EVALUATION OF SEROLOGICAL TECHNIQUES

IN THE DIAGNOSIS OF

ACTIVE INVASIVE AMOEBIASIS

BY

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This dissertation is dedicated

to the memory of my

late parents

Mr. Mahomed Sathar (8/8/77)

Mrs. Ajrah Sathar (16/7/90)

ETHICAL CONSIDERATIONS

Informed consent was obtained from all participants in this study. Where the patient was a minor, informed consent was obtained from the parent or legal guardian.

All research protocols relating to this study were approved by the Ethics Committee of the Faculty of Medicine, University of Natal.

CONTENTS

ABSTRACT

Early diagnosis of invasive amoebiasis could improve the outcome of treatment and decrease the fatality rate.Amoebic dysentery (AD) and amoebic liver abscess (ALA) are endemic in the Natal coastal area, including the peri-urban areas of Durban. A definitive diagnosis of invasive amoebiasis relies on the demonstration of the causative organism, Entamoeba histolytica, in either stool specimens in the case of AD or the liver aspirate in ALA. While this is relatively simple in the case of AD, it is much more difficult in patients with ALA. However, the demonstration of specific antibodies to E. histolytica in serological tests has proved to be useful in establishing tissue invasion by the parasite, but these tests do not adequately distinguish between specific circulating antibodies produced during active amoebic infection and those persisting after previous infection, the indirect fluorescent antibody test (IFAT) is an exception. Although the IFAT was shown to be useful in differentiating between past and present infection by monitoring specific IgM-anti-E. histolytica antibodies, the test has had limited use in the diagnostic laboratory. Therefore, an enzyme-linked immunosorbent assay (ELISA) was evaluated. Most ELISAs have measured either IgG-anti-E. histolytica antibodies or total

immunoglobulins. In this dissertation the ELISA was evaluated for its ability to distinguish between past and current amoebic infection by detecting specific IgG- and IgM-anti-*E. histolytica* antibodies.

Using a soluble antigen of *E. histolytica*, the ELISA was compared with the amoebic gel diffusion test (AGDT). In 100 patients with clinically diagnosed ALA and 144 controls, the IgG-ELISA had a sensitivity of 99% (99/100) and a specificity of 91,7% (132/144). In these same patients, the IgM-ELISA had a sensitivity of 64% and a specificity of 97,9%. There was an excellent correlation between the AGDT and IgG-ELISA ($r = 0,99$).

Although the IgM-ELISA was specific (97,9%), it was not sensitive enough (64%) to differentiate between past and current amoebic infection. This prompted another study in which the ELISA was compared to the AGDT and the IFAT. The IFAT is a useful test in diagnosing invasive amoebiasis, but the interpretation of immunofluorescence, especially IgM, was subjective.

To improve the diagnostic potential of the IgM-ELISA, the plasma membrane of axenic cultures of *E. histolytica* strain HK9 was used as the antigen to detect specific IgG and IgM

antibodies. The sensitivities of the IgM- and IgG-ELISA in 22 ALA patients were 91% (20/22) and 95% (21/22), respectively. In 22 controls without invasive amoebiasis the specificities of the IgM- and IgG-ELISA were 95% (21/22) and 91% (20/22), respectively. There was an excellent correlation (r = 0,96) between the IgG-ELISA and the AGDT.

The use of the plasma membrane antigen improved the diagnostic potential of the IgM-ELISA in differenting between past and current amoebic infection.

PREFACE

The experimental work in this thesis was carried out in the Department of Medicine, University of Natal and the Research Institute for Diseases in a Tropical Environment (RIDTE) of the South African Medical Research Council (SAMRC), Durban, under the supervision of Professor A.E. Simjee and Mr. B.L.F. Bredenkamp.

This thesis is the candidate's own original work and has not been submitted in any form to another University. Selected results from this thesis have been presented at Scientific Meetings and published in Scientific Journals. Research workers who were closely associated in these studies are either co-authors in these publications or are duly acknowledged in the text.

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CHAPTER 1

1.1. INTRODUCTION

E. histolytica is a protozoan parasite. Amoebiasis has been defined as 'the state of an individual harbouring *E. histolytica*' (WHO, 1969). Although the parasite usually behaves as a commensal and induces no signs or symptoms in the majority, it can result in invasive amoebiasis in some (Walsh, 1986). Invasive amoebiasis is characterised either by amoebic dysentery or extra-intestinal lesions (mainly ALA); the latter occurs when there is blood-borne dissemination of the amoebae (Manson-Bahr and Apted, 1982). The reason why certain individuals or communities are more susceptible to invasive amoebiasis is not known. Some researchers believe that all *E. histolytica* strains are potentially capable of invasion but are usually restrained by host-mediated defence mechanisms (WHO, 1969). Others feel that there are two distinct species of *E. histolytica* (Editorial, 1979); one of which is always invasive and the other a harmless commensal; these two strains can be distinguished by characteristic isoenzyme (pathogenic and non-pathogenic zymodemes) patterns (Sargeaunt *et al*, 1978).

The protozoan has cystic and trophozoite stages. The cysts (10-20um in size) are typically quadrinucleate and indicate the infective stage (Manson-Bahr and Apted, 1982). Trophozoites do not transmit the disease because they cannot survive for any length of time outside the body and if ingested live, are most probably destroyed by gastric juices (Manson-Bahr and Apted, 1982).

Entamoeba histolytica has been grouped into pathogenic and non-pathogenic zymodemes by isoenzyme electrophoresis based on the electrophoretic mobility of certain of its isoenzymes. Both the zymodemes have been isolated from asymptomatic individuals, but only the pathogenic zymodemes cause the clinical disease (Jackson et al, 1985). Pathogenic strains are characterised by the presence of a relatively fast migrating phosphoglucomutase (PGM) isoenzyme which localises at a position termed beta (β) and the absence of an alpha (α) band, together with a faster migrating pair of hexokinase (HK) isoenzymes. Non-pathogenic zymodemes show the presence of an alpha band in PGM and slow running bands in HK (Sargeaunt et al, 1982 and 1987). From the point of view of treatment (Gathiram and

Jackson, 1987) as well as the interpretation of serological tests (Jackson et al, 1985, Sargeaunt et al, 1987) such differentiation is considered to be vital (Jackson et al, 1985; Sargeaunt et al, 1987).

It was estimated by Walsh (1986) that approximately 480 million people are infested with E. histolytica worldwide. Based on the assumption that the serum antibodies against the trophozoite indicate past or present invasive amoebiasis, Walsh (1986) calculated that annually 48 million people (approximately 10% of infected individuals) suffer from invasive amoebiasis. She (Walsh, 1986) estimated that ALA accounts for 2-20% of all cases of invasive amoebiasis. Mortality from ALA has been reported to be between 2-10% and the annual world mortality attributable to amoebiasis has been estimated to be 40-110 thousand (WHO, 1985). On a global scale, E. histolytica is the third leading cause of death from parasitic infections after malaria and schistosomiasis (Walsh, 1986).

Microscopical identification of Entamoeba histolytica continues to be the 'gold standard' for the diagnosis of amoebiasis (Healy, 1986). The detection of erythrophagous trophozoites in stool specimens or tropho-

zoites in liver aspirates indicates active invasive amoebiasis. However, stool microscopy is difficult and subjective (Healy, 1986). Where morphological diagnosis is difficult, a demonstrable immune response may help confirm or refute microscopic findings (Powell et al, 1965/1966).

1.2. LITERATURE REVIEW OF IMMUNODIAGNOSTIC TESTS FOR INVASIVE AMOEBIASIS

In any infectious disease the most reliable method of diagnosis is by identifying the responsible organism either by microscopy or culture. Local experience with culture showed it to be four times more effective than microscopy in identifying E. histolytica (Gathiram et al, 1984). Gathiram et al (1984) showed that direct microscopy of the amoebic pus revealed E. histolytica in 21% of all ALA patients, and when culture techniques alone were employed E. histolytica was identified in 73% of pus specimens. Culture combined with microscopy increased positive identification to 81%, thereby making the amoebic aetiology of ALA unquestionable (Gathiram et al, 1984). Culturing the amoebae is useful in diagnosing ALA but this is difficult except in the research situation. The technique requires an

experienced microscopist and technical expertise; furthermore, specimens must reach the laboratory within 12 hours because the amoebae cannot survive outside the human host for longer periods (Gathiram et al, 1984).

However, the body does develop a specific immune response and detection of antibodies can be used as indirect evidence of ALA (Salata and Ravdin, 1986). The earliest attempts at detecting circulating antibodies to E. histolytica were made using the complement fixation (CF) bentonite flocculation (BF) intradermal (ID) and amoeba immobilisation tests (Sadun, 1976). These tests lacked sensitivity and specificty; they were tedious to perform and technically difficult; the antigens used in these tests were obtained from E. histolytica grown in association with other microflora which made standardisation of antigens difficult (Sadun, 1976). These problems were overcome with the successful cultivation of E. histolytica in an axenic medium (Diamond, 1968). The preparation of suitable antigens from such amoebae was a turning point in the field of diagnostic serology, because it provided antigenic material which was more defined and reproducible, thereby increasing the sensitivity and specificity of

the serological tests. More sensitive and specific methods, viz. the amoebic gel diffusion test (AGDT); indirect haemagglutination test (IHA); counter-current immuno-electrophoresis (CCIE); radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are discussed in more detail.

Before discussing the various serological tests that are currently available in amoebiasis, the following comments need to be made since interpretation of results obtained with these different tests is dependent upon understanding these concepts.

1. In general, in invasive amoebiasis, whether it is amoebic dysentery (AD) or amoebic liver abscess (ALA), the tests are positive in the range of 95%-100% for ALA and 60%-95% for AD.
2. In non-endemic areas, in both asymptomatic cyst passers and non-cyst passers, the tests are positive in less than 5% of individuals.
3. In areas endemic for amoebiasis, the following results are obtained:
   a) Asymptomatic cyst passers: in general, seropositivity is in the range of 20%-50%.
   b) In non-cyst passers: seropositivity is obtained in 10%-30%.

These latter groups are important because a positive result obtained with these tests is not thought to represent a non-specific reaction. The explanation for this is believed to be as follows:

i) Amoebic antibodies are known to persist for several years after treatment (Krupp and Powell, 1971b and Joyce and Ravdin, 1988).

ii) Asymptomatic cyst passers of pathogenic zymodemes are strongly seropositive whilst asymptomatic cyst passers of non-pathogenic zymodemes are either weakly or moderately positive or seronegative (Jackson et al, 1985).

1.2.1. Indirect Haemagglutination Test

Use of the indirect haemagglutination test was first described by Kessel et al (1961). The procedure was subsequently modified using antigens derived from axenic cultures of E. histolytica (Krupp, 1969 and Om Prakash et al, 1970).

The IHA test has a sensitivity of 91%-100% in cases of ALA, and 92%-100% in acute amoebic dysentery (Kessel et al, 1965; Maddison et al, 1965a/b, 1968a; Milgram et al, 1966; Om Prakash et al, 1969 and Krupp and Powell, 1971a). In endemic areas, resuslts vary in asympto-

matic cyst passers from 0%-45% (Maddison et al, 1968a; Krupp and Powell, 1971a and Gandhi et al, 1987), and 0%-32% in hospitalised patients with diseases other than amoebiasis who did not pass cysts (Maddison et al, 1968a; Krupp and Powell, 1971a and Vinayak et al, 1975).

The advantages of the IHA test are:

i) It does not require specialised equipment (Ambroise-Thomas, 1980);

ii) It is simple to perform (Ambroise-Thomas, 1980);

iii) The test can be completed in less than 4 hours (Draper and Lillywhite, 1984);

iv) The use of microtitre plates which facilitates visual reading, substantially reduces the volume of reagents required to perform the test (Sadun, 1976).

The disadvantages of the IHA test are:

i) Its relatively poor reproducibility and lack of specificity (Sadun, 1976);

ii) Variable results observed with IHA depends on whether the erythrocytes are treated with tannic acid, formalin, other aldehydes or chromium chloride before the amoebic antigen is adsorbed onto the cell surface (Sadun, 1976 and Draper and

Lillywhite, 1984). Moreover, cells of one animal species provide more reproducible results with a given antigen than cells of another donor species (Sadun,1976);

iii) The test reagents and fragile erythrocytes remain stable for 2 weeks only (Kessel *et al* 1965; Sadun, 1976 and Draper and Lillywhite, 1984).

1.2.2. Amoebic Gel Diffusion Test

The amoebic gel diffusion test (AGDT) was first described by Atchley *et al* (1963). The AGDT has a sensitivity of 91-100% in cases of ALA and 88-97% in amoebic dysentery (Maddison, 1965; Maddison *et al*, 1965a/b, 1968a; Powell *et al* 1965, 1966; Morris *et al* 1970b; Krupp and Powell 1971a; Monroe *et al*, 1972; Vinayak *et al*, 1975; Patterson *et al*, 1980; Jackson *et al*, 1984 and Shetty *et al*, 1988). In asymptomatic cyst passers of *E. histolytica* the prevalence of seropositivity varies from 0% in non-endemic areas (Patterson *et al*, 1980) to 39%-50% in endemic areas (Maddison, 1965; Maddison *et al*, 1965a and Krupp and Powell 1971a). The higher seropositivity observed with asymptomatic cyst passers in endemic areas may be due to higher prevalence of pathogenic *E. histolytica* in these

areas compared to non-endemic areas (Jackson et al, 1985 and Gathiram and Jackson, 1987). In a semi-rural area south of Durban, the overall prevalence of pathogenic and non-pathogenic zymodemes has been shown to occur in approximately 10%, pathogenic zymodemes occur in approximately 1% of apparently asymptomatic individuals (Gathiram and Jackson, 1987). The proportions of seropositive responses ranged from 31% for carriers of non-pathogenic zymodemes to 100% in carriers of pathogenic zymodemes (Jackson et al, 1985). In endemic areas a seropositivity of 10%-20% was observed in hospitalised patients with no clinical evidence of amoebiasis and who were not cyst passers (Maddison, 1965; Maddison et al, 1965a/b, 1968b; Powell et al, 1965, 1966; Krupp and Powell 1971a and Vinayak et al, 1975). In similar groups of patients a positivity of less than 0,9% were observed in non-endemic areas (Monroe et al, 1972, and Patterson et al, 1980). The higher prevalence of seropositivity reported in endemic areas in individuals without invasive amoebiasis may be due either to a past infection with E. histolytica, as amoebic antibodies are known to persist for 2 years or longer after successful treatment (Krupp and Powell, 1971b and Joyce and Ravdin, 1986), or to a higher

prevalence of pathogenic E. histolytica in an endemic environment (Jackson et al, 1985). A negative AGDT result is significant in that it makes a diagnosis of ALA unlikely. The advantages of the AGDT are:

i) Its reproducibility and low cost;

ii) It is interesting to note that the AGDT is useful in detecting asymptomatic cyst passers of pathogenic E. histolytica (Jackson et al, 1985). This is not due to any specific characteristic of the AGDT, but to its ability to detect higher antibody concentrations;

iii) Jackson et al (1985) using the AGDT and indirect fluorescent antibody test (IFAT) concluded that a strong positive serological response is highly indicative of the presence of a pathogenic zymodeme.

The disadvantages of the AGDT are:

i) It requires a certain degree of technical expertise in its performance (Elsdon-Dew et al, 1971);

ii) Interpretation of results is subjective (Elsdon-Dew et al, 1971);

iii) The test has a clinical disadvantage in that a

result is obtainable 24-48 hrs after receipt of a specimen; an unequivocal negative result is obtained in 48 hours (Morris et al, 1970a and Monroe et al, 1972). Repetition of the test for confirmation takes even longer (Morris et al, 1970a and Monroe et al, 1972).

1.2.3. Latex Agglutination and Cellulose Acetate Membrane Tests

With the exception of IFAT, CCIE and ELISA most serological test results cannot be reported on the same day when blood specimens are received. This can be of clinical disadvantage when an urgent decision needs to be made (delaRey Nel et al, 1989). Under such circumstances there is a 'need for a simple quick test' to confirm clinical impressions (Elsdon-Dew, 1971). To this end the latex agglutination (LA) (Morris et al, 1970b) and the cellulose acetate membrane precipitin (CAPT) (Stamm and Phillips, 1977) tests were developed.

Morris et al (19709a/b) using the LA test reported a sensitivity of 98% for ALA and 96% for amoebic dysentery. The test has the advantage of being rapid with results being provided within 5-10 minutes. The test is qualitative and interpretation can be subjective.

Furthermore, the inability to standardise the reagents for the LA test and the high cost of the Serameba kit (Ames Co., USA), made the test inaccessible to Third World laboratories with limited funds (Stamm et al, 1973 and Mahajan et al, 1976a). The CAPT is similar to the AGDT, but it utilises cellulose acetate membrane instead of agarose as the support medium. The only advantage of the CAPT has over the AGDT is the speed with which the results are available, 4 hours compared to 24-48 hours with the AGDT (Stamm and Phillips,1977). The membrane is easier to handle than agar; however, the membrane must be stained to show arcs of precipitin (Stamm and Phillips, 1977).

1.2.4. Immunoelectrophoresis

The technique of immunoelectrophoresis (IEP) was first applied to amoebiasis by Maddison (1965) to demonstrate precipitating antibodies in the sera of two ALA patients. A more extensive study using the IEP in amoebiasis was undertaken by Savanat and Chaicumpa (1969) to assess its value in the diagnosis of invasive amoebiasis. Savanat and Chaicupma (1969) reported seropositives of 96,8% in patients with ALA and 66,7% in those with amoebic dysentery from an area endemic

for amoebiasis in Thailand. In addition, Savant and Chaicumpa (1969) noted that 0,6% of blood donors and 7,4% of individuals with unrelated diseases were seropositive. Krupp and Powell (1971b) in 448 patients (with proven ALA and amoebic dysentery) and Savanat and Chaicumpa (1969) in 6 ALA patients used the IEP to differentiate between precipitin arcs present during active disease and those persisting after treatment. In their respective studies they noted that certain antigen-antibody arcs were lost with treatment. Their results suggested that the IEP test may be of some value in differentiating between past and recent infection. However, such studies using the IEP test were not pursued any further.

The only known advantage of the IEP is that it offers better resolution of the precipitin bands than the AGDT (Savant and Chaicumpa, 1969). The disadvantages of the assay are:

i) It requires relatively large quantities of reagents (Ambroise-Thomas, 1980);

ii) It takes a long time to complete (72 hours) (Ambroise-Thomas, 1980);

iii) The assay is cumbersome to perform and requires expensive equipment, which has restricted its general use routinely.

1.2.5. Counter-current immunoelectrophoresis

The use of counter-current immunoelectrophoresis (CCIE) in amoebiasis was first described by Sepulveda *et al* (cited by Ambroise-Thomas, 1980). The sensitivity of CCIE was reported to be 95-100% for ALA and 47-88% for amoebic dysentery (Krupp, 1974; Mahajan *et al*, 1976a; Alper *et al*, 1976; Tosswill *et al*, 1980; Garcia *et al*, 1982 and Sharma *et al*, 1984; Samrejrongroj and Tharavanij, 1985). In all of these studies, the CCIE was reported to be 100% specific, including the study of Krupp (1974). However, in this particular study, the AGDT was also 100% specific. This makes it virtually impossible to assess the true value of the CCIE because it is well known that in the Durban area where the work was done, the specificity of the AGDT for active amoebiasis is only 80%-90% (Krupp and Powell, 1971a/b). Thus the real value of the CCIE is not known. The ability of CCIE to detect asymptomatic cyst passers of *E. histolytica* has not been evaluated.

The advantages of CCIE are:

i) It is economical in respect to reagents and antigen used;

ii) The assay is rapid; it can be completed in 1-2 hours after receipt of the specimen (Mahajan et al, 1975). To improve accuracy, results are read at 24 hours (Mahajan et al, 1975 and Alper et al, 1976).

The disadvantages of the CCIE are:

i) It requires expensive equipment and some technical expertise;

ii) Weak precipitin lines are occasionally difficult to distinguish from artifacts that are often seen close to the antiserum well (Kohler et al, 1982).

1.2.6. Indirect Fluorescent Antibody Test

Jeanes (1966) and Goldman (1966) simultaneously described the use of immunofluorescence in amoebiasis. The sensitivity of the indirect fluorescent antibody test (IFAT) for the diagnosis of ALA varies between 96%-100% and 54%-95% for amoebic dysentery. In areas endemic for amoebiasis the IFAT is seropositive in 9%-24% of asymptomatic cyst passers and in up to 12% of hospitalised patients (Jeanes, 1966; 1969; Ambroise-

Thomas and Truong, 1972; Ambroise-Thomas 1976 and Jackson et al, 1984).

One of the main advantages of the IFAT is the use of anti-immunoglobulins to detect different classes of human immunnoglobulins (Jackson et al, 1984). These authors (Jackson et al, 1984) reported that the IgG-IFAT was seropositive in all 35 (100%) ALA patients and in 19% (3/16) of hospitalised patients with diseases other than amoebiasis. The IgM-IFAT was seropositive in 83% (29/35) of ALA patients and in 6% (1/16) of patients with other diseases (Jackson et al, 1984). Jackson et al (1984) used the AGDT and the IFAT in a longitudinal study of patients with ALA. They found that in more than 50% of patients with invasive amoebiasis, specific IgM antibodies became negative sooner (within 3-6 months) than the IgG. These authors (Jackson et al, 1984) proposed that when the IgM-IFAT is negative even though the IgG-IFAT and AGDT may remain positive, active disease is absent. The presence of specific IgM (even in low titres) indicates current tissue invasion by E. histolytica. Similar observations with regard to IgM antibodies were reported by others (Ambroise-Thomas and Truong 1972 and Ambroise-

Thomas, 1976).

