STUDIES ON THE ANTIGENS OF *ENTAMOEBA HISTOLYTICA* SCHAUDINN, 1903

By

William David Strachan, B.Sc., B.A.

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in the

Faculty of Science at the University of Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

"If in the stools of the dysenteric, substances of a fleshy appearance are seen, the disease will be mortal".

Hippocrates
(460-377 B.C.)

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LIST OF ABBREVIATIONS

A	-	ampere (amps)
ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
DEAE	-	diethylaminoethyl
dH_2O	-	distilled water
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
EITB	-	electroimmunotransfer blotting
EtOH	-	ethyl alcohol
FACS	-	fluroescence activated cell sorting
FCS	-	foetal calf serum
FITC	-	fluoroscein isothiocyanate
g	-	gravity
GUPM	-	Glasgow University Protozoology Monoclonal
HAT	-	hypoxanthine aminopterin thymidine
HEPES	-	hydroxyethyl ethanesulphonic acid
HGPRT	-	hypoxanthine guanosine phosphoribosyl transferase
HK	-	hexokinase
IFAT	-	indirect fluorescent antibody test
Ig	-	immunoglobulin
kD	-	kilodalton
M	-	moles l^{-1}
Mab	-	monoclonal antibody
MEM	-	minimal essential medium
min	-	minute
NAD	-	nicotinamide adenine dinucleotide
OD	-	optical density
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PEG	-	polyethylene glycol
PGM	-	phosphoglucomutase
PTFE	-	polytetrafluoroethylene

rbc	-	red blood cell
RNA	-	ribonucleic acid
RT	-	room temperature
s	-	seconds
SAPU	-	Scottish Antibody Production Unit, Law Hospital, Lanark
SDS	-	sodium dodecyl sulphate
Tris	-	tris(hydroxymethyl)aminomethane
TBS	-	Tris buffered saline
V	-	volts

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SUMMARY

Twenty one monoclonal antibody-secreting hybridoma cell lines were produced from fusions between mouse myeloma cells and spleen cells from mice immunised with axenically-cultivated Entamoeba histolytica. These monoclonal antibodies (Mabs) were used in the subsequent study of the antigens for which they were specific.

The subcellular locations of these antigens within E. histolytica trophozoites were visualised by use of the Mabs in indirect fluorescent antibody tests (IFAT). Three of the Mabs recognised antigens located on the surface of the amoebae; confirmed by positive results in IFAT experiments using live E. histolytica. These Mabs recognised only a percentage of cells (the percentage was different for each Mab) present in cultures. This heterogeneity of antigen expression within a culture was not a stable property of the cells, since cloned lines were also heterogeneous when tested. The phenomenon, which occurred in all isolates recognised by these Mabs, may be related to the cell cycle or, more likely, be due to subpopulations of cells arising in the cultures. Two of the Mabs were specific for antigens related to the vacuolar system of the amoeba while the remainder recognised other internal components of the amoeba.

The Mabs were tested for their ability to recognise antigens in IFAT tests using other protozoan cell types, including other members of the genus Entamoeba. Two of the Mabs recognised all cell types tested, including a mammalian cultured cell line. Eight Mabs failed to cross-react with any of the cells tested, while a further nine reacted with either Entamoeba invadens (a reptile pathogen) or Entamoeba moshkovskii (free-living in sewage) or both. These experiments demonstrated a high degree of antigenic relatedness between some members of the quadrinucleate cyst group of the genus

Entamoeba; E. histolytica, E. invadens and E. moshkovskii, but not E. hartmanni. The identical pattern of Mab cross-reaction obtained with Entamoeba coli (from man) and Entamoeba muris (from mice) reflected the morphological indistinguishability of these two members of the octonucleate cyst group of Entamoebae.

Fifteen isolates of E. histolytica, other than strain 200:NIH (against which these Mabs were raised), were tested in IFAT experiments using the Mabs. In general, very little antigenic heterogeneity was observed. The failure of the three surface-staining Mabs to recognise some strains may have been due to an undetectably low number of positive cells. Apart from these, only three other Mabs failed to recognise all isolates. In subsequent IFAT experiments involving a larger sample of E. histolytica isolates, two of these Mabs, 22.3 and 22.5, were shown to react specifically with amoebae belonging to pathogenic zymodemes. The nature of the pathogen-specific antigens recognised by these Mabs, is not known.

Approximate molecular weights of some of the antigens recognised by these Mabs, were determined using the Mabs as probes in electro-immunotransfer blotting of amoebic lysates previously subjected to polyacrylamide gel electrophoresis. Values were obtained using thirteen of the Mabs and ranged from 12 kD to over 300 kD with the majority between 95 and 130 kD.

Mabs found not to cross-react with other tested human protozoan parasites (selected on the basis of previous IFAT experiments) were tested for their ability to detect E. histolytica antigen as primary capture reagents in a double sandwich enzyme-linked immunosorbent assay (ELISA). Two Mabs, 24.7 and 29.3, consistently detected antigen (amoebic lysate) down to a concentration of 0.1 μg total protein per ml of sample, indicating their potential usefulness in an immunodiagnostic test for amoebiasis.

CHAPTER 1 INTRODUCTION

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1.1 GENERAL INTRODUCTION

It is estimated, based on published reports, that there are nearly 500 million human infections with Entamoeba histolytica worldwide (Walsh, 1986b). Of these infections, which occur mainly in the developing world, only 8-10% cause clinical disease, the remainder being asymptomatic. With an estimated mortality of between 40,000 and 110,000 (Walsh, 1986a), amoebiasis has been ranked third among parasitic causes of death, behind only malaria and schistosomiasis (Walsh, 1986a).

Amoebiasis can be defined as infection with the protozoan parasite Entamoeba histolytica Schaudinn, 1903, with or without overt clinical symptoms (W.H.O., 1969). Dysentery (of which amoebiasis is one cause) and its potentially infectious nature, was recognised as long ago as the 4th century B.C. and was discussed by Hippocrates (Adams, 1939). Lösch (1875) found the parasite in the stools of a patient (from Arkhangel'sk in N. Russia) suffering from chronic dysentery and although colitis could be reproduced in dogs given the patient's stool orally or rectally, he did not believe that the amoebae were the causative agent. Invasion of tissues by amoebae was described by Koch in 1887 and detailed clinical and pathological descriptions of amoebic dysentery and amoebic liver abscesses were provided four years later by Councilman and Lafleur (1891). Cysts of the parasite were first recognised by Quincke and Roos (1893) and in 1903 the organism was named Entamoeba histolytica by Schaudinn (1903) because of its apparent ability to lyse tissue. The organism was first cultured in vitro by Boeck and Drbohlav (1925) and the perseverance of Diamond (1961, 1968, 1978) resulted in the successful axenic culture of E. histolytica.

1.2 TAXONOMIC CLASSIFICATION

E. histolytica belongs to the subkingdom and phylum Protozoa; the subphylum Sarcodina (motility dependent on pseudopodia); the superclass Rhizopoda (class Lobosea); and the order Amoebida (Levine et al., 1980). Separated from the free-living amoebae, family Valkampfidae - genus Naegleria and family Acanthamoebidae - genus Acanthamoeba, the family Entamoebidae contains the genera Endolimax and Iodamoeba as well as the genus Entamoeba, which includes the species E. histolytica and others further discussed in Chapter 3.

1.3 MORPHOLOGY AND GENERAL BIOLOGY OF E. HISTOLYTICA

E. histolytica can exist in two basic forms; the trophozoite and the cyst. The trophozoites, or feeding stages, show extreme pleomorphism when viewed under the light or electron microscope (when preservation is adequate) and can vary considerably in size. When the diameters of fixed rounded trophozoites are measured, amoebae obtained directly from liver or intestinal lesions are generally large (20-40 μm) while those found in non-dysenteric stools or in culture are smaller (7-30 μm). The diameters of living trophozoites are larger than their fixed counterparts.

Active living cultured trophozoites, and indeed carefully fixed amoebae, are elongated with protruding lobopodia and a trailing uroid. Under the scanning electron microscope most of the cell surface has a wrinkled appearance, with circular endocytotic stomata and filopodia more frequently observed in axenically-grown amoebae (Martinez-Palomo, 1976). The circular stomata are of two types; 0.2-0.4 μm diameter openings corresponding to micropinocytotic vesicles and 2-6 μm diameter openings corresponding to macropinocytotic vesicles. Filopodia are thin filamentous surface projections about 0.1 μm in diameter and several microns in length. They can occur at any site on the cell surface but are more common at the basal surface, particularly around the uroid (Lushbaugh and

Pittman, 1979), an area at the posterior of actively progressing trophozoites consisting of irregular folds of membrane. Foreign particles, cells and bacteria have been observed attached to this region but it is not definitely known whether the uroid participates in endocytotic or exocytotic processes (Lushbaugh and Pittman, 1979).

The plasma membrane of E. histolytica trophozoites is approximately 10 nm thick and has a classic trilaminar unit membrane appearance in thin section transmission electron microscopy (Martinez-Palomo et al., 1976). This membrane is covered by a surface coat, composed of short filaments, 6-10 nm in length, detectable by either ruthenium red or alcian blue staining used together with osmium tetroxide in electron microscopy (Pinto da Silva et al., 1975). The actual thickness of the coat seems to be dependent on the origin of the amoebae, appearing thicker in amoebae isolated from intestinal lesions and grown monoxenically than in amoebae grown under axenic conditions (Lushbaugh and Miller, 1974). However, the loosely-bound nature of the coat components and the adsorption of serum and other components to the surface make such differences difficult to interpret (Martinez-Palomo et al 1980). The plasma membrane and surface coat will be mentioned in more detail in Chapter 2.

The bulk of the cytoplasm, when viewed in thin sections, appears to consist of vacuoles of variable size, ranging from 0.5-9.0 μm in diameter within a cytoplasmic matrix (Martinez-Palomo, 1982). A system of tubules and vesicles, approximately 20 nm in diameter, superficially resembling the smooth endoplasmic reticulum of mammalian cells can also be found in the cytoplasm. Although it is likely that the vacuoles represent components of the phagolysosomal system (since some contain ingested food particles) and the vesicles, components of the intracellular synthetic apparatus, the exact functional significances of these organelles have not been elucidated (Martinez-Palomo, 1982).

Other cytoplasmic inclusions encountered in electron microscopic studies include ribosomes, both free and in ordered helical arrays.

The significance of such arrays remains unclear. In cysts and resting cultured trophozoites such helices may aggregate into large crystalline inclusions (constituting the "chromatoid body" visible under the light microscope) which may represent ribosomal precursors (Barker and Swales, 1975) or may be related to storage of ribosomes during periods of reduced metabolic activity (Kusamrarn, 1975).

Dense cylindrical bodies, often arranged in rosettes, have frequently been described in the cytoplasm of both cultured trophozoites and amoebae obtained directly from lesions (Feria-Velasco and Treviño, 1972; Bird and McCaul, 1976). These bodies are up to 250 nm in length and 90 nm in diameter, are surrounded by a thin incomplete membrane and their morphological characteristics have led to the suggestion that they may be rhabdoviruses (Bird and McCaul, 1976). Various filamentous and polyhedral DNAviruses have been described in cultured amoebae (Mattern et al., 1972), although such particles are not observed in healthy axenic cultures (Diamond and Mattern, 1976). No evidence of a relationship between virus presence and amoebic virulence has so far been obtained (Mattern et al., 1979; Hickman (pers. comm.), 1987).

In contrast to free-living amoebae, whose cytoskeleton has been extensively studied, very little is known about the structural organisation of the cytoskeleton of E. histolytica. Short microfilaments, 7 nm in diameter, have been identified with the electron microscope, located immediately below the plasma membrane (Martinez-Palomo, 1974). In phagocytic channels, a fibrogranular material can be observed but few definite fibrillar structures can be identified even when specimens are fixed under conditions known to minimise depolarisation of actin filaments during fixation (Martinez-Palomo et al., 1976). Immunofluorescence studies using anti-actin antibodies have failed to show structured microfilaments in Entamoeba (Aust-Kettis et al., 1977) which can be identified in similar experiments using vertebrate fibroblasts. Microtubules have only been identified in the nuclei of dividing trophozoites (Martinez-Palomo, 1982). Several authors have found occasional

microtubule-like structures in the cytoplasm of E. histolytica (Rosenbaum and Wittner, 1970; Michel and Schupp, 1975) but their diameter (36 nm), general ultrastructure and rare occurence suggests the possibility that they may be filamentous viruses.

The nuclear morphology of iron haematoxylin-stained amoebae has been used in the identification of E. histolytica in clinical laboratories for many years (Dobell, 1919). Although a number of these morphological characteristics are known to be artefacts, the majority have been corroborated by electron microscopic studies. The nucleus of the trophozoite is 4-7 μm in diameter with uniformly sized chromatin clumps usually evenly distributed on the inside of the nuclear membrane. The endosome is a 0.5 μm diameter, spherical mass located in the central part of the nucleus and generally appears less dense than the peripheral material. Albach et al. (1980), using radiolabelled nucleotides, showed that the "peripheral chromatin" is rich in RNA, and may be the counterpart of the nucleolus in other eukaryotes, while the endosome is a site of DNA condensation. Intranuclear bodies in Entamoeba are a common occurrence (Zaman, 1973) though their function is not known.

The mechanism of nuclear division is not understood but it is known that it occurs without dissolution of the nuclear membrane (Cervantes and Martinez-Palomo, 1980) and that intranuclear microtubular spindles occur in dividing cells (Martinez-Palomo, 1982).

All published electron microscopic studies confirm that E. histolytica possesses no mitochrodria, rough endoplasmic reticulum and no centrioles. The existence of a golgi apparatus is disputed. While two groups of workers describe such a structure (Eaton et al., 1970; Proctor and Gregory, 1972) most workers have found no evidence for golgi membranes (reviewed by Martinez-Palomo, 1982).

The exact life cycle of E. histolytica in the human is not known and the most detailed knowledge has come from the work of Dobell (1928) on cultures of a strain obtained from a monkey. He describes four

consecutive stages: trophozoite, precyst, cyst and metacyst. It is thought that under adverse conditions in the lower colon, trophozoites develop into precystic and cystic stages, which are followed by two nuclear divisions producing two to four nuclei within the cyst. Asymptomatic carriers, convalescent patients and dysenteric patients between periods of active disease, are capable of shedding up to 15 x 10^6 cysts per day in their faeces (Feachem, et al., 1983). Mature quadrinucleate cysts may survive outside the host for several weeks in a moist environment and are responsible for faecal-oral transmission of the infection via food, water, fomites or direct person-person contact (Feachem, 1984).

After ingestion, the quadrinucleate cysts reach the large intestine where excystation occurs and a quadrinucleate metacystic amoeba is thought to emerge from the cyst and undergo a further nuclear division to produce eight uninucleate trophozoites (Dobell, 1928).

The investigation of the biochemical requirements for encystation have been hampered by the fact that E. histolytica in culture only encysts in xenic media. A reliable axenic medium for the encystation of Entamoeba invadens (a species pathogenic in reptiles) has been developed (Rengpien and Bailey, 1975) and it appears that the depletion of carbohydrate sources and a decrease in osmolarity of the medium are essential triggers (Balamuth, 1962; Rengpien and Bailey, 1975).

Cysts of E. histolytica are round or slightly oval in shape and are 8-20 μm in diameter. The cyst wall is 125-150 μm thick and, when viewed under the electron microscope, appears to be composed of 2-3 nm diameter fibrils in a tight mesh (Chavez et al., 1976). It is known to contain chitin (Arroyo-Begovich et al., 1978). One to four nuclei and chromatoid bodies can be seen within a cytoplasm similar in structural organisation to that described for trophozoites.

Since the large intestine provides an essentially anaerobic environment for the trophozoite and since a low redox potential is required for *in vitro* growth, *E. histolytica* has previously been considered to be an obligate anaerobe. However amoebae are able to consume oxygen and will grow in an atmosphere containing up to 5% oxygen (Weinbach and Diamond, 1974) and could be better described as "micro-aerophilic". The biological significance of this oxygen utilisation is not known; since the organism possesses no mitochondria or tricarboxylic acid cycle. Electrons are thought to be transferred from reduced substrates to oxygen via a series of carriers including flavins, iron-sulphur proteins and ubiquinone (Weinbach, 1981).

Carbohydrates provide the main source of energy for *E. histolytica*; glucose or glucose-containing polymers being essential (Charoenlarp *et al.*, 1968). Glucose uptake occurs via a specific transport mechanism, which is the rate limiting step in the utilisation of glucose, while pinocytosis plays a relatively insignificant rôle (Serrano and Reeves, 1975). Glucose is metabolised to pyruvate via the Embden-Meyerhoff pathway with ethanol and CO_2 being the principal end products (Lo and Reeves, 1978). The glycolytic process differs most noticably from other eukaryotes in the use of inorganic pyrophosphate as a high energy substrate (ATP, in most other eukaryotes) in several reactions (Reeves, 1968; Reeves *et al.*, 1974). This peculiarity, shared by a number of other organisms, mostly bacteria (Klemme, 1976), and the absence of glutathione metabolism (Fahey *et al.*, 1984) demonstrate the primitive nature of this organism which in terms of metabolism resembles anaerobic and microaerophilic bacteria more closely than other eukaryotes.

1.4 HOST SPECIFICITY

Although man is the major host and reservoir of infection of *E. histolytica*, a few other mammalian species have been reported to be naturally or experimentally infected with this organism.

Asymptomatic natural infections have been recorded in Macaque monkeys and pigs, and those in dogs and rats may be symptomatic (Hoare, 1962). Among non-human primates, only Macaque monkeys have been shown to spontaneously harbour E. histolytica in nature (Dobell, 1931) but zoo infections have occurred in a number of other species (Fremming et al., 1955; Frank, 1982). Although the normal intestinal site of infection in most hosts is the colon, in leaf-eating monkeys, gastric amoebic ulcers occur due to the neutral pH of the anterior regions of the stomach (Frank, 1982; Loomis et al., 1983).

Both infection with colonic or hepatic involvement and asymptomatic cyst excretion have been described in dogs (Ganapathy and Alwar, 1957). Natural infections have been reported in North Africa, India, South East Asia, China and the United States (Hoare, 1962). Although the potential exists for transmission from dogs to humans, it is not widely recognised. The source of these canine infections is not known, but it is likely to be human faeces. It has also been postulated that natural infections in rats play a rôle in the transmission of human amoebiasis (Neal, 1951).

Experimental infections have been reported in a number of hosts, however rodents, particularly young rats (Neal and Harris, 1977), guinea-pigs (Rees et al., 1954) and hamsters (Jarumilinta and Maegraith, 1961) have been the most widely used models. Attempts to develop a model naturally infected via the oral route have been hampered by the inability to produce large quantities of amoebic cysts in culture. Some progress has been made however, using trophozoites in conjunction with cimetidine, a gastric acid secretion inhibitor (Owen, 1984).

1.5 VIRULENCE OF E. HISTOLYTICA

Despite the worldwide distribution of amoebic infections, invasive disease seems to be more frequent in certain geographical areas (Elsdon-Dew, 1971), although epidemic outbreaks may occur anywhere

(Deschiens, 1965). This observation and the finding that in 90% of individuals (on global average), E. histolytica exists as a commensal, (Walsh, 1986) has led to years of discussion concerning the underlying biology.

Dobell in 1919 stated that "E. histolytica is always a destroyer of tissue, but by no means always a producer of disease", and regarded all apparently healthy carriers to suffer some form of intestinal ulceration. He later suggested that the amoeba could, in fact, be a commensal (Dobell, 1931) although some later authors still favoured this hypothesis (Faust et al., 1970). However asymptomatic carriers frequently show negative serology and show no evidence of intestinal invasion as demonstrated by rectosigmoidoscopic examination (Meza et al., 1986).

Brumpt (1925) suggested that two species of morphologically similar Entamoebae infected man. One species (E. dysenteriae) was restricted to warm climates and was responsible for invasive amoebiasis, while another (E. dispar) had a cosmopolitan distribution and caused asymptomatic luminal amoebiasis (Brumpt, 1949). This hypothesis has received renewed interest in the light of Sargeaunt's recent studies (see below).

A third, widely supported hypothesis, proposes that the species E. histolytica is composed of an unknown number of different strains with varying degrees of virulence (Neal, 1966; Elsdon-Dew, 1968; W.H.O., 1969). Crowding, poor hygiene and thus repeated faecal-oral transmission is believed to increase the virulence of E. histolytica in areas with a high incidence of invasive disease (Swellengrebel, 1961).

Virulent strains are generally defined as those isolated from patients with active amoebiasis while avirulent strains are those isolated from proven asymptomatic carriers (Sepulveda, 1982). Although no morphological differences could be determined in light

and electron microscopic studies (Kazuko-Kawashima et al., 1973; Trevino-Garcia Manzo et al., 1973) several biological differences have been found and since used as virulence markers:

1) Trophozoites of pathogenic strains have a higher susceptibility to agglutinate in the presence of concanavalin A than trophozoites of carrier strains (Martinez-Palomo et al., 1973; Trissl et al., 1977).

2) Pathogenic trophozoites also show a greater erythrophagocytic capability (both in terms of rate and maximum number of red cells ingested) than those of carrier strains (Trissl et al., 1978).

3) Trophozoites from asymptomatic carriers have been shown to be non-pathogenic when inoculated into the livers of adult and new-born hamsters while inoculation with pathogenic strains produces amoebic abscesses (Tanimoto-Weki et al., 1974).

4) Strains from invasive cases of amoebiasis and strains from carriers can be differentiated on the basis of the electrophoretic mobilities of certain enzymes (Sargeaunt et al., 1978; Sargeaunt and Williams, 1979). (This will be further discussed below). It should be noted that variations in virulence and virulence-related properties can occur between trophozoites within one cultured isolate of E. histolytica due to the genetic heterogeneity of subpopulations (Orozco et al., 1986).

Sargeaunt et al (1980a; 1982; 1984) have carried out an extensive survey of isolates of E. histolytica from several continents using starch gel electrophoresis of amoebic enzymes. More than twenty different mobility patterns have been identified and isolates displaying identical patterns, grouped into zymodemes. Amoebae cultured from well characterised cases (showing clinical signs and positive serology) of invasive amoebiasis can be grouped into 8 different zymodemes. These show a β band with absence of an α band in the enzyme phosphoglucomutase, and "fast-running" bands in hexokinase, except for zymodeme XIII which has slow hexokinase bands.

Isolates from probable carriers, showing no clinical signs and negative serology, produce other patterns without the features described above. Amoebae belonging to pathogenic zymodemes have been isolated from asymptomatic, cyst-passing individuals showing both positive and negative serology (Meza et al., 1986; Sargeaunt, 1987). However no amoebae belonging to "non-pathogenic zymodemes" have ever been isolated from cases of invasive amoebiasis (Sargeaunt, 1987).

Axenically-grown trophozoites of isolates, originally from sympomatic cases and belonging to the same pathogenic zymodeme, have been shown to differ considerably in their virulence, assayed by various methods. For example strain HK-9 in comparison to strain HM1:IMSS has been found to possess a reduced capibility to (a) induce hepatic abscesses in hamsters (Diamond et al., 1974; Mattern et al., 1982) (b) destroy tissue culture monolayers (Mirelman et al., 1983) (c) demonstrate erythrophagocytosis (Mirelman et al., 1983) and (d) produce soluble toxic substances (Bos, 1979; Kobiler et al., 1981). It has also been found that the virulence of axenically-grown strains of E. histolytica can be increased by a variety of procedures: (1) extensive subculturing and repeated hamster liver passage (Bos, 1979; Mattern et al., 1982) (2) the addition of cholesterol to the culture medium (Bos and van de Griend, 1977) (3) association with living bacteria (Wittner and Rosenbaum, 1970).

For many years it was thought that cultured E. histolytica trophozoites were unable to produce a pathogenic effect unless grown in association with bacteria, in spite of the frequent observation that liver abscesses produced in man usually contained no other microorganisms. This belief was further supported by the work of Phillips et al. (1972) who reported the failure of axenically-grown strains to produce intestinal lesions in germ-free guinea pigs. Virulence was regained after co-culture with bacteria. However other work showed that axenic amoebae were capable of producing both intestinal and hepatic lesions in hamsters (Ghadirian and Meerovitch, 1979; Tanimoto et al., 1971).

The rôle of bacteria in the augmentation of amoebic virulence is unclear but it is known that living bacteria are necessary (Wittner and Rosenbaum, 1970) and that even short term associations (one hour) of axenically-grown amoebae with certain types of bacteria markedly enhance their virulence (Bracha and Mirelman, 1984). It has been suggested that ingested bacteria may protect the amoebae from the toxic effects of hydrogen peroxide and also help to lower the redox potential within the cell (Bracha and Mirelman, 1984). Mirelman et al. (1984) showed that although association with bacteria was capable of increasing the virulence of a particular E. histolytica strain, as determined by tissue culture cell monolayer destruction and hepatic abscess formation in hamsters, the isoenzyme electrophoretic pattern of the amoebae remained unchanged. Experiments involving the long-term association of amoebae belonging to non-pathogenic zymodemes with bacteria isolated with pathogenic amoebae also produced no change in isoenzyme electrophoretic pattern (Sargeaunt, 1987). Based on these studies and others by Sargeaunt and colleagues (Sargeaunt and Williams, 1978; Sargeaunt et al., 1980b) it has been proposed that zymodemic analysis would suffice as the basis for deciding whether drug treatment is necessary in infected individuals (Nanda et al., 1984; Editorial, 1985; Allason-Jones et al., 1986; Goldmeier et al., 1986).

This philosophy has recently been challenged by the work of Mirelman et al. (1986a, 1986b). For many years, the study of virulence of E. histolytica has been hampered by the fact that no amoebae belonging to non-pathogenic zymodemes can be grown in the absence of bacterial flora. The first successful axenization of such an isolate was achieved (Mirelman et al., 1986b) using antibiotic suppression of bacterial growth and X ray-irradiated bacteria as a temporary supplement for growth in Diamond's axenic medium (Diamond, 1978). A gradual increase in virulence, assayed by in vitro mammalian cell monolayer destruction and liver abscess formation in hamsters, was observed after transfer to this medium without viable bacteria. Such changes were accompanied by an alteration in isoenzyme electrophoretic pattern from one characteristic of a non-pathogenic zymodeme (Z I) to one characteristic of a pathogenic zymodeme (Z II).

In order to exclude the possibilty that such a result was due to culture conditions selecting for the growth of a subpopulation within the original culture, the same experiment was performed using a cloned culture of a non-pathogenic strain (Mirelman et al., 1986b). The same concomitant change in zymodeme and increase in virulence were observed. A reverse conversion to a non-pathogenic zymodeme was also accomplished by subculturing the newly converted pathogenic clone reassociated with the original bacterial flora. These results and similar ones in other laboratories (B. Andrews, pers. comm.) suggest that isoenzyme electrophoretic patterns of E. histolytica may not be a constant and stable property of the organism, as has been previously found in other protozoa such as Paramecium (Rowe et al., 1970) and Tetrahymena (Allen, 1968). Whether such changes in E. histolytica, achieved in these experiments by quite considerable artificial manipulation, can actually occur in vivo is not known. The subject is currently an area of debate but most epidemiological features, including Sargeaunt's extensive studies, seem to point to the relative stability of zymodeme and virulence.

Recent work by Burchard and Mirelman (1988) has further suggested that virulence does not necessarily correlate with a pathogenic zymodeme. These authors found that two out of four strains of amoebae from asymptomatic carriers and belonging to non-pathogenic zymodemes were as virulent as known pathogenic strains. Virulence was assayed both in vitro, by measuring rates of destruction of monolayers of baby hamster kidney cells, and in vivo, by assessing their capacity to form hepatic abscesses in hamsters, and caecal ulcerations in rats. Such results demonstrate the difficulty of using experimental assays for virulence to obtain clear information on the pathogenicity of E. histolytica in man. An animal model that more closely approximates the natural conditions of growth and infectivity in the human intestine would help to dispel much of the uncertainty of current studies.

A number of other amoebic properties are likely to be important virulence factors since they aid in the survival of trophozoites in the host by evading or surviving the host's immune response. Reed et al. (1983) found that isolates belonging to non-pathogenic zymodemes from asymptomatic carriers were all susceptible to complement-mediated lysis (alternative pathway). Nine of eleven pathogenic strains were resistant to lysis under identical conditions while two were susceptible. Although correlation with virulence was not complete it is interesting to note that all five of the strains isolated from liver abscesses were resistant to lysis indicating that such a property may indeed by a necessary factor in extraintestinal spread of the disease. Other factors suggested, include resistance to destruction by neutrophils (Guerrant et al., 1981) and the removal of bound host antibodies by capping (Aust-Kettis and Sundqvist, 1978; Calderon et al., 1980). Attempts to correlate these two factors with zymodeme have not been made.

Clearly a variety of variable host factors may possibly affect the outcome of the host-parasite relationship and thus the apparent virulence of the amoeba. Proposed factors include sex (Alvarez-Alva and de la Loza-Saldivar, 1971), age (Fuchs et al., 1988), nutritional status (Gutierrez et al., 1970) and immunosuppression (Ghadirian and Meerovitch, 1981). A definite genetic component of host susceptibility has been identified in mice (Ghadirian and Kongshavn, 1984), while in man, it has been noticed that expression of certain HLA antigens was significantly higher in amoebiasis patients than in a control population (Kretschmer et al., 1985). In summary, it seems likely that all isolates of E. histolytica possess some pathogenic capability, the expression of which depends on a complex interaction between host, parasite and bacterial flora.

1.6 PATHOGENESIS

The pathogenesis of invasive amoebiasis requires four basic events:
(1) Adherence of E. histolytica trophozoites to the colonic mucus layer; multiplication at this site resulting in intestinal colonisation.
(2) Disruption of the mucus layer and intestinal barrier by the secretion of enzymes or toxins.
(3) Lysis of intestinal epithelial cells and other cell types, resulting in colonic ulceration and possibly distant organ invasion (eg. liver).
(4) Resistance to host immune responses.
In asymptomatic carriers of E. histolytica where the amoebae exist as commensals within the lumen of the colon, trophozoites adhere to the mucus layer and multiply, but further pathogenic processes do not occur.

Lectin-like molecules have been identified in E. histolytica and are known to be necessary for (a) phagocytosis of bacteria in the luminal phase of the parasite (Bracha et al., 1982), (b) adhesion to the colonic mucus layer and the surface of intestinal epithelial cells (Ravdin et al., 1985a) and (c) adhesion prior to contact-dependent killing of target cells (Ravdin et al., 1980). Galactose-inhibitable adherence of E. histolytica to bacteria (Bracha and Mirelman, 1983) and a variety of mammalian cells including erythrocytes, mononuclear cells and cultured epithelial lines (Ravdin et al., 1980; Salata et al., 1982) has been demonstrated. The activity of this recently purified 260 kD lectin (Petri et al., 1987b) is also inhibited by N-acetyl-D-galactosamine (GalNAc) (Ravdin et al., 1980) and more effectively by galactose (Gal) terminal glycoproteins such as asialofetuin (Petri et al., 1987b). Chadee et al. (1987) found that human colonic mucins, rich in such sugars, are

high-affinity ligands for the E. histolytica Gal/GalNAc adherence lectin. Although colonic mucins probably facilitate intestinal colonisation by E. histolytica, it is thought that they may be an important defence mechanism, preventing the amoebae from attaching to and lysing intestinal epithelial cells (Ravdin, 1989). Infected individuals are known to mount an antibody response against this lectin (Petri et al 1987a) and both monoclonal antibodies and polyclonal antisera raised against it inhibit adherence of the amoebae to cultured mammalian cells (Petri et al., 1987b; 1989).

Other amoebic adherence proteins are known to exist. A 220 kD lectin whose activity is inhibitable by N-Acetyl-D-glucosamine (GluNAc) (Kobiler and Mirelman, 1980) has recently been isolated (Rosales-Encina et al., 1987). However unlike the situation with the 260 kD lectin described above, monoclonal and polyclonal antibodies, raised against this protein, and the specific monosaccharide (free GluNAc) only partially inhibited amoebic adherence to tissue culture cells (Leitch et al., 1988; Ravdin and Guerrant, 1981). Arroyo and Orozco (1987) have produced monoclonal antibodies which inhibit erythrophagocytosis by E. histolytica and recognise a 112 kD protein in electroimmunotransfer blotting experiments.

Experiments with animal models of colonic amoebiasis suggest that, prior to mucosal invasion by E. histolytica trophozoites, there is a depletion of intestinal mucus, diffuse inflammation and disruption of the epithelial barrier (Chadee and Meerovitch, 1985). Although this depletion of mucus may provide the potentially invasive amoeba with more direct access to the epithelium, this may not be sufficient. E. histolytica lysates do contain enzymes capable of degrading protein in hog gastric mucin-impregnated agarose gels (Spice, W., pers. comm.) but whether or not they possess a specific mucinase complex similar to that found in Vibrio cholerae (Stewart-Tull et al., 1986) is not known.

A variety of enzymes and toxins have been implicated as possible mediators of the tissue-invasive activity of E. histolytica. No one component has been shown to be necessary and sufficient for the virulent capacity of the amoeba but it is likely that if one of these factors is impaired, virulence may be affected. These factors are also likely to be important, after the initial invasion, in the formation of both intestinal and extraintestinal pathological lesions.

A neutral protease described by Keene et al. (1986) was shown to degrade fibronectin, laminin and type I collagen suggesting a possible rôle for this enzyme in disrupting cell-cell adhesion and extracellular matrix integrity during initial invasion. A β-N-acetylglucosaminidase purified by Lundblad et al. (1981) is secreted by E. histolytica trophozoites and may also have a rôle in degrading glycoprotein bonds between adjacent epithelial cells. Other enzymic activities of the amoeba which may help to effect extracellular tissue matrix breakdown include hyaluronidase (Jarumilinta and Maegraith, 1969) and collagenase activity (Muñoz et al., 1982), the latter requiring direct contact between the amoeba and substrate. A serum-sensitive protease, initially postulated as a cytotoxin by Lushbaugh et al. (1979) is not cytolethal, but has a reversible cell-releasing effect on tissue culture cells (Lushbaugh et al., 1984; 1985). The protease described by Keene et al. (1986) has a similar effect in such in vitro studies.

Amoebic secretion of an enterotoxin or cytotoxin has been postulated (Pittman et al., 1973), although animal studies and human pathology show that damage to the mucosa only occurs in areas in direct contact with amoebae (Prathap and Gilman, 1970; Proctor and Gregory, 1972). Using indomethacin to impair prostaglandin-mediated mucosal protection, Udezulu et al. (1981) demonstrated enterotoxic activity in soluble fractions of sonicated amoebae. This heat-labile enterotoxin activity caused no other noticeable pathological changes

and interestingly was not detectable in the non-pathogenic "E. histolytica-like" Laredo strain. A 30 kD amoebic protein with heat-labile enterotoxin activity in indomethacin pretreated rats, has been partially purified by Feingold et al. (1985). Whether living amoebae secrete such enterotoxic substances during invasion is not known.

Lynch et al. (1982) and Young et al. (1982) have reported the existence of an ion channel-forming protein ("amoebapore") that is present within E. histolytica, and can be secreted into the extracellular medium. The protein can spontaneously incorporate into mammalian cell membranes causing depolarization and in experiments using lipid vesicle membranes and artificial bilayers, it appears to induce rapid cation permeability. The release of amoebapore from cultured trophozoites can be stimulated by exposure to calcium ionophore, concanavalin A or Escherichia coli lipopolysaccharide. It is not known if amoebapore actually participates in the cytolytic activity of E. histolytica but it is interesting to note that in E. invadens, a species highly pathogenic in reptiles (with similar lesion pathology to human amoebiasis), the pore forming activity was much lower than that of E. histolytica, similar to that of the non-pathogens, "E. histolytica-like" Laredo strain and Entamoeba moshkovskii (Keller et al., 1988).

Ravdin et al. (1980) demonstrated that E. histolytica exerts a rapid contact-dependent cytolytic effect on target cells in vitro which is followed by phagocytosis of the dead target cell. It is believed that this activity is essential to penetration of the mucosa and the formation of tissue lesions characteristic of amoebiasis (Martinez-Palomo, 1982). Most of the work investigating this phenomenon has been performed using established tissue culture cell lines of epithelial origin (Ravdin and Guerrant, 1981; Martinez-Palomo et al., 1985) although fibroblasts (Ravdin and Guerrant, 1981) and leucocytes (Guerrant et al., 1981) have been used. The contact-dependent cytolytic mechanism requires lectin-mediated adherence between the amoeba and the target cell, is

temperature-dependent, 37 °C being optimal, and requires amoebic microfilament function (Ravdin and Guerrant, 1981).

The exact cytotoxic factor or factors involved are not known but a number of possible candidates exist. Morphological observations initially suggested that a "surface-active lysosome" was released on contact with host cells, liberating lytic enzymes (Eaton et al., 1970). However the fusion of such organelles with the plasma membrane during interaction with target cells has not been confirmed in other studies (Martinez-Palomo, 1982; Martinez-Palomo et al., 1985).

Phospholipase A (PLA) enzymes may be involved, since pharmacological inhibitors of these enzymes inhibit parasite cytolysis of Chinese hamster ovary cells (Ravdin et al., 1985b). E. histolytica is known to contain two PLA enzymes: a Ca^{2+}-dependent enzyme with optimal activity at pH 7.5, and a Ca^{2+}-independent enzyme optimal at pH 4.5 (Long-Krug, 1985). The Ca^{2+}-dependent enzyme is highly associated with the surface plasma membrane and its in vitro enzymic activity is inhibited by known pharmacological inhibitors of PLA enzymes. This enzyme and the free-fatty acids released from membrane phospholipids via its action, and been proposed as major factors in the cytopathogenic activity of E. histolytica (Said-Fernandez and Lopez-Revilla, 1988).

Free intracellular Ca^{2+} concentrations in target cells show a marked irreversible increase within 30-300 s after contact with an amoeba (Ravdin et al., 1988) followed by death of the cell. Interestingly, immunoaffinity-purified Gal/GalNAc adherence lectin produced a similar rise in target cell intracellular Ca^{2+} concentration. Although plant lectins are capable of causing transient increases (Hesketh et al., 1983; 1985) the magnitude and irreversibility of the increase in this case has led Ravdin et al. (1988) to suggest that the lectin involved has a cytotoxic activity. The 13 kD ionophore described by Lynch et al. (1982) and Young et al. (1982) is a

possible mediator of this calcium flux and the possibility that the lectin contains amoebapore as a subunit cannot be ruled out (Ravdin et al., 1988).

Amoebic phagocytosis of target cells which can occur in the absence of lectin-mediated adhesion and cytolysis (Ravdin and Guerrant, 1981) is also important in the pathogenesis of amoebiasis. The level of phagocytic activity appears to correlate with in vivo virulence (Trissl et al., 1978) and it is thought that the mechanical activities of the amoeba are important in the separation and displacement of neighbouring cells during mucosal invasion and subsequent spread (Martinez-Palomo, 1987).

1.7 DISEASE SYNDROMES

The outcome of infection with E. histolytica can range from asymptomatic infection to fatal disease depending on the balance of the host-parasite relationship, due to factors discussed above (Section 1.5).

