DISCUSSION
The increasing number of reports of primary amoebic meningo-encephalitis and granulo amoebic meningo-encephalitis since 1958 from various parts of the world, reflects greater awareness among the clinicians of the pathogenic potential of free-living amoebae, improved diagnostic facilities for examination of cerebrospinal fluid and increasing prevalence of opportunistic infections.

Studies on the immunological aspects of amoebic infections are desirable since these will enable us in long run to understand the protective cellular immune response against these pathogens. From a public health view point, there is a need to develop reliable diagnostic test for the rapid identification of pathogenic species of amoebae and to employ chemotherapeutic measures for the control of disease. Preliminary studies conducted so far suggest that some of the tests employed for surface antigens of amoebae for example- indirect immunofluorescence test, immobilization agglutination, immunoperoxidase staining, Con A test (Van Dijck et al. 1974, De Jonkheere et al. 1975) could be improved and applied for specific diagnosis of PAME and GAE cases. There is need for developing definitive and supporting tests for detection of pathogens at early stages of infection. Since at present the disease is generally diagnosed at a stage when chemotherapy is of little value.

There are many lacunae in our understanding of the immunology of endogenous amoebiasis. A number of reports, dealing with serodiagnostic methods, which are currently employed in the serodiagnosis and seroepidemiology of endogenous amoebiasis, have been published. Except for the few workers (Ortiz- Ortiz et al. (1973,1974), Harris and Bray, 1971 and Gold et al. 1978, Khan et al. 1982) only a little work has been done in the field of cellular immunity against E. histolytica.
Thus in the present work dynamics of immune responses against these aetiological agents of amoebiasis have been elucidated.

Incidence of Entamoeba histolytica:

Results of the three surveys carried out at Central Drug Research Institute, Lucknow, indicate that -

1) Amoebiasis still constitutes a major health problem in India, inflicting a considerable percentage of population in both urban and rural areas. A reliable diagnosis of asymptomatic and clinical cases would be most desirable for the control of the disease.

2) Inspite of the introduction of effective and powerful chemotherapeutic agents like metronidazole, amoebiasis continues to prevail without any abatement in its incidence. Apparently adequate public health control measures have not been implemented to check the spread of infection.

In the present study there was a slight variation in the incidence of *E.histolytica* as obtained in three surveys (Table I) which might be a reflection of the seasonal variation in the incidence of amoebiasis.

Studies on axenic cultivation of *E.histolytica*

The development of axenic media for growth of *E. histolytica* has greatly facilitated the biochemical, pathological and immunologic investigation of *E.histolytica*.

In the present study five commonly used axenic media were compared to get an idea as to which medium supported the best growth of *E.histolytica* (Table II,III,IV,V,VI).

The best growth supporting medium for axenic *E. histolytica* was found to be modified TPS-1 or TPS-2 devised by Dutta and Yadava (1972) (Table II).
The limitations of Diamond's TPS-1 medium have been demonstrated by Singh, Das and Dutta (1973). They have shown that combination of L-cystein HCl and ascorbic acid in TPS-1 medium gradually leads to a shift in O-R potential towards the positive side. When the medium is stored for two weeks at room temperature, amoebae die out without multiplication in 3-5 days even with an inoculum of 5000 amoebae/ml of the modified TPS-1 medium containing 0.2 % cystein without ascorbic acid maintained strongly negative O-R potential for several weeks on storage. In this modified TPS-1 medium Singh, Das and Dutta (1973) were able to grow amoebae with an inoculum of 250 amoebae/ml of the medium and Dutta (1976) with an inoculum of 25 amoebae/ml of the medium. These findings provide evidence for suitability of modified TPS-1 medium for growth of axenic E. histolytica as it maintains strongly negative O-R potential and can be stored for several weeks.

Studies on Axenic Cultivation of *N. aerobia*

In the present study a comparison of the growth of *N. aerobia* was made in Nelson's medium prepared with two different (foetal calf and bovine) sera as medium components. Results indicated that bovine serum was also capable of supporting the growth of *N. aerobia* though the yield of amoebae was $1.5 \times 10^8$ amoebae/100 ml as compared to $2 \times 10^8$ amoebae/100 ml, when foetal calf serum was used. (Table VII). Bovine serum was found to support the growth of amoebae for 3 to 4 subcultures. Although O'Dell and Steven's (1973) failed to grow *N. aerobia* with bovine serum, Haight and John (1980) found normal calf serum supporting better growth of *N. aerobia* as compared to foetal calf serum.

