against recombinant EhCP1 (expressed in pRSETA, Invitrogen) and rEhCP5 (expressed in pBAD/Thio-TOPO, Invitrogen). Rabbits were injected subcutaneously three times with 100 µg of each recombinant proteinase with Titermax (Sigma), and the IgG fraction was purified using Protein A chromatography (Pharmacia). Production of monoclonal antibodies to EhCP3 and a polyclonal anti-peptide antibody to EhCP2 has been previously described [242]. To test the specificity of the antibodies, 1.0 µg of each recombinant antigen (rEhCP1, 2, 3, and 5) was electrophoresed on 12% gels, transferred to nitrocellulose, reacted with a 1-5 µg/ml dilution of each antibody, and detected with goat anti-mouse or anti-rabbit IgG alkaline phosphatase and BCIP/NBT (5-bromo, 4-chloro, 3-indolylphosphate/ nitroblue tetrazolium, Zymed, San Francisco, CA) [243].
Analysis of Thio-proEhCP1 and purified, active rEhCP1 by SDS-PAGE (Coomassie and silver stains), western blots and gelatin gels. Fractions of Thio-pro-EhCP1 or refolded rEhCP1 were analyzed by SDS-PAGE and stained with Coomassie blue or silver. Aliquots of refolded rEhCP1 were boiled (5-10 min) in the presence of E-64 (100 µM, sample buffer with SDS and BME was added, and the samples were boiled again for 5-10 min. Samples were electrophoresed on 12% polyacrylamide-SDS gels and stained with Coomassie blue or with the SilverExpress silver staining kit (Invitrogen, Carlsbad, CA). For Western blots, 12% SDS-gels were transferred to PVDF membranes (Millipore, Boston, Mass.) using a Hoefer-Semiphor transfer unit, for 2h at a constant current of 50 mA. Membranes were blocked for 1h with 5% milk-TBS-Tween and probed for 2h with absorbed-polyclonal rabbit anti-rEhCP1 Ab (1:500). Membranes were washed 5 times with TBS-Tween and probed with Goat-anti-rabbit-IgG-AP conjugate (1: 10,000). Membranes were washed again 5-10 times with TBS-Tween and developed with BCIP/NBT solution (Zymed, San Francisco, CA). Refolded rEhCP1 was also analyzed in gelatin substrate gels (12% polyacrylamide-SDS polymerized with 0.1 % porcine gelatin). Aliquots of refolded rEhCP1, which had been previously inhibited for 15 min with 100 µM E-64 (and non-inhibited enzyme aliquots) at room temperature, were mixed with non-reducing sample buffer (w/o BME) and loaded onto a 0.1% gelatin gel and electrophoresed at no more than 75 V (constant voltage) for 2 ½ h. Then, gelatin gels were washed for 30 min with a 2.5% Triton-TBS solution, and 3 more times with TBS (10 min each time). Gels were incubated for 4h or overnight at 37 °C with a buffer containing: 2mM
EDTA, 25mM Tris, and 5 mM DTT, pH 7.5. Finally, gels were stained with Coomassie blue for at least 2h and de-stained appropriately.

Michaelis constant (Km) of active, purified rEhCP1 for the synthetic peptide substrates Z-Arg-Arg-AMC and Z-Ala-Arg-Arg-AMC. Aliquots of refolded, purified and active rEhCP1 were assayed for 5 minutes at 25°C in a total volume of 200 µl of a buffer containing: 25mM Tris, 2mM EDTA, 2mM DTT, pH 7.5 and increasing concentrations of the synthetic peptide substrates Z-Arg-Arg-AMC or Z-Ala-Arg-Arg-AMC (from 1 to 15 µM) were added. Enzyme activity, slope curves and were calculated with Ascent software. Each substrate concentration was assayed in quadruplicate. Initial velocities were estimated from RFUs, with the aid of a 7-AMC standard curve. The final data was processed and analyzed with Microsoft Excel and the Enz-fitter programs.

Scanning the specificity of the rEhCP1 active site with tetra-peptide combinatorial libraries. Two synthetic combinatorial libraries were used to determine the substrate specificities of the S1-S4 sub-sites of active, purified EhCP1 as described previously [244]. A bifunctional fluorophore, ACC (7-amino-4-carbamoylmethylcoumarin), incorporating a site for peptide synthesis and another site for attachment to a solid support was used to prepare the fluorogenic substrates. By using an acid-labile Rink linker between the ACC group and the resin, Fmoc(9-fluorenlymethoxycarbonyl)-base solid phase synthesis techniques were used to produce efficiently the substrate libraries as described earlier [245]. To determine P1
specificity, a P1 diverse library consisting of 20 sub-libraries was used. In each sub-library, the P1 position contained one native amino acid (cysteine was replaced by norleucine), and the P2, P3 and P4 positions were randomized with equimolar mixtures of amino acids (in each case, cysteine was omitted and replaced by norleucine) for a total of 6859 tetrapeptide substrates per sub-library. Aliquots of $8.9 \times 10^{-9}$ mol from each sub-library were added to 20 wells of a 96-well Microfluor-1-U-bottom plate (Dynex Technologies, Chantilly, VA, USA) for a final concentration of 13 nM of each compound per well. To determine P2, P3 and P4 specificity, a P1-lysine fixed library was used. In this library, the P1 position is fixed with lysine and the P2, P3 and P4 positions were spatially addressed with 19 amino acids (cysteine was omitted and replaced by norleucine), whereas the remaining two positions were randomized. Aliquots of $9.0 \times 10^{-9}$ mol from each sub-library were added to 60 wells (361 compounds/well) for a final concentration of 250 nM of each compound per well. Hydrolysis reactions were initiated by the addition of diluted aliquots (1:30) of purified active rEhCP1 and monitored fluorometrically with a Molecular Devices SpectraMax Gemini spectrofluorometer, with excitation at 380nm and emission at 460 nm. Assays were performed at 25°C in 100mM sodium phosphate, pH 6.5, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.01% Brij-35 and 1% DMSO.

**The pH profile of purified active rEhCP1.** The pH optimum of purified active enzyme was determined by pre-incubation of diluted rEhCP1 aliquots with 100 µl of buffers containing: 25mM sodium acetate, 2mM EDTA (pH 4.0-6.5) or 25 mM Tris, 2mM EDTA, pH 7.0-9.0) for 30 min at 25°C in fluorometric 96-well plates.
Enzyme activity was assayed kinetically by adding an additional 100 µl per well of the appropriate pH buffer plus 20 µM of the synthetic peptide Z-Arg-Arg-AMC, in a Fluoroskan-Ascent Fluorometer (Labsystems, USA). Enzyme activity, slope curves and RFUs were calculated. The effect of each pH buffer on the catalytic activity of purified active rEhCP1 was determined in quadruplicate.

**Cleavage of human complement component 3 (C3) by purified, active rEhCP1.** Native C3 cleavage was performed by a modification of a previously published method [246]. One µg of native human C3 (Quidel, San Diego, California) was incubated with increasing amounts of active purified rEhCP1, which had been previously incubated in the presence and/or absence of 40 µM E-64, for 60 min at 37°C, in a buffer containing 2mM EDTA, 50 mM NaH₂PO₄, pH 6.5. At the end of the incubation period, additional E64 was added to each sample, and the samples were incubated for 15 min at 25°C. The samples were boiled for 5-10 min; reducing sample buffer was added to each sample and boiled for 5-10 min. The C3 cleavage products were analyzed by SDS-PAGE in 7.5 % gels, and stained with Coomassie blue.

