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Entamoeba histolytica

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5.1 INTRODUCTION

Entamoeba histolytica is the cause of high levels of morbidity and mortality throughout the developing world, having been ranked as the third most important parasitic agent of disease after malaria and schistosomiasis. In 1981, it was estimated that up to 500 million people are infected annually [265]. The majority of infections are asymptomatic and apparently non-pathogenic. In most of these cases, spontaneous eradication of the parasite is thought to occur within several months [28, 153]. Some 10%, however, go on to develop invasive amoebiasis [201] in which symptoms range from mild and transient diarrhoea to fulminating colitis characterized by the passage of bloody stools. In some cases dissemination via the bloodstream may result in the production of extra-intestinal abscesses in other organs, principally the liver. Invasive amoebiasis is held to account for around 75 000 deaths per year [79].

5.1.1 Life-cycle and diagnosis

E. histolytica has a life-cycle consisting of two stages, the transmissive cyst and the vegetative trophozoite. Cysts are ingested orally from contaminated food or water, and excyst in the large bowel. Colonization of the mucosal surface by the trophozoites may subsequently lead to tissue invasion and disease; the cycle is completed by encystation and excretion of cysts. Diagnosis of amoebic infection is mainly carried out by light

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microscopic examination of faecal smears or formalin–ether concentrates of stool specimens [80]. The quadrinucleate cysts of *E. histolytica* distinguish this species from other lumen-dwelling amoebae, although there is no morphological basis for discriminating between pathogenic and non-pathogenic forms, other than by the detection of haematophagous trophozoites in some stool specimens once pathogenesis is already well under way. Both luminal and systemic amoebiasis are commonly treated with metronidazole or, in the former, with diloxanide furoate [187, 201].

5.2 PATHOGENICITY OF AMOEBIC INFECTION

An issue of fundamental importance in the study of amoebiasis is whether amoebae isolated from symptomatic and asymptomatic infections represent genotypically distinct groups, or two potentially interconvertible phenotypes of the same organism [1, 143, 222]. While recent investigations of the molecular biology of this organism (reviewed in section 5.2) strongly suggest separate genetic identities for these two groups, reports that at least some non-pathogenic isolates are capable of converting to the pathogenic form *in vitro* merit further detailed examination [145, 148].

The eventual resolution of this question will not only increase our basic understanding of amoebiasis, but will also be of practical benefit to the clinical management of infected individuals. Thus, if it were shown that carriers of non-pathogenic amoebae have no future risk of disease, treatment in the majority (90%) of cases would be rendered unnecessary, and would permit substantial reductions in drug expenditure [8, 87]. Conversely, if it were shown that a harmless commensal could become invasive as a result of unknown changes in the luminal environment, pre-emptive treatment of asymptomatic cystpassers might be considered more appropriate [143, 222].

5.2.1 Historical perspectives

Controversy has surrounded the question of pathogenicity in *E. histolytica* since the early 20th century. The trophozoite was recognized in two forms, a small, luminal amoeba termed 'minuta' which gave rise to cysts [61] and a larger, haematophagous form associated with tissue pathology. In the 'unicist' hypothesis advanced by Dobell [56] and labelled 'Promethean' by Elsdon-Dew [62], all infections with *E. histolytica* were thought to result in damage to the gut. To account for asymptomatic cases, the rate of host repair was assumed to be sufficient to suppress any outward manifestations of disease. The advent of *in vitro* culture [22] and the observation that the amoebae could be grown in a medium containing bacteria and

starch, contributed towards Dobell's later recognition of the commensal nature of some infections [57], although the original idea continued to be enthusiastically endorsed by some workers until as recently as 1970 [43, 44, 47, 65].

The opposing view, expressed by Kuenen and Swellengrebel [108] and by Mathis and Mercier [133], was that the 'minuta' amoeba represented a commensal stage which could, under appropriate conditions, produce the pathogenic tissue form. Only the former was considered to be capable of giving rise to cysts, while the latter was placed outside the normal cycle of transmission and infection.

In 1925, Brumpt, recognizing the wide geographical variations in the proportion of infected individuals who would subsequently develop invasive disease, proposed the existence of two morphologically indistinguishable species which he termed *E. dysenteriae* and *E. dispar*, to represent pathogenic and non-pathogenic *E. histolytica* respectively [30]. This became known as the 'dualistic' hypothesis, but failed to gain wide acceptance owing to the lack of any practical basis for differentiation between the two amoebae.

More recently, the 'pluralist' hypothesis propounded by Hoare [82], Neal [155], Elsdon-Dew [62, 63] and others has attributed regional variations in the levels of disease to the existence of a range of strains all belonging to the single species *E. histolytica*, but varying in pathogenicity from the asymptomatic commensal to the highly virulent. This approach bears similarities to the original concept of Kuenen and Swellengrebel [108].

5.2.2 Recent developments

Little further progress was made in this debate until Martinez-Palomo *et al.* reported in 1973 that only pathogenic amoebae were agglutinable with low concentrations of concanavalin A [130], thereby providing the first concrete evidence in support of Brumpt's notion of two species. Subsequently, Sargeaunt and Williams [227, 228] showed that amoebae isolated from symptomatic and asymptomatic infections produced specific electrophoretic banding patterns using two isoenzymes, hexokinase and phosphoglucomutase. This observation was demonstrated with 6000 isolates collected worldwide [224], and the patterns were found to be stable over long periods in culture [229].

Over the last 10 years a range of other cellular, biochemical and immunological properties have been used to differentiate pathogenic from non-pathogenic *E. histolytica*, although until relatively recently, few have shed any light on the basis of pathogenicity in this parasite. The growing consensus that *E. histolytica* comprises two genetically discrete groups of

organisms was challenged in the late 1980s by Mirelman *et al.* [145, 148], with a report that cloned isolates defined as non-pathogenic by isozyme analysis could undergo an apparent conversion to the pathogenic phenotype. One of the distinguishing features of pathogenic amoebae is their ability to grow in the absence of bacteria, and it was while attempting to derive an axenic, non-pathogenic cell line that the switch in phenotype was observed. The procedure was based on the antibiotic-mediated removal from xenic culture of associated faecal flora, combined with the addition of gamma-irradiated bacteria as a substitute food source. The change was accompanied by the acquisition of pathogen-related activities such as cytotoxicity against Chinese hamster ovary (CHO) cell monolayers and the production of hepatic lesions in hamsters. Similar 'conversions' have now been reported from other laboratories [11] (Diamond, personal communication), although the event has not yet been demonstrated *in vivo* [205]. Such efforts are hampered by the lack of any suitable animal model for amoebic colitis.

The renewed uncertainty surrounding the relationship between pathogenic and nonpathogenic *E. histolytica* has generated a substantial amount of research effort over the past two years aimed at resolving this question through the application of recombinant DNA techniques. As a result, not only is it becoming increasingly clear that the two forms of infection should indeed be attributed to separate organisms, but an overall picture of the molecular basis of pathogenicity has also begun to emerge. These developments will be reviewed in the following sections.