The application of bovine serum in axenic cultivation of *N. aerobia* may greatly decrease the cost of medium since it is available free of cost.
Studies on the amoebic antigen

The nature and source of antigen employed contribute significantly to the sensitivity and specificity of immunological procedures.

E.histolytica Antigen:

In the past years antigen employed for immunological tests was prepared from E.histolytica growing with bacterial associates in culture. This antigen containing bacterial proteins was not suitable for standardization of many immunodiagnostic techniques (WHO 1969); obviously the use of crude amoebic antigen containing significant quantities of non- E.histolytica proteins was the chief cause of conflicting results in the serological studies on amoebiasis (De Blai and Magaud- Borzi, 1958). For example in gel-diffusion precipitin test, Atchley et al. (1963) observed that the sensitivity of test depended on concentration of amoebic protein in antigen preparation. Sen et al (1961) and Maddison (1965) found non-specific precipitin lines (Welchii bands) against bacterial proteins which were mingled with amoebic proteins in antigen. Thus in this study antigen used was prepared exclusively from axenically grown E.histolytica. Method for cultivation of axenic E.histolytica was of Diamond (1968) as modified by Singh et al. (1974).

E.histolytica antigen was also fractionated on Sephadex G-200 to get the pure components of antigen.

Free-living amoebic antigen-

In the last decade after discovery of pathogenicity of free-living amoebae surface antigens were used as diagnostic tool for these amoebae. However, surface antigens could be of use only after availability of amoebae either in brain sections after death or in CSF cultures, extraction of which is quite dangerous.
Some workers have used bacteria associated cultures for preparation of antigen which are not suitable for standardization of any immunological test as they gave misleading results due to mingled bacterial proteins. Some of the workers (Cursons et al. 1980) though prepared antigens from axenically grown amoebae, did not sonicate and estimate the protein contents of antigen. They used antigen on the basis of concentration of amoebae per ml which is indeed not an accurate method for immunology. In the present study for more reproducible results, antigens were prepared from axenically grown cultures of N. aerobia and A. culbertsoni using methods of Nelsons (1970) and Visvesvara & Balamuth (1975), respectively.

A standard antigen prepared from axenic amoebae should contain a known amount of total amoebic proteins. Protein content of any amoebic antigen depends on the growth phase of amoebae at which they are harvested. It could vary from .76 mg to 1.009 mg of protein/million for E. histolytica, .14 mg to .24 mg for A. culbertsoni, .07 to .13 mg for N. aerobia as shown in Table VIII.

For the purpose of present study three amoebic antigens were also sonicated and lyophilized in small aliquots. Storage of antigens was done at -70°C to prevent any alteration in their immunological reactivity.

In the present investigation, with purified and improved antigens prepared from axenic E. histolytica, N. aerobia and A. culbertsoni, it was possible to obtain reproducible results with maximum specificity in different immunologic tests like IHA and gel diffusion for humoral antibodies and skin test and migration inhibition for CMI.
In the present study three fractions FI, FII and FIII of axenic *E. histolytica* were obtained on Sephadex G-200 column chromatograph (Table XIII). Many other workers Sahgal *et al.* 1982, Sawhney *et al.* 1980 have also reported three fractions available on column chromatography of axenic *E. histolytica* antigen whereas four fractions have been reported with bacteria associated antigen of *E. histolytica* (Ali Khan *et al.* 1968).

Out of the three fractions it was found that FI has the highest immunological reactivity which is in fair agreement with the work of other workers (Sahgal *et al.* 1982, Sawhney *et al.* 1980; Krupp, 1974). The reactivity of fractions was evaluated in IHA test and skin test. In IHA test it was found that sensitizing ability of FI had been greatly enhanced and at a protein concentration of as low as 5.0 μg/ml, it gave the same titers as obtained with 40.0 μg/ml proteins of whole amoebic antigen Table XIV. This is consistent with the findings of Sahgal *et al.* (1982) who tested the lowest concentration of FI only upto 10 μg/ml in IHA and found reproducible results whereas Sawhney *et al.* (1980) have also reported that FI in concentration as low as 5 μg/ml could show IHA titers as obtained with 40 μg/ml of whole antigen.

In the skin test it was found that FI in very low concentration (5 μg) was able to produce a pronounced erythema with high infiltration of monocytes as obtained with 10 μg of whole antigen whereas other two fractions exhibited only minor skin reactions when tested in guinea-pigs (Table XV).

These findings suggest that crude antigen should be replaced by only Fraction I for accurate estimation of immunological status of individuals in different immunodiagnostic tests as it contains the maximum antigenic
reactivity and exerts a response similar to that elicited by the whole antigen. It has also been reported (Boonpucknavig et al. 1967) that the antigenic activity is mainly confined to the particles of microsomal density in *E. histolytica* and *F. in again in our preparation might have contained this material.

