The invasiveness of *Entamoeba histolytica* – a continuing enigma

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Introduction

In 1875 Lösch described the case of a young farmer who had been admitted to his clinic in St Petersburg, Russia, two years earlier. The man had been suffering from chronic dysentery and Lösch found such large numbers of amoebae in his faeces that he became convinced that they were responsible for the disease. He named the organism *Ameba coli* and showed that it produced colonic ulceration and dysentery in dogs. This patient was the first known to have died from amoebiasis and subsequent work by a number of investigators established that the organism had a simple life cycle, involving a motile trophozoite and a resistant, usually quadrinucleate, cyst and could spread away from the intestine; the name *Entamoeba histolytica* also became established.¹ Other, non-pathogenic, species of amoeba (such as *E coli*) were also defined and the unique invasiveness of *E histolytica*—its ability to penetrate the wall of the human large bowel and disseminate via the blood stream to the liver and other organs—has been the subject of investigation ever since. In recent times the pace of this investigation has noticeably quickened and the purpose of this short paper is to summarise some recent developments in our understanding of how and why *E histolytica* spreads through the body. It is in no sense exhaustive and the still indispensable monograph edited by Ravdin² and more recent reviews^{3–6} should be consulted for further details. As the title indicates, much about this fascinating organism remains obscure.

Entamoeba histolytica and *E dispar*

Open any but the most recent of medical textbooks and you will probably find the following statement in some form or other: “There are approximately 500 million new infections with *E histolytica*, world-wide, each year, of whom 10% at most will suffer from clinical disease.⁷ These 50 million or so patients will be affected by invasive amoebiasis in which the organism disseminates from the lumen of the large bowel causing ulceration of the mucosa and bloody diarrhoea (amoebic dysentery), more rarely invades the liver (causing hepatic amoebiasis, commonly known as amoebic liver abscess) and occasionally attacks other organs. This invasive disease kills between 40 and 100 thousand people each year, almost all in developing countries. The remaining 450 million cases of amoebiasis will comprise seronegative, asymptomatic cyst passers...”⁷

For many years it was far from clear whether the outcome of infection resulted from differences in host or parasite, but since the pioneering observation of Martinz-Palomo *et al*,⁸ it has become more and more apparent that there are fundamental biochemical, immunological and genetic differences between the organisms recovered from patients with invasive disease and those parasitising asymptomatic cyst passers. The proposal⁹ that the invasive and non-invasive organisms were best regarded as separate species (rRNA sequence differences suggest that they are about as different as humans and mice) with the name *E histolytica* retained for the one causing clinical disease and the designation *E dispar* revived for the amoeba recovered from asymptomatic cyst passers is now almost universally accepted and is adopted here. It is important realise that the cysts of the two species are morphologically indistinguishable.

This new insight is far more than simple taxonomic nit-picking for it means that much of the amoebiasis literature needs to be reinterpreted—to give but two examples, it now seems likely that the majority of those infected with *E histolytica* will suffer some degree of illness and an experiment looking for genomic differences between “invasive and non-invasive strains” of *E histolytica* has probably been comparing two different species.

Pathogenicity and virulence

These two terms are often used loosely and/or synonymously but are probably best defined for our purposes¹⁰ as follows:

- disease is a measurable departure from the normal condition;
- a pathogen is an organism capable of causing disease;
- virulence is the relative capacity of a pathogen to cause disease.¹¹

Thus, in principle, pathogenicity is an unqualifiable term—an organism either causes disease or it does not—while virulence can range from low to high depending on the severity of the resulting illness. Of course, it is very unusual for any pathogen to cause a uniform level of damage in everyone infected and low virulence can mean either mild disease in most patients or more severe illness in a minority. Only under experimental conditions is it usually possible to say whether these different results relate to host variability, the existence amongst a pathogen of strains of differing virulence, or both.

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In the case of the *Entamoeba*, *E. dispar* is regarded by some as non-pathogenic and by others as a pathogen of almost no virulence.^{12 13} *E. histolytica* is clearly a pathogen but the proportion of those infected who are asymptomatic is unclear. Ravdin⁶ suggests that it may be as high as 90% but a figure this large makes it very hard to understand how Sargeant and Williams were able to obtain a near-perfect correlation between the detection (by culture) of organisms with a "pathogenic" zymodeme (that is, *E. histolytica*) and the presence of clinical symptoms in nearly 6000 samples from different parts of the world.¹⁴ Extensive studies by Jackson *et al* in South Africa have shown that these asymptomatic carriers may proceed to frank illness or may spontaneously lose the infection; significantly, however, all cases were seropositive.^{15–17} Long term carriage of *E. dispar* without seroconversion undoubtedly occurs and it is possible that highly immunogenic parasite molecules can only gain access to the host's immune system if at least minimal damage to the mucosa has taken place.