Advantages of the IFAT are:

i) The whole parasite is used as the antigen; this gives ease of preparation (Draper and Lillywhite, 1984).

ii) Antigen coated slides used for the IFAT are stable for more than 6 months when stored at -20°C (Thomas et al, 1981 and Ambroise-Thomas and Truong, 1972).

iii) IFAT results are available 1-2 hours after receipt of a specimen.

iv) Different classes of immunoglobulins can be measured (Jackson et al, 1984).

The disadvantages of the IFAT are:

i) That interpretation of immunofluorescence is subjective and depends on the technical expertise of the microscopist (Sadun, 1976).

ii) The test requires a fairly intricate microscope (Ambroise-Thomas, 1976).

iii) The assay is difficult or even impossible to automate (Ambroise-Thomas, 1976).

Time-resolved fluoroimmunoassay (TR-FIA) (Aceti et al, 1987) is a modification of the IFAT. The assay uses a

chelate of the lanthanide europium as the label, instead of fluorescien isothiocynate. The chelate reduces non-specific reactions and background fluorescence (Aceti et al, 1987). The assay was positive in 90% of patients with invasive amoebiasis; it was positive in 1,6% of patients with diseases other than amoebiasis, and in 18% of sera obtained from other individuals. In the latter, no clinical data were available; presumably they had no clinical evidence of active amoebiasis (Aceti et al, 1987). Unlike conventional IFAT, TR-FIA detects antibodies to soluble antigens adsorbed to microtitre plates. The assay is more quantitative and less subjective than IFAT (Aceti et al, 1987). The application of TR-FIA in diagnosing invasive amoebiasis, especially in areas endemic for amoebiasis needs to be evaluated. . However, the use of an expensive specialised equipment, viz. a fluorimeter, may restrict its use in poorer laboratories.

1.2.7. Enzyme-linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) reported by Engvall and Perlmann (1971) was first applied to amoebiasis by Bos et al (1976). The assay is sensitive (97-100%) and specific (96-97%) for the diagnosis of

ALA (Yang and Kennedy, 1979; Agarwal *et al*, 1981; Lin *et al*, 1981; Tandon, 1981; Mohapatra and Sen, 1983; Baveja *et al*, 1984 and Gandhi *et al*, 1987). Variable results have been observed for amoebic dysentery (63-100%), but fewer papers have been published on this form of amoebiasis (Agarwal *et al*, 1981; Tandon, 1981; Mohapatra and Sen, 1983 and Kumar *et al*, 1985). The ELISA is positive in 2-10% of subjects from endemic areas who have no clinical evidence of amoebiasis. (Yang and Kennedy, 1979; Bos *et al*, 1980; Agarwal *et al*, 1981 and Baveja *et al*, 1984).

The seropositivity of the test for the detection of asymptomatic cyst passers of *E. histolytica* varies between 0% to 50% (Lin *et al*, 1981 and Gandhi *et al*, 1986, 1987). However, the ability of the ELISA to detect such individuals has not been comprehensively evaluated.

The ability of the ELISA to differentiate between past and current infection by detecting specific IgM-anti-*E. histolytica* antibodies has not been evaluated. Researchers using the ELISA have measured either specific IgG-anti-*E. histolytica* antibodies (Yang and Kennedy, 1979; Agarwal *et al*, 1981; Mohapatra and

Sen, 1973; Baveja et al, 1984 and Gandhi et al, 1987) or total anti-E. histolygica immunoglobulins (Bos et al 1980; Lin et al, 1981 and Samrejrongroj and Tharavanij, 1985).

The advantages of the ELISA are:

i) It is a simple, rapid and sensitive test (O'Sullivan et al, 1979) free from the hazards of radioisotopes (Voller et al, 1977a).

ii) The reagents are stable and minute quantities are used (Voller et al, 1977b and Draper and Lilly-white, 1984).

iii) The assay has an objective numerical readout and it can be fully automated (Mathews et al, 1984).

iv) The entire assay can be performed in 2 1/2 hours, the antigen sensitised plates have been shown to remain stable on storage (Yang and Kennedy, 1979 and Lin et al, 1981).

Disadvantages of the ELISA:

i) Single evaluations in solid state microtitre plates are not practical. Test sera must be assayed in batches. However, the availability of strips and single wells is a major improvement on the ELISA.

ii) Serial dilution of test sera to determine the diagnostic endpoint titre can become labour intensive

and tedious. However, the use of single test dilution can obviate this disadvantage.

iii) A disadvantage of the ELISA is that it utilises an expensive plate reader. This may limit its use in poorer Third World Countries in the Tropics, where it is most needed.

Whilst serological tests are of great value, they do not adequately differentiate between present and a previous amoebic infection. In this dissertation the IFAT is re-evaluated; an indirect ELISA is evaluated using crude soluble and plasma membrane antigens of E. histolytica, for detecting specific IgG and IgM-antibodies, to assess its value in differentiating past from current amoebic infection.

Patients with amoebic dysentery respond well to treatment, and are usually discharged from a tertiary hospital like King Edward VIII, before the results of stool microscopy, serology or biopsy reach the clinician (Solomon et al, 1984). On the other hand, patients with amoebic liver abscess (ALA) are usually admitted to hospital and are treated under supervision. Therefore, in this dissertation more emphasis has been placed in evaluating serological tests to aid the diagnosis of ALA.

CHAPTER 2

EVALUATION OF THE INDIRECT FLUORESCENT ANTIBODY TEST (IFAT) IN THE SERODIAGNOSIS OF AMOEBIC LIVER ABSCESS (ALA)

Systemic humoral immune response in patients with invasive amoebiasis is characterised by specific circulating antibodies predominantly of the IgG class (Trissl, 1982). IgG-anti-*E. histolytica* antibodies persist over a long period or decline slowly after treatment (Osisanya and Warhurst, 1980; Knobloch and Mannweiler, 1983; Jackson *et al*, 1984 and Pinon *et al*, 1987). IgG is the major class of antibody detected by most serological tests for invasive amoebiasis (Maddison *et al*, 1968b; Savanat and Chaicumpa, 1969 and Dasgupta, 1974). A drawback of most serodiagnostic tests is that persisting IgG antibodies can be detected in patients long after diagnosis, therapy and presumed cure have taken place (Healy, 1986). Therefore, most of the conventional serological tests for invasive amoebiasis are unable to differentiate between past and current amoebic infection (Healy, 1986).

The results of earlier researchers (Bray and Harris, 1977; Harris *et al*, 1978 and Osisanya and Warhurst, 1980) indicate that monitoring specific IgM-anti-*E. histolytica* antibodies may constitute an important feature of current diagnosis

since they persist for a shorter period. The presence of IgM antibodies in invasive amoebiasis was confirmed in the early stages of ALA using class specific antisera in the IFAT (Jackson et al, 1984). The regression of IgM antibodies in less than 3-6 months in more than 50% of patients with invasive amoebiasis suggests that detecting specific IgM-anti-E. histolytica antibodies may be more valuable in diagnosing current amoebic infection (Jackson et al, 1984).

This study re-evaluates the indirect fluorescent antibody test (IFAT) for the diagnosis of amoebic liver abscess (ALA).

2.1. MATERIALS AND METHODS

2.1.1. Antigen

A 48 hour axenic culture of E. histolytica strain HK9 was supplied by the RIDTE in screw-capped glass test tubes. The glass tubes were cooled in ice water for 10 minutes to dislodge the amoebae adherent to the glass surfaces. The amoebic suspension was transferred to a sterile, cold 10ml glass conical centrifuge tube and pelleted by centrifugation (Beckman Model J6B centrifuge; JS4.2 rotor, 800rpm (160xg) at 4°C, 10 mins). The amoebae were washed with cold Ringers solution by centrifugation at 800 rpm (160xg) at 4°C. The washing procedure was repeated twice. The sediment from the

final wash was suspended in 1ml of cold Ringers solution. Ten microlitres (10ul) of the suspension was pipetted into each well of a 12-well Teflon-coated multitest slide (Flow Laboratories Inc., McLean, Va). The slides were air-dried in an oven at 60°C for 1 hour. Slides were stored at -20°C and allowed to reach room temperature before being used for IFAT.

2.1.2. Serology

2.1.2.1. Indirect Fluorescent Antibody Test

The IFAT was initially performed according to the method described by Jackson et al (1984). However, non-specific background fluorescence was observed in all samples tested. Thereafter, attempts were made to reduce the non-specific background fluorescence by including bovine serum albumin (BSA), a blocking agent, at various concentrations (1%-6%),in the serum diluent. In addition, the conjugates were centrifuged to remove any debris that may have enhanced the non-specific background fluorescence. No pellet was visible. Rhodamine, which is dessicated normal bovine serum conjugated with Lissamine Rhodamine, was used as a counterstain to prevent non-specific binding of the conjugated fluorescein. These attempts to reduce non-

specific background fluorescence were unsuccessful.

The IFAT of Jackson et al (1984) was modified. In the modified assay, Tris saline (TS) buffer, pH 8,0 replaced phosphate buffered saline (PBS)-Tween 20 + 2% normal rabbit serum as the serum diluent. TS-Tween 20 (TST) replaced PBS-Tween 20 (PBST) as the washing buffer. Specific FITC conjugates obtained from Sigma Chemicals (St. Louis, MO, U.S.A.) replaced those obtained from Miles-Yeda (Rehovot, Israel). TST replaced PBS as the conjugate diluent. All incubations in the modified IFAT were performed at 4°C as opposed to room temperature.

2.1.2.2. Performance of the Modified Indirect Fluorescent Antibody Test.

The test sera were diluted in Tris saline (TS) buffer, pH 8.0. Ten microlitres (10ul) of serial two-fold dilutions of the test sera beginning at 1/25 were applied to the antigen-coated slide in duplicate. Slides were incubated at 4°C for 30 minutes in a humid container. The slides were stacked in a staining rack and placed in a staining jar. The slides were washed by continuous stirring in TST for 5 minutes. This washing procedure was repeated three times with fresh

TST at each wash. The slides were allowed to air-dry at room temperature. Goat-antihuman IgG (gamma (γ) chain specific) FITC conjugate or goat-antihuman IgM (mu-(u) chain specific) FITC conjugate (Sigma Chemicals, St. Louis, MO, U.S.A.) was diluted 1/20 in TST. Ten microlitres (10ul) of the conjugate was applied to each well. The slides were incubated at 4°C for 30 minutes. Thereafter, the slides were washed as described above in three changes of TST of 5 minutes each. Glass cover slips were mounted on the slides with 10% glycerol in 0,01M phosphate buffered saline (PBS), pH 7,1. The slides were read in an incident light fluorescence microscope ('Axioskop' H, DIC, Zeiss, West Germany). The reciprocal of the highest dilution of the serum that fluoresced was defined as its end point titre. Included in each slide in the IFAT was a negative serum from a normal healthy subject, a positive serum from a clinically diagnosed ALA patient, a well with diluent (TS) substituted for serum and a well containing only the conjugated antibody were also included. The fluorescence was read in duplicate relative to a negative control serum.

2.1.3. Study Population

The initial part of the study included 58 patients; the sera from 33 ALA patients were obtained from Dr. E.M. Irusen. All patients were diagnosed by ultrasonic detection of a liver defect; in addition all patients had a positive AGDT (20 hrs). Sterile amoebic pus was aspirated from 23 patients. Stool microscopy was positive for E. histolytica in 17 patients and cultured from 18. In these 18 patients the zymodemes of the isolates were determined by isoenzyme electrophoresis (Jackson et al, 1985). They were all pathogenic. The 25 normal healthy subjects served as controls. They had no clinical evidence of amoebiasis. Their AGDT were negative and their stools negative for E. histolytica. All sera were stored in aliquots of 200ul at -20°C. No serum specimen was thawed more than once.

In the second part of the study, sera were obtained from 28 subjects studied previously (Sathar et al, 1988). These sera were randomly selected. They included 10 of 100 ALA patients; 10 of 23 normal healthy African subjects and 8 of 103 hospitalised patients with various diseases other than amoebiasis. These 28 sera were coded by Mr. M. Naicker and Dr. R.

Nadar of the Gastrointestinal Unit, Department of Medicine. The IFAT on these sera (28) was performed by the author (MAS) and Mrs. C.B. Anderson of the RIDTE, without foreknowledge of the patients diagnosis.

2.2. RESULTS

The distribution of the IFAT end point titres for both IgG and IgM are shown in Figs. 2.1 and 2.2 (page 31), respectively. IgG-anti-E. histolytica antibody titres of the 33 ALA patients ranged from 1/1600 to 1/12800 (Fig. 2.1). They did not overlap those of the negative control group. The IgG titres of the normal healthy controls ranged from 0 to 1/400 (Fig. 2.1). Only 1 of 25 normal healthy control sera had an endpoint titre of 1/400 (Fig. 2.1). Anti-E. histolytica IgM titres of the 33 ALA patients ranged from 1/25 to 1/400 (Fig. 2.2). The IgM titres of the healthy control sera ranged from 0 to 1/100 (Fig. 2.2). Of the 33 ALA sera 29 had IgM titres greater than (>) 1/25 whereas only 5 of 25 control sera had titres greater than (>) 1/25 (Fig. 2.2). Using a titre of >1/500 in the IgG-IFAT to indicate seropositivity similar to that used by Jackson et al (1985), all 33 (100%) ALA patients were seropositive. There were no false positives in the control group (Fig. 2.1) With the IgM-IFAT, 88% (29/33) of

ALA patients were positive when a titre of greater than (>) 1/25 was considered to be seropositive; there was a false positive rate of 20% (Fig. 2.2). The end point titres of 1/500 and 1/25 for IgG-IFAT and IgM-IFAT were used to differentiate seropositive from sero-negative subjects.

Results of the IgG-IFAT and IgM-IFAT among the various study groups are shown in Table 2.1. The IgG-IFAT WAS positive in all 10 cases of ALA (100%). No seropositives were recorded in either the 10 healthy controls or the 8 patients who had no clinical evidence of amoebiasis (Table 2.1). There was total agreement between the two independent observers in the interpretation of IgG immunofluorescence for the 28 patients.

TABLE 2.1: RESULTS. OF IgG-IFAT AND IgM-IFAT IN THE VARIOUS STUDY GROUPS

STUDY GROUPS	TOTAL NO.	NO. POSITIVE	
		IgG-IFAT	IgM-IFAT
Amoebic liver abscess	10	10	5
Normal healthy controls	10	0	0
Hospitalised patients with other diseases	8	0	3

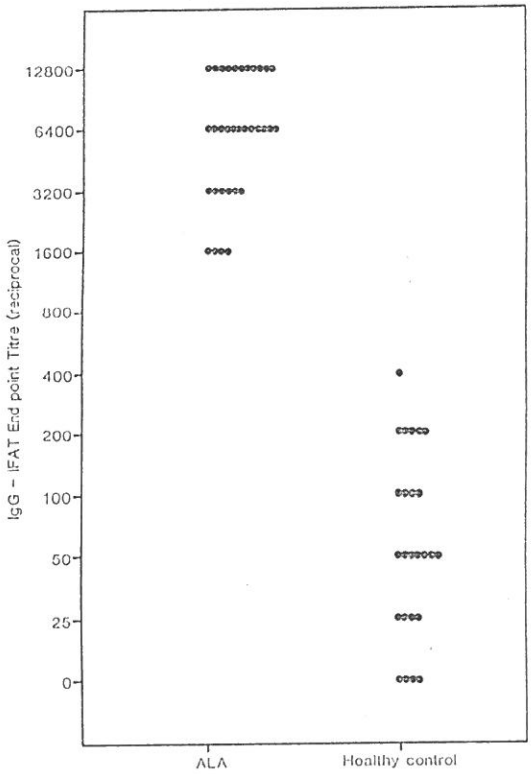

FIGURE 2.1 IgG-IFAT results in ALA patients and healthy controls

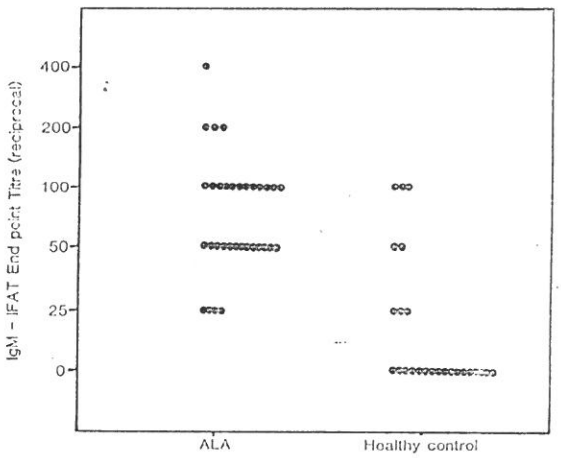

FIGURE 2.2 IgM-IFAT results in ALA patients and healthy controls

The IgM-IFAT was positive in 5 of 10 (50%) ALA patients (Table 2.1). In 8 patients without any clinical evidence of amoebiasis 3 were positive by the IgM-IFAT test. The 10 healthy controls were negative by the IgM-IFAT (Table 2.1). There was 89% (25/28) agreement between the two independent observers in the interpretation of IgM immunofluorescence.

2.3. DISCUSSION

The sensitivity (100%) of the modified IgG-IFAT performed either with or without prior knowledge of the patients disease condition is similar (96%-100%) to that reported by others for the diagnosis of ALA (Jeanes, 1966, 1969; Ambroise-Thomas and Truong, 1972; Ambroise-Thomas, 1976 and Jackson et al, 1984). However, the specificity (100%) of the modified IgG-IFAT was higher (87.5%) than that reported by Jackson et al (1984). The positivity of 12,5% observed by Jackson et al (1984) in hospitalised patients with diseases other than amoebiasis may be due to a past infection with E. histolytica as was observed with the AGDT (Powell et al, 1965). In this experiment, hospitalised patients with diseases other than amoebiasis were randomly selected from a group of patients whose AGDTs were negative. This may account for the higher specificity (100%) recorded with the IgG-IFAT. It is well documented in this endemic area for amoebiasis, that in 15-20% of such individuals, the AGDT is seropositive (Powell et al, 1965 and Jackson et al, 1985). There was 100% agreement between the two independent observers in the interpretation of the intensity of the fluorescence recorded as a numerical value from +1 to +4 for IgG-IFAT.

The specificity (83%) of the IgM-IFAT when performed on sera which were coded was not markedly different (80%) to when the test was performed on sera where the diagnosis of the patient's condition was known ; however, the specificity was lower than that of 93,75% reported by Jackson et al (1984). The sensitivity (88%) of the modified IgM-IFAT was not markedly different to that (83%) reported by Jackson et al (1984), when the assay was performed on known serum samples. However, when the IgM-IFAT was performed coded, its sensitivity was reduced to 50%. The interpretation of IgM immunofluorescence was subjective. The results of the more experienced observer were recorded. The overall agreement between the two independent observers in the interpretation of the intensity of the fluorescence recorded as +1 to +4 for the IgM-IFAT was 89%.

It is difficult to make a comparison between the modified IFAT and the IFAT of Jackson et al (1984), especially with regard to their specificity. The IFAT test was different to that of Jackson et al (1984); in addition, Jackson et al (1984) determined the specificity of the IFAT using hospitalised patients with

diseases other than amoebiasis. In the present study specificity was determined using normal healthy subjects as controls. In the former group of patients, 15-20% are known to be seropositive, due to persisting antibodies (Krupp and Powell, 1971b). This probably accounts for the discrepancies observed between the two methods with regard to their specificity.

The results not only underlines the subjectivity of the IFAT, especially the interpretation of IgM immunofluorescence, but stresses the importance of performing the IFAT without prior knowledge of the patient's disease. In addition, the interpretation of immunofluorescence must be based on the observations of two or more independent, skilled microscopists. Based on these observations, a simple quantitative and a less subjective test needs to be evaluated to detect specific IgM-anti-E. histolytica antibodies to differentiate between past and current invasive amoebiasis.

CHAPTER 3

ENZYME-LINKED IMMUNOSORBENT ASSAY

The major class of antibodies detected by most serological tests used in invasive amoebiasis is IgG (Savanat and Chaicumpa, 1969 and Dasgupta, 1974). In an endemic area, detecting specific IgM antibodies might be more useful in determining whether or not there is active/current amoebic infection, since IgM antibodies persist for a shorter time. Jackson et al (1984), using the IFAT, showed that monitoring specific IgM-anti-E. histolytica antibodies was of some value in differentiating past from current infection. However, the application of the IFAT in the routine diagnosis of amoebiasis has been limited by the fact that its performance requires expertise. Furthermore, the interpretation of immunofluorescence is subjective and thus the results are not reproducible. There is a need for a more reproducible test. The ELISA obviates the disadvantages of the IFAT and fulfils the criteria of reproducibility, ease of performance and non-subjectivity (O'Sullivan et al, 1979 and Voller et al, 1976).