Studies on Experimental Induction of Delayed Hypersensitivity in Guinea Pigs Against *E. histolytica*, *N. aerobia* and *A. culbertsoni*

Studies on the experimental induction of cellular immune response reveal that a dose of 200 μg protein of *E. histolytica* and 50 μg for *N. aerobia* and *A. culbertsoni* was optimum. That such a small amount of antigen could elicit a delayed hypersensitivity reaction was suggested by the work of Lunde et al. (1969) on *E. histolytica*. Results of present study further indicate that amount of antigen more than optimum, would yield only partially or weakly positive results, although a pronounced reaction could be obtained with as little as 10 μg of the sensitizing antigen. This is in fair agreement with the results of Lunde et al. (1969) & Haq et al. (1982) who also reported weak immune reactions with larger doses and suggested use of smaller doses in sensitization. In addition the IHA titers of sera obtained from animals sensitized with 200 μg of *E. histolytica* antigen and 50 μg of *N. aerobia* and *A. culbertsoni* were also highest among the entire groups made for each amoeba.

It can be inferred from these studies as also from those of Lunde et al. (1969) and Haq et al. (1982) that pathogenic amoebic antigens do elicit a functional cellular immune response when injected in very small amount after emulsifying with an adjuvant like Freund's complete adjuvant. However, for human administration the use of some other suitable adjuvant like muramyl dipeptide (MDP) should be explored.
Studies on skin reaction in intradermal test -

Guinea pigs are best suited model for skin test though other small animals like mice, rats, and hamsters can also be used (Akpon et al. 1967). In the present study guinea pigs have been used as experimental model.

The delayed hypersensitivity a (DTH) skin reaction an in vivo parameters of cell mediated immunity, is the result of complex events including antigen recognition, lymphocyte macrophage interaction, release of biologically active lymphocyte mediators (lymphokines) and changes in so that mononuclear cells vascular permeability are infiltrated. These are difficult to dissociate and quantitate separately.

It is difficult to evaluate the skin test results because three distinct and separate types of immunologic reactions may take place. First is immediate reaction which is associated with IgE antibody, develops within minutes after antigen challenge and reaches a maximum in 15-30 minutes with a characteristic and fades out in 1-2 hours. This reaction was not seen in the present study with the doses of antigens employed.

Second is Arthus type, which is mediated by IgG, antibody, develops after 4-5 hours and may persist up to 24 hours or longer and then fades out, which was also not seen in the present study.

The third type of reaction is true delayed hypersensitivity reaction which is not antibody mediated and specifically requires 24 to 48 hours to develop and is not detectable before 24 hours (Lunde et al. 1969, Krupp, 1974).

Inflammatory cells in each type of skin test can be characterized histologically. In immediate IgE reaction (Type I) the cellular infiltration is predominantly eosinophilic. In the Arthus Ig G1 reaction polymorphonuclear neutrophils are main cell type and in third type i.e. true delayed type of skin reaction, large mononuclear cells predominate.
In the present study for proper study of skin reaction and to make sure that reaction was of delayed type, observations were taken at 15 minutes, 4 hours, 24 hours, 48 hours, 72 hours, and 96 hours after intradermal injection of challenging antigen. It was found that at 15 minutes and 4 hours there was no sign of erythematous oedema, whereas at 24 hours a characteristic wheel and flare of about 1 to 1.5 cms. with induration appeared and persisted up to 72 hours. At 96 hours sloughing occurred from injection sites as shown in tables XVI, XVII, XVIII. In this manner, it has been confirmed that cell mediated immunity exists in amoebiasis for all the three pathogenic amoebae.

In spite of a standard test for diagnosis of amoebiasis, intradermal test is subject to serious inherent limitations. This test lacks specificity and has some disadvantages. In the test an introduction of antigen into the host is involved which increases the infection danger, moreover, accurate estimation of the hypersensitive state is not possible as several body factors as blood circulation, body temperature physical activity and simultaneous humoral reactions may alter the course of CMI reaction. Several in vitro systems have been developed to monitor the function of immunocompetent lymphocytes since these are the cells (along with macrophages) involved in the effector limb of CMI. Thus in the present study an in vitro correlate technique for amoebiasis has also been applied with skin test for better understanding of CMI response in amoebiasis.