5.3 GENETIC ORGANIZATION

E. histolytica has traditionally been regarded as a primitive, anaerobic organism, lacking mitochondria and many other organelles normally associated with higher eukaryotic cells [122, 131]. There is no extra-nuclear DNA, and the absence of evidence for sex in *E. histolytica* has precluded conventional genetic analysis, although recent *in vitro* studies have suggested that genetic exchange may occur [21, 225, 238].

5.3.1 Nuclear division and organization

Nuclear division, which is apparently not always accompanied by cell division [142], occurs by a pleuromitotic process [161], in which the nuclear membrane remains intact and microtubules form 'V'-shaped arrays rather than a conventional spindle. Four stages in nuclear division have been recognized: interphase, early anaphase, late anaphase and telophase, but a metaphase is apparently absent [161]. A nuclear bud appears during

prophase, which enlarges and finally separates to produce the daughter nucleus after approximately 120 minutes.

Electron microscopic studies of the nuclear distribution of nucleic acids in *E. histolytica* have been carried out on autoradiographed sections of pulse-labelled amoebae [4, 5, 7], and on sections stained with phosphotungstic acid [142]. A region of DNA containing peripheral chromatin, originally reported by Sharma *et al.* [234], has been identified as a site of extensive RNA synthesis. This is thought to represent a functional equivalent of the nucleolus [3, 142], a similar arrangement having been observed in *Amoeba proteus* [67]. A centrally located endosome, containing tightly packed DNA, may be involved in DNA condensation prior to division [3, 164]. Meza *et al.* [142] have speculated that the endosome could be brought into association with the peripheral chromatin during replication and transcription. RNA, possibly heteronuclear, and DNA are also distributed throughout the nucleus, the latter mainly during interphase.

5.3.2 Chromosomes

In common with many protozoa, the absence of a nuclear condensation stage during division has hindered analysis of the amoeba karyotype. In electron microscopic studies using colchicine-synchronized cultures, Solis *et al.* [239] have reported four or five electron dense bodies which could correspond to chromosomes, while in 1988 Orozco *et al.* [161], using the same system, observed 12–16 chromosome-like structures associated with the microtubular arrays.

The pulsed field gel electrophoresis (PFGE) technique of Schwartz and Cantor [232], by which large (100–2000 kb) DNA molecules can be resolved on agarose gels, has enabled studies on chromosome sizes and numbers in many protozoan parasites to be conducted [64, 90, 102, 220, 263], although the chromosomes of *E. histolytica* have not, for the most part, proved amenable to this procedure [142]. However, densitometric analysis of PFGE gels did reveal up to nine bands over a size range of 280–2000 kb, which were interpreted as comprising about 16 large and 31 small chromosomes [261]. These results, though preliminary, provide an important basis for further characterization of the *E. histolytica* karyotype.

5.3.3 Organization of chromatin

Organization of the peripheral chromatin has been shown to correspond broadly with that found in other cells, although certain differences have been observed. In higher eukaryotes, extended DNA fibres have a regular

beaded appearance arising from the presence of nucleosomes, which are 10 nm diameter particles consisting of histones associated with DNA sequences of between 160 and 250 bp [106, 252]. Limited digestion with micrococcal nuclease produces a ladder of DNA fragments on agarose gels corresponding to multimers of the nucleosomal unit.

Electron microscopy of phosphotungstic acid stained chromatin spreads from *E. histolytica* revealed nucleosomes spaced at often irregular intervals [257]. Mild digestion yielded a smear on ethidium bromide stained agarose gels, and radioactive DNA probes for both actin and ribosomal genes also produced a smear on Southern blots. Only a non-transcribed probe from the extra-chromosomally situated ribosomal episome (section 5.2.7) gave a clear ladder, leading the authors to conclude that little of the DNA in amoeba chromatin is nucleosome protected.

The nucleosomal proteins extracted from *E. histolytica* chromatin differed substantially from typical histones in molecular weight. In addition, synthetic oligonucleotides based on consensus sequences for the H3 and H4 histones failed either to detect histone-encoding clones in a genomic library or to permit amplification by PCR of homologous sequences from total DNA, suggesting that histone genes in this parasite have diverged considerably from the highly conserved sequences of other eukaryotes [257].

5.3.4 Ribosomal RNA

Ribosomal RNA species in *E. histolytica* are known to be highly labile, due to the tight association of RNAse with ribonucleoprotein complexes [14, 183]. Using diethylpyrocarbonate to inhibit RNAse activity during the isolation procedure, Heebner and Albach [81] described rRNA species from the axenic strain HK-9 with sedimentation coefficients of 4S, 17S and 25S in ratios of 1:2:3. The 25S rRNA dissociates readily at 55 °C into products of 5.8S, 16S and 17S, indicating that the molecules are non-covalently bound *in vivo* [15, 23]. An additional 5S rRNA has been detected by denaturing polyacrylamide gel electrophoresis [6]. Ribosomes are commonly organized into helical arrays in the cytoplasm of both cysts and trophozoites, as observed by electron microscopy [60, 211].

5.3.5 Characterization of DNA

The characterization of nucleic acids in *E. histolytica* was facilitated by the development in 1968 of axenic culture methods [53], whereby amoebal DNA could be studied in isolation from the DNA of original bacterial associates. Caution should be exercised, however, in the extrapolation of

information concerning gene expression under axenic conditions to amoebae living in the xenic environment of the gut lumen where pathogenesis is initiated, especially in the light of the known influence of certain bacteria in modifying amoebal virulence [144, 147, 179, 271].

DNA content, %GC and ploidy

Most studies of the DNA content of *E. histolytica* trophozoites have used the diphenylamine method of Burton [33], and reported values are subject to considerable variation. For example, DNA content for strain HK-9 ranged from 0.45–1.5 pg/cell [75, 113], while another widely used strain, NIH:200, had 0.4 pg/cell [207]. In contrast, the DNA content of strain HM2 was reported to fluctuate between 3.0–8.9 ± 0.48 pg/cell [113]. While such relatively high values require confirmation, it has been suggested that changes in ploidy [113], possibly through the production of multinucleate trophozoites, as noted both in our own laboratory and elsewhere [113, 142], could be contributory factors. Pathogenic *E. histolytica* isolated from lesions in experimental animals has been reported to contain four times more DNA/cell than the same strain grown in culture [124].

While interstrain variations in the GC content of DNA are also evident from a number of studies, more consistency has been found in comparisons of the same strain by different workers. Based on thermal denaturation measurements, values quoted for HK-9 range from 22.4–24% [74, 113, 207], compared with 25.4–27.3% for NIH:200 [75, 207] and 27% for strains HM2 and HM3 [113]. A surprisingly high figure of 38% was calculated for the widely used HMl strain by Perez-Mutul [168], although this was obtained by buoyant density measurement which appears to produce somewhat higher values than thermal denaturation [207].

On the basis of GC measurements, Gelderman *et al.* [75] estimated the size of the HK-9 haploid genome to be $10^{7.5}$ (3.16×10^7) base pairs, a figure which has been supported by Valdes *et al.* [261] whose calculations were founded on the numbers and sizes of chromosomes in strain HM-1 detected by PGF electrophoresis. Taking this value to be equivalent to 0.033 pg of DNA per haploid genome, Byers [34] used Gelderman's figure of 0.45 pg of DNA per HK-9 trophozoite to propose a ploidy level of 14n.