1.7.1 Asymptomatic infection

The majority of human infections with E. histolytica (~90%) are completely asymptomatic (Walsh, 1986b). Trophozoites live as commensals on the wall, and in the lumen, of the colon and up to 15 million cysts per day can be shed in the faeces (Feachem et al., 1983). It is these patients that represent the main source of human infection. The ratio of asymptomatic to symptomatic cases varies between different populations. In endemic areas, such as Mexico, it has been estimated that there are four or five asymptomatic carriers to every one case of invasive amoebiasis (Sepulveda, 1982). In more temperate zones, where the overall prevalence is low, the proportion of carriers is much higher. The higher concentration of invasive amoebiasis in certain geographical areas, in particular South East Asia, the Indian subcontinent, South East Africa and Central America, has been attributed to the distribution of certain pathogenic strains of E. histolytica (Sargeaunt et al., 1984). High population density, poor hygiene and sanitation, and consequently high transmission rates in such areas, have also been implicated (Swellengrebel, 1961). Most asymptomatic carriers are believed to eliminate the parasite from the gut within twelve months (Nanda et al., 1984).

1.7.2 Symptomatic intestinal infection

Intestinal amoebiasis occurs when virulent trophozoites breach the mucosal barrier and cause lesions in the intestinal wall. The form of the disease depends on the site and severity of the lesions. The most frequent sites of primary lesions are the caecal and rectosigmoidal regions, where intestinal flow is slowest. Less frequent sites include the ascending colon, the rectum and appendix

(Brown and Neva, 1983). Lesions can occur in other sites in the colon as the infection progresses.

The mildest form is characterised by moderate malaise, abdominal discomfort with irregular colicky pain and weakness. Constipation may alternate with mild diarrhoea. Acute intestinal amoebiasis has been observed one to four weeks after the ingestion of cysts (Bundesen et al., 1936) but incubation periods of up to one year have been recorded (Craig, 1917). The onset may be insidious and preceded by the symptoms described above, or overt, with severe dysentery. Numerous small stools are passed containing blood, mucus and shreds of necrotic mucosa and these symptoms are usually accompanied by acute abdominal pain and tenderness and sometimes fever (Adams and MacLeod, 1977a). Trophozoites of E. histolytica, rather than cysts are found in the stools.

Initially the ulceration is superficial with minimal necrosis and cell infiltration at the site of invasion. The ulcerations may extend laterally and downwards to form the classic "flask-shaped" ulcer which extends through the mucosa and muscularis mucosae into the submucosa. Such lesions appear to occur in only a minority of cases (Prathap and Gilman, 1970). An amorphous, granular material surrounds trophozoites in tissue lesions (whether intestinal or extraintestinal) and consistent with the fact that E. histolytica can exert a cytolytic effect on leucocytes (Guerrant et al., 1981), intact inflammatory cells are found only at the periphery of lesions (Prathap and Gilman, 1970; Griffin and Juniper, 1971). Evidence in animal models suggests that lysis of inflammatory cells and the consequent release of their contents may be involved in the production of focal necrosis of the mucosa (Chadee and Meerovitch, 1985). Complications of acute intestinal amoebiasis include amoebic appendicitis, fulminating colitis, intestinal perforation and haemorrhage. Secondary bacterial infections of lesions are common.

Chronic intestinal amoebiasis is characterised by recurrent attacks of dysentery with intervening periods of mild to moderate gastrointestinal disturbances with constipation. Liver enlargement and localized abdominal tenderness may be present (Haider and Rasul, 1975).

Chronic ulceration of the intestinal mucosa can cause the formation of a non-fibrotic proliferation, termed amoeboma (Brandt and Perez-Tamayo, 1970; Prathap and Gilman, 1970). These psuedotumoural lesions occur predominantly in the caecum, sigmoid colon and rectum and are often mistaken for malignant growths (Brown and Neva, 1983). These lesions are infrequent, occuring in an estimated 0.5-1.5% of all patients presenting with amoebic colitis, and respond well to anti-amoebic therapy (Ravdin, 1988).

1.7.3 Extraintestinal infection

Extraintestinal amoebiasis may develop within days of the onset of symptomatic intestinal amoebiasis, or follow up to several years afterwards. Indeed in up to 50% of cases it may occur in the absence of any history of intestinal infection (Adams and MacLeod, 1977b).

The liver is the commonest site of spread of intestinal amoebiasis and dissemination from the primary focus appears to occur most frequently via the hepatic portal system (Carrera, 1950; Aikat et al., 1979). Hepatic amoebiasis is characterised by an enlarged, tender liver with pain ranging from vague right upper-quadrant discomfort to localized tenderness between the ribs, on palpation. Elevation and immobility of the right side of the diaphragm and severe pain referred to the right shoulder frequently occurs. Fever is common and may be either low grade or acute. A leucocytosis with increased neutrophils helps to distinguish the disease from viral hepatitis. Mortality from amoebic liver abscess is estimated at approximately 20% (Sepulveda and Martinez-Palomo, 1982) where adequate diagnosis and treatment is not available but with modern

hospital facilities the mortality rate can be as low as 2% even in endemic areas (Martinez-Palomo, 1987).

In a hamster model of liver abscess formation, early lesions consist of a small rounded mass of necrosed hepatocytes and E. histolytica trophozoites surrounded by a neutrophil infiltrate (Martinez-Palomo, 1987). As the lesions enlarge, macrophages and epithelioid cells replace other leucocytes and granulomata develop, with subsequent fusion to form large lesions. In human patients, although granulomatous lesions can occur, they were not reported in a large study performed by Aikat et al. (1979). Amoebic abscesses contain proteinaceous debris, rather than the pus of true bacterial abscesses and it has been proposed (as for intestinal lesions) that the products released from lysed polymorphoneclear cells contribute to the necrosis (Ravdin and Guerrant, 1982).

Pulmonary amoebiasis, although infrequent is the second commonest extraintestinal site of amoebiasis. Lesions usually occur from direct extension of a liver abscess through the diaphragm and pleura (Adams and MacLeod, 1977b). Haematogenous spread of amoeba is responsible for the rare occurrence of abscesses in brain and spleen. Other rare complications include fistular and perineal cutaneous lesions, ulcerative vaginitis, cervicitis and balanitis.

An interesting feature of most amoebic lesions, is the lack of proliferation of connective tissue and their ability to heal without scar formation. This is particularly noticeable in liver and cutaneous lesions in which treatment can result in remarkable anatomical and functional recovery (Perez-Tamayo, 1986). No adequate explanation exists, although it has been postulated (Dennis and Chadee, 1988) that the lack of macrophages in amoebic lesions may be significant, since macrophages are involved in the stimulation of collagen deposition in wound healing (Freundlich et al., 1970).

1.8 IMMUNE AND NON-IMMUNE MECHANISMS DIRECTED AGAINST E. HISTOLYTICA

The characterisation of amoebic antigens responsible for stimulating host immune responses against the parasite, will be dealt with in subsequent chapters.

1.8.1 Non-immune mechanisms

Colonic mucins are thought to be a major host defence against intestinal invasion by E. histolytica (Dennis and Chadee, 1988; Ravdin, 1989). Not only are they high-affinity receptors for the Gal/GalNAc lectin (Chadee et al., 1987), but they also appear to bind released parasite toxins (McGowan et al., 1982; Lushbaugh et al., 1979). Physical trapping of E. histolytica trophozoites by mucus also occurs (Leitch et al., 1988).

Normal human milk has been shown to have an amoebicidal effect in vitro that is not due to the presence of secretory immunoglobulin A (s-Ig A) (Gillin et al., 1983). The active component, which had a similar effect on Giardia lamblia (another intestinal protozoan parasite) is believed to be bile salt-stimulated lipase.

1.8.2 Evidence for protective immunity in amoebiasis

In most cases, non-invasive asymptomatic infections are apparently spontaneously cleared within twelve months (Nanda et al., 1984). It is not known, however, whether this clearance is due to a specific antiamoebic response and followed by resistance to further challenge. Indeed epidemiological studies demonstrating an increasing prevalence of asymptomatic infections with age suggest a lack of such resistance (Oyerinde et al., 1977).

Some resistance to intrarectal reinfection following cure has been documented in dogs (Thompson and Lilligren, 1949; Swartzwelder and Avant, 1952) and similar results have been obtained using intrahepatically-inoculated hamsters following drug treatment (Vazquez-Saavedra et al., 1973). However the relevance of such

results to the human situation is doubtful, since hamsters and dogs are far from being perfect animal models.

Studies in humans have been based on epidemiological data. In one uncontrolled study, performed in Mexico City in a population at high risk of reinfection, a 0.29% recurrence of hepatic abscess was demonstrated over a period of five years (de Leon, 1970). This rate is well below the expected rate in the total population (Kagan, 1974; Gutierrez et al., 1976). Further evidence of the rôle of the immune system in resistance to infection has come from the observation that there is an increased severity of invasive amoebiasis when it is associated with immunosuppression. Several authors have reported the activation of previously undiagnosed amoebiasis and the exacerbation of existing hepatic lesions following corticosteriod treatment (Mody, 1959; El-Hennawy and Abd-Rabbo, 1978; Stuiver and Goud, 1978). Amoebiasis is known to be more severe and frequently fatal in pregnant women (Abioye, 1973; Constantine et al., 1989) in whom depression of cell-mediated immunity is known to occur (Weinberg, 1984).

1.8.3 Humoral immunity

Patients with invasive amoebiasis normally develop a high titre of circulating antibodies specific for E. histolytica within one to two weeks of the onset of symptoms (Kotcher et al., 1970; Krupp, 1970; Patterson et al., 1980). These antibodies are predominantly of the IgG class (Lee et al., 1970). Individuals with asymptomatic infection less frequently develop a systemic antibody response (Kotcher et al., 1970) although frequencies of up to 78% have been recorded (Savanat et al., 1974). Although this may be due to a response to luminal amoebic antigens, it has been suggested that positive serological results in asymptomatic carriers are due to subclinical invasion by the parasite (Trissl, 1982). This is supported by the studies of Sargeaunt, who reports that while asymptomatic patients harbouring E. histolytica belonging to pathogenic zymodemes may or may not be serologically positive,

patients with non-pathogenic amoebae are always serologically negative (Sargeaunt, 1988).

In general, positive results in serological tests are regarded as an indication of current or prior invasive disease (Elsdon-Dew, 1970; Trissl, 1982). There is no evidence that antibody titre correlates with clinical status of the patient or with severity of lesions (Krupp and Powell, 1971; Trissl, 1982), but higher serum titres are generally associated with a recent onset of invasive disease (Krupp, 1970). Antibody levels are known to decrease after recovery but can persist at detectable levels for up to 11 years (Healy et al., 1974; Patterson et al., 1980). The relevance of the systemic humoral response to E. histolytica is questionable since invasive amoebiasis is known to recur even in the presence of high antibody titres (Krupp, 1970; Krupp and Powell, 1971).

Several studies have demonstrated the presence of locally secreted copro-antibodies in patients with invasive intestinal amoebiasis (Shaalan and Baker, 1970; Mahajan et al., 1972; Martinez-Cairo et al., 1979). In contrast to circulating antibodies, these antibodies, predominantly of the IgA class (Martinez-Cairo et al., 1979), persist only for a short time. It has been suggested that secretory IgA antibody to the Gal/GalNAc amoebic adherence lectin could be a potential defence mechanism against intestinal colonisation by preventing parasite adhesion to the colonic mucus blanket (Ravdin, 1989).

Several authors have reported the ability of E. histolytica trophozoites in vitro to rapidly aggregrate and shed or ingest human antiamoebic antibodies that have bound to the cell surface (Aust-Kettis and Sundqvist, 1978; Calderon et al., 1980; Aust-Kettis et al., 1981). These results suggest a possible mechanism by which the amoeba may evade the humoral immune response.

E. histolytica trophozoites incubated in vitro with human sera have been shown to be vulnerable to complement-mediated lysis via both the classical and alternative pathways (Sepulveda et al., 1973; Ortiz-Ortiz et al., 1978). Depletion of complement in hamsters using cobra venom factor caused an increased susceptibility to the formation of hepatic abscesses (Ghadirian and Meerovitch, 1982c). The studies of Reed et al. (1983) suggest that amoebic resistance to complement-mediated lysis may be an important prerequisite for invasion. All tested isolates belonging to non-pathogenic zymodemes were uniformly susceptible to lysis while nine out of eleven pathogenic isolates (including all five isolates obtained from liver abscesses) were resistant.

1.8.4 Cellular Immunity

1.8.4.1 Polymorphonuclear neutrophils

E. histolytica trophozoites have been shown capable of lysing human neutrophils in vitro without loss of amoebic viability (Geurrant et al., 1981; Salata et al., 1982), although some axenic strains of low virulence (with a reduced ability to lyse cultured epithelial cells) were killed by the neutrophils. Apart from being a mechanism for avoiding one particular arm of the body's defence mechanism, lysis of neutrophils and the release of their contents may contribute to the tissue necrosis characteristic of amoebic lesions (as previously stated; 1.7.2) (Chadee and Meerovitch, 1985).

1.8.4.2 Cell-mediated immunity

Clinical observations in patients receiving corticosteroid treatment have indicated the importance of cell-mediated immune mechanisms in invasive amoebiasis (Section 1.8.2). Similarly, using a hamster model, it has been shown that procedures which depress cell-mediated immunity, such as corticosteroid treatment, neonatal thymectomy, ionising radiation treatment, and antimacrophage and antithymocyte globulin treatment, cause enhanced formation of hepatic abscesses (Ghadirian and Meerovitch, 1981; 1982b; Ghadirian et al., 1983).

Stimulation of the macrophage system, on the other hand, by immunisation with bacille Calmette-Guerin (BCG) had the opposite effect on such lesions (Ghadirian and Meerovitch, 1982b).

Peripheral blood lymphocytes and peritoneal mononuclear cells from hamsters immunised intradermally and intrahepatically with live amoebae, killed E. histolytica trophozoites in vitro (Ghadirian and Meerovitch, 1982a). In humans, Guerrero et al. (1976) found that the in vitro interaction between E. histolytica trophozoites and lymphocytes obtained from patients recovering from treated liver abscess, resulted in lysis of the amoebae. However, lymphocytes obtained from patients with acute hepatic amoebiasis or from uninfected controls were themselves lysed by the amoebae. The importance of the macrophage and T-lymphocyte in the response against E. histolytica has been further indicated by the work of Salata and co-workers. While they found that T-lymphocytes from treated hepatic abscess patients did not display a spontaneous cytotoxic effect on cultured trophozoites (c.f. above), if the lymphocytes were stimulated for five days with a soluble amoebic protein preparation, the amoebae were killed (Salata et al., 1986). Exposure of T-lymphocytes from non-infected control patients to the amoebic antigen was ineffective in producing an amoebicidal response. This group has also shown that cured liver abscess patients show an in vitro T-lymphocyte proliferative response to E. histolytica antigen, producing lymphokines (including gamma-interferon) that can stimulate human monocyte-derived macrophages to kill trophozoites in vitro (Salata et al., 1986; Salata et al., 1987).

Several clinical studies have measured delayed type hypersensitivity reactions to intradermal amoebic antigen and demonstrated that patients with acute hepatic amoebiasis showed an absence or reduction in reactions while reactiveness returned after treatment (Kretschmer et al., 1972; Ortiz-Ortiz et al., 1975; Ganguly et al., 1979, 1981). In one study, a specific state of unresponsiveness to amoebic antigens was suggested, since skin reactions against unrelated

antigens were positive at all stages of the disease (Kretschmer et al., 1972). Confirmatory evidence for this state has been obtained in experiments studying the in vitro responses of lymphocytes from early hepatic amoebiasis patients (Ortiz-Ortiz et al., 1975). The reason for this suppression is not known. Recent evidence in an animal model shows that constituents of a crude extract of E. histolytica are capable of exerting an inhibitory effect on a variety of macrophage functions including responsiveness to lymphokines, oxidative metabolism and phagocytic ability (Denis and Chadee, 1988).

In summary, it seems that effective afferent and efferent cell-mediated immune responses that develop after invasive amoebiasis represent the major host defence against E. histolytica. Although antibody and complement may have a rôle in preventing invasion by certain strains of amoebae, and antibody levels can be used as an indicator of invasive disease, humoral immune systems do not appear to provide protective immunity.

1.9 TREATMENT

The drug of choice for severe intestinal and hepatic amoebiasis is Metronidazole, a 5-Nitroimidazole. If oral treatment with this drug fails, intramuscular or deep subcutaneous infections of emetine hydrochloride or dehydroemetine dihydrochloride are recommended (Brown and Neva, 1983). The choice of treatment of asymptomatic carriers, if it is performed (Editorial, 1985; Sargeaunt, 1987), varies from country to country. Drugs such as diloxanide furoate and diiodohydroxyquin (either alone or with tetracyclines) are active against amoebae in the lumen of the intestine. These drugs are effective in the treatment of carriers and are also frequently used in the treatment of invasive intestinal disease, in conjunction with those mentioned above, since emetine and dehydroemetine are only active against amoebae in the tissues and not against those in the intestinal lumen (Knight, 1980). The use of diiodohydroxyquin in conjunction with metronidazole treatment of such cases is also recommended (Brown and Neva, 1983) for the same reason.

Treatment of amoebiasis is not without risk; metronidazole and emetine, although effective, have numerous unpleasant side-effects and metronidazole has been shown to be mutagenic in bacteria (Voogd *et al.*, 1974) and carcinogenic in rats (Roe, 1979) while emetine has a serious cardiotoxic effect (Klatskin and Friedman, 1948). Such information demonstrates the need for the development of less harmful antiamoebic agents.

1.10 AIMS OF THE PROJECT

The aim of this project was to study some of the antigens of E. histolytica using monoclonal antibodies raised against whole trophozoites. Chapter 2 describes the production of the monoclonal antibodies using hybridoma technology (a technique pioneered by Kohler and Milstein (1975)). The subcellular locations of the antigens, for which these monoclonal antibodies are specific, are discussed. Chapter 3 examines the specificity of the antibodies for E. histolytica and discusses possible phylogenetic relationships between members of the genus Entamoeba. Chapter 4 examines the antigenic heterogeneity of different isolates of E. histolytica using the monoclonal antibodies as probes. In Chapter 5, preliminary attempts at immunochemical analysis of the antigens recognised by the monoclonal antibodies, are discussed. Chapter 6 describes efforts to investigate the suitability of the monoclonal antibodies as antigen-capture agents in an enzyme-linked immunosorbent assay for amoebic antigen. Chapter 7 describes work investigating the possibility that two of the monoclonal antibodies recognise, in indirect fluorescent antibody tests, antigens specific to isolates of E. histolytica belonging to pathogenic zymodemes. This work has been previously published as Strachan et al. (1988).

CHAPTER 2 PRODUCTION OF MONOCLONAL ANTIBODIES

2.1 INTRODUCTION

2.2 MATERIALS AND METHODS

2.2.1 Axenic *in vitro* culture of *Entamoeba histolytica*

2.2.1.1 Medium

2.2.1.2 Culture

2.2.2 Production of monoclonal antibodies

2.2.2.1 Immunisation of mice

2.2.2.2 Culture of myeloma cells

2.2.2.3 Preparation of spleen cells from an immunised mouse

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2.4 DISCUSSION

CHAPTER 2 PRODUCTION OF MONOCLONAL ANTIBODIES

2.1 INTRODUCTION

The immunochemical characterisation of an individual parasite antigen requires, at some stage, the ability to distinguish that antigen from others that may be present in the organism. Previous study of Entamoeba histolytica antigens has involved the use of amoeba-specific antibodies. However antisera raised against whole cells contain antibodies against many of the constituent antigens. Hybridoma technology, pioneered by Köhler and Milstein (1975), provides a method of obtaining antibodies which are specific for a single antigenic determinant and such monoclonal antibodies (Mabs) are now routinely used to identify and purify single antigens from complex mixtures.

Monoclonal antibody-secreting hybridomas are produced by fusing antibody-producing spleen cells with myeloma cells. As spleen cells rapidly die in vitro, the myeloma cells, which are adapted to grow in culture, are required to confer immortality on the hybridoma. Various methods have been used to induce fusion of these cells (reviewed by Goding, 1986) including treatment with Sendai virus, lysolecithin and polyethylene glycol (PEG). PEG treatment (Pontecorvo, 1976) has been the most widely used and this method appears to give the most reproducible results. When spleen cells are mixed with myeloma cells in the presence of PEG, fusion of the cells takes place in a random fashion. If the fusion products and unfused cells are allowed to grow, the unfused myeloma cells will overgrow other cells leading to the inability to select hybrids secreting the desired Mabs. To overcome this, mutant myeloma cells have been isolated which lack hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme involved in the salvage pathway of purine synthesis. If the de novo pathway of synthesis is blocked by aminopterin, these cells cannot synthesise DNA (Littlefield, 1964).

Spleen cells, when fused with these mutant myeloma cells supply the resultant hybrids with HGPRT, and therefore using a medium containing aminopterin supplemented with thymidine and hypoxanthine (HAT medium) selection for growth of hybrid cells ("hybridomas") can be achieved. Myeloma cells which have further mutated and do not synthesise or secrete immunoglobulin chains of their own have now been isolated (Kearney et al., 1979). Fusion of spleen cells with these myeloma cells results in hybridomas which secrete biologically active Mabs containing no myeloma-derived immunoglobulin chains. Cloning of individual cells can be performed after initial screening for antibody in spent medium (supernatant) from hybridoma-containing wells. Theoretically Mabs can then be obtained in unlimited quantities from cell culture supernatants or from ascitic fluid from hybridoma-bearing mice.

In this work monoclonal antibodies were produced by hybrid cells from fusions between mouse myeloma cells and spleen cells from mice immunised with axenically-cultured Entamoeba histolytica trophozoites. The techniques described here for the production of hybridoma cells have been based on the method used by Crowe (1983) adapted and modified from protocols published by Norwood et al. (1976); Goding (1980) and Pearson et al. (1980).

The subcellular locations of the antigens against which the Mabs were directed were examined in experiments using the indirect fluorescent antibody test (IFAT) as a preliminary to further investigation of the antigens.

2.2 MATERIALS AND METHODS

2.2.1 Axenic *in vitro* culture of *Entamoeba histolytica*

2.2.1.1 Medium

Amoebae were grown in TYI-S-33 medium developed by L.S. Diamond (Diamond *et al.*, 1978). Complete medium was prepared in 100 ml volumes from:

87 ml TYI broth
10 ml Heat inactivated horse serum
3 ml Vitamin-Tween 80 solution

TYI broth

Trypticase (Lab. Impex, Twickenham)	2 g
Yeast extract (Oxoid, Basingstoke)	1 g
Glucose (Sigma, Poole)	1 g
NaCl (Sigma, Poole)	200 mg
K_2HPO_4 (Sigma, Poole)	100 mg
KH_2PO_4 (Sigma, Poole)	60 mg
L-Cysteine HCl (Sigma, Poole)	100 mg
L-Ascorbic Acid (Sigma, Poole)	20 mg
Ferric Ammonium citrate (Mallinskrodt)	2.28 mg
Distilled H_2O	50 ml

The final volume was adjusted to 87 ml and the pH adjusted to 6.8 with 1M NaOH.
It was then clarified by passage through one layer of Whatman No.1 filter paper, autoclaved for 15 min at 121 °C, and stored at room temperature.

Vitamin-Tween 80 mixture

All chemicals were obtained from Sigma (Poole).
Four stock solutions, A, B, C and D were prepared.

A - vitamin mixture 107

B - vitamin B12 solution

C - Thioctic Acid solution

D - Tween 80 solution

A) Vitamin mixture 107

Five primary stock solutions (1 - 5) were prepared.

1) Water soluble B vitamins

Solution (i) 62.5 mg niacin
125 mg para-aminobenzoic acid
dissolved in boiling distilled H_2O and brought to a total volume of 150 ml.

Solution (ii) 62.5 mg niacinamide
62.5 mg pyridoxine HCl
62.5 mg pyrodoxal HCl
25 mg thiamine HCl
25 mg calcium panthothenate
125 mg i-inositol
1.25 g choline chloride
dissolved in distilled water and brought to a total volume of 150 ml.

Solution (iii) 25 mg Riboflavin added to 75 ml of distilled H_2O and dissolved with the aid of 0.1M NaOH added dropwise. The total volume was then brought up to 100 ml.

Solutions (i), (ii) and (iii) were combined and brought to a total volume of 500 ml with distilled H_2O.

2) Biotin solution

30 mg D-Biotin was dissolved in 200 ml distilled H_2O with the aid of 0.1M NaOH and brought to a final volume of 300 ml with distilled H_2O.

3) Folic Acid solution

30 mg Folic Acid was dissolved in 200 ml of distilled H_2O with the aid of 0.1 M NaOH and the final volume brought up to 300 ml with distilled H_2O.

4) Lipid soluble vitamins A, D and K

Solution (i) 300 mg vitamin D2 (calciferol) was dissolved in 63 ml of 95% Ethyl alcohol. To this, 300 mg of vitamin A (retinol) were added and dissolved.

Solution (ii) 60 mg vitamin K (menadione sodium bisulphite) were dissolved in 300 ml of a 5% (v/v) aqueous solution of Tween-80.

Solution (i) was then combined with solution (ii) and the total volume brought up to 3 L with distilled H_2O.

5) Vitamin E solution

25 mg of vitamin E (alpha tocopherol acetate) were dissolved in 250 ml of distilled H_2O. A working mixture of vitamin mixture 107 was then prepared by combining the 5 primary stock solutions in the following proportions:

500 ml	Solution 1
250 ml	Solution 2
250 ml	Solution 3
2500 ml	Solution 4
250 ml	Solution 5

This mixture was stored at -22 °C

B) Vitamin B12 solution

40 mg vitamin B12 was dissolved in 100 ml distilled H_2O.

C) Thioctic Acid solution

100 mg DL-6,8-Thioctic Acid in 100 ml of absolute ethanol.

D) Tween-80 solution

50 g Tween-80 was dissolved in 100 ml of absolute ethanol.

The final working solution of the vitamin-Tween-80 mixture was obtained by combining the 4 stock solutions (A - D) in the following proportions:

1000 ml	A
12 ml	B
4 ml	C
4 ml	D
180 ml	distilled H_2O

The mixture was filtered through a 0.2 µm polycarbonate filter and stored as 3 ml aliquots at - 22 °C in the dark.

Horse serum

Batch tested, mycoplasma screened, sterile horse serum was obtained from Gibco (Paisley). It was heat inactivated at 56 °C for 30 min before use.

The complete TYI-S-33 medium was stored in the dark at 4 °C and used within 5 days after preparation.

2.2.1.2 Culture

Stock cultures of E. histolytica, strain 200:NIH (ATCC:30458) (Tobie, 1949) were maintained in Lux 16 x 125 mm plastic tissue culture tubes (Flow, Irvine) filled to within 5 mm of the top with TYI-S-33 medium. For bulk growth Lux 50 ml plastic tissue culture flasks (Flow, Irvine) were used. Tubes and flasks containing amoebae were kept incubated at 35.5 °C and stock cultures were subcultured at approximately 48 h intervals. Subculturing was performed by chilling the tubes and fresh medium on ice for 3 minutes. The tubes were inverted several times to release attached amoebae and 1 ml of the resulting suspension, containing approximately 10^5 cells, was transferred to tubes containing fresh (chilled) medium.

2.2.2 Production of monoclonal antibodies

2.2.2.1 Immunisation of mice

Tubes of amoebae in late log phase growth (36-48 h cultures) were cooled in ice water for 3 min and gently inverted several times to dislodge adherent amoebae. The tubes were centrifuged at 200 g for 5 mins at room temperature, and the cells resuspended in 13 ml of Phosphate-buffered saline (PBS) (pH 7.4) and a sample counted in a haemocytometer. After a second wash, 10^6 amoebae were suspended in 0.2 ml of PBS. One 6-8 week old female Balb/c mouse was injected intraperitoneally with 10^6 amoebae and the procedure repeated 4 weeks later. After a further 2 weeks 10^6 amoebae were administered intravenously (via the caudal vein) and 3 days later the mouse was sacrificed and the spleen was removed for hybridoma production.

2.2.2.2 Culture of myeloma cells

Media

RPMI incomplete medium

This was used as a stock solution for culture medium and, in various procedures for washing cells. It consisted of RPMI 1640

(Gibco-Europe, Paisley) with 25 μm HEPES (Sigma, Poole) at pH 7.2. Ten litre batches of this medium were prepared from powder and filter sterilised (0.22 μm pore size). The prepared medium was stored for up to six months at 4 °C.

RPMI complete medium

This medium was used as the growth medium for myeloma and hybridoma cells. The following stock solutions were produced or purchased and stored as stated.

Gentamycin sulphate	10 mg/ml	(Sigma, Poole), 4 °C
Sodium bicarbonate	7% w/v	(BDH, Poole), 4 °C
Fungizone (Amphotericin B)	250 μg/ml	(Flow, Irvine), - 20 °C
L-Glutamine	200 mM	(Sigma, Poole), - 20 °C
2-Mercaptoethanol	0.1 M	(Sigma, Poole), 4 °C
Foetal Calf serum (FCS)		Heat inactivated, 56 °C, 30 min, screened for the ability to support growth of myeloma cell line P3-X63-Ag8-653 (Flow, Irvine), - 20 °C

To produce 100 ml of RPMI complete medium the following amounts of the stock solutions were mixed:

80 ml	RPMI incomplete medium
2 ml	Gentamycin sulphate
1 ml	7% Sodium Bicarbonate
0.8 ml	Fungizone
1 ml	L-Glutamine

50 µl 2-Mercaptoethanol

15 ml Heat inactivated Foetal Calf serum

RPMI complete medium was stored at 4 °C for not more than 7 days.

Culture of myeloma cells

The P3-X63-Ag8-653 mouse myeloma cell line was used, (Flow, Irvine). The cells were grown at 37 °C in 5% CO_2 in a humidified atmosphere in RPMI complete medium.

The cells were kept at log phase growth in Lux tissue culture flasks (Flow, Irvine) at a density of approximately 1-5 x 10^5 cells per ml medium. Fresh RPMI complete medium was added to the flask daily. For fusion, 10^7 myeloma cells were required. Immediately prior to fusion the cells were harvested by gentle shaking of the flask to release the attached cells followed by centrifugation at 200 g for 5 min (RT). The required myeloma cells were resuspended in 10 ml of RPMI incomplete medium.

2.2.2.3 Preparation of spleen cells from an immunised mouse

The mouse was killed by gentle cervical dislocation and briefly immersed in 70% alcohol. The spleen was removed under aseptic conditions and placed in 5 ml of RPMI incomplete medium at RT and teased apart using forceps. After disruption the remaining larger clumps were allowed to sediment for 1 minute. The cell-containing supernatant was passed through hypodermic needles of decreasing bore (21 G, 23 G, 26 G) in a 10 ml syringe. The spleen cell suspension was transferred to a 50 ml centrifuge tube filled with RPMI incomplete medium and centrifuged at 200 g for 5 min (RT). The pellet was washed in a further 50 ml of RPMI incomplete medium as above, and the final pellet resuspended in 10 ml RPMI incomplete medium. The spleen cells were counted in a haemocytometer and the viability assessed by phase-contrast microscopy (always > 90%).

2.2.2.4 Fusion of cells

Solutions

Polyethylene Glycol (PEG)
Solution A

10 g PEG (Molecular wt. 1540) (Sigma, Poole) was autoclaved at 121 °C, for 20 min. After cooling to 60 °C, 11 ml of RPMI incomplete medium and 3 ml of Dimethyl sulphoxide (DMSO) (Sigma, Poole) were added. This gave 41.6% PEG/15% DMSO solution.

Solution B

5 g PEG 1540 was autoclaved at 121 °C for 20 min. After cooling to 60 °C, 15 ml of RPMI incomplete medium were added. This gave 25% PEG solution.

These solutions were stored for up to 6 months at 4 °C.

HAT medium

Hypoxanthine Aminopterin Thymidine solution (x50) (Flow, Irvine) was stored at - 20 °C. 2 ml of 50x HAT were added to 73 ml of RPMI complete medium to make 1.3x HAT medium.

All solutions and cell suspensions were pre-warmed to 37 °C.

1) 10^8 spleen cells and 10^7 myeloma cells were mixed in a 50 ml conical plastic centrifuge tube and the volume made up to 50 ml with RPMI incomplete medium.

2) The mixture was centrifuged at 200 g for 5 min, at RT, and the supernatant completely removed.

3) 0.5 ml of PEG solution A was added using a 1 ml pipette over a 30 second period, the pellet being disrupted with the tip of the pipette, and gently rocked for a further 30 seconds.

4) 0.5 ml of PEG solution B was added. The pellet was resuspended in this solution for 2-3 min using the pipette tip.

5) 40 ml of RPMI complete medium was added dropwise over a 10 min period with constant rocking of the tube, after which a further 60 ml of the same medium was added. The resultant suspension was distributed into wells of 5 Nunc flat-bottomed microtitre plates (Gibco, Paisley), 200 µl/well, and incubated at 37 °C, 5% CO_2 in a humidified atmosphere.

6) 24 hours after the fusion, 150 µl of supernatant from each well was removed and replaced with 150 µl of 1.3x HAT medium containing 10^4 freshly isolated Balb/c peritoneal exudate cells per ml. The plates were then reincubated for 9 days without feeding.

7) On day 10 post fusion, the plates were observed using an inverted microscope. Wells containing plaques of hybridoma cells were recorded and 20 µl samples of supernatant were removed for screening (see below). Cells secreting Mabs against E. histolytica were removed using a pasteur pipette and transferred to wells in a 24-well Linbro plate (Flow, Irvine) containing 1 ml of RPMI complete medium and 10^4 Balb/c peritoneal exudate cells set up 24 hours previously to allow conditioning of the medium. The original microtitre plate wells were refilled with 200 µl of RPMI complete medium.

2.2.2.5 Cloning of Hybridoma cells

After 24-48 hours growth in 24-well plates the cells were removed for cloning by limiting dilution.

Cells were resuspended using a pasteur pipette and counted in a haemocytometer. Six columns of a microtitre plate were used to clone cells from one of the above wells. The microtitre plate was prepared

24 hours prior to cloning by placing 150 µl of RPMI complete medium, containing 10^4 peritoneal exudate cells per ml, in each well. The following numbers of cells were placed in the microtitre plate:

Wells in column	1 -	10^2	hybridoma cells
Wells in column	2 -	10	hybridoma cells
Wells in column	3 + 4 -	1	hybridoma cells
Wells in column	5 -	10^{-1}	hybridoma cells
Wells in column	6 -	10^{-2}	hybridoma cells

Supernatants from wells in columns 3-6 showing growth of hybridomas by day 10 were screened by IFAT. Mab-secreting cells were transferred to 24-well plates and recloned by the same technique. Positive clones were then transferred from 24-well plates to 6-well Linbro plates (Flow, Irvine) for 2-7 days depending on growth rate, and then into tissue culture flasks. When a total of 3×10^6 cells in log phase growth was reached the cells were harvested and centrifuged at 200 g for 5 min (RT), resuspended in 90% foetal calf serum (Gibco, Paisley), 10% DMSO (Sigma, Poole) placed in 1.8 ml cryotubes (Gibco, Pailsey) and stored under liquid nitrogen.

2.2.2.6 Production of Ascitic fluids

Six to eight week old female Balb/c mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethyl-pentadecane) (Sigma, Poole). Seven to ten days after pristane treatment the cultured hybridomas were harvested by centrifugation (200 g, 5 min, RT) and resuspended in RPMI incomplete medium at a density of 2×10^7 cells/ml. Each mouse received 0.5 ml of the cell suspension intraperitoneally via a 23 G hypodermic needle. The animals were inspected daily for swelling of the abdomen (indicating fluid production) and ascitic fluid was removed using a 5 ml syringe and a 19 G hypodermic needle. Cells were removed by centrifugation at 500 g for 10 min, RT, and aliquots stored frozen at -40 °C. Some samples of ascitic fluid were kept refrigerated at 4 °C with 0.02%

sodium azide and with 1 µl of 0.1 M Phenylmethylsulphonyl Fluoride (PMSF) in acetone added per ml of ascitic fluid.

2.2.2.7 Screening of supernatants by indirect fluorescent antibody test using fixed cells

Culture tubes containing amoebae in late log phase growth were cooled in ice water for 3 min, inverted several times and centrifuged at 200 g for 5 min (RT). The pellet was washed twice in PBS (pH 7.4) and centrifuged as above. The washed cells were resuspended in 0.2 ml 10% horse serum in PBS (pH 7.4) and one drop placed in each well of a multispot microscope slide (Hendley, Essex). The slides were air dried, fixed in methanol (5 min) and could be stored with silica gel (Sigma, Poole) at - 22 °C. For screening, 5 µl quantities of supernatant were placed in reaction zones and the slides incubated for 30 min at room temperature in a humidified atmosphere. After two washes in PBS (pH 7.2) fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (whole molecule) antiserum was applied at 1/100 dilution in PBS containing Evans blue (1/10,000) and the slides incubated for a further 30 min. Control wells received 5 µl of complete RPMI medium in place of culture supernatant.

Finally the slides were washed twice in PBS and mounted in 50% w/v glycerol/PBS and reaction zones examined using a Leitz Ortholux II microscope with incident light fluorescence, an HB 50 high pressure mercury vapour lamp, a TK 510 dichroic mirror, 2 x KP 490 (exciting) and a K 515 (suppressing) filter. Photographs were taken on Fujichrome 1600 ASA film.

2.2.3 IFAT using live amoebae

In this protocol Phosphate Buffered Saline (pH 7.45) was used according to the method of Diamond (1968).

NaCl	6.5 g
K_2HPO_4	2.8 g
KH_2PO_4	0.4 g
distilled H_2O	1 litre (pH 7.45)

All materials and solutions were maintained on ice at below 4 °C. Trophozoites in late log phase growth were harvested, washed twice in cold PBS (pH 7.45) and resuspended at a final concentration of 2 x 10^4/ml in 500 µl of diluted ascitic fluid in TYI broth (1/100). (Ascitic fluids used in these experiments contained neither sodium azide nor proteinase inhibitors). After 30 min incubation on ice the cells were washed twice in 10 ml of PBS (centrifugation at 200 g for 5 min at 4 °C) then resuspended in 500 µl of TYI broth with 5 µl of FITC-conjugated rabbit anti-mouse IgG (whole molecule) antiserum. The cells were incubated on ice for 30 min, washed twice in PBS, and samples placed on microscope slides for examination using incident light fluorescence as previously described.

2.2.4 Determination of Immunoglobulin subclasses

The immunoglobulin subclasses of the monoclonal antibodies produced were determined by IFAT experiments using methanol-fixed amoebae similar to those described above. In these experiments however the second antibody used was an FITC-conjugated rabbit antiserum raised against a particular murine immunoglobulin subclass (Nordic) Anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM antisera were used.