**Cleavage of human IgG by purified, active rEhCP1.** Native human IgG cleavage was performed by a modification of a previously published method [205]. Briefly, native human IgG (Sigma, St. Louis, Mo. USA) was incubated with increasing amounts of purified active rEhCP1 which had been previously incubated in the presence and/or absence of 80 µM E64 for 16 h at 37°C, in a buffer containing 2mM EDTA, 50 mM NaH₂PO₄, pH 6.5. At the end of the incubation period, additional
E64 was added to each sample, and then incubated for 15 min at 25 °C. The samples were boiled for 5-10 min; reducing sample buffer was added to each sample and boiled for 5-10 min. The human IgG cleavage products were analyzed by SDS-PAGE in 12% gels, and stained with Coomassie blue, or transferred to a PVDF membrane (Immobilon P, Millipore, Boston, Mass, USA) and probed with Goat anti-human IgG HRP conjugate, diluted 1: 20,000 (Zymed, San Francisco, CA) and developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

**Cleavage of human pro-IL-18 by purified, active rEhCP1.** Soluble recombinant human pro-IL-18 cleavage was performed by a modification of a previously published method [213]. Affinity-purified recombinant human pro-IL-18 [247] was incubated with increasing amounts of purified active rEhCP1 which had been previously incubated in the presence and/or absence of 40 µM E64 for 1-3 h at 37°C, in a buffer containing 2mM EDTA, 50 mM NaH$_2$PO$_4$, pH 7.5, 5mM DTT. At the end of the incubation period, E-64 was added to each sample, and incubated for an additional 15 min at 25 °C. The samples were then boiled for 5-10 min; reducing sample buffer was added to each sample and boiled for 5-10 min. The human pro-IL-18 cleavage products were analyzed by SDS-PAGE in 12% gels which were blotted to PVDF membranes (Immobilon P, Millipore, Boston, Mass, USA), the blots were probed first with Monoclonal IL-18 Ab (1: 10,000; US Biological) for 90 min at Room Temperature (25 °C); after washing extensively with TBS-Tween solution, the blots were probed with either Goat anti-mouse IgG AP or Goat anti-mouse IgG HRP conjugates, diluted 1: 10,000 (Zymed, San Francisco, CA) for 60 min at RT and
developed either with NBT-BCIP buffer solution (Zymed, San Francisco, CA) or the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

**Effect of proteinase inhibitors on active rEhCP1.** Purified active rEhCP1 was activated for 15 min with 5mM DTT, incubated for an additional 20 min at 25 °C with a variety of proteinase inhibitors, such as E-64 (cysteine proteinase inhibitor, irreversible) Pefablock (serine proteinase inhibitor, irreversible), K11777 (cysteine proteinase inhibitor, irreversible) and then assayed using the conditions described above (200µl total volume, 10µM Z-Arg-Arg-AMC used as substrate, etc.).

**Culture of Entamoeba histolytica trophozoites.** *Entamoeba histolytica* strain HM-1: IMSS was grown axenically in TYI-S-33 medium [248] and sub cultured every 48-72 h.

**Isolation of released amebic cysteine proteinases (Conditioned medium).** Released proteinases were prepared from *Entamoeba histolytica* trophozoites by washing confluent axenic *Entamoeba histolytica* cultures twice with DPBS (Dulbecco’s PBS) and resuspending them in DPBS-Cys⁺⁺ (Dulbecco’s PBS plus 40mM Cysteine, 0.5 mM MgCl₂, 0.12 mM CaCl₂, pH 7.2) to a final cell concentration of 5×10⁶ Eh cells/mL. This cell suspension was incubated for 90 min at 37 °C with occasional mixing. *E. histolytica* cell viability was tested by the trypan blue exclusion method, at the beginning and the end of the incubation period, and was found to be higher than 98%. Released proteinases were collected from the supernatant. The
supernatant was filtered through a 0.45 um membrane and dialyzed against Dulbecco's PBS for 2hr at 4°C. If the released proteinases were going to be used in ELISA assays (see below), the proteinase activity was inhibited with E-64 (40 µM), filtered again through a 0.45µm membrane, and used immediately to coat ELISA plates or stored frozen at -70 °C, until used.

Effect of the vinyl sulfone cysteine proteinase inhibitors K11777 and WRR 483, on active rEhCP1 and released amebic proteinases. Purified active rEhCP1 or released cysteine proteinases (conditioned medium, prepared as described above) were incubated for 30-60 min at 25° C with 1-100 µM of the irreversible, vinyl sulfone cysteine proteinase inhibitors, K1777 [238] or 0.001-1.0 µM WRR483 (synthesized by Dr. Bill Roush, Scripps Research Institute, Florida, USA). Cleavage of the substrate Z-Arg-Arg-AMC was followed fluorometrically as detailed above and the corresponding IC50s calculated.

Effect of the vinyl sulfone cysteine proteinase inhibitors K11777 and WRR- 483 on the viability and released proteinase activity of E. histolytica (HM-1) trophozoites. E. histolytica log phase trophozoites were harvested and washed twice with DPBS to eliminate bovine serum. Washed trophozoites were resuspended in TYI-S-33 medium w/o serum to a final concentration of 1 x 10⁶ trophozoites/mL and incubated with 0, 1 and 20 µM K11777 (or 0, 1 and 10 µM WRR 483) for 30 min at 37° C. Cell viability was determined by the trypan blue exclusion method at t = 0 min, and t = 30 min. Culture supernatants from each experimental condition were
harvested by centrifugation and screened for proteinase activity by assaying aliquots with the synthetic peptide substrate Z-Arg-Arg-AMC, in a Fluoroskan-Ascent Fluorometer (Labsystems, USA).

Quantification of extracellular amebic cysteine proteinases by ELISA. Axenic HM-1 trophozoites (5 X 10^6/ml) were incubated in Dulbecco’s PBS-Cysteine as described above for released proteinases. Ninety-six well ELISA plates were coated first with 1.0-50.0 ng of purified recombinant EhCP1, EhCP2, EhCP3, or EhCP5 for standard curves. EhCP1, EhCP2, EhCP3, and EhCP5 were all expressed as recombinant proteins and purified as previously described [242]. Monoclonal antibody to EhCP3 (ACP1) and polyclonal anti-peptide antibody to EhCP2 (ACP2) were made as previously described [242]. Polyclonal antibodies were raised against recombinant EhCP1 (expressed in pRSETA, Invitrogen) and EhCP5 (in pBAD/Thio-TOPO, Invitrogen) and purified by nickel chromatography. Rabbits were injected subcutaneously three times with 100 μg of each recombinant proteinase with Titermax (Sigma), and the IgG fraction was purified using Protein A chromatography (Pharmacia). The same 96 well ELISA plates were coated with 50 μL (or less) of a 1:50 and/or 1:100 dilution of dialyzed released proteinases (for 2h at 37 °C or overnight at 4 °C). Wells were blocked for 1 h with 3% BSA (100 μL), then mono-specific polyclonal antibody raised against recombinant forms of EhCP1, EhCP2 and EhCP5, and monoclonal Ab for recombinant EhCP3 were added as the primary antibody diluted in 3% BSA. The primary antibody (100 μL) was added and incubated in the wells for 90 min at room temperature (RT). Then wells were washed at least 5 times
with PBS-Tween buffer. The secondary antibody (100 µL) was added to wells at a dilution of 1: 10,000 (in PBS-Tween buffer). The secondary antibody (Goat anti-rabbit IgG-Horse Radish Peroxidase-conjugate or Goat anti-mouse IgG-HRP-conjugate) was incubated in the wells for 30-60 min at RT. Wells were washed at least 5 times with PBS-Tween buffer and at least 2 times with PBS buffer alone. All measurements of standards were done in duplicate, and conditioned medium determinations were done in triplicate. The amount of each amebic proteinase in ng/µL from conditioned medium was determined [243].