Many recent attempts to understand how *E. histolytica* is able to invade have compared its proteins and enzymes, genes and mRNAs with less virulent amoebae; these "controls" have included organisms which we now know to be different species (*E. dispar* and *E. moshkovskii*, the former "Laredo" or "*E. histolytica*-like" strains¹⁸). Alternatively, comparisons have been made amongst a range of laboratory isolates of *E. histolytica* growing axenically (that is, in the absence of bacteria or other living organisms) which have high or low virulence when measured in vitro or in laboratory animals. There are weaknesses with both approaches: applying the first, how to tell which of the many detectable differences are actually significant while in the second case the use of axenic cultures brings its own problems. All large bowel amoebae normally live in an intimate relation with a very complex bacterial flora; the process of axenisation involves intense and prolonged selection pressure,¹⁹ which is known to affect proteinase levels,^{20 21} the expression of epitopes^{22 23} and virulence in vitro.²⁴ It is not actually known whether in vitro or small animal measures of virulence detect differences which are significant in human disease nor, in fact, whether naturally occurring *E. histolytica* differ in virulence at all—variation in disease severity could result from host factors and human volunteer studies²⁵ have shown that cysts from a single patient can produce both dysentery and asymptomatic amoebiasis in different recipients.

How does *E. histolytica* invade?

The process whereby a luminal parasite arrives in the liver is conventionally divided into six stages: adherence, target cell killing (cytotoxicity), dissolution of the basement membrane, ingestion of cell fragments and erythrocytes (phagocytosis), egress into the circulation, and finally, establishment of foci of infection in the liver. Our knowledge of the molecular mecha-

nisms underlying these processes declines steadily going from first to last.

Adherence

E. histolytica trophozoites express a number of molecules which mediate attachment to enterocytes, of which by far the best studied is the galactose/N-acetyl galactosamine inhibitable lectin (GIL). It is not known whether this adherence is simply a precursor to invasion or a necessity to prevent the parasite being swept away with the bowel contents, but the presence of a homologue of the GIL gene in *E. dispar* would support the latter role. The GIL is a heterodimeric molecule comprised of a heavy (170 kDa) and light (35 kDa) subunit; at least five heavy and possibly six light subunit genes are known,²⁶ but at present all known biological functions seem to be located on the heavy subunit. These functions are extraordinarily diverse and include, as well as adherence, involvement in contact mediated cytotoxicity and resistance to complement lysis (see later), making GIL a key virulence factor. Yet anomalies remain; the carbohydrate recognised is present in colonic mucin as well as on the surface of epithelial cells and mucin prevents the in vitro binding of *E. histolytica* to target cells very effectively.²⁷ In the gerbil model, mucus depletion precedes epithelial erosion²⁸ and a heat stable secretagogue has been detected in axenic cultures of *E. histolytica*.^{29 30} It is plausible that the latter activity is involved in the invasion process by facilitating adherence of trophozoites to the mucosa. Whether *E. dispar* produces this as yet uncharacterised molecule is not known. It is likely, however, that the GIL homologue of the latter can mediate adherence to colonic epithelium as two anti-GIL monoclonal antibodies which enhance lectin mediated adherence to target cells recognise the only epitopes (Nos. 1 & 2) which are also present on *E. dispar* trophozoites.^{31 32} The cloning of secretagogue and lectin genes from *E. dispar* will clearly shed much new light on the early stages of pathogenesis.

Other molecules mediating adherence have been described and may be less studied rather than less important than GIL; the ability of galactose to inhibit almost all binding and cytotoxicity argues against the former. A monoclonal antibody directed against a surface glycoconjugate inhibited binding and cytotoxicity³³; a 112 kDa adhesin³⁴ also possesses proteinase activity.³⁵ No corresponding functional molecule can be detected in *E. dispar*.³⁶ Finally, a 220 kDa lectin³⁷ also exists whose binding is inhibited by polymers of N-acetyl glucosamine.³⁸ These last two molecules and GIL will mediate the binding of *E. histolytica* to red blood cells and erythrophagocytosis, which are much studied properties of this parasite (see later). Although in vitro both *E. dispar* and the non-pathogenic *E. coli* ingest erythrocytes, in vivo haematophagous trophozoites are found only in the faeces of patients with invasive disease caused by *E. histolytica*. The benefits which this species obtains from its ability to penetrate the mucosa are obscure at best (see later); however, it is possible that ingestion of red cells

provides a source of iron, which is an absolute requirement for *E. histolytica*.^{39, 40} Other molecules may be involved in binding to erythrocytes, as the process is most effectively inhibited by lactose⁴¹ and not all avirulent *E. histolytica* mutants with a reduced ability to bind CHO cells are deficient in adherence to red blood cells.⁴²