Genetic complexity and repetitive DNA

Little information is available concerning genetic complexity and gene number in *E. histolytica*. From the T_m data of Gelderman [75], Byers [34] has inferred that approximately 50% of the genome may be composed of

repetitive sequences. Assuming a DNA content per cell of 0.4 pg, which is equivalent to approximately 4×10^8 base pairs, and an average gene size of 2000 bp, Torres-Guerrero *et al.* [257] have calculated a maximum number of 200 000 genes. However, the proposed ploidy level of 14n would reduce this figure to about 14 000 genes per haploid genome. Furthermore, if only 50% of the genome is composed of single copy sequences, this figure would be reduced to 7000, while if *E. histolytica* is to any extent typical of higher eukaryotes, in which less than 20% of single copy sequences are transcribed into mRNA [253, 257], the total number of genes could be as few as 1500.

Repetitive DNA in *E. histolytica* was first visualized by Cruz-Hernandez *et al.* [48], who reported the presence of bands in total DNA which had been digested with the restriction enzymes *Eco*RI and *Hin*DIII and electrophoresed on agarose gels. Subsequently, Bhattacharya *et al.* [17], using the same enzymes, conducted a comprehensive study of repetitive DNA from five *E. histolytica* strains and other *Entamoeba* species and detected characteristic banding patterns in every case. Southern blots hybridized with ribosomal probes from *Plasmodium berghei* produced signals corresponding to the prominent bands observed on gels, from which it was concluded that the ribosomal DNA accounts for a large proportion of repetitive DNA and at least 10% of total DNA in *E. histolytica*.

DNA purification

It has been noted by several authors that the DNA of *E. histolytica*, like ribosomal RNA, is difficult to purify intact and in sufficient quantities for useful analysis [3, 48, 59, 142, 168]. The trophozoites are slow growing and osmotically fragile [53], while co-purification of carbohydrate and potent nucleases during conventional phenol extraction presents further problems. As a result, many workers have adopted the preparation of nuclei as a preliminary step in DNA isolation [59, 84, 250].

5.3.6 Gene sequence analysis

Perhaps partly because of these technical factors, the first report of gene cloning in this parasite did not appear until 1987, when Edman *et al.* [59] and Huber *et al.*[84] independently described the sequence of actin. This is one of the most abundantly transcribed genes in *E. histolytica*, comprising 1% of clones in a representative cDNA library [84], while actin itself accounts for 10% of total cell protein [69]. Analysis of cDNA and genomic sub-clones revealed a number of interesting features, including the

absence of introns, and an unusual codon bias involving a large number (83%) of silent 3rd position base changes to A or T. This has given rise to a discrepancy between amino acid and nucleotide sequence homologies with human cytoplasmic actin (89% compared with 69% respectively, by Edman *et al.* [59]). The length of the 3′ non-transcribed region was found to be variable in a number of clones and both typical and atypical polyadenylation signals were reported. The 5′ non-transcribed region was comparatively short (11 nucleotides) and was not variable in length [59]. These observations, taken together with the appearance of multiple bands on Southern blots of strain HM-1, suggest the existence of a multi-gene family of between four and seven members [59, 84].

Analysis by Huber *et al.* [85] of apoferredoxin, another abundantly transcribed *E. histolytica* gene, disclosed similarities with the actin genes, including the absence of intervening sequences, a short 5′ non-transcribed sequence and a strong 3rd position codon bias for A/T, characteristics also shared by a gene encoding a 125 kDa surface antigen [58] (section 3.2.1).

5.3.7 Characterization of ribosomal genes

Cloning of the highly abundant DNA species previously reported to contain ribosomal genes [17] led to the discovery by Huber *et al.* [86] and Bhattacharya *et al.* [18] that these sequences are carried on a 24.6 kb extra chromosomal DNA molecule present in about 200 copies per genome. A similar arrangement has been reported in the free living soil amoeba *Naegleria gruberi* [39] and for several other schizopyrenoid amoebae [40]. The episomal genes were mapped to two large inverted repeat regions of at least 5.2 kb, each of which represents a single ribosomal cistron. Inverted repeats have also been detected on linear ribosomal elements in other organisms including *Tetrahymena* and *Physarum polycephalum* [95, 264]. The palindromic arrangement of these genes is thought to guard against recombinational events [163]. Intervening regions of repeated, non-coding DNA were also detected in the *E. histolytica* episome both upstream and downstream of the coding regions, possibly serving a regulatory function during transcription [76, 206]. Evidence for the existence of at least one chromosomal copy of the ribosomal genes has been found by Bhattacharya *et al.* [18] and by Valdes *et al.* [261] from Southern blots of low concentration agarose gels and PFGE gels respectively.

An AT-rich origin of replication was identified in the episome downstream of the ribosomal inverted repeats which was able to confer stable ARS (autonomous replication sequence) activity on an ARS-deficient yeast vector, YIp5 [77]. The isolation of an amoebal ARS is an important step towards the development of a genetic transformation system for *E. histolytica*.

5.3.8 Molecular differentiation of pathogenic and non-pathogenic strains

Ribosomal sequences

The presence of homologous ribosomal elements in non-pathogenic *E. histolytica* was described by Garfinkel *et al.* [72]. A genomic clone, H6.6, spanning the entire length of the ribosomal cistron, produced distinct banding patterns when used to probe *Eco*RI-digested DNA from pathogenic and non-pathogenic isolates. Two were specific for the former and one for the latter, suggesting that pathogenic *E. histolytica* may consist of two or more genotypic subsets. Interestingly, when radioactively labelled H6.6 was used to probe Southern blots of the two strains reported previously by Mirelman *et al.* [145, 148] as having undergone phenotypic conversion, the banding patterns obtained were identical to those recognized for clinical pathogenic isolates.

Furthermore P145, another episomal clone derived from non-coding DNA downstream of the inverted repeats, was found to hybridize exclusively to DNA of pathogenic isolates under high stringency conditions after a short exposure. This was the first example of a DNA sequence specific to pathogenic *E. histolytica*, and was reported together with an analogous probe, B133, isolated from a genomic library of a non-pathogen and with exclusive specificity for this form [72].

A faint hybridization signal (approximately seven-fold weaker) was, however, obtained on long exposure when P145 was hybridized to non-pathogenic DNA, and *vice versa*. Although the 43.6% sequence homology between the two probes might have been sufficient to account for this observation, Garfinkel *et al.* [72] instead proposed that each form of *E. histolytica* harbours one or a few master copies of the corresponding sequences. The subsequent report by Mirelman *et al.* [146] that the pathogen-specific element P145 could be amplified by PCR from the DNA of the cloned, non-pathogenic strain SAW1734R, lent support to this contention, leading the authors to propose selective gene amplification as a possible mechanism for previously observed phenotypic conversions. Further to this result, sequence comparisons between P145 and the PCR product obtained from the non-pathogen would now prove useful in confirming the precise identity of the latter.