The measurement of specific IgM antibodies with the ELISA has been shown to be valuable in differentiating current from past infections in other parasitic infections, viz. giardia-

sis (Goka et al, 1986) and toxoplasmosis (Naot and Remington, 1980). However, the ability of the assay to detect specific IgM-anti-E. histolytica antibodies in an endemic environment has not been evaluated. Comparative studies of serological tests which have used the ELISA to detect either specific IgG antibodies or total immunoglobulins to E. histolytica indicate that the assay is simple to perform. The ELISA has been shown to be as sensitive as the other serodiagnostic tests employed in the diagnosis of ALA, e.g. the IFAT, IHA, AGDT and CCIE (Yang and Kennedy, 1979; Agarwal et al, 1981; Bos et al, 1980; Mohapatra and Sen, 1983; Samrejrongroj and Tharavanij, 1985 and Gandhi et al, 1987).

Quantitative measurements of antibody concentrations are generally reported in terms of titres based on doubling dilutions of serum smaples. Titration to the end point when large numbers of sera have to be tested can become laborious and time consuming (Voller et al, 1977b and 1978). It would be much easier to determine a diagnostic optical density (OD) cut-off point which will differentiate normal healthy individuals from ALA patients using a single dilution of the test serum. The indirect method of the enzyme-linked immunosorbent assay (ELISA) (Voller et al, 1976, 1977b and 1978) with modification (Baigh et al, 1986) was applied to detect IgG

and IgM antibodies to E. histolytica.

Rheumatoid factor (RF) and antinuclear antibodies (ANF) in the presence of specific IgG antibodies can produce false-positive results in the various assays developed for specific IgM (Naoat and Remington, 1980; Kohler et al, 1982). For this reason and because of the value of detecting IgM-anti-E. histolytica antibodies all the sera were tested for RF and ANF. The AGDT was used as the reference test.

3.1. MATERIALS AND METHODS

3.1.1. Chemicals

All chemicals were analytical grade unless specified otherwise. Tris (hydroxymethyl) aminomethane, polyoxy-ethylene sorbitan-monolaurate (Tween 20), peroxidase conjugated immunoglobulin G (IgG)(gamma-chain specific) goat anti-human (IgG) and peroxidase conjugated IgM (mu-chain specific) goat anti-human (IgM) were obtained from Sigma Chemicals Co., St. Louis, MO, USA.

Sodium chloride (NaCl), sodium azide (NaN_3), sodium carbonate (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$) and hydrochloric acid (HCl) were obtained from BDH Chemicals Ltd., Poole, England. All chemicals were prepared in glass distilled water.

3.1.2. Antigen

Soluble E. histolytica antigen was obtained from the Research Institute for Diseases in a Tropical Environment (RIDTE) of the South African Medical Research Council (SAMRC), Congella, Durban. The antigen was prepared from a mixed culture of E. histolytica, strains HK-9 and NIH-200 grown axenically according to the technique described by Diamond (1968). The antigen was prepared using the method described by Jackson et al (1983). It had a protein concentration of 9,7mg/ml.

3.1.3. Antigen Carrier

Griener flat-bottom polystyrene microtitre (PS) plates (Dynatech, M129B, T & C Scientific Supplies, Durban), were purchased from stocks at the Natal Blood Transfusion Services (NBTS).

3.1 4. Enzyme Substrate

Chromogenic substrate (18,4mM $Na_2B_4O_7$ $19H_2O$ 30,5mM succinic acid (0,36g), 3,77mM O-phenylenediamine (40mg) 40mM urea, hydrogen peroxide (40mg), and distilled water to 100ml) (Conradie et al, 1980) was obtained from the Natal Blood Transfusion Services (NBTS) and the Institute of Immunology, Durban, South Africa.

3.1.5. Optical Density

Optical densities (OD) were read in a Titertek Multiskan micro-ELISA plate reader (Flow Laboratories Inc., McLean, Va) at 492nm.

3.1.6. Study Population

Optimal conditions for the IgG- and IgM-ELISAs were determined by titrating a positive (ALA) and a negative (normal health) reference sera. The ALA patient had been diagnosed on clinical features, the presence of an amoebic liver lesion on ultrasound examination, the aspiration of sterile pus and a positive amoebic gel diffusion test (AGDT). The control serum was obtained from a healthy subject who had no clinical evidence of amoebiasis and whose AGDT and stool microscopy were negative.

Sera from 93 subjects were assayed for the presence of anti-*E. histolytica* antibodies using the AGDT and ELISA. The study population included:

i) 30 African patients who had clinically diagnosed ALA. The diagnosis was based on ultrasonography, response to metronidazole, a strongly positive AGDT (20 hrs) and bacteriologically

sterile pus which was aspirated from 13 patients.

ii) 23 Healthy African subjects who had no clinical evidence of amoebiasis and in whom the AGDT and stool microscopy was negative.

iii) 30 hospitalised patientss who had diseases other than amoebiasis served as controls; they included patients with tuberculosis (10) and typhoid (20). The AGDT was negative in all of them.

iv) Also included in the control group were 10 subjects who had one of a range of unrelated parasites isolated from their stool, viz. Trichuris sp (2), Taenia sp (2), Ascaris sp (3), hookworm sp (1), Schistosoma haematobium (1) and Balantidium .coli (1). None of the control subjects had any evidence of amoebiasis. The AGDT was negative in all of them, except for the subject from whom a hookworm sp. was isolated.

The OD values of each study group was expressed as its mean +/- standard deviation (mean +/- SD). The OD cut-off points were determined by using the mean +/- SD of the normal healthy controls.

3.1.7. Serology

3.1.7.1. Amoebic gel diffusion test

The AGDT was examined for the development of precipitin bands over a period of 48 hours. Positivity of the AGDT expressed as 20 hours or 40 hours indicate when precipitin bands were observed. A 20 hour positive AGDT is regarded as a strong positive reaction and a 40 hour positive AGDT as a weak positive reaction.

3.1.7.2. Enzyme-linked Immunosorbent Assay

The assay was performed as follows:

1. The amoebic antigen was diluted in 0,05M Na_2CO_3 - $NaHCO_3$ buffer, pH 9,6. One hundred microlitres (100ul) of this antigen solution was dispensed into each of the 96 wells of the microtitre plate.
2. Plates were incubated in a humid container at room temperature for 2 hours.
3. Plates were washed with 300ml of 0,05M Tris-saline (TS) containing 0,05% Tween 20 (TST), followed by 200ml of distilled water in an ELISA shower (Conradie et al, 1981).
4. The excess washing solution was emptied by 'flicking' out the contents of the microtitre plate. The plates were dried by padding onto

absorbent material (Softex).

5. Plates were used within 24 hours. They were either used immediately or stored at 4°C overnight.
6. Fifty microlitres (50ul) of the test serum was added to two wells. Sera were tested in duplicate. Tris saline (TS) was used as the diluent for the sera. Both bovine serum albumin (BSA) and Tween 20 were used as blocking agents.
7. Plates were incubated in a humid container at 45°C for 1 hour.
8. Plates were washed in the ELISA shower with 500ml of TST and dried as in step 4 by 'flicking' out the excess washing fluid and padding the plate dry onto absorbent material (Softex).
9. Fifty microlitres (50ul) of either IgG or IgM conjugate diluted in TST at the manufacturer's recommended dilution of 1/1000, was added to each well.
10. Plates were incubated at 45°C for 1 hour in a humid container.
12. Fifty microlitres (50ul) of chromogenic substrate was added to each well and the plate incubated in the dark at room temperature.
13. The enzymatic reaction was stopped by adding 100ul

of 1,5N HCl to each well.

14. The intensity of the colour reaction was measured at 492nm using a Titertek Multiskan micro-ELISA plate reader.

3.1.7.3. Autoantibodies

Sera of ALA patients and normal healthy controls were tested for antinuclear antibodies (ANF) and rheumatoid factor (RF). The test for ANF was performed by the Natal Institute of Immunology, Department of Medicine. The sera were tested for RF using a 2-minute indirect haemagglutination (IHA) slide test (Wamplle Laboratories, N.J., U.S.A.).

3.2. RESULTS

Optimal antigen concentration occurred between 6,25ug/ml and 12,5ug/ml of protein for IgG- (Fig. 3.1, page 47) and at 6,25ug/ml for IgM-ELISA (Fig 3.2, page 47). To conserve antigen, it was decided to coat microtitre plates with 6,25ug/ml of amoebic protein. Plates coated with amoebic antigen and stored at 4°C overnight in a sealed plastic bag containing silica gel were stable (Figs. 3.3 and 3.4, page 47, respectively).

In both the IgG- and IgM-ELISA (Fig. 3.5 and 3.6, page 49 respectively), good titration curves were obtained

at 45°C after 1 hour of incubation which gave a good differentiation between the ALA patient and the normal healthy control. An added advantage of the ELISA is that simultaneous dual screening of IgG and IgM antibodies under identical experimental conditions saves considerable operator time. Therefore, both sera and conjugated antisera were incubated at 45°C for 1 hour in all further experiments.

The absorbence product was linear with time for the first 10 minutes for both IgG and IgM (Figs. 3.7 and 3.8, page 48, respectively). The reactivity of the ALA serum in the IgG-ELISA (Fig. 3.7) goes beyond the scope of the spectrophotometer. In order to minimize NSB of the conjugate and to reduce the signal to noise ratio, 5 minutes substrate reaction time was chosen, which gave a good differentiation between positive and negative sera for both IgG (Fig. 3.7) and IgM (Fig 3.8).

The reaction was stopped with 1,5N HCl in both the IgG and IgM-ELISAs. The substrate reaction product was stable for 1 hour with no marked colour change (Fig. 3.9, page 50).

A clear distinction between the healthy control and the

ALA patient was observed in the IgM-ELISA when BSA was excluded from the serum diluent (Fig. 3.10, page 50). Subsequently all test sera were diluted in TS.

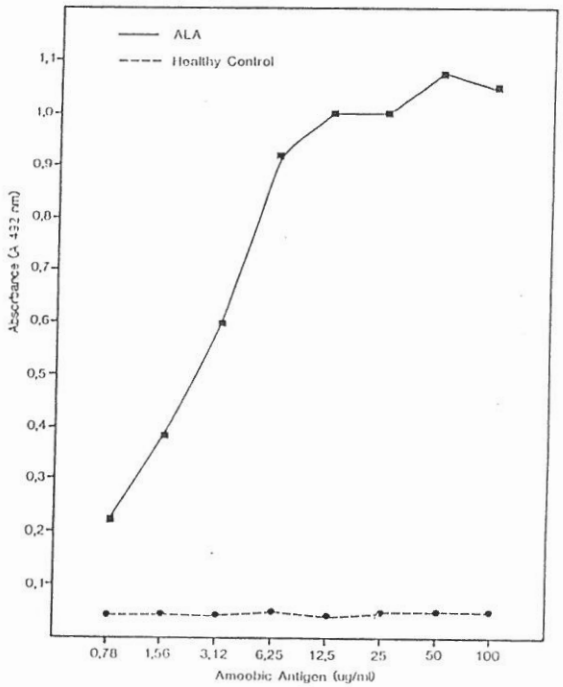

FIGURE 3.1 Optimal amoebic antigen concentration for IgG-ELISA

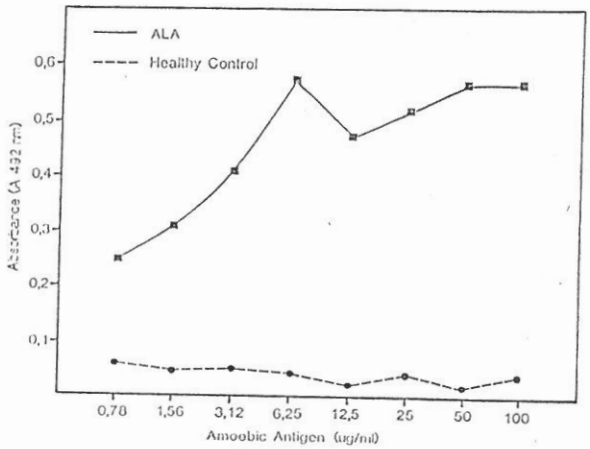

FIGURE 3.2 Optimal amoebic antigen concentration for IgM-ELISA

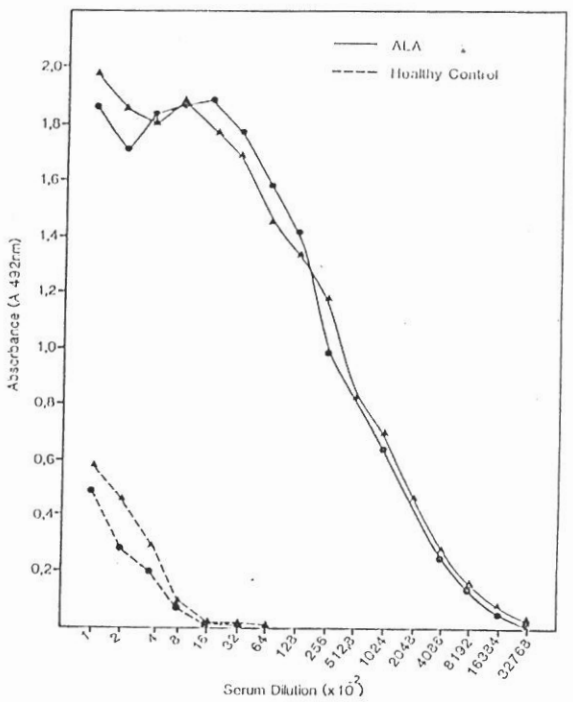

FIGURE 3.3 Antigenic stability of microtitre plates coated for 2 hours at room temperature in the IgG-ELISA.

(▲) plates used immediately

(●) stored overnight at 4°C

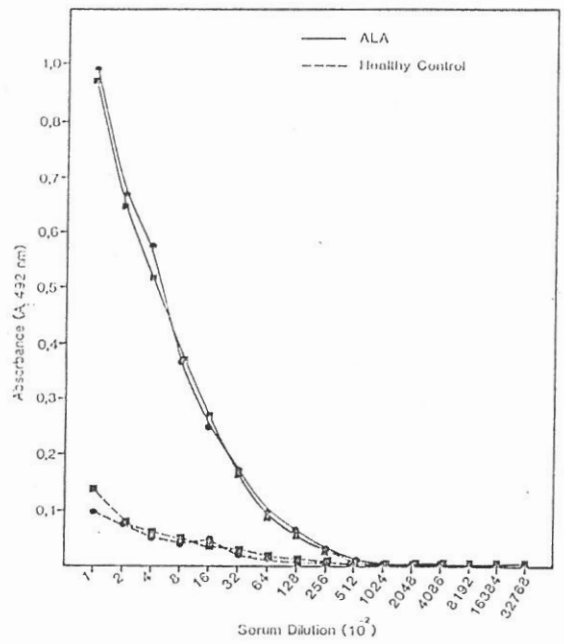

FIGURE 3.4 Antigenic stability of microtitre plates coated for 2 hours at room temperature in the IgM-ELISA.

(■) plates used immediately

(●) stored overnight at 4°C

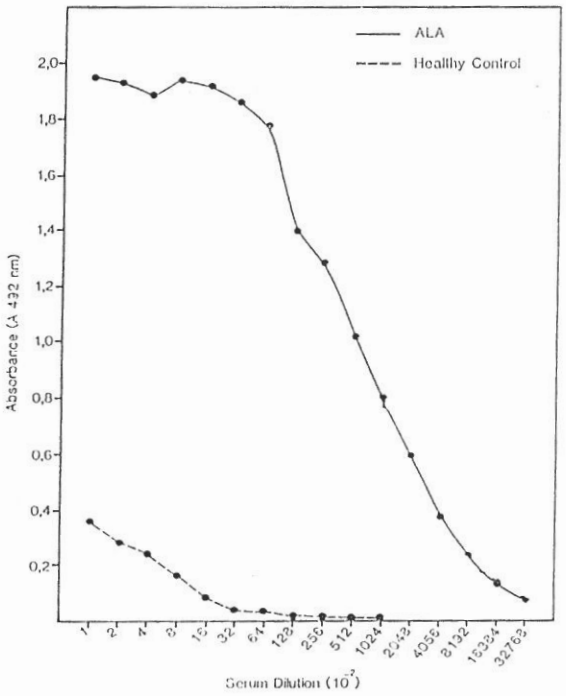

FIGURE 3.5 Effects of temperature and duration (1 hour) of serum and conjugate incubation on the IgM-ELISA
(●) 45°C;

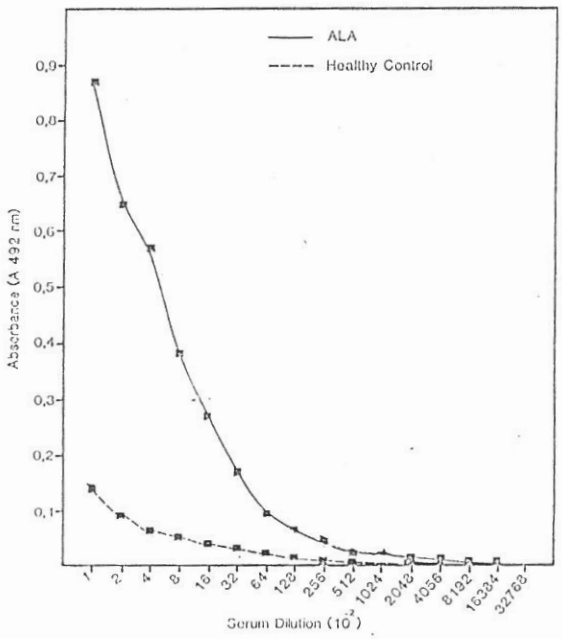

FIGURE 3.6 Effects of temperature and duration (1 hour) of serum and conjugate incubation on the IgM-ELISA
(■) 45°C;

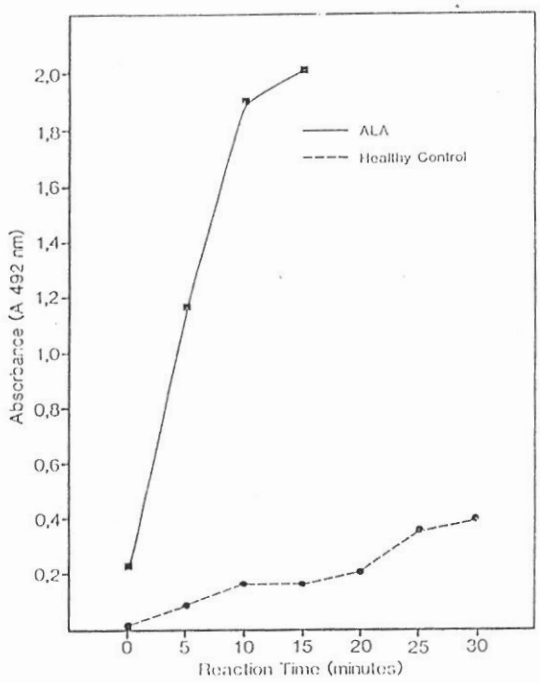

FIGURE 3.7 Optimal reaction time in the IgM-ELISA

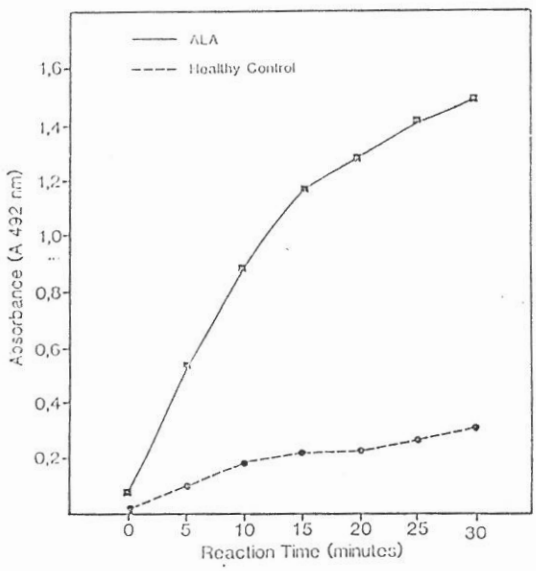

FIGURE 3.8 Optimal reaction time in the IgM-ELISA

Serum titration curves for IgG- and IgM-anti-E. histolytica antibodies are illustrated in Figs. 3.11 and 3.12 (page 50 respectively). The end point IgG-anti-E. histolytica antibody titre was approximately 1/819200 for the ALA patient and approximately 1/3200 for healthy control (Fig. 3.11). In the IgG-ELISA (Fig. 3.11), higher 'background noise' occurred at serum dilutions of 1/100 to 1/800. A single serum dilution of 1/6400 gave the biggest differentiation between the positive and negative control sera with minimum 'background noise'.

The end-point IgM-anti-E. histolytica antibody titre was approximately 1/3200 for ALA and 1/400 for the healthy control (Fig. 3.12). In the IgM-ELISA minimum 'background noise' was observed at serum dilutions of 1/400 to 1/800 (Fig. 3.12). At higher dilutions of 1/1600-1/3200 the erratic OD readings are difficult to evaluate. A good distinction between the positive and negative control sera was observed at a serum dilution of 1/400.