Target cell killing

One of the most studied properties of *E. histolytica* (and the one which gives it its name) is its ability to destroy tissues. The killing of nucleated mammalian cells is an essential first step in this process. Although the number of studies is tiny, it seems that *E. dispar* largely lacks the ability to kill tissue derived cells,^{36, 43, 44} however, when the targets were neutrophils Bos⁴⁵ found no difference between "patient" and "carrier" strains; Leippe *et al.*⁴⁶ reported that *E. dispar* had about one third of the ability of *E. histolytica*. As for cytotoxic mechanisms, the problem is not finding one, but understanding why there are so many.

It is clear that although cytotoxicity is contact dependent,⁴⁷ the GIL is involved in killing as well as adherence. Even if adherence in the presence of galactose is brought about by centrifugation, CHO cells are not killed⁴⁸ and a monoclonal antibody directed against epitope 1 actually decreases cytotoxicity.⁴⁹ However, affinity purified lectin itself does not kill CHO cells, despite causing a rise in intracellular calcium concentrations.⁴⁹ An irreversible increase (about 20-fold) in the intracellular concentration of this ion in CHO cells occurs within seconds of contact with live trophozoites although cell death occurs only five to 15 minutes later. Pretreatment of target cells with EDTA or verapamil prevents this effect, which is also inhibited by galactose.⁵⁰ Lectin-target cell contact also leads to an increase in cytokine (interleukin-8) production, probably mediated via intracellular Ca^{2+} concentrations.⁵¹

These results suggest a signalling role for GIL, rather than a directly lethal one and this is not unreasonable because: (1) the predicted amino acid sequence of the heavy subunit is compatible with a membrane spanning location^{52, 53}; (2) each published *hgl* sequence contains several potential phosphorylation sites; (3) there is evidence for the participation of parasite protein kinase C in the cytotoxic process⁵⁴; and (4) adherence of liposomes containing a variety of glycosphingolipids bearing glycans with galactose and N-acetyl galactosamine termini (presumably involving binding to the GIL) stimulates rapid polymerisation of *E. histolytica* actin.⁵⁵

The message that contact has been established presumably causes the amoeba to activate a specific killing mechanism; several have been suggested and will be discussed later. It has also been suggested that target cells may actually be ordered to commit suicide. There is very preliminary evidence that *E. histolytica* induces apoptosis⁵⁶ and that GIL is transferred to the target cell following adherence.⁵⁷ It is not known whether these two processes are linked.

Of the amoeba's own putative killing mechanisms, by far the best studied are the family of pore forming peptides known collectively as amoebapores. Molecules of this class with molecular masses of 30^{58, 59} 13–15^{60, 61} and 8 kDa⁶² have been described but only the last has been investigated intensively and sequenced and is the one usually meant when amoebapore is discussed. Whether the larger molecular masses represent polymers or aggregates of amoebapore (or simply anomalous migration in gels) is not definitely known, but they may well do so. Amoebapore (actually a mixture of at least three isoforms) has recently been reviewed extensively^{63, 64} and I shall only consider here whether the molecule has a significant role in killing nucleated target cells. Two facts suggest that it does not: bacteria are killed by low concentrations (2 μ M) which do not seriously damage eukaryotic cells⁶⁴; and a homologous peptide is produced by *E. dispar*. It seems reasonable to assume that the function of amoebapore is to prevent ingested bacteria from multiplying. However, higher concentrations (particularly of the minor C isoform) are capable of killing tumour cell lines.⁶⁵ The *E. dispar* molecule is present in reduced amounts⁶⁶ and is of lower specific activity,⁴⁶ and amoebapore has structural similarities to natural killer (NK) lysin⁶⁷ which is assumed to contribute to the cytotoxicity exhibited by NK and T cells.⁶⁸ The amoebapores are apparently stored in (or as) cytoplasmic granules (together with another, lysozyme-like antibacterial protein⁶⁹), which could be released after target cell adhesion had occurred, producing a lethally high local concentration. The original function of the molecule may have been adapted in *E. histolytica* to serve a new, aggressive role.