Genotypic divergence

In 1989, Tannich *et al.* [250] described the sequence of a clone isolated from an HM1 cDNA expression library using an antiserum pre-enriched for pathogen-specific antibodies. The clone, cEH-Pl, was then used to screen a non-pathogen cDNA library under low stringency conditions,

from which a homologous clone, cEH-NPl, was purified. Sequencing of the two clones revealed a 12% divergence in the inferred amino acid sequence, and Southern blot hybridizations with a range of pathogenic and non-pathogenic isolates of *E. histolytica* produced banding patterns specific for each form.

A number of other recent reports concerning gene sequences from both pathogenic and non-pathogenic *E. histolytica* [58, 251] (sections 5.2.1 and 5.3.2) have supported the earlier proposal of Sargeaunt and co-workers that the two forms are genetically distinct. In addition, recent results from our laboratory have disclosed the existence of a pathogen-specific RFLP lying within the ribosomal gene cistron (45a); further work is in progress to identify the precise nature of this difference. Details of divergent sequences, where they may relate to the molecular basis of pathogenicity, will be discussed in the next section.

The growing evidence for genotypic divergence is such that it is becoming increasingly difficult to envisage a model of genetic organization that could also accommodate phenotypic conversion. An approach towards resolving this apparent conflict would be use PCR to establish whether gene sequences specific for each form could be amplified in their respective counterparts. The successful application of PCR by Tannich *et al.* [248] to amplify specific fragments of the gene described previously [250] from both forms of the organism suggests that it should also be possible to design primers capable of detecting any variant sequences present in low copy number.

5.4 MOLECULAR PATHOGENESIS

Pathogenesis in amoebiasis is produced by complex interactions involving the invasive processes of the parasite [186], the virulence of the infecting isolate [271], the physiological [54, 152] and immune status of the host [258], and bacterial associates in the gut lumen [177, 178, 180, 181]. While aspects of all of these determinants have been studied at the cellular level, molecular analysis has so far been largely confined to the mechanisms of host tissue invasion.

As its name implies, *E. histolytica* has the potential to express a wide range of destructive properties, enabling it to penetrate deep into the intestinal submucosa and to form necrotic lesions in solid organs [37, 243, 244]. The following stages in parasite invasion can be defined:

1. Binding of trophozoites to carbohydrate moieties on host epithelial cells (or secretions) via cell surface adhesins and receptors;
2. Detachment and cytolysis of target cells;
3. Phagocytosis [159, 208] and intracellular degradation of host cellular

components, including neutrophils [51, 68, 219] and erythrocytes [191, 259];
4. Translocation through the tissue by pseudopodial action [129, 235].

Investigations of the molecular basis of invasion have concentrated on the first two stages, adhesion and tissue destruction, and are consequently of greater interest in the present context. This section will also describe a number of recently isolated genes encoding novel cell surface molecules with potential involvement in pathogenesis, making particular reference, where possible, to comparisons between pathogenic and non-pathogenic *E. histolytica*.

5.4.1 Adhesion to host tissues

Several lectin-like activities have been detected in *E. histolytica*, indicating the presence of receptors with specificities for a number of different carbohydrate groups. Kobiler and Mirelman identified a 220 kDa membrane-bound lectin, with high affinity for *N*-acetyl glucosamine-containing molecules such as chitotriose and hyaluronic acid [105, 140]. Antibodies raised against the lectin partially inhibited binding of trophozoites to erythrocytes and mammalian cells and prevented ingestion of red blood cells [210]. Similar inhibitory effects were also reported using antibodies to an immuno-dominant 112 kDa surface adhesion [12]. Trophozoite adherence to target cells also depends on the maintenance of microfilament function, as demonstrated by the inhibition of amoebic adherence to CHO cells at low temperatures [141,190] and by cytochalasins B and D [191].

The Gal/GalNAc lectin

The most intensively studied receptor of *E. histolytica* is the 170 kDa galactose/*N*-acetyl galactosamine-inhibitable lectin first described by Ravdin and Guerrant [191] and proposed as a potential immunoprotective reagent due to its high immunogenicity [189] and its central role in pathogenesis [173–175, 186, 197]. Ravdin and Guerrant [191] demonstrated the specificity of this lectin for galactose and *N*-acetyl-galactosamine (GalNAc) residues by the ability of these sugars to inhibit binding of trophozoites to CHO cells *in vitro*; cytotoxicity was also blocked, although phagocytosis was reported to be unaffected [191, 193]. Similar results were obtained using fixed rat and human colonic mucosa [194]. In addition, galactose inhibited adherence to bacteria and erythrocytes [191] and to rat and human colonic mucins [35, 38].

Purified lectin was found to prevent amoebic adherence to CHO cells in

a galactose inhibitable and competitive manner [175]. The nature of the carbohydrate receptors recognized by the lectin was confirmed with a range of CHO glycosylation-deficient mutants; binding to a cell line lacking O- and *N*-linked Gal and GalNAc residues was virtually abolished, while enhancement was observed in mutants with a higher terminal galactose content [200]. The 170 kDa lectin is linked by disulphide bonds to a 35 kDa subunit which is non-immunogenic and does not participate in sugar binding [170], although a possible identity with the fibronectin receptor reported by Talamas-Rohana and Meza [247] has been suggested [125]. Polyclonal and monoclonal antibodies raised against the 170 kDa lectin were effective in preventing adherence to CHO cells [170, 198], although in a later study, Petri *et al.* [176] reported that two out of a panel of six monoclonal antibodies (Mabs) increased binding; such activation could be due to changes in the conformation or state of aggregation of the molecule. This result was reflected in the finding that while individual sera from 25 of 44 patients with amoebic liver abscess inhibited trophozoite adherence to CHO cells, a further 16 sera increased binding.

The lectin epitopes recognized by the two enhancing Mabs were present on both pathogenic and non-pathogenic amoebae, whereas the remaining four were only found in pathogenic isolates; Mabs to three of these were inhibitory, while one had no effect [171]. Based on these antigenic differences, the authors were able to classify 50 isolates of *E. histolytica* as pathogenic or non-pathogenic by radioimmunoassay. The presence of a functional lectin in the non-pathogenic amoebae suggests that the molecule is important in colonization of the intestine in addition to its role in pathogenesis.

Beside its potential application in clinical diagnosis, immunization with the Gal/GalNAc lectin has also been shown to confer immunity in the gerbil liver abscess model. In a series of trials, Petri and Ravdin [174] reported complete protection in 67% of immunized animals. However, an effective vaccine would presumably exclude either of the antigenic determinants shown by Petri *et al.* [171] to enhance trophozoite adherence, thus ruling out the use of whole lectin as an immunogen.

Gal/GalNAc lectin genes

Gene sequences for the Gal/GalNAc lectin from pathogenic *E. histolytica* derived independently by Tannich *et al.* [249] and Mann *et al.* [125] are 12.5% divergent, suggesting the possible existence of two or more genes. This proposal is supported by variations observed in *N*-terminal amino acid sequences [126] and by the appearance of multiple bands on Southern blots of genomic DNA probed with a full length cDNA clone [249]. A single 4 kb message was detected by Northern blotting.