2.2.5 Cloning of E. histolytica

Axenically-cultured E. histolytica (Strain 200:NIH), cooled to 4 °C, were diluted in TYI-S-33 medium to a concentration of 40/ml. Aliquots of 25 µl of amoebic suspension were dispensed under aseptic conditions into sterile Benjamin glass haematocrit tubes (Harshaw, Daventry) and the tubes sealed in a Bunsen flame. The tubes were examined microscopically, by two observers, for the presence of single amoebae and such tubes incubated at 35.5 °C. After adequate growth the haematocrit tubes were immersed in 70% ethanol for 3 min,

subsequently opened under aseptic conditions and transferred to 16 x 125 mm plastic tissue culture tubes containing 11 ml of TYI-S-33 medium and incubated at 35.5 °C as described above.

2.2.6. Erythrophagocytosis by E. histolytica

Two drops of fresh whole mouse blood obtained via tail bleed from a Balb/c mouse were added to a late log phase culture of E. histolytica (strain 200:NIH) in a plastic tissue culture tube. The tube was inverted 5 times to mix and incubated at 35.5 °C for 20 min. The cells were subsequently harvested as previously described and methanol-fixed antigen slides were prepared.

2.3 RESULTS

2.3.1. Hybridomas

In three fusions using whole E. histolytica (strain 200:NIH) as antigen, 279 hybrid cultures were obtained, of which 61 secreted anti-E. histolytica antibodies detectable by IFAT screening. A number of indirect immunofluorescence assay reactivity patterns were distinguished during the primary screening and 21 hybrids were successfully cloned, maintained in culture and stored frozen. The hybrids were assigned a figure code or GUPM number (Glasgow University Protozoology Monoclonal), the first figure of which corresponded to the number of the fusion from which it was produced (ie. fusion 22, 24 or 29). Of the 21 monoclonal antibody secreting hybridomas produced, 4 produced antibodies of the IgM class and 17 produced antibodies of the IgG class. Of these IgG secreting hybridomas 15 produced IgG_1 subclass antibodies, one produced IgG_{2a} and one IgG_{2b}. (See Table 2.1).

2.3.2 Immunofluorescence patterns

Five basic types of staining pattern were observed in IFAT experiments using methanol-fixed amoebae. (See Table 2.1).

Surface: Monoclonals 22.1, 22.6 and 29.6 produced surface staining patterns confirmed by positive results in experiments using live amoebae (see below). Such patterns were characterised by a bright ring of fluorescence around the boundary of the cell, the remainder of the cell showing a less intense uniform fluorescence (Plate 2.1). Filopodia and other surface protrusions were clearly visible. Mab 22.1 stained virtually all of the cells observed although occasionally negative cells were seen. Such cells were not considered fixation artefacts since, under normal light, they were morphologically identical to positive cells. Mabs 22.6 and 29.6

stained only a percentage of cells. Some cells showed no staining at all (red background staining only) while others showed an intermediate range of patterns involving small discrete areas of surface fluorescence (Plate 2.2). The pattern of fluorescence appeared to show no obvious correlation with particular morphology when observed under normal light, or with cell size, although no detailed counts were made (see Plates 2.3 and 2.4). While Mab 22.1 consistently produced just less than 100% positive staining, the percentages obtained with 22.6 and 29.6 were found to vary during long term culture of strain 200:NIH when cells were tested after intervals of several months. With 22.6, the percentage of positive cells varied between 52% and 98%, while with 29.6 the percentage varied between 17% and 59%. Accurate time-sampling experiments were not performed.

Internal vacuolar: Monoclonal antibodies 22.5 and 22.7 produced fluorescence of internal components which appeared to be internal vacuoles of variable size. With Mab 22.5 all cells observed were stained in this manner (Plate 2.5) but with 22.7, only a percentage of cells were stained with many of these showing a more generalised internal fluorescence as well (Plate 2.6). With both these Mabs the size of the apparent vacuoles varied considerably with the state of the cells. Some large cells, which appeared highly vacuolated when viewed under normal light, had large fluorescent vacuoles while others had more numerous smaller ones. Using both Mabs the vacuolar fluorescence obtained was rarely evenly distributed throughout the cytoplasm but frequently concentrated in certain areas leaving other parts of the cytoplasm free of fluorescent material (Plate 2.5). Occasionally in experiments using 22.7, cells were observed with a ring of fluorescence at the margin of the cell (centre cell, Plate 2.6), similar to the surface fluorescence described above, but live amoebae-IFAT experiments did not confirm its surface nature (see below). Methanol-fixed slides of amoebae which had internalised (phagocytosed) erythrocytes were used in IFAT experiments using Mabs 22.5 and 22.7. It was observed that the vacuolar fluorescence did

not surround the internalised erythrocytes which were clearly visible, stained dark red by the background stain, but remained in other regions of the cytoplasm.

Internal granular: Ten Mabs (22.3, 22.4, 24.1, 24.3, 24.5, 24.7, 24.8, 29.9, 29.10 and 29.11) produced internal granular fluorescence patterns distributed evenly throughout the cell.

Using Mabs 24.5, 24.7 and 24.8 course granular fluorescence patterns were consistently produced (Plate 2.7) but with the remaining Mabs the size of the fluorescent particles varied with the state of vacuolation of the cells. If the cells were highly vacuolated (when viewed under normal light), a coarse pattern was produced and with less vacuolated cells, finer fluorescent granules were observed (Plate 2.8).

Internal diffuse: Mabs 22.8, 24.2, 24.4, 24.6 and 29.3 produced internal diffuse fluorescence patterns. Experiments using Mab 24.6 produced uniform diffuse fluorescent staining (Plate 2.9). With the other Mabs the patterns obtained varied with the state of vacuolation of the amoebae. Amoebae showing few vacuoles when observed under normal light were uniformly fluorescent while highly vacuolated amoebae showed discontinuous ("patchy") fluorescent patterns (Plate 2.10).

Perinuclear fluorescence: Mab 29.5 produced a unique fluorescent staining pattern consisting of spots around the position of the nucleus which was stained dark red by the counterstain (Plate 2.11). The spots appeared as blebs on the surface of the amoebae but in live IFAT experiments no staining was observed (see below). In cells in the process of division, showing two nuclei, fluorescent blebs were observed around both nuclei. In certain cells, that seemed to contain no nuclei, the fluorescent blebs were more generally distributed over the entire cell.

2.3.3 IFAT experiments using live amoebae

All Mabs were tested for their activity against live E. histolytica. Only 22.1, 22.6 and 29.6 gave positive results. Bright uniform surface fluorescence, characterised by a bright ring at the periphery of the cells, was observed with all three Mabs when the amoebae were maintained at < 4 °C (Plate 2.12). If, after the experiment, the amoebae were allowed to warm to room temperature a surface redistribution or "capping" of fluorescent material was observed (Plate 2.13). The fluorescence was, over a period of approximately 5 min, concentrated on one area of the cell surface and eventually shed in some cases, while in other amoebae the fluorescent material appeared to become internalised. As in experiments using fixed amoebae, Mab 22.1 appeared to produce almost 100% fluorescence while 22.6 and 29.6 stained only a lower percentage of cells, with non-fluorescent cells and occasional faintly fluorescent cells observed. Accurate counts of positive and negative cells were not performed.

2.3.4 IFAT experiments using methanol-fixed cloned amoebae

Methanol-fixed amoebae derived from two clones of strain 200:NIH were used in IFAT experiments with Mabs 22.6 and 29.6. In each case a heterogeneous staining pattern was observed. As with original 200:NIH, some cells were brightly fluorescent (characteristic surface fluorescence), some were negative, while a small number showed an intermediate pattern. In experiments with 22.6 the majority of cells were fluorescent while with 29.6 the majority of cells were negative. The same was true for both clones. Too few cells were observed to enable accurate counting of positive and negative cells.

GUPM NO.	SUBCLASS	FLUORESCENT PATTERN IN IFA EXPERIMENTS USING:	
		METHANOL FIXED AMOEBAE	LIVE AMOEBAE
22.1	G2b	SURFACE (%)	SURFACE (%)
22.3	G1	INTERNAL; GRANULAR	-
22.4	G1	INTERNAL; GRANULAR	-
22.5	G1	INTERNAL; VESICLES	-
22.6	M	SURFACE (%)	SURFACE (%)
22.7	G1	INTERNAL / SOME INT. P.M.	-
22.8	G1	INTERNAL; DIFFUSE	-
24.1	G1	INTERNAL; GRANULAR	-
24.2	G1	INTERNAL; DIFFUSE	-
24.3	G1	INTERNAL; GRANULAR	-
24.4	G1	INTERNAL; DIFFUSE	-
24.5	G1	INTERNAL; GRANULAR	-
24.6	G2a	INTERNAL; DIFFUSE	-
24.7	G1	INTERNAL; GRANULAR	-
24.8	G1	INTERNAL; GRANULAR	-
29.3	G1	INTERNAL; DIFFUSE	-
29.5	M	INTERNAL;PERINUCLEAR	-
29.6	M	SURFACE (%)	SURFACE (%)
29.9	M	INTERNAL; GRANULAR	-
29.10	G1	INTERNAL; GRANULAR	-
29.11	G1	INTERNAL; GRANULAR	-

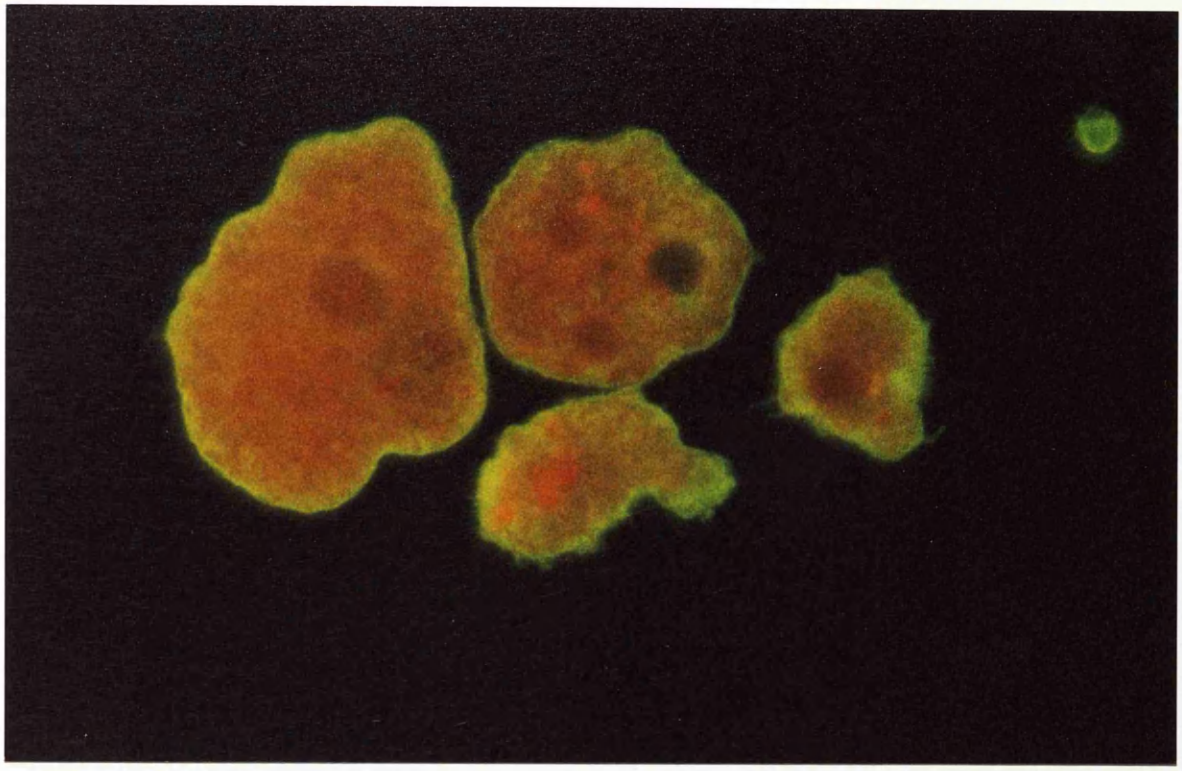
TABLE 2.1: IMMUNOGLOBULIN SUBCLASSES OF MONOCLONAL ANTIBODIES PRODUCED AND FLUORESCENT PATTERNS OBTAINED IN IFA EXPERIMENTS USING METHANOL-FIXED AND LIVE E. HISTOLYTICA (STRAIN 200:NIH)

ABBREVIATIONS: % = NOT ALL CELLS POSITIVE
INT. P.M. = INTERNAL PLASMA MEMBRANE

Green fluorescence was apparent for all positive results.

PLATE 2.1 Photomicrograph. E. histolytica, methanol-fixed (x 1000). Indirect fluorescent antibody test using GUPM 22.1. Filopodia visible on cell on right.

PLATE 2.2 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 22.6.



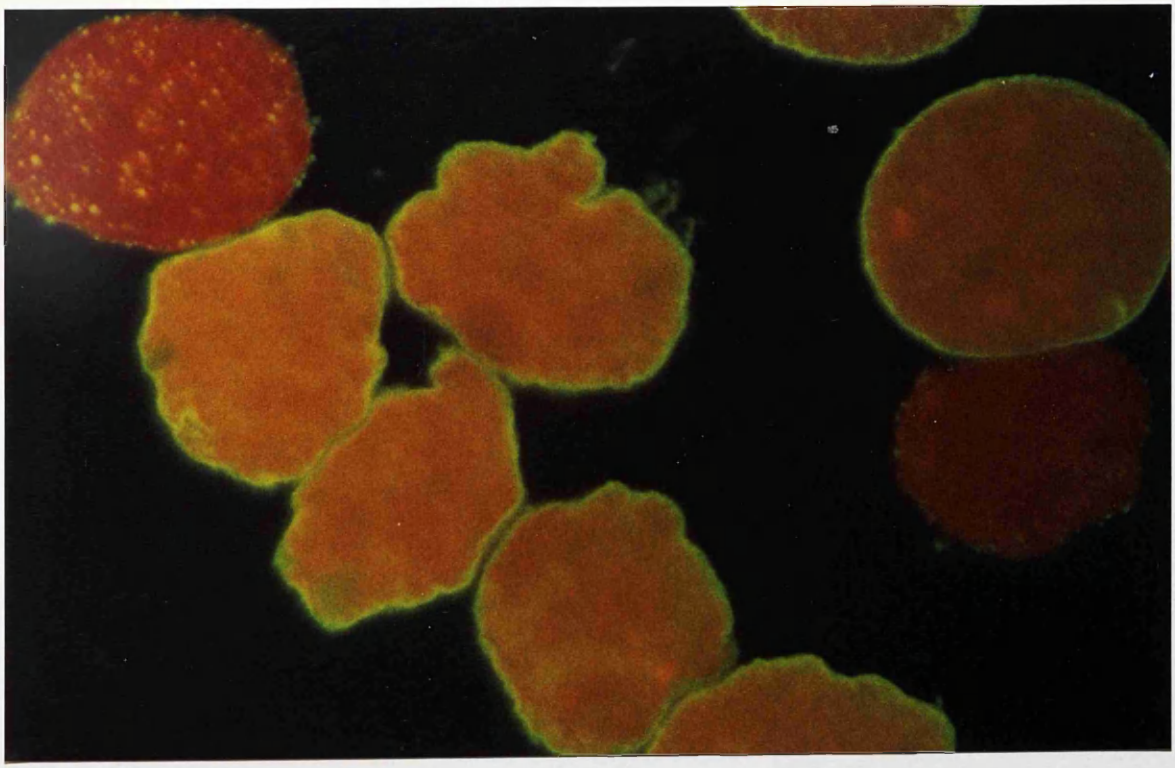
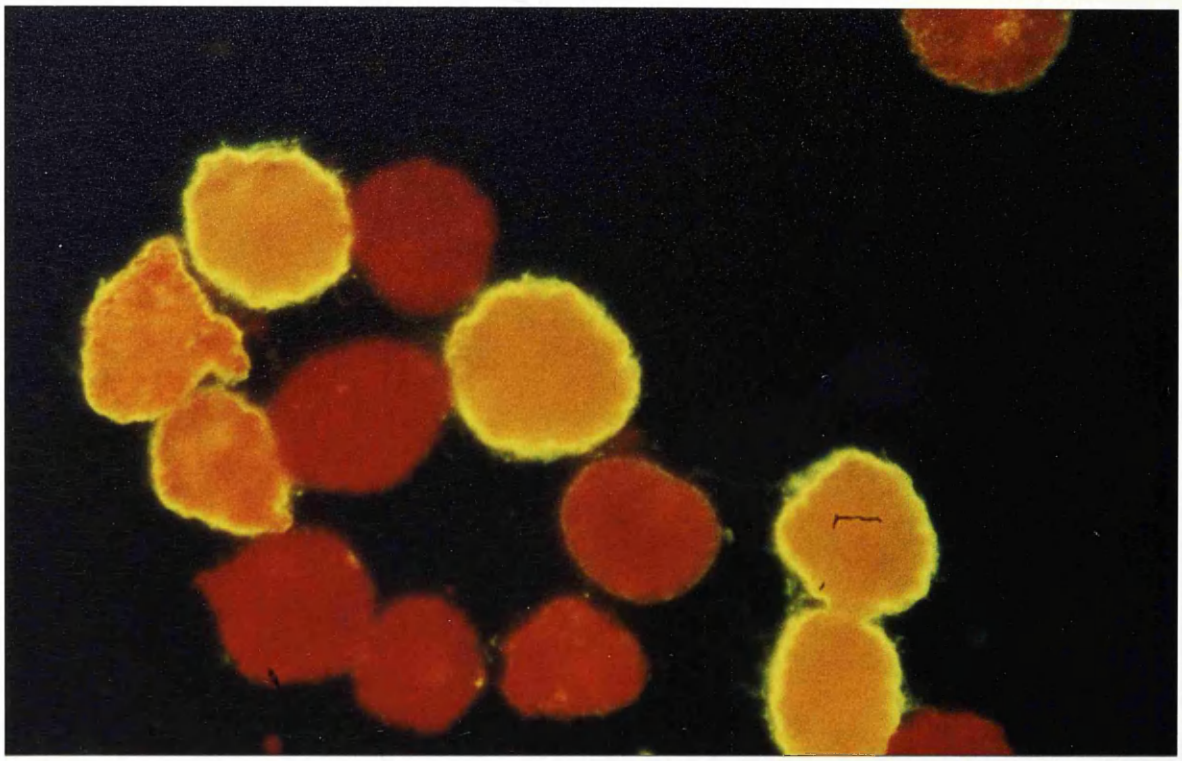


PLATE 2.3 Photomicrograph. E. histolytica, methanol-fixed (x 500). IFAT using GUPM 22.6.

PLATE 2.4 Photomicrograph. E. histolytica, methanol-fixed (x 500). Normal light, same field as Plate 2.3.



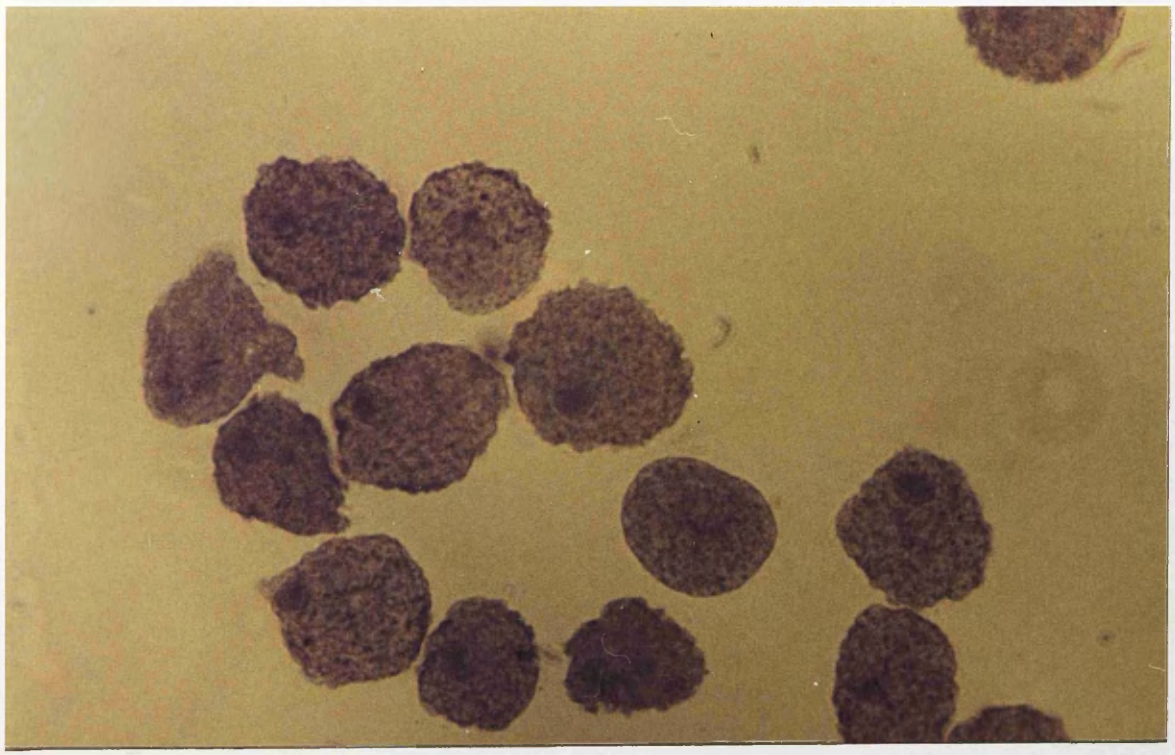
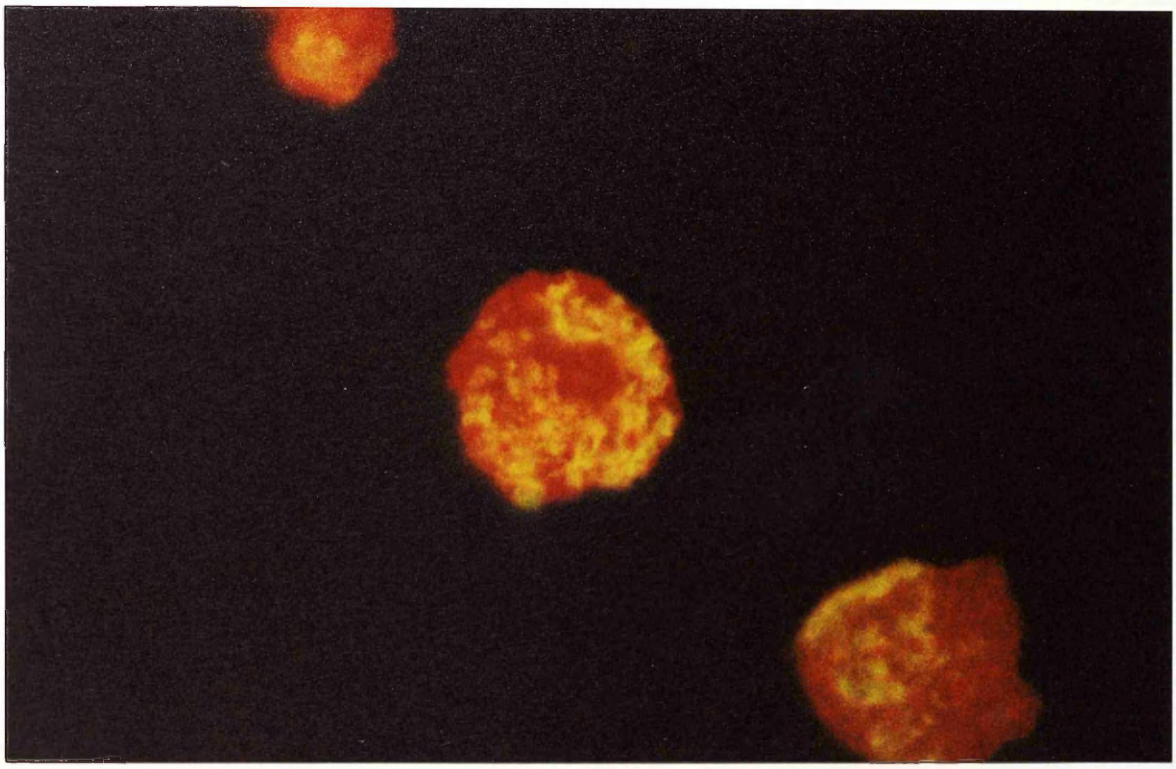


PLATE 2.5 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 22.5.

PLATE 2.6 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 22.7.
Lower cell-negative, centre cell-marginal fluorescence.



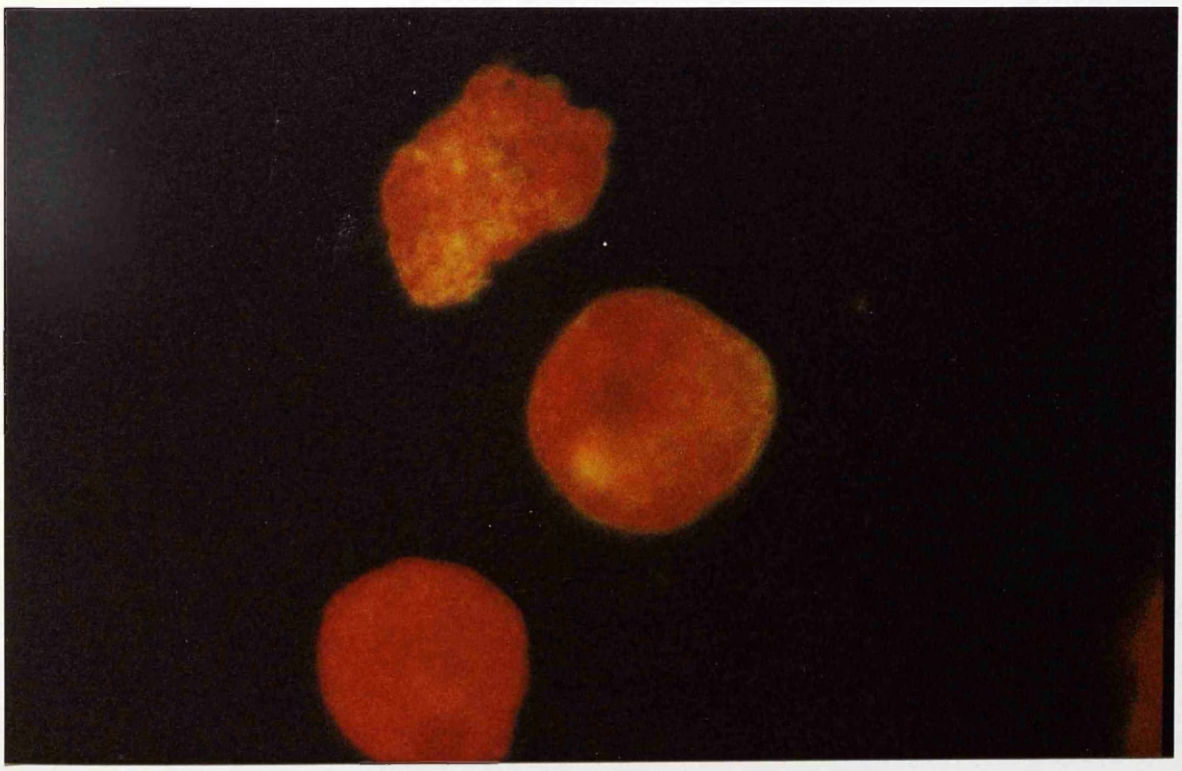
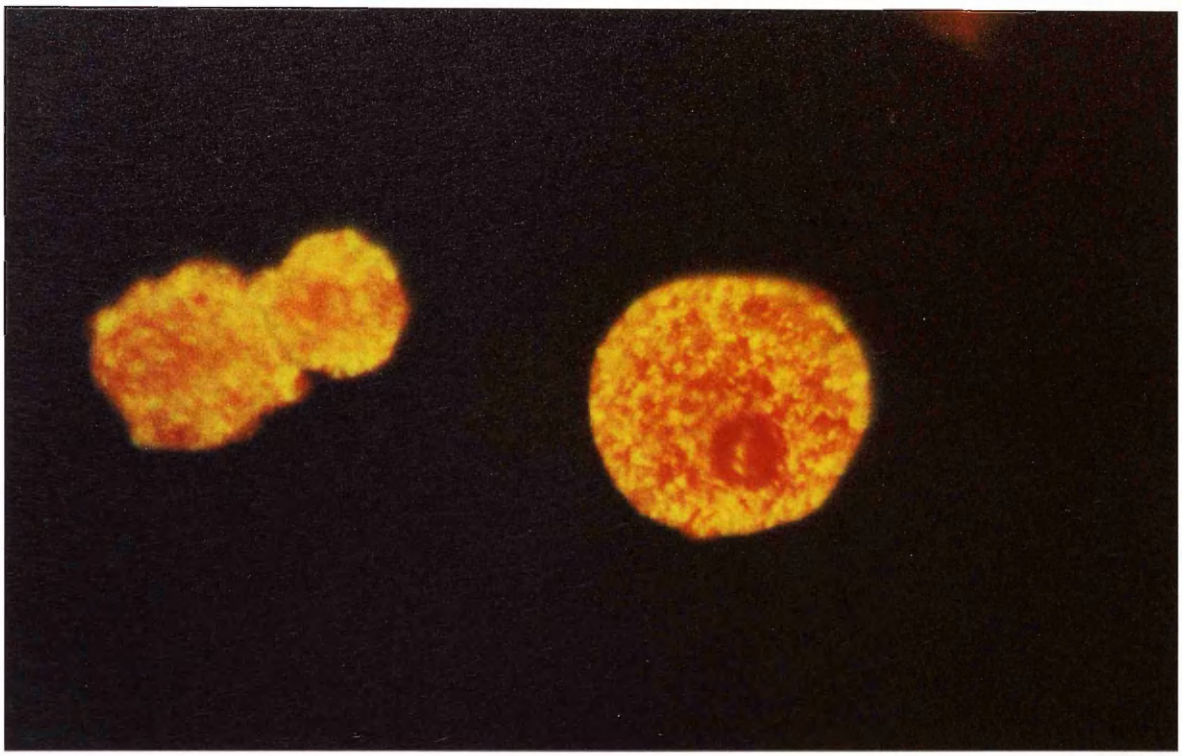


PLATE 2.7 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 24.7.

PLATE 2.8 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 22.4.



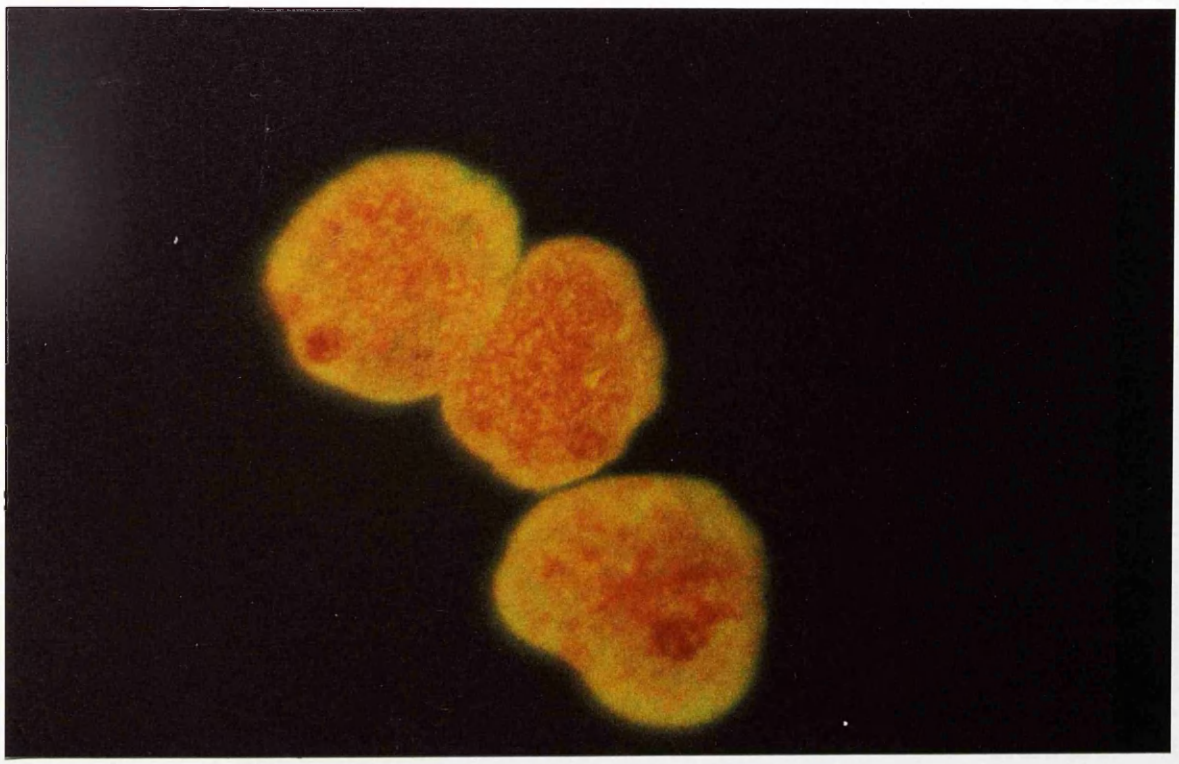
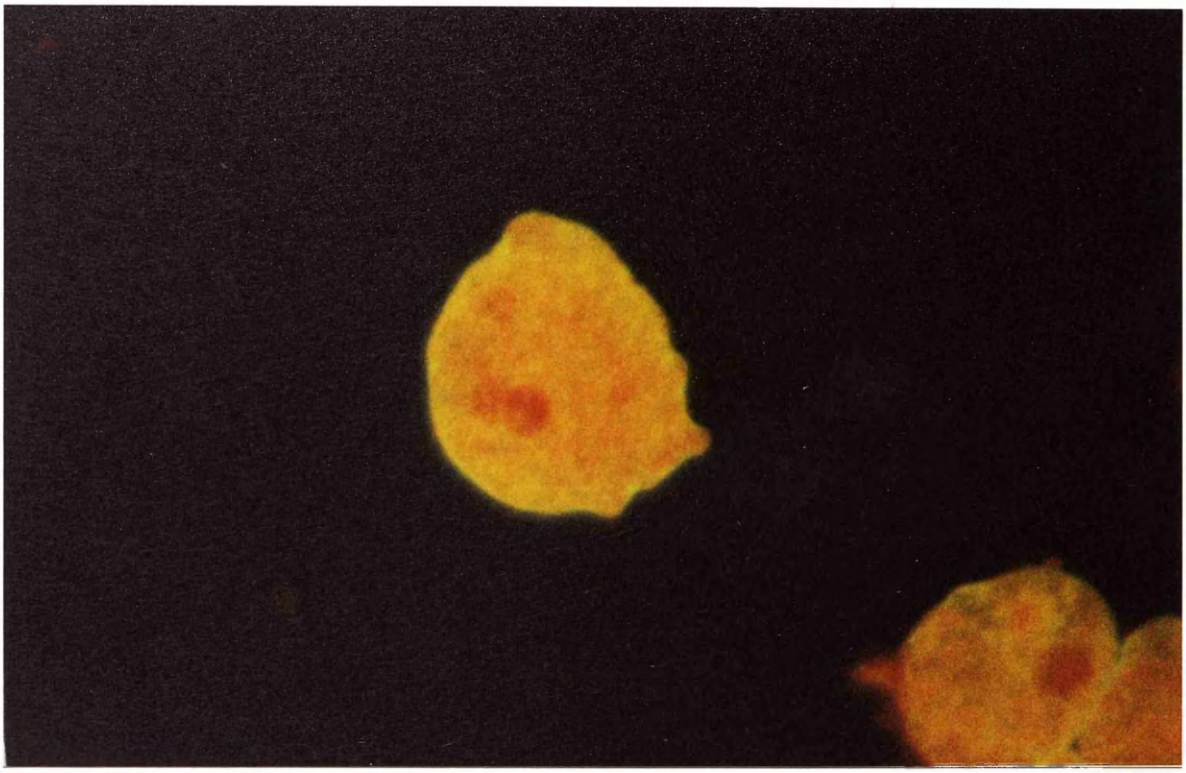


PLATE 2.9 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 24.6.

PLATE 2.10 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 24.2.
Lowest cell showing uniform fluorescence. Cell on left showing discontinuous ("patchy") fluorescence.



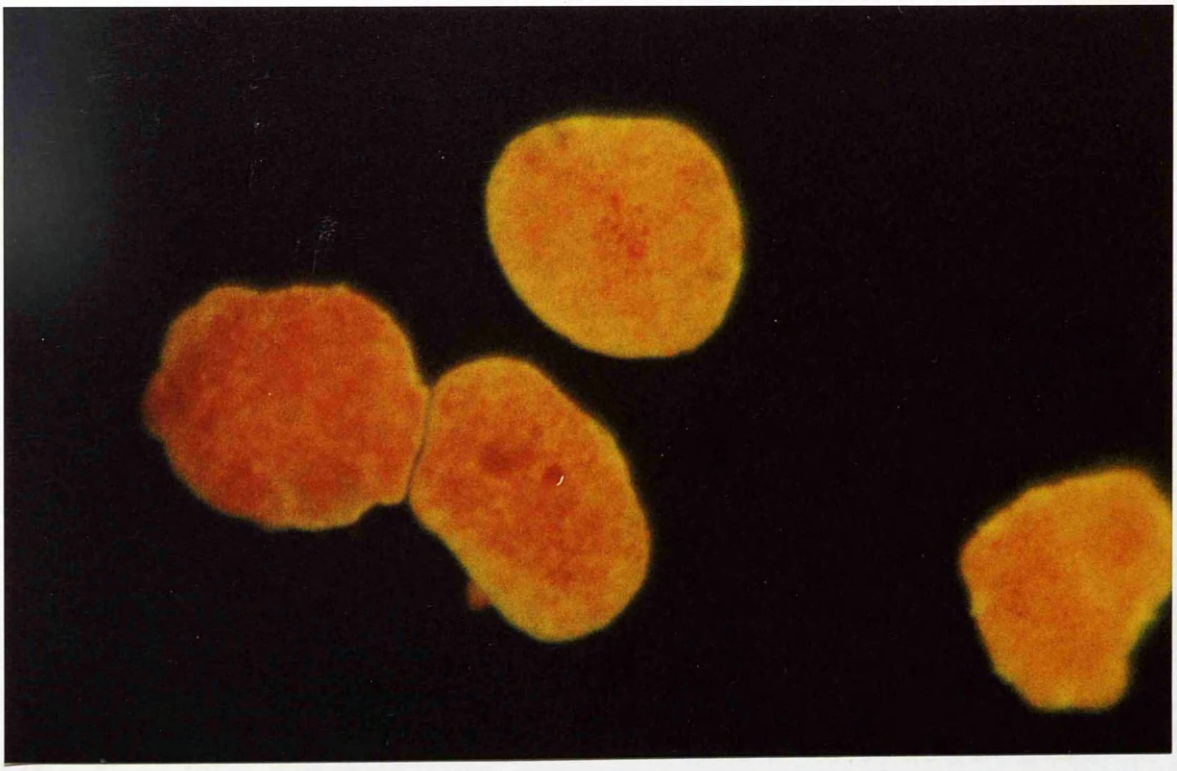


PLATE 2.11 Photomicrograph. E. histolytica, methanol-fixed (x 1000). IFAT using GUPM 29.5.
Larger cell possessing two nuclei. Smaller cell possessing one nucleus.