Other possible cytotoxic effectors include a phospholipase A enzyme, initially identified as a haemolytic activity in whole amoeba lysates,^{70, 71} which is calcium dependent and membrane bound.^{72, 73} López-Revilla and Said-Fernández⁷⁰ correlated the level of activity with virulence. A second phospholipase A enzyme was detected in the soluble fraction and is calcium independent.⁷² Cytotoxicity, but not adherence, was blocked by Rosenthal's inhibitor and phosphatidylcholine (inhibitors of phospholipase A₂); destruction of the target cell membrane by the generation of lysophospholipids and free fatty acids was proposed as the mode of action of this enzyme.^{74, 75} A different and novel origin for the haemolytic activity of *E. histolytica* has been proposed by Hagblom *et al.*⁷⁶; these authors have identified three small open reading frames in the multicopy episomal circular DNA elements in which the parasite's rRNA genes are found.^{77, 78} When expressed in *Escherichia coli* haemolytic activity is produced; the expression products also react with anti-whole *E. histolytica* antiserum. These results were greeted with much scepticism because it could not be shown that the genes were actually expressed in the amoeba; however, this may now have been achieved for two of them.⁷⁹ The presence of an open reading frame between (and in the opposite orientation to) two rRNA genes has also been reported from

another protozoan (*Giardia lamblia*⁸⁰). High concentrations of extracts from expressing bacteria were lethal to CaCo-2 cells but expression in *E histolytica* itself will need to be confirmed before they can be accepted as yet another cytotoxic effector. Whatever these peptides are, they do not seem to be related to two 25 kDa haemolytic molecules described recently.⁸¹

Proteolytic activity

Enzymic degradation of extracellular matrix components, basement membrane proteins and connective tissue has long been considered to play a central role in tissue invasion by *E histolytica*.^{82–83} Most, if not all, of these activities reported from in vitro studies are mediated by collagenase and cysteine proteinases, and levels of expression have been correlated widely with virulence.^{84–87}

In 1982 Muñoz *et al*⁸⁴ described the destruction of human collagen films by *E histolytica* trophozoites, showing a higher specificity for type I collagen over type III. The EDTA inhibitable, contact dependent activity was located in the membrane fraction of freeze thawed lysates and was not secreted. A second collagenolytic activity was also detected in medium conditioned by growth of the same strain.⁸⁸ More recently, collagenase has been localised to dense granules and shown to be secreted actively on contact with its substrate,^{89–90} again focusing attention on the role of contact mediated signal transduction in invasion.

Cysteine proteinases, which constitute a large proportion of the overall proteolytic activity found in whole cell lysates of axenic *E histolytica*,^{91–93} have been investigated intensively. The partial purification of an acid proteinase has been reported,^{94–95} but little is known of its substrate specificity, subcellular location or possible involvement in tissue destruction.

The much better known cathepsin B-like neutral cysteine proteinase is usually said to be actively secreted by trophozoites.^{96–97} However, Spice and Ackers²⁰ found that the amounts present in culture medium could be accounted for by the numbers of autolysed trophozoites. Multiple bands are found on substrate SDS-PAGE gels but at present it is not clear how many should be attributed to different gene products, multiple forms of the same enzyme or purification artefacts. Levels of cysteine proteinase expression and secretion are widely regarded as key virulence determinants,^{98–100} although this view has been questioned.¹⁰¹ Quantitative analysis of mRNA showed a level of expression 10- to 100-fold higher in three *E histolytica* isolates compared with two *E dispar* strains.¹⁰² This finding is in broad agreement with previous comparative estimates of proteinase activity.^{98–103} Citing the intracellular sorting system of yeast, Tannich *et al*¹⁰² hypothesised that the over-production of a primarily intracellular proteinase by *E histolytica* could activate a secretory pathway, thereby adventitiously conferring on it the ability to degrade extracellular tissue components and become

an invasive parasite. In most cases, however, axenic cultures of *E histolytica* have been compared with xenic ones of *E dispar* and we have shown that the former have considerably higher levels of proteinase activity than xenic cultures of either species.^{20–21} This suggests that, as with *Dictyostelium*, culture conditions can have a profound effect on the levels of expression of these enzymes (but see¹⁰⁴).

Three cysteine proteinase genes have been well characterised, although a recent abstract¹⁰⁴ has raised the total number to six. Two (possibly four) are present in both *E histolytica* and *E dispar*.¹⁰² Most interestingly, one (ACP1, CP3) has been claimed to be present only in *E histolytica*,¹⁰⁵ although later work has not confirmed this.¹⁰⁶ The sequence of this gene is very different from those of the other two and if its uniqueness is confirmed, questions of its evolutionary origin and specific functions will need to be addressed.