Both of the reported genes lack intervening sequences, and indicate a protein of 1270–1276 amino acids with a molecular weight of approximately 143 kDa. Glycosylation may account for the higher value of 170 kDa obtained from preparations of the purified lectin, since amoebae pre-incubated with the glycosylation inhibitor tunicamycin lost their ability to adhere to CHO cells [126]. The derived protein sequence predicts a short, conserved C-terminal cytoplasmic tail of approximately 40 residues. This region contains putative phosphorylation sites and may participate in signal transduction [125]. A short, adjacent hydrophobic domain is proposed as a transmembrane region. This is followed by a large, extracellular portion of more than 1200 residues, and is variously reported to contain nine [249] and sixteen [126] glycosylation sites. The initial two-thirds is cysteine-rich, possibly conferring resistance to proteolysis [126]. A short, hydrophobic signal peptide is located at the *N*-terminal. Significant homologies with other known genes were not found, and no sequences for the corresponding non-pathogenic lectin gene have yet been reported. Future definition of sequences encoding pathogen-specific epitopes can be expected to facilitate the development of recombinant vaccines.

5.4.2 Other surface proteins

In addition to the elucidation of adherence mechanisms in *E. histolytica*, interest in the identification of other cell surface properties with possible involvement in pathogenicity has led to a number of recent reports describing membrane proteins of as yet undisclosed function [20, 58, 240, 242, 254–256]. Those for which gene sequences have already been characterized are discussed below.

125 kDa immunodominant antigen

Edman *et al.* reported a highly immunoreactive 125 kDa antigen from lysates of both pathogenic and non-pathogenic *E. histolytica* [58]. A monoclonal antibody prepared against the partially purified antigen induced cap formation with live trophozoites, indicating a surface location for the molecule. A monospecific antibody eluted from phage lysates of a cDNA clone encoding part of the sequence for this antigen gave the same result.

Analysis of two genomic clones for this gene, named M17, revealed an open reading frame of 3345 bp, which was in broad agreement with the mRNA size of approximately 3000 bp obtained by Northern blotting. The 5′ flanking region of the gene was found to contain only 17 nucleotides, in common with the short non-transcribed 5′ sequences previously observed

in both actin and ferredoxin genes [59, 84, 85]. Additional similarities were found in the presence of an unusual putative promoter sequence of TATTTAAA, in contrast to the more common TATAAATA motif, and a 5′-ATTCA-3′ sequence present at the transcriptional start site of both the M17 and actin genes. Southern blotting indicated that the gene was present as a single copy.

The inferred amino acid sequence of M17 revealed that part of it was almost identical to the clone reported previously by Tannich *et al.* [250]. Although both were initially isolated from the pathogenic strain HM-1, a 1% divergence in amino acid sequence was detected. Comparison with a homologous gene from a non-pathogen revealed a 12.1% divergence at the amino acid level, in good agreement with the finding of Tannich *et al.*, and a 10.3% difference in nucleotide sequence. The non-pathogens used in the two studies differed in amino acids by 1.3%.

The sequence reported by Edman *et al.* is rich in asparagine (8.2%), threonine (8.1%), serine (7.6%) and tyrosine (6.3%) residues, and contains 17 *N*-linked glycosylation sites. A hydrophobic, 35 residue *N*-terminal region may be membrane-located, while the polar C-terminal portion is possibly exposed. A stretch of 31 amino acids in this region showed close homologies with the beta chains of human fibronectin receptor and mouse integrin, although the functional significance of this association remains to be determined. No other sequence similarities were found.

Serine-rich protein

A gene encoding a serine-rich protein with an inferred molecular weight of 25 kDa was isolated by Stanley *et al.* [240] by differential screening of an HM-1 cDNA library with cDNA probes derived from HM-1 and the closely related Laredo amoeba. Many of the 52 serine residues, out of a total of 233, are present in tandemly-repeated motifs of 8 and 12 amino acids; the repeats occur between residues 82 and 194, and are preceded by a highly charged region. A hydrophobic domain is situated at each end of the protein, possibly indicating membrane insertion sites.

No homologies were found with other sequences at either the nucleotide or amino acid levels, although the protein bears a strong structural resemblance to the malarial circumsporozoite protein [101], with respect to the presence of multiple dodecapeptide repeats preceded by a charged region, and terminal hydrophobic domains.

An antiserum raised against the recombinant protein identified bands of 46 and 52 kDa on Western blots of *E. histolytica* membrane preparations, but the molecule was apparently absent from the soluble fraction. Post-translational modification was suggested to account for the approximately

two-fold discrepancy between predicted and actual sizes of the protein. The antiserum reduced binding to CHO cells by 70%, implying a possible role in adhesion.

Cysteine-rich protein

Torian *et al.* have described a 29 kDa surface protein from axenic trophozoites of *E. histolytica* with an unusually high cysteine content [254]. A 700 bp cDNA clone encoding the molecule was selected from an expression library of strain H-302 : NIH using a rabbit antiserum pre-enriched for surface antigens. The clone hybridized with three other axenic strains but gave no signal with several other protozoan species. Non-pathogenic *E. histolytica* were not included.

Polyclonal and monoclonal antibodies to the fusion protein detected a 29 kDa band on Western blots of axenic amoeba whole lysates, suggesting that a length of sequence encoding some 40 *N*-terminal amino acids was missing from the original clone. Western blots of pathogenic isolates grown in polyxenic culture also produced a 29 kDa band with some, but not all of the Mabs tested, suggesting variation in the expression of certain epitopes, while two non-pathogens were weakly immunoreactive.

Immunoprecipitation of ^{125}I-surface-labelled amoebae using a mouse Mab gave a 29 kDa band on autoradiographs; localization of the antigen on the cell surface was also indicated by uniform staining of fixed trophozoites using indirect immunofluorescence. Analysis of the deduced amino acid sequence of the antigen showed that cysteine accounted for 7% of the 227 residues present, and that almost half of these occurred within the first 18 *N*-terminal residues, including two doublets and a triplet. No functional significance has yet been assigned to this configuration, although Torian *et al.* [254] report that a similar motif is present in a host cell binding protein of a porcine coronavirus and in a wool keratin protein. A six amino acid hydrophilic sequence was identified as a potential immunogenic epitope. It is not yet known whether the adjacent *N*-terminal portion of the sequence presumed missing from the cDNA clone contains a similarly high proportion of cysteine.

5.4.3 Histolysis

Target cell killing by *E. histolytica* trophozoites, observed *in vitro*, takes place within minutes of initial contact [192], and incubation of amoebae with a mammalian cell monolayer at a cell ratio of 1 : 1 can result in complete destruction in approximately 60 minutes [160]. The sequence of events accompanying epithelial cell detachment and lysis was described by Martinez-Palomo *et al.* [129] using time-lapse microcinematography and

electron microscopy. Amoebae adhered strongly to an MDCK cell monolayer, shown by distortion of microvilli; this was followed by the opening of intercellular junctions, surface blebbing, disruption of the plasma membrane, cell detachment and phagocytosis. In addition, portions of host cell cytoplasm were directly pinched off and engulfed. Initial attachment was marked by a lowering of transepithelial resistance, as measured in an Ussing chamber [129].

This, and other studies on the physical interactions between amoebae and target tissues, has underlined the importance of amoebic motility and microfilament function to attachment [142], phagocytosis [159] and target cell displacement by pseudopodial action [235]. The structural approach has been complemented by a large number of investigations over the past 14 years aimed at establishing the biochemical basis of histolysis. While yielding considerable insights, such studies have needed to rely extensively on somewhat artificial *in vitro* systems.

