DISCUSSION

The clinical syndromes of amoebiasis that result from the varied host and parasite conditions range from asymptomatic infection to a relentless, invasive, disseminated fatal disease, the main consequences of which are amoebic colitis, dysentery and liver abscess. Based on the assumption that the presence of serum antibodies against the trophozoite exist only in individuals with past or present invasive amoebiasis, Walsh (1982) calculated that annually 48 million people, (approximately 10% of infected individuals) suffer from invasive amoebiasis expressed as intestinal (dysentery) or extraintestinal lesions (mainly liver abscess). The values of annual world mortality attributable to *E. histolytica* has been estimated to be 40-100,000 (Walsh, 1986) which are of interest in raising problems regarding the nature of the disease and the capacity of the host to mount defences against the parasite. There is evidence that natural immunity against recurrent invasive amoebiasis exists (DeLeon, 1970; Sepulveda and Martinez-Palomo, 1984) but currently no vaccine is available to prevent amoebic disease in the developing world.

The complexity of the clinical spectrum of human amoebiasis and the high variability in laboratory methods used to detect *E. histolytica* infections have made it difficult to obtain reliable epidemiological data. Thus more sensitive, specific and standardized methods are needed to accurately identify infections with this parasite. The level of anti-amoebic antibodies is relatively higher in liver abscess cases, but it cannot differentiate past or silent from present infection. Thus the demonstration of antigen in amoebic pus, in tissues, or in the circulation (free or complexed form) would be an ideal choice for diagnosing an individual case of
amoebiasis. Presence of increased serum immune complexes have been used as a tool for diagnosing patients with amoebic liver abscess and chronic cyst passers (Onyemelukwe and Onyewotu, 1981; Nuti et al 1982), but sequential studies regarding formation and fate of such immune complexes at different stages of amoebic disease is still lacking.

Since the liver tissue of the hamster is very susceptible to direct intrahepatic inoculation of *E. histolytica* trophozoites (Reinertson and Thompson, 1951; Neal and Vincent, 1956) and since the same animal has also been utilized to study the cell mediated immunity as well as serological responses against *E. histolytica* (Ortiz-Ortiz et al 1973; Gold et al 1978), golden hamster has been used in the present work for establishment of hepatic abscess model on which various studies have been performed. A major thrust of the present dissertation was to study the kinetics of the *E. histolytica* induced immune complexes (if any) over a period of time as well as the detection of specific *E. histolytica* antigen in complexed form as a precise diagnostic approach in amoebic disease.

As evident from data, the successful induction of hepatic amoebiasis in golden hamster was achieved using an *E. histolytica* strain isolated locally (from Calcutta). Taking into account the well known fact that the virulence of cultured *E. histolytica* varies according to the strain (Diamond et al 1974; Orozco et al 1978) and culture conditions (Phillips, 1973), the strain was grown in both axenic and monoxenic conditions. It was observed that when trophozoites from both these culture systems were inoculated with increasing numbers to hamsters, higher virulence indices were achieved with lesser number of monoxenic amoebae than its axenic counterpart. The correlation between manifestation of virulence and size of
inoculum was found to be more pronounced in monoxenic amoebae. An explanation of the dose-effect may be the assumption of strain heterogeneity where the virulent and non-virulent trophozoites exist within the population of one strain. In an axenic environment, selection may result in a lower frequency of virulent individuals, so that a large inoculum is necessary to induce necrosis. Presence of Crithidia would in this hypothesis, favour the selection of virulent individuals, so that low inocula are sufficient for manifestation of pathogenicity.

In this context, presence of heterogeneity in axenic culture of E. histolytica has been indicated by the existence of differences in adhesion, phagocytosis and virulence of clones isolated from strain HMI-IMSS (Orozco et al 1985).

The higher virulence of monoxenic trophozoites as observed in this study are in line with the earlier findings on the higher virulence of bacteria associated or Crithidia associated E. histolytica in culture than the axenic one (Wittner and Rosenbaum, 1970; Bos and Hage, 1975).