**Labeling *Entamoeba histolytica* (HM-1) trophozoites with LysoTracker Red-DND-99 and EhCP1 polyclonal antibodies.** For IFA, *Entamoeba histolytica* trophozoites were grown to semi-confluency in 6mL tubes and labeled with LysoTracker-Red DND-99 (LT) at a dilution of 1:500 (12µL of LT/6mL of TYI-S-33 medium) for 16 hours (overnight), as previously reported [249]. LT labeled trophozoites were transferred to chamber slides (LabTek, 8 chamber slides) and incubated for 60 min at 37 °C to allow attachment of the cells to the slide surface. The medium was removed carefully from each chamber and washed once with warm (37 °C) DPBS. Trophozoites were fixed with warm 3.7% paraformaldehyde for 10 min at 37 °C. Washed 3 times with warm DPBS, and then permeabilized with 0.5% Triton-X-100 in DPBS for 5 minutes at RT. Permeabilized trophozoites were washed 3 times with DPBS at RT and blocked with 1% BSA in DPBS for 30 min, at RT. Blocked cells were incubated for 60 min with EhCP1-Ab (diluted to 1:200 in 1% BSA in DPBS at RT), then washed 3 times with DPBS at RT. Trophozoites were labeled by
incubation with goat anti-rabbit IgG-Alexa 488 conjugate (1:500) for 45 min at RT. Cells were washed at least 4 times with DPBS at RT. Finally, chambers were very carefully removed from the slides, and any excess buffer removed by blotting the corners of the slide with absorbent paper (Kimwipes). Slides were mounted with Prolong Gold (Molecular probes, Eugene OR, USA), covered with cover slips and dried overnight at RT in the dark. Controls included trophozoites labeled with Lysotracker Red DND-99, but not labeled with EhCP1-Ab, with and w/o conjugate, and cells labeled only with EhCP1 antibody.

For confocal microscopy, *E. histolytica* trophozoites were washed twice in PBS and fixed in 10% formalin for 15 min at RT, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% BSA in PBS. Trophozoites were incubated with a mouse monoclonal antibody to EhCP3 (1:200) and a polyclonal rabbit antibody to EhCP1 (1:200), followed by Alexa 488-conjugated goat anti-mouse and Alexa 594-conjugated goat anti-rabbit Antibody (1:200 dilution, Molecular Probes, Eugene, Oregon, USA). Samples were imaged with a Zeiss 510 Laser scanning confocal microscope and argon/krypton laser. Image analysis was performed with LSM and Adobe Photoshop.

For immunoelectron microscopy, trophozoites were chilled, washed three times with DPBS (Dulbecco’s PBS) and fixed in 1% glutaraldehyde/ 4% paraformaldehyde. The samples were processed and embedded in glycol-metacrylate [250]. Ultra thin sections from the fixed and embedded samples were prepared and reacted initially with rabbit polyclonal EhCP1-Ab (1:200) and mouse monoclonal anti-EhCP3 (1:200). After washing, samples were incubated with anti-rabbit IgG-
conjugated with gold particles (15 nm diameter, Ted Pella Inc., Redding, CA) and/or anti-mouse IgG-conjugated with gold particles (20 nm diameter, Ted Pella Inc., Redding, CA) as the second antibody. Sections were washed and contrasted with uranyl acetate and lead citrate. Observations were carried out in a Zeiss EM910 Transmission Electron Microscope. Isotype matched antibodies and the conjugates alone were used as controls.

**Amebic infection of a human intestinal xenograft model.** Human intestinal xenografts were transplanted subcutaneously into the backs of SCID mice and allowed to mature for 10-15 weeks. Xenografts were infected by injection with *Entamoeba histolytica* trophozoites (1×10^6^ log phase axenic HM-1 trophozoites in 100 µL of DMEM or TYI-S-33 media) into the lumen. An equivalent volume of cell culture medium alone (100 µL) was injected into an identical xenograft to serve as a negative control. Other xenografts were injected with 1×10^6^ *E. histolytica* trophozoites, which had been previously incubated in the presence of the irreversible vinyl sulfone cysteine proteinase inhibitors K11777 (20 µM) or WRR 483 (10µM). A total dose of 1.5 mg of inhibitor was administered I.P. to each mouse. Twenty-four hours after infection SCID mice were euthanized, and the xenografts harvested. The mucous plug from each xenograft was removed, and both were frozen in liquid nitrogen and stored at -80°C. Part of the xenograft tissue was fixed in 10% formalin, embedded in paraffin, and sections were stained with hematoxylin/eosin or periodic acid Schiff. The pathology of the xenografts was screened to insure all specimens extracted for PCR all had an intact muscularis layer and the mucus plug removed.
Quantification of amebic invasion in human intestinal xenografts by real time PCR. Visually quantifying amebic invasion directly from tissue sections of infected human colon xenografts is a subjective and labor-intensive task. To address this problem, we developed a very sensitive and effective quantitative real time PCR assay that enabled us to measure amebic invasion in xenografts infected with *E. histolytica*. A standard curve to measure the amount *E. histolytica* trophozoites found in infected human intestinal xenografts was developed by extracting DNA from 25-50 mg of human intestinal xenograft tissue to which a known number of *E. histolytica* trophozoites (10-100,000), had been added. DNA from human intestinal xenografts (infected with *E. histolytica* trophozoites [virulent strain HM-1: IMSS] pre-incubated with or w/o K11777/WRR 483) was extracted with the PUREGENE DNA purification kit (Gentra Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. Real time PCR reactions were carried out using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 50 (or 25) µL, with 100ng (contained in 10 or 5 µL) of the extracted DNA (from xenografts and standards) and 100 nM (final concentration) of both the forward primer (5’- AAATCAATTGTGAAGTTATTGGAGTGA-3’) and the reverse primer (5’- TCCTACTCCTCCTTTTACTTTATCTGTGCT-3’), to amplify a 92 bp region of the *E. histolytica* peroxiredoxin gene. Real Time PCR was performed under the following conditions: polymerase activation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 1 minute (annealing/extension), and finally 1 cycle at 95°C for 15 seconds, 60°C for 30 seconds, 95°C for 15 seconds. Each PCR assay included two negative controls: a water/reagent/no-template control and a non-infected
xenograft DNA (100 ng) control, a positive control (*E. histolytica* genomic DNA standards) and the intestinal xenograft DNA experimental samples run in triplicate. The PCR reactions were run in an Applied Biosystems PE-5700 PCR machine (or the Applied Biosystems 7300 Real Time PCR System) and the resulting data was analyzed and processed using the GeneAmp 5700 SDS software program (or the Applied Biosystems 7300 SDS Software/Sequence detection software 1.3.1. RQ study application). The presence of any PCR reaction inhibitor in each of the human intestinal xenograft DNA samples was tested by the quantitative measure of amplifiable human genomic DNA with primers specific for the detection of human *Alu* sequences. Briefly, 0.1 ng of xenograft DNA was amplified with 100 nM of sense: 5’ ACG CCT GTA ATC CCA GCA CTT 3’ and antisense: 5’ TCG CCC AGG CTG GAG TGC A 3’, human *Alu* primers, under the same PCR conditions as described above. Negative controls, positive controls and the experimental samples were run in duplicates. The Ct values from each sample were averaged and the standard deviation (S.D.) and/or standard error (S.E.) calculated.
Chapter 5

Expression, Purification and Characterization of rEhCP1.