The later stages of invasion

For reasons of space these can be dealt with only briefly. Amoeboid movement is clearly vital in passing from the lumen to the circulation. Phagocytosis (of killed epithelial cells and erythrocytes) is often regarded as a virulence determinant in its own right as well as a marker of a fully functional cytoskeleton. Erythrophagocytosis has been correlated with pathogenicity¹⁰⁷ and phagocytosis deficient mutants seem to be avirulent in animal models¹⁰⁸; however, this has not been confirmed by more recent results.^{109–110} Functional studies of locomotion and phagocytosis are well under way^{55–111–115} and a number of actin binding proteins have been recognised.^{116–118} Genes for actin,^{119–120} myosin II¹²¹ and profilin¹²² have been sequenced. Although *E histolytica* actin has a number of peculiarities—for example, the DNase I binding site is conserved but the protein does not bind the enzyme, in general nothing exotic has been seen so far.

It seems implausible that trophozoites can reach the liver from the bowel without being taken there by the portal circulation and therefore it is axiomatic that they are not killed en route. Activation of the alternative complement pathway is an obvious non-specific, host defence mechanism and it was extremely satisfying when Reed *et al*¹²³ showed that while all *E dispar* isolates (and old, laboratory adapted *E histolytica* isolates in axenic culture) were lysed, freshly isolated strains of *E histolytica* in xenic culture were apparently largely resistant. This fact alone could have been enough to explain the non-invasiveness of *E dispar*. The mechanism of resistance is not understood fully but seems to be linked to the cleavage of C3 by the parasite's cysteine proteinase.¹²⁴ A recent review¹²⁵ should be consulted for more details. Perhaps not surprisingly the ubiquitous GIL also seems to be involved; the molecule shares sequence similarities and antigenic cross-reactivity with the human complement regulatory protein CD59¹²⁶ and purified GIL caused 90% inhibition of *E histolytica* lysis mediated by C5b-9.¹²⁷

This logically pleasing picture has been spoiled by the studies of Hamelmann *et al.*^{128 129} These authors found that non-pathogenic isolates (that is, *E. dispar*) were nearly unaffected by exposure to alternative pathway activation; freshly isolated pathogenic strains (*E. histolytica*) were susceptible to lysis and became more so on long term cultivation, with or without bacteria.^{128 129} *E. histolytica* acquired complement resistance on hamster liver passage or on growth in media containing increasing concentrations of normal human serum, apparently as a result of the presence of trypsin sensitive surface molecule(s).¹³⁰ Genes specific for the resistant phenotype have been isolated by subtractive hybridisation.¹³¹ There seems to be a conflict between the results obtained by the two groups which needs to be resolved before the role of complement resistance in invasiveness can be assessed.

The events which occur when *E. histolytica* trophozoites reach the liver are almost completely unknown, not least because we have no animal model in which spontaneous spread from the intestine to that organ occurs. We do not know whether specific binding or random lodgment takes place; whether the amoeba, which certainly metabolises oxygen in vitro, also utilises it in vivo; or whether it flourishes only in small anoxic areas produced by earlier injury¹³²; why men are so much more frequently affected than women; or what proportion of hepatic abscesses (if any) resolve spontaneously is uncertain. (The event appears superficially similar to tumour metastasis and several groups are trying to see if the vast knowledge of that process can shed any light on amoebiasis^{133 134}.)

Why?

Perhaps most curiously of all, we do not know why *E. histolytica* invades. Intestinal amoebae which do not invade are generally much more commonly found in parasitological surveys and *E. histolytica* is absent from some populations (for example, male homosexuals in developed countries except Japan) in which other intestinal protozoa are common. Transmission seems to be exclusively via the resistant cysts but diarrhoea results in the appearance of fragile trophozoites (and presumably fewer cysts) in the faeces; no onward transmission from an hepatic abscess seems to be possible. Hepatic invasion may well be an accidental, unfavourable and relatively rare consequence of the ability to invade the gut wall (although the existence of specific, hepatotropic strains which pass straight to the liver has also been suggested¹³⁵). However, in a recent careful review Clark¹³⁶ could not identify any obvious evolutionary benefits from the ability to penetrate the intestinal mucosa. He suggests that humans may be an abnormal host for *E. histolytica*, to which it has only recently transferred from, say, a ground dwelling Old World primate. There is no better alternative theory available at present. Those of us who continue to hope that invasiveness will eventually turn out to be adaptive can only be grateful that the

same author, by finally persuading *E. dispar* to grow axenically,¹³⁷ has provided us with the ideal tool with which to tackle the problem.

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