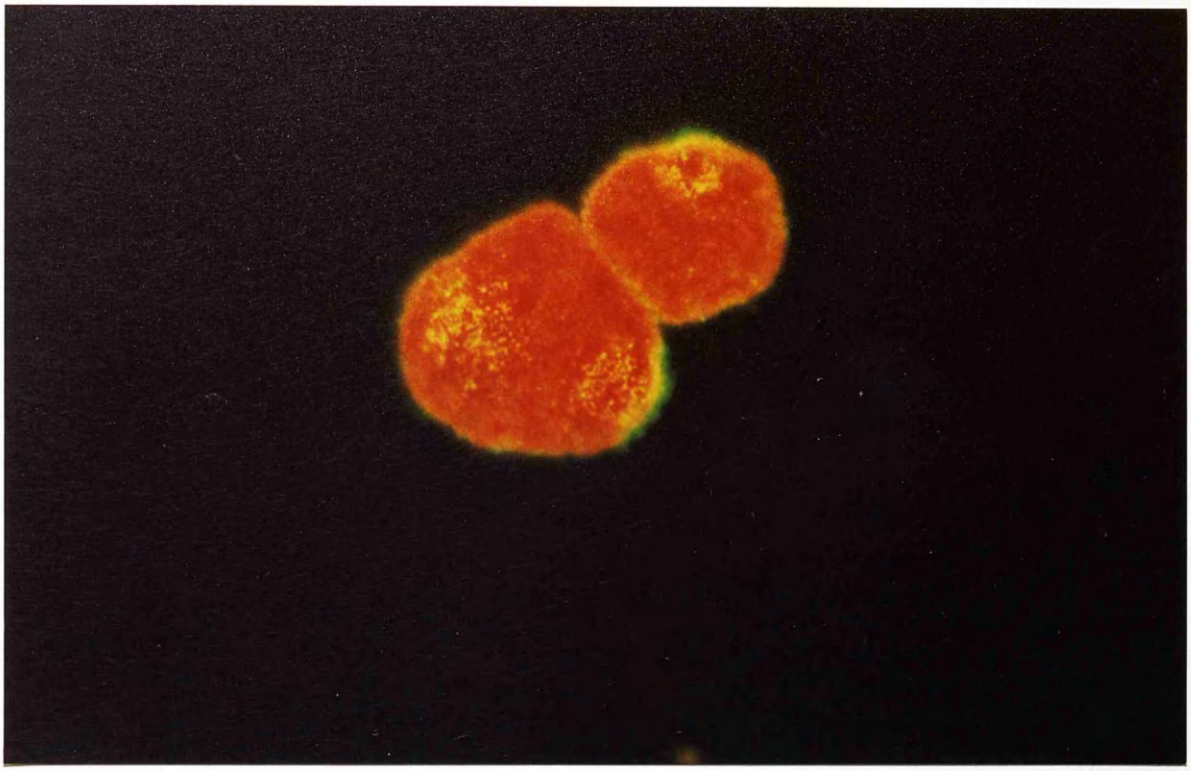
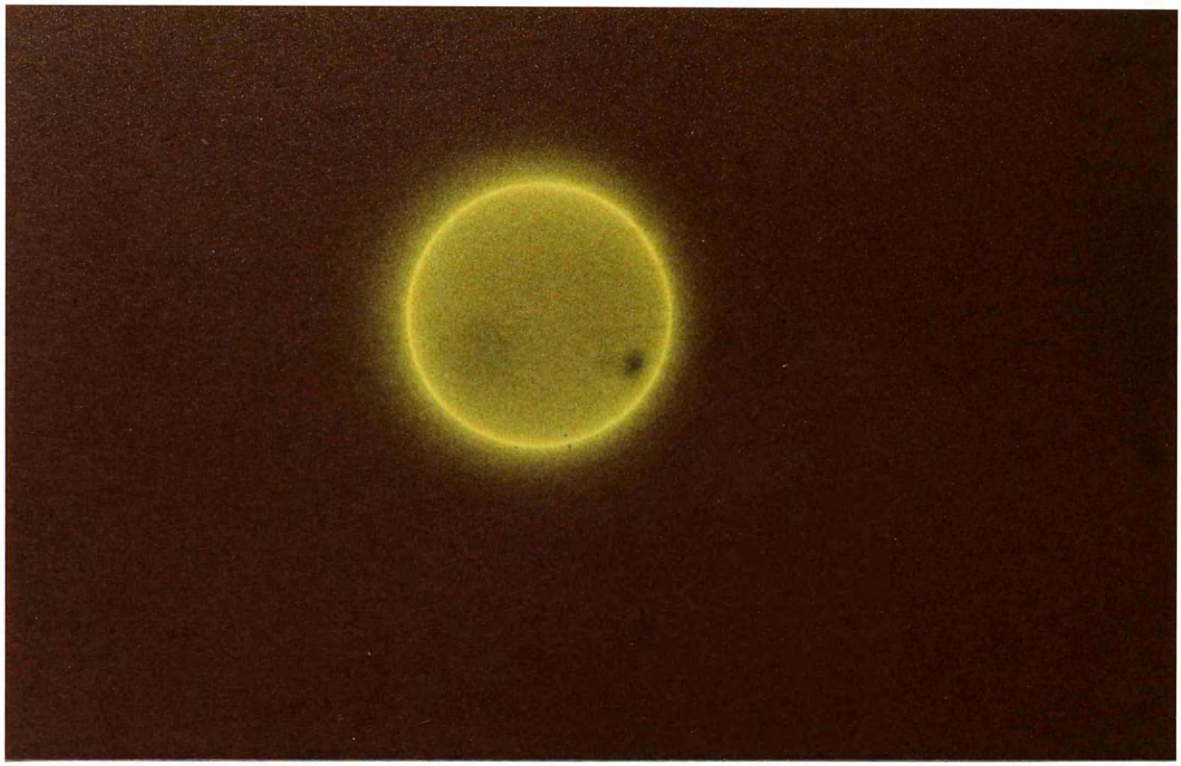
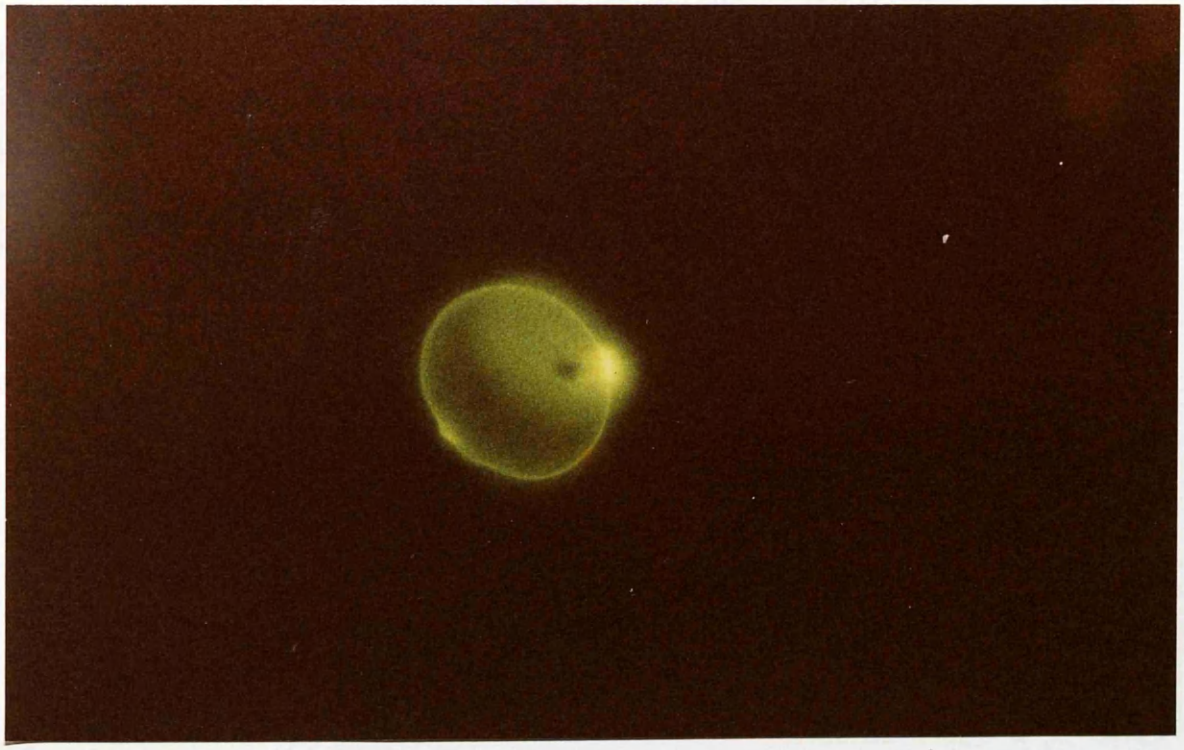


PLATE 2.12 Photomicrograph. E. histolytica, live cell (x 1000).
IFAT using GUPM 22.1.
Cells maintained at 4 °C.

PLATE 2.13 Photomicrograph. E. histolytica, live cell (x 1000).
IFAT using GUPM 22.1.
Cells warmed to room temperature. Concentration of fluorescent material on right-hand side of amoeba.





2.4 DISCUSSION

Monoclonal antibodies produced by 21 mouse hybridomas were used to localise antigens of Entamoeba histolytica. The incidence of the various immunoglobulin classes and subclasses among the monoclonals approximately reflected the subclass composition of normal mouse serum immunoglobulin, as expected (Goding, 1986). The preponderance of IgG_1 noted here was similar to that found by other authors preparing a series of monoclonal antibodies against whole protozoan cells screened by a similar indirect fluorescent antibody technique (IFAT) (Hall et al., 1983; Ravdin et al., 1986).

The three methods most commonly used for hybridoma supernatant screening are the Indirect Fluorescent Antibody Test (IFAT), primary binding Enzyme-linked Immunosorbent Assay (ELISA) and primary binding Radioimmunoassay (RIA). Of these, IFAT is generally considered most efficient since it does not select for Mabs directed only against antigens which are capable of non-covalently adsorbing to the polystyrene microhaemagglutination plates used in ELISA tests, although Voller et al. (1976) state that lysed sonicated E. histolytica material does adsorb satisfactorily. However, in one recent study involving E. histolytica where both IFAT and ELISA were used to screen hybridoma supernatants, 24 of the 35 positive supernatants were positive by ELISA while 19 were positive by IFAT and 8 positive by both methods (Ravdin et al., 1986). In the work presented here IFAT was used for primary screening since the equipment was available and routinely used in this laboratory and because this method yielded immediate information on the localisation of the antigens concerned.

Since the monoclonal antibodies were raised against whole trophozoites they were expected to recognise a number of different antigens. Five groups of antibodies have been distinguished by the subcellular locations of their corresponding antigens determined by IFAT staining.

One group of three Mabs was directed against exposed surface antigens confirmed by fluorescence in IFAT experiments using live amoebae. The cell surface of E. histolytica consists of, the surface coat and the plasma membrane, both of which contain antigenic material. The surface coat or glycocalyx of axenically-grown E. histolytica appears, in electron microscope studies using osmium tetroxide and ruthenium red fixation, to consist of short filaments, 6-10 nm in length (Martinez-Palomo, 1982) though the glycocalyx itself can be 20-30 nm thick on bacteria-associated trophozoites isolated from intestinal lesions (Lushbaugh and Miller, 1974). The nature of the coat material is largely unknown but, based on ruthenium red and alcian blue staining, the presence of mucopolysaccharides has been claimed (Feria-Velasco et al., 1972; Lushbaugh and Miller, 1974). The coat may contain exposed mannose or glucose residues since it binds the lectin concanavalin A (Con A) (Martinez-Palomo et al., 1973).

Following interaction with specific molecules (both lectins and antibody), surface coat components redistribute and are accumulated at the uroid (Calderon et al., 1980) whence they are endocytosed or released into the medium. In indirect immunofluorescence experiments using patient sera and a fluorescein-conjugated anti-human antiserum Aust-Kettis et al. (1981) found that 34% of the material was internalised while 56% was released, most of that as antigen-antibody complexes. In contrast, using FITC-conjugated human patient sera in direct immunofluorescence experiments the amoebae tended to shed the material, without capping, over a longer period of 3-4 h. Since cross-linking and redistribution of a single layer of antibodies relies on the multivalency of the antigens (Aust-Kettis and Sundqvist, 1978) the authors concluded that because a double layer of antibodies was required for capping, the antigens possess only one determinant. This would seem unlikely since the serum used was polyspecific. However Calderon et al (1980) were able to induce capping with a single layer of antibodies using a polyspecific antiserum at higher concentration.

This same capping phenomenon was observed, in this work, using Mabs 22.1, 22.6 and 29.6 in live indirect immunofluorescence experiments. The Mabs were not used in direct immunofluorescence experiments (ie. directly conjugated to fluorescent compounds). However since monoclonal antibodies are by definition monospecific they would not be expected to induce capping in such experiments unless the antigen concerned contained repeating epitopes. Con A-induced capping mimics that achieved with a double layer of antibodies since the antigens concerned contain multiple ligand molecules (mannose or glucose) (Aust-Kettis and Sundqvist, 1978). The shedding or internalisation of surface antigen-antibody complexes and also the constant release of surface coat molecules into the extracellular environment (Pinto da Silva et al., 1975) have been proposed as possible methods of interfering with the effective mediation of humoral and cellular immune mechanisms against invading amoebae (Aust-Kettis and Sundqvist, 1978; Martinez-Palomo, 1982).

The plasma membrane, with which the surface coat molecules are inextricably linked, is a highly dynamic structure. When grown in vitro, the amoebae exhibit a high rate of pinocytosis (Reeves, 1984), thought to be the main method of solute internalisation since facilitated diffusion appears only to exist for glucose (Reeves, 1984) and pyrimidines (Booden et al., 1978). The rate has been reported to be between 6 and 9 $\mu l min^{-1} ml^{-1}$ (Serrano and Reeves, 1975) which Gitler and Mirelman (1986) extrapolate to suggest that the amoebae internalise their total surface membrane approximately every 20 min, and that the plasma membrane is rapidly recirculated. The plasma membrane contains antigenic proteins (Aust-Kettis et al., 1983; Parkhouse et al., 1978) as well as a highly immunogenic aminophosphoglycolipid (Gitler et al., 1984) and a chemically distinct lipophosphopeptidoglycan (Rivera et al., 1985) which nevertheless cross-reacts with purified antilipid antibodies (Gitler and Mirelman, 1986). Such antilipid antibodies were also shown to induce rapid capping in live amoebae (Gitler and Mirelman, 1986).

The plasma membrane proteins of axenically grown E.histolytica have recently been well characterised by Rosenberg and Gitler (1985) who recognised a total of 34 proteins separated by gel electrophoresis, 16 of which are exofacial as determined by labelling with lactoperoxidase (Hubbard and Cohn, 1975). Monoclonal antibodies 22.1, 22.6 and 29.6 may be directed either against antigens (protein or carbohydrate moieties) of the surface coat or against antigens (protein, carbohydrate or possibly even lipid moieties) of the plasma membrane. Further characterisation of these antigens will be considered in Chapter 5. It is also possible that the antigens against which these Mabs are directed are medium components which have adhered to the surface of the amoebae (Noya-Gonzalez et al., 1980). This seems unlikely since Entamoeba invadens, a related species pathogenic in reptiles, grown in identical medium gave negative results in IFAT experiments (see Chapter 3) although it is possible that adherence mechanisms specific to E. histolytica exist.

Since this work was performed there have been several reports of monoclonal antibodies raised against E. histolytica, some of which describe their use in IFAT experiments. Previously, Lopez et al. (1982) had created 15 Mab-secreting stable mouse hybridomas, immunising with sonicated whole amoebae and screening by ELISA. No further characterisation was performed. Ximenez et al. (1985) used a membrane preparation (Aley et al., 1980) to immunise and produced 6 hybridomas screened by ELISA. One of these was used in IFAT experiments giving characteristic surface fluorescence using live amoebae fixed, post-experiment, in formaldehyde. Torian et al. (1987), immunising with live amoebae, further characterised Mabs produced by 7 hybridomas selected from a total of 20 created cell lines. Using formalin-fixed amoebae all 7 produced patterns characterised by an even distribution of fluorescence over the entire body of the amoebae. Only 3 of the 7 produced similar fluorescent patterns using live amoebae. Ravdin et al. (1986a) produced 19 Mabs giving positive results in IFAT experiments using acetone-fixed cells but gave no details of fluorescence patterns observed. Most recently

Meza et al. (1987) immunising with a known amoeba surface protein produced Mabs which induced capping phenomena in IFAT experiments using live amoebae.

None of the above authors report the heterogeneity of fluorescence among amoebae that was observed in IFAT experiments using the surface-specific Mabs in this work. However this phenomenon has been reported by Aust-Kettis and Sundqvist (1978) using infected patient sera in direct and indirect immunofluorescence experiments. Antigen expression was found to vary with the cell cycle, being at a minimum during logarithmic growth and reaching a maximum during the stationary phase, suggesting that a metabolically active amoeba has low cell surface antigenicity (Aust-Kettis and Sundqvist, 1978). Another possible explanation, however, might be that prolonged culture of the amoeba (since 1949 for strain 200:NIH used both by these authors and in this work) had resulted in disappearance or alteration of certain antigens, as suggested by Bos (1978), such that antigens were presented during log phase that were not recognised by the patient serum. It may also be possible that during logarithmic phase there is increased adherence of serum components to the amoeba surface (Noya-Gonzalez et al., 1980). It is not known whether such variations in antigen expression occurs in vivo but, if so, they could contribute to evasion from immune attack by the parasite (Cohen, 1982).

The heterogeneity of surface antigen expression among cultured amoebae reported in this work did not seem to be a stable property of the cells concerned since cloned lines also exhibited heterogeneity. Time-sampling cultures were not performed to determine whether the heterogeneity had a cell cycle basis, but there was no obvious correlation between fluorescence and cell size which suggests that the phenomenon may have a different basis. Heterogeneity in single antigen expression within cultured isolates of Trichomonas vaginalis, using a monoclonal antibody, has been reported (Alderete et al., 1986a). Using the Mab in IFAT and flow cytofluorometric experiments

it was found that some isolates showed no reaction and remained negative during extended culture while others were heterogeneous (Alderete et al., 1986b). In a finding similar to that presented here for Entamoeba, these authors observed that clones of positive T. vaginalis cells (separated by Fluorescence Activated Cell Sorting - FACS) became negative or heterogeneous. This occurred over extended culture and the authors concluded that the perturbations were not coupled to single cell cycle events. It was also reported that Mab reactivity corresponded with patient serum reactivity, tested by antibody-dependent complement mediated lysis, suggesting that there is some coordination of antigen expression (Alderete et al., 1987) both in vitro and in experimental subcutaneous infections in mice. Interestingly, cells showing no Mab reactivity demonstrated an enhanced cytotoxic effect on cultured HeLa cells in vitro.

It would be interesting to determine whether such coordination in phenotypic variation is occurring in E. histolytica surface antigen expression. Relevant experiments would include detailed study of synchronised cloned cultures (Austin and Warren, 1983) using directly FITC- and TRITC-(tetramethylrhodamine isothiocyanate) conjugated Mabs to study, simultaneously, the expression of the antigens recognised by both 22.6 and 29.6 in individual cells. Preliminary efforts to prepare cells for use in FACS (results not shown) were hampered by the fragility of the cells. Correlation with cytopathogenicity could also be examined since an effective in vitro assay has been developed (Ravdin et al., 1980). Antigenic variation in vitro and in vivo has also been reported in Giardia lamblia (Nash et al., 1988; Aggarwal and Nash, 1988). When clones of an isolate possessing a particular surface antigen were exposed to an antigen-specific cytotoxic monoclonal antibody, a small number of trophozoites expressing new surface antigens survived. Exposure of these trophozoites to a cytotoxic Mab against one of these, resulted in the survival of G. lamblia possessing another set of antigens. The antigenic variation described in these anaerobic protozoa (E. histolytica, T. vaginalis and G. lamblia) is not as profound as the extensively

studied antigenic variation of the variant surface glycoproteins of the genus Trypanosoma (reviewed by Vickerman and Barry, 1982). Unlike African trypanosomes which possess a surface coat composed of one antigen, the exposed surface of these anaerobes consists of a more complex mixture of antigens. Antigenic variation in trypanosomes and in bacteria of the genus Borrelia (Kehl et al., 1986) is known to prolong infection by evading host immune mechanisms.

In order to investigate the functional significance of the surface antigens, against which the Mabs are directed, various further experiments could be performed:
(a) Use of the Mabs in in vitro assays to determine whether or not they can cause immobilisation (Zaman, 1960) or classical complement mediated lysis of trophozoites (Aust-Kettis and Utter, 1984).
(b) Use of the Mabs in in vitro cytotoxicity assays to determine whether or not they are capable of inhibiting the adherence of trophozoites to cultured cells or the cytotoxic effect of the trophozoites on these cells. Arroyo and Orozco (1987) and Meza et al. (1987) have independently produced Mabs, against two different lectin-like surface molecules, which partially inhibit amoebic adherence to cultured cells in vitro. Ravdin et al. (1986a) have also produced such inhibitory Mabs which appear to be directed against an amoebic adherence lectin (Petri et al., 1987b).

Since surface-exposed antigens are those most likely to be shed and stimulate the immune system (Cox, 1968) and induce effector mechanisms possibly lethal to the parasite (Trissl, 1982) and since the cytopathogenic effect of the amoeba depends on surface contact, it is these antigens which have received most attention from investigators. However there is evidence to suggest that there are a larger number of more immunogenic antigens present in the cytoplasmic vesicle fractions than in plasma membrane fractions (Boonpucknavig et al., 1967). Later work has shown that a soluble cytoplasmic fraction contains more antigens recognised by human patient sera than either

of these membrane fractions (Mathews et al., 1986). Aside from their possible serodiagnostic value, the internal antigens of E. histolytica may be of interest due to their functional significance. Although the main cytotoxic event relies on target cell membrane/amoeba membrane adherence (Ravdin et al., 1980) the putative mediators of cytolysis and other processes in amoebic pathogenicity must have their origin within the parasite. Some of these factors may be in membrane-bound vacuoles such as proteinases (Lushbaugh et al., 1984), a collagenase (Muñoz et al., 1982), lysodiacyl-phospholipids produced by amoebic phospholipase A action (Long-Krug et al., 1985) and an, as yet unidentified, lysosomal constituent (Ravdin et al., 1986b). Others may be in aggregated states within the cytoplasm as is proposed for a powerful ion-channel forming protein described by Lynch et al. (1982).

Eighteen of the twenty-one Mabs produced in this work were directed against internal antigens, confirmed by negative results in IFAT experiments using live amoebae. The cytoplasm of E. histolytica has a rather undifferentiated appearance at both the light and electron microscope levels and consequently little detail could be seen of the fluorescent patterns obtained which were divided into four more or less distinct groups; vacuolar, diffuse, granular and perinuclear fluorescence. Ultrastructural studies of E. histolytica (Martinez-Palomo, 1982; Miller et al., 1961) and E. invadens (Deutsch and Zaman, 1959) show that the cytoplasm is devoid of many of the differentiated organelles found in most eukaryotic cells; mitochondria, golgi apparatus, centrioles, cytoplasmic microtubules or recognisable rough endoplasmic reticulum.

A large proportion of the cytoplasm is occupied by a heterogeneous population of membrane-bound vacuoles and vesicles, of variable size (0.5 μm - 9.0 μm) and shape though most appear circular in profile in thin sections. Two of the Mabs, 22.5 and 22.7, appear to be directed against components of this system. Considering the continuous rapid rate of endocytosis of this parasite (Serrano and Reeves, 1975) there

can be little doubt that a significant proportion of the vacuoles is derived from internalised plasma membrane. Freeze-fracture studies (Martinez-Palomo et al., 1976) demonstrate the existence of two morphologically distinct populations of cytoplasmic vacuoles: the most numerous having a smooth contour, while the others appear crenated. The significance of such differences is not known. The exact sequence of vacuole processing after internalisation is likewise unclear, but internalised fluid-phase markers are rapidly released, demonstrating rapid membrane recycling (Aley et al., 1984). Whereas lysosomes in other eukaryotic cells contain soluble acid phosphatase within the vesicle, in Entamoeba the enzyme is membrane-associated (Aley et al., 1980; Martinez-Palomo, 1982) although other enzymes, such as β-D-glucosaminidase occur in both membrane-associated and soluble forms (Rosenberg and Gitler, 1985). Primary lysosomes (containing hydrolases that have not yet been active) have not been clearly differentiated from secondary lysosomes although Aley et al. (1984) described small highly acidic vesicles which may be relevant.

The vacuoles recognised by Mabs 22.5 and 22.7 were of variable size and did not appear to be recently internalised membrane, as demonstrated by IFAT experiments using erythrocyte-fed trophozoites. With hindsight the amoebae should have been sampled at various times after erythrocyte phagocytosis to determine whether or not the membranes surrounding the ingested erythrocyte became fluorescent after a longer period. Such a result would have suggested that the vacuoles concerned are indeed lysosomal. The peripheral fluorescent pattern observed in occasional cells with Mab 22.7 did not appear to involve surface-exposed antigens since no fluorescence was observed in live IFAT. However, the number of cells showing this peripheral fluorescence using fixed amoebae, was so small that such cells may not have been present in the smaller sample observed in live IFAT. These observations show that the antigen for which 22.7 is specific is probably associated with the membrane of certain vacuoles and, in some cells, becomes associated with the plasma membrane, though whether it is exposed on either face of the membrane is uncertain.

The presence of lysosomal membrane-associated enzymes associated with the plasma membrane has been reported (Aley et al., 1980; Rosenberg and Gitler, 1985) and is thought to be due to membrane recycling (Gitler and Mirelman, 1986). Alderete et al. (1986a) found that while many isolates of Trichomonas vaginalis expressed a certain membrane antigen on their surface (recognised by a Mab in IFAT), some isolates synthesised the protein but did not express it. A similar situation may be occurring within subpopulations of the cultured E. histolytica used here. The fluorescent pattern obtained with 22.5 was always restricted to vacuoles within the cytoplasm. Since no plasma membrane fluorescence was observed, it is possible that the antigen concerned is located within the lumen of the vacuoles and is extruded from the cell as a vesicle/plasma membrane fusion occurs (Gitler and Mirelman, 1986). Alternatively these vacuoles might represent a population not involved in the lysosomal system, though considering their size and number this seems unlikely.

Two groups of Mabs were directed against cytoplasmic antigens apparently not associated with the vacuolar system. Indeed the presence of large numbers of vacuoles in some trophozoites used in IFAT experiments caused the cytoplasmic fluorescence to become locally more concentrated causing a coarser pattern in the case of Mabs producing granular fluorescence, and a less uniform pattern with those producing diffuse fluorescence.

Although the lack of observable detail in the cytoplasm makes it difficult to speculate on the nature of the antigens concerned, there are several more obvious features which are possible candidates; the tubular system, the cytoskeleton and ribosomal arrays.

A lattice of smooth tubules and vesicles, of approximately 20 nm in diameter is occasionally found in the cytoplasm of E. histolytica in thin sections and by freeze-fracture electron microscopy (Martinez-Palomo, 1982). The vesicles themselves are a more common occurence and have a membrane thickness of 6 nm compared with that of

the vacuolar and plasma membranes of 10 nm. It is possible that the granular patterns observed with some Mabs may be due to their recognising components of this system. It is thought that the system may be involved with synthesis, packaging and processing of extracellular amoebic products but there is no evidence to support this (Martinez-Palomo, 1982).

In spite of the detailed ultrastructural studies that have been performed, very little is known about the structural organisation of the cytoskeleton of E. histolytica. Actin filaments are the only filaments to have been identified in the cytoplasm with certainty, by electron microscopy (Martinez-Palomo et al., 1974) and using immunocytochemistry by Aust-Kettis et al. (1987) who, using an anti-human actin antiserum, produced a "star-like" IFAT pattern with brightest fluorescence at the centre of the cell or more occasionally patterns consisting of fluorescent "bunches or whirls". Such a pattern was not obtained with any of the Mabs produced in this work.

In ultrastructural studies most ribosomes of E. histolytica are not observed free in the cytoplasm or membrane-associated but appear in ordered helical arrays approximately 300 nm in length and 40 nm in diameter. The helices themselves are frequently found in association with membrane systems within the cytoplasm (Rosenbaum and Wittner, 1970). Antibodies against protein constituents of these ribonucleoprotein bodies might be expected to produce granular fluorescence patterns in IFAT experiments.

Mab 29.5 produced a unique pattern of apparent surface blebs in IFAT experiments using fixed amoebae but gave no fluorescence using live cells, indicating that the antigen concerned may be located on the internal face of the plasma membrane. Where a nucleus was present, such blebs were consistently associated with the nuclear region while in the absence of a nucleus they had a more random distribution throughout the cell. This makes it tempting to speculate that the antigen concerned might be related to cytoskeletal specialisations on

the internal surface of the plasma membrane involved in anchoring the nucleus to the rest of the cytoskeleton. Little is known of the method of nuclear division in E. histolytica but that it occurs without dissolution of the nuclear membrane (Cervantes and Martinez-Palomo, 1980). Presumably such a process requires substantial interaction with the cytoskeletal system. No structures resembling these blebs have been reported in ultrastructural studies of Entamoebae.

More accurate localisation of antigens using these monoclonal antibodies could be performed using colloidal gold in double or direct labelling immunoelectronmicroscopy of frozen sections of E. histolytica (Geuze et al., 1981; de May, 1983). This technique has been successfully used in the study of antigen expression in other parasites such as variant surface glycoprotein in Trypanosoma brucei (Tetley et al., 1987).

The 21 monoclonal antibodies produced here were used throughout the subsequent investigations.

CHAPTER 3 SPECIES SPECIFICITY OF MONOCLONAL ANTIBODIES PRODUCED

3.1 INTRODUCTION

3.2 MATERIALS AND METHODS

3.2.1 Cells

3.2.2 IFAT using fixed cells

3.2.3 IFAT experiments using live E. invadens

3.3 RESULTS

3.4 DISCUSSION

3.1 INTRODUCTION

Individuals undergoing immunodiagnostic tests for infection with Entamoeba histolytica, whether for clinical or epidemiological purposes, are frequently host to a number of other protozoan gastro-intestinal parasites. This is particularly true of groups experiencing a greater than average degree of faecal-oral route contact, such as homosexual men (McMillan, 1988) and the mentally retarded (Thacker et al., 1979). Of the various protozoa capable of inhabiting the human intestine, listed in Table 3.1, only seven are definitely known to cause disease; E. histolytica, Giardia lamblia, Balantidium coli, Isospora belli, Sarcocystis hominis, Sarcocystis suihominis and Cryptosporidium spp.

Since it was intended that the monoclonal antibodies produced in this work should be tested for their usefulness as immunodiagnostic reagents (see Chapter 6) it was decided to test their specificity for E. histolytica by checking for cross-reactivity with other protozoan parasites and other cultured cell types available.

Members of the genus Entamoeba Casagrandi and Barbagallo, 1895, occur as parasites of the digestive tract in a wide range of vertebrates although one species is found in invertebrates. In addition, one species, E. moshkovskii Tshalaia, 1914, appears to be free-living in sewage. The classification of the species within this genus is based on the number of nuclei in their mature cysts, either eight, four, or one (Neal, 1966). Examples of these groups are listed in Table 3.2. The division into four- or eight-nucleate cyst groups is also associated with a difference in nuclear morphology (in the position of the endosome and distribution of peripheral "chromatin") (Neal, 1966), though the one-nucleate cyst species are not distinguishable by nuclear structure alone.

TABLE 3.1: THE MORE COMMON INTESTINAL PROTOZOAN PARASITES OF MAN.
* denotes infection can cause disease symptoms

Amoebae	
	*Entamoeba histolytica**
	Entamoeba coli
	Entamoeba hartmanni
	Endolimax nana
	Iodamoeba bütschlii
Flagellates	
	Chilomastix mesnili
	Enteromonas hominis
	Retortamonas intestinalis
	*Giardia lamblia**
	Dientamoeba fragilis
	Pentatrichomonas hominis
Ciliates	
	*Balantidium coli**
Coccidia	
	*Isospora belli**
	*Sarcocystis hominis**
	*Sarcocystis suihominis**
	Cryptosporidium spp.*

Within the quadrinucleate-cyst amoebae there is a group of species which, at the light microscope level, are morphologically very similar: E. invadens Rodhain, 1934, E. histolytica, E. hartmanni von Prowazek, 1912, E. moshkovskii and E. histolytica-like amoebae. These last, also known as Laredo-type amoebae, include strains isolated from humans that substantially differ from most E. histolytica in several physiological attributes but have not been granted separate species status (reviewed by Martinez-Palomo, 1982).

These species/types can be distinguished by various features apart from host/habitat and pathogenicity; E. hartmanni cysts are consistently smaller than E. histolytica cysts and have distinct nuclear morphology (Burrows, 1964). E. invadens, E. moshkovskii and "E. histolytica-like" amoebae are characterised by their ability to grow well at 27 °C (Richards et al., 1966; Neal, 1966) and their tolerance of a hypotonic medium (Richards et al., 1966; Goldman, 1969). Studies comparing E. histolytica with the other amoebae of the mophologically similar group have revealed specific differences in isoenzyme patterns (Reeves and Bischoff, 1968; Sargeaunt et al., 1980b) and in antigenic composition (Goldman, 1969; Krupp, 1966).

The Mabs were tested for their ability to recognise antigens in various members of the genus Entamoeba in IFAT tests. It was hoped that such experiments would yield information on the antigenic relatedness of these species to E. histolytica, which might be extrapolated to examine phylogenetic relationships within the genus.

No. of Nuclei in Cyst	Species	Host/Habitat
8	E. coli	man
	E. gallinarum	domestic fowl
	E. muris	rats, mice
	E. baretti	turtles
4	E. histolytica*	human
	E. histolytica-like	human
	E. hartmanni	human
	E. moshkovskii	in sewage
	E. invadens*	reptiles
	E. terrapinae	turtles
	E. knowlesi	tortoises
	E. pyrrhogaster	salamanders
	E. ranarum	amphibians
	E. philippinensis	roach, (Panesthia javanica)
	E. aulostomi	leeches (and other invertebrates)
1	E. bovis	cattle
	E. polecki	pigs, man
	E. suis	pigs
	E. ovis	sheep
No cyst formation	E. gingivalis	man, oral cavity
	E. canibuccalis	dog, oral cavity
	E. equibuccalis	horse, oral cavity

TABLE 3.2 Species of Entamoeba, showing host/habitat and number of nuclei in mature cyst. For octonucleate, uninucleate and non-cyst-forming groups not all known species are shown.

* denotes pathogenic

3.2 MATERIALS AND METHODS

3.2.1. Cells

E. invadens: Isolate GU had been maintained in this Department for several years although the original source of the isolate is not known. Isolates TRM, ZM, TR1 and TR2 were obtained from Dr D.C. Barker, Molteno Institute, University of Cambridge. TRM, TR1 and TR2 were isolated in Madagascar in 1963 from different individuals of Testudo radiata. ZM was also isolated in Madagascar in 1963 from Charanodon madagascarensis. These isolates were all maintained at 25 °C in Jones' Horse Serum and Marmite (JHSM) medium containing 5% heat inactivated horse serum (Gibco, Paisley) and supplemented with rice starch (Jones, 1946). Isolate LSH was obtained from Dr Ann Chayen, Department of Medical Protozoology, London School of Hygiene and Tropical Medicine (LSHTM), London, although the original source is not known. This isolate was maintained in axenic culture in TYI-S-33 medium at 25 °C.

E. moshkovskii: An isolate of E. moshkovskii (SAW 698) was provided by P.G. Sargeaunt, Department of Medical Protozoology, LSHTM. It was isolated from sewage in Mexico City in 1979 and had been maintained in Robinson's Medium at 37 °C (Robinson, 1968). E. coli Grassi, 1879, and E. hartmanni were obtained from P.G. Sargeaunt, LSHTM, both as constituents of isolate MH 661, isolated from a male homosexual patient at the Middlesex Hospital, London in 1986. This isolate was maintained in Robinson's Medium.

E. muris Grassi, 1879, was obtained fresh from the intestine of a naturally infected Balb/c mouse. The mouse was killed by gentle cervical dislocation and the abdomen opened. The contents of the colon and caecum were removed, mixed with an equal volume of PBS (pH 7.4) and sieved through an Endecott filter (mesh size 250 μm) to remove large particles. The filtrate was centrifuged at 200 *g* for 3 min (RT) and the pellet resuspended and washed again in PBS.

The final pellet was resuspended in a small volume of PBS containing 10% horse serum.

Pentatrichomonas hominis Davaine, 1860, was provided by Dr A Yule, Department of Medical Protozoology, LSHTM, and grown in modified Diamond's medium (Diamond, 1957).

Trichomonas vaginalis Donné, 1836, was provided by Dr G H Coombs of this Department, and grown in modified Diamond's medium.

Giardia lamblia Stiles, 1915, was provided by Dr H V Smith, Department of Bacteriology, Stobhill General Hospital, Glasgow and was grown in TYI-S-33 medium.

Trypanosoma (Trypanozoon) brucei rhodesiense: Bloodstream forms were provided by Dr A J Scott of this Department. The cells were obtained from infected mouse blood following Diethylaminoethyl (DEAE)-cellulose ion-exchange chromatography (Lanham and Godfrey, 1970). Procyclic forms of the same species were provided by Cathy Cameron, of this Department, and grown in Minimal Essential Medium (MEM) with 10% Foetal calf serum.

Trypanosoma (Nannomonas) congolense Broden, 1904, bloodstream forms were provided by Frances Devaney, of this Department, and prepared from mouse blood as for T. brucei rhodesiense above. Procyclic forms of T. congolense were provided by Dr Kay A K Hendry, of this Department and were grown in MEM, as above.

Acanthamoeba castellani and Naegleria gruberi were provided by Cathy Cameron. A. castellani was grown axenically in Neff's medium (Neff, 1957) while N. gruberi was grown on non-nutrient agar with Klebsiella aerogenes as a food source.

Chinese Hamster ovary (CHO) cells were obtained from Flow Labs, Irvine and grown in Hams' F-12 medium with 10% FCS.

3.2.2 Indirect fluorescent antibody test

Cells grown in Robinsons' medium (E. hartmanni, E. coli and E. moshkovskii) were prepared for IFAT by removing a small quantity of liquid from the bottom of the agar slope by pipette, and gently mixing to form a suspension.

All other cell types apart from E. muris as previously described, were washed in PBS (pH 7.4), centrifuged at 200 g for 3 min and resuspended in a small volume of PBS containing 10% horse serum.

Drops of all cell suspensions were placed in wells of multispot slides (Hendley, Essex) and allowed to air-dry. Following fixation in methanol (5 min) the slides could be used immediately or stored desiccated at -22 °C.