Although the purification of individual native cysteine proteinases from Entamoeba histolytica trophozoites has been previously reported by several groups [26-28,141,150,251], these techniques have proven to be difficult and unreliable because the main cysteine proteinases expressed by cultured trophozoites (EhCP1, EhCP2 and EhCP5) behave very similarly during chromatographic separation (ion-exchange chromatography and gel filtration) and, because of the high number of cysteine proteinase genes present in the E. histolytica genome (more than 40 genes) [102,139,155,252]. Therefore, the expression of recombinant amebic cysteine proteinases becomes an important tool to purify, and biochemically characterize these important virulence factors. In order to find a suitable expression system for E. histolytica cysteine proteinases, our group has evaluated their expression in E. coli using the pCheY vector [170], which had been used successfully to express cruzain from T. cruzi, the pRSET and pBAD-Thio-TOPO vectors, which have been used to express EhCP1-5 in E. coli [242], and the vector pAcGP67B for expression of EhCP2-3 in baculovirus [242]. Most recently, we have expressed active recombinant EhCP5 in Pichia pastoris [213], which has been a useful system for large-scale production of a number of proteinases from T. cruzi, T. brucei, and Schistosoma [253]. Of all of the cysteine proteinase genes, discovered so far, only two, ehcp1 and ehcp5, are unique to
Entamoeba histolytica and not expressed in the morphologically identical and non-invasive Entamoeba dispar. In addition, EhCP1 is one of the highest expressed cysteine proteinases in axenically cultured trophozoites and one of the main released proteinases, as shown by studies done by our group [243] and by Tannich’s group in Germany [139]. Therefore, the studies described in this dissertation have focused on EhCP1 and have tested the hypothesis that Entamoeba histolytica Cysteine Proteinase 1, EhCP1, which is unique to E. histolytica, plays a crucial role in amebic invasion.

Specific Aim 1 tested the hypothesis that E. histolytica Cysteine proteinase 1 (EhCP1) differs in its specificity for substrates from the other E. histolytica Cysteine proteinases.

a.) Expression, and purification of active recombinant EhCP1 to homogeneity.

Our initial efforts were devoted to express EhCP1 in Pichia pastoris, a yeast expression system that we had used successfully to express EhCP5 [213]. This system has the advantage that the recombinant proteins are secreted into the growth medium in a native conformation, so no refolding protocols are necessary. Expression of active EhCP1 in the Pichia pastoris system was unsuccessful, possibly due to a base pair mutation we identified in the cloned sequence, which might have affected expression and secretion of the proteinase. When the correct sequence was expressed, the proteinase was active, but only low amounts were expressed (data not included).

We next decided to use a bacterial expression system and cloned the sequence of the pro-mature region of EhCP1 into the bacterial expression vector pBAD-Thio-
TOPO, as described in Material and Methods (Chapter 4). Figure 5.1 shows the map of the expression plasmid pBAD-Thio-pro-EhCP1.

Figure 5.1- pBAD-Thio-pro-EhCP1 plasmid. Map of the expression plasmid pBAD-Thio-pro-EhCP1 used for the recombinant expression of EhCP1.

The fusion protein (Thio-pro-EhCP1) was extracted directly from bacterial cells or from inclusion bodies under denaturing conditions (6M urea), purified by nickel chelation chromatography, and eluted with imidazole.

Expression of Thio-pro-EhCP1 in *E. coli* resulted in ~ 10 mg/L of a 50 KDa protein, which corresponds well with the predicted molecular weight for Thio-pro-
EhCP1 of 50.2 KDa (36.3 KDa for the zymogen form of EhCP1 plus 13 KDa of the Thioredoxin peptide and 0.9 Kda of the 6X His tag). As shown in figure 5.2.

![Figure 5.2- Expression and purification of Thio-pro-EhCP1. Recombinant Thio-pro-EhCP1 was purified by Ni-affinity chromatography and analyzed by 12% SDS-PAGE. Thio-pro-EhCP1 was visualized by: (A) Coomassie blue staining, (B) immunoblot probed with polyclonal rabbit anti-EhCP1 Ab, and (C) immunoblot probed with mouse monoclonal anti-Thioredoxin Ab.](image)

The Thio-pro-EhCP1 peak fractions were pooled and concentrated, and denatured Thio-pro-EhCP1 was refolded initially using 16 different conditions, which were based on our modification [240] of a protein-refolding screen first published by Armstrong et al [241].
Active rEhCP1 was detected by cleavage of the fluorogenic peptide substrate Z-Arg-Arg-alpha-methylcoumarin (Z-RR-AMC). Refolded active rEhCP1 was purified by ion-exchange chromatography with a Hi-Trap-Q column (Amersham Biosciences, UK), and as shown in Figure 5.3, analysis of the peak fractions revealed the presence of just one polypeptide band with a MW of 30 KDa by silver stain of 12% SDS gels (A), gelatin zymography (B) and western blots (C) probed with polyclonal anti-EhCP1 Ab.

**Figure 5.3- Purification of refolded active rEhCP1.** Hi-Trap-Q purified refolded and active rEhCP1 was analyzed by: (A) SDS-PAGE (12% gels, silver stained), (B) gelatin zymography (12%SDS-PAGE/0.1% gelatin, run under non-reducing conditions), and (C) immunoblot (rabbit polyclonal anti-EhCP1 antibody).
This protocol yielded pure and homogeneous rEhCP1, which was then used to:

b.) **Determine the specificity of rEhCP1 for synthetic and physiological (biological) substrates.**

The synthetic peptide substrate specificity of rEhCP1 was determined as described in Materials and Methods (Chapter 4). Figure 5.4 shows the substrate specificity of refolded active rEhCP1. Fluorogenic peptide substrates such as Z-Arg-Arg-AMC and Z-Ala-Arg-Arg-AMC were readily cleaved by this proteinase (Typical cathepsin B substrates), whereas substrates such as Z-Phe-Arg-AMC (Typical cathepsin L substrate) and Z-Phe-Ala-Arg-AMC were not cleaved at all.

![Cleavage of synthetic substrates by refolded EhCP1](image)

**Figure 5.4- Cleavage of synthetic substrates.** Purified active rEhCP1 was assayed in a total volume of 200 µl of substrate buffer (50mM Na Phosphate 2mM EDTA, 5mM DTT, pH= 6.5), and 10 µM Synthetic Substrate (Z-RR-AMC, Z-ARR-AMC, Z-FR-AMC, and Z-FAR-AMC), as described in Materials and Methods.
This singular preference of active rEhCP1 for synthetic peptide substrates with arginine (Arg= R) at the P1 and P2 positions, was studied further, in collaboration with Dr. Charles Craik (UCSF), by scanning the catalytic site of rEhCP1 with two synthetic tetra-peptide combinatorial libraries to determine the substrate specificities of the S1-S4 sub-sites of the active enzyme, as described in Material and Methods (Chapter 4) [244,245,254].

**Figure 5.5** shows the results of mapping the specificity of rEhCP1 active site with the tetra-peptide combinatorial libraries; the most striking finding is the almost absolute requirement of Arginine at the P2 position for catalytic function.
Figure 5.5- Substrate specificity determined by synthetic combinatorial libraries. A P1 complete diverse library, and P2, P3 and P4 sub-libraries of the P1-Lysine fixed library were used to determine the substrate specificities of rEhCP1. Activities were displayed as percentages of the maximum at each position. Amino acids are represented by the single-letter code (n is norleucine). Error bars represent the S.D. from the results of duplicate experiments.

Biological substrate specificity of rEhCP1. To determine if rEhCP1 had the same biological substrate specificity as the secreted native proteinases, human complement component 3 (C3), IgG and Pro-IL-18 were incubated with equal amounts of active purified rEhCP1 or native *E. histolytica* secreted proteinases. Figure 5.6 shows that rEhCP1 cleaves C3 in an identical fashion as the secreted native
proteinases, by cleaving the $\alpha$-subunit of human C3 and generating a product which is about 10 kDa smaller: the $\alpha'$-subunit.