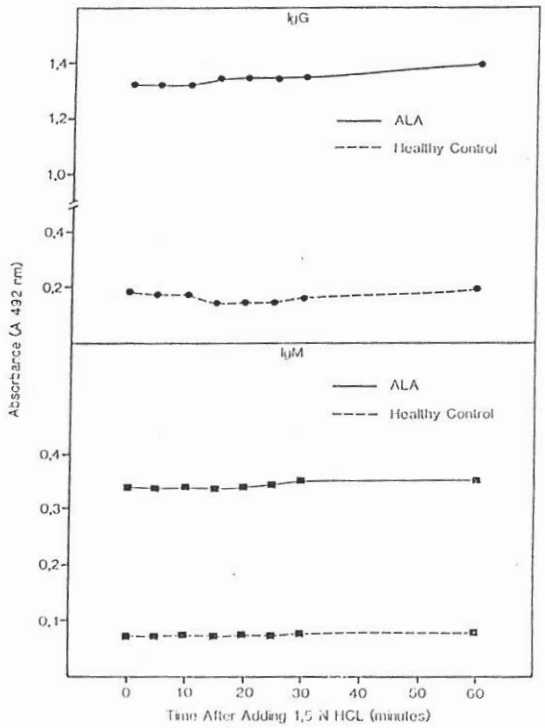

FIGURE 3.9 Stability of the substrate reaction product in the IgG- and IgM-ELISAs

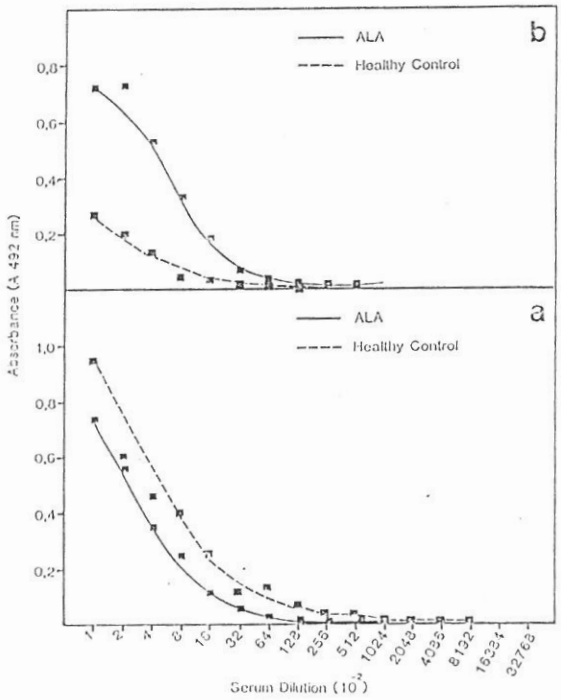

FIGURE 3.10 Non-specific binding observed with BSA (a) and (b) without BSA in the IgM-ELISA.

(a) 1% BSA-PBS; (b) PBS

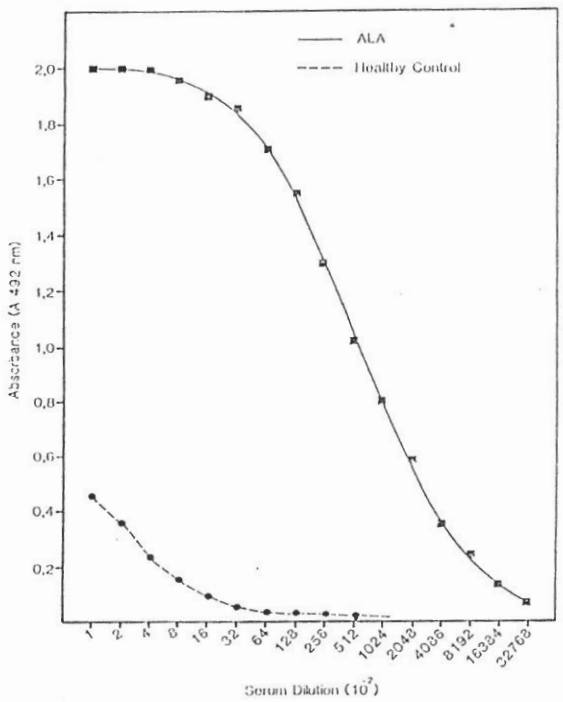

FIGURE 3.11 Typical ELISA titration curves for IgG-anti-*E. histolytica* antibody

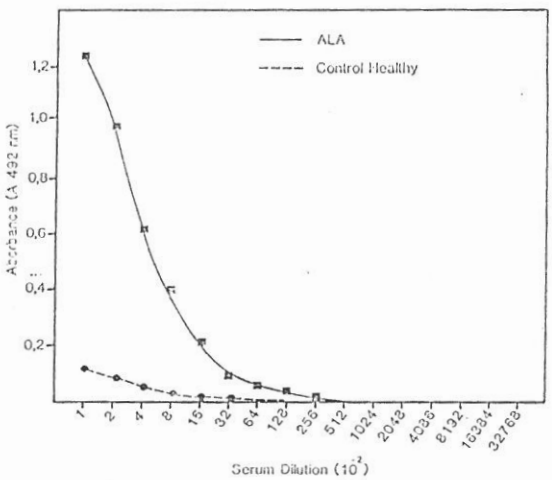

FIGURE 3.12 Typical ELISA titration curves for IgM-anti-*E. histolytica* antibody

To minimise 'background noise' and to obtain a good differentiation between seropositive and seronegative sera, a single test dilution of 1/6400 and 1/400 were chosen for the IgG- and IgM-ELISAs, respectively. These dilutions were used to determine the diagnostic OD cut-off points for the IgG- and IgM-ELISAs. The mean absorbance values obtained in the IgG- and IgM-ELISA assays for the various study groups are presented in Tables 3.1 and 3.2, page 53, respectively. The 'positive/negative cut-off' line which distinguished seropositive from seronegative sera and which gave maximum positivity amongst the various groups was determined by using the mean OD value of healthy controls plus three standard deviations (SD) of the mean for the IgG-ELISA (Table 3.1) and mean plus two standard deviations of the mean for the IgM-ELISA (Table 3.2).

The IgG-ELISA at a cut-off point of mean OD + 3SD study (0,153) gives maximum positivity amongst the various groups (Table 3.1). Use of a higher cut-off point, e.g. 0,250 would seem more logical as it would exclude most of the false positives while still retaining the sensitivity of the test. Using an OD of 0,250, the IgG-ELISA was positive in 99% of ALA patients, and only

one control patient in whom a hookworm was isolated was seropositive (Table 3.1).

TABLE 3.1: MEAN ABSORBANCE (492nm) VALUES OBTAINED BY IgG-ELISA AMONG HEALTHY SUBJECTS AND VARIOUS GROUPS OF PATIENTS AT DIFFERENT CUT-OFF POINTS DETERMINED FROM HEALTHY CONTROLS

STUDY GROUPS	NO	Mean A492nm ± SD	PERCENT (%) POSITIVE			
			Mean ± 1SD A = 0,079	Mean ± 2SD A = 0,116	Mean ± 3SD A = 0,153	A = 0,250
1 AMOEBIC LIVER ABSCESS (ALA)	30	0,873 ± 0,321	100	100	100	99
2 HOSPITALISED PATIENTS WITH OTHER DISEASES						
A Tuberculosis	10	0,074 ± 0,030	30	10	0	0
B Typhoid	20	0,153 ± 0,050	95	80	55	0
3 PARASITIC INFECTION	10	0,085 ± 0,030	30	20	10	10
4 NORMAL HEALTHY CONTROLS	23	0,042 ± 0,037	13	9	4	0

TABLE 3.2 MEAN ABSORBANCE (492nm) VALUES OBTAINED BY IgM-ELISA AMONG HEALTHY SUBJECTS AND VARIOUS GROUPS OF PATIENTS AT DIFFERENT CUT-OFF POINTS DETERMINED FROM HEALTHY CONTROLS

STUDY GROUPS	NO	Mean A492nm ± SD	PERCENT (%) POSITIVE			
			Mean ± 1SD A = 0,268	Mean ± 2SD A = 0,344	Mean ± 3SD A = 0,420	A = 0,350
1 AMOEBIC LIVER ABSCESS (ALA)	30	0,471 ± 0,233	83	63	43	63
2 HOSPITALISED PATIENTS WITH OTHER DISEASES						
A Tuberculosis	10	0,115 ± 0,078	10	0	0	0
B Typhoid	20	0,231 ± 0,099	35	15	10	15
3 PARASITIC INFECTION	10	0,152 ± 0,077	10	0	0	0
4 NORMAL HEALTHY CONTROLS	23	0,192 ± 0,076	22	0	0	0

In the IgM-ELISA, mean + 2SD (0,350) of normal healthy controls was taken as the cut-off point to distinguish seropositive from seronegative subjects (Table 3.2). Using an OD of 0,350, 63% of patients with ALA were positive and only 3 control patients who had typhoid were positive. At a lower OD cut-off (0,268), the sensitivity is good (83%) but specificity of the IgM-ELISA is reduced. Therefore, 0,350 was chosen as the OD cut-off point, increasing the specificity of the assay at the expense of sensitivity (Table 3.2). None of the sera of ALA patients or healthy controls was positive for RF or ANF.

The reproducibility of the assay was examined over a period of approximately 3 weeks using the same batch of reagents. Sera of an ALA patient and a normal healthy control were tested at single dilutions of 1/6400 for IgG- and 1/400 for IgM- E. histolytica antibodies. It should be noted that during this period the specimens were stored in aliquots of 200ul at -20°C. No specimen was thawed more than once for each determination. The results are shown in Table 3.3, page 55.

Table 3.3 REPRODUCIBILITY OF IgG and IgM-ELISA (RESULTS OVER A PERIOD OF 3 WEEKS)

	IgG (OD 492nm)		IgM (OD 492nm)	
DAY	ALA	NORMAL HEALTHY CONTROL	ALA	NORMAL HEALTHY CONTROL
1	1,480	0,140	0,837	0.128
6	1,482	0,133	0,827	0,113
8	1,311	0,165	0,840	0,104
14	1,382	0,130	0,766	0,108
21	1,313	0,145	0,692	0,110
X	1,393	0,142	0,792	0,112
SD	0,08	0,012	0,06	0,009
CV (%)	6	9	8	8

The co-efficient of variation (CV%) of duplicate testing of the positive and negative control sera ranged from 1% to 5%. In most instances they were less than 5%. The CV (%) between assays ranged from 6% to 9% (Table 3.3).

3.3. DISCUSSION

With ELISA the solid phase is a critical element of the test system since the antigen must be bound to the carrier for the assay to be performed (Shekarchi et al, 1984). Insufficient binding of the antigen decreases the sensitivity of the test and frequently increases the 'background noise'. Uneven binding decreases the reproducibility of the test (Sorensen and Bordbeck, 1986). The polystyrene (PS) plate (M129B) gives a good discrimination between the two reference sera. The binding capacity of the carrier is a function of both its chemical and physical nature and is thus subject to many changes during manufacture and storage (Shekarchi et al, 1984). Variations in binding to microtitre plates have been found to occur between plates made from the same plastic by different manufacturers and even between lots or batches produced by a single manufacturer (Shekarchi et al, 1984). It is important that the solid phase takes up adequate amounts of the antigen in a consistent reproducible manner (Sherkarchi et al, 1984). Conradie et al (1981 and 1983) after extensive trials, obtained a co-efficent of variation (CV) of less than 10% with the Dynatech M129B PS micro-

titre plates. Using these plates (Dynatech M129B) similar results were obtained in the present study, thus confirming the observations made by Conradie et al (1981 and 1983). Therefore, Dynatech M129B PS plates were obtained from Dr. Conradie's large stocks at NBTS.

For most proteins sensitisation of microtitre plates is achieved with protein concentrations of 1-10ug/ml in carbonate-bicarbonate buffer, pH 9,6 (Voller et al, 1977b and 1978). Adsorption occurs rapidly, being completed within 1-2 hours at 20°C-25°C (Voller et al, 1977b and 1978). For convenience overnight coating at 4°C is often satisfactory (Voller et al, 1977b and 1978). Good results were obtained with plates coated with 6,25ug/ml of amoebic protein, incubated at room temperature for 2 hours. Antigen-coated plates stored overnight at 4°C were stable, however, the stability of the antigen-coated plates over a longer period of time was not determined.

Increasing the temperature of incubation of the serum and conjugated antiserum speeds up the reaction time and decreases the binding time (Gilman and Docherty, 1977). Increasing the temperature above 45°C is disad-

vantageous since specific activity is decreased, presumably due to a combination of temperature denaturation effects, disassociation of conjugated antiserum as well as release of the antigen from the microtitre plate, due possibly as a result of structural changes in the plastic (Gilman and Docherty, 1977).

The chromogenic substrate is light sensitive; therefore, incubation was performed in the dark (Voller et al, 1977b and 1978). The substrate reaction product was stable for 1 hour. The latter is important since inherent colour changes could occur when considerable time is required in the testing of large numbers of sera (Bullock and Walls, 1977 and Bidwell et al, 1977).

In the ELISA there is always a tendency for proteinaceous material present in the test serum to attach in a non-specific manner to the solid phase (Kohler et al, 1982). Such non-specific binding may be eliminated or reduced to an acceptable level by the addition of a 'blocking' agent to the serum diluent (Vogt et al, 1987). This is a collective term for various additives, e.g. bovine serum albumin (BSA) (Vogt et al, 1987) or non-ionic detergents, e.g. Tween 20 (Kenny and

Dunsmoor, 1987) that have no active part in the immunochemical reactions of the assay. The reliability of Tween 20 as an effective blocking agent is uncertain (Gardas and Lewartowska, 1988) as different brands and batches of microtitre plates differ in their ability to retain non-ionic detergents (Kenny and Dunsmoor, 1987). Cardas and Lewarkokska (1988) have shown that effective binding of proteins to microtitre plates can be enhanced by using detergents with a high critical micelle concentration (CMC). To reduce non-specific binding (NSB) both BSA and Tween 20 were included in the serum diluent but these did not significantly improve the IgG-ELISA. Bullock and Walls (1977) reported that eliminating the blocking step saves time and reduces costs while still reducing 'background noise' to a minimum, without sacrificing sensitivity and specificity. When BSA was excluded from the serum diluent, especially in the IgM-ELISA, it resulted in a clear separation between positive and negative sera. Recently Mahomed and Esen (1989) showed that a blocking agent and a blocking step are not needed in the ELISA.

To optimize the efficiency and economy of the ELISA, the detection of the test material (IgG/IgM-anti-*E.*

histolytica antibodies) in the test sample (serum) can usually be made on a single dilution of that test sample (Voller et al, 1976, 1977b, and de Savigny and Voller, 1980). If a single dilution of the test serum is to be used then it is important to select a dilution high enough so that the OD readings can be made in the effective part of the curive, i.e. at a dilution at which high titre serum would fall on the upper part of the descending curve (Sherkarchi et al, 1984). At lower dilutions, the erratic OD readings of high titre serum would be difficult to evaluate. Therefore, a working dilution of 1/6400 was chosen for IgG and 1/400 for IgM.

The various methods of processing and reporting ELISA results have been reviewed by de Savigny and Voller (1980), Karpinski et al (1987), Crofts et al, (1988) and Parkinson et al (1988). No single method has been found to be satisfactory. Bos et al (1976) used serial dilutions of the test serum to determine the diagnostic titre. They (Bos et al, 1976) compared the OD values of the test sera to that of normal serum. Felgner (1977) on the other hand, used the OD value of pooled healthy sera as the cut-off point for diagnosis. They

(Felgner, 1977) considered an OD greater than that of the pooled serum as being diagnostic. Agarwal et al (1981) used mean OD + 2SD (standard deviation) of healthy controls as the cut-off point. Knobloch et al (1982), expressed their results as multiples of normal activity (MONA). In this dissertation the methods used by Agarwal et al (1981), Sakata et al (1985), Lin et al (1986) and Gandhi et al, (1987) were used to determine the OD cut-off points, i.e. mean + 2 or 3 SD of healthy controls. The subject from whom a hookworm sp was isolated and who had a positive AGDT was also seropositive with the IgG-ELISA. This subject might have had a past unrecognised E. histolytica infection with a pathogenic zymodeme, since anti-amoebic antibodies are known to persist for several years (Krupp and Powell, 1971b).

Having optimised the ELISA to detect specific IgG- and IgM-anti-E. histolytica antibodies, the sensitivity and specificity of the assay needs to be determined and evaluated in well documented cases of ALA and appropriate control groups.

CHAPTER 4

EVALUATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) IN THE SERODIAGNOSIS OF AMOEBIC LIVER ABSCESS (ALA)

In an endemic area such as Durban, diagnosis of amoebic liver abscess (ALA) is always considered in the differential diagnosis of hepatomegaly, especially when the liver is tender (Powell et al, 1965 and Adams and MacLeod, 1977). In non-endemic areas, however, because of the infrequency of ALA, the diagnosis of this very treatable disease can be missed. A reliable serological test could be used to obviate this problem. An important differential diagnosis of ALA is pyogenic liver abscess. The latter, especially in endemic areas, is less common than ALA and, therefore, may be less likely to be diagnosed. The ultrasonography and clinical features (toxic pyrexial illness with tender hepatomegaly and leucocytosis) in pyogenic liver abscess are similar to ALA. These patients' serology for ALA is invariably negative and if untreated could have a high mortality. Thus in this situation a rapid reliable negative serological test for ALA is of value. Even when ALA is diagnosed not all patients require aspiration and thus the diagnosis remains presumptive (Adams and MacLeod, 1977). If serology in these patients is positive, the clinician awaits response to therapy, before

deciding whether aspiration should be performed or not (delaRey Nel et al 1989). If, on the other hand, amoebic serology is negative, another diagnosis is considered and aspiration and/or liver biopsy is indicated. Therefore serological tests for ALA can be of considerable supportive value in various clinical situations. Early and rapid diagnosis of ALA could improve the outcome of treatment and decrease the fatality rate (Adams and MacLeod, 1977).

Extensive studies in our environment have shown the AGDT test to be a sensitive serological test when used routinely in the differential diagnosis of ALA. Negative serology not only excludes amoebiasis (Powell et al, 1965) but positive serology indicates a past or present infection with a pathogenic E. histolytica (Jackson et al, 1985). Previous comparative studies of serological tests with enzyme-linked immunosorbent assay (ELISA) systems which detected antibodies to E. histolytica of both the IgG class or total immunoglobulin indicated that the assay is more sensitive and specific than the other serodiagnostic tests employed in the diagnosis of invasive amoebiasis (Yang and Kennedy, 1979; Bos et al, 1980; Agarwal et al, 1981, and Baveja et al, 1984).

The ELISA was evaluated for its ability to differentiate past from current tissue invasion by E. histolytica. The sensit-

ivity and specificity of the ELISA was evaluated using sera from patients with ALA and appropriate control groups. The serological response of carriers of pathogenic and non-pathogenic zymodemes of E. histolytica was also assessed by ELISA in an attempt to relate serology to the zymodeme type. The assay was compared to the AGDT.

4.1. MATERIALS AND METHODS

4.1.1. Antigen

The E. histolytica antigen, a mixture of strains HK9 and NIH200 with a protein concentration of 9,7mg/ml was prepared as described by Jackson et al (1983).

4.1.2. Serology

The AGDT and the ELISA were performed as outlined by Jackson et al (1983) and Sathar et al (1988), respectively.

4.1.3. STUDY POPULATION

Sera from 295 subjects were assayed by the AGDT and the ELISA. The study population comprised the following groups:

One hundred (100) patients with confirmed ALA. The diagnosis of ALA was made on clinical features, demonstration of a liver abscess by ultrasound examination,

positive AGDT and a favourable response to metronidazole; in addition, bacteriologically sterile pus was aspirated from 49 of these patients. Twenty-three healthy African subjects who were either University or Hospital staff served as controls. They had no clinical evidence of amoebiasis. In addition these subjects had a negative AGDT, as well as negative stool microscopy for E. histolytica. Also included in the study were 121 hospitalized patients with various diseases other than amoebiasis, of whom 103 were seronegative by the AGDT and 8 were seropositive. A further 10 control subjects had collagen vascular diseases and were positive for one or more autoantibodies. In these patients amoebiasis was excluded clinically and by a negative AGDT. The 8 patients who were AGDT positive did not have clinical amoebiasis on the basis of clinical examination which included sigmoidsocopy, stool microscopy and ultrasonography of the liver.

Sera from 51 healthy African subjects were obtained from a study performed in an area endemic for amoebiasis south of Durban (Jackson et al, 1985 and Gathiram and Jackson, 1985). The stools of these

subjects had been cultured for E. histolytica and the isolates subjected to isoenzyme electrophoresis to determine their zymodemes (Jackson et al, 1985 and Gathiram and Jackson, 1985). These subjects were grouped into those who were culture negative for E. histolytica (n=30) and those who had pathogenic (n=11) and non-pathogenic (n=10) zymodemes.

4.2. RESULTS

Using an optical density (OD) of 0,250 as the cut-off value to separate seropositive from seronegative sera, the IgG-ELISA has a sensitivity of 99% and a specificity of 91,7% (Fig. 4.1, page 69). The IgM-ELISA has a sensitivity of 64% and a specificity of 97,9% at an OD cut-off value of 0,350 (Fig. 4.2, page 69). Comparative results of the IgG-ELISA and AGDT and the IgM-ELISA and AGDT among the various groups are presented in Table 4.1 and Table 4.2 (page 70), respectively.