Ascitic fluids containing Mabs were used diluted in PBS at two $\log_3$ dilutions above their endpoint obtained in IFAT titration experiments using E. histolytica strain 200:NIH as antigen. Slides of antigen were warmed to room temperature and 5 µl quantities of diluted ascitic fluids were placed in each well and the slides processed as previously described in Section 2.2.2.7. Control wells received 5 µl of a 1/100 dilution of normal mouse serum.

3.2.3. IFAT experiments using live E. invadens

IFAT experiments using live E. invadens isolate ZM were performed using Mabs 29.5 and 22.6 as previously stated for E. histolytica.

3.3 RESULTS

All 21 Mabs were used in IFAT experiments using methanol-fixed cells of all species and cell lines described in Section 3.2.1. The fluorescent patterns observed were recorded and are summarised in Table 3.3. The results of experiments involving N. gruberi, A. castellani, species of trypanosomes and CHO cells are not shown in the table. The results of IFAT experiments involving isolates of E. invadens are summarised in Table 3.4.

Two Mabs, 29.5 and 29.9, gave positive results with all cell types tested. Mab 29.5 produced perinuclear fluorescent patterns in most cells except N. gruberi and CHO cells where the fluorescence appeared to be localised beneath the plasma membrane. Only in other members of the genus Entamoeba was the perinuclear fluorescence similar to that observed in E. histolytica, consisting of apparent membrane "blebs". No fluorescent cells were observed in IFAT experiments using live E. invadens (strain ZM). Mab 29.9 produced internal granular fluorescent patterns in all amoebae tested (including N. gruberi and A. castellani) but more diffuse patterns in all flagellates and CHO cells.

Eight Mabs (22.1, 22.3, 22.5, 22.7, 22.8, 29.6, 29.10 and 29.11) gave negative results with all cell types tested.

Mab 22.4 reacted with all members of the genus Entamoeba, apart from E. hartmanni, and also reacted with P. hominis. In each case a granular internal fluorescence pattern was produced. Mab 22.6 gave positive results with four out of the six isolates of E. invadens tested (ZM, TRM, TR1 and TR2) but with no other cell type. In each case only a percentage of cells were fluorescent. Most positive cells showed specks of fluorescence on their surface while a minority showed typical membrane fluorescence as seen with

E. histolytica (Section 2.3.2). The external surface location of the antigen concerned was confirmed by positive results in IFAT experiments using live E. invadens (strain ZM). Mab 24.1 reacted with only one isolate of E. invadens (GU) giving internal granular fluorescence. It also reacted with T. brucei rhodesiense, both bloodstream and procyclic forms, with the flagellum being the only fluorescent part of the cell. Mab 24.1 did not react with T. congolense or any other flagellate tested. Mabs 24.2 and 24.4 reacted with only the GU isolate of E. invadens and with the isolate of E. moshkovskii used, giving a diffuse internal fluorescence pattern.

Mab 24.3 reacted with E. moshkovskii, E. muris and E. coli producing an internal granular fluorescence pattern. Mabs 24.5, 24.7 and 24.8 reacted with all six isolates of E. invadens tested but with no other cell type. All three Mabs produced internal granular fluorescence patterns.

Mab 24.6 reacted only with E. moshkovskii producing an internal diffuse fluorescence pattern.

Mab 29.3 reacted with all six isolates of E. invadens and with E. moshkovskii and produced a diffuse fluorescence pattern in each case.

TABLE 3.3: IFA EXPERIMENTS USING METHANOL-FIXED PROTOZOA;
ENTAMOEBA INVADENS
ENTAMOEBA MOSHKOVSKII (E. MOSK.)
ENTAMOEBA MURIS
ENTAMOEBA COLI
ENTAMOEBA HARTMANNI
PENTATRICHOMONAS HOMINIS
TRICHOMONAS VAGINALIS
GIARDIA LAMBLIA

FLUORESCENT PATTERNS OBSERVED:
I = INTERNAL
S = SURFACE
- = NEGATIVE RESULT
S/- = SURFACE IN CERTAIN ISOLATES, NEGATIVE IN OTHERS
I/- = INTERNAL IN CERTAIN ISOLATES, NEGATIVE IN OTHERS
(SEE TEXT FOR DETAILS)

GUPM NO.	SPECIES							
	E.invadens	E.mosk.	E.muris	E.coli	E.hartmanni	P.hominis	G.lamblia	T.vaginalis
22.1(S)	-	-	-	-	-	-	-	-
22.3	-	-	-	-	-	-	-	-
22.4	I	I	I	I	-	I	-	-
22.5	-	-	-	-	-	-	-	-
22.6(S)	S/-	-	-	-	-	-	-	-
22.7	-	-	-	-	-	-	-	-
22.8	-	-	-	-	-	-	-	-
24.1	I/-	-	-	-	-	-	-	-
24.2	I/-	I	-	-	-	-	-	-
24.3	-	I	I	I	-	-	-	-
24.4	I/-	I	-	-	-	-	-	-
24.5	I	-	-	-	-	-	-	-
24.6	-	I	-	-	-	-	-	-
24.7	I	-	-	-	-	-	-	-
24.8	I	-	-	-	-	-	-	-
29.3	I	I	-	-	-	-	-	-
29.5	I	I	I	I	I	I	I	I
29.6(S)	-	-	-	-	-	-	-	-
29.9	I	I	I	I	I	I	I	I
29.10	-	-	-	-	-	-	-	-
29.11	-	-	-	-	-	-	-	-

GUPM NO.	ISOLATES OF ENTAMOEBA INVADENS					
	GU	LSHTM	TRM	ZM	TR1	TR2
22.1(S)	-	-	-	-	-	-
22.3	-	-	-	-	-	-
22.4	I	I	I	I	I	I
22.5	-	-	-	-	-	-
22.6(S)	-	-	S%	S%	S%	S%
22.7	-	-	-	-	-	-
22.8	-	-	-	-	-	-
24.1	I	-	-	-	-	-
24.2	I	-	-	-	-	-
24.3	-	-	-	-	-	-
24.4	I	-	-	-	-	-
24.5	I	I	I	I	I	I
24.6	-	-	-	-	-	-
24.7	I	I	I	I	I	I
24.8	I	I	I	I	I	I
29.3	I	I	I	I	I	I
29.6(S)	-	-	-	-	-	-
29.10	-	-	-	-	-	-
29.11	-	-	-	-	-	-

TABLE 3.4 : FLUORESCENT PATTERNS OBTAINED IN IFA EXPERIMENTS USING ISOLATES OF ENTAMOEBA INVADENS.

I = INTERNAL
S% = SURFACE, NOT ALL CELLS POSITIVE. (CONFIRMED BY IFA USING LIVE AMOEBAE)
- = NEGATIVE RESULT

SEE TEXT FOR DETAILS OF STRAINS

3.4 DISCUSSION

The specificity of the 21 monoclonal antibodies for E. histolytica, was determined by testing for cross-reactivity with a range of other cell types in IFAT experiments. These cells included members of the genus Entamoeba, other parasitic and free-living protozoa and one mammalian cell line. Such tests comprised part of the initial assessment of the suitability of these Mabs for use in an antigen-capture assay in immunodiagnosis. However it must be emphasised that the Mabs were used here in immunofluorescence tests with methanol-fixed whole-cell antigen, whereas antigen-capture assays in which they may be useful, such as ELISA (Chapter 6), employ native, often soluble, antigen. Therefore the absence of cross-reaction in these experiments does not necessarily exclude cross-reaction with the same cells under different conditions. Additionally, these Mabs may cross-react with other, non-protozoan antigens that may be present in samples tested in a diagnostic assay, for example serum proteins in body fluids and various commensal and pathogenic intestinal bacteria present in faeces.

Eight of the Mabs (22.1, 22.3, 22.5, 22.7, 22.8, 29.6, 29.10 and 29.11) failed to cross-react with any of the cell types used. A further nine (22.6, 24.1, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8 and 29.3) cross-reacted with E. invadens or E. moshkovskii or both of these. Since E. invadens seems only to infect certain reptiles (Ratcliffe and Gieman, 1938) and since attempts to infect man (Rogova, 1958) and other mammals (de Carneri, 1958) with E. moshkovskii have been unsuccessful, these Mabs may yet be of use in E. histolytica antigen detection in human-derived samples, as these two species of amoebae would not be present to create confusion.

Most other work involving Mabs raised against E. histolytica has reported only those that fail to cross-react with other protozoan species. However Lopez et al. (1982) reported that one out of six Mabs described, cross-reacted in ELISA with E. moshkovskii

(although no other cell types were tested) and Ximenez et al. (1985) found that five out of six Mabs produced, cross-reacted with E. invadens, E. moshkovskii and the "E. histolytica-like" Laredo strain.

Cross-reactions with monoclonal antibodies cannot always be taken to suggest a functional or evolutionary relationship between the antigens involved, since identical or closely similar epitopes may occasionally occur on otherwise unrelated antigens, as has been suggested by Nigg et al. (1982) for the cross-reaction between viral src-c-peptide and β-tubulin. This is particularly true if the epitopes concerned are carbohydrate moieties but cross-reaction may also occur with protein epitopes. If, however, cross-reactions occur between apparently related species then there is a higher probability that such cross-reactions do reflect an evolutionary relationship, where a larger number of shared epitopes are to be expected. There are two distinct explanations for the physical basis of the more capricious Mab cross-reactions (Lane and Koprowski, 1982). One interpretation involves two antigens which share a small and precise detail of their surface topology. Such an epitope might not be detectable using conventional antisera in which most of the antibodies would be directed against determinants specific to one or other of the antigens. In the second interpretation the two epitopes have dissimilar structures but interact with the same antibody molecule since antibody binding sites are known to be polyfunctional (Richards et al., 1975). Obviously a mid-way situation may exist where an epitope on one antigen may be partly homologous to an epitope on another.

The antigens recognised by Mabs 29.5 and 29.9 are likely to be conserved antigens since the cross-reactions occurred throughout a wide range of species. The suggestion that Mab 29.5 recognises an element of the cytoskeleton (Section 2.4) is strengthened here by the observation that a perinuclear fluorescence pattern was observed in most cell types. The cross-reaction observed with Mab 24.1 and T. brucei rhodesiense is more likely to represent the

fortuitous presence of a similar epitope since it occurred only in one species of unrelated (except by Phylum!) protozoan. It is interesting to note that Mab 24.1 recognised the flagellum of the trypanosome, since Dulbecco et al. (1981), who found a cross-reaction between an anti-Thy 1 Mab and intermediate filaments, suggested that the large concentrations of cytoskeletal proteins in the cell may be a factor in the detection of such cross-reactions by IFAT.

Phylogenetic relationships between species have been determined using conventional antisera (Ibrahimi et al., 1979; Prager et al., 1979) to detect cross-reactive and non cross-reactive epitopes and while a single Mab against a single epitope is not an effective tool for this kind of investigation it was felt that a larger panel of Mabs against a wider range of antigens might be useful in drawing at least preliminary conclusions. A negative result in these species cross-reaction experiments may signify an absence or change in the epitope for which the Mab is specific or absence of the antigen altogether. The cross-reactions observed with Mab 29.5 and 29.9 have been mentioned and will not be discussed further.

None of the Mabs (other than 29.5 and 29.9) cross-reacted with Naegleria gruberi and Acanthamoeba castellani. This finding supports that of Sen et al. (1961b) who found that anti-E. histolytica antiserum failed to react with N. gruberi antigen in precipitin tests. Adam (1964), using the immobilisation test, (which by nature, examines surface-exposed antigens), showed that antisera prepared against E. histolytica, E. invadens and E. moshkovskii failed to have an immobilising effect on four strains of A. castellani. Such antigenic difference is not suprising given these free-living amoebae are morphologically and biochemically very distinct from members of the genus Entamoeba (Bowers and Korn, 1968; Gutteridge and Coombs, 1977).

E. coli and E. muris both showed cross-reactions with the same two Mabs, 22.4 and 24.3. This unique pattern of cross-reactivity is

interesting since both species belong to the octonucleate cyst group and are morphologically indistinguishable from each other (Neal, 1950) differing only in their host-restrictions. It would be interesting to discover if this pattern of reactivity occurred with other members of the group eg. E. gallinarum and E. barretti. Immunological cross-reactivity between E. coli and E. histolytica has been reported by Goldman (1954) using antisera to both species in IFAT experiments. However the heterologous fluorescence was removed after absorption with the appropriate species.

The cross-reactivities observed with members of the quadrinucleate cyst group were more complex. E. hartmanni failed to cross-react with any of the Mabs, except 29.5 and 29.9. This is in contrast to the findings of Goldman et al. (1960) who, using anti-E.histolytica antiserum in IFAT experiments, reported that cross-reaction with E. hartmanni occurred to the same extent as cross-reaction with E. invadens and E. coli. There are various possible explanations for this anomaly. Since the Mabs used in this work are merely a random sample of the total mouse response to E. histolytica, it is possible that, by chance, those hybridomas involving plasma cells producing antibodies that do cross-react with E. hartmanni, were not selected during screening. Another possibility is that significant antigenic heterogeneity might exist between isolates of E. hartmanni, although antigens cross-reacting with other members of the genus would most likely be conserved (as observed in E. coli and E. muris). Finally it is possible that the small amoeba present in isolate MH 661 was not E. hartmanni, although its nuclear morphology (similar to E. histolytica) and small size, suggested that it was. It is interesting to note that Mab 22.4 showed a cross-reaction with the flagellate Pentatrichomonas hominis, and yet none with E. hartmanni, nor with the other trichomonad tested, Trichomonas vaginalis. The significance of this finding is not known.

The large number of Mabs cross-reacting with E. invadens and E. moshkovskii suggest that these two species are antigenically closely related to E. histolytica. However the two species do differ in terms of the individual Mabs that recognise them. Apart from 29.5 and 29.9, six other Mabs cross-reacted with E. moshkovskii; 22.4 and 24.3 (as with E. muris and E. coli), 24.2, 24.4, 24.6 and 29.3. Interestingly, E. moshkovskii was the only cell type other than E. histolytica which was recognised by 24.6.

A certain amount of heterogeneity of cross-reactions was observed among the six isolates of E. invadens tested. Five Mabs reacted with all six of the isolates; 24.5, 24.7 and 24.8 (cross-reacting only with E. invadens), 22.4 (which also recognised other species of Entamoeba except E. hartmanni) and 29.3 which also cross-reacted with E. moshkovskii. Isolate GU also cross-reacted with 24.2 and 24.4 in common with E. moshkovskii and uniquely with 24.1. All four Madagascar isolates of E. invadens (TRM, ZM, TR1, TR2) gave surface fluorescence with Mab 22.6, of a pattern similar to that observed in E. histolytica. No isolate of E. invadens gave a positive reaction with Mab 24.3 which cross-reacted with E. moshkovskii, E. muris and E. coli. These results demonstrate antigenic heterogeneity among isolates of E. invadens and the importance of testing more than one isolate of a certain species when attempting to determine antigenic relatedness. Unfortunately this was not accomplished with any of the species other than E. invadens.

Several other workers have examined cross-reactions between the antigenic structures of E. histolytica and other members of the quadrinucleate cyst groups, although only in some of these were E. moshkovskii and E. invadens examined simultaneously. Using antisera against E. histolytica in IFAT, Goldman et al. (1960) found there was much stronger cross-reaction with E. invadens than with E. moshkovskii. Zaman (1960) showed that anti-E. histolytica antiserum was capable of immobilising E. invadens but not E. moshkovskii trophozoites, an observation supported by the

finding of cross-reacting surface antigens in E. histolytica and E. invadens in the work presented here. In contrast, Ali Khan and Meerovitch (1968), using a variety of techniques, (indirect haemagglutination, gel precipitation and immunoelectrophoresis) demonstrated that anti-E. histolytica antiserum cross-reacted more strongly with E. moshkovskii than with E. invadens. Conflicting results have also been reported in experiments using anti-E.invadens antiserum. Siddiqui and Balamuth (1966) in IFAT experiments showed a stronger cross-reaction with E. moshkovskii than with E. histolytica, while Ali Khan and Meerovitch (1968) in indirect haemagglutination tests showed the converse. It is agreed, however, that both E. invadens and E. moshkovskii are more closely related antigenically to E. histolytica-like strains such as the Laredo and Huff strains (E. moshkovskii in particular) than they are to normal E. histolytica (Siddiqui and Balamuth, 1966; Krupp, 1966; Goldman and Cannon, 1968) reflecting their relationship in terms of in vitro culture characteristics.
It would be interesting to test E. histolytica-like strains with the panel of Mabs produced here to shed further light on the antigenic relatedness of the members of the quadrinucleate cyst group. In particular, such experiments may help to investigate a theory (de Carneri, 1968) that E. moshkovskii and Laredo-type strains originated from a common quadrinucleate cyst ancestor living in decaying organic matter and that E. invadens and E. histolytica evolved from this Laredo-type strain.

In summary, experiments with these Mabs have demonstrated differences between octonucleate and quadrinucleate cyst groups of Entamoebae and within the latter have demonstrated a certain number of common antigens although results obtained with one isolate of E. hartmanni seem to contradict previous work.

CHAPTER 4 ISOLATE SPECIFICITY OF MONOCLONAL ANTIBODIES

CHAPTER 4

ISOLATE SPECIFICITY OF MONOCLONAL ANTIBODIES

4.1 INTRODUCTION

The immunodiagnosis of a particular infectious disease normally relies on the successful detection of either antigen(s) or a host response to antigen(s) common to all isolates of the causative species. In some cases, where isolates of the organism are known to differ in their pathogenic capability, a more useful approach might involve antigens specific to those members of the species causing disease. Such a situation may occur in amoebiasis (see below).

Sargeaunt and his colleagues, by analysis of the isoenzyme profiles of trophozoites from thousands of asymptomatic and symptomatic cases around the world, have characterised at least 20 distinct zymodemes (Sargeaunt and Williams, 1978, 1979; Sargeaunt et al., 1982, 1987). Based on the serology and clinical status of the isolate source, these zymodemes have been grouped into two categories: pathogenic and non-pathogenic. Isolates belonging to non-pathogenic zymodemes are derived from asymptomatic carriers with negative serology, while the pathogenic zymodemes are usually found in patients with positive serology and a history of clinical symptoms. Sargeaunt has never reported the occurrence of a non-pathogenic zymodeme in conjunction with a pathogenic one in the same host (Sargeaunt, 1985). In longitudinal culture studies in the presence of bacteria from pathogenic or non-pathogenic zymodemes, no evidence of zymodemic alteration from pathogenic to non-pathogenic, or vice versa, could be demonstrated (Sargeaunt, 1985, 1987). Thus it has been proposed that isoenzymes are stable biochemical markers that distinguish two different E. histolytica subspecies and that patients harbouring non-pathogenic zymodemes require no drug treatment (Lancet , 1985; Sargeaunt, 1987). A non-treatment policy is already in practice in

populations where the occurrence of infection with pathogenic zymodemes is rare, for example in male homosexuals in London (Allason-Jones et al., 1986; Goldmeier et al., 1986). This policy, and the "stable zymodeme" hypothesis underlying it, has been called into question by the findings of Mirelman et al. (1986) who detected virulence and zymodeme changes of cloned cultures during axenization. This phenomenon has been observed in other laboratories (B. Andrews, personal communication) and suggests that although harsh changes, such as those experienced by the amoebae during the axenization process, probably do not occur in vivo, the possibility for transformation within the host does exist. However the bulk of the epidemiological evidence does favour the "stable zymodeme/virulence" hypothesis and an immunodiagnostic test detecting such virulent amoebae would be an improvement over the need for isolation, culture and zymodeme analysis.

Thus comparison of the antigenic composition of different isolates of E. histolytica has been attempted by various workers in order to detect (a) antigens common to all isolates and (b) antigens specific for pathogenic isolates. Early work by Krupp (1966) using 10 pathogenic isolates and 3 antisera in immunoelectrophoresis (IEP) produced 4 precipitin arcs common to all isolates. Later work (Krupp, 1977) using 2 axenic isolates with 148 patient antisera in IEP, showed a common repertoire of 14 antigens recognised by patient sera (but not all sera) from a world-wide range of geographical locations. Subsequent work by Chang et al. (1979) using 2-dimensional-IEP and Mathews et al. (1986) using EITB showed a large number of shared antigens between axenically-grown pathogenic strains. It should be noted that much of the reported work on the study of virulence and virulence-markers in E. histolytica has been performed comparing virulent axenic strains with so-called "avirulent" axenic strains which are, in fact, only less virulent members of pathogenic zymodemes

and were originally isolated from symptomatic patients (Trissl et al., 1978; Mattern et al., 1978; Orozco et al., 1980).

In this study, 19 monoclonal antibodies raised against axenically-grown E. histolytica strain 200:NIH, were used in IFAT experiments against a variety of both long-term and recent isolates of various zymodemes and differing geographical origin, in order to identify common and perhaps pathogen-specific antigens.

4.2 MATERIALS AND METHODS

4.2.1 Cells

Details of the isolates of E. histolytica used, including date of isolation, country of origin, patient status and isoenzyme characteristics are presented in Table 4.1. Characterisation of isoenzymes (Glucose phosphate isomerase - E.C.5.3.1.9, Phosphoglucomutase - E.C.2.7.5.1, L-Malate:$NADP^+$ Oxidoreductase - E.C.1.1.1.40 and Hexokinase - E.C.2.7.1.2) and zymodeme classification was performed by P.G. Sargeaunt, Department of Medical Parasitology, London School of Hygiene and Tropical Medicine (Sargeaunt and Williams, 1979; Sargeaunt et al., 1982; Sargeaunt, 1987). In two cases where full isoenzyme characterisation was not performed, hexokinase mobility alone was analysed in agarose mini-gels (Strachan et al., 1988). Pathogenicity (ie., whether pathogenic or non-pathogenic) was determined according to the isoenzyme mobility pattern and clinical presentation of the patient (Sargeaunt, 1988).

Isolates 200:NIH (ATCC 30458), HK9 (ATCC 30015) and HM1:IMSS are all widely grown laboratory strains and were originally obtained from Dr L.S. Diamond, National Institutes for Health, Bethesda, Md.
The Loon Lake isolate of E. histolytica was provided by Dr Eileen Proctor, Department of Medical Microbiology, University of British Columbia, Vancouver, B.C. All four of these isolates were grown axenically in TYI-S-33 medium in this laboratory as described in Section 2.2.1.

The remaining isolates were grown with bacteria in Robinson's medium on agar slopes with rice starch (Robinson, 1968).
Six isolates with the prefix SAW were provided by P.G. Sargeaunt and originally isolated by Sargeaunt and J.E. Williams at the London School of Hygiene and Tropical Medicine.

Isolates MH651 and MH716 were isolated by P.G. Sargeaunt from stool samples obtained from male homosexual outpatients at the Middlesex Hospital, London.
Isolates SGH:1 and SGH:2 were provided by Dr H.V. Smith, Department of Bacteriology, Stobhill General Hospital, Glasgow. They were isolated from E. histolytica cyst-positive stools sent to the hospital for parasitological examination. Information on patient history and possible sources of infection was not available.
Isolate PRC:1 was provided by Professor W. Hu and Dr T. Zhang, Division of Infectious Diseases, Department of Medicine, 2nd Hospital of Tianjin Medical College, Tianjin, Peoples' Republic of China. The cells were sent as methanol-fixed smears on microscope slides, stored with desiccant. Information on patient status and isolate pathogenicity was not available.

Isolates C29 and MP:1 were provided by Dr J.P. Ackers, Department of Medical Parasitology, London School of Hygiene and Tropical Medicine.

4.2.2 IFAT experiments

Axenically-grown isolates were prepared for IFAT as described in Section 2.2.2.7. Isolate PRC:1 was prepared as stated. All other isolates grown in Robinson's medium were prepared at source. Samples were withdrawn from the bottom of the agar slope by pipette and gently mixed to suspend amoebae. Drops of this suspension were then placed in wells of multispot slides and left to air dry. After fixation in Methanol (5 min) the slides were stored desiccated at -22 °C.

For isolates 200:NIH, HK9, HM1 and Loon Lake full titrations of the Mabs were performed using $\log_3$ dilutions from 1 to 12 (ie. 1/3 to 1/531441). For all other isolates, ascitic fluids containing Mabs were used at two $\log_3$ dilutions above endpoint dilution determined using strain 200:NIH as antigen.

After warming the slides to room temperature, 5 µl quantities of ascitic fluid dilution were added to wells and the slides processed as described in Section 2.2.2.7. Negative controls used were (a) Mouse monoclonal GUPM 18.2 raised against Trypanosoma brucei rhodesiense variant surface glycoprotein used at 1/50, followed by FITC conjugate, and (b) FITC conjugate alone. Experiments were repeated at least once.

4.3 RESULTS

IFAT experiments with 19 Mabs were performed using 17 different isolates of E. histolytica. Mabs 29.5 and 29.9, previously found to recognise antigens in a variety of unrelated cell types (Chapter 3), were not used in these experiments. No amoebae were fluorescent with either of the negative controls although in some isolates, fluorescent bacteria were observed with both controls. The results of these experiments are summarised in Table 4.2. Where a negative result was obtained, the Mabs were tested at a series of lower dilutions to confirm. This was not possible with isolates SAW 142 and SAW 1719 due to the small number of slides available and the low number of amoebae on each slide.

Thirteen of the Mabs (22.4, 22.7, 22.8, 24.1, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3, 29.10 and 29.11) gave positive results with all isolates tested. In all cases the fluorescent patterns observed were identical to those obtained with strain 200:NIH (Section 2.3.2) although the presence of large food vacuoles in polyxenically-grown amoebae occasionally interfered with the pattern. Mab 22.7 continued to stain only around 90% of cells and the pattern observed was one of internal vacuolar fluorescence. No plasma membrane fluorescence was observed.

Mab 22.1 gave positive results in all isolates except SGH:1 where, in repeated experiments, no fluorescent cells were seen. In most cases plasma membrane fluorescence was observed, similar to that seen in strain 200:NIH (Section 2.3.2), but in isolates MP:1 and C29 some of the cells showed a general internal fluorescence, similar to the internal diffuse pattern obtained using Mab 24.6 for example.

Mab 22.6 failed to label cells in isolates MP:1 and SAW 142. In the remainder of the isolates, plasma membrane fluorescence was observed

although in isolates SAW 1760 and SAW 1774, cells showing internal fluorescence without membrane fluroescence were observed.

Mab 29.6 failed to label cells in isolates SAW 1719, SAW 142 and SGH:1. Plasma membrane fluroescence was observed in most of the remaining isolates, although in isolates MP:1 and SAW 1766 internal fluorescence was observed in some of the cells. In isolate C29 no plasma membrane fluorescence was observed and all positive cells showed only diffuse internal fluorescence. IFAT experiments using live amoebae to confirm the surface localisation of the antigens concerned were not performed using these isolates of E. histolytica.

With all three of these Mabs (22.1, 22.6 and 29.6) only a percentage of cells were positive, whether showing surface or internal fluorescence. This was true of all isolates tested where positive cells were observed. The actual percentage of cells stained varied considerably between isolates. Unfortunately, accurate determination of these values was not possible in isolates SAW 1766, SAW 1719 and SAW 142 due to the very low numbers of cells present on the microscope slides. Among the polyxenically-grown isolates: Mab 22.1 staining ranged from 96% in MH 716 to 36% in SAW 1760 and indeed even lower in SAW 142. Mab 22.6 staining ranged from 37% in SAW 1760 to < 0.1% in SAW 408. Mab 29.6 staining ranged from 69% in SAW 1774 to 3% in SAW 408. Among the axenically-grown isolates the percentages of positive cells were generally higher: Mab 22.1 stained just below 100% of cells in all four isolates. Mab 22.6 staining ranged from 98% in one sample of 200:NIH to 48% in HK9. Mab 29.6 staining ranged between 74% in HK9 to 17% in 200:NIH.

Mabs 22.3, 22.5 and 24.3 were the only antibodies recognising purely internal antigens that failed to stain all isolates tested. Mab 24.3 gave positive results with all isolates except SGH:2. Mab 22.3 failed to recognise C29, MH 651, MH 716, PRC:1 and SGH:1. Mab 22.5 failed to

recognise these five isolates and in addition failed to recognise SAW 1719 and SAW 142.

End point Mab titres were obtained using isolates HK9, HM1:IM55 and Loon Lake (data not shown) and were found to be within one $\log_3$ dilution of these obtained using 200:NIH.

TABLE 4.1

Information on isolates used in experiments.
Assigned Pathogenicity Status inferred by Zymodeme/Hexokinase Mobility.
Abbreviations: NIH - National Institutes of Health, Bethesda, Ma., USA.
LSHTM - London School of Hygiene and Tropical Medicine, Keppel St., London.
SGH - Stobhill General Hospital, Glasgow.
TMS - Tianjin Medical School, Tianjin, Peoples Republic of China.
UBC - Dept. of Medical Microbiology, University of British Columbia, Canada.

HK - Hexokinase
P - Pathogenic
NP - Non-Pathogenic

N.A.- Information not available
N.D.- Not Done

ISOLATE	DATE OF ISOLATION	SOURCE	COUNTRY OF ORIGIN	PATIENT STATUS	CULTIVATION MEDIUM	ZYMODEME/ HEXOKINASE MOB.	PATHOGENICTY
200:NIH	1949	NIH	USA	RECTAL ULCER	AXENIC (TYI)	Z II	P
HK9	1951	NIH	KOREA	DYSENTERY	AXENIC (TYI)	Z II	P
HM1:IMSS	1967	NIH	MEXICO	RECTAL ULCER	AXENIC (TYI)	Z II	P
LOON LAKE	N.A.	UBC	CANADA	DYSENTERY	AXENIC (TYI)	Z II	P
SAW142	1979	LSHTM	INDIA	ASYMPTOMATIC	POLYXENIC	Z III	NP
SAW408	1979	LSHTM	MEXICO	DYSENTERY	POLYXENIC	Z II	P
SAW1719	1984	LSHTM	AUSTRALIA	ASYMPTOMATIC	POLYXENIC	Z I	NP
SAW1760	1985	LSHTM	LONDON(TRAVELLER)	DYSENTERY	POLYXENIC	Z XIX	P
SAW1774	1985	LSHTM	LONDON(TRAVELLER)	DYSENTERY	POLYXENIC	Z XIV	P
SAW1766	1985	LSHTM	LONDON(TRAVELLER)	DYSENTERY	POLYXENIC	Z XIV	P
MH651	1986	LSHTM	LONDON(HOMOSEXUAL)	ASYMPTOMATIC	POLYXENIC	Z III	NP
MH716	1986	LSHTM	LONDON(HOMOSEXUAL)	ASYMPTOMATIC	POLYXENIC	Z III	NP
SGH:1	1985	SGH	GLASGOW	N.A.	POLYXENIC	SLOW HK	NP
SGH:2	1986	SGH	GLASGOW	N.A.	POLYXENIC	N.D.	N.A.
PRC:1	1985	TMS	TIANJIN,P.R.C.	N.A.	POLYXENIC	N.A.	N.A.
C29	1985	LSHTM	INDONESIA	ASYMPTOMATIC	POLYXENIC	SLOW HK	NP
MP:1	1987	LSHTM	MALAYSIA	DYSENTERY	POLYXENIC	FAST HK	P

TABLE 4.2 : FLUORESCENT PATTERNS OBTAINED IN IFA EXPERIMENTS USING ISOLATES OF ENTAMOEBA HISTOLYTICA (METHANOL-FIXED).

SEE TABLE 4.1 FOR DETAILS OF ISOLATES

ABBREVIATIONS: S = SURFACE
I = INTERNAL
% = NOT ALL CELLS POSITIVE

GUPM NO.	ISOLATES OF ENTAMOEBA HISTOLYTICA									
	200:NIH LOON LAKE HK9 SAW408	SAW1760 SAW1774	SGH:2	SAW1766	MP1	C29	MH651 MH716 PRC:1	SAW1719	SAW142	SGH:1
22.1	S%	S%	S%	S%	S%/I%	S%/I%	S%	S%	S%	-
22.3	I	I	I	I	I	-	-	I	I	-
22.4	I	I	I	I	I	I	I	I	I	I
22.5	I	I	I	I	I	-	-	-	-	-
22.6	S%	S%/I%	S%	S%	-	S%	S%	S%	-	S%
22.7	I%	I%	I%	I%	I%	I%	I%	I%	I%	I%
22.8	I	I	I	I	I	I	I	I	I	I
24.1	I	I	I	I	I	I	I	I	I	I
24.2	I	I	I	I	I	I	I	I	I	I
24.3	I	I	-	I	I	I	I	I	I	I
24.4	I	I	I	I	I	I	I	I	I	I
24.5	I	I	I	I	I	I	I	I	I	I
24.6	I	I	I	I	I	I	I	I	I	I
24.7	I	I	I	I	I	I	I	I	I	I
24.8	I	I	I	I	I	I	I	I	I	I
29.3	I	I	I	I	I	I	I	I	I	I
29.6	S%	S%	S%	I%/S%	I%/S%	I%	S%	-	-	-
29.10	I	I	I	I	I	I	I	I	I	I
29.11	I	I	I	I	I	I	I	I	I	I

TABLE 4.2

4.4 DISCUSSION

The detection and study of differences between isolates from asymptomatic and symptomatic hosts of E. histolytica from different geographical regions, is important in increasing our understanding of the pathogenesis and epidemiology of amoebiasis (Guerrant, 1986). Monoclonal antibodies represent specific tools for use in such a study and may subsequently be useful as a basis for an immunodiagnostic test.

In a large study of E. histolytica antigens Krupp (1977) used 148 patient sera from Africa, Asia, Central America and Canada in IEP with axenic amoebae as antigen. One particular precipitin arc was produced using all sera while up to thirteen other arcs occurred less regularly. From this work, it is difficult to draw conslusions on the antigenic composition of the amoebae originally stimulating the response in the patient since, as the author states, individual response to infection must vary with length of exposure to infection, repeated amoebic infections, concurrent infections and nutritional and genetic factors. In her earlier study, Krupp (1966) had demonstrated antigenic differences between pathogenic E. histolytica isolates and the non-pathogenic E. histolytica-like Huff and Laredo strains but non pathogenic E. histolytica isolates from asymptomatic patients were not tested.

In this study 19 monoclonal antibodies raised against axenically-grown strain 200:NIH (in culture since 1949) were used in IFAT experiments against 3 other long-term cultured axenic isolates and 13 more recent isolates grown with mixed intestinal flora. The amoebae were isolated in S.E. Asia, N.E. Asia, India, Australia, Central America, Canada and the U.K., although the exact origin of 5 of the U.K. infections is not known since at least 3 of the source patients were known to have travelled widely. However, the isolates obtained from male homosexuals in London are thought to be indigenous infections. The unavailability of African isolates for testing was unfortunate, since

approximately 17% of the world's infections occur in this continent (Walsh, 1986). Nine of the isolates were designated as pathogenic and six as non-pathogenic based on the clinical status of the patient from which they were obtained, and their isoenzyme profile. Full isoenzyme characterisation of isolate SGH:1 was not performed but the advanced bands of hexokinase (fast HK) obtained in agarose mini-gels were concluded to indicate its pathogenic nature (Mirelman et al., 1986).

In general, there was little variation in Mab reactivity between strains, with 13 out of the 19 Mabs reacting with all isolates. Unfortunately end point titre values for each Mab with each isolate were not obtained due to the scarcity of antigen slides of many of the isolates. This was particularly true for isolates SAW 142, SAW 1719 and SAW 1766 where only a few poorly-fixed amoebae were present in each well, since the cultures from which these samples were taken were old (late stationary phase). End point titre values would have provided more detailed information on isolate differences but would have been difficult to interpret since titre variation can be caused by both differences in the epitope structure and differences in antigen concentration. It is possible, that by developing Mabs using 200:NIH as immunising antigen, antibodies against antigens common to all isolates were being selected for. Prolonged culture of E. histolytica is thought to result in the disappearance or masking of certain antigens (Bos, 1978) and it is interesting to note that the three axenically-grown isolates had Mab endpoint titres in IFAT experiments similar to 200:NIH. These four isolates, and polyxenically-grown SAW 408, show an identical pattern of reactivity with the panel of Mabs and are all members of zymodeme II. Though this result may be significant, no other correlation between Mab reactivity and zymodeme was noticed. There is also evidence to suggest that the zymodeme to which an amoeba belongs is not a stable characteristic. Aside from the non-pathogenic to pathogenic transformation reported by Mirelman et al. (1986) under severe artificial conditions, Gathiram and Jackson (1987) suggest that the zymodeme changes (within the pathogenic group) observed in

longitudinal studies of asymptomatic patients were due to a change in amoebic isoenzyme expression occurring *in vivo* (and probably not due to mixed infection or re-infection).

Variation in isolate reactivity was observed with the surface-specific Mabs, with 22.1 failing to react with one isolate, 22.6 failing to react with two isolates and 29.6 failing to react with three isolates. However all these Mabs stained only a percentage of cells in each experiment (as previously discussed in Chapter 2). Thus, failure to react may be due to failure to detect a low percentage of cells expressing antigen; for example with SAW 408, only 2 fluorescent cells were counted out of at least 3000 observed. Therefore the results of such experiments must be interpreted with caution especially with isolates SAW 142, SAW 1719 and SAW 1766 where a total of under 50 cells were observed in experiments using each Mab. A similar explanation may account for the failure to detect cells stained with Mab 22.7 demonstrating plasma membrane fluroescence as observed with very small numbers of 200:NIH cells in Chapter 2.

No correlation was observed between percentage of cells stained and pathogenicity, with different pathogenic isolates showing both low and high percentage staining with all surface-specific Mabs. Indeed the large variation in percentage staining between isolates further supports the theory that the variations in expression observed in Chapter 2 are not due to single cell cycle events but perhaps due to cell sub-populations in the cultures as occurs in *Trichomonas vaginalis* (Alderete *et al.*, 1987) and in *Giardia lamblia* (Nash, 1988).

The internal (non-membrane) fluorescence observed in some cells in certain isolates in experiments with 22.1, 22.6 and 29.6 suggests that in some cases the antigen is present, but not expressed on the plasma membrane. This phenomenon has been reported in *T. vaginalis* by Alderete *et al.* (1986a) where a sub-population of cells, negative in live fluorescence experiments, were found to contain the protein detected in lysates using a Mab in EITB experiments. Amoebae showing

surface fluorescence would also be expected to contain the antigen internally, either in the cytoplasm or in elements of the vesicular system, since it must be synthesised within the cell. However such internal staining would be masked by the surface fluorescence. Immunogold experiments, as suggested in Section 2.4, would perhaps provide more detailed information on surface antigen localisation and synthesis.