**Figure 5.6- Cleavage of C3 by rEhCP1.** C3 (2 µg) was incubated with PBS (Control=C), 50 or 10 RFUs of active rEhCP1 for 60 min in PBS, or 50 RFUs pre-incubated with 100 µM E-64. Samples were separated by 10% SDS-PAGE and stained with Coomassie blue. Bands consistent with the $\alpha$ and $\alpha'$ subunits of C3 are indicated with arrows.

Like wise, human IgG cleavage by active rEhCP1 generates identical products as amebic secreted proteinases, and as shown in **Figure 5.7**. Active rEhCP1 cleaves specifically the $\gamma$-heavy chain; this cleavage is inhibited by E-64.
Figure 5.7- Degradation of human IgG by rEhCP1. Human IgG (2 μg) was digested with released proteinases or purified rEhCP1 (1400 r fus) for 16 h at 37°C and the resulting immunoblots probed with goat-antihuman IgG HRP-conjugate. The cleavage was inhibited by pre-incubating the proteinases for 30 min at Room Temperature with 80 μM E-64. Molecular weight markers are labeled on the left and the heavy (H) and light (L) chains of IgG on the right. RB: reaction buffer, CP: cleavage product.

Active rEhCP1 degrades human pro-IL-18 in a time and dose dependent manner (Figure 5.8), as was previously shown for secreted amebic proteinases and recombinant EhCP5 [213].
Figure 5.8- EhCP1 degrades human Pro-IL-18. Recombinant pro-IL-18 (0.4 μg) was incubated for 1h at 37°C in buffer alone, with native proteinases from conditioned medium (CM, 750 RFUs) or with 200-800 RFUs of rEhCP1. The degradation of pro- IL-18 was completely inhibited by pre-incubation with 40 μM E-64.

c.) Characterization of rEhCP1 (pH profile of rEhCP1)

pH optimum of rEhCP1. The pH profile of active rEhCP1 was investigated by using a dual buffer system (Acetate buffers from pH 4.0-6.5, and Tris buffers from pH 7.0-9.0), as described in Materials and Methods (Chapter 4). Recombinant EhCP1 was found to have a pH optimum of 6.0 (Figure 5.9), retaining most of its catalytic activity in the pH range of 5.5 to 7.5. A 50% or higher reduction in the catalytic activity is seen at pH values ≤ 5.0 or ≥ 8.0. The use of a different buffer system
(Citrate/Phosphate buffer), to determine the pH optimum of rEhCP1 yielded the same results (Data not shown).

**Figure 5.9 - pH profile of rEhCP1.** Refolded, active rEhCP1 was assayed at room temperature in a total volume of 200 µl of 25 mM Acetate (from pH: 4.00 to 6.50), 2 mM EDTA, 5 mM DTT, 10 µM Z-RR-AMC and/or 25 mM Tris (from pH 7.00 to 9.00), 2 mM EDTA, 5 mM DTT, 10 µM Z-RR-AMC (in 96 well plates), using a Labsystems Fluoroskan-Ascent fluorometer (10 min kinetic assay).

**d.) Determination of the Michaelis constant (Km)**

The Michaelis constant (Km) of active, purified rEhCP1 for the synthetic peptide substrates Z-Arg-Arg-AMC and Z-Ala-Arg-Arg-AMC, was calculated by assaying aliquots of purified and active rEhCP1 as described in Materials and Methods (Chapter 4). **Figures 5.10 and 5.11** are the Lineweaver-Burk plots from which the Km for Z-RR-AMC and Z-ARR-AMC was calculated.
Figure 5.10- Km for Z-RR-AMC. Lineweaver-Burk (LB) plot for the calculation of the Michaelis constant (Km) for Z-RR-AMC a synthetic peptide substrate of rEhCP1.

Figure 5.11- Km for Z-ARR-AMC. Lineweaver-Burk (LB) plot for the calculation of the Michaelis constant (Km) for Z-ARR-AMC a synthetic peptide substrate of rEhCP1.
**Figure 5.12** is a 7-AMC standard curve (7-Aminomethylcoumarin), the end product resulting from the cleavage of the synthetic peptide substrates. The standard curve was used to estimate the Vo values.

![7-AMC standard curve graph](image)

**Figure 5.12- 7-AMC (Aminomethylcoumarine) standard curve.** This curve was used to estimate the Vo values used in calculations of the Michaelis constant for the synthetic peptide substrates Z-RR-AMC and Z-ARR-AMC.

The resulting Michaelis constants of the rEhCP1 synthetic substrates Z-RR-AMC and Z-ARR-AMC calculated from their respective Lineweaver-Burk plots were: 1.99 µM and 1.87 µM respectively.
Chapter 6

Release and Intracellular Localization of EhCP1

Quantification of Extracellular Proteinase Release

Specific Aim 2 tests the hypothesis that EhCP1 differs in its release or localization from other cysteine proteinases.

We first identified the major released cysteine proteinases by ELISA. We implemented and validated a quantitative ELISA assay, developing standard curves with recombinant amebic cysteine proteinases EhCP1, EhCP2, EhCP3 and EhCP5 and specific antibodies developed to detect them (Rabbit polyclonal: EhCP1, EhCP2 and EhCP5; and mouse monoclonal: EhCP3). Standard curves for each proteinase were made by coating plates with 6.5-50 ng of recombinant protein and detecting it with specific antibody. The amount of each released proteinase in *Entamoeba histolytica* conditioned medium (CM) (prepared as described in Materials and Methods, Chapter 4) was then determined based on the standard curves. Figure 6.1 summarizes the results obtained with this quantitative ELISA.
Quantitation of *Entamoeba histolytica* released proteinases from conditioned medium (CM)

![Bar graph showing the quantitation of released proteinases.](image)

**Figure 6.1- Released amebic proteinases.** Measurement of *E. histolytica* released proteinases in conditioned medium (CM) by quantitative ELISA.

The most abundant cysteine proteinase released by *E. histolytica* is EhCP2 followed by EhCP5 and EhCP1; EhCP3 was almost undetectable (Figure 6.1).

**Intracellular Localization of EhCP1**

Immunofluorescence (IFA) labeling of intracellular EhCP1 was performed in permeabilized trophozoites using polyclonal antibody, as described in Materials and Methods (Chapter 4). Most of the intracellular pool of EhCP1 is localized inside large cytoplasmic vesicles, as is clearly seen in figure 6.2A. Figure 6.2B shows the phase contrast image figure 6.2A.
Figure 6.2- Localization of EhCP1 by IFA. Immunofluorescence labeling of intracellular EhCP1 (white arrow) in log phase trophozoites (A) and a DIC (Differential Interference Contrast or Nomarski) image of the same field (B).

Confocal Microscopy. Because localization is difficult in round trophozoites, we next evaluated the localization of EhCP1 compared to a known intracellular proteinase, EhCP3, by confocal microscopy. As shown in figure 6.3, EhCP1 and EhCP3 localize to different intracellular compartments. EhCP1 seems to be contained mainly in large secretory vesicles, whereas EhCP3 is localizes to a population of smaller secretory vesicles.
**Figure 6.3 EhCP1 and EhCP3 do not co-localize.** Confocal image of log phase *E. histolytica* trophozoites labeled with EhCP1 polyclonal Ab (color green, white arrow) and EhCP3 monoclonal Ab (color red, light blue arrow).