Tissue dissolution by *E. histolytica* results from a combination of cytolethal, contact dependent mechanisms, and non-cytotoxic proteolytic enzymes which act on components of the extracellular matrix; the latter are secreted, with the exception of collagenase (section 5.3.2). There is so far little evidence to support the existence of secreted cytotoxins as previously suggested [26, 128, 169], although non-enzyme based enterotoxic activities capable of inducing fluid secretion in *in vivo* gut models have been reported [36, 136, 137].

5.4.4 Cytotoxic mechanisms

Calcium influx

The role of calcium in cytolysis was studied by Ravdin *et al.* [199], who reported that pre-treatment of CHO cells and human neutrophils with the slow Na–Ca ion channel inhibitor, verapamil, reduced amoebic killing but was ineffective when used to pre-treat trophozoites. Tetrodotoxin, a fast sodium channel inhibitor had no effect on either cell type, implying that the process was calcium dependent. This was later supported in experiments using the fluorescent calcium probe Fura-2 [195]. Thirty to three hundred seconds after initial contact with the trophozoite, a substantial and irreversible rise in target cell calcium concentration was followed by an increase in non-specific membrane permeability and cell death. No change in overall or local calcium levels were detected in the amoebae. The cytotoxic effect of calcium influx is widely known [195], and it is thus also likely to contribute to cell killing by trophozoites. Calcium influx was inhibited by galactose, suggesting a mediating role for the Gal/GalNAc lectin.

Phospholipase A

A phospholipase A enzyme, initially identified as a haemolytic activity in whole amoeba lysates [114, 215], is calcium dependent and membrane bound [112]. Lopez-Revilla and Said-Fernandez [114] correlated the level of activity with virulence. A second phospholipase A enzyme was detected in the soluble fraction and is calcium independent [112]. Killing of target CHO cells was inhibited with the chelating agents EDTA and EGTA, and with specific phospholipase A antagonists such as Rosenthal's inhibitor and phosphatidylcholine [196]. 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 [196, 216].

Protein kinase C

Protein kinase C is a ubiquitous enzyme with a central role in intracellular signal transduction [73, 156]. Weikel *et al.* [269] investigated the possible involvement of an amoebal protein kinase C in the regulation of cytolysis by observing the effects of phorbol 12-myristate 13-acetate (PMA), a specific activator of this enzyme, on the rate of CHO cell destruction by the pathogenic strain HM-1. Exposure of amoebae to 10^{-6}–10^{-7}M PMA by either brief pre-incubation (5 min), or direct addition to the assay system, resulted in a 100% enhancement of cell killing. Cytotoxicity was significantly reduced by sphingosine, a specific inhibitor of protein kinase C. PMA did not enhance cytolytic activity in two avirulent clones of HM-1. While these results indicate the involvement of a protein kinase C in the regulation of cytolysis by *E. histolytica*, the mechanisms involved in this process have yet to be defined.

Amoebapore

A membrane pore forming protein of 13–15 kDa, named 'amoebapore', was described by Lynch *et al.* [123] and Young *et al.* [273]. The molecule has been detected in highly dense, sub-cellular particles in axenic trophozoites [213], and exists as a dimer in two isoforms [212]. Using synthetic unilamellar lipid vesicles, Rosenberg *et al.* [212] demonstrated spontaneous insertion by amoebapore into synthetic unilamellar lipid vesicles, producing large pores which were non-selective for ions and were resistant to changes in transmembrane potential. Although studies on cytotoxicity have not been conducted to date, a reported correlation between amoebapore levels and strain virulence [100], together with the involvement of similar molecules in pathogenesis by other parasites [158, 272] and in cell killing by T-lymphocytes [96] suggests a likely role in cytotoxicity for amoebapore.

Non-cytotoxic activities

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* [16, 89, 97, 99, 119, 138, 150, 154]. 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 widely correlated with virulence [13, 70, 71, 97, 98, 117, 118, 150].

Collagenolysis

In 1982, Munoz *et al.* [150] described the destruction of human collagen films by HM-1 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. In contrast, a second collagenolytic activity was detected in conditioned medium by Gadasi and Kessler [70] using the same strain, and could be eluted from plasma membranes with 0.5 M NaCl, suggesting that the enzyme was not integrally bound.

Cysteine proteinases

Cysteine proteinases constitute a large proportion of the overall proteolytic activity found in whole cell lysates of axenic *E. histolytica* [13, 167, 231]. The partial purification of an acid proteinase has been reported [138, 230], but little is known of its substrate specificity, sub-cellular location or possible involvement in histolysis. The more widely reported neutral cysteine proteinase secreted by trophozoites [26, 66, 99], is serum inhibitable [26, 119] and has been reported to induce fluid secretion in ligated intestinal loops *in vivo* [66, 119, 260].

It is at present unclear whether the various molecular weights reported for this activity, including values of 16 kDa [118], 26–29 kDa [115, 231] and 56 kDa [99, 203], should be attributed to different molecules with similar properties, multiple forms of a single enzyme [115] or purification artefacts [99].

The enzyme(s) described by Keene *et al.* [99] and Schulte and Scholze [231] degrade(s) fibronectin, laminin and human collagen types I, IV and V, resembling cathepsin B in its ability to cleave the synthetic substrate Z-Arg-Arg-NHMec [118], (although Luaces and Barrett [115] also noted several dissimilarities with this enzyme). Destruction of the cell anchoring proteins fibronectin and laminin is likely to account for the non-lethal rounding-up and detachment of epithelial cells from *in vitro* monolayers

frequently noted by Lushbaugh *et al.* [119–121] and others [24, 25, 135, 136, 190].

Keene *et al.* [99] proposed that the collagenolytic activity corresponded to that reported by Gadasi and Kessler [70], and that the enzyme may act in concert with the membrane-associated metallocollagenase of Munoz *et al.* [150]. The completely purified cysteine proteinase degraded human glomerular basement membrane [115]. Ortiz-Ortiz *et al.* [162], and later Reed *et al.* [202, 204], reported that a cysteine proteinase activates the alternative and classical pathways of complement by cleavage of the C3 component. Other possible functions such as plasminogen activation and elevation of vascular permeability were suggested by Keene *et al.* [99]. The sensitivity of this enzyme to serum, and its functional dependence on a reducing environment, imply that a close physical association between the amoeba and its target tissue *in vivo* is probably required for effective digestion [99].

Secretion of a cysteine proteinase activity by non-pathogenic isolates of *E. histolytica* was described by Reed *et al.* [203], and was found to be significantly lower than for pathogens; similar results were obtained by de Meester *et al.* [49] using an iodinated diazopeptidyl inhibitor of cysteine proteinases to label enzymes from whole lysates of the two forms. Reed *et al.* [203] identified the 56 kDa molecule as being pathogen-specific.

Cysteine proteinase genes

An antiserum raised against the 27 kDa cysteine proteinase purified by Schulte and Scholze [231] was used by Tannich *et al.* [251] to isolate a cDNA clone from an HM-1 expression library. Nucleotide and inferred amino acid sequences of this clone, and a homologous clone derived by hybridization to a non-pathogenic cDNA library, were found to diverge by 16% and 17% respectively. Sequence alignment with a papain gene revealed that all residues known to participate in the presumed active site are present in each form.