Since it has been observed that progressive liver necrosis was more evident with increasing number of inoculated trophozoites from monoxenic culture, determination of the right inoculum size was found to be inevitable that would permit long term survival of the infected animals having reproducible liver lesions. To achieve this, hamsters were intrahepatically inoculated with different doses of monoxenic trophozoites and sacrificed at 3 days interval till 15th post inoculation day. A higher mortality of infected hamsters from 9th day onwards was noted after inoculation with higher number (75,000) of monoxenic amoebae. Post-mortem observations of individual animal detected severe liver lesions as the main reason for the
expected mortality. On the other hand, inoculation with lower number (25,000) of monoxenic trophozoites was unable to produce lesion in majority of the animals. Though ideally in the selection of optimum dose of infection for any experimental lesion involving a microbe, ID$_{50}$ (50% of the infect amoeba) should be determined. In the present study, ID$_{50}$ could not be determined since minimum margin of difference in the production of lesions was not even one log. The liver lesions in the infected animals when present was found to be small and discrete thus hindering the use of 25,000 particular inoculum size in the present study further. However, an inoculum size of 50,000 monoxenic trophozoites per hamster was found to be optimum for induction of consistent and reproducible liver lesions from 6th day onwards. The overall survival of the infected animals up to the 15th day enabled the use of this inoculum size for induction of hepatic amoebiasis in golden hamsters as well as permitted the monitoring of serological and immunological observations on such animals for a maximum period of 41 days.

The signs and symptoms of an amoebic infection can provide the means to obtain a clinical diagnosis and the ultimate determination of an amoebic infection involves identification in the laboratory either directly by demonstrating the etiologic agent, or indirectly by demonstrating antibody or antigen in serum or other body fluids. The value of serologic testing in cases of suspected amoebic liver abscess is widely recognised. The inability to detect amoebae in abscess fluid or even to obtain fluid for laboratory examination stimulated the investigation and perfection of serologic tests for amoebiasis of which the major type of immunodiagnostic testing for amoebiasis has been the detection of specific antibody in serum.
In seroepidemiologic studies, 81-100% of patients with invasive colonic amoebiasis or amoebic liver abscess develop circulating IgG antibodies specific for *E. histolytica* while such antibody response was found less frequently in individuals with asymptomatic infection (Kessel et al 1965; Thompson et al 1968; Kotcher et al 1970; Krupp, 1970; Juniper et al 1972; Patterson et al 1980). Moreover, a positive serologic finding has been accepted as indicative of current parasite invasion or prior invasive disease (Elsdon-Dew, 1970; Trissl, 1982). It has been suggested that higher serum titres are associated with more recent onset of invasive disease (Healy et al 1970; Krupp, 1970). On the other hand there is no evidence that the titre of antibody to *E. histolytica* correlates with clinical outcome (Krupp and Powell, 1971; Trissl, 1982).

The present study was intended to clarify the immunological reaction of hamsters to *E. histolytica* infection on a time sequence basis. In this model, the exact time of exposure was known, whereas in human hepatic amoebiasis a similar study cannot be initiated until symptoms appear. The results in the present study showed that a suboptimum inoculum size i.e. $2.5 \times 10^4$ trophozoites of *E. histolytica* triggered poor antibody response in hamsters. Measurable amount of antibody as measured by IHA appeared only on 15th day post inoculation. Although IHA appeared to be sensitive and titres of 1:32 and above were specific for antiamoebic antibodies the absence of liver lesions in majority of the animals restricted to draw a conclusion. Animals which were inoculated with trophozoites but did not have any lesion showed IHA titre similar to those obtained in uninfected controls. Results of testing 63 sera from hamsters inoculated in the liver with higher numbers of *E. histolytica* and from 18 uninoculated controls by IHA showed that detectable amounts of antibody appeared as early as 6th day post inoculation and generally increased to a high level.
within 15th day post inoculation. By the end of 3rd day post inoculation, the mean titres of the test sera were not exceeded than those of controls but they rose considerably between 9th and 15th days and thereafter remained at a plateau. A slight decline in the antibody titre was observed on 31st to 41st day after infection. The early occurrence of some large lesions and persistence of small lesions 3-4 weeks after inoculation probably reflected the variation of virulence of the organism inoculated. Norman et al (1979) studied 298 sera from hamsters infected with E. histolytica and suggested that although an IHA titre of 1:16 and above were specific for amoebic lesions, only those of 1:128 and above were indicative of liver infections in the experimentally infected hamsters. It has also been observed by the above workers that the mean titres of their test sera generally increased to a high level within 20 days and persisted for the duration of the experiment (58 days). In the present study it has been observed that an IHA titre of 1:64 could be considered specific for liver lesion in one week old hamsters. Since Norman et al (1979) used older hamsters, this along with the difference in E. histolytica strain might attribute to the discrepancies. Significant positive correlation between the lesion scores and IHA antibody titres was noticed throughout the study period. However, correlation was more pronounced during 3rd to 9th (r=0.75; p<0.005) and 21st to 41st (r=0.85; p<0.005) post inoculation days. Correlation was slightly less (r=0.67; p<0.005) during the 12th to 18th post inoculation days. Since the macroscopic scoring of liver lesions is not complementary with the semiquantitative IHA titre determination, the relationship between individual lesion score and the IHA titre could not be ascertained in this study. However, it has been observed that persistence of lesions having viable organisms was essential for generation of specific antibody which can be detected after 1 week of inoculation. On the otherhand, no positive antibody titre was
ever detected in the animals which were inoculated with *E. histolytica* but were without any lesion in the liver.