**Electron microscopy.** In collaboration with Dr. Bibiana Chavez-Munguía (CINVESTAV, IPN, México City, México) localization of the proteinases was also determined by immunoelectron microscopy. Log phase *E. histolytica* trophozoites were labeled with polyclonal EhCP1 Ab (15 nm gold particles) and monoclonal anti-EhCP3 (20nm gold particles) (as described in Materials and Methods). **Figure 6.4** shows that EhCP1 is localized mainly in large secretory vesicles. In contrast, EhCP3 localizes to smaller vesicular compartments, confirming the observations from the confocal microscopy images.
Figure 6.4- Localization of EhCP1 and EhCP3 by immuno-EM. Axenically grown *E. histolytica* (HM-1: IMSS) were fixed with paraformaldehyde and embedded in LR-white. Fixed and embedded samples were reacted initially with anti-EhCP1Ab (Polyclonal Ab, Rabbit IgG) (15 nm gold particles, black arrow) and EhCP3 Ab (Monoclonal Ab, Mouse IgG) (20 nm gold particles, blue arrow). Samples were analyzed by Transmission Electron Microscopy (TEM). Magnification: 20,000 X.

Given that rEhCP1 has a slightly acidic pH optimum, we were interested in investigating if the vesicles, which contain EhCP1, were also acidic. In order to answer this question live *E. histolytica* trophozoites were first labeled with the fluorescent lysosomal dye LysoTracker Red DND-99 (which fluoresces red at acid pH), and after fixation, a second labeling with polyclonal anti-EhCP1 (described in Materials and Methods, Chapter 4). Although *E. histolytica* trophozoites have numerous acidic intracellular compartments (*Figure 6.5A*), EhCP1 seems to localize to both acidic and non-acidic compartments as shown in the merged image (*Figure 6.5C*).
Figure 6.5- EhCP1 localizes to acidic and non-acidic vesicles. Log phase E. histolytica trophozoites were double labeled with LysoTracker red DND-99 and EhCP1 polyclonal Ab. (A) Trophozoites labeled with LysoTracker Red DND-99; (B) Trophozoites labeled with EhCP1 polyclonal Ab; (C) Image resulting from merging (A) and (B); and (D) DIC (Nomarsky) image of trophozoites from the same field.
Specific Aim 3: tests the hypothesis that chemical or genetic inhibition of EhCP1 would block or significantly diminish invasion. We initially tested if specific irreversible cysteine proteinase inhibitors have an effect on amebic invasion. We decided to use K11777 (N-methylpiperazine-urea-Phe-homophenylalanine-vinylsulfone-benzene), an irreversible vinyl sulfone cysteine proteinase inhibitor (or peptide-mimetic or pseudopeptide substrate analogue inhibitor). The chemical structure of K11777 is shown in Figure 7.1. We picked this inhibitor because of its activity against multiple other parasite cysteine proteinases and the extensive amount of toxicity and pharmacokinetic testing that had been performed. K11777 was designed and first synthesized by Dr. J. Palmer [255]. McKerrow’s group had demonstrated efficacy of K11777 in the treatment of Chagas’ disease in two different animal models (mouse and dog) [159,237], and its effect in the growth and pathogenicity of Leishmania tropica [256]. A recent study has shown that K11777 blocks blood-brain barrier traversal by the African trypanosome Trypanosoma brucei gambiense [257]. Biotransformation and toxicology studies, [238] have shown that K11777 is much safer than other families of currently used cysteine proteinase
inhibitors. The compound is entering clinical trials for treatment of Chagas’ disease (J. McKerrow, personal communication).


The IC50 of K11777 for secreted proteinases from conditioned medium (CM) and purified rEhCP1. *E. histolytica* HM-1 conditioned medium (CM) and purified rEhCP1 were prepared as described in Materials and Methods (Chapter 4), and aliquots of each were incubated for 30-60 min (at RT) with increasing concentrations of K11777. Residual enzyme activity was determined as described in materials and methods (Chapter 4). The IC50 of K1777 for native released proteinases
in CM and rEhCP1 was 15.0 and 11.5 μM respectively. **Figures 7.2 and 7.3** show typical titrations of HM-1 CM and rEhCP1 with K11777.

**Figure 7.2- IC50 of K11777 for released amebic proteinases.** Released amebic proteinases from conditioned medium (HM-1 CM) were incubated with increasing concentrations of the cysteine proteinase inhibitor K11777 and the concentration which inhibited 50% of the activity calculated based on the regression curve. Results are shown for a typical experiment out of four that were performed. The average calculated IC50 for secreted amebic proteinases was: 15.0 μM.
The irreversible vinyl sulfone cysteine proteinase inhibitor K11777 blocks amebic invasion in the human intestinal xenograft model of amebic colitis. Stanley’s group has shown that the early pathology of invasion by \textit{E. histolytica} trophozoites can be reproduced in human intestinal xenografts engrafted on SCID mice \cite{258}. In order to determine if K11777 could block amebic invasion, we infected human intestinal xenografts transplanted subcutaneously into the backs of SCID mice with \textit{Entamoeba histolytica} (HM-1). \textit{E. histolytica} trophozoites (1x 10^6, axenic strain HM-1) were pre-incubated for 30 min with and without 20µM K11777 in growth medium (TYI-S-33) without serum and injected into the lumen of 10-12 week old xenografts. An equivalent volume of TYI-S-33 medium without serum (100 µl) was injected into identical xenografts, which served as negative controls. At 24 h, the grafts were harvested, embedded in paraffin, and sections stained with
hematoxylin/eosin or periodic acid Schiff (PAS). **Figure 7.4** shows the pathology from xenografts infected with *E. histolytica* trophozoites alone in which there are undermined ulcers containing trophozoites interspersed with normal mucosa, mimicking human disease (B). Sections of intestinal xenografts, which were infected with *E. histolytica* trophozoites, preincubated with 20µM K11777 show an intact mucosa with minimal invasion and inflammation (C), very similar to sections of uninfected intestinal xenografts (A).

![Figure 7.4- Pathology of human intestinal xenografts infected with amebic trophozoites.](image)

To quantify the extent of invasion, we developed a real-time PCR assay to determine the number of trophozoites in tissue. A standard curve was made by adding whole trophozoites (10-100,000) to 25-50 mg of uninfected human intestine, the DNA
extracted using the PURE GENE genomic DNA purification kit (Gentra Systems), and the number of copies of the amebic peroxiredoxin gene determined. The assay could detect as few as 10 trophozoites. Figure 7.5 shows a typical standard curve constructed for the quantification of the number of *E. histolytica* trophozoites found in human intestinal xenografts.

![Figure 7.5: Standard curve used for the quantification of *E. histolytica* trophozoites in infected human intestinal xenograft tissue.](image)

The actual number of invading trophozoites in xenograft tissues was determined by PCR using the standard curve. *E. histolytica* (HM-1) trophozoites were pre-incubated for 30 min (at 37 °C) with or w/o 20 μM of the vinyl sulfone inhibitor, K11777, before injection into the xenografts. At 24 hr, the xenografts were harvested,
DNA was extracted from 50-100 mg of each intestinal xenograft, and the number of trophozoites/mg of tissue determined by real-time PCR as previously described. As shown in figure 7.6, pre-incubation of *E. histolytica* trophozoites with the cysteine proteinase inhibitor reduced amebic invasion into the human intestine by >80% (p<0.0001).

![Figure 7.6- Effect of K11777 on amebic invasion. K11777 reduces *E. histolytica* invasion of human intestinal xenografts. Median ± S.E from three xenografts (per experimental condition) (p<0.0001).](image)

K11777 blocks amebic invasion by inhibiting the secreted cysteine proteinases, including native EhCP1, despite having IC50s in the low micromolar range for secreted amebic cysteine proteinases (CM) and rEhCP1 (15 and 11.5 µM respectively). Dr. Bill Roush of Scripps Florida synthesized a derivative of K11777 specifically designed to block the amebic proteinases that depend on Arginine in the P2 position. WRR483 is also a vinyl sulfone, but instead of having phenylalanine
(Phe) at the P2 position like K11777, WRR 483 has arginine (Arg). Figure 7.7 compares the chemical structures of both K11777 and WRR 483.