The AGDT was strongly positive (20 hrs) in all 100 patients with ALA and the 11 symptomless carriers of pathogenic E. histolytica, the test was weakly positive (40 hrs) in 2 of 10 symptomless carriers of non-pathogenic zymodemes. Only 8 of 121 hospitalised patients were seropositive by the AGDT (Table 4.1). All

30 subjects from the endemic area who were culture negative for E. histolytica, were seronegative by the AGDT (Table 4.1).

The IgG-ELISA was positive in 99 of 100 ALA patients and 10 of 11 symptomless carriers of pathogenic zymodemes (Table 4.1). Four symptomless carriers of non-pathogenic zymodemes were detected by the IgG-ELISA (Table 4.1). Six of 30 subjects from the endemic area who were culture negative for E. histolytica were positive by the IgG-ELISA (Table 4.1). In the 121 patients without any clinical evidence of amoebiasis, 12 were seropositive by the IgG-ELISA (Table 4.1).

Sixty-four (64) of 100 patients with ALA and none of the 11 symptomless carriers of pathogenic E. histolytica were seropositive using the IgM-ELISA (Table 4,2). One of 10 symptomless carriers of non-pathogenic zymodemes were detected by the IgM-ELISA (Table 4.2). One of 30 subjects from the endemic area who was culture negative for E. histolytica (Table 4.2); In the 121 patients without any clinical evidence of amoebiasis, 3 were seropositive by the IgM-ELISA (Table 4.2).

Using Pearson's correlation co-efficient, an excellent correlation was obtained between the IgG-ELISA and the AGDT (r=0,99).

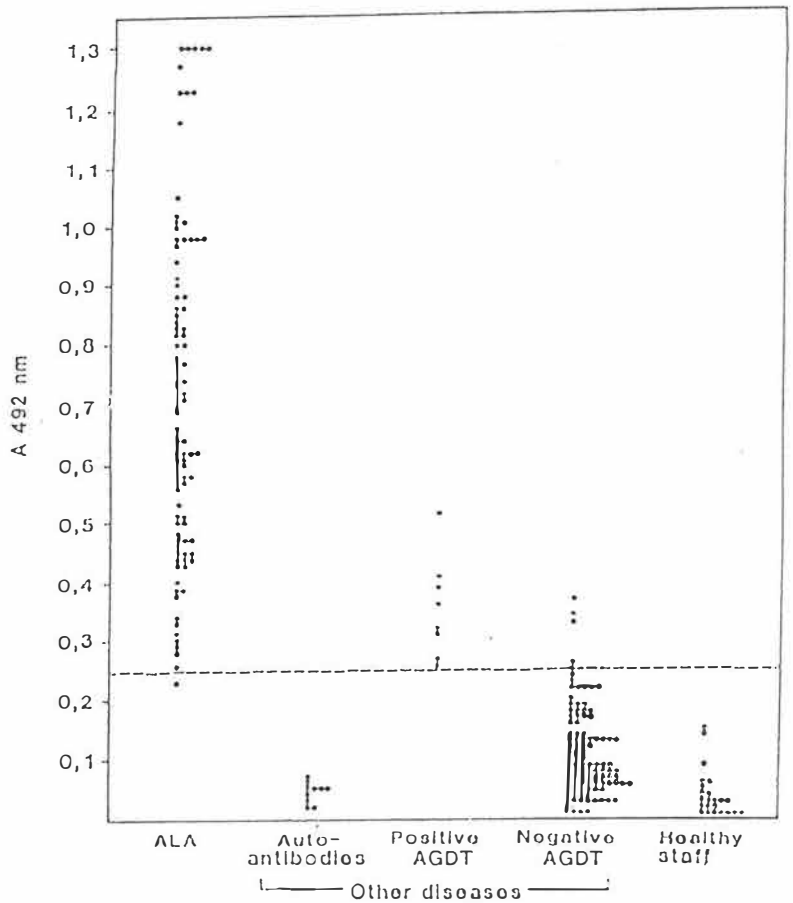

FIGURE 4.1 ANTI-AMOEBIC IgG RESULTS OBTAINED WITH ELISA

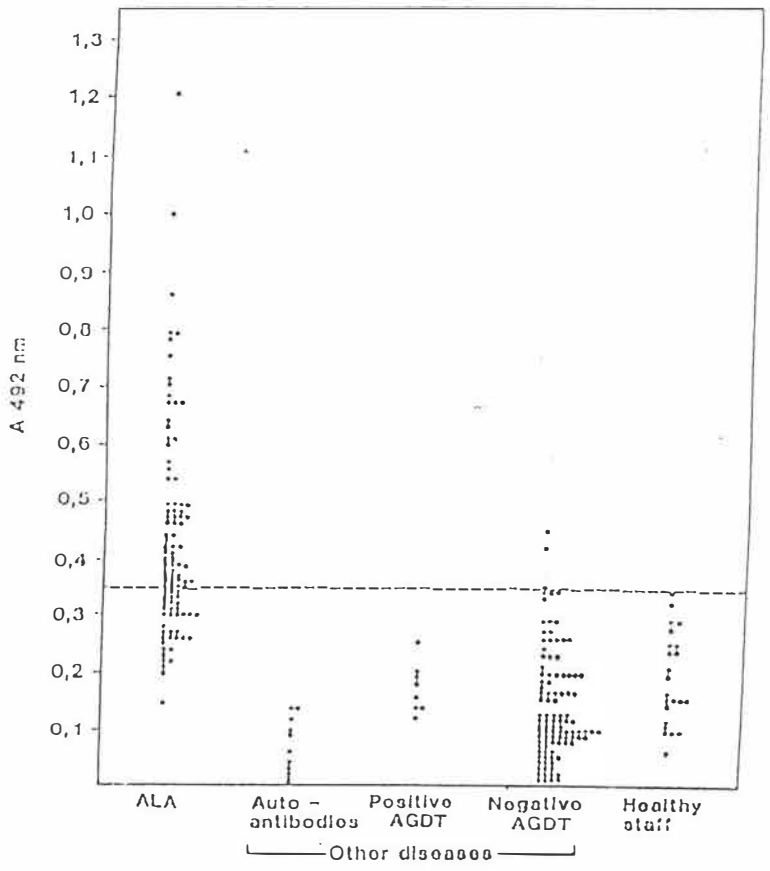

FIGURE 4.2 ANTI-AMOEBIC IgM RESULTS OBTAINED WITH ELISA

TABLE 4.1 COMPARATIVE RESULTS OF IgG-ELISA and the AGDT

STUDY GROUPS	TOTAL NO.	NO. POSITIVE	
		IgG-ELISA	AGDT
Amoebic liver abscess	100	99	100
Healthy controls	23	0	0
Hospitalised patients with other diseases:			
a) AGDT negative	103	4	0
b) AGDT positive	8	8	8
c) Collagen disease (with one or more auto-antibodies positive)	10	0	0
Subjects from an endemic area:			
a) No amoeba cultured	30	6	0
b) Symptomless carriers of E. histolytica:			
i) Pathogenic	11	10	11
ii) Non-pathogenic	10	4	2

TABLE 4.2 COMPARATIVE RESULTS OF IgM-ELISA and the AGDT

STUDY GROUPS	TOTAL NO.	NO. POSITIVE	
		IgM-ELISA	AGDT
Amoebic liver abscess	100	64	100
Healthy controls	23	0	0
Hospitalised patients with other diseases:			
a) AGDT negative	103	3	0
b) AGDT positive	8	0	8
c) Collagen disease (with one or more auto-antibodies positive)	10	0	0
Subjects from an endemic area:			
a) No amoeba cultured	30	1	0
b) Symptomless carriers of E. histolytica:			
i) Pathogenic	11	0	11
ii) Non-pathogenic	10	1	2

4.3. DISCUSSION

The sensitivity of the IgG-ELISA for the diagnosis of ALA (99%) is comparable (97-100%) to results reported by others (Yang and Kennedy, 1979; Agarwal et al, 1981; Lin et al, 1981; Tandon, 1981; Mohapatra and Sen, 1983; Baveja et al, 1984 and Gandhi et al, 1987). The IgM-ELISA is specific for clinical ALA (97,9%) but lacks sensitivity (64%). Researchers generally agree that titres of IgM-anti-E. histolytica antibodies when present are less than that of IgG (Boonpucknavig and Nairn, 1967; Bray and Harris, 1977 and Osisanya and Warhurst, 1980). This seems to indicate that the IgM-ELISA is unlikely to be of much clinical value until its sensitivity is improved.

In this study a seropositivity of 8,3% was observed with the IgG-ELISA in patients without amoebiasis, a figure similar to that observed by others with the AGDT (Maddison, 1965; Powell et al, 1965 and Maddison et al, 1965b). This is unlikely to be due to non-specific reactions since the IgG-ELISA was negative in patients with auto-antibodies and other diseases. However, this 'background noise' may reflect a past infection with E. histolytica as amoebic antibodies are known to persist

for several years after treatment (Krupp and Powell, 1971b).

Jackson et al (1985) observed that the seropositivity of symptomless carriers of pathogenic zymodemes of E. histolytica were similar to patients with the clinical disease. Subjects with a strong positive serological reaction were usually associated with a pathogenic zymodeme and a negative or weakly positive serological reaction was associated with a non-pathogenic zymodeme. The positivity of the serological tests in these carriers of pathogenic zymodemes is not a non-specific response, but indicates subclinical tissue invasion (Jackson et al, 1985). Results with the IgG-ELISA in cyst passers of pathogenic zymodemes confirm the findings of Jackson et al (1985). The IgM-ELISA is negative in the majority of subjects from the endemic area, possibly indicating an inactive disease.

A higher seropositivity was observed with the IgG-ELISA when compared to the AGDT in hospitalized patients with other diseases, in subjects from an endemic area whose stools were negative for E. histolytica and in symptomless carriers of non-pathogenic zymodemes. This could be due to the higher sensitivity of the ELISA when

compared to other immunodiagnostic tests as has been suggested by the work of others (Bos et al, 1980; Lin et al, 1981; Agarwal et al, 1981; Gandhi et al, 1986, 1987 and Proctor et al, 1987). Except for Proctor et al (1987) none of these researchers characterised the zymodeme of their E. histolytica isolate. This is due to the fact that isoenzyme electrophoresis was not available to the earlier researchers. The IgG-ELISA is as sensitive as the AGDT for the diagnosis of ALA.

Whilst the sensitivity (99%) and specificity (91,7%) of the IgG-ELISA is excellent, the sensitivity of the IgM-ELISA needs to be improved. The use of more specific antigens rather than soluble complex antigens of E. histolytica could improve the sensitivity and specificity of the ELISA, (Mathews et al, 1986 and Schulz et al, 1987), especially the IgM-ELISA. The different amoebic strains used and the methods of preparing the antigen could also influence the sensitivity of the ELISA. The immunogenicity and specificity of the various fractions of E. histolytica are being analysed by various researchers (Mathews et al, 1986, Ortiz-Ortiz et al, 1986 Joyce and Ravdin, 1986 and Petri et al, 1987).

It is only as regards the speed with which the result is provided that this assay would appear to have an advantage over the AGDT. The IgG-ELISA results can be provided in 2 1/2 hours compared with a minimum of 24 hours by the AGDT. The prompt availability of the IgG-ELISA results combined with an improved IgM-ELISA could allow the clinical to start specific investigations earlier. Early diagnosis and prompt treatment of an ALA could reduce morbidity and mortality whilst late or misdiagnosis could be fatal (Adams and MacLeod, 1977 and Editorial, 1978).

These findings have been published by the South African Medical Journal, 1988, 74: 625-628, a copy of which has been included in the dissertation.

Evaluation of an enzyme-linked immunosorbent assay in the serodiagnosis of amoebic liver abscess

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Summary

An indirect enzyme-linked immunosorbent assay (ELISA) was evaluated for the detection of anti-amoebic IgG and IgM antibodies to assess its value in distinguishing past from current infection in invasive amoebiasis, particularly in amoebic liver abscess (ALA) patients. Using sera from 295 individuals, the ELISA was also compared with the amoebic gel diffusion (AGD) test. In 100 patients the IgG-ELISA at a single test dilution of 1/6 400 had a sensitivity of 99% for clinically diagnosed ALA. In these same patients the IgM-ELISA at a single dilution of 1/400, had a sensitivity of 64% and a specificity of 97,9%. No cross-reactions were observed in sera from patients with collagen vascular disease. In 121 patients without clinical invasive amoebiasis, 8 were AGD-positive and 12 were IgG-ELISA-positive, giving the latter assay a specificity of 91,7%. This is thought to be due to past infection with *Entamoeba histolytica.* In symptomless carriers of pathogenic zymodemes, 10/11 were seropositive by the IgG-ELISA and 11/11 by the AGD test. There was an excellent correlation between the IgG-ELISA and the AGD test (r = 0,99). The IgG-ELISA is a sensitive, specific, simple and rapid test. It has the clinical advantage that results are obtainable 2½ hours after receipt of the specimen, compared with the 24 - 48 hours required for the AGD test. The prompt availability of IgG-ELISA results could prove advantageous for implementation of early therapy. The IgM-ELISA was not found to be sensitive enough to be used as an index of active amoebic infection.

S Afr Med J 1988; 74: 625-628

Walsh[1] estimated in 1981 that 480 million people harboured *Entamoeba histolytica* in their intestinal tracts; in some there is intestinal and hepatic invasion.[2] Amoebiasis is regarded as the third most important cause of death from parasites after malaria and schistosomiasis.[1]

In an endemic area such as Durban, diagnosis of amoebic liver abscess (ALA) is always considered in the differential diagnosis of hepatomegaly, especially when the liver is tender. In non-endemic areas, however, because of the infrequency of ALA, the diagnosis of this very treatable disease can be missed. A reliable serological test could be used to obviate this problem. An important differential diagnosis of ALA is pyogenic liver abscess. The latter, especially in endemic areas, is less common than ALA and, therefore, may be less likely to be diagnosed. The ultrasonographic and clinical features (toxic pyrexial illness with tender hepatomegaly and leucocytosis) in pyogenic liver abscess are similar to ALA. These patients' serology for ALA is invariably negative and if untreated could have a high mortality. Thus in this situation a rapid reliable negative serological test for ALA is of value. Even when ALA is diagnosed not all patients require aspiration and thus the diagnosis remains presumptive.[3] Therefore serological tests for ALA can be of considerable supportive value in various clinical situations.

Early and rapid diagnosis of ALA could improve the outcome of treatment and decrease the fatality rate.[3] With the availability of antigen from axenic cultures of *E. histolytica,* emphasis has been placed on serological tests as aids in the rapid diagnosis of invasive amoebiasis. The various tests used in invasive amoebiasis include the indirect haemagglutination (IHA),[4] indirect fluorescent antibody (IFA),[5] counter immuno-electrophoresis (CIE),[6] micro-immunoelectrophoresis (MIE),[7] amoebic gel diffusion (AGD)[8,9] and latex agglutination (LA).[10]

Extensive studies in our environment have shown the AGD test to be a sensitive serological test when used routinely in the differential diagnosis of ALA. Negative serology not only excludes amoebiasis,[9] but positive serology indicates a past or present infection with a pathogenic *E. histolytica.*[11] Previous comparative studies of serological tests with enzyme-linked immunosorbent assay (ELISA) systems which detected antibodies to *E. histolytica* of both the IgG class or total immunoglobulin indicated that the assay is more sensitive and specific than the other serodiagnostic tests employed in the diagnosis of invasive amoebiasis.[12-15]

In the search for a test that is sensitive, specific, rapid and easy to perform, and that holds the potential of distinguishing past from current infection by specifically measuring IgM antibodies to *E. histolytica,* we performed a comparative evaluation of the ELISA technique with the AGD test.

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Materials and methods

Chemicals

All chemicals were analytical grade. Tris (hydroxmethyl) aminomethane, polyoxyethylenesorbitan monolaurate (Tween 20), peroxidase conjugated immunoglobulin IgG (γ-chain-specific goat anti-human IgG) and peroxidase conjugated immunoglobulin IgM (μ-chain specific goat anti-human IgM) were obtained from Sigma Chemicals (St Louis, Missouri, USA).

Sodium chloride (NaCl), sodium azide (NaN_3), sodium carbonate (Na_2CO_3), hydrochloric acid (HCl) and sodium hydrogen carbonate ($NaHCO_3$) were obtained from BDH Chemicals (Poole, England).

Antigen

The *E. histolytica* antigen, a mixture of strains NIH200 and HK9, with a protein concentration of 9,7 mg/ml was prepared as described previously.[16]

Serology

The AGD test was performed as described by Jackson *et al.*[16]

ELISA technique

The indirect method for the assay of antibodies by ELISA[17] with modifications[18] was used to measure anti-amoebic IgG and IgM antibodies.

Wells of polystyrene microtitre plates, F-form (Dynatech) M129, T & C Scientific Supplies, Durban) were coated with amoebic antigen 100 µl and protein concentration 6,25 µg/ml in coating buffer (0,05M carbonate buffer, pH 9,6) and incubated for 2 hours at room temperature in a humid container. Plates were washed with 500 ml of 0,05M Tris-0,1M NaCl buffer (TS) pH 8,0 containing 0,05% (w/v) Tween 20 (TST) in a mictotitre plate washer.[19] Washing fluid was shaken off and the plates were dried by padding on absorbent material (e.g. Softex). Test sera (50 µl) appropriately diluted in TS was added to duplicate wells. Plates were incubated for 1 hour at 45°C in a humid container.

Plates were washed with 500 ml of TST, dried, and incubated with 50 µl of a 1/1 000 dilution of horse radish peroxidase conjugated IgG or IgM in TST for 1 hour at 45°C. After washing, with 500 ml of TST, plates were dried, 50 µl of chromogenic substrate (H202/OPD)[18] (Natal Blood Transfusion Services, Durban) was added to each well and plates incubated for 5 minutes in the dark at room temperature. The reaction was stopped by adding 100 µl of 1,5N HCl. The optical density was measured at 492 nm using a Titertek Multiscan Micro-ELISA plate reader (Flow Laboratories).

Study population

Sera from 295 subjects were assayed by the AGD test and ELISA. The study populations (Table I) were as follows: (*i*) 100 patients with confirmed ALA of whom 49 were aspirated, the remaining 51 patients were diagnosed as ALA on clinical features, ultrasonography and a positive AGD test and all responded to metronidazole with complete amelioration of clinical features; (*ii*) 23 healthy black subjects who were either university of hospital staff; (*iii*) hospitalised patients with various diseases other than amoebiasis, of whom 103 were negative by the AGD test and 8 positive; a further 10 had collagen vascular diseases and were positive for one or more auto-antibodies (the 8 patients who were AGD positive did not have clinical amoebiasis on the basis of clinical examination, which included sigmoidoscopy, stool microscopy and ultrasonography of the liver); and (*iv*) sera from 51 healthy black subjects described in an earlier study performed in an endemic area south of Durban;[11] stools were cultured for *E. histolytica* and isolates subjected to iso-enzyme-electrophoresis to determine their zymodemes[11] (subjects were grouped into those who had no amoebae (30) and those who had pathogenic (11) and non-pathogenic (10) zymodemes).

Results

Initial experiments using checkerboard titrations were performed to optimise the concentrations of the reagents used in the assay. The optimal concentration of *E. histolytica* antigen required to coat microtitre plates was found to be 6,25 µg/ml. To minimise 'background noise' and to obtain a good discrimination between patients and controls, a test dilution of 1/6 400 was chosen for IgG and 1/400 for IgM. At a cut-off point of optical density (OD) 0,250 the IgG-ELISA had a sensitivity of 99% and a specificity of 91,7% (Fig. 1). At a cut-off point of OD 0,350 the IgM-ELISA had a sensitivity of 64% and a specificity of 97,9% (Fig. 2). As regards the IgM-ELISA, the greatest difference between patients and controls was observed at a dilution of 1/400 with a higher specificity at OD 0,350. At lower serum dilutions of IgM or at a lower OD cut-off, both sensitivity and specificity were markedly reduced with no observable difference between patients and controls. Thus a dilution of 1/400 was chosen. Comparative results of IgG-ELISA and AGD, IgM-ELISA and AGD, among the various groups are presented in Tables I and II respectively.