Identification and characterization of host and parasite components in circulating immune complexes (IC) appear to be especially important for understanding their pathogenic significance in disease. Antigen antibody complexes are regularly found in patients with certain disorders and there is compelling evidence for the view that such complexes may play a pathogenic role (W.H.O., 1977). Soluble immune complexes (IC) circulating in the blood stream can be detected by a variety of methods and these are widely used in clinical immunology to aid the study of immunopathology of diseases, the assessment of disease progression and the patient's response to therapy (Zubler and Lambert, 1978). Moreover, there are instances, where IC were found in sera but neither free antigen nor antibody could be detected and in these patients the isolation and identification of the IC might be of diagnostic importance (Jones and Orlans, 1981). A WHO study compared 18 methods for detecting circulating IC (CIC) (Lambert et al., 1978). Five of the assays used Clq, based on either the fluid phase or solid phase binding to Clq of aggregates containing IgG. All of these methods detected CIC using radio labelled anti-IgG or radio labelled Clq (Hay et al., 1976).

Immune complexes are formed in dynamic equilibrium with their constituent molecules, therefore any attempt to isolate them will shift this equilibrium leading to their partial dissociation. This unstability of complexes after separation, presents problems in their isolation and limits sequential purification steps. However, the speed of dissociation varies between different equilibrium systems depending on several factors such as size. It seems however, that the prime factor affecting complex stability is the avidity of the antibodies to
its corresponding antigen (Mannik, 1982). In accordance with many parasitic infections (Zubler & Lambert, 1978) although increased soluble immune complexes have been documented in circulation of amoebic liver abscess patients and chronic cyst passers, attempts to clarify the sequelae of circulating immune complex (CIC) in hamster model of amoebiasis have not been described. Thus one of the intents of the present study was to evaluate the sequential change in the circulating immune complex (CIC) level of *E. histolytica* infected hamsters by the Clq binding assay (Zubler and Lambert, 1976).

In the present study the detection of CIC by Clq binding ability was performed in a sequential fashion. The induction of immune complex in golden hamsters was attempted using different doses of infecting inoculum of monoxenic *E. histolytica* trophozoites. Inoculation of 25,000 trophozoites resulted in discrete lesions in a few animals only. The percentage binding of Clq was also very poor in infected animals and was not considered positive for the presence of immune complex in sera, because similar binding pattern was also observed in hamsters those served as control. Use of 50,000 trophozoites was found to be optimum for consistent as well as persistent liver lesions in the present study. Presence of CIC was detected in the infected hamsters receiving 50,000 monoxenic trophozoites. The mean Clq binding pattern exceeded the control values from 12th day onwards. Presence of immune complex in sera was more pronounced from 15th to 18th day as detected by higher percentage binding of Clq which subsequently declined from 21st day post inoculation and subsided below the control value on 41st day post inoculation. Interestingly the pattern of antibody titre followed the same observed trend of Clq binding.