**Studies on Migration Inhibition Test**

Recent reports indicate the potential usefulness of in vitro correlates of cell mediated immunity in man (Rocklin et al. 1970). Their advantage over the skin test is that they can be easily quantitated and no entrance of antigen in the host system is involved.
There are two separate methods for performing migration inhibition assays. Two populations of cells namely - lymphocytes and macrophages are involved in this test.

In the direct method, the whole procedure takes place in an incubating chamber. Here sensitized lymphocytes present in the packed cells of the capillaries get stimulated in presence of antigen and they liberate lymphokine namely migration inhibition factor in the medium. This lymphokine in turn inhibits the migration of macrophages from capillary tubes (Ortiz, Ortiz, 1973a).

In the indirect method, sensitized lymphocytes from any immune competent source are cultured with antigen for 24 hours. The culture supernatants containing lymphokine MIF is added in the chamber containing cells packed capillary filled with incubating medium. Here this lymphokine inhibits the migration of cells. Here lymphokine and macrophages can be obtained from different species (Gold et al, 1978).

In the present study, direct method was used for testing the MIF. It was found that with E. histolytica antigen migration inhibition test was 50% on day 8 post sensitization which increased to 90% on day 90 post sensitization. (Table XX).

These results are in agreement with the work of Ortiz. Ortiz et al. (1973a).

In the present investigation a correlation between skin test and macrophage migration inhibition test was found as both the tests were positive on day 8 as compared to humoral antibodies which appeared later in the system.

Studies on Precipitin Test

It was found that precipitin antibodies appear in serum after 15 days post-sensitization. The concentration of precipitin was highest in the sera of animals sensitized
with 200 µg of E. histolytica antigen and 50 µg of free-living amoebae antigen. These precipitins persisted throughout the period of experiment. There was no trace of detectable precipitin antibodies, on 8th day post sensitization except in sera of guinea pigs sensitized with 50 µg of A. culbertsoni. (Tables XXII, XXIII, XXIV).

These results reveal that precipitin antibodies appear later in the immune response and they also become lower in concentration after some time for all the three amoebae as against early institution and persistence of cellular immunity. This shows that there is no apparent correlation between appearance of precipitin antibodies and stimulation of cell mediated immune response as judged by skin testing and migration inhibition test.

Studies on IHA Test

In the present study it was found that agglutinin antibodies appeared in the serum of sensitized guinea pigs 8 days post sensitization, although their titer was very low. These agglutinins persisted throughout the period of experiment for all the three amoebae.

Cross Reactivity Among the three pathogenic amoebae

It was found in the present study that there is no cross reactivity among the three pathogenic amoebae as judged by the four immunological tests (skin test, M.I. test, IHA and GDP) Tables XIX, XXI, XXV, XXIX. This shows that three amoebae namely E. histolytica, N. aerobia, and A. culbertsoni are dissimilar in their antigenic reactivity, more over this negative cross reactivity is evident for the specificity of these immunological tests applied.

Immunological studies in infected animals

It was observed that cell-mediated immunity do not play any important role in protection of experimental animals against the infection of A. culbertsoni or N. aerobia as revealed by faint skin test in infected guinea pigs.
observed on fourth day post infection and later disappeared. Similarly mice infected with live trophozoites of N. aerobia did not show any significant foot pad reaction (Table XXXIII). These findings suggest that suppression of CMI might be the one of causes for 100% and early fatality of these infections in experimental animals.

However, humoral immunity was developed in guinea pigs infected with either N. aerobia or A. culbertsoni. Though only agglutinins could be detected by IHA test (Table XXXIII) and precipitins were not found when sera of infected guinea pigs were tested in gel diffusion precipitin test against homologous antigens. Thus it can be inferred from present investigation that IHA can be employed as a diagnostic tool for early detection of these fatal infections, while gel diffusion precipitin test was found not suitable.

Studies on pathogenicity of free-living amoebae in experimental animals

In the present study we found that both A. culbertsoni and N. aerobia are highly pathogenic to mice and guinea pigs inoculated either intranasally or intracerebrally in mice and intranasally in guinea-pigs.

They invade the brain tissue extensively as revealed by the histopathology of brain (Fig. XVII). In N. aerobia infections varying degree of necrosis and haemorrhage seen. Focal areas of demyelination have been observed. In some areas clusters of amoebae were seen with little or no cellular reaction.

In Acanthamoeba culbertsoni infection focal necrosis, haemorrhages and abscess formation have been observed in mouse brain. Chronic inflammatory cells predominate with granulomatous reactions and multinucleated giant cells. Martinez et al. (1980) have reported aneurysm formation. Both trophozoites and cysts were found within the white
and grey matter without any reaction around them.