Quantitative analysis of mRNA using a 28-mer oligonucleotide synthesized from a region conserved in both sequences, showed a level of expression 10- to 100-fold higher in three pathogenic isolates compared with two non-pathogens. This finding is in broad agreement with the previous comparative estimates of proteinase activity made by Reed *et al.* [203] and de Meester *et al.* [49], and suggests that quantitative rather than qualitative differences may account for the effects observed. Citing the intracellular sorting system of yeast, Tannich *et al.* [251] hypothesized that the over-production of a primarily intracellular proteinase by pathogenic amoebae could activate a secretory pathway, thereby adventiously conferring the ability to degrade extracellular tissue components.

Southern blotting of digested genomic DNA with the pathogenic cDNA clone gave a multiple banding pattern, indicating the possible existence of several gene copies. Sequence analysis of the clone revealed a putative preproenzyme containing a hydrophobic leader sequence and a region equivalent to 80 amino acids which could be cleaved off to produce the active enzyme. The predicted molecular weight of 24.1 kDa for the mature protein was in sufficient agreement with that of the purified enzyme [251].

Using PCR based on consensus sequences for eukaryote cysteine proteinases, Eakin *et al.* [188] obtained a partial gene sequence from axenic amoebae which shared only 43% homology with the corresponding portion of the sequence reported by Tannich *et al.* This gene may encode the acid proteinase described earlier [138, 230], or another neutral cysteine proteinase.

5.5 AMOEBIASIS IN AIDS

5.5.1 Sexually-transmitted amoebiasis

E. histolytica is a common sexually transmitted parasite among western male homosexuals. Surveys conducted in Berlin [270], London [8], New York [182], San Francisco [127] and Seattle [185] have recorded prevalence rates of 16.3%, 20%, 20%, 28.6% and 30%, respectively.

Sexually acquired amoebiasis is generally assumed to be asymptomatic [226, 267]. Most isolated parasites produce non-pathogenic isoenzyme patterns [8, 132, 184], and infected individuals are reported to be seronegative for anti-amoebal antibodies [139, 270]. However, several authors have argued that non-pathogenic *E. histolytica* may cause morbidity in homosexuals, questioning the presumption of Allason-Jones *et al.* [9] that treatment is not required in such cases [107, 166, 172, 241]. McMillan *et al.* [139] found that, while the incidence of diarrhoea in a group of 35 infected homosexuals was not significantly higher than in a non-infected control group, the histology of the rectal mucosa was abnormal in 63% of the former, and enteric symptoms persisted for a longer period.

Furthermore, positive serology, which is indicative of invasive disease, has been reported in up to 20% of Japanese male homosexuals [245, 246], and pathogenic amoebae have been isolated from four symptomatic and seropositive subjects [157]. Evidence of sexually acquired, pathogenic *E. histolytica* in homosexuals has also been recorded in San Francisco [165], Italy [2] and the UK [32].

It would appear, therefore, that sexually transmitted *E. histolytica* is associated largely, though not exclusively, with the non-pathogenic form. While the reasons for this are at present unclear, it is possible that hitherto

unknown differences in transmission patterns between the two types of amoebae may be involved.

5.5.2 *E. histolytica* in AIDS

In view of the incidence of both non-pathogenic and pathogenic *E. histolytica* in male homosexuals, it is perhaps surprising that virtually no cases of invasive amoebiasis have been reported in AIDS patients, despite evidence that immunity to invasive amoebiasis is T-cell mediated [217], and previous observations that the severity of amoebiasis is increased in immunosuppressed individuals [93, 110].

In several recent studies, sexually acquired *E. histolytica* isolates cultured from HIV infected patients were exclusively non-pathogenic by isoenzyme analysis, and in no case was any correlation found with enteric symptoms [9, 31, 205]. The absence of amoebic disease in homosexual AIDS patients has been borne out in other surveys of parasite-related morbidity [109, 237].

While this phenomenon may be related in part to the small numbers of pathogenic strains endemic in the west, studies undertaken in Africa [41, 42, 233], where invasive amoebiasis is endemic, have likewise failed to detect amoebic disease in AIDS patients, with the exception of one report [262]. These observations prompted Lucas [116] to describe amoebiasis as a 'missing disease' in AIDS, and led Curry *et al.* [46] to suggest the existence of a negative association.

5.5.3 Interactions with HIV

Induction of HIV replication

A mitogenic activity for human lymphocytes, present in pathogenic *E. histolytica* extracts, was described by Diamantstein *et al.* [52] and Kettis and Sundquist [103]. The level of this activity was subsequently correlated with virulence *in vitro* and was shown to be associated with the Gal/GalNAc inhibitable lectin [218]. It is not known whether this property is also expressed by non-pathogens. Conclusive evidence that the Gal/GalNAc lectin is responsible for observed T-cell proliferation is not yet available, although similar effects produced by other lectins such as Con A and phytohaemagglutinin [111, 149] indicate a likely involvement.

Based on observations that T-lymphocyte proliferation is an essential precursor of HIV replication [274], and that the onset of AIDS can be correlated with infection by intestinal parasites and other sexually transmitted diseases [268], Croxson *et al.* [45] investigated the possibility

that the reported mitogenic activity of *E. histolytica* could also induce replication of HIV *in vitro*.

Using crude sonicates of the pathogenic strain HK-9, together with appropriate controls, these workers were able to detect only a low grade lymphoblastic response in long-term cultures of peripheral blood mononuclear cells (PBMC) derived from 15 HIV-infected individuals. However, virus replication was demonstrated in five of these cases, indicating the potential of *E. histolytica* as an agent of HIV induction. The relatively low level of mitogenic activity observed in HK-9 sonicates, compared with the earlier finding of Salata and Ravdin [218], could in part be explained by the differences in virulence of the strains used, since HK-9 is known to be less virulent than HM-1 [71, 151]. Given that most, if not all amoebic infection in AIDS is apparently caused by non-pathogenic isolates, it would now be of considerable interest to establish whether this form is also capable of inducing HIV replication.

Detection of HIV in trophozoites

Reports during the 1970s of the occurrence of viruses in *E. histolytica* [19, 55, 83, 134] has led to speculation that amoebae could act as vectors for HIV. To test this hypothesis, Brown *et al.* [29] incubated HIV-infected HUT-78 cells with HM-1 trophozoites, which were found to lack CD4-like receptors. Following phagocytosis of the infected cells, HIV was detected immunocytochemically in amoebae for up to 48 hours, but in 15 separate experiments, co-culture of HIV-harbouring trophozoites with uninfected PBMC at a 1:1 ratio failed to result in transmission. In a further experiment, amoebae isolated from the stools of seven AIDS patients and one with AIDS related complex were tested for HIV p18 and p25 core antigens; 2% of trophozoites from one case were positive for HIV, but, as before, it was not possible to demonstrate transmission to uninfected PBMC. These results suggest that the uptake of HIV by *E. histolytica* is a non-specific event, and that, once internalized, the virus is not transmissible.