TABLE I. COMPARATIVE RESULTS OF IgG-ELISA AND AGD TEST

Study groups	Total No. of subjects	No. positive IgG ELISA	No. positive AGD
Amoebic liver abscess	**100**	**99**	**100**
Healthy hospital staff	**23**	**0**	**0**
Hospitalised patients with other diseases			
AGD-negative	**103**	**4**	**0**
AGD-positive	**8**	**8**	**8**
Collagen diseases (with one or more auto-antibodies positive)	**10**	**0**	**0**
Subjects from an endemic area			
No amoeba cultured	**30**	**6**	**0**
Symptomless carriers of *E. histolytica*			
Pathogenic	**11**	**10**	**11**
Non-pathogenic	**10**	**4**	**2**

TABLE II. COMPARATIVE RESULTS OF IgM-ELISA AND AGD TEST

Study groups	Total No. of subjects	No. positive IgM ELISA	No. positive AGD
Amoebic liver abscess	**100**	**64**	**100**
Healthy hospital staff	**23**	**0**	**0**
Hospitalised patients with other diseases			
AGD-negative	**103**	**3**	**0**
AGD-positive	**8**	**0**	**8**
Collagen diseases (with one or more auto-antibodies positive)	**10**	**0**	**0**
Subjects from an endemic area			
No amoeba cultured	**30**	**1**	**0**
Symptomless carriers of *E. histolytica*			
Pathogenic	**11**	**0**	**11**
Non-pathogenic	**10**	**1**	**2**

The AGD test was strongly seropositive (20 hours) for all 100 patients with ALA, as well as for the 11 symptomless carriers of pathogenic *E. histolytica* and weakly positive (40 hours) in 2/10 symptomless carriers of non-pathogenic *E. histolytica.*

The IgG-ELISA was positive in 99/100 ALA and 10/11 symptomless carriers of pathogenic zymodemes (Table I), while 64/100 and 0/11 patients with ALA and symptomless carriers of pathogenic *E. histolytica* respectively were positive by the IgM-ELISA (Table II). Two additional symptomless carriers of non-pathogenic zymodemes were detected by the IgG-ELISA (Table I) and 1/10 by the IgM-ELISA (Table II).

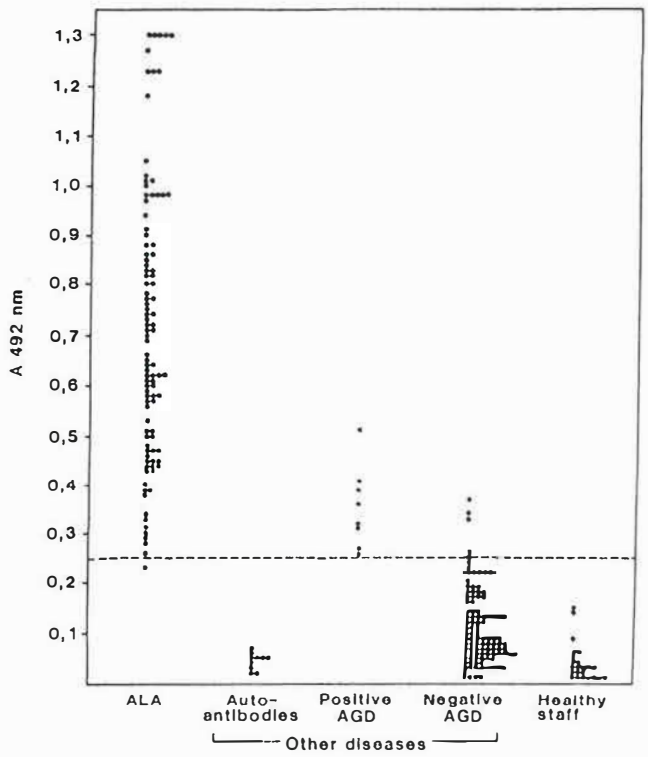

Fig. 1. Anti-amoebic IgG results obtained with ELISA.

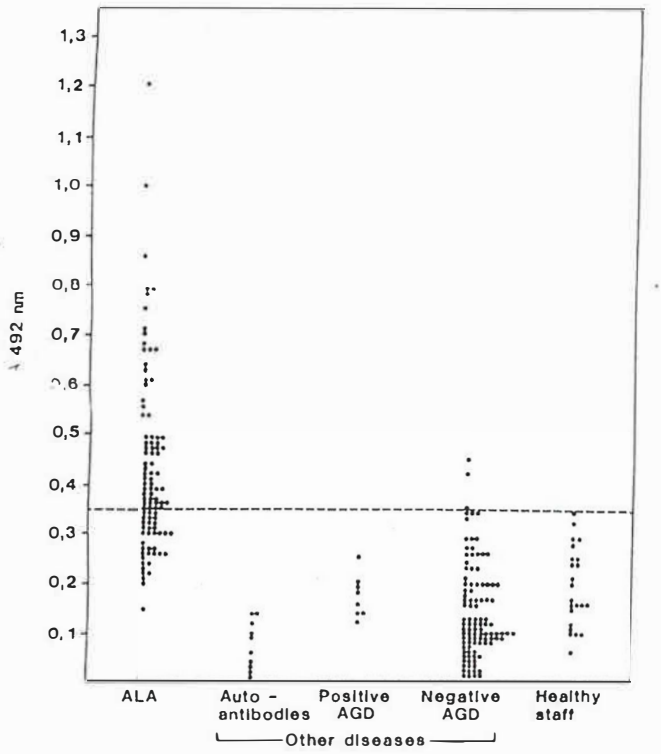

Fig. 2. Anti-amoebic IgM results obtained with ELISA.

The 30 subjects from an endemic environment from whom no amoebae were cultured were seronegative using the AGD test. However, 6/30 were positive by the IgG-ELISA (Table I) and 1/30 by the IgM-ELISA (Table II).

In the 121 patients without any clinical evidence of amoebiasis, 8 were positive by the AGD test, 12 by the IgG-ELISA and 3 by the IgM-ELISA. None of the 10 patients with collagen vascular disease were positive by any of the three tests.

Using Pearson's correlation co-efficient, we obtained an excellent correlation ($r = 0,99$) between the AGD test and the IgG-ELISA.

Discussion

The sensitivity of the IgG-ELISA for clinical ALA in this study (99%) is comparable (97 - 100%) to those reported by others.[13-15,20-22] The IgM-ELISA appears to be specific for clinical ALA (97,9%) but lacks sensitivity (64%). Researchers generally agree that the detectable anti-amoebic IgM titres are below those of IgG[23-25] and this seems to indicate that the assay is unlikely to be of much clinical value until its sensitivity is improved.

What then is the place of the IgG-ELISA in the diagnosis of ALA and in particular, how does it compare with the AGD test? In the past 20 years, the AGD test has proved a very reliable and inexpensive test for the diagnosis of ALA. The test is invariably negative in those patients who do not have ALA. Therefore, a negative result with the AGD rules out invasive amoebiasis and thus another diagnosis needs to be considered. Positive serology indicates that the abscess may be due to *E. histolytica*, but in our endemic area 10 - 15% of individuals with no clinical evidence of invasive amoebiasis are seropositive using the AGD test.[8,9,26] This is thought to be due to past infection with *E. histolytica* since amoebic antibodies are known to persist for long periods after successful treatment.[7] We observed a seropositivity of 8,2% with the IgG-ELISA, a figure similar to that observed by others.[8,9,26] This was not thought to be due to nonspecific reactions since the IgG-ELISA was negative in patients with auto-antibodies. Serological tests are positive in invasive amoebiasis both in amoebic dysentery[8] and ALA.[9] It is necessary to comment on a positive test in asymptomatic carriers of pathogenic zymodemes. We feel that the work of Jackson *et al.*[11] has shown that the positivity of the test is not a nonspecific response, but appears to indicate subclinical invasion. Thus these patients are included under invasive amoebiasis.

A higher seropositivity was observed with the IgG-ELISA when compared with the AGD test in hospitalised patients with other diseases, in subjects from an endemic area whose stools were negative for *E. histolytica*, and in symptomless carriers of non-pathogenic zymodemes. This could be due to the higher sensitivity of the ELISA when compared with other immunodiagnostic tests for ALA as has been suggested by the work of others,[13-15,20-22] or it could reflect a nonspecific background noise.[27] If indeed it is the latter, it makes the IgG-ELISA a less suitable test than the AGD. However, the immunogenicity and specificity of the various fractions of the amoebic antigen are still matters of speculation.[28-32] The different amoebic strains used and the methods of preparing the antigen could influence the sensitivity of the tests. Whether the antibodies detected by ELISA are similar or different from that detected by the AGD test has not been determined.[14,15]

Elsdon-Dew *et al.*[33] emphasised 'a need for a simple, quick test' and stated that 'the AGD test suffers from one clinical disadvantage . . . one cannot report an unequivocal negative reaction in less than 48 hours'.

The latex agglutination test (Scrameba) was developed to aid the rapid diagnosis of ALA,[10] giving an answer within 30 minutes, but the disadvantage was its high cost.[34,35] The IgG-ELISA is rapid and simple. It is only as regards the speed

with which the result is provided that this assay would appear to have an advantage over the AGD test. IgG-ELISA can be provided in 2½ hours compared with a minimum of 24 hours by the AGD test. The prompt availability of the IgG-ELISA results could allow a clinician to start specific investigations and treatment earlier.

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CHAPTER 5

SPECIFIC IgG- AND IgM-ANTIBODY RESPONSE TO *ENTAMOEBA HISTOLYTICA* (HK9) SOLUBLE AND MEMBRANE FRACTIONS

Generally, the tendency has been to use soluble complex antigens derived from the whole amoebae when evaluating the ELISA. For invasiveness to occur direct contact of *E. histolytica* trophozoites with the host's cells is necessary (Ravdin, 1989); and surface or plasma membrane antigens would be the first to interact with the hosts immune system. Therefore, it would seem logical to use these antigens in sero-diagnostic tests, especially in the ELISA.

The difference in sensitivities between the IgM-ELISA (Sathar *et al*, 1988) and the IgM-IFAT (Jackson *et al*, 1984) may be due to differences in the physical state of the antigen used. In the former assay IgM antibodies were detected to soluble complex antigens compared to insoluble, whole parasite surface antigens in the latter assay. Whilst the interpretation of immunoflourescence, especially that of IgM-IFAT is subjective, the IgM-ELISA using soluble complex antigens of *E. histolytica* is not sensitive enough (64%) to determine current infection (Sathar *et al*, 1988). Using the ELISA to detect specific *E. histolytica* antibodies in studies with a

limited number of ALA patients (2-4), serologically active antigens have been shown to be associated with the plasma membrane (Mathews et al, 1986 and Schulz et al, 1987). These limited studies show that surface or plasma membrane antigens of E. histolytica may have a role to play in the ELISA.

This study was performed in order to determine whether the use of membrane or other subcellular fractions of E. histolytica as antigens, would improve the diagnosis potential of the IgM-ELISA.

5.1. MATERIALS AND METHODS

Forty-eight (48) hour cultures of E. histolytica strain HK9 grown axenically were obtained from the RIDTE, Durban. Separation of the amoeba into its subcellular fractions was performed by the method described by Aley et al (1980). Each fraction was solubilised by the method described by Mathews et al (1986).

The protein content of the various fractions were determined by the Lowry method (1951) using human serum albumin (HSA) (Sigma Chemicals Co., St. Louis, MO) as the standard. The antigens were stored frozen at -20°C in 100ul aliquots. These aliquots were used once only and were not repeatedly thawed or frozen.

5.1.2. Serology

AGDT was performed according to the method described by Jackson et al (1983). The ELISA (Sathar et al, 1988) was used to determine the specific IgG and IgM response of each membrane and soluble fraction of E. histolytica.

To reduce the NSB to acceptable levels when plasma membranes and internal membranes were used as the antigen in the IgM-ELISA; the test sera were diluted in 3% (w/v) gelatin (Biorad Laboratories, California) Tris saline (TS). To further reduce any possible non-specific binding of the conjugate, F(ab')2 fragment of the enzyme conjugate was used (goat-anti-human IgM u-chain specific, F(ab')2 fragment, Sigma Chemicals, St. Louis, MO, USA).

5.1.3. Study Population

Positive control sera were obtained from two patients with amoebic liver abscess (ALA) who were clinically diagnosed by ultrasonography, aspiration of sterile pus, positive AGDT (20 hr) and a favourable response to metronidazole. A negative control serum was obtained from a volunteer of the University staff who had no clinical evidence of amoebiasis and whose AGDT and

stool microscopy for E. histolytica were negative. The sera were tested at a single serum dilution of 1/100.

5.2. RESULTS

The protein concentrations in the various fractions were as follows: soluble fraction, 210ug/ml; plasma membrane, 164ug/ml; internal membranes, 160ug/ml and non-vesiculated membranes and debris, 120ug/ml. The protein content of the standard antigen was 9,7mg/ml. The data obtained with the IgM-ELISA using soluble, plasma membrane, internal membrane and vesiculated membrane fractions as the antigens are reported here.

The IgM titration curves of the negative and the two ALA sera obtained with the various antigens for the IgM-ELISA are shown in Figures 5.1 - 5.4 (page 80) and Fig. 5.5 (page 81). As can be seen from these figures, serum A was more reactive than serum B with each antigen preparation. Another common feature in all of the antigen fractions is the rather high non-specific binding (NSB). This is lowest in the standard antigen (Fig. 5.1), nevertheless, it is considerably higher than that reported elsewhere (Fig. 3.1, page 47), and highest in the case of non-vesiculated membrane and debris (Fig. 5.5, page 81). In the case of the soluble

fraction (Fig.5.2), the plasma membrane (Fig. 5.3) and the internal membrane (Fig. 5.4), the high NSB did not prevent a clear distinction between the positive sera and the negative control. However, particularly favourable titration curves were observed with the plasma membrane antigen (Fig. 5.3) and the internal membrane antigen (Fig. 5.4). Due to the higher NSB observed with non-vesiculated membranes and debris in the IgM-ELISA (Fig 5.5, page 81) this fraction was not further investigated.

Following the use of 3% gelatin and the F (ab')2 fragments of the conjugate the NSB was reduced to acceptable levels in the IgM-ELISA using plasma membranes or internal membranes of E. histolytica as the coating antigens (Figs 5.6 and 5.7, page 81, respectively). The optimum concentration of the plasma membrane antigen was deemed to be that which gave minimal signal to noise ratio. To conserve antigen, a protein concentration of 5ug/ml of plasma membrane antigen was considered optimal for the IgM-ELISA (Fig. 5.6).

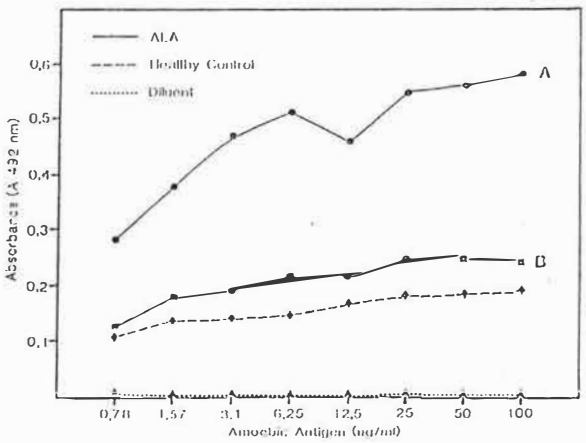

FIGURE 5.1 Immunoreactivity of the standard antigen with the IgM-ELISA

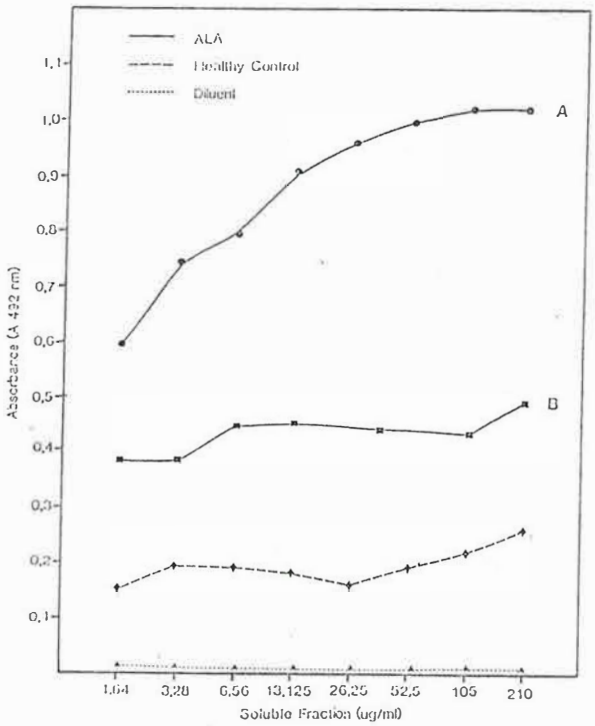

FIGURE 5.2 Immunoreactivity of the soluble fraction of *E. histolytica* with the IgM-ELISA

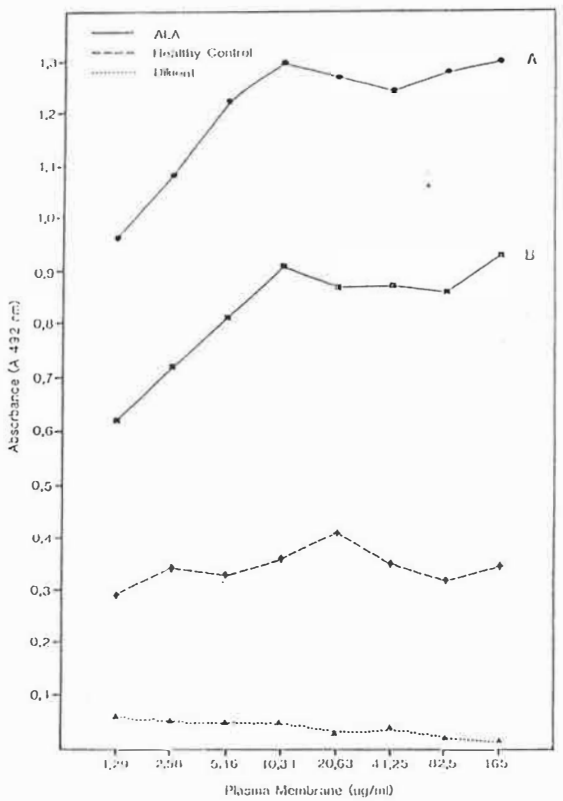

FIGURE 5.3 Immunoreactivity of the plasma membrane fraction of *E. histolytica* with the IgM-ELISA

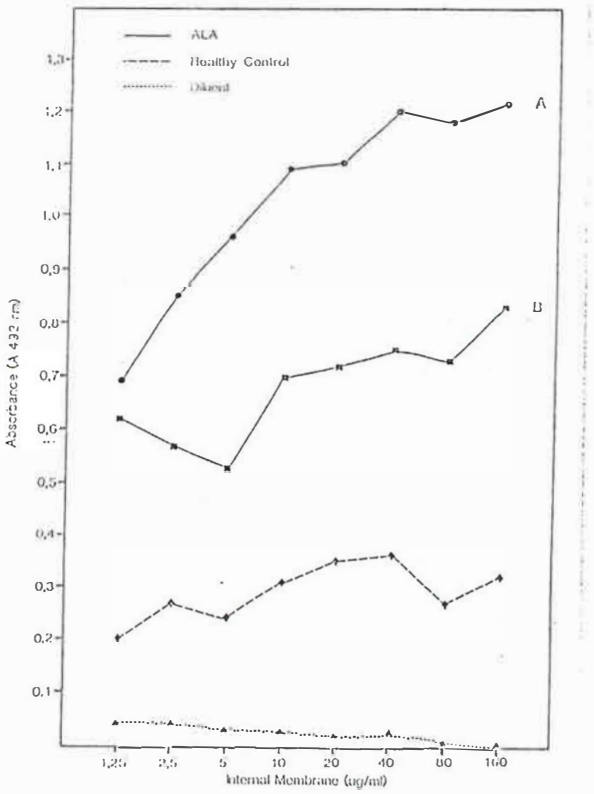

FIGURE 5.4 Immunoreactivity of the internal membrane fraction of *E. histolytica* with the IgM-ELISA

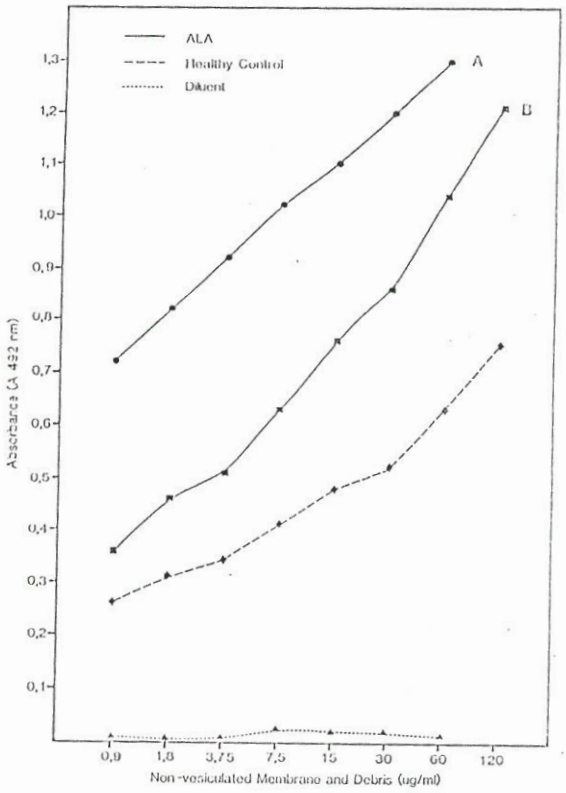

FIGURE 5.5 Immunoreactivity of the non-vesiculated membrane and debris fractions of *E. histolytica* with the IgM-ELISA

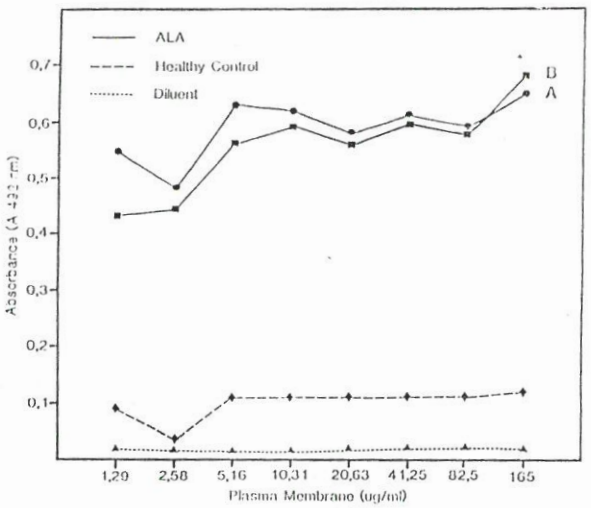

FIGURE 5.6 Immunoreactivity of the plasma membrane fraction of *E. histolytica* in the IgM-ELISA using 3% gelatin-TS as the serum diluent.