Many workers (Culbertson 197, Cerva, 1971b, Carter, 1972, Duma, 1972, Jadin 1973, Singh & Das, 1970) have shown the invariable pathogenicity of these species of amoebae. Culbertson et al. (1972) obtained cellulocutaneous invasion and generalized visceral lesions in guinea-pigs by subcutaneous or intramuscular injections of several thousand N. fowleri. In the present study, guinea pigs were fatally infected with both N. aerobia & A. culbertsoni (axenic) and no cellulocutaneous lesions were observed although an eye infection was seen in some guinea pigs but it was healed up after few days. Singh (1975) had also reported fatal infection of guinea-pigs but he used bacteria associated amoebae.

In the present study, it was found that mice from 10g to 20g show hundred percent mortality and mice weighing more than 20g were not very much affected as only 50-60% mortality was observed. Use of 10-12g mice had been suggested by many workers for study of pathogenicity in experimental infections (Misra & Sharma, 1980) have reported that mice weighing more than 15g were not suitable for testing pathogenicity although in present experiment it was found that mice of 15-20g weight showed 100% mortality with pathogenic strains of N. aerobia (ATCC-73) and A. culbertsoni (A-1).

Serological studies in amoebic cases and random population

1. IHA test -

Result of the present study indicate that indirect haemagglutination test possesses a high degree of specificity for diagnosis of amoebic liver abscess patients. A positive IHA test in 100% of the 14 amoebic liver abscess cases (Group IV) showing 2623.0 GMRT compared well with findings of Kessel et al. (1965), Milgram et al. (1966), Krupp (1970) and Sharma et al.
who obtained 100, 96, 87 and 100% positive IHA tests respectively, among amoebic liver abscess cases.

The specificity of the test is further showed by results obtained in the non-amoebic hospital patients and healthy subjects. Amongst 26 hospital patients (Group I) who did not present any laboratory or clinical evidence of amoebiasis only 1 gave positive IHA results, indicating usefulness of this test in that, it is not influenced by the presence of other non-amoebic pathogens protozoa, helminths or bacteria in the gut (Krupp, 1970, Vinayak et al. 1974, Sharma et al. 1978a). Similarly among 36 healthy subjects, Group IV) only 2 subjects were found to be positive in IHA test. Asymptomatic intestinal amoebiasis cases also showed insignificant results in IHA showing absence of antibodies in these cases (Table XXXIV).

**Immunoglobulins levels**

The results of present investigation reveal that of all the immunoglobulins IgG, is most affected in amoebic liver abscess cases, whereas levels of immunoglobulins in asymptomatic intestinal amoebiasis cases were not altered significantly from the normal subjects. These findings are in agreement with results of Ganguli et al. (1978) and Abioye et al. (1972) who also reported elevated IgG levels in ALA cases. However, Ravi et al. (1975) did not find increased levels of IgG in patients with amoebiasis, but did find increased levels of IgM and IgA. The elevated IgG levels can be partly attributed to higher amoebic antibody titers as indicated by high reciprocal IHA titers obtained in sera of amoebic liver abscess cases, as compared to insignificant IHA titers in healthy as well as in asymptomatic intestinal amoebic cases in the present investigation (Table XXXVI). It has been reported that passive cutaneous anaphylactic, haemaglutinating, complement fixing, immuno precipitating and immobilizing activities reside in the IgG fractions (Yap et al. 1969, Maddison et al. 1968). Moreover, the intimate involvement of IgG
in amoebic liver abscess cases is proved by the rapid fall in its concentration during chemotherapy.

Serum proteins

It was observed in the present investigation, that there was a significant change in serum protein of amoebic liver abscess cases examined. A decrease in albumin concentration, i.e. hypoalbuminaemia and increase in globulin concentration, i.e. hyperglobulinaemia was present (Table XXXVII). These findings are consistent with findings of El Zaydi et al. (1976), Kamat et al. (1968), Santhangopalan et al. (1964) and Powell (1959).

According to El Zaydi et al. (1976) hypoalbuminaemia might result from hepatic cell necrosis, fever, vomiting, anorexia which accompany liver abscess. Powell (1959) attributed the disturbance of protein metabolism to the proteolytic enzymes of E_histolytica.

In the present investigation $\gamma_1$, $\alpha_1$ and $\alpha_2$ globulins fractions were highly elevated while $\beta$ globulin was not significantly affected. Significant rise in gamma globulin could be attributed to acquisition of immunity, as a part of gamma globulin comprises specific amoebic antibodies. Santhangopalan (1964) et al. have ascribed the raised gamma globulin to serum reaction.