![Figure 7.7- Comparison of the chemical structures of the vinyl sulfone cysteine proteinase inhibitors K11777 and WRR 483.](image)

The IC50 of WRR 483 for released proteinases from conditioned medium (CM) and purified rEhCP1. *E. histolytica* HM-1 conditioned medium (CM) and purified rEhCP1 were prepared as described in Materials and Methods (Chapter 4), and aliquots of each were incubated for 30 min (at Room Temperature) with increasing concentrations of WRR 483 (0.001-0.1 µM). Residual enzyme activity was determined as described in materials and methods (Chapter 4). Figures 7.8 and 7.9 show typical titrations of HM-1 CM and rEhCP1 with WRR 483.
Figure 7.8- IC50 of WRR 483 for released proteinases. Secreted amebic proteinases from conditioned medium (HM-1 CM) were incubated with increasing concentrations of the cysteine proteinase inhibitor WRR 483. The average calculated IC50 for secreted amebic proteinases was: 0.017 µM (17 nM).

Figure 7.9- IC50 of WRR 483 for rEhCP1. The average calculated IC50 for rEhCP1 was: 0.008 µM (8 nM).
The irreversible vinyl sulfone cysteine proteinase inhibitor WRR 483 blocks amebic invasion in the human intestinal xenograft model of amebic colitis.

In order to determine if WRR 483 could also block amebic invasion, we infected human intestinal xenografts transplanted subcutaneously into the backs of SCID mice with *Entamoeba histolytica* (HM-1). *E. histolytica* trophozoites (1x 10^6, axenic strain HM-1) were pre-incubated for 30 min with and without 10µM WRR 483 in TYI-S-33 w/o serum and injected into the lumen of 10-12 week old xenografts. An equivalent volume of TYI-S-33 medium was injected into identical xenografts, which served as negative controls. At 24 h, the grafts were harvested, embedded in paraffin, and sections stained with hematoxylin/eosin (H & E) or periodic acid Schiff (PAS) (Tissue section photographs not shown). DNA was also extracted from 50-100 mg of each intestinal xenograft, and the number of trophozoites/mg of tissue determined by real-time PCR as previously described. As shown in figure 7.10, pre-incubation of *E. histolytica* trophozoites with the cysteine proteinase inhibitor WRR 483 reduced amebic invasion into the human intestine by > 95% (p<0.0001).
As described in Chapter 4 (Materials and Methods), we tested for the presence of any PCR reaction inhibitor in each of the human intestinal xenograft DNA samples by the quantitative measure of amplifiable human genomic DNA with primers specific for detection of human \textit{Alu} sequences. The real time PCR reactions were performed under the same conditions as were used for the \textit{E. histolytica} peroxiredoxin primers. The average Ct obtained from these PCR assays was 16.04±0.72 (S.E.). Therefore, each specimen had equivalent human xenograft DNA and no inhibitors.
Chapter 8

Discussion

Experimental work by our group and others clearly shows that released amebic cysteine proteinases play a fundamental role in invasion of the host’s target tissues by *E. histolytica* trophozoites. The most recent analysis of the *E. histolytica* genome has revealed that more than 40 genes encode cysteine proteinases \[102,139\]. Studies on the expression of the cysteine proteinase genes have shown that only three, *ehcp1*, *ehcp2*, and *ehcp5*, account for more than 90% of the cysteine proteinase specific transcripts \[81,139\] and more than 95% of released amebic proteinases *in vitro* \[263\]. This dissertation has focused on EhCP1 because it is unique to *E. histolytica* with no homologous gene in the closely related but noninvasive *E. dispar* \[81\]. It is also one of the highest expressed and released cysteine proteinases \[139,243,274\]. Because of the difficulty in separating the large number of similar native cysteine proteinases, we focused on the expression of active, recombinant enzyme. Our research group has previously expressed active EhCP2 and EhCP3 in baculovirus \[242\] and EhCP5 in *Pichia pastoris* \[213\]. In the work presented here, we have expressed the pro-enzyme of EhCP1 in *E. coli* as a thioredoxin fusion with a 3’ histidine tag which auto-catalytically processed itself to the mature, active enzyme during refolding \[240,241\] (Figures 5.2 and 5.3). Refolded rEhCP1 was found to be already active, as it could be assayed directly with synthetic peptide substrates, without pre-incubation at low pH or in the presence of thiol-reagents (DTT, BME, or cysteine). This distinguishes rEhCP1
from previously purified recombinant parasite cysteine proteinases [213,242,255,268,270,271,272,276]. Purified refolded rEhCP1 has a pH optimum of 6.0, although 50% or more of its catalytic activity is retained across a pH range of 5.5 to 8.5. This broad pH range is consistent with the release of native EhCP1 in the neutral environment of the large bowel [266].

We have shown previously by homology modeling that the structures of EhCP2, 3, and 5 are characteristic of cathepsin Ls with the ERFNIN motif, but their preference for substrates with positively charged amino acids resembles cathepsin Bs [242]. Recombinant EhCP1 has a similar preference for cathepsin B substrates (Figure 5.4), cleaving preferentially peptide substrates such as Z-RR-AMC and Z-ARR-AMC, but being virtually inactive against Z-FR-AMC and Z-FAR-AMC. Native released amebic proteinases demonstrated the same substrate specificity (data not shown). In order to understand this singular substrate specificity of rEhCP1, we mapped the substrate preference of the active site cleft of rEhCP1 by using two positional scanning synthetic tetra-peptide combinatorial libraries [244,245,255]. The almost absolute preference for Arginine (Arg) at the P2 position shown by rEhCP1 (Figure 5.5), is unique among Clan CA proteinases. This unusual substrate specificity has been the basis for the rational design of highly specific and potent amebic cysteine proteinase inhibitors, such as WRR 483 (Chapter 7).

Recombinant EhCP1 cleaves a number of key physiologic substrates in an equivalent fashion as the native amebic proteinases (and recombinant amebic cysteine proteinases described so far). Active rEhCP1 readily cleaves the α chain of human complement component C3 (Figure 5.6), which we have previously shown mimics the
cleavage of C3 convertases, producing an active C3b molecule [246]. The cleavage site is actually one amino-acid residue distal to native convertases, placing an Arginine in the P2 position for rEhCP1 [246]. Our lab has previously shown that the released C3a anaphylatoxin fragment is further degraded by the amebic cysteine proteinases [146], which could impair the host’s inflammatory response. Human IgG is also cleaved by refolded active rEhCP1 (Figure 5.7), yielding identical products as we have previously shown with purified amebic proteinases [205]. This observation is consistent with clinical and epidemiological findings that systemic anti-amebic IgG responses do not protect from re-infection with *E. histolytica* [36,37,262]. Our research group has previously demonstrated that rEhCP5, the only other cysteine proteinase unique to *E. histolytica*, degraded pro-IL-18, a finding that implicates amebic cysteine proteinases in the observed lack of neutrophils in amebic lesions [213]. Now we have found that rEhCP1 produces a similar dose- (Fig. 5.8) and time-dependent degradation of pro-IL-18. The absence or reduced levels of IL-18 will affect the induction of Interferon Gamma (IFN-γ) which is indispensable for the activation of macrophages, the main cell capable of killing *E. histolytica* trophozoites. Degradation of pro-IL-18 by rEhCP1 seems to suggest a role for EhCP1 in limiting the action of pro-inflammatory signals synthesized by cells of the target tissue: colonic epithelial cells. These results strongly suggest the involvement of released EhCP1 in impairing the host’s inflammatory response to *E. histolytica* invasion.