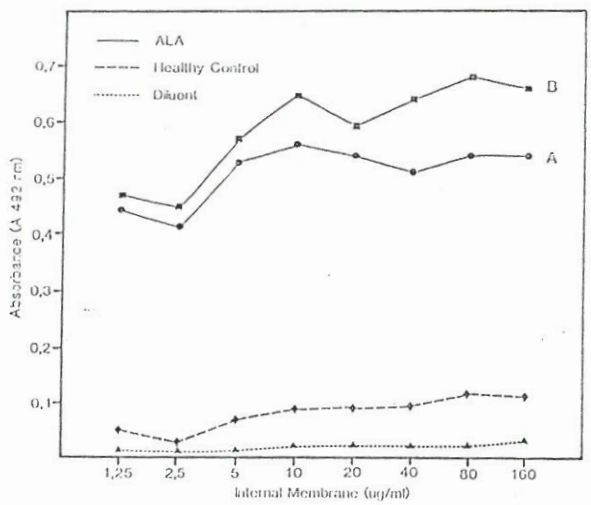

FIGURE 5.7 Immunoreactivity of the internal membrane fraction of *E. histolytica* in the IgM-ELISA using 3% gelatin-TS as the serum diluent

5.3. DISCUSSION

There was no added advantage in using the plasma membrane or soluble fractions of E. histolytica in the IgG-ELISA. On the contrary, using the soluble complex antigen derived from ruptured E. histolytica (Chapter 4) gave excellent sensitivity (99%) and specificity (92%). Therefore, the use of this antigen to detect IgG-anti-E. histolytica antibodies with the ELISA is adequate.

In preparing the standard soluble antigen, the insoluble material is discarded (Jackson et al, 1983). This insoluble material contains the membrane fractions of E. histolytica. The soluble antigen preparation used in the ELISA previously, (Chapter 4) may not have included sufficient antigens to detect specific IgM-anti-E. histolytica antibodies. The improved titration curves observed with the the IgM-ELISA when the plasma membrane fraction was employed as the antigen, may be due to more IgM-epitopes being available to react with IgM antibodies. Sufficient quantities of the antigen may have been bound to the solid phase, therefore, reacting more strongly.

Previously, the exclusion of bovine serum albumin (BSA) (Fig. 3.10 page 50) in the IgM-ELISA improved the specificity of the assay. In this experiment the addition of 3% gelatin as a blocking agent (Vogt et al, 1987) in the serum diluent (TS) reduced NSB of the negative control serum. In addition, the use of the F(ab')2 fragments of the enzyme conjugate would appear to have minimised NSB (Fig 5.3 vs Fig.5.6, and Fig 5.4 vs Fig 5.7).

The reactivity of the sera to the internal and plasma membrane fractions (Figs 5.3 and 5.4, and Figs 5.6 and 5.7) were similar. This is in keeping with current thinking that E. histolytica is constantly turning over its plasma membrane, interchanging its outer membrane with fresh internal membrane (Aley et al, 1980; Aust-Kettis, 1983 and Mathews et al, 1986). Recently, Ostoa-Saloma et al (1989) provided further support for the rapid membrane turnover theory. These researchers used the method of Aley et al (1980) to fractionate E. histolytica into different subcellular fractions to determine the subcellular locations of proteolytic enzymes. Their results suggest that the proteinase composition of the internal vesicles and internal mem-

branes are similar to that of the plasma membrane (Ostoa Saloma et al, 1989).

Contact by Entamoeba histolytica with the host's cells is a prerequisite for invasiveness (Ravdin, 1989). Therefore, a reasonable inference is that surface or membrane antigens have an important role to play in the ontogeny of an immune response to the invading organisms. Consequently, a great deal of attention has been focussed on the antigens of the surface membrane of E. histolytica.

At the time of writing this dissertation no single antigen has been identified as having greater diagnostic potential than any other. Indeed it is probable that most, if not all the antigens identified as being immunogenic on the surface of E. histolytica (Meza et al, 1987; Joyce and Ravdin, 1988; Petri et al, 1989; Torian et al, 1989 and Reed et al, 1989) are present in the plasma membrane fraction used in the study described in this Chapter. In addition, the fact that good yields of the plasma membrane fraction can be produced by a relatively simple, differential centrifugation (Aley et al, 1980), as opposed to the elab-

orate purification schemes described by others (Meza et al, 1987; Torian et al, 1987 and Petri et al, 1987), leads one to conclude that the plasma membrane fraction is entirely adequate for use in a practical ELISA for detecting specific IgM-antibodies to invading E. histolytica. In addition, the sensitivity of the serodiagnostic test may be enhanced by monitoring the immune response to a number of antigens (plasma membrane) rather than to any one antigen.

Although future research may identify a unique and exceptionally potent antigen, it is questionable whether it would find application in serodiagnosis in the field, in view of the likely high cost. In Third World Laboratories where funds are limited and First World technology is absent, it may be more economical to use the plasma membrane fraction in the ELISA. Evaluation of the ELISA to detect specific IgG- and IgM-anti-E. histolytica antibodies using a plasma membrane fraction has been limited (Mathews et al, 1986 and Schulz et al, 1987). These researchers investigated 2 and 4 ALA patients, respectively. The results of these limited studies suggest that the assay needs to be evaluated in a more comprehensive study using

a larger series of ALA patients and appropriate control groups.

CHAPTER 6

DETECTION OF *Entamoeba histolytica* IMMUNOGLOBULINS G AND M TO PLASMA MEMBRANE ANTIGEN BY ENZYME-LINKED IMMUNOSORBENT ASSAY

In areas where inavsive amoebiasis is not endemic (Stamm *et al*, 1976), the detection of specific IgG-anti-*E. histolytica* antibodies would be considered to be diagnostic of current amoebic infection. On the other hand, in areas endemic for invasive amoebiasis, detecting specific IgM-anti-*E. histolytica* antibodies would be more useful in determining whether or not there is active/current amoebic infection. Most ELISAs used in diagnosing invasive amoebiasis have measured either specific IgG or total immunoglobulins. These antibodies have been detected to soluble, complex antigens of *E. histolytica.* The ability of the ELISA to detect specific IgM-anti-*E. histolytica* antibodies in endemic areas of amoebiasis is not sensitive (64%) enough to differentiate past from current invasive amoebiasis (Sathar *et al*, 1988). Another approach to improve the diagnostic potential of serological tests would be to use specific antigens of *E. histolytica.* The antigen preparations used to date have been highly complex and of a poorly defined composition (Yang and Kennedy, 1979; Agarwal *et al*, 1981; Bos *et al*, 1980;

Mohapatra and Sen, 1983; Gandhi et al9, 1987 and Sathar et al, 1988). Mathews et al (1986) using the ELISA to detect IgG-anti-E. histolytica antibodies, observed that the immune sera of 2 ALA patients recognized specific antigens associated with the plasma membrane of E. histolytica. Schulz et al (1987) using antigens derived from monoaxenic cultures of E. histolytica showed that the IgM antibodies of one ALA patient reacted with a 10-15 KDa protein component of the plasma membrane. These limited studies show that specific antigen preparations of E. histolytica may play a significant role in the detection of specific antibodies in the serodiagnosis of invasive amoebiasis.

In this study, the plasma membrane of E. histolytica was used as the antigen in the enzyme-linked immunosorbent assay (ELISA) to detect specific IgG and IgM antibodies in serum samples obtained from 61 subjects.

6.1. MATERIALS AND METHODS

6.1.1. Antigen

The plasma membrane of axenically cultured E. histolytica strain HK9 was isolated by the methods of Aley et al (1980).

6.1.2. Serology

The performance of the ELISA was similar to that of Sathar et al (1988), except for two modifications, viz:

i) The test sera were diluted 1/100 in 3%-Gelatin (Bio-Rod Laboratories, Richmond, California) - Tris Saline (TS).

ii) F(ab')2 fragments (Sigma Chemical Co., St. Louis, MO, USA) of horseradish peroxidase conjugated goat anti-human IgG (gamma-chain specific) and IgM (mu-chain specific) were used as the developing antibodies.

The AGDT was performed as described by Jackson et al (1983).

6.1.3. Study Population

Serum samples from 61 subjects were collected and stored in aliquots of 200ul at -20°C. All sera tested were thawed once only to detect specific IgM and IgG antibodies using the ELISA. All sera were coded and tested. The study population included the following subgroups:

i) Twenty-two (22) patients with ALA. The diagnosis was made on the basis of clinical features,

ultrasonography, a positive AGDT, a favourable response to metronidazole and aspiration of sterile pus from 10 patients.

ii) Twelve (12) healthy volunteers employed at the University of Natal or King Edward VIII Hospital who had no clinical evidence of amoebiasis. Their sera were negative for anti-E. histolytica antibodies by the AGDT and their stools were negative for E. histolytica on microscopy.

iii) Ten (10) hospitalised patients from the medical wards of King Edward VIII Hospital with various diseases other than amoebiasis. Amoebiasis was excluded by the absence of clinical symptoms and a negative AGDT.

iv) Seventeen (17) healthy subjects from an area endemic for amoebiasis; the AGDT response of these subjects had been determined previously (Jackson et al, 1985). Single stool samples from all subjects were cultured in Robinson's culture medium (Robinson, 1968) and in those cases where E. histolytica was isolated the zymodeme, pathogenic or non-pathogenic, was determined by previously described methods (Gathiram and Jackson, 1987). In six (6) of 17 subjects, E.

istolytica could not be cultured from the stool samples; three (3) were AGDT positive and three (3) AGDT negative. There were six (6) asymptomatic carriers of non-pathogenic E. histolytica, two (2) of whom were AGDT positive and four (4) AGDT negative; the remaining five (5) were asymptomatic carriers of pathogenic E. histolytica, all of whom were AGDT positive.

6.1.4. Statistical Analysis

Pearson's correlation co-efficient was used to determine the correlation between the AGDT and the ELISA. The Student t-test was used to determine the difference in OD values between the different study groups.

6.2. RESULTS

The criteria established previously (Chapter 3), were used to determine the absorbance cut-off between seropositive and seronegative results, i.e. mean + 2SD (standard deviation) and mean + 3SD of normal healthy controls for IgG and IgM, respectively. A single serum dilution of 1/100 was used for all samples tested. An absorbance value of 0,250 for IgG and 0,200 for IgM was used as the cut-off to separate seropositive from seronegative samples.

Distribution of individual results obtained with the IgG- and IgM-ELISA for the various groups are represented in Figs. 6,1, 6.2 and 6.3, page 95 respectively. All healthy controls were seronegative by the IgG- and IgM-ELISA as well as the AGDT. Nineteen (19) of the 20 patients (95%) with ALA were seropositive by the IgG-ELISA (Fig. 6.1) and in 18 (90%) the IgM-ELISA gave positive results (Fig. 6.2). Two (2) patients with ALA had a negative AGDT at the time of admission to the hospital; both of them proved to be seropositive by the IgG-ELISA (Fig 6.1) , and in one of them, the IgM-ELISA was also positive (Fig 6.2). There was a significant difference ($P < 0,001$) in the OD values between ALA patients and normal healthy controls with the IgG- and IgM-ELISA. No significant difference was observed between the latter group and hospitalised patients with other diseases.

The distribution of absorbance values obtained with IgG- and IgM-ELISA on sera obtained from asymptomatic subjects from an area endemic for amoebiasis are represented in Fig. 6.3. All culture-negative subjects were found to be seropositive by IgG-ELISA; notably, the highest absorbance (A.492nm) values were observed

from the three (3) cases who were also positive by the AGDT. The IgM-ELISA was positive in only one of the culture-negative controls. All 5 carriers of pathogenic zymodemes were seropositive by the IgG-ELISA; however, none of these subjects was seropositive by IgM-ELISA. Only one of six (6) sera from carriers of non-pathogenic zymodemes proved to be positive by the IgM-ELISA; of the three (3) subjects who were IgG-ELISA positive, two (2) also had a positive AGDT. Of the 10 hospitalized patients without clinical evidence of amoebiasis, two were positive by the IgG-ELISA (Fig. 6.1) and the one by IgM-ELISA (Fig. 6.2). No significant difference in the OD values were observed between normal healthy controls and subjects from an endemic area with the IgM-ELISA. However, there was a significant difference ($P < 0,02$) in the OD values obtained with the IgG-ELISA between normal healthy controls and subjects in whom E. histolytica could not be cultured from their stool. There were also significant differences between normal healthy controls and asymptomatic individuals with pathogenic ($P < 0,001$) and non-pathogenic ($P < 0,05$) zymodemes. There was an excellent correlation between the IgG-ELISA and AGDT ($r=0,96$). The IgG- ELISA had a sensitivity of 95% and

a specificity of 91%; the IgM-ELISA had a sensitivity of 91% and a specificity of 95%, for the diagnosis of ALA.

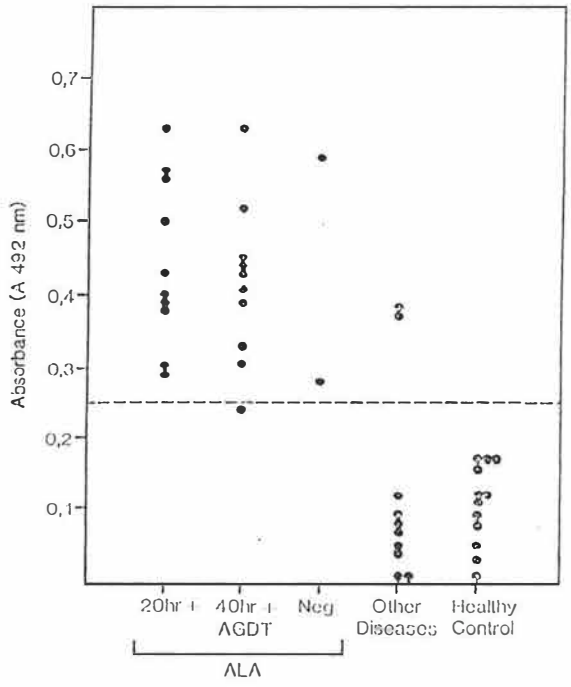

FIGURE 6.1 IgG-ELISA results of ALA patients and controls. ALA patients were grouped by AGDT results into 20 hour (strong) positive; 40 hour (weak) positive and negative

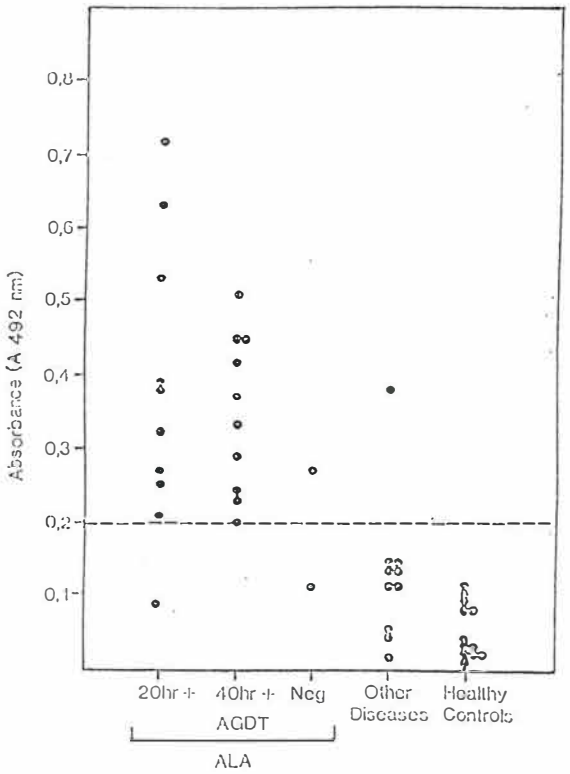

FIGURE 6.2 IgM-ELISA results of ALA patients and controls. ALA patients were grouped by AGDT results into 20 hour (strong) positive; 40 hour (weak) positive and negative

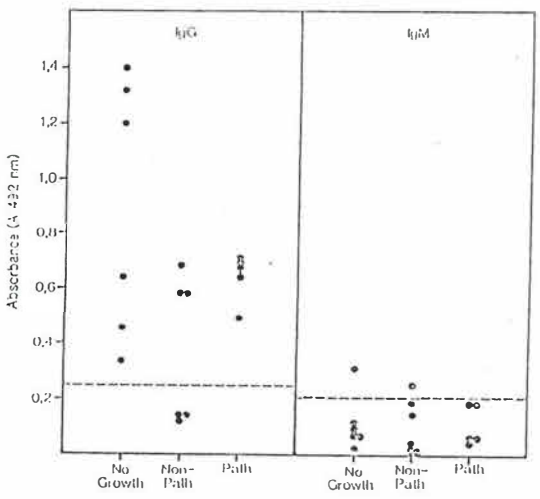

FIGURE 6.3 IgG- and IgM-ELISA results of healthy subjects from an area endemic for amoebiasis

6.3. DISCUSSION

Previously reported studies using the ELISA to diagnose invasive amoebiasis employed poorly defined soluble antigens of E. histolytica (Yang and Kennedy 1979 and Bos et al, 1980). This is the first study in which the solubilised plasma membrane of E. histolytica was used in an ELISA to detect specific IgG- and IgM-antibodies in a large group of well-documented cases of clinically diagnosed ALA patients and appropriate control groups, including asymptomatic carriers of pathogenic and non-pathogenic amoebae.

Use of the plasma membrane antigen of E. histolytica did not improve the sensitivity of the IgG-ELISA (95%) compared to that reported with soluble, complex antigen (99%) (Chapter 5). Further purification of the whole, soluble extract of E. histolytica would be of no further advantage in improving the sensitivity of the IgG-ELISA. By contrast, use of the plasma membrane antigen in the ELISA to detect specific IgM-anti-E. histolytica antibodies effectively improved the sensitivity of the IgM-ELISA from 64% (Chapter 4), to 91%. In comparison to a sensitivity of 68% reported by Torian et al (1989) and 80% reported by Reed et al

(1989), the higher sensitivity (95%) of the IgG-ELISA in this study in diagnosing ALA may be due to the presence of some, if not all of the specific, multiple defined E. histolytica surface antigens (37 KDa-220 KDa) which have been isolated by other researchers (Meza et al, 1987; Petri et al, 1987 and 1989; Torian et al, 1989; Joyce and Ravdin, 1988 and Reed et al, 1989) rather than to the absence of any one antigen.

The success of the IgM-IFAT (Jackson et al, 1984) in distinguishing past from current amoebic infection was due to the detection of specific IgM-antibodies to insoluble surface antigens of E. histolytica. The improved sensitivity (91%) of the IgM-ELISA in the present study is due to the detection of specific IgM antibodies directed to epitopes on the surface membrane.

Occasionally, the AGDT is either weakly positive (40 hr) or negative for ALA patients on admission; this is thought to be due to inadequate antibody production (Jackson et al, 1985), since the AGDT invariably becomes positive when repeated a few days after hospital admission (Powell et al, 1965; Krupp and Powell, 1971a and Gathiram et al, 1989). Interest-

ingly, the IgG-ELISA of the two ALA patients whose AGDTs were negative on admission, were positive. The IgM-ELISA was positive in one ALA patient and negative in the other. When the AGDT was repeated five days later, it was seropositive in both these patients. The IgG and IgM-ELISA in both these patients were positive on the repeat blood samples (data not shown). With the ELISA a current diagnosis would have been confirmed. This clinical advantage of the ELISA could be useful in the early diagnosis and treatment of ALA.

The higher seropositivity observed with the IgG-ELISA compared to the AGDT in hospitalised patients with other diseases is due to the higher sensitivity of the ELISA compared to other serodiagnostic tests (Yang and Kennedy, 1979; Bos et al, 1980; Agarwal et al, 1981 and Knobloch and Mannweiler, 1983), and it reflects a past infection with E. histolytica, as amoebic antibodies are known to persist for several years after treatment (Krupp and Powell, 1971b and Joyce and Ravdin, 1988).