Application of this classical but non specific method
for detection of CIC in an animal model of amoebiasis in the present study indicated the graded outcome of the increasing level of CIC with the disease process. It is generally accepted that immune complex trigger classical and alternate pathways of complement activation. Classical pathway activation is initiated by binding of Clq to a suitable antibody (IgG1-3, IgM) in the immune complex and is favoured by large immune complexes with multiple epitopes, slight antibody excess, high antibody affinity antibodies and polymerised antibodies like rheumatoid factors. On the other hand, alternate pathway activation follows with certain complexed antigen or if antibody binding enables the antigen to activate alternate pathway. It has also been suggested that the classical pathway activation may prevent immune complex precipitation and deposition and alternate pathway proteins can solubilize immune complex proteins (Sethi and Mayer, 1987). Studies of Reed et al (1983, 1986) have documented the role of *E. histolytica* trophozoites or its components to activate both the classical and alternative pathway of complement, where the virulent organisms were able to escape the complement mediated lysis. In this context, the involvement of complement activating *E. histolytica* antigen in immune complex cannot be ruled out. It can also be assumed that complement activation by such immune complexes lead to (a) the survival of virulent trophozoites in the tissues, which may help in the progression of lesion, and (b) hypocomplementaemia to facilitate immune complex diseases (Schifferli and Peters, 1983).

Since the Clq protein binds with the immunoglobulin portion of the IC it is unable to demonstrate the etiologic inducer of the observed circulating immune complex level (CIC). Although the CIC level was detected in the infected hamsters by Clq binding assay in the present study, it was uncertain whether the detected CIC was due to *E. histolytica* infection.
Thus it was necessary to apply a specific method which can detect specific *E. histolytica* component in the immune complex. This would also be useful as a diagnostic method where the antigen is not available in free or detectable form. A specific radio immunoassay was developed by Pillai and Mohimen (1982) for the detection of circulating *E. histolytica* antigen in the sera of patients with different forms of amoebiasis. Further studies using solid phase RIA also provided evidence that the circulating *E. histolytica* antigen is usually present in the form of an immune complex (Mohimen et al, 1989). Using a micro-ELISA, Vinayak et al (1986) also confirmed the presence of specific circulating immune complexes in amoebic liver abscess pus. In order to ascertain the nature of CIC observed in infected hamsters, the solid phase RIA method of Mohimen et al (1988) has been applied in the present study with minor modifications. In place of anti human antibody, here anti hamster antibody raised in rabbits was used. This method had the advantage of not detecting any free antigen, and therefore it was reasonable to assume that any antigen detected would be in the form of immune complex.

The method was unable to detect any specific antigen in the form of circulating immune complex up to 15th day post inoculation in hamsters inoculated with 25,000 trophozoites. The antigen in the CIC was detected only after 15th day post inoculation in hamsters inoculated with 50,000 trophozoites and having persistent liver lesions. When the level of CIC containing specific *E. histolytica* antigen was monitored in hamsters infected with 50,000 trophozoites from 3rd to 41st day post inoculation, a definite pattern emerged. A rise in CIC level was observed from 12th day post inoculation which reached a peak on 18th day and then gradually declined. This observed trend in CIC level was also found to follow the variation of Clq binding. Thus confirming the existence of CIC response in
hamsters. A significant correlation was also evident ($P<0.005$) when the lesion score of individual animal was compared to the CIC level as monitored by radioactive counts per minute values (CPM). The data in the present study thus demonstrated the formation of IC in experimentally induced amoebic liver abscess and confirmed the involvement of specific E. histolytica antigen in the formation of CIC. Sequential observations demonstrated that it was between 12th to 18th day after infection that the antibody titre as well as the CIC level were maximum which slowly subsided to control values near about the 41st day post inoculation. Significant correlation ($p<0.001$) was also observed between the antibody titre and CIC level both of which were also dependent on the presence of consistent liver lesions.