The failure of Mab 24.3 to recognise isolate SGH:2 is suprising since this monoclonal gave positive results with all other E. histolytica isolates and with E. coli, E. muris and E. moshkovskii (Chapter 3). Of the other Mabs recognising internal antigens, only 22.3 and 22.5 failed to react with some of the isolates. Failure of both 22.3 and 22.5 to react coincided in 5 isolates while only 22.5 failed to react with SAW 142 and SAW 1719. Interestingly, 6 of these 7 isolates have been designated as non-pathogenic by virtue of their isoenzyme characteristics and, except in the case of SGH:1, the clinical status of the source-patient. Mabs 22.3 and 22.5 both reacted with all pathogenic isolates used. It seems therefore, that 22.5 staining correlates perfectly with pathogenicity for all isolates where pathogenic/non-pathogenic status has been determined. Based on this, it could be suggested that isolate PRC:1 might belong to non-pathogenic zymodeme, although this information was not available. The correlation of 22.3 staining and pathogenicity is not perfect since this Mab recognises two non-pathogenic isolates. However the correlation of 22.3 and 22.5 IFAT results in thirteen out of fifteen isolates suggests that further investigation is required. Subsequent work using both 22.3 and 22.5 is presented in this thesis in Chapter 7.

The reactivity of E. histolytica-specific Mabs with various isolates of the species has not been extensively reported by other workers. Ortiz-Ortiz et al. (1986) found Mab MC 004 reacted with four axenically-grown strains of E. histolytica but not with the E. histolytica-like Laredo strain. Torian et al. (1989), using a Mab

against a 96 KD surface membrane antigen, were able to purify the antigen from 4 axenically-grown isolates and could detect it in several polyxenically-grown pathogenic and non-pathogenic clinical isolates.

A panel of nine Mabs raised against Trichomonas vaginalis have been used in IFAT experiments to examine antigenic heterogeneity in a total of 88 isolates from four cities in the United States (Krieger et al., 1985). The individual antibodies reacted with between 22% and 76% of the isolates although some antibodies recognised none out of 15 isolates from one particular location indicating substantial geographical variation in antigenic composition. In contrast, the work reported here with E. histolytica shows quite a high degree of antigenic similarity and relatively little geographical variation.

From this work it is clear that the surface-specific Mabs, 22.1, 22.6 and 29.6, are not suitable individually for use in an immunodiagnostic test, since expression of the corresponding antigens is so variable. Apart from Mab 22.4 which recognises commensal species of Entamoeba (Chapter 3), the other 12 of the 13 Mabs reacting with all isolates tested may be suitable for use in an assay detecting both pathogenic and non-pathogenic types of E. histolytica.

CHAPTER 5 IMMUNOCHEMICAL ANALYSIS OF ANTIGENS

CHAPTER 5 IMMUNOCHEMICAL ANALYSIS OF ANTIGENS

5.1 INTRODUCTION

Until the advent of techniques for the axenic cultivation of E. histolytica (Diamond, 1961; Diamond, 1968; Diamond et al., 1978) attempts to characterise the antigenic composition of the amoeba gave unsatisfactory results because trophozoites were grown in association with intestinal bacteria or other protozoa (Sen et al., 1961b).

Early work using immunoelectrophoresis (IEP) revealed 6-8 distinct precipitin bands with rabbit antiserum (Lunde and Diamond, 1969). Later, Krupp (1977) detected a basic pattern of 14 antigens reacting with sera from patients from different geographical locations. The use of 2-dimensional immunoelectrophoresis by Chang et al (1979) provided an increase in resolution and detected up to 32 precipitin peaks using immune rabbit antisera.

Homogenates of axenic trophozoites have also been subjected to column chromatographic fractionation, with the main serological activity, as detected by immunodiffusion and indirect haemagglutination, being present in the high molecular weight fractions (Sawhney et al., 1980). Protection against infection in hamsters and guinea-pigs (by intrahepatic inoculation) can be induced most effectively by these fractions (Ghadirian et al., 1980; Sawhney et al., 1980).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) is perhaps the most widely used single method of protein analysis. Introduced by Shapiro et al. in 1967 and modified by Laemmli in 1970, it has since been used in numerous studies involving the characterisation of many proteins, including the antigens of E. histolytica. When proteins are heated to 100 °C for 2-5 min in the presence of SDS and reducing agents they unfold and bind approximately 1.4 g SDS per gram of protein. The unfolding is probably not complete and a considerable degree of secondary structure

is likely to be retained. The native charge of the protein is however masked by the strong negative charge so that the mass:charge ratio becomes constant for virtually all proteins and the electrophoretic mobility in acrylamide is inversely proportional to the logarithm of the molecular weight (Weber and Osborne, 1969).

SDS-PAGE of E. histolytica homogenates (Aust-Kettis et al., 1983; Mathews et al., 1986) and detergent lysates (Joyce and Ravdin, 1988) stained for total protein have revealed up to 60 recognisable bands (Aust-Kettis et al., 1983) all within a 10-200 kD apparent molecular weight range. In a detailed study of plasma membrane proteins Rosenberg and Gitler (1984) identified 34 proteins, ranging from 13 to 205 kD, of which 16 were externally exposed (ie. could be labelled using ^{125}I-lactoperoxidase).

Attempts have been made to identify the clinically relevant antigenic components of E. histolytica by various methods. Parkhouse et al. (1978) used patient sera to immunoprecipitate membrane glycoproteins, identifying 1 major and 3 minor bands on SDS-PAGE. Using immunoadsorption (involving purified patient IgG covalently bound to CNBr-Sepharose) Aust-Kettis et al. (1983) identified 7 antigens in trophozoite homogenates. Most other studies have used electroimmunotransfer (or "Western") blotting in which proteins are probed with antibody after electrophoretic separation. The heterogeneous mixture of proteins is separated by SDS-PAGE and the proteins eluted electrophoretically by a transverse electric field onto a membrane which binds protein tightly, most commonly nitrocellulose (Towbin et al., 1979). The membrane is then probed with either labelled antibody, or antibody followed by a labelled second antibody. Aust-Kettis et al. (1983) detected 11 antigens recognised by rabbit immune serum and 8 using human patient serum of which 7 were surface membrane antigens. Mathews et al. (1986) probing with human serum, detected at least 20 distinct bands in a soluble (cytosol) fraction alone and a lower number of more reactive bands in internal and plasma membrane fractions.

In this work antigens of E. histolytica were analysed by SDS-PAGE and electroimmunotransfer blotting (EITB) using the 21 monoclonal antibodies described in Chapter 2.

5.2 MATERIALS AND METHODS

5.2.1 Cells

E. histolytica: Strain 200:NIH and the Loon Lake isolate were used in these experiments. Both were grown in TYI-S-33 medium at 35.5 °C in Lux plastic tissue culture tubes (Flow, Irvine).

E. invadens: The LSH isolate, described in Section 3.2, was used in these experiments. Cells were grown axenically, in TYI-S-33 medium at 25 °C in plastic tissue culture tubes.

5.2.2 Preparation of Lysates

This method was used for both E. histolytica and E. invadens. Tubes containing amoebae in late log phase growth were cooled for 5 min in ice water then gently inverted several times to dislodge adherent cells. Following centrifugation at 200 g for 5 min the amoebae were washed twice in PBS (pH 7.45) (see Section 2.2.3) and finally resuspended in ice cold PBS at a concentration of 4×10^6/ml.

A solution of 1% (w/v) Nonidet P40 (BDH, Poole) in PBS containing 1 mM ethylenediamine tetra-acetic acid (EDTA) and 1 mM iodoacetic acid (IAA) was cooled on ice. Immediately prior to use, 1 µl of a 0.1 M solution of phenylmethylsulphonyl fluoride (PMSF) in acetone was added per 0.5 ml of detergent solution. Equal volumes of cell suspension and detergent solution were gently mixed in a glass centrifuge tube by pipette and incubated on ice for 15 min. The mixture was then centrifuged at 200 g for 5 min at 4 °C and the supernatant removed and centrifuged in an ultracentrifuge at 11800 g for 5 min at 4 °C. The supernatant was stored at -22 °C.

5.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as described by Laemmli (1970) using 7.5%, 10% and 5-15% - gradient separating gels and 5% stacking gels. Gels were run under both reducing and non-reducing conditions.

Sample Buffer - Reducing:

62 mM	Tris-HCl (pH 6.8)
2% (w/v)	SDS
7.5% (w/v)	Glycerol
5% (v/v)	2-Mercaptoethanol
0.02% (w/v)	Bromophenol Blue

Sample Buffer - Non-Reducing:

62 mM	Tris-HCl (pH 6.8)
2%	SDS
7.5%	Glycerol
10 mM	Iodoacetamide
0.02%	Bromophenol Blue

NP40 lysate samples (and samples of TYI-S-33) medium were diluted 1 in 4 in appropriate sample buffer and boiled for 10 min prior to electrophoresis. If both non-reduced and reduced (ie. molecular weight markers) samples were electrophoresed in the same gel a 1/10 volume of 1.5 M iodoacetamide was added to the reduced samples after boiling to destroy excess reducing agent. Samples were electrophoresed at approximately 10-15 V/cm length of gel (30 mA) until the dye front had reached the bottom of the gel.

Molecular weight markers used in these experiments were obtained from Sigma (Poole).

Myosin	205 kD
β-Galactosidase	116 kD
Phosphorylase B	97.4 kD
Bovine Albumin	66 kD
Egg Albumin	45 kD
Carbonic Anhydrase	29 kD
Trypsinogen	24 kD
β-Lactoglobulin	18.4 kD
Lysozyme	14.3 kD

Gradient gels (5-15%) were fixed and stained for total protein with Coomassie Brilliant blue 0.25% (w/v) in 45% (v/v) methanol, 10% (v/v) glacial acetic acid overnight at RT. Gels were destained in 25% (v/v) methanol, 7.5% (v/v) glacial acetic acid until a clear background was obtained. Gels were dried in a vacuum gel drier.

5.2.4 Electro-immunotransfer blotting (EITB)

Following SDS-PAGE, the separate proteins were electrophoretically transferred onto nitrocellulose paper (Biorad) according to the method of Towbin et al. (1979). The transfer was performed at 60 V, 0.21 A for 5 hr in a Tris/ Glycine/Methanol buffer:

Tris	9.09 g
Glycine	43.2 g
Methanol	600 ml
distilled water	2.4 l (pH 8.2)

Following transfer the nitrocellulose sheet was incubated overnight at 4 °C in 0.1 M Tris-HCl (pH 7.6) containing 13% (w/v) glycine to block spare protein-binding sites on the paper. The sheet was then cut into 5 mm-wide strips. Strips bearing transferred molecular weight markers and one strip of transferred lysate proteins were stained for total protein in 0.5% (w/v) amido black prepared in 5% (v/v) acetic acid 50% (v/v) methanol for 5 min. Following 1 min washing in water the strips were destained in 5% (v/v) acetic acid 50% (v/v) methanol until a clear background was obtained. The remainder of strips, bearing transferred lysate proteins, were incubated in 1/100 dilutions of Ascitic fluids in 0.1 M Tris-HCl (pH 7.6) containing 2% (w/v) glycine (diluting buffer) for 30 min at RT with gentle agitation. Following washing for 30 min in 3 x 50 ml of 0.1 M Tris-HCl (pH 7.6) containing 0.1% (v/v) Triton-X 100 (Sigma, Poole) (wash buffer) the strips were incubated in diluting buffer containing 1/250 horseradish peroxidase-conjugated goat anti-mouse antibody (HRP-GAM) (SAPU, Law Hospital, Lanark) for 30 min at RT. Following 30 min washing in wash

buffer, antibody binding was visualised by treating the strips with a solution of peroxide containing 4-chloro-1-napthol (0.018% (w/v) 4-chloro-1-napthol in 6% methanol in wash buffer plus 0.025 ml 40% H_2O_2 per 100 ml). The reaction was stopped by rinsing the strips in several changes of dH_2O. HRP-GAM-treated strips and amido black-stained strips were photographed using Ilford FP4 125 ASA plate film. Controls included a) strips incubated in a 1/100 dilution of a Mab raised against Plasmodium chabaudi followed by conjugate b) strips incubated in conjugate alone and c) strips subjected to chloronapthol colour development only.

5.3 RESULTS

5.3.1 SDS-PAGE

Proteins in NP-40 lysates of E. histolytica (strain 200:NIH) and E. invadens (isolate LSH) were separated by polyacrylamide gel electrophoresis under reducing conditions on 5-15% gradient gels. The approximate molecular weights of visible bands were calculated, using the known molecular weights of marker proteins, and are presented in Table 5.1. The protein profiles of the two species showed several bands of similar intensities at similar or identical molecular weights. In both species the bulk of proteins detectable by this method had molecular weights in the range 20-130 kD. The most prominent protein bands within the lysates of E. histolytica had approximate apparent molecular weights of 130 kD, 102 kD, 53 kD, 49 kD, 42 kD and 20 kD.

5.3.2 Electroimmunotransfer Blotting (EITB)

5.3.2.1 EITB using E. histolytica antigens

All 21 Mabs were used to probe nitrocellulose blots of 200:NIH whole cell NP40 lysates previously electrophoresed on 7.5% and 10% polyacrylamide gels. The apparent molecular weights obtained under reducing (i.e. with mercaptoethanol) conditions are presented in Table 5.2. Fourteen of the Mabs recognised antigens in these experiments (Plates 5.1 and 5.4). The antigens detected ranged in apparent molecular weight from 12 to 190 kD with the majority around 100 kD. Of the Mabs recognising surface antigens in intact amoebae, only 22.1 detected bands of protein on these blots. This Mab detected one band at approximately 58 kD (the most intense band), one at 95 kD and several high molecular weight bands greater than 300 kD (Plates 5.1 and 5.2). This Mab also consistently produced a smear of stain on blots between the high molecular weight bands and the 58 kD band. Mab 29.11 similarly detected multiple bands at 12 kD, 16 kD and 45 kD (Plates 5.2 and 5.4). The remaining twelve Mabs detected single bands of

E. histolytica	E. invadens
230	230
130	125
102	102
74	
72	
60	60
53	52
49	48
42	41
39.5	39.5
35	36
33	33
29	
28.5	
	25
	24
	23
22	
21	
20	20
18.5	
17	
16	
15	15
14	

TABLE 5.1: Approximate apparent molecular weights (kD) of major proteins of E. histolytica and E. invadens NP40 lysates, detectable by Coomassie Blue staining of SDS-Polyacrylamide 5-15% gradient gels run under reducing conditions. The weights of the most prominent bands are underlined. Bands, apparently related in terms of staining intensity and similar molecular weights are shown joined by dotted lines.

protein. Mabs 22.8, 24.2 and 24.4 all detected bands of 100 kD. The intensity of the staining and thickness of the band was identical using Mabs 24.2 and 24.4 but different with 22.8 which recognised a thinner band (Plate 5.2). Mabs 24.5, 24.7 and 24.8 all recognised bands of 97 kD that were identical in thickness and intensity of staining (Plates 5.2 and 5.3). Mabs 22.4, 22.7, 24.3, 29.3 and 29.9 recognised single bands at 115 kD, 190 kD, 130 kD, 120 kD and 125 kD respectively. A photograph of the protein band recognised by Mab 29.9 is not shown.

When the SDS-PAGE was performed under non-reducing conditions only 4 Mabs recognised bands on the corresponding blots (Plate 5.5). However in the two experiments performed under these conditions the resolution of protein bands was not good (Total protein Eh track in Plate 5.5). Mab 22.4 recognised a protein of 230 kD. Mabs 24.2 and 24.4 both recognised a band at 100 kD and Mab 29.3 recognised a band at 120 kD.

5.3.2.2 EITB using E. invadens antigen

All 21 Mabs were used to probe nitrocellulose blots of LSH whole cell NP40 lysates previously electrophoresed on 7.5% polyacrylamide gels under reducing conditions. Only one Mab gave a positive result: Mab 29.3 recognised a band at 120 kD (Plate 5.6).

GUPM NO. (and subclass)	MOLECULAR WEIGHT (kD)
22.1 (G2b)	58 95 (+ 3 HMW bands >300)
22.3 (G1)	-
22.4 (G1)	115
22.5 (G1)	-
22.6 (M)	-
22.7 (G1)	190
22.8 (G1)	100
24.1 (G1)	-
24.2 (G1)	100
24.3 (G1)	130
24.4 (G1)	100
24.5 (G1)	97
24.6 (G2a)	-
24.7 (G1)	97
24.8 (G1)	97
29.3 (G1)	120
29.5 (M)	-
29.6 (M)	-
29.9 (M)	125
29.10 (G1)	-
29.11 (G1)	12 16 45

TABLE 5.2 : APPROXIMATE APPARENT MOLECULAR WEIGHTS (KILODALTONS) OF ANTIGENS OF ENTAMOEBA HISTOLYTICA (STRAIN 200:NIH) DETECTED USING MONOCLONAL ANTIBODIES ON NITROCELLULOSE BLOTS OF WHOLE CELL LYSATES FOLLOWING SDS-PAGE (REDUCING CONDITIONS).

PLATE 5.1

ELECTROIMMUNOTRANSFER BLOT (EITB) following SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (sds-PAGE) (10% gel-reducing conditions) of *E. histolytica* strain 200:NIH lysate.

Eh - Nitrocellulose strip stained for total protein with amido black (0.5%w/v).

22.4 - Strip probed with GUPM 22.4

22.8 - Strip probed with GUPM 22.8

22.1 - Strip probed with GUPM 22.1

Approximate molecular weight values in kilodaltons.

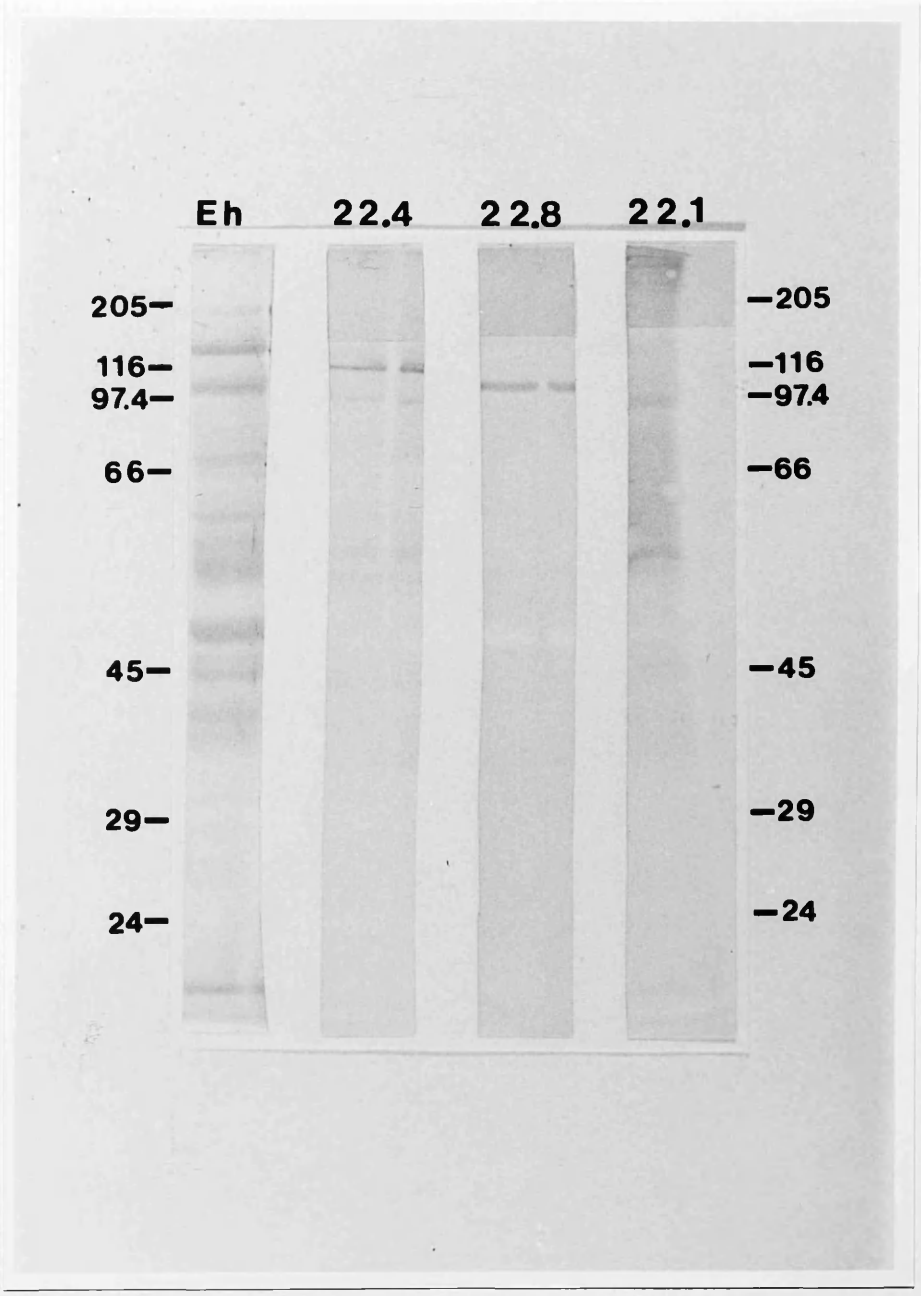

Eh
22.4
22.8
22.1
205
116
97.4
66
45
29
24
205
116
97.4
66
45
29
24

PLATE 5.2

EITB following SDS-PAGE (7.5% gel-reducing conditions) of *E. histolytica* strain 200:NIH lysate.

Eh - Strip stained for total protein with amido black (0.5%w/v)

22.1 - Strip probed with GUPM 22.1

22.8 - Strip probed with GUPM 22.8

24.2 - Strip probed with GUPM 24.2

24.3 - Strip probed with GUPM 24.3

24.4 - Strip probed with GUPM 24.4

24.5 - Strip probed with GUPM 24.5

24.7 - Strip probed with GUPM 24.7

24.8 - Strip probed with GUPM 24.8

29.3 - Strip probed with GUPM 29.3

29.11 - Strip probed with GUPM 29.11

Approximate molecular weight values in kilodaltons.

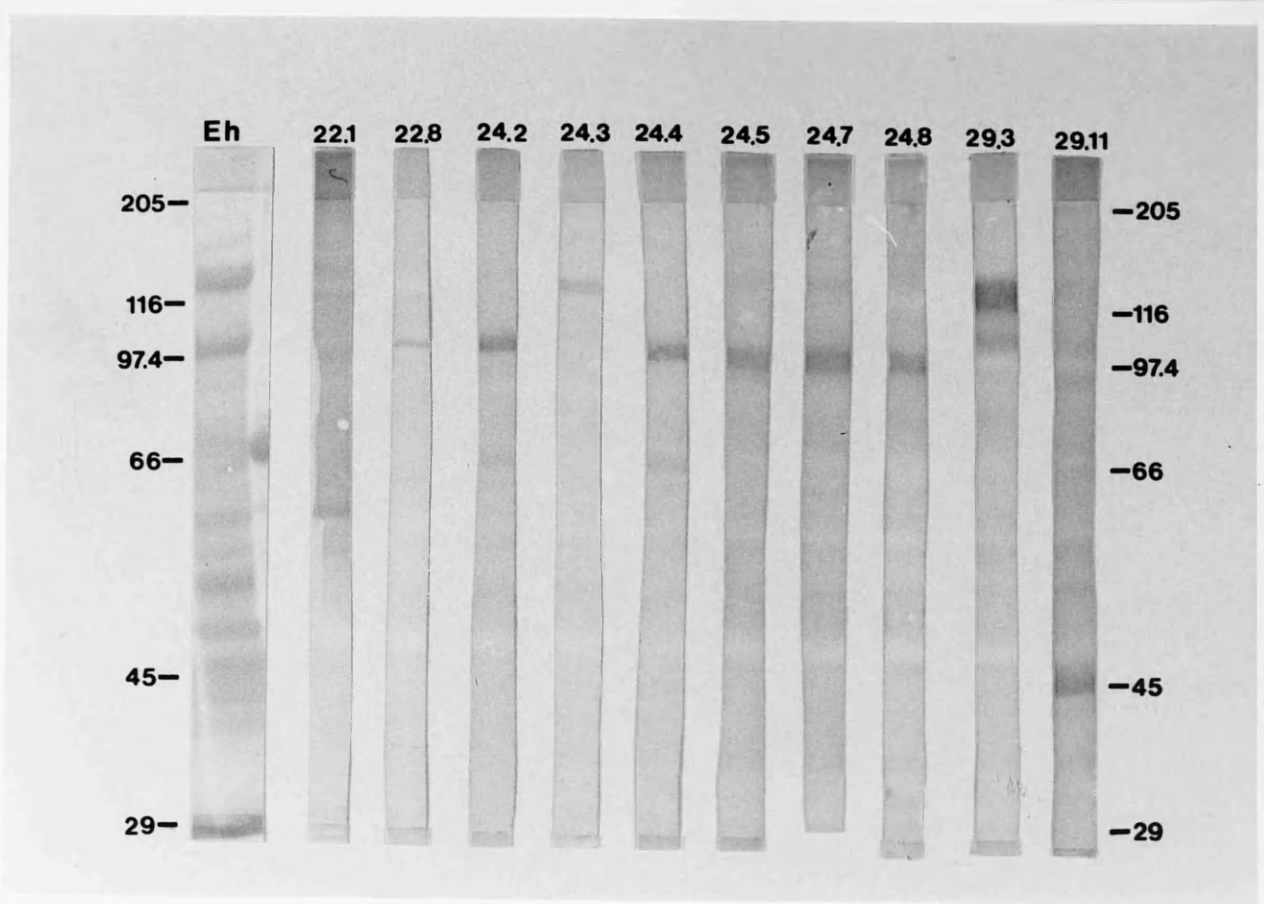

Eh
22.1
22.8
24.2
24.3
24.4
24.5
24.7
24.8
29.3
29.11
205
116
97.4
66
45
29
205
116
97.4
66
45
29

PLATE 5.3

EITB following SDS-PAGE (10% gel-reducing conditions) of *E. histolytica* strain 200:NIH lysate.

Eh - Strip stained for total protein with amido black (0.5% w/v)

22.4 - Strip probed with GUPM 22.4

22.7 - Strip probed with GUPM 22.7

22.8 - Strip probed with GUPM 22.8

24.5 - Strip probed with GUPM 24.5

24.7 - Strip probed with GUPM 24.7

24.8 - Strip probed with GUPM 24.8

Approximate molecular weight values in kilodaltons.

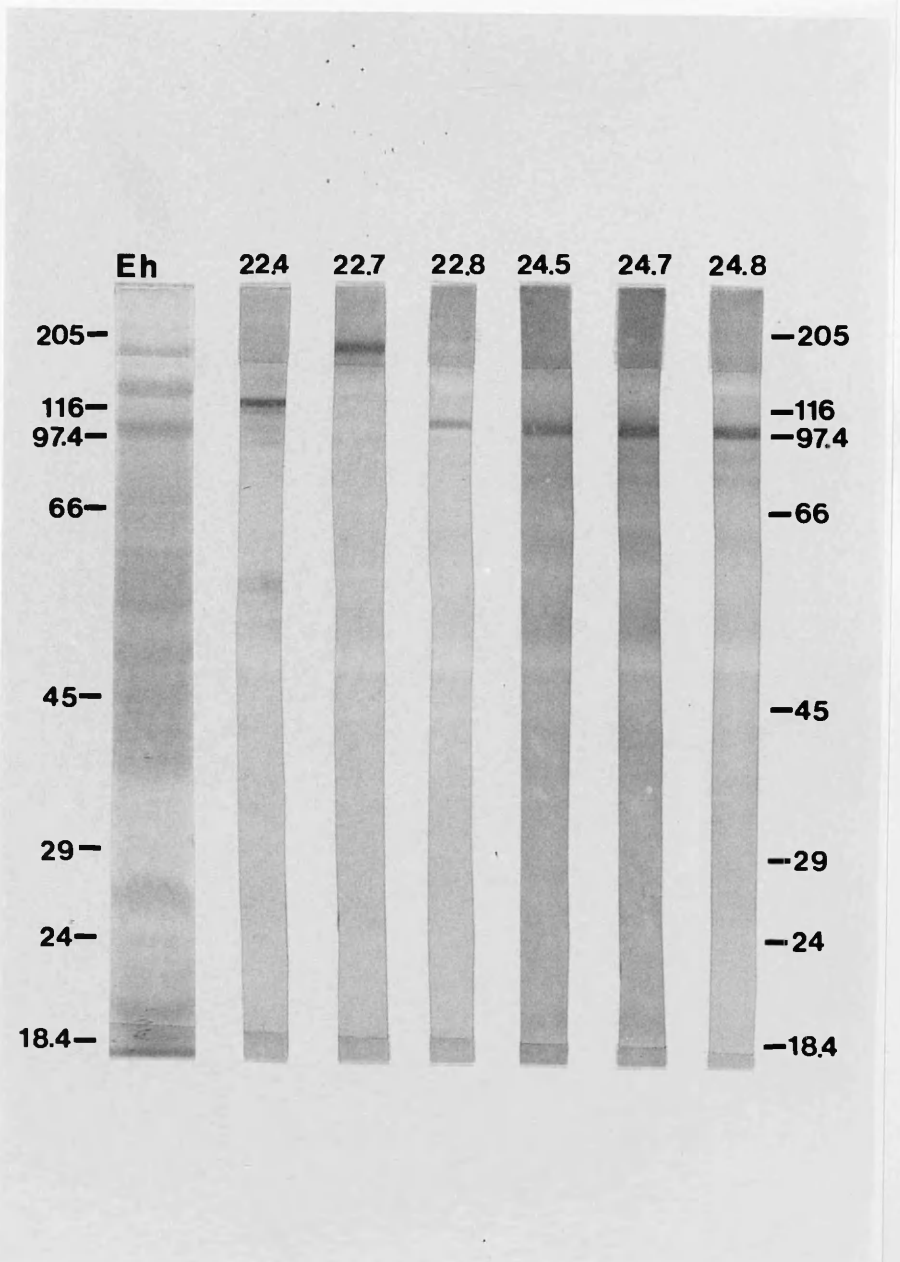

Eh
22.4
22.7
22.8
24.5
24.7
24.8
205
116
97.4
66
45
29
24
18.4

PLATE 5.4

EITB following SDS-PAGE (10% gel-reducing conditions) of *E. histolytica* strain 200:NIH lysate.

Eh - Strip stained for total protein with amido black (0.5% w/v)

29.3 - Strip probed with GUPM 29.3

29.11 - Strip probed with GUPM 29.11

Approximate molecular weight values in kilodaltons.

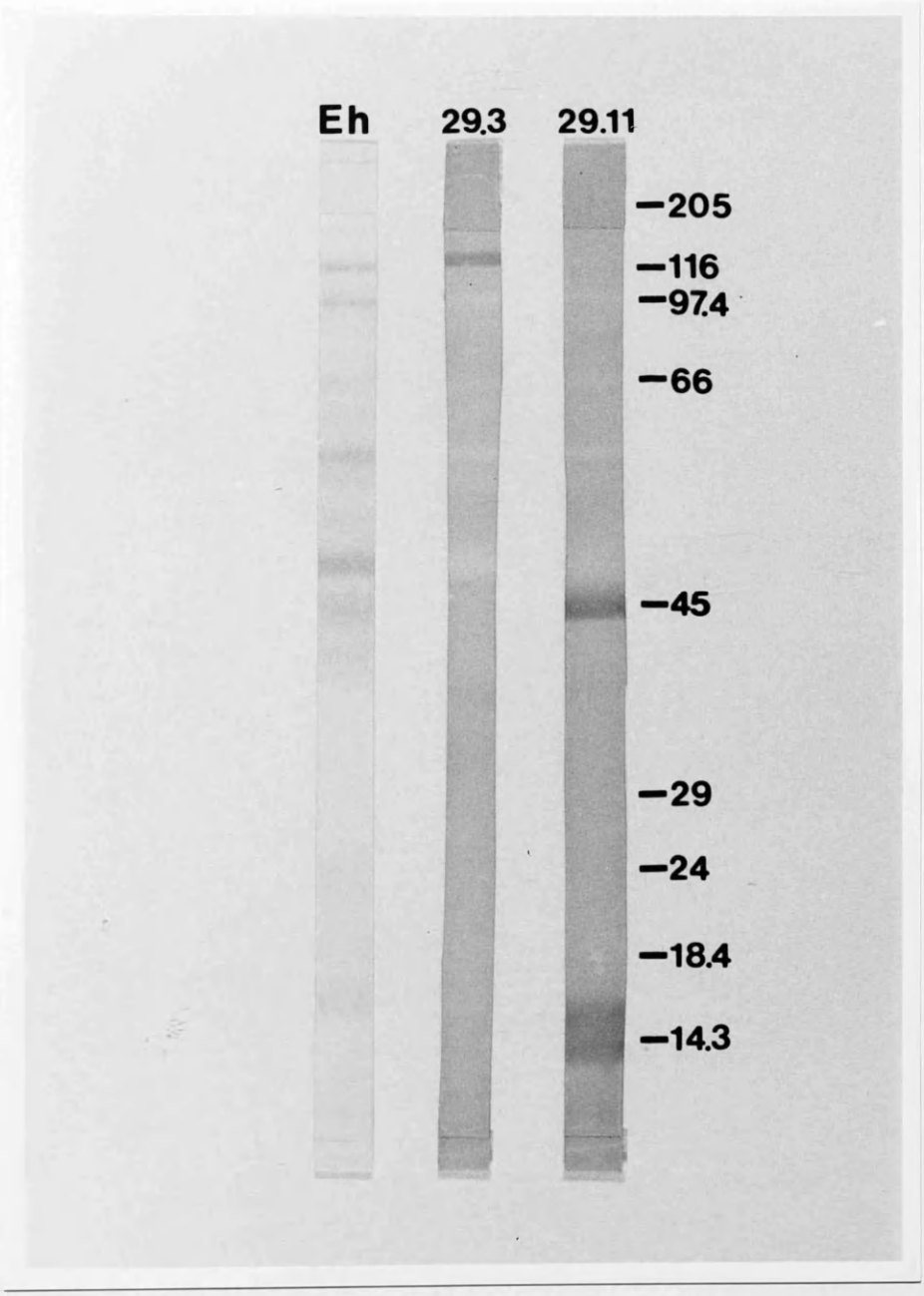

Eh
29.3
29.11
205
116
97.4
66
45
29
24
18.4
14.3

PLATE 5.5

EITB following SDS-PAGE (7.5% gel - non-reducing conditions) of *E. histolytica* strain 200:NIH lysate.

Eh - Strip stained for total protein with amido black (0.5% w/v)

22.4 - Strip probed with GUPM 22.4

24.2 - Strip probed with GUPM 24.2

24.4 - Strip probed with GUPM 24.4

29.3 - Strip probed with GUPM 29.3

Approximate molecular weight values in kilodaltons

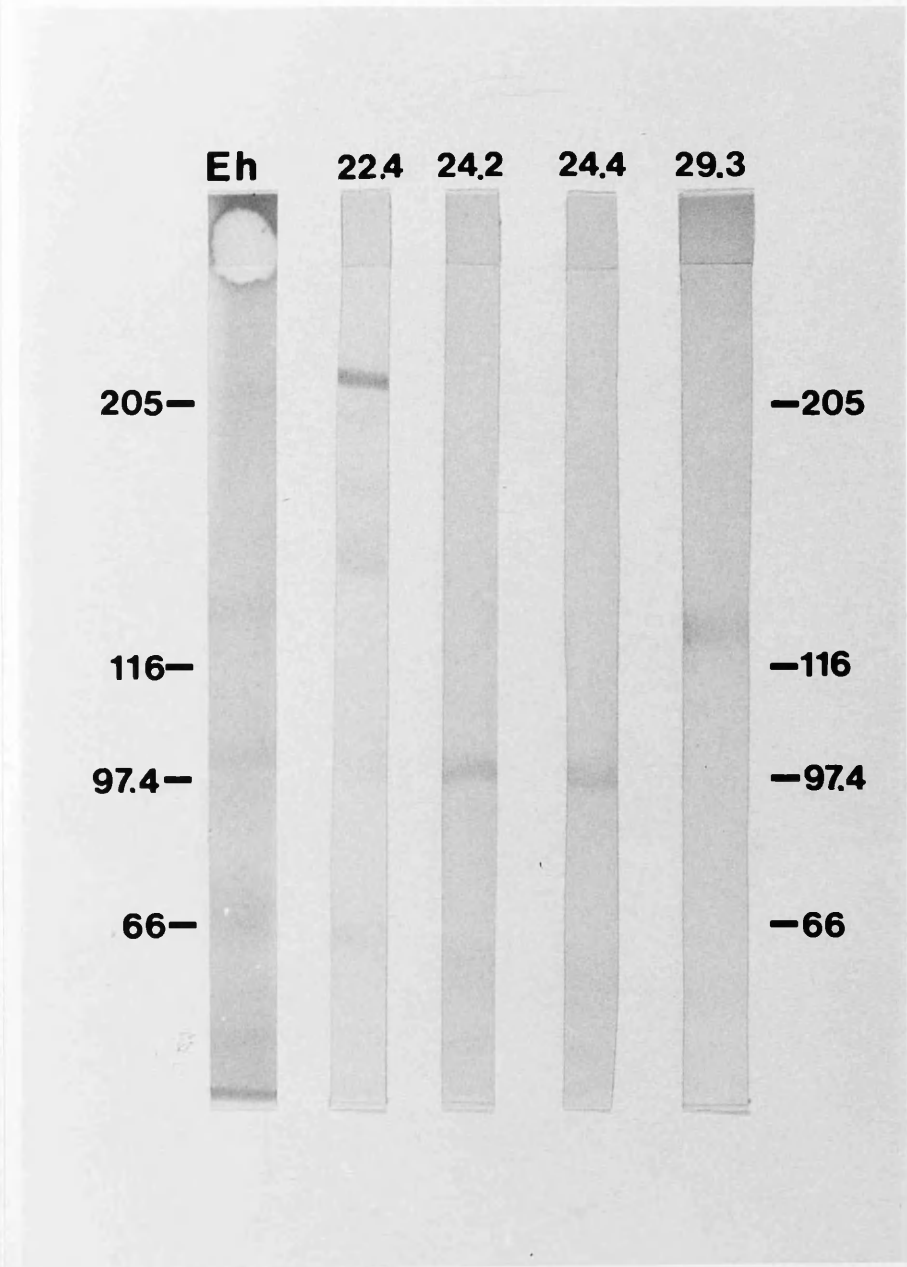

Eh
22.4
24.2
24.4
29.3
205
116
97.4
66
205
116
97.4
66

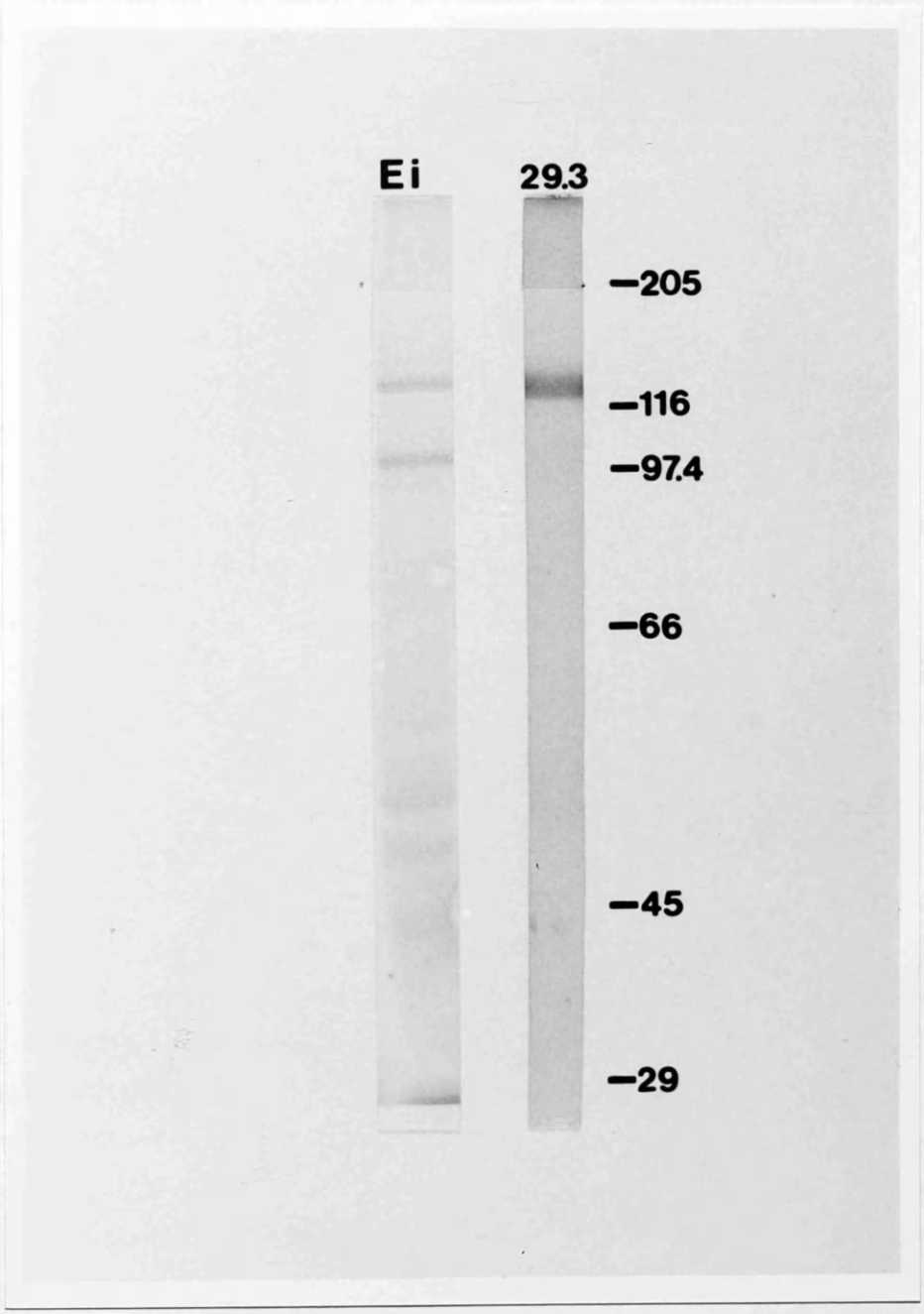
PLATE 5.6

EITB following SDS-PAGE (7.5% gel-reducing conditions) of *E. invadens*, LSH strain, lysate.