The intracellular localization is known for only a few amebic cysteine proteinases. EhCP5 [141] EhCP2 [242], and EhCP112 [268] are membrane-associated, while EhCP3 is intracellular [242]. By using fluorescence, confocal and
immunoelectron microscopy, we found that EhCP1 localizes to large cytoplasmic vesicles, a site distinct from the vesicles containing EhCP3 (Figures 6.1, 6.2 and 6.3). The EhCP1 containing vesicles are acidic and non-acidic compartments, as shown by immuno-fluorescence double labeling experiments performed with EhCP1 Ab and the fluorescent lysosomal dye LysoTracker Red DND-99 (Figure 6.5). Previous studies performed by our research group have shown that EhCP2 and EhCP3 are quickly recruited to phagocytic vesicles during phagocytosis [242]. Recent proteomic analyses of the *E. histolytica* phagosome have revealed the ubiquitous presence of EhCP1 (and the other major amebic Cysteine Proteinases) in pre-phagocytic, non-acidic vesicles (PPV: Pre-Phagosomal Vacuoles) and in mature phagosomes. In these studies, the presence of the amebic cysteine proteinases was confirmed by biochemical, cell fractionation and immuno-fluorescence (IFA) studies similar to ours [242,249,277-280]. The sub-cellular localization is consistent with the pH optimum of rEhCP1 of 6.0-6.5 (Figure 5.9).

Animal models of amebiasis have been problematic as only humans and higher primates are naturally susceptible to infection. Cysteine proteinase inhibitors such as E-64 and an EhCP5 antisense construct, which blocked expression of multiple cysteine proteinases, inhibited amebic liver abscess formation in SCID mice [147] and hamsters [148]. The trophozoites had to be injected directly into the liver however, which bypasses the normal route of infection through the colon. To avoid this limitation, we closely mimicked human infection by using the human xenograft model pioneered by Stanley’s group [258]. When fetal intestinal loops are implanted under the skin of SCID mice, they develop physiologically normal colonic mucosa.
Following intra-luminal injection of invasive *E. histolytica* trophozoites, classic flask-shaped ulcers formed in the human graft (Figure 7.4).

Cysteine proteinases are an attractive target for agents designed to disrupt invasion by *E. histolytica*. To test the proof of principle that EhCP1 and released cysteine proteinases are critical for invasion of the human bowel, we evaluated the effects of the inhibition of EhCP1 (and the other released cysteine proteinases) by two novel vinyl sulfone inhibitors, K1777 (IC50 of 11.5 μM against rEhCP1; figures 7.1, 7.2 and 7.3) and WRR 483 (IC50 of 8 nM against rEhCP1; figures 7.8 and 7.9). We initially chose K11777 because it already had undergone extensive pharmacokinetic, bioavailability and toxicity testing [238, 260] and is approaching Phase I clinical trails for the treatment of Chagas’ disease. Pre-incubation with the inhibitor dramatically reduced intestinal invasion (Figure 7.4). We also quantified invasion by real time-PCR (q-PCR), with a standard curve based on the detection of the amebic peroxiredoxin gene sequence (Figure 7.5) [180, 259]. Our results show for the first time that a specific cysteine proteinase inhibitor (K11777) blocks amebic invasion of the human colon by more than 80% (Figure 7.6). These findings are consistent with previous studies on human colonic xenografts, in which inoculation of virulent trophozoites that had been transfected with an anti-sense vector to EhCP5 resulting in reduced expression of multiple cysteine proteinases caused less inflammation and damage to the intestinal mucosa [29, 258]. K11777 blocks amebic invasion by inhibiting the released cysteine proteinases (which include native EhCP1) despite having IC50s in the low micromolar range for secreted amebic cysteine proteinases (CM) and rEhCP1 (15 and 11.5 μM respectively; figures 7.2 and 7.3).
The unique requirement of EhCP1 for arginine in the P2 position and the demonstrated ability of a specific cysteine proteinase inhibitor (K11777; figures 7.1 and 7.7) to block invasion of the bowel, lead to the design and synthesis of new vinyl sulfone inhibitors based on the K11777 scaffold with structural features to target more specifically the amebic cysteine proteinases (EhCP1). Dr. William Roush from Scripps Research Institute, Florida synthesized WRR483, a vinyl sulfone with arginine (Arg) at the P2 position instead of phenylalanine (Phe) (Figure 7.7). This inhibitor (WRR 483) has IC50s which are about one thousand times lower for rEhCP1 and released amebic proteinases than K11777 (WRR 483 IC50 for rEhCP1 is 0.008 µM and 0.017 µM for released amebic proteinases; Figures 7.8 and 7.9).

WRR 483 has been studied by Dr. James McKerrow’s group (Department of Pathology and the Sandler Center for the study of parasitic diseases, UCSF) as an inhibitor of cruzain (the main cysteine proteinase of Trypanosoma cruzi) and as an anti-trypanosomal agent. WRR483 has proven to be at least as effective as K11777 in in vitro assays vs. T. cruzi. Also, WRR 483 has proven highly effective in curing mice of T. cruzi infections when administered orally (J. McKerrow, unpublished results). WRR483 is able to cross cell membranes, in spite of the presence of the highly polar arginine residue in its structure. Pharmacokinetic and toxicology studies of WRR 483 are now in progress at UCSF.

In order to determine if WRR 483 could block amebic invasion, we infected human intestinal xenografts transplanted subcutaneously into the backs of SCID mice with Entamoeba histolytica (HM-1). Invasion was evaluated by quantification of an amebic specific peroxidase gene in the human bowel tissues and by histology, as
described in Materials and Methods (Chapter 4). And, as shown in figure 7.10, pre-incubation of *E. histolytica* trophozoites with the cysteine proteinase inhibitor WRR 483 reduced amebic invasion of human intestinal xenografts by more than 95%.

To the best of our knowledge, WRR483 is one of the most potent and selective inhibitors of EhCP1 and native amebic cysteine proteinases. WRR 483 contains a P2 arginine (Arg) residue that reflects the specificity of EhCP1 for arginine (Arg) in the P2 position. WRR 483 is 16-fold more active against EhCP1 than human cathepsin B and 4-fold more than human cathepsin L (Reed Lab, data not shown). Thus, a future goal would be to design, synthesize and identify protozoal cysteine proteinase inhibitors with IC50s in the low nanomolar range which have at least 10 to 50-fold selectivity for the parasite cysteine proteinases versus the human cathepsins B and L. Further studies with these novel compounds, would then allow their development as therapeutic agents to treat protozoal infections.

As in previous published work from our laboratory, we show that the expression of active recombinant enzymes is an indispensable tool, which has allowed us to dissect the role of individual amebic cysteine proteinases in the virulence of this important protozoan parasite. Further studies with these recombinant enzymes will be also helpful to address the question of whether *E. histolytica* releases a higher level of cysteine proteinases facilitating invasion, in contrast to non-invasive *E. dispar*, or whether the key determinants of its invasiveness are the unique proteinases, EhCP1 and/or EhCP5. We are expecting that our continuing efforts to design, synthesize and test specific inhibitors of these unique cysteine proteinases will shed yet more light on their roles in the pathogenesis of amebiasis. In the meantime, our current studies have
proven that specific chemical inhibition of amebic cysteine proteinases will block *E. histolytica* invasion of the human bowel and have identified a new promising scaffold inhibitor (WRR 483), whose design was based on the substrate specificity of the amebic cysteine proteinases (rEhCP1).
References