The biological properties of immune complexes having direct relevance in disease are their rate of formation as well as clearance from the circulation, their complement combining capacity and their ability to form deposits in tissues (Jones and Orlans, 1981). It has also been postulated that deposition of CIC in glomeruli with the subsequent activation of complement and other mediators of inflammation causes acute glomerulonephritis (Wilson and Dixon, 1986). CIC containing specific virulence proteins of bacteria have been demonstrated in acute glomerulonephritis due to acute infection with Shigella flexneri type 1B (Musher et al 1990). Immune complexes cause a number of clinical manifestations in systemic lupus erythematosus (SLE) and in other immune complex disease. The pathogenic immune complexes are either deposited from the circulation or by the local formation of antigen-antibody complexes. In local formation of immune complexes, the antigens are part of the target organ or unrelated antigens are selectively deposited in the organ and antibodies from the circulation react with these antigens. Involvement of one organ
is usually observed in this type of disease mechanism. On the contrary, when pathogenic immune complexes are present in circulation, usually more than one organ is involved due to the deposition of CIC (Mannik, 1982).

One of the objectives of the present work was to study the fate of the CIC in experimental hepatic amoebiasis and the deposition of immune complexes in various tissues of golden hamsters infected with *E. histolytica*. Four tissues namely liver, kidney, spleen and heart were chosen and immune complexes containing specific *E. histolytica* antigen was detected by the solid phase RIA using affinity depleted and affinity purified *E. histolytica* antibody. Since the model used in the present study involves direct intra hepatic inoculation of trophozoites, the unaffected parts of the liver tissue were taken for the detection of immune complexes. It is quite evident from the data that all the four tissues were found negative for immune complex up to the 15th day post inoculation using varying number of trophozoites as infecting inoculum. Among the four tissues tested, deposited immune complexes having *E. histolytica* antigen was more prominent in liver which tended to increase from 21st day post inoculation to reach maximum on 41st day post inoculation. The kidney was also found positive for immune complex albeit in a lower order of magnitude than the liver. However, increased immune complex levels was encountered on 31st day post inoculation which continued till the end of the study period i.e. 41st days. Apart from liver and kidney, the spleen and heart tissues remained negative for immune complexes throughout the study period suggesting the preferential deposition of *E. histolytica* induced immune complexes in liver and kidney of infected hamsters. Significant correlation was observed (p<0.005) between the extent of liver lesions and the increased immune complex levels indicating the need of continuous supply of
antigen in formation and deposition of immune complexes. The data also demonstrates that immune complexes appeared in circulation from 12th day post inoculation onwards, reached maximum on 18th day post inoculation and declined thereafter. On the other hand, deposition of immune complexes in tissues started from 21st day post inoculation onwards till the 41st day of the stipulated study period.

The present study was the first of its kind detecting the specific *E. histolytica* antigen in the form of immune complexes in the sera and tissues of golden hamsters. Furthermore, the formation of *E. histolytica* induced immune complexes in circulation and the preferential deposition of such immune complexes in certain tissues was evaluated for the first time in experimentally induced hepatic amoebiasis.

Formation of immune complexes generally depends on ratios of antigen to its respective antibody. Such ratios ultimately indicate the size of the immune complexes which in turn is responsible for the fate of formed immune complexes. Based on the ratios, three states can be assumed, i.e. immune complexes formed in the condition of antigen excess, where the clearance of immune complexes from circulation was slow; where the antibody is in excess, the immune complexes were not precipitated but the clearance was slow and where there is a state of equivalence between antigen and antibody, there is rapid clearance of immune complexes by monocyte - macrophage system (Fudenberg et al 1978).

In this study, higher titres of antibody was noticed throughout the study period and it can be assumed that the immune complexes formed in the infected animals was in the presence of excess antibody. On the other hand, in the absence of quantitative antigen assay, the progressive and persistent
lesion scores having living *E. histolytica* might be considered as a evidence of continuous supply of antigen and thus the state of antigen excess at certain stage of immune complex formation cannot be ruled out. Deposition of immune complexes in tissues results from the impairment of clearance by the cells of reticuloendothelial system. In this study, liver was inoculated directly by *E. histolytica* trophozoites which might result in the impairment of the general phagocytic ability of the cells thus hindering the uptake of immune complexes in a non specific way. Since there was a continuous supply of antigen in the liver, presumably monocyte-macrophage system appeared to become saturated due to increasing amounts of immune complexes in the circulation and liver was no longer capable of clearing excess amounts of immune complexes. Therefore, the increased amounts of immune complexes were found in the circulation and due to defective clearance system in the liver, this excess immune complex gets deposited in the liver and to some extent, in the kidney.