Ei - Strip stained for total protein with amido black (0.5% w/v)

29.3 - Strip probed with GUPM 29.3

Approximate molecular weight values in kilodaltons


Ei
29.3
205
116
97.4
66
45
29

5.4 DISCUSSION

The electrophoretic patterns of proteins from detergent lysates of axenically grown E. histolytica and E. invadens were analysed by SDS-PAGE and certain individual antigens recognised by Mabs in EITB ("Western-blotting"). It must be stressed at this point that the molecular weights described here are "apparent molecular weights" and since inaccuracies in estimations occur, especially in the case of glycosylated proteins (Goding, 1986), comparisons between antigens detected here and those reported by other workers are made only tentatively.

Gradient polyacrylamide gels stained with Coomassie Blue for total protein revealed 22 distinct bands for E. histolytica and 15 bands for E. invadens all in the range 14-230 kD apparent molecular weight. There were twelve protein bands, in both species, that appeared to correspond in terms of similar apparent molecular weight and intensity of staining; this further supports the existence of substantial interspecific antigenic cross-reaction discussed in Chapter 3. A number of the more prominent bands observed here with E. histolytica do seem to have possible counterparts in other reports. Both Aley et al. (1980) and Aust-Kettis et al. (1983) detect major bands at 130 kD, ~ 100 kD, and several between 43-48 kD similar to those described here.

Using the 21 Mabs in EITB, it was found that 14 of these recognised antigens on nitrocellulose immune replicas of E. histolytica lysates following SDS-PAGE under reducing conditions. There are various reasons why 7 Mabs failed to react in these experiments. Firstly, the relevant antigens may not have been present in the detergent lysates produced. Although Nonidet-P40 is thought to solubilize most proteins (Goding, 1986; Johnstone and Thorpe, 1982) there are certainly some antigens of E. histolytica that would not be present in these lysates. Additionally some antigens may be glycolipids, such as the aminoglycophospholipid isolated by Calef and Gitler (1984), which would probably

be absent from detergent lysates. It is possible that the antigens against which Mabs 22.6 and 29.6 are directed are lipid-based molecules, such as those located within the plasma membrane. Secondly, the relevant antigen may be present on the nitrocellulose but in a denatured form such that the conformational determinant is not recognised by the monoclonal antibody. This inability to detect SDS-denatured antigen has been reported by several authors (Turner, 1983; Conelly et al., 1985). It is thought that during EITB as the protein binds to the nitrocellulose, much of the denaturing SDS continues to the anode allowing the protein to regain more of its native configuration (Goding, 1986), but removal of all SDS would be unlikely. It is to be expected that a certain proportion of Mabs raised against native antigens will not recognise SDS-denatured antigen in EITB, but since the screening method utilised here required that the Mabs be capable of recognising antigen fixed in methanol, this perhaps lowered that proportion (Goldstein et al., 1982). Three out of the four IgMs among the Mabs did not give results in EITB, reflecting perhaps the lower affinity binding of this subclass failing to recognise partially denatured antigen.

Mab 22.1 detected several bands on blots as well as a smear of staining between the bands. Multiple bands in EITB have been reported by other workers (Ortiz-Ortiz et al., 1986) and a number of underlying explanations are possible: (a) The Mab may be recognising common determinants (especially if carbohydrate) on a series of related cell proteins or on subunits (separated under the reducing conditions) of one protein. (b) The Mab may be recognising the products of degradative proteolysis of a protein by the amoeba's own proteinases during production of the lysate. This phenomenon has been reported by Reichlin (1975) with human serum albumin. (c) The Mab may be recognising precursors of a protein during biosynthesis and processing in the cell as reported by Holder and Freeman (1982) for an antigen of Plasmodium falciparum. Since Mab 22.1 recognises an antigen expressed on the surface of the amoeba and also internally in some cells (Chapter 4), it is possible that these bands may represent stages, from synthesis within the cytoplasm to expression at the plasma

membrane. Analysis of purified plasma membrane and other subcellular fractions by EITB may yield further information relating to this phenomenon. The significance of the smear obtained in blots is not known but its restriction to the area between discrete bands suggests that it may be due to the protein being heavily glycosylated because of irregular binding of SDS and a consequently variable charge:mass ratio (Goding, 1986).

Rosenberg and Gitler (1985) identified a major plasma membrane glycoprotein of 57 kD and one of 100 kD among a total of 34. More recently Torian et al. (1987) described 5 Mabs recognising a protein of 96 kD present on the surface membrane of E. histolytica. This antigen is believed to be a glycoprotein since binding of the Mabs is sensitive to oxidation by periodate although periodate treatment may also affect the polypeptide structure of the protein (Cecil, 1963; Bergamini et al., 1983). Calderon and Avila (1986) isolated rabbit antibody-induced caps of E. histolytica and identified a 98 kD surface protein enriched in the cap. It is possible that these reported surface proteins of approximately 98 kD apparent molecular weight represent the same molecule and that Mab 22.1 is also recognising it. The Mabs produced by Torian et al. (1987) however, did not recognise any labelled antigens of other molecular weights in immunoprecipitation experiments. Other workers using the same ^{125}I-lactoperoxidase labelling method (Krupp et al., 1983; Joyce and Ravdin, 1988) failed to detect surface proteins of either ~ 57 kD or ~ 98 kD. However Joyce and Ravdin (1988) did detect an internal 59 kD glycoprotein antigen recognised in EITB experiments using all 11 of the human patient sera tested. This further demonstrates the difficulty of comparing immunochemical analyses from independent sources.

Recently, three independent groups of workers have detected specific surface proteins of functional significance. Petri et al. (1987b) have detected and isolated the galactosamine/N-acetyl-D-galactosamine-inhibitable lectin found to consist of 170 kD and 35 kD subunits which migrate as a single band of 260 kD under non-reducing conditions (Petri et al., 1989). Rosales-Encina et al (1987) isolated a 220 kD

lectin whose effect is inhibitable by the trimer or tetramer of N-acetyl-D-glucosamine. Arroyo and Orozco (1987) have identified a 112 kD protein, Mabs against which were capable of partially inhibiting amoebic adherence to erythrocytes and the cytopathogenic effect on cultured cell monolayers. All three of these antigens are recognised by antibodies present in human immune sera. No details of surface-related antigens of molecular weight greater than 300 kD, such as those detected here by Mab 22.1, have so far been reported in the literature.

The multiple bands of low molecular weight detected by Mab 29.11 may have a similar basis to the phenomenon observed with 22.1 discussed above. The antigens detected by 29.11 however, are internal and not expressed on the surface of the amoeba. A major internal antigen of 43 kD, recognised by 8 out of 11 sera from cured liver abscess patients, has been reported by Joyce and Ravdin (1988).

Mab 22.7, which in IFAT experiments recognised internal vacuolar structures and occasionally the inner surface of the plasma membrane (Chapter 2), detected a protein band with an apparent molecular weight of 190 kD. Although Rosenberg and Gitler (1985) report a 195 kD glycoprotein present in the membrane but apparently not exposed on the external surface it is not known whether it also occurs in internal membranes. Mathews et al. (1986) detected antigens of approximately 230 kD, 175 kD and 145 kD using human immune sera in EITB analysis of both internal and plasma membrane fractions of amoebae demonstrating the antigenic similarity of the two membrane systems.

Mabs 22.8, 24.2 and 24.4 all recognised antigens of approximately 100 kD in EITB under non-reducing conditions. Under reducing conditions, 24.2 and 24.4 detected an antigen of 100 kD while 22.8 failed to detect any bands of protein. Considering this result, the identical EITB band pattern obtained with 24.2 and 24.4 (different from 22.8), and taking into account the identical behaviour of these two Mabs in IFAT experiments using E. histolytica and E. invadens (Chapters 2 and 3), it is not unreasonable to suggest that they both

recognise the same single chain polypeptide of approximately 100 kD molecular weight. They may be directed against different epitopes of the same antigen but the possibility exists that they arose from the same clone due to cross-contamination of cultures during early hybridoma growth although every precaution was taken against this. Mab 22.8 appears to recognise a different 100 kD polypeptide.

A similar situation occurred with Mabs 24.5, 24.7 and 24.8, which produced identical patterns on blots, recognising an antigen of approximately 97 kD. The same possibility that these Mabs might be identical due to cross-contamination of cell lines, also exists. This question could be answered by testing the ability of the Mabs to recognise the same fragments in EITB experiments using affinity-purified antigen that has been exposed to limited proteolytic digestion (eg. using trypsin). The sensitivity of the Mabs' antigen binding capacity to antigen that has received prior Proteinase K or periodate treatment (see below) would also yield information on whether or not the epitopes recognised are different or identical.

Mab 29.3 recognised an antigen with an apparent molecular weight of 120 kD under both reducing and non-reducing conditions, indicating that the molecule within the cell is a single polypeptide. Interestingly, a protein of identical molecular weight was detected by this Mab in EITB experiments using an E. invadens lysate under reducing conditions supporting the cross-reaction noted with this species in IFAT experiments (Chapter 3).

Mab 22.4 detected an antigen of 115 kD under reducing conditions that migrated as a band of 230 kD under non-reducing conditions suggesting that the in vivo protein might possibly be a dimer consisting of two identical 115 kD subunits.

Finally, Mab 24.3 detected a protein of 130 kD which might possibly correspond to the prominent band of the same molecular weight noticed in gradient SDS-polyacrylamide gels and EITB strips stained for total protein (Plate 5.2).

With the limited experimental evidence presented here it is difficult to draw conclusions on the significance of these antigens, detected by the Mabs, in terms of their function or their role in stimulating an immune response in infected individuals. The latter might be investigated by using affinity purified antigen (using the Mabs) in EITB experiments and probing blots with human immune serum. Further immunochemical analysis would involve investigation of the biochemical nature of the antigens. Protein antigens are likely to be sensitive to proteases, such as proteinase K or trypsin, destroyed by heat (100 °C) and to survive treatment by periodate. The converse is true for carbohydrate antigens although none of these tests is absolutely diagnostic. For example, some proteins are extremely resistant to proteolysis (Hardman et al., 1981) while certain amino-acids such as tyrosine and tryptophan are oxidised by periodate (Geoghegan et al., 1980). In spite of this, abrogation Mab binding following some of these antigen treatments may give a prelimary suggestion of the nature of the epitope involved and thus the antigen.

Attempts could also be made to characterise antigens where the Mabs failed to produce results in EITB experiments. These would involve (a) immuno-precipitation of metabolically-labelled or iodinated (in surface antigens) antigen followed by SDS-PAGE and autoradiography or (b) Affinity chromatography or immunoadsorption experiments using Mab covalently linked to solid phase gel.

CHAPTER 6

THE USE OF MONOCLONAL ANTIBODIES IN AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR E. HISTOLYTICA ANTIGEN

6.1 INTRODUCTION

The definitive diagnostic test for amoebiasis is generally regarded to be detection of the organism in faeces or tissues (Healy, 1978). In asymptomatic cases this involves the detection of cysts in the faeces, while diagnosis of symptomatic intestinal amoebiasis relies on the identification of trophozoites containing ingested erythrocytes (Elsdon-Dew, 1969). Numerous fixatives, stains and concentration techniques are available for use in the direct identification of amoebic cysts and trophozoites. There are, however, certain problems associated with microscopic examination: (i) the method is time-consuming and requires trained personnel (ii) the excretion of parasites is not continuous and their distribution in the faecal specimen may be uneven (Despommier, 1981). For this reason, it is recommended that at least three separate stool specimens be examined. (iii) Successful detection relies on the morphological integrity of the trophozoites or cysts. (iv) A number of "interfering" substances are capable of causing reduction, or temporary elimination, of numbers of amoebae in the stool (Juniper, 1969). Such substances include broad-spectrum antibiotics, antacids, sulphonamides and various antidiarrhoeal preparations.

The diagnosis of hepatic amoebiasis by direct observation is complicated by the fact that: (i) liver abscesses frequently occur after or in the absence of intestinal symptoms (Adams and MacLeod, 1977b) and (ii) attempts to aspirate amoebic liver abscesses are not always successful and amoebae are not always detected in the sample (Healy, 1986). In the light of these and the problems associated with stool sample examination, the use of immunological techniques for the diagnosis of amoebiasis has become widespread in the last 25 years. These techniques have been of great value both for the diagnosis of invasive disease in individual patients, and for assessing the extent of invasive amoebiasis in populations by seroepidemiological survey. Immunodiagnostic tests can be of two basic types: (a) Those involving the detection of specific

anti-E. histolytica antibodies in sera, and (b) those involving the detection of E. histolytica antigen in patient samples (usually stools or sera).

There are about eleven different types of immunodiagnostic test for the presence of specific circulating antibody currently in use in various parts of the world (Healy, 1986). However, those in most widespread use are: cellulose acetate membrane precipitation (CAP), Ouchterlony immunodiffusion (ID), indirect fluorescent antibody test (IFAT), indirect haemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA). The ELISA (or enzyme immunoassay-EIA) method has received particular attention over recent years and has resulted in the production of a number of commercially available kits. A problem common to these serodiagnostic tests is the persistence of circulating antibody in patients long after therapy and presumed cure (Healy et al., 1974) and the consequent inability to distinguish between current infection and past disease.

The detection of amoebic antigen in patient samples implies current infection with E. histolytica. Several authors have reported the successful detection of circulating immune complexes in the serum of patients. Pillai and Mohimen (1983) using radioimmunassay found immune complexes in the sera of all 21 patients with amoebiasis and none in the sera of 22 uninfected control patients or 6 drug-cured dysenteric cases. This procedure involved the polyethylene glycol (PEG) precipitation of the immune complexes and took approximately 24 hours. More recently, Vinayak et al. (1986) reported the use of a more rapid (11 hours) ELISA that detected immune complexes in the sera of 14 out of 15 confirmed amoebic liver abscess patients and 4 out of 7 suspected cases. None of 9 sera from cases of non-amoebic hepatitis or 10 sera from controls, contained detectable complexes. These results show promise for the development of a simple, rapid, non-radioactive procedure for routine laboratory diagnosis of amoebiasis. Although reasonable success has been reported in the detection of amoebic antigen in liver abscess aspirates using counter immunoelectrophoresis (Mahajan and Granguly, 1980) and ELISA techniques (Bhave et al., 1985), the need for successful aspiration remains a disadvantage.

Root et al. (1978) described an ELISA method for the detection of amoebic antigen in faeces. The IgG fraction of rabbit anti-E. histolytica antisera was adsorbed onto 3 discs (positive and negative controls and sample) of a patented material mounted on a polystyrene slide. A suspension of faeces in buffer solution was applied to one of the discs and the slide agitated for 12-16 hours. After washing, peroxidase-conjugated antiamoebic IgG was added to each disc and the slide incubated for 2 hours at 20 °C. After further washing, colour development using 3-amino-5-ethylcarbazole was performed together with the inclusion of discs not treated with conjugated IgG, to test for endogenous peroxidase activity of the sample. Problems associated with the test included the requirement that faeces had to be fresh or at most, stored at 4 °C for only a few hours, and the presence of peroxidase in the faecal sample. This method formed the basis of a commercial kit (Immunozyme E. histolytica Reagent Kit, Millipore Corporation). The first extensive trial of this ELISA was reported by Palacios et al. (1978), in which doubts about the specificity of the system were raised. Although 155 of 159 samples containing cysts or trophozoites of E. histolytica were ELISA-positive, only 307 out of 562 microscopy-negative samples were ELISA-negative. Although these results may have been due to false positives, it is possible that the numbers of organisms present in the stools were below the level of microscopic detection. Randall et al. (1984) using the same kit in a study in male homosexuals, reported no ELISA-positive/ microscopy-negative results, but found that only 5 out of 12 microscopy-positive samples were ELISA-positive. In contrast, these results show a high specificity but a lower sensitivity, which, the authors suggested, may be due to a lower reactivity of cysts (present in the stools of these patients) as compared with trophozoites, against which the rabbit antiserum used in the test, was raised.

Grundy (1982) described the use of plastic microtitre plates in an ELISA test, again using rabbit anti-E. histolytica antiserum as a capturing antibody. Of the five samples tested that were positive for E. histolytica by microscopic examination, the three containing

trophozoites were ELISA-positive, while the two containing cysts were negative. This evidence supports the above suggestion by Randall et al. (1984), since here again, axenically-grown trophozoites were used as immunising antigen for production of the rabbit antiserum.

The use of a monoclonal antibody antibody in an ELISA test for E. histolytica in faecal samples was described by Ungar et al. (1985). Polystyrene microtitre plates were coated with a commercially produced monoclonal antibody raised against the HK9 axenically-grown strain. After incubation with diluted faecal sample and appropriate washes, rabbit antiserum against HK9 was added, followed by an alkaline phosphatase-conjugated goat anti-rabbit antiserum. Out of 22 specimens containing E. histolytica trophozoites and/or cysts, 18 were positive by ELISA; the 4 ELISA-negative stools had been stored frozen for up to 3 months, a finding similar to the loss of antigenicity following freezing described by Root et al. (1978). Only three of 186 microscopy-negative stools were ELISA-positive and all stools containing other species of amoebae were ELISA-negative. The test described was comparatively rapid (approximately 5 h), specific and sensitive and, in contrast to the previously mentioned test, showed good reactivity with the cyst stage of the parasite.

Some of the monoclonal antibodies described in earlier chapters were tested as "antigen capture" antibodies in an ELISA similar in procedure to that described by Ungar et al. (1985).

6.2 MATERIALS AND METHODS

6.2.1 Preparation of E. histolytica antigen

Culture tubes containing approximately 10^6 amoebae (strain 200:NIH) in late log phase growth were cooled in ice water for 5 min, inverted several times to remove adherent cells and centrifuged at 200 g for 5 min at RT. The pellet was washed twice in PBS (pH 7.4) and the cells resuspended in 1 ml of PBS in a 1.8 ml microcentrifuge tube. This suspension was subjected to three freeze-thaw cycles in liquid nitrogen. Following centrifugation at 11,800 g for 10 min at 4 °C, the supernatant was assayed for protein content using the method of Lowry et al. (1951) and stored at -22 °C.

6.2.2 Preparation of rabbit anti-E. histolytica antiserum

One New Zealand White rabbit was immunised intramuscularly with 2 x 10^6 washed E. histolytica (strain 200:NIH) in Freund's complete adjuvant and boosted on days 14 and 28 with the same dose in Freund's incomplete adjuvant. Blood was collected on day 35 and serum prepared.

This serum was subjected to affinity chromatography to remove any cross-reactivity with mouse immunoglobulin. A column of mouse monoclonal antibody against Trypanosoma brucei rhodesiense (GUPM 7.1) coupled to cyanogen bromide activiated Sepharose 4B (Pharmacia) was prepared according to the manufacturer's instructions using 10 mg protein per ml of gel. A 5 ml aliquot of immune rabbit serum was applied to an equal volume of coupled gel and the column washed with 20 ml of PBS (pH 7.4). The eluate was collected and concentrated by osmotic pressure dialysis using polyethylene glycol, 40000 D molecular mass (Sigma, Poole).

6.2.3 Enzyme-linked immunosorbent assay (ELISA)

A double antibody ELISA was used, involving anti-E.histolytica monoclonal antibody adsorbed to the solid phase (microtitre plate). After the addition of test material, the presence of antigen was detected using Rabbit anti-E.histolytica antiserum followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum. Optimal concentrations of these reagents were determined by titration.

The standard protocol was as follows: Microtitre plate (Immuno II, Nunc) wells were coated with 50 µl of Mab-containing ascitic fluid, diluted 1/1000 in 0.05 M sodium carbonate/bicarbonate buffer pH 9.8, overnight at 4 °C. The plate was washed three times with PBS containing 0.05% Tween-20 (Sigma, Poole) (PBS-Tween) before use. E. histolytica lysate was diluted in PBS-Tween containing 0.25% w/v BSA (diluting buffer), and 50 µl aliquots added to duplicate wells at the following concentrations of total protein: 50 µg/ml, 10 µg/ml, 1 µg/ml 0.1 µg/ml and 0.01 µg/ml. Control wells received 50 µl of diluting buffer. After incubation for 30 min at 37 °C, the plate was washed three times with PBS-Tween and 50 µl aliquots of Rabbit anti-E.histolytica antiserum, diluted 1/200 in diluting buffer, were added to each well. After a further incubation for 30 min at 37 °C, followed by three washes, 50 µl of alkaline phosphatase-conjugated goat anti-Rabbit antiserum (Sigma, Poole), diluted 1/500 in diluting buffer, was added to each well. Following incubation for 30 min (37 °C) and three washes, colour was developed by the addition of 50 µl p-nitrophenyl phosphate substrate solution (Sigma, Poole) (1 mg/ml in 10% Diethanolamine, pH 9.8) to each well. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 50 µl 3 M NaOH to each well, and the optical density at 405 nm was measured using a Titertec Multiscan Colourimeter (Flow, Irvine).

Protocol B contained the following alterations to the standard protocol: (i) The E. histolytica antigen was diluted in PBS-Tween containing 5% w/v Marvel* (Cadbury's) instead of normal diluting buffer. (ii) Incubation steps involving E. histolytica antigen,

* Dried skimmed milk

Rabbit antiserum and anti-Rabbit conjugate were performed for 30 min at room temperature with the plate on a rotary shaker (240 cycles per min).

Protocol C contained the following alteration: A separate incubation step was added between Mab coating and antigen incubation. This involved the addition of 75 µl of PBS-Tween containing 5% Marvel to each well and incubation for 30 min at 37 °C, followed by washing three times in PBS-Tween. All other steps performed as standard protocol.

6.2.4 Preparation of faecal extract

Samples of parasitologically-negative human faeces were thoroughly mixed in an equal volume of PBS (pH 7.4). The mixture was passed through an Endecott filter, 250 µm mesh size, to remove large particles, followed by passage through Whatman No.1 filter paper. This filtrate was filter sterilised through 0.22 µm Millex filters (Millipore) and stored as 3 ml aliquots at -22 °C.

6.2.5 Preincubation of E. histolytica antigen in human faecal extract

Amoebic lysate was incubated in human faecal extract at a concentration of 50 µg/ml for 7 days at 4 °C. This antigen was used in ELISA experiments, protocol C in place of antigen in diluting buffer. Control wells received 50 µl of faecal extract in place of diluting buffer in the antigen incubation step.

6.3 RESULTS

Seventeen Mabs (22.1, 22.3, 22.5, 22.6, 22.7, 22.8, 24.1, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3, 29.6, 29.10, 29.11) were tested as "antigen-capture" reagents in a double layer indirect ELISA for soluble E. histolytica antigen, using the standard protocol described. Antigen was used at five different concentrations and the tests carried out in duplicate wells. The mean of the two optical density values was calculated and the results are presented in Figure 6.1. A dilution of antigen was considered positive if it yielded an optical density value greater than twice that of diluting buffer alone (control wells).

Mabs 24.7 and 29.3 were able to detect amoebic antigen at a level of 0.1 µg/ml while eight other Mabs (22.3, 22.6, 22.8, 24.2, 24.4, 24.5, 24.6 and 24.8) were able to detect antigen down to a level of 1 µg/ml. Varying the dilutions of the Mab in coating solutions from 1/100 to 1/4000 produced very little difference in both maximum and minimum optical density values obtained (results not shown).

Nine Mabs (22.8, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3 and 29.10) were used in experiments in which the standard ELISA protocol (protocol A) was altered. Protocol B involved shaking during incubation steps, while protocol C involved a separate blocking step after coating and prior to sample addition. The results of these experiments are presented in Figure 6.2. The alterations to the standard protocol (A) employed in protocol B had little or no effect on the sensitivity of the antigen detection. The addition of a separate blocking step in protocol C caused, in general, a reduction of background signals (diluting buffer controls) and an increase in the sensitivity of antigen detection, although no statistical analyses were performed. Consequently seven Mabs (22.8, 24.2, 24.4, 24.5, 24.6, 24.7 and 29.3) were able to detect amoebic antigen down to a level of 0.1 µg total protein per ml.

Nine Mabs (22.8, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3 and 29.10) were used in ELISA experiments, protocol C, to detect ameobic antigen that had been previously incubated in human faecal extract for 7 days. Five Mabs (22.8, 24.2, 24.5, 24.8 and 29.3) were able to detect antigen down to a level of 0.1 μg/ml total protein, while Mab 24.7 detected antigen to a level of 0.01 μg/ml. The results of these experiments have not been presented graphically.

FIGURE 6.1

Titration curves of Optical Density at 405 nm (OD_{405}) versus *E. histolytica* lysate protein concentration using Mabs in double sandwich ELISA under optimum conditions.

Mabs 22.1, 22.3, 22.5, 22.6, 22.7, 22.8, 24.1, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3, 29.6, 29.10 and 29.11 used to coat microtitre plates at 1/1000 dilution.

E. histolytica lysate antigen diluted in PBS-Tween with 0.25% BSA

Dotted line = OD_{405} obtained with diluted buffer in place of *E. histolytica* antigen.

See Section 6.2 for details of method.

FIGURE 6.1

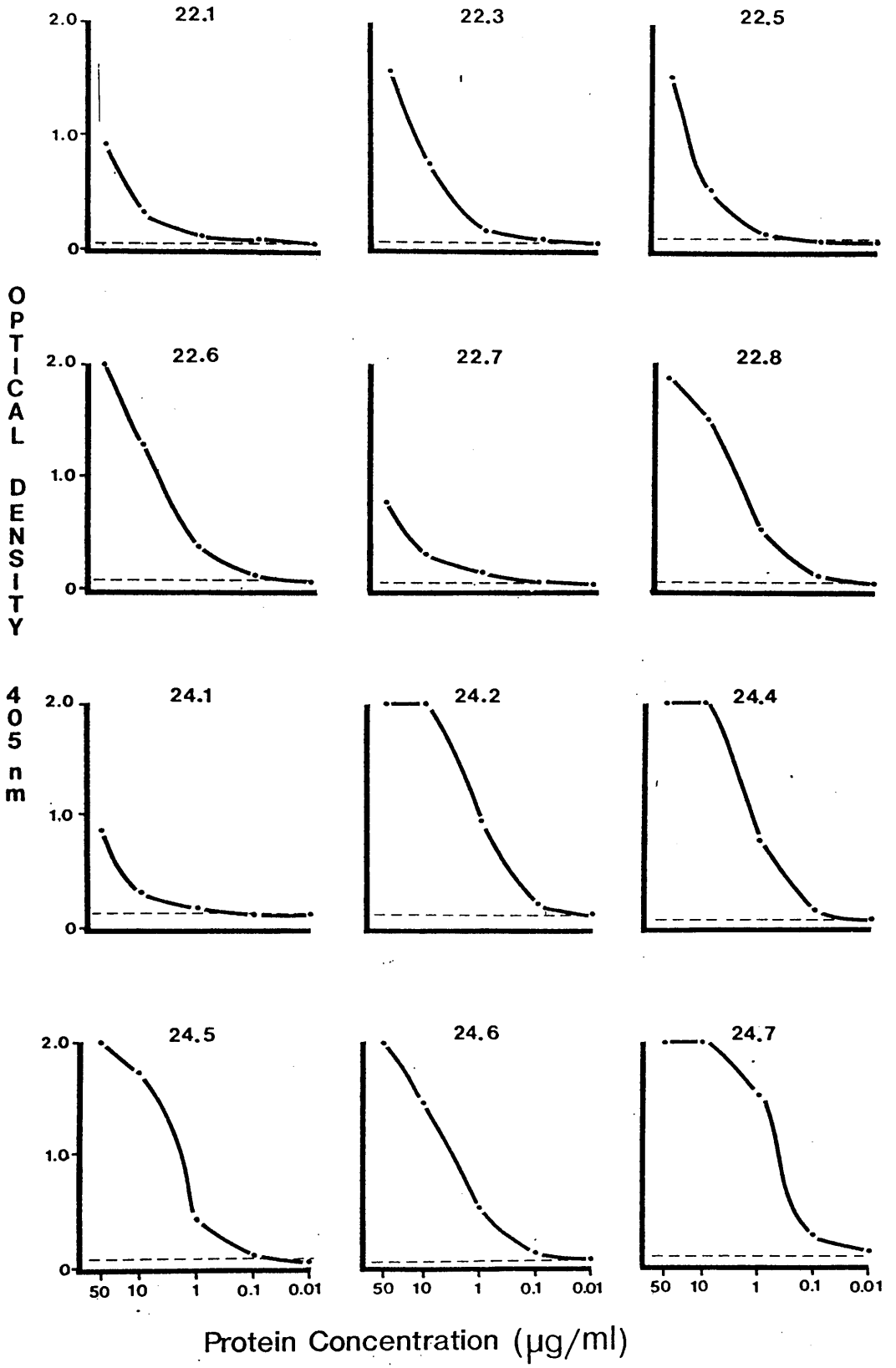

22.1
22.3
22.5
22.6
22.7
22.8
24.1
24.2
24.4
24.5
24.6
24.7
2.0
1.0
0
OPTICAL DENSITY
405 nm
50 10 1 0.1 0.01
Protein Concentration (µg/ml)

FIGURE 6.1 continued

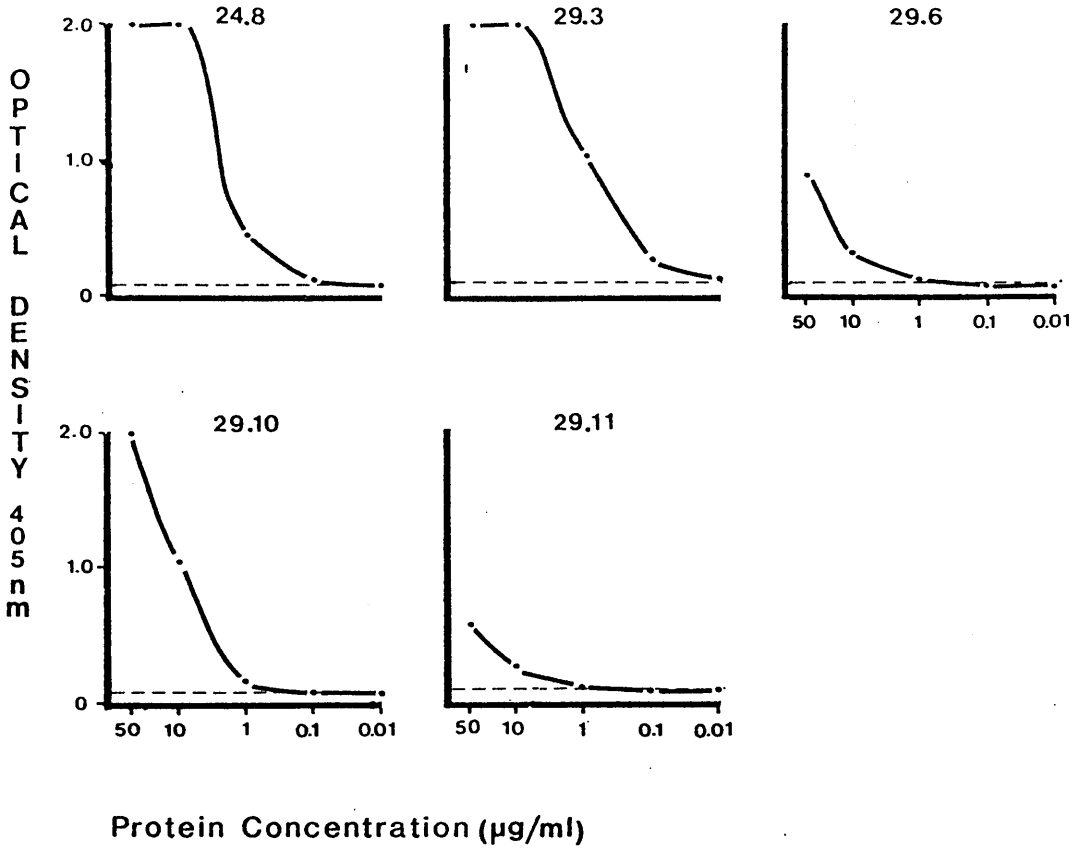


FIGURE 6.2

Titration curves of Optical Density 405 nm (OD_{405}) versus *E. histolytica* lysate protein concentration using Mabs in double sandwich ELISA under three sets of conditions.

Mabs 22.8, 24.2, 24.4, 24.5, 24.6, 24.7, 24.8, 29.3 and 29.10 used to coat microtitre plates at 1/1000 dilution.

Protocol A - Standard protocol

Protocol B - Incubations performed on rotary shaker

Protocol C - Separate pre-antigen blocking step (5% Marvel)

Dotted line = OD_{405} obtained with diluting buffer in place of *E. histolytica* antigen.

See Section 6.2 for details of methods.