5.6 MOLECULAR DIAGNOSIS

5.6.1 Standard procedures

The laboratory diagnosis of amoebiasis depends on the microscopic identification of trophozoites or cysts in stools, and on the detection of anti-amoebal antibodies in blood. A comprehensive account of diagnostic procedures is given by Walsh [265]. The limitations of these techniques are numerous, and to some extent account for the lack of reliable

epidemiological data from several regions of the world. For example, stool examination depends extensively on the detection of cysts rather than trophozoites, which tend to be present only during periods of acute dysentery. The numbers of cysts shed may vary widely, requiring at least three separate samples to achieve 80–90% sensitivity [91], which is both time consuming and often impracticable.

Secondly, lymphocytes with ingested red blood cells may readily be mistaken for haematophagous trophozoites, especially in old or fixed samples where the important criterion of amoebic motility cannot be applied [104]. Thirdly, *E. histolytica* cysts can only be distinguished from those of some other commensal amoebae by the number of nuclei present, requiring the skills of highly trained and experienced staff who may not always be available [214, 236]. Fourthly, since the cysts of pathogenic and non-pathogenic strains are morphologically identical, differentiation between the two forms requires prior culture of amoebae over several days in order to obtain sufficient numbers for isoenzyme analysis [223].

5.6.2 Immunological methods

Immunological detection of *E. histolytica* antigen in stools by ELISA has been widely reported [10, 50, 78, 209], although in every case, the immunological reagents employed are raised against trophozoites rather than cysts, since the latter cannot be produced *in vitro*. Given that trophozoites are absent from the stools of the majority of infected individuals, such assays are of limited use in diagnosis, as shown in the relatively low sensitivities reported. In a recent study, Jain *et al.* [88] found that, while 100% of faecal specimens containing trophozoites were positive by ELISA, only 40% of cyst-passers were correctly diagnosed. A higher detection rate of 80% was reported by Kabil *et al.* [92] for asymptomatic carriers, but this test also produced false positives in 30% of samples containing unrelated parasites.

Diagnosis based on positive serology is hampered by the inability to distinguish between past and present infections. Furthermore, detection of antibodies in cases of amoebic colitis, which comprise the majority of pathological conditions, is only 50–80% sensitive, compared with 85–90% in patients with liver abscess [266].

5.6.3 Molecular diagnosis

The application of molecular biology techniques to the diagnosis of amoebic infection provides an opportunity to improve on the sensitivity, specificity and rapidity of conventional procedures. Two recent reports have exploited the abundance of non-transcribed sequences derived from

the high copy number extrachromosomal episome of *E. histolytica* (section 5.2.7). Samuelson *et al.* [221] independently isolated the 145 base pair tandemly repeated episomal element reported elsewhere by Garfinkel *et al.* [72] as being specific for pathogenic amoebae. When used as a radiolabelled probe under low stringency conditions, however, trophozoites and cysts of both pathogenic and non-pathogenic isolates were detected in unfractionated stool samples spotted onto nylon membranes. A sensitivity of 1.0 and a specificity of 0.93 were recorded in a blind trial with 123 clinical samples, 25 of which were positive for *E. histolytica* as previously judged by microscopy. In separate experiments, hybridization signals were obtained after overnight autoradiography with as few as eight cultured trophozoites per spot; the level of detection in faecal material was estimated to be approximately ten-fold lower.

Bracha *et al.* [27] utilized the same probe, P145, together with B133, the corresponding non-pathogenic element, previously reported to discriminate between the two forms at high stringency [72], to classify 81 clinical isolates obtained from several regions of the world. Specific hybridization signals were obtained with as few as 200 cultured amoebae spotted onto nylon membranes. However, detection of either form in stools was not attempted.

Tannich and Burchard [248] also developed a test to distinguish pathogenic from non-pathogenic trophozoites, based on the gene previously described by Tannich *et al.* [250] and Edman *et al.* [58] as encoding a 125 kDa surface antigen of *E. histolytica* (sections 5.2.8 and 5.3.2). A 482 base pair fragment of the gene containing identical 3′ and 5′ ends was amplified from each form of the parasite by PCR. Specific internal sequence differences were then detected by digestion with selected restriction enzymes, followed by agarose gel electrophoresis. PCR products could be obtained directly from cell lysates of as few as ten cultured trophozoites, although it is unclear whether yields from this number of amoebae were sufficient to perform restriction analysis. The application of this test in direct faecal analysis has yet to be demonstrated.

An ideal molecular diagnostic method for amoebiasis needs to be sufficiently sensitive to detect *E. histolytica* cysts in stools and also specific enough to differentiate between pathogenic and non-pathogenic amoebae. The test described by Samuelson *et al.* meets the first criterion, so eliminating the need for prior culture of isolates. From the results of Bracha *et al.* it is reasonable to expect that the same probe applied at high stringency would identify only pathogenic cysts, thus fulfilling the second criterion, although whether this would be accompanied by a critical loss of sensitivity remains to be determined. Reliance on the preparation of radioactive probes has obvious limitations for use in the field, where non-isotopic labelling methods would be more practicable.

The PCR-based test of Tannich and Burchard, while dispensing with DNA hybridization, nevertheless depends on the availability of equipment to perform PCR and gel electrophoresis, and has yet to be demonstrated with cyst template DNA. However, provided that the technical difficulties associated with performing PCR in the presence of faecal contaminants can be overcome, this approach could prove suitable for use in relatively specialized centres.

5.7 CONCLUSION

Studies on the molecular biology of *E. histolytica*, though only recently initiated, have already yielded some important insights into the genetic organization and pathogenic mechanisms of this parasite. Perhaps the most significant development has been the demonstration of genomic differences between pathogenic and non-pathogenic forms, providing definitive support for Brumpt's original notion of two species. In the light of this evidence, chemotherapy for non-pathogenic *E. histolytica* can be assumed to have little advantage, although further investigation is needed into reports of mucosal damage associated with such infections in homosexual men.

The application of molecular biological methods in amoebiasis research will also improve our understanding of many other aspects of genetic organization, including the *E. histolytica* karyotype, the control of replication and gene expression, ploidy, the differentiation of trophozoites into cysts, and genetic exchange. The development of a transfection system will provide a particularly powerful tool for addressing these and other questions.

The study of pathogenic mechanisms in *E. histolytica* at the cellular level is being increasingly complemented by molecular analysis, with particular reference to parasite–target cell binding, cytolysis and tissue dissolution. This approach should also prove valuable in determining the molecular basis of amoeba–bacterium relations, virulence, interactions with the host immune system and the control and co-ordination of pathogenic processes.

Contrary to some expectations, there is no evidence that amoebiasis is an opportunistic infection in AIDS. However, the reported mitogenicity of parasite antigens for T-lymphocytes, and the potentiation of HIV replication require closer evaluation.

DNA probes have been reported which are capable of detecting *E. histolytica* cysts and trophozoites in stools. This technique offers several advantages over conventional microscopy, particularly with regard to speed and sample throughput. Discrimination between pathogenic and non-pathogenic forms has also been demonstrated for small numbers of cultured trophozoites; the eventual development of a pathogen-specific

DNA test for use on faecal specimens will be of considerable benefit in both epidemiological and clinical applications.

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