Studies in experimental animals have shown that circulating, large latticed immune complexes containing IgG antibodies are efficiently removed by Kupffer cells (Mannik, 1982). The removal of immune complexes is also influenced by the nature of antigens and antibodies in the complexes and by the functional capacity of the Kupffer cells. The rate of immune complex formation in turn depends on the rate of availability of specific antigens. Information is lacking regarding the rate of immune complex formation in human disease or in murine models of SLE (Fleuren et al 1980). Studies using mice, rabbits or monkeys (Mannick et al 1971; Haakenstad and Mannick, 1976; Bockow and Mannick, 1981) demonstrated that large latticed immune complexes were removed relatively quickly while the small latticed immune complexes persisted longer in circulation. The maximum deposition of immune complexes in
liver of infected hamsters might be due to the fact that the liver accounted for the overwhelming majority of removal of circulating large latticed complexes in mice, rabbits and monkeys (Arend and Mannick, 1971; Bockow and Mannick, 1981). On the other hand, the deposition of immune complexes in kidney might be due to the longer persistence of large latticed immune complex in circulation due to decreased hepatic uptake. There is experimental evidence that impairment in the interaction of IgG with the Fc receptor of mono nuclear phagocytic cells caused increased glomerular deposition (Haakenstad et al 1979).

Antigen-antibody complexes are known to be involved in the pathogenesis of a variety of diseases (Theofilopoulos and Dixon, 1979). Evidence for this has been obtained by using several reproducible assays aimed at determining immune complexes in body fluids, although at present these assays do not conform to agreed standards. While a comparison of various methods quantifying reference preparations of aggregated immunoglobulins showed good correlations between some of the tests, conflicting results were obtained when specific sera were assayed. Thus, sera collected from patients with different diseases were found to be positive for immune complexes in one assay but negative in another (Lambert et al 1978). This is quite likely considering the different principles underlying the assays as well as the different composition of the immune complexes encountered in different diseases. Most methods currently in use are antigen non specific quantifying either the antibody or the complement components attached to the immune complexes thus imposing limitations. One of the means to overcome these limitations is by specifically measuring the antigen moiety involved in the formation of immune complexes. Although CIC are an established feature of most infections diseases (Zubler and Lambert, 1978), detection of circulating
specific antigen (in these complexes) has generally proved a formidable task.

In earlier studies (Ortiz-Ortiz et al 1974), circulating antigen-antibody complexes have not been found in patients with amoebic liver abscesses, although Ruiz Castaneda et al (1976) suggested that circulating amoebic antigen is present during some phases of invasive amoebiasis. The above studies also suggested that lack of immune complexes is one of the reasons for the absence of immunopathologic lesions from patients with invasive amoebiasis. In recent years, development of new methodologies have undoubtedly detected immune complexes in amoebic liver abscess patients by specifically detecting the antigen moiety (Pillai and Mohimen, 1982; Vinayak et al 1986). The presence of such immune complexes in experimental amoebiasis has been documented in the present dissertation using the specific detection method developed by Pillai and Mohimen (1982) and Mohimen et al (1989) for detection of immune complexes in human amoebiasis. They have observed (a) preferential deposition of amoebic immune complex in liver and colonic mucosa, and (b) tissue deposited immune complex did not always correlated with the circulating immune complex status. Similar trend was also noted in the present experimental model. Preferential deposition of immune complexes in certain tissues in infected hamsters might have some pathophysiologic implications which needs further study. In this context, golden hamster hepatic abscess model can be explored as an immune complex disease model.

The present study although documented the kinetics over a limited period of observation of E. histolytica induced immune complexes in experimental liver abscess, the exact nature of antigen, antibody and the complement status yet to be evaluated. Even though the study has provided some evidence of
formation and deposition of immune complexes having specific *E. histolytica* antigen much more remains to be known. As more information is obtained, undoubtedly new approaches will become available to enhance the understanding of human immune complex disease. Besides the diagnostic applicability, improved understanding of the pathophysiology of immune complexes in experimental animals will also permit more incisive enquiry into human diseases.