FIGURE 6.2

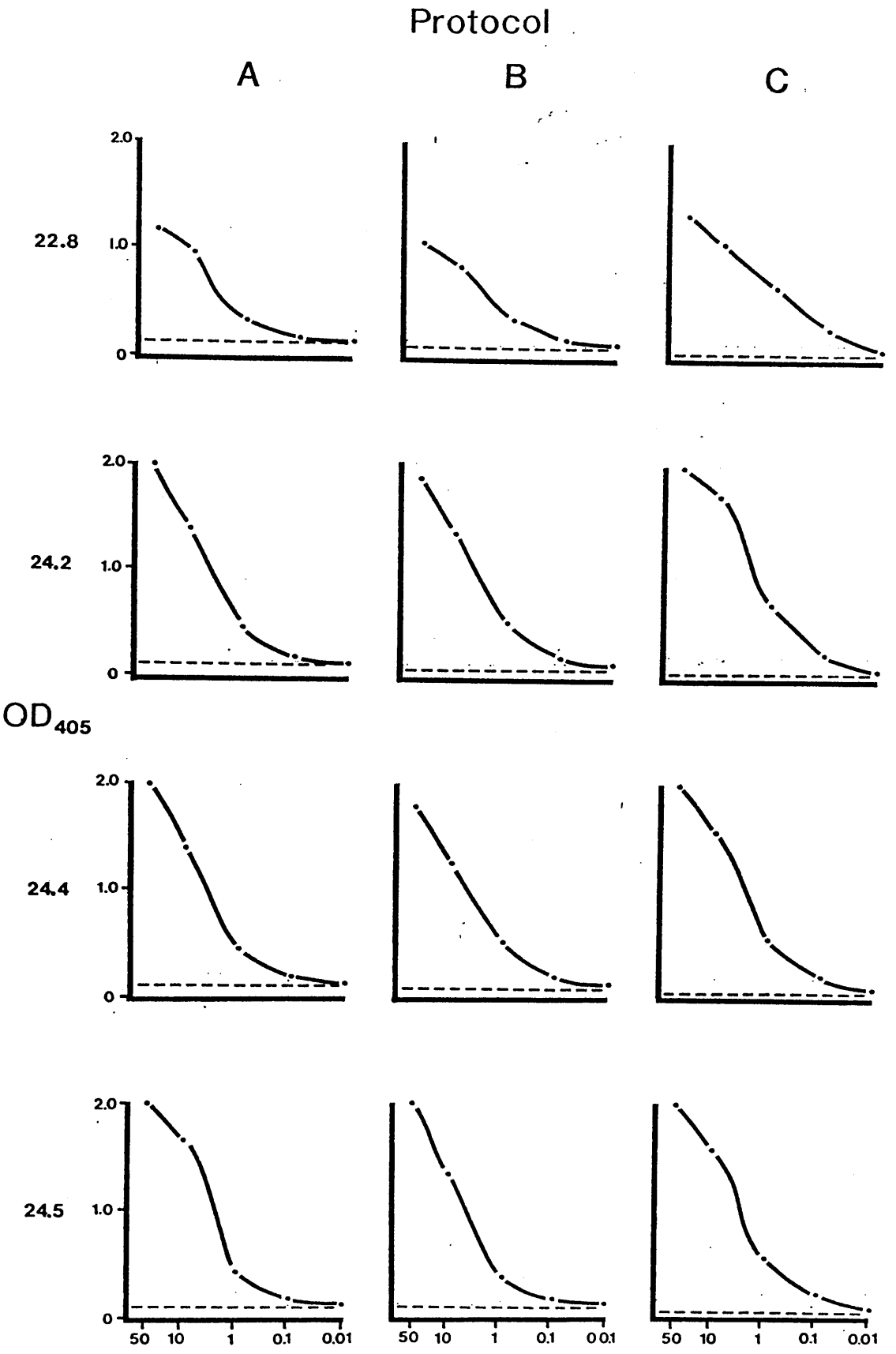

Protocol
A
B
C
22.8
24.2
24.4
24.5
OD405
2.0
1.0
0
50
10
1
0.1
0.01
Protein Concentration (µg/ml)

FIGURE 6.2 continued

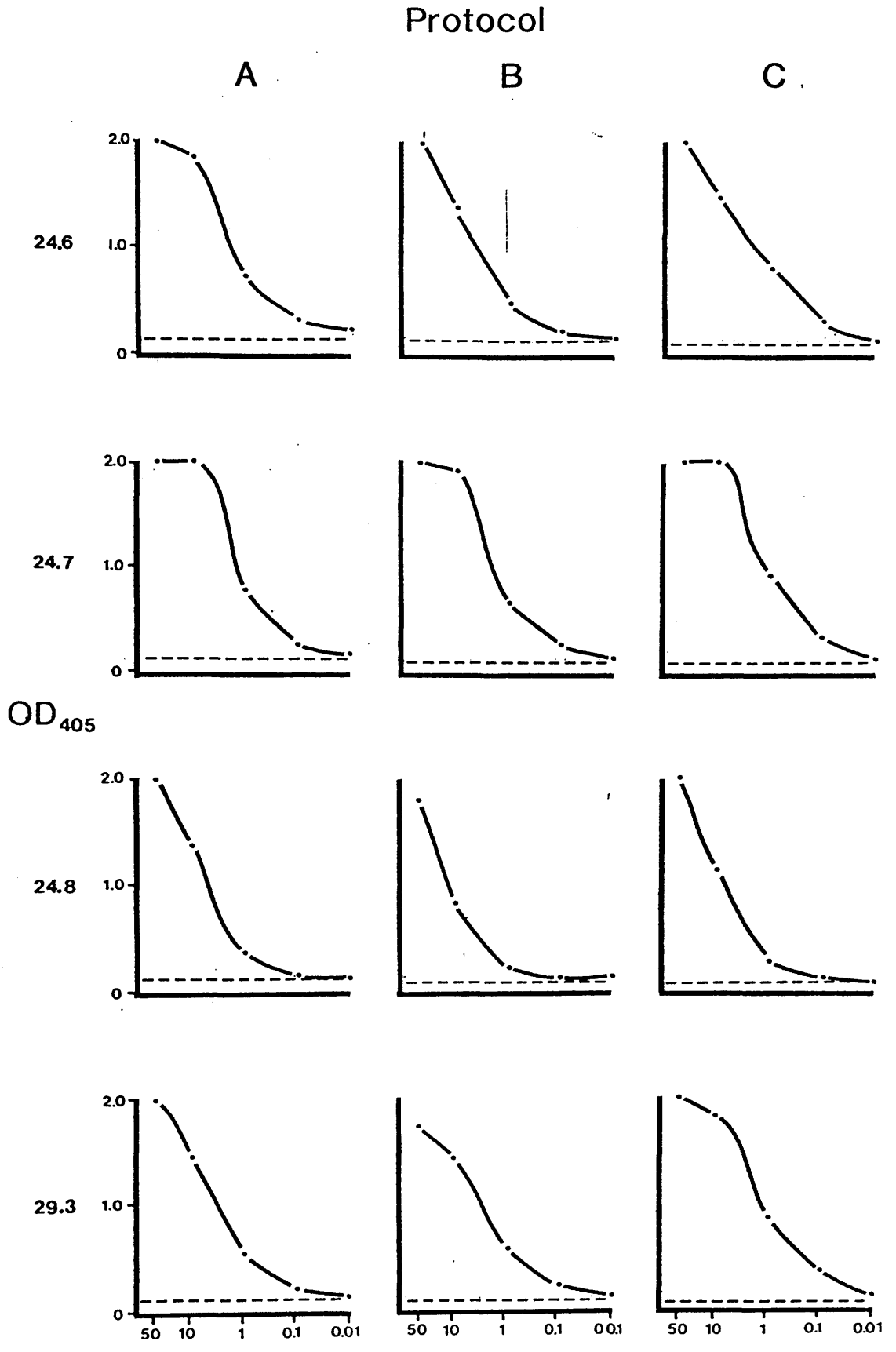
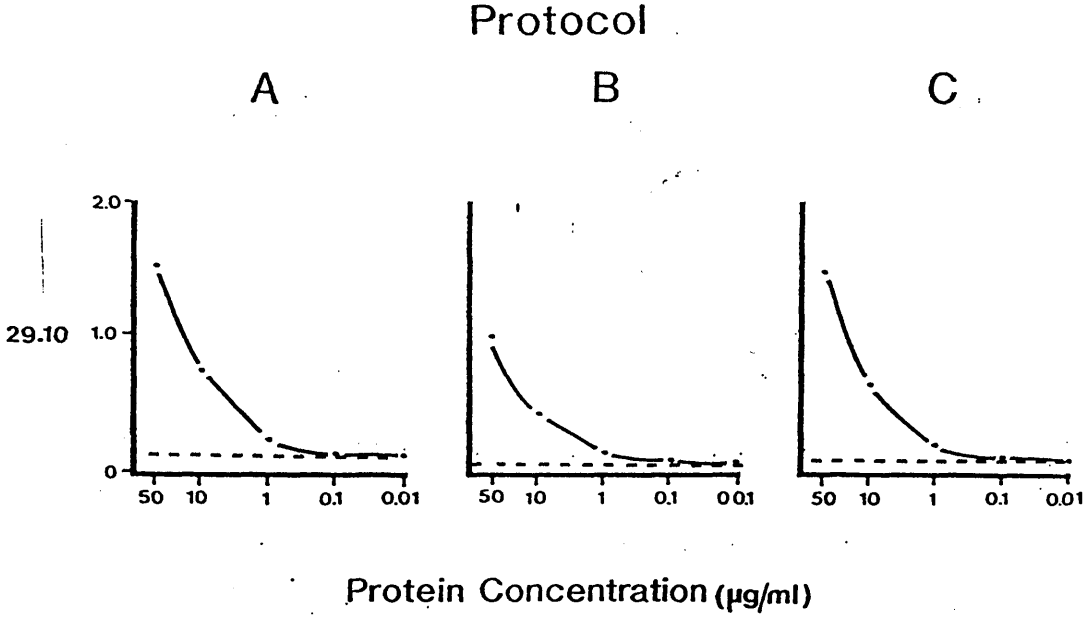


FIGURE 6.2 continued



6.4 DISCUSSION

Previous attempts to develop ELISA tests for the detection of E. histolytica antigen in patient samples have, with the exception of one, used polyclonal antisera. These assays have been lengthy to perform and, in the case of one widely used assay for ameobic antigen in faeces (Root et al., 1978; Palacios et al., 1978), shown inadequate specificity. Ungar et al. (1985), using the specificity of a monoclonal antibody in combination with an animal derived antiserum, developed an apparently successful ELISA with a considerably shorter assay duration. In the work described in this Chapter, Mabs raised against axenically-grown E. histolytica (strain 200:NIH) that were shown not to cross-react with other human protozoan parasites in IFAT experiments (Chapter 3), were used as "antigen capture" antibodies in a rapid (3 hour) double sandwich indirect ELISA for laboratory prepared 200:NIH antigen.

Two of the Mabs (24.7 and 29.3), when used in the assay, were able to detect antigen down to a level of 0.1 μg total amoebic protein per ml of sample. When a separate 30 min blocking step was added before antigen incubation, a further five Mabs were capable of this level of detection. These results compare very favourably with the results reported by previous workers; Root et al. (1978) showed that their method had a sensitivity of 0.23 μg/ml while the assay described by Grundy (1982) had a higher sensitivity of 0.15 μg/ml. Ungar et al. (1985) gave no precise figures for amoebic protein detection. If, however, an approximation of 10^6 organisms being equivalent to 1 mg of protein is used, their highest reported sensitivity of 40 organisms per ml would correspond to detection of 0.04 μg/ml.

The poor performance of several of the Mabs in this assay has several possible causes: (a) the antigens for which they are specific may be present only in small amounts in the lysate or may have been denatured during the lysate preparation. (b) The antigens bound by the Mabs may be recognised by antibodies present only in very small

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The poor performance of several of the Mabs in this assay has several possible causes: (a) the antigens for which they are specific may be present only in small amounts in the lysate or may have been denatured during the lysate preparation. (b) The antigens bound by the Mabs may be recognised by antibodies present only in very small

amounts in the rabbit antiserum. (c) The Mabs may be of low affinity, so that antigen is only weakly bound or is easily displaced from the antigen binding site during washing stages. This last, is probably the most likely explanation, since low optical densities were obtained even in conditions of antigen excess (Steward and Lew, 1985). While the addition of a separate blocking step, designed to reduce non-specific binding of antigen to the plate, seemed to increase the sensitivity of the assay, attempts to maximise antigen monoclonal and antibody-antibody interactions by mixing on a rotary shaker did not seem to have any effect.

Results obtained with antigen previously incubated in human faecal extract for 7 days were encouraging. Faecal components do not seem to cross-react or interfere with at least six of the Mabs, and the antigens for which these Mabs are specific do not seem sensitive to any protease activities in the extract, which can be a problem in faecal antigen detection (Green, 1986).

Most of the Mabs produced in this work, the exceptions being 22.3 and 22.5, have not been tested for their reactivity with cysts of E. histolytica (the only reliable source being asymptomatic carrier faeces). Since they were raised against trophozoites of the parasite, it is possible that, like 22.3 and 22.5, they do not recognise cyst antigens. E. histolytica trophozoite antigens have, however, been shown to cross-react with Entamoeba invadens cyst antigens (Yap et al., 1970) and the Mab utilised by Ungar et al. (1985) was certainly capable of recognising E. histolytica cysts. These Mabs may therefore have more use in the detection of circulating immune complexes which would involve trophozoite-derived antigens. Such a test would, by nature, be specific for current invasive disease; whereas faecal antigen detection, unless pathogen-specific Mabs were utilised (Chapter 7), would detect both asymptomatic and symptomatic (invasive) amoebiasis. Unfortunately the Mabs specific for pathogenic amoebae (22.3 and 22.5) did not perform well in the ELISA procedure described here.

In summary, several of the Mabs appear promising as reagents in an ELISA specific for amoebic antigen, although the type of assay in which they might be suitable (faeces or serum detection of antigen), can only be determined by the extensive testing of patient samples.

CHAPTER 7

IMMUNOLOGICAL DIFFERENTIATION OF PATHOGENIC AND NON-PATHOGENIC ISOLATES OF ENTAMOEBA HISTOLYTICA.

This work was performed in the Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, London. Financial support for this work was provided by the Wellcome Trust and the World Health Organisation.

This work has been previously published as: Strachan, W.D., Spice, W.M., Chiodini, P.L., Moody, A.H. and Ackers, J.P. (1988). Immunological differentiation of pathogenic and non-pathogenic isolates of Entamoeba histolytica.
Lancet, i: 561-563.

7.1 INTRODUCTION

While there are up to 500 million human infections with Entamoeba histolytica worldwide (Walsh, 1986b), in the vast majority of cases the parasite lives as a harmless commensal confined to the lumen of the large intestine. However, in a small but very variable proportion, invasion and dissemination occurs, causing symptomatic amoebiasis - classically and most frequently invasive intestinal disease or amoebic liver abscess. There has been much debate over the years between those who believe that all amoebae are potentially capable of invasion and are restrained by some host-mediated defence mechanisms (Faust et al., 1970), and those who feel that two kinds (or even two species) of Entamoeba are involved, one of which is always (potentially) invasive and the other incapable of causing disease (Brumpt, 1949). (This argument should not be confused with the similar-sounding disagreement over the pathogenic potential of the "small race" of E. histolytica, now universally accepted as a different and non-pathogenic species, E. hartmanni (WHO, 1969)). The argument for all E. histolytica being potential pathogens restrained by the host's immune system, was supported by exacerbations which seemed to occur during pregnancy (Abioye, 1973) and following treatment with corticosteroids (Kanani and Knight, 1969) and was widely accepted. However, in 1982 some of the first evidence of a fundamental difference between E. histolytica isolates obtained from symptomatic patients and asymptomatic cyst-passers was presented by Martinez-Palomo et al. (1973), who demonstrated differences in agglutinability by the lectin Concanavalin A. Since then other differences have been found which seem to be inherent in the organism, the best validated of which are the isoenzyme results of Sargeaunt and Williams (Sargeaunt et al., 1978; Sargeaunt and Williams, 1979). These authors have shown that particular band patterns obtained after starch-gel electrophoresis with the enzymes hexokinase (HK) and phosphoglucomutase (PGM) correlate very closely with the clinical findings in the patients from whom the organisms were isolated. In particular, E. histolytica isolates from patients

with invasive disease possess two advanced HK bands ("fast") while in isolates from asymptomatic cyst-passers the two bands are less advanced ("slow"). The same results have been obtained by other groups using different electrophoretic media: agarose (Mirelman et al., 1983), polyacrylamide (Mathews et al., 1983), and cellulose acetate (Moss and Mathews, 1987).

These results, and in particular their practical application, have been discussed in two recent Lancet editorials (Editorial, 1985; Editorial, 1986), with particular reference to the question of whether it is necessary or desirable to treat patients shedding cysts of a non-invasive zymodeme. In some populations where the carriage of invasive zymodemes appears to be extremely rare (for example male homosexuals in London (Goldmeier et al., 1986)) a non-treatment policy is already practised (Allason-Jones et al., 1986). This policy has recently been questioned by Mirelman and his co-workers, following their discovery that it is possible, by certain (admittedly very artificial) manipulations of the bacterial flora which accompany most cultures of E. histolytica, to change not just the isoenzyne pattern, but also the in vitro and in vivo virulence of non-invasive isolates of the parasite (Mirelman et al., 1986a,b). The significance or otherwise of these experiments is currently the subject of vigorous debate (Mirelman, 1987; Sargeaunt, 1987). However, the epidemiological results presented over the years by Sargeaunt and his co-workers leave little doubt that, in the majority of cases, the outcome of an infection with E. histolytica does correlate with the zymodeme of the infecting organism. This correlation clearly does have important clinical implications, as well as opening up new approaches for research. If, however, determination of the invasive potential is ever to be applied to routine samples, a method which is simpler, quicker and cheaper than culture (for up to seven days to obtain sufficient organisms) followed by isoenzyme electrophoresis, will need to be developed. A method is presented here, based on immunofluorescence using monoclonal antibodies, which may partially meet this need.

7.2 MATERIALS AND METHODS

The monoclonal antibodies (Mabs) 22.3 and 22.5 were members of a large group prepared by standard procedures from BALB/c mice immunised with axenically-grown trophozoites of the laboratory-adapted strain of E. histolytica 200:NIH, and utilised in the form of ascitic fluids obtained from pristane-primed mice. 200:NIH (ATCC 30458) was first isolated from sigmoidoscopic material in 1949 (Tobie, 1949) and has since been adapted to axenic (Bacteria-free) culture but retains a pathogenic zymodeme (Z II) (Sargeaunt et al., 1980b). It had already been established that some of the monoclonal antibodies (including 22.3 and 22.5) were specific for E. histolytica (Chapter 3), and since it was suspected that these two Mabs were specific for invasive isolates (Chapter 4), the trial described here was set up.

All faecal samples, found by routine examination at the Hospital for Tropical Diseases (St Pancreas, London), to contain cysts of E. histolytica, were stored at 4 °C for not more than six days, before being inoculated into Robinson's medium (Robinson, 1968) (but using 50% less starch than in the original formula) and cultivated at 37 °C. After the initial overlay change, sub-cultures were made as necessary until sufficient growth was obtained for isoenzyme examination (usually within seven days). Several older isolates of known invasiveness, which had been cryopreserved for up to four years, were re-established in the same medium. When enough organisms (about 10^5) were present, the amoebae from a single 5 ml bottle (plus starch and bacteria) were harvested by centrifugation, washed, resuspended in 20 µl of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM iodoacetate and 10 µg/ml N-p-Tosyl-L-lysine chloromethyl ketone, pH 8.0) and lysed by freezing (in liquid nitrogen) and thawing three times. Centrifugation (12,000 g for 1 min) yielded the supernatant which was used for isoenzyme assay. Only HK was examined, and lysates were run in agarose mini-gels submerged in 0.1 M borate buffer pH 8.0 (Mirelman et al., 1986a) at 50 V for 90 minutes. Enzyme bands were visualised

by the method used by Sargeaunt and Williams (1979), except that no overlay was used, and development was performed in a tank. Two strains of known invasiveness and zymodeme, TE (invasive, zymodeme II) and C29, (non-invasive, zymodeme I), were included as controls. Under these conditions the distinction between fast bands, indicating invasiveness, and slow bands was clear.

Reaction of the amoeba with the Mabs was assessed by indirect fluorescent antibody test. Samples of the bottom starch layer of 2-day Robinson's medium cultures were removed and gently mixed. Drops of suspension were added to the wells of the multispot PTFE-coated slides (Henley) and air dried. Slides were fixed in methanol (5 min) and stored with desiccant at -22 °C. Tripling dilutions of ascitic fluid were applied to wells and the slides incubated in a humidified atmosphere at room temperature for 30 min. Following two washes in phosphate-buffered saline (PBS) pH 7.2, fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse immunoglobulin (Amersham) was applied, diluted 1:50 in PBS with 1/10,000 Evans Blue, and the slides incubated for a further 30 min. Finally the slides were washed twice with PBS and mounted in 50% glycerol (Citifluor) in PBS. Wells were examined using a Leitz microscope with incident-light fluorescence, using a HB50 high pressure mercury vapour lamp, a TK 510 dichroic mirror, a 2 x KP 490 (exciting) and a K 515 (suppressing) filter. End-point dilutions were recorded as the highest producing recognisable specific fluorescence. All cultures were confirmed as E. histolytica by morphology and staining with the species-specific Mab 22.8. Immunofluorescence results were read before the HK gels were run; subsequently clinical details and the results of amoebic serology (where available) were supplied by Dr P L Chiodini, Consultant Parasitologist, Hospital for Tropical Diseases.

7.3 RESULTS

Fifty two faecal samples containing cysts of E. histolytica were obtained, from which 25 successful cultures (48%) were established although one (G) grew insufficiently to enable isoenzyme analysis. Results from these isolates, together with those for six cryopreserved isolates and four invasive, laboratory-adapted, axenic strains, are shown in Table 7.1. The pattern of staining produced by the two Mabs was not entirely similar; 22.3 produced a granular internal fluorescence, while 22.5 produced an internal vacuolar staining pattern (Section 2.3.2).

The two Mabs, 22.3 and 22.5 appear to bind exclusively to isolates of E. histolytica possessing fast mobility HK bands. All five of the isolates obtained from patients showing positive serology were recognised. Results from IFAT experiments using isolates possessing slow mobility HK bands were all negative at a Mab dilution of 1/9 ($\log_3$ titre = 2), while fast mobility HK isolates were positive to $\log_3$ titres 7 - 12.

LEGEND TABLE 7.1

COMPARISON OF CLINICAL FINDINGS, HEXOKINASE MOBILITY AND IFAT STAINING WITH THE TWO MONOCLONAL ANTIBODIES 22.3 AND 22.5 FOR VARIOUS ISOLATES OF ENTAMOEBA HISTOLYTICA.

The first four strains are long-established invasive isolates which have been adapted to grow without bacteria in axenic medium; the next six are cryopreserved clinical isolates recultured in Robinson's medium, and the remaining strains, fresh clinical isolates.

All clinical samples contained E. histolytica (cysts unless otherwise stated), and in addition: E.h + rbc = haematophagous trophozoites; G.l = Giardia lamblia (G. intestinalis); E.c = Entamoeba coli; I.b = Iodamoeba buetschlii; E. hart = Entamoeba hartmanni; E. nana = Endolimax nana; Z = zymodeme.

Ocas.diar.: occasional diarrhoea

E.h antibody: IFAT = Reciprocal slide immunofluorescence titres using fixed 200:NIH organisms as antigen; CAP = Precipitin reaction on cellulose acetate membrane (+ = Positive result; - = Negative result).

Mab IFAT titres are $\log_3$ dilutions
NA: Information not available
ND: Not done
IG: Insufficient growth for isoenzyme analysis

TABLE 7.1

Isolate	Date of isolation	Clinical details	E.h antibody IFAT	E.h antibody CAP	Hexokinase	Mab IFAT titres 22.3	Mab IFAT titres 22.5
NIH:200	1949	Axenic; ATCC 30458 ; Z II	ND	ND	Fast	7	8
HK9	1951	Axenic; ATCC 30015 ; Z II	ND	ND	Fast	7	8
HM 1: IMSS	1967	Axenic; ZII	ND	ND	Fast	7	8
Loon Lake	NA	Axenic; ZII	ND	ND	Fast	7	8
165	3.83	Not available	ND	ND	Fast	8	10
0478	4.83	Dysentery; ZII	160	-	Fast	9	10
TE	7.83	Amoebic dysentery; E.h + rbc	320	+	Fast	8	10
C29	3.85	Asymptomatic; ZI	-	-	Slow	Neg	Neg
8008	1.86	Asymptomatic	ND	ND	Slow	Neg	Neg
8672	1.86	Asymptomatic	ND	ND	Slow	Neg	Neg
SI	5.87	E.h + rbc	ND	ND	Fast	9	12
238	5.87	Asymptomatic	ND	ND	Slow	Neg	Neg
B	6.87	Asymptomatic	ND	ND	Slow	Neg	Neg
E	7.87	Intermittent diarrhoea	ND	ND	Slow	Neg	Neg
G	7.87	Asymptomatic	ND	ND	IG	Neg	Neg
I	7.87	Dysentery, enlarged liver	160	+	Fast	11	11
J	7.87	Asymptomatic	ND	ND	Slow	Neg	Neg

CONTINUED/

TABLE 7.1 CONTINUED

Isolate	Date of isolation	Clinical details	E.h antibody IFAT	E.h antibody CAP	Hexokinase	Mab IFAT titres 22.3	Mab IFAT titres 22.5
K	7.87	Asymptomatic	-	-	Slow	Neg	Neg
N	7.87	Ocas.diar.; E.h troph; G.l; E.c	-	-	Slow	Neg	Neg
R	7.87	Abdominal symptoms	320	ND	Fast	7	7
T	7.87	Asymptomatic	ND	ND	Slow	Neg	Neg
Y	7.87	Asymptomatic	ND	ND	Slow	Neg	Neg
Z	7.87	Asymptomatic; E.c; I.b; E.hart	ND	ND	Slow	Neg	Neg
AA	7.87	V. loose stool; I.b	ND	ND	Slow	Neg	Neg
AA2	7.87	Asymptomatic; I.b;	ND	ND	Slow	Neg	Neg
AC	7.87	Intermittent diarrhoea	ND	ND	Slow	Neg	Neg
AE	7.87	Asymptomatic	ND	ND	Slow	Neg	Neg
AK	8.87	E.h troph. + rbc	160	-	Fast	8	12
AL	8.87	Asymptomatic	ND	ND	Slow	Neg	Neg
AM	8.87	Asymptomatic	ND	ND	Slow	Neg	Neg
AO	8.87	Asymptomatic; E.c; E.nana	ND	ND	Slow	Neg	Neg
AQ	8.87	Asymptomatic; G.l	ND	ND	Slow	Neg	Neg
AS	8.87	Asymptomatic	ND	ND	Slow	Neg	Neg
AU	8.87	Asymptomatic	ND	ND	Slow	Neg	Neg
AX	8.87	Recurrent diarrhoea; G.l	ND	ND	Slow	Neg	Neg

7.4 DISCUSSION

It is clear from Table 7.1 that Mabs 22.3 and 22.5, when used in IFAT experiments, recognise only those isolates belonging to pathogenic zymodemes. Although full zymodeme characterisation of many of these isolates has not been performed (involving the isoenzymes Glucose phosphate isomerase, Phosphoglucomutase, L-malate: $NADP^+$ Oxidoreductase as well as Hexokinase), all isolates possessing fast mobility HK bands are considered to belong to pathogenic zymodemes (Sargeaunt, 1987). Isolates belonging to the rare zymodeme XIII do show "slow" (non-pathogenic) HK bands but in conjunction with phosphoglucomutase bands characteristic of pathogenic status (Sargeaunt, 1987). However, this zymodeme has not yet been isolated from symptomatic cases of amoebiasis, or cases showing positive serology, and cannot yet be classified as pathogenic or non-pathogenic (Jackson *et al.*, 1985). Thus it is likely that all isolates used here that showed slow HK bands, and failed to react with the Mabs, belonged to non-pathogenic zymodemes. The disease-causing potential of the Mab-positive/"fast" HK isolates is confirmed by patient clinical symptoms and antiamoebic antibody titres, when available (amoebic serology is not normally requested for asymptomatic patients).

The nature of the antigens for which these Mabs are specific are not known; neither Mab was able to detect antigen on electroimmuno-transfer blots of *E. histolytica* lysates after SDS-PAGE (Chapter 5). It is unlikely that both are directed against the same antigen however, since the patterns obtained with each Mab in IFAT experiments were markedly different. The antigens may be directly related to virulence or, as is likely with isoenzyme virulence markers, be indirectly linked in some way. Whether the non-pathogenic isolates fail to express these antigens or express them in altered forms unrecognisable to the Mabs remains to be answered.

The practical utility of these findings will largely depend on the resolution of current controversies over the treatment of asymptomatic cyst-passers; but clearly they have the potential to provide the basis for a relatively cheap and rapid method for determining the invasive potential of a clinical isolate of E. histolytica. The Mabs do not appear to react directly with E. histolytica cysts, so that preliminary culture is still required, but sufficient organsisms for the production of IFAT slides can often be obtained within two days, rather than the seven days often needed for preparing the lysate for isoenzyme analysis. Unfortunately, the 22.3 cell line has ceased producing antibody; but now that it is clear that antigenic differences between invasive and non-invasive organisms do exist, attempts to produce further Mabs with the same specificity are in progress.

CHAPTER 8 GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

Twenty one monoclonal antibody-secreting hybridoma cell lines were produced from fusions between mouse myeloma cells and spleen cells from mice immunised with axenically-cultured E. histolytica trophozoites. These Mabs were used as probes to study the amoebic antigens against which they were directed. With hindsight, a slightly more rational approach to the characterisation of amoebic antigens might have yielded more useful information. This would have involved the identification of immunologically relevant antigens recognised by human patient serum, and their subsequent isolation. The antigens studied in this work represent a more or less random selection of the total antigenic composition of E. histolytica.

The indirect fluorescent antibody test (IFAT) provided a rapid and effective technique to determine the subcellular locations of the antigens and was utilised extensively in this study. When IFAT experiments were performed using a variety of other protozoan, (and one mammalian) cell types, it was found that two of the Mabs, 29.5 and 29.9, reacted with all cells tested, suggesting that they were recognising conserved antigens. The remaining 19 Mabs produced a variety of staining patterns in E. histolytica trophozoites in IFAT experiments. Although preliminary attempts at immunochemical analysis of the antigens recognised by the Mabs was performed, the functional significance of these antigens was not investigated in this study.

Three of the Mabs recognised antigens exposed on the surface of the amoeba; results confirmed by the fluorescence of live trophozoites used in IFAT experiments. These antigens may either be components of the surface plasma membrane of the amoeba, or part of the glycocalyx external to it. When reduced SDS-denatured lysates of E. histolytica

were electrophoresed and subjected to electroimmunotransfer blotting (EITB), one of these Mabs, 22.1, detected a series of antigen bands with a variety of approximate molecular weights. Surface antigens with approximate molecular weights corresponding to two of these bands (58 kD and 95 kD) have been detected by other authors (Rosenberg and Gitler, 1985; Calderon and Avila, 1986; Torian et al., 1987). A smearing of stain between the bands suggests that the antigen may be a glycoprotein (Goding, 1986). Though other possible explanations exist, it is attractive to suggest that these multiple bands might correspond to precursors of a surface located antigen during intracellular processing, as reported for an antigen of Plasmodium falciparum (Holder and Freeman, 1982). Although the detection of such internal precursors would probably be obscured by surface membrane fluorescence in IFAT experiments using fixed amoebae, it was noted, that in certain isolates of E. histolytica tested, amoeba showing only internal fluorescence did occur. The purely internal location of antigens more frequently located on the surface of the amoebae was also detected using Mabs 22.6 and 29.6 in a number of isolates (though never occurring with all three Mabs in the same isolate). This phenomenon has been reported in Trichomonas vaginalis (Alderete et al., 1986a), in which it is due to a subpopulation within the culture failing to express the antigen on the cell surface.

A heterogeneity of surface antigen expression among cultures and cloned lines of amoebae was detected using all three of these Mabs. Although such variation in expression might be coordinated with cell cycle events, reported in E. histolytica by Aust-Kettis and Sundqvist (1978), it seemed to have features more in common with the antigenic heterogeneity described in T. vaginalis by Alderete et al. (1986a,b). Variation in the numbers of cells expressing surface antigen occurred over extended culture and these authors concluded that the changes were not coupled to single cell cycle events. It is not known

whether this type of variation in antigen expression occurs *in vivo* in *E. histolytica*, as it does in *T. vaginalis* (Alderete *et al.*, 1987).

Mabs 22.1, 22.6 and 29.6 recognised surface-located antigens in most isolates of *E. histolytica* tested; although negative results may have been due to undetectably low proportions of cells expressing the particular antigen. Two of these antigens, recognised by 22.1 and 29.6, were specific to *E. histolytica*, while the antigen recognised by 22.6 was also detected in *Entamoeba invadens* among a range of related and unrelated gut protozoa tested.

Two Mabs, 22.5 and 22.7 appeared to recognise antigenic components of the vacuolar system specific to *E. histolytica*. Mab 22.7, which recognised an antigen of approximately 190 kD molecular weight in EITB, also produced plasma membrane staining in a very small proportion of observed amoebae. Whether or not this antigen is exposed on the surface of live amoebae, is not certain. A 195 kD plasma membrane protein, apparently not exposed on the surface, has been identified by Rosenberg and Gitler (1985). Mab 22.5 recognised an antigen of unknown molecular weight, detectable only in pathogenic isolates of *E. histolytica* (see below).

The remaining fourteen Mabs recognised cytoplasmic components of the amoeba. Only four of these detected antigens specific to *E. histolytica*, while the other ten detected antigens shared mainly by other members of the genus *Entamoeba*. One of the *E. histolytica*-specific antigens, recognised by Mab 22.3, was detected in all pathogenic isolates tested in a detailed study (Chapter 7) though some conflicting results involving non-pathogenic isolates had been obtained in earlier work (Chapter 4). The other *E. histolytica*-specific antigens included a 100 kD antigen detected by 22.8, a series of antigens of approximately 12 kD, 16 kD and 45 kD molecular weights (on EITB after SDS-PAGE under reducing conditions) detected by 29.11.

Mab 22.4 recognised an antigen of 230 kD, detectable in several other species of Entamoeba (and Pentatrichomonas hominis), which is possibly a dimer composed of two 115 kD subunits (from EITB after SDS-PAGE under reducing and non-reducing conditions). Mab 24.3 recognised a 130 kD monomeric antigen, undetectable in one isolate of E. histolytica, that was shared also by Entamoeba moshkovskii, while 29.3 detected a 120 kD monomeric antigen also detected in both E. moshkovskii and all tested isolates of E. invadens. One pair of Mabs, 24.2 and 24.4, appeared to recognise the same antigen, producing identical IFAT staining patterns, EITB results (100 kD) and species cross-reactions (E. moshkovskii and E. invadens). This result, as discussed, may have arisen due to contamination of cell lines during hybridoma culture. A similar phenomenon was observed using Mabs 24.5, 24.7 and 24.8. These Mabs recognised a 97 kD E. histolytica antigen also detected in E. invadens. Mabs 24.1 and 24.6 detected internal antigens of unknown molecular weight also detected in one isolate of E. invadens, in the case of 24.1, and E. moshkovskii in the case of 24.6. Except in the case of Mab 29.3, where a 120 kD antigen was detected in EITB experiments using both E. histolytica and E. invadens lysates, it is not known whether Mabs detecting antigens in other protozoan species were recognising antigens of the same approximate molecular weight as those in E. histolytica.

Studies with other protozoan species (Chapter 3) showed that these Mabs recognised antigens restricted mainly to the genus Entamoeba; except 29.5 and 29.9 which recognised every cell type tested, only two Mabs reacted with species other than the Entamoebae. The cross-reaction of Mab 24.1 with the flagellum of only Trypanosoma brucei rhodesiense, among several flagellate species tested, is not likely to represent the existence of an evolutionarily-conserved antigen but rather the fortuitous possession of cross-reacting epitopes. While only eight of the Mabs were specific for E. histolytica, a further eight recognised only E. invadens or E. moshkovskii or both, out of the range of cells tested. Since E. invadens seems restricted to reptilian hosts (Ratcliffe and

Gieman, 1938), and E. moshkovskii seems non-infective to man (Rogova, 1958), the potential of these eight Mabs for use in a diagnostic test for human amoebiasis cannot be excluded. The cross-reaction of these eight Mabs with E. invadens and E. moshkovskii demonstrated the close relationship, in terms of antigenic composition, between these morphologically similar members of the quadrinucleate-cyst group of Entamoeba. However, one member of this group, Entamoeba hartmanni, showed no cross-reactions (except with 29.5 and 29.9); in contrast to the findings of Goldman et al. (1960) who reported that anti-E. histolytica antiserum cross-reacted with E. hartmanni to the same extent as it did with E. invadens. The identical patterns of Mab cross-reactivity obtained with Entamoeba coli (a commensal species in man) and Entamoeba muris (a commensal in mice) reflected the morphological indistinguishability of these two members of the octonucleate-cyst group of Entamoeba (Neal, 1950).

The heterogeneity of Mab cross-reactions observed among the various isolates of E. invadens tested, demonstrates the importance of testing for cross reactions in more than one isolate of a certain species. Very little heterogeneity in antigenic composition was observed among the fifteen isolates of E. histolytica tested. Apart from two Mabs (22.3 and 22.5) which detected antigens only in pathogenic isolates (Chapter 7), only the three surface-staining Mabs (as previously discussed) and Mab 24.3 failed to react with all the isolates. The failure of 24.3 to react with one particular isolate is surprising since it recognised E. moshkovskii, E. coli and E. muris.

Since Mab 22.4 was capable of recognising the human commensal amoeba, E. coli, it was concluded that twelve of these thirteen Mabs that detected all tested isolates of E. histolytica, were potentially useful antigen capture agents in an immunodiagnostic assay for detecting both pathogenic and non-pathogenic E. histolytica in human samples. Current W.H.O. directives (T. Vesikari, pers. comm.) favour the development of an assay able to detect the presence of only

pathogenic amoebae in human samples, both as an epidemiological tool and as a possible aid to targeting treatment towards asymptomatic patients at risk from invasive disease. Mabs 22.3 and 22.5 might be useful as the basis for such a test. Unfortunately the pathogen-specificity of these Mabs had not been determined when preliminary investigation of the Mabs' diagnostic potential was carried out.

Seventeen Mabs were tested as antigen capture reagents in a double sandwich enzyme-linked immunosorbent assay for amoebic antigen. Seven of these were capable of detecting antigen down to a level of 100 ng/ml total amoebic protein. The sensitivity of this assay was improved by the addition of a separate blocking step immediately after coating of the assay plates with Mab, before sample incubation. Incubation of amoebic lysate in human faecal extract for 7 days caused no apparent loss of reactivity of at least six of the antigens concerned.

In extensive studies examining the correlation of isolate isoenzyme profile with the clinical and serological status of the source-patient, Sargeaunt and his colleagues have grouped isolates of E. histolytica into two distinct categories: pathogenic and non-pathogenic (Sargeaunt and Williams, 1978, 1979; Sargeaunt et al., 1984; Sargeaunt, 1987). Amoebae belonging to non-pathogenic zymodemes are isolated only from asymptomatic patients with negative serology, while amoebae belonging to pathogenic zymodemes are isolated mainly from symptomatic patients, although asymptomatic carriers of these zymodemes occur in endemic areas (Sargeaunt, 1987). Although other markers for pathogenicity apparently exist (Section 1.5), isoenzyme electrophoretic mobility analysis was, until recently, the only method widely tested in clinical isolates of E. histolytica. The work discussed here (Chapter 7) and in Strachan et al. (1988), represents the first published report of antigenic differences between pathogenic and non-pathogenic isolates. More

recently, differences at the DNA level have been discovered. Using two sets of DNA probes derived from tandemly repeated sequences present in extrachromosomal circular elements from both pathogenic and non-pathogenic isolates, the two groups of amoebae could be differentiated in hybridization experiments (Garfinkel et al., 1989; Huber et al., 1989).

There are two opposing hypotheses to explain the basis of pathogenicity in E. histolytica. One hypothesis proposes that pathogenic and non-pathogenic isolates represent distinct subspecies of this parasite (Sargeaunt, 1987). Long-term studies of asymptomatic carriers of non-pathogenic amoebae (Allason-Jones et al., 1986) and in vitro studies in which the bacterial flora of polyxenically-cultured non-pathogenic amoebae was altered, showed no evidence of changes from non-pathogenic to pathogenic zymodemes (Sargeaunt, 1987). This evidence of zymodeme stability has been used to propose a non-treatment policy of patients harbouring amoebae belonging to non-pathogenic zymodemes (Allason-Jones et al., 1986). The second hypothesis, that zymodemes and pathogenicity are not such stable characteristics, is based on the recent work of Mirelman and his colleagues (Mirelman et al., 1986a,b). These authors found that during the attempted axenization of a cloned non-pathogenic isolate, the isoenzyme mobility pattern changed from a non-pathogenic one to a pathogenic one. This change was accompanied by a concomitant increase in in vitro and in vivo virulence. Furthermore reassociation of this converted strain with its original flora resulted in a return to a non-pathogenic isoenzyme pattern and a decrease in virulence, while association with bacterial flora from a polyxenically-cultivated pathogenic isolate resulted in no such reversion. Whether such changes could actually occur in vivo is not known, but the possibility does exist.

Garfinkel et al. (1989) have shown that, in these converted strains, the hybridisation patterns obtained with the pathogen-specific and non-pathogen-specific DNA probes correlated prefectly with isoenzyme

pattern. Recent unpublished results using Mabs 22.3 and 22.5 in IFAT experiments with these strains and others prepared by the same technique in a different laboratory, have shown a similar correlation. During the axenization process, Mab recognition occurs at the same time as the isoenzyme pattern changes from one characteristic of a non-pathogenic zymodeme to one characteristic of a pathogenic zymodeme. Although the molecular mechanisms occurring during conversion and change in zymodeme are not known, these results suggest a coordination of processes within the amoeba. Studies on the hexokinase enzymes of pathogenic and non-pathogenic strains of E. histolytica have suggested that electrophoretic differences might be due to post-translational modifications of the proteins (Bracha et al., 1987). Since these enzymes are unlikely to be virulence factors in themselves it is possible that such changes are the consequences of a more general activation of metabolic processes within the amoeba. Further work involving the characterisation of the antigens for which Mabs 22.3 and 22.5 are specific, is being continued.

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