The serological responses of asymptomatic subjects are interesting. The asymptomatic carriers of pathogenic zymodemes all produce IgG antibodies to the surface membrane of E. histolytica, suggesting that these indi-

viduals have at some stage experienced subclinical tissue invasion. The seropositivity (AGDT and IgG-ELISA) of asymptomatic carriers of pathogenic zymodemes (100%) is not significantly different to that of patients with clinically active disease (ALA) (91%-95%). In the former group of individuals, the absence of an IgM response would indicate inactive disease, compared to a positive IgM response in patients with active disease (ALA). The specificity of the IgM-ELISA for excluding active disease is indicated by the negative serological responses obtained from the sera of healthy carriers of non-pathogenic zymodemes and culture negative controls from the endemic area. The positive results obtained by the IgG-ELISA in culture negative controls and carriers of non-pathogenic zymodemes possibly indicate past infection with pathogenic zymodemes, confirming that the ELISA may be a more sensitive test than the AGDT for the diagnosis of past infection.

Recently it was shown that more than one-third of ALA patients continue to pass cysts of pathogenic E. histolytica following successful treatment; there was recurrence of symptoms in some with high antibody tit-

res (AGDT) (Irusen et al, 1989). It would be interesting, therefore, to monitor the IgG and IgM antibody response of asymptomatic cyst passers of pathogenic zymodemes, using the ELISA, during an extended follow-up study. The ELISA could prove useful, since the seropositivity (IgG-ELISA and AGDT) of asymptomatic carriers of pathogenic zymodemes is comparable to ALA, whilst the IgM-ELISA in the former, is negative. Thus while the IgG-ELISA like the AGDT would be of importance in ascertaining the prevalence of pathogenic zymodemes in the community, inclusive of past and present infections, (the interpretation of which must be made with caution), the sensitivity (91%) and specificity (95%) of the IgM-ELISA would make it of value for diagnosing current tissue invasion by E. histolytica.

These findings have been published by the Journal of Clinical Microbiology 1990; 28; 332-335, a copy of which has been included in the dissertation.

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Detection of *Entamoeba histolytica* Immunoglobulins G and M to Plasma Membrane Antigen by Enzyme-Linked Immunosorbent Assay

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Sixty-one serum specimens from 22 patients with clinically diagnosed amoebic liver abscess (ALA), 10 hospitalized patients with a variety of diseases other than amoebiasis, 12 normal healthy controls, and 17 subjects from an amoebiasis-endemic area were assayed by enzyme-linked immunosorbent assay (ELISA). The plasma membrane fraction of axenic cultures of *Entamoeba histolytica* HK9 separated from other subcellular fractions by differential centrifugation was used as the antigen to detect specific immunoglobulin G (IgG) and IgM antibodies. Using a single serum dilution of 1/100 and optical densities at 492 nm of 0.200 and 0.250 as the cutoff values for the IgM and IgG ELISAs, their respective sensitivities in 22 ALA patients were 91% (20 of 22) and 95% (21 of 22). In 22 patients (10 hospitalized and 12 normal healthy controls), the specificities of the IgM and IgG ELISAs were 95% (21 of 22) and 91% (20 of 22), respectively. All five asymptomatic carriers of pathogenic *E. histolytica* were seropositive by the IgG ELISA and the amoebic gel diffusion test (AGDT). The AGDT was positive for three of six culture-negative controls, while the IgG ELISA was positive for all six. For six asymptomatic carriers of nonpathogenic zymodemes, the AGDT was positive for two, and the IgG ELISA was positive for three. There was an excellent correlation ($r = 0.96$) between the IgG ELISA and the AGDT. Only one of six culture-negative controls, none of the asymptomatic carriers of pathogenic *E. histolytica*, and one of six carriers of nonpathogenic *E. histolytica* were seropositive by the IgM ELISA, thus highlighting the specificity of the IgM ELISA in the diagnosis of ALA. It is believed that the use of plasma membrane fractions has improved the diagnostic potential of the IgM ELISA.

The measurement of specific immunoglobulin M (IgM) antibodies is valuable in distinguishing current and past infections in giardiasis (7) and toxoplasmosis (18). Antibodies to *Entamoeba histolytica* have been shown to persist for at least 3 years after cure of amoebic liver abscess (ALA) (11). At present, there is no serological test that can reliably distinguish active from past tissue invasion by this parasite. The amoebic gel diffusion test (AGDT) is a sensitive test for ALA and is positive in 96% of cases (20). However, a positive AGDT is given by 15 to 20% of the population of the amoebiasis-endemic areas around Durban who have no evidence of active amoebiasis and is thus of limited value in distinguishing current infection (10). A previous study of the specific IgM response using the indirect immunofluorescent-antibody test showed it to be of some value in diagnosing current infection, since the IgM antibody response lasted approximately 6 months (8). We have previously reported that the measurement of specific IgM antibodies by an enzyme-linked immunosorbent assay (ELISA) technique that employed soluble antigens of *E. histolytica* was specific (97.9%) but lacked sensitivity (64%) (24). Monitoring specific circulating antigen of *E. histolytica* is of particular importance; it would allow a more accurate assessment of the infection process than does the detection of antibodies, but this requires further evaluation (5, 17). Direct contact of *E. histolytica* trophozoites and host cells is necessary for invasiveness (21). Thus, it seems logical that surface membrane or plasma membrane antigens would be the first to interact with the host immune system during infection and thereby might constitute the major component in the serological response. By using an ELISA to detect specific IgG-*E. histolytica* antibodies, serologically active antigens have been shown to be associated with the plasma membrane (15). In the present study, the plasma membrane fraction of axenically cultured *E. histolytica* was used as the antigen in an attempt to improve the diagnostic potential of the IgM ELISA.

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MATERIALS AND METHODS

Antigen preparation. The contents of several simultaneously grown tubes of 48-h axenically cultured *E. histolytica* HK9 were harvested and pooled. The packed cells from the final wash were counted and appropriately diluted to give 2.5×10^7 cells per ml of buffer (1).

The plasma membrane fraction of *E. histolytica* HK9 was obtained by differential centrifugation (1). Briefly, washed trophozoites were incubated with concanavalin A. This stabilized the plasma membranes as large sheets, facilitating separation by differential centrifugation. Dissociation of concanavalin A with α-methyl-D-mannopyranoside followed by additional homogenization vesiculated the plasma membranes. This preparation was solubilized (15), and its protein concentration was determined (14).

Study population. Serum samples from 61 subjects collected on hospital admission over a period of about 2 years were stored in aliquots of 200 μl at −20°C. All sera tested were thawed once only to detect specific IgM and IgG antibodies by using the ELISA method (24); all sera were tested in a blind coded manner. The study population included the following subgroups. (i) Twenty-two patients

with ALA. The diagnosis was made on the basis of clinical features, ultrasonography, a positive AGDT, a favorable response to metronidazole (24), and aspiration of sterile pus from 10 patients. (ii) Twelve healthy volunteers employed at the University of Natal or King Edward VIII Hospital who had no clinical evidence of amoebiasis and whose AGDTs and stool microscopies were negative for *E. histolytica*. (iii) Ten hospitalized patients with various diseases other than amoebiasis. Amoebiasis was excluded by the absence of clinical symptoms and a negative AGDT. (iv) Seventeen healthy subjects from an amoebiasis-endemic area; the AGDT responses of these subjects had been determined previously (10). Single stool samples from all subjects were cultured in Robinson culture medium (22), and in those cases in which *E. histolytica* was isolated, the zymodeme (pathogenic or nonpathogenic) was determined by a previously described method (6). For six subjects *E. histolytica* could not be cultured from the stool samples; three were AGDT positive, and three were AGDT negative. There were six asymptomatic carriers of nonpathogenic *E. histolytica*, two of whom were AGDT positive and four of whom were AGDT negative; the remaining five were asymptomatic carriers of pathogenic *E. histolytica*, and all five were AGDT positive.

IgG and IgM ELISAs. The ELISA was a modification of that used previously (24). Wells of F-form polystyrene microtiter plates (Dynatech M129; T & C Scientific Supplies, Durban, South Africa) were coated with 100 μl of 0.05 M carbonate–bicarbonate (Na_2CO_3-$NaHCO_3$) buffer, pH 9.6, which contained a protein concentration of 5 μg of plasma membrane antigen per ml. Antigen-coated plates were used immediately. Plates were incubated for 2 h at room temperature in a humid container. They were washed with 300 ml of 0.05 mM Tris–0.1 M NaCl buffer (TS), pH 8.0, containing 0.05% (wt/vol) Tween 20 (TST), followed by 200 ml of glass-distilled water. At each wash, microtiter plates were flooded with an excess of wash fluid in a specially constructed ELISA shower (Natal Blood Transfusion Services, Durban, South Africa) (4). TST was shaken off, and the plates were dried by padding them onto absorbent material. Test sera (50 μl) diluted 1/100 in 3% gelatin (Bio-Rad Laboratories, Richmond, Calif.)–TS were added to duplicate wells. Plates were incubated for 1 h at 45°C in a humid container and washed with 500 ml of TST. Fifty microliters of a 1/1,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (γ-chain specific) or IgM (μ-chain specific) $F(ab')_2$ fragments (Sigma Chemical Co., St. Louis, Mo.) in TST was added, and plates were incubated at 45°C for 1 h. After being washed with 500 ml of TST, the plates were dried, and 50 μl of chromogenic substrate (3) (18.4 mM sodium borate, 30.5 mM succinic acid, 3.77 mM *O*-phenylenediamine, 4.0 mM hydrogen peroxide, pH 5.0) was added to each well. The plates were then incubated for 5 min in the dark at room temperature. The reaction was stopped by the addition of 100 μl of 1.5 N HCl. The optical density (OD) was read at 492 nm by using a Titertek Multiskan Plate Reader (Flow Laboratories, Inc., McLean, Va.). Selection of the optimal test dilution (1/100) was based on titration curves derived from data based on serial twofold dilutions of positive (ALA) and negative (normal healthy) control sera. The cutoff OD at 492 nm to separate seropositive from seronegative samples was based on the mean plus or minus two standard deviations (0.250) for IgG and on the mean plus or minus three standard deviations (0.200) for IgM of normal healthy controls. A single serum dilution of 1/100 was used for all samples tested.

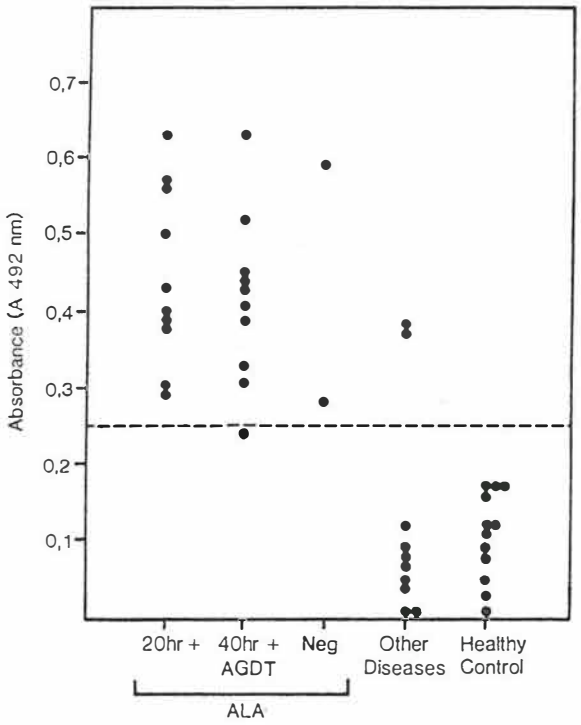

FIG. 1. IgG ELISA results for ALA patients and controls. 20hr+, Strong-positive AGDT result; 40hr+, weak-positive AGDT result; Neg, negative AGDT result.

AGDT. The AGDT described elsewhere (9) was examined for the development of precipitin bands over a period of 48 h. Positivity of the AGDT (expressed at 20 or 40 h) indicates when precipitin bands were observed.

Statistical analysis. Data were analyzed by using the Pearson correlation coefficient and the Student *t* test.

RESULTS

A_{492} values obtained with the IgG and IgM ELISAs are shown in Fig. 1, 2, and 3. All healthy controls were seronegative by the IgG and IgM ELISAs as well as by the AGDT. Nineteen of the twenty patients (95%) with ALA who had a positive AGDT on admission were also seropositive by the IgG ELISA (Fig. 1), and for 18 (90%) the IgM ELISA gave positive results (Fig. 2). Two patients with ALA had a negative AGDT at the time of admission to the hospital; both of them proved to be seropositive by the IgG ELISA (Fig. 1), and for one the IgM ELISA was also positive (Fig. 2). There was a significant difference ($P < 0.001$) in the OD values between ALA patients and normal healthy controls with the IgG and IgM ELISAs. No significant difference was observed between the latter group and hospitalized patients with other diseases.

The distribution of A_{492} values obtained with IgG and IgM ELISAs on sera obtained from asymptomatic subjects from an amoebiasis-endemic area are represented in Fig. 3. All culture-negative subjects were found to be seropositive by the IgG ELISA; notably, the highest A_{492} values were observed for the three subjects who were also positive by the AGDT. The IgM ELISA was positive for only one of the culture-negative controls. All five carriers of pathogenic zymodemes were seropositive by the IgG ELISA; however, none of these subjects were seropositive with the IgM

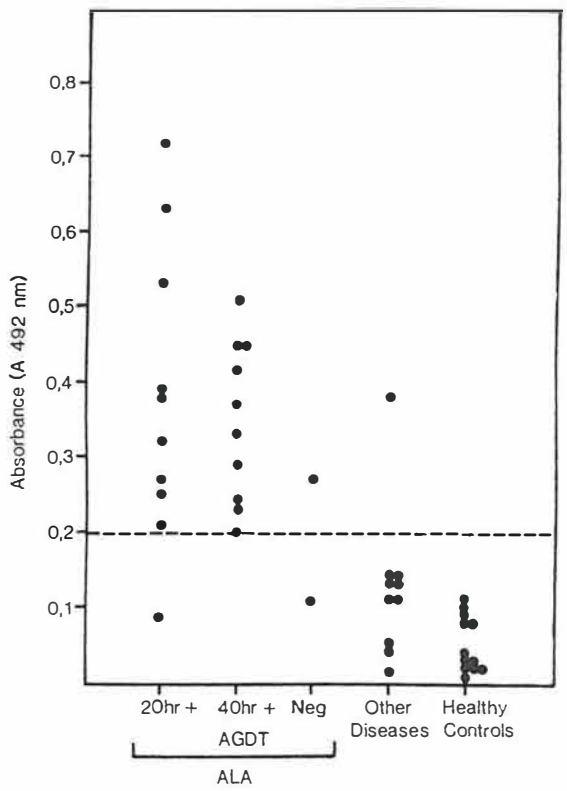

FIG. 2. IgM ELISA results for ALA patients and controls. 20hr+, Strong-positive AGDT result; 40hr+, weak-positive AGDT result; Neg, negative AGDT result.

ELISA. Only one of six sera from carriers of nonpathogenic zymodemes proved to be positive by the IgM ELISA; of the three subjects who were IgG ELISA positive, two had a positive AGDT. Of the 10 hospitalized patients without clinical evidence of amoebiasis, two were positive by the IgG ELISA (Fig. 1) and one was positive by the IgM ELISA (Fig. 2). No significant differences in the OD values were observed between normal healthy controls and subjects from an endemic area with the IgM ELISA. However, significant differences in OD values were observed with the IgG ELISA between normal healthy controls and culture-negative controls ($P < 0.02$) and between normal healthy controls and symptomless cyst passers of pathogenic ($P < 0.001$) and nonpathogenic ($P < 0.05$) zymodemes. There was an excellent correlation between the IgG ELISA and the AGDT ($r = 0.96$). The IgG and IgM ELISAs were found to have sensitivities of 95 and 91% and specificities of 91 and 95%, respectively, for the diagnosis of ALA.

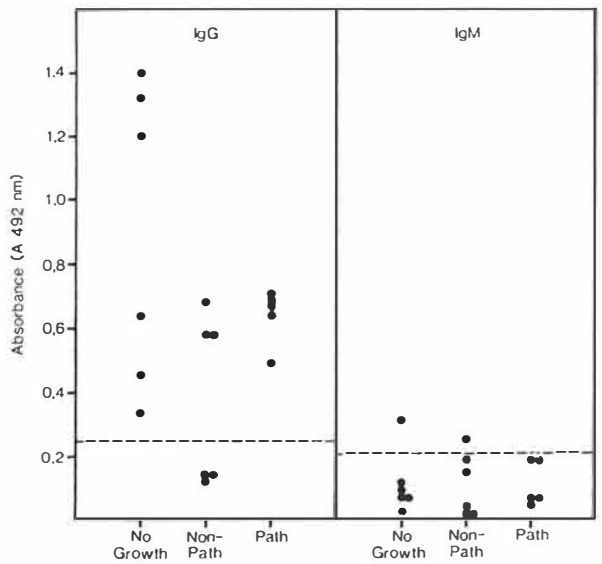

FIG. 3. IgG and IgM ELISA results for healthy subjects from an amoebiasis-endemic area. No growth, Culture-negative subjects; Non-Path, carriers of nonpathogenic zymodemes; Path, carriers of pathogenic zymodemes.

DISCUSSION

Previously reported studies using the ELISA in the serological diagnosis of invasive amoebiasis have used poorly defined soluble antigens (2, 24, 26). More recently, highly antigenic membrane fractions of 27 to 220 kilodaltons have been identified by using either monoclonal or polyclonal antibody probes (11, 15, 16, 19, 25). In particular, two such antigens are recognized by the immune sera of ALA patients. One is a 170-kilodalton galactose-*N*-acetyl galactosamine (Gal/Gal NAc)-inhibitable lectin, and the other is a 90- to 96-kilodalton surface antigen which binds the Gal/Gal NAc-inhibitable lectin (11, 19, 25). The improved sensitivity and specificity of the IgM ELISA in the serodiagnosis of ALA in the present study was possibly due to the detection of specific IgM antibodies directed to epitopes on the surface membrane which were not previously detected when a crude, soluble *E. histolytica* extract was used as the antigen (24).

Occasionally, the AGDT is negative on admission for ALA patients; this is thought to be due to inadequate antibody production, since the AGDT invariably becomes positive when repeated a few days after hospital admission (10, 13, 20). In other comparative serological studies, the ELISA was found to be positive on hospital admission in cases of ALA when other precipitin tests were negative (12, 23). This clinical advantage of the ELISA could be useful in the early diagnosis and treatment of ALA.

The serological responses of asymptomatic subjects were interesting. The asymptomatic carriers of pathogenic zymodemes all produced IgG antibodies to the surface membrane of *E. histolytica*; however, the absence of an IgM response in them would indicate the chronicity of infection and the absence of deep tissue invasion. The specificity of the IgM ELISA for excluding deep tissue invasion is also indicated by the negative serological responses obtained from the sera of healthy carriers of nonpathogenic zymodemes and culture-negative controls from the endemic area. The positive results obtained by the IgG ELISA for culture-negative controls and carriers of nonpathogenic zymodemes possibly indicates past infection with pathogenic zymodemes. Thus, while the IgG ELISA (like the AGDT) would be of importance in ascertaining the prevalence of pathogenic zymodemes in a community, inclusive of past and present infections, the specificity of the IgM ELISA would make it of value for diagnosing current tissue invasion.

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CONCLUSIONS AND RECOMMENDATIONS

The sensitivities and specificities of the IgG- and IgM-enzyme-linked immunosorbent assays (ELISA) using soluble complex antigen of Entamoeba histolytica were 99%/64% and 92%/98%, respectively. The amoebic gel diffusion (AGDT) is as sensitive (91-100%) as the IgG-ELISA in the serodiagnosis of amoebic liver abscess (ALA). It is only with regard to the speed with which IgG-ELISA results are made available to the clinician that the ELISA has an advantage over the AGDT. Thus the IgG-ELISA like the AGDT, could be used to detect invasive amoebiasis, past and present, in the community.

The IgM-ELISA was not sensitive enough (64%) to differentiate past from current amoebic infection; use of the plasma membrane antigen of E. histolytica improved the diagnostic potential of the IgM-ELISA by improving its sensitivity from 64% to 91%. Thus the IgM-ELISA could prove valuable in differentiating past from current amoebic infection. Use of the plasma membrane antigen in the IgG-ELISA offered no added advantage over the soluble complex antigen which is less expensive and more easily prepared. To detect specific IgG-anti-E. histolytica antibodies, it is more economical to use the soluble complex antigen, whilst the detection of specific IgM-anti-E. histolytica would require the use of the

plasma membrane antigen.

The ability of the IgM-ELISA to differentiate between past and present infection needs to be further evaluated in a longitudinal study of patients with invasive amoebiasis in order to determine how soon after treatment IgM antibodies are not detected in the sera.

Several investigators have demonstrated that the IgG-anti-E. histolytica antibodies or total immunoglobulins recognised surface antigens of MW27 KDa-220 KDa (Mathews et al, 1986; Petri et al, 1987; Joyce and Ravdin, 1988; Torian et al, 1989/1990 and Reed et al, 1989). Attempts to identify antigens specific for IgM-anti-E. histolytica antibodies have not been performed. The observations recorded in this dissertation indicate that such antigens are located on the plasma membrane of E. histolytica. Because of the improved sensitivity of the IgM-ELISA with the plasma membrane, identification, isolation and purification of these antigens is warranted. Such investigations may constitute an important step in improving the serodiagnosis of invasive amoebiasis.

The ELISA is a relatively cheap, simple, rapid and sensitive test. With the availability of purified antigens, it is highly likely that the ELISA may become the method of choice in the future.

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