PHASE II

DISCUSSION
Phase I of the study highlighted the identification of a major 58 KD amoebic polypeptide of E. histolytica. This was one of the three amoebic polypeptides present in circulating immune complexes (CICs). Phase II of the study was carried out with a view to develop monoclonal antibody directed at 58 KD amoebic polypeptide for its potential use in detecting 58 KD antigen in sera of amoebic patients in order to differentiate an ongoing clinical amoebic disease from a past exposure.

Several monoclonal antibodies were produced by fusion of PAIOP3 cells and sensitized splenocytes obtained from CAE immunized Balb/c mice. The hybridization experiments resulted in successful fusion leading to development of growing stable hybrids in 14(19.4%) of the 72 wells (Table 1). Of these 6 (42.8%) were found to be secreting anti-amoebic antibodies (Table II) and amongst these, 2(33.3%) secreted anti 58 KD antibodies (Table II). The hybrid cells these from two wells (P1P4 and P2C2) were cloned by the limiting dilution procedure (Table III). Finally, anti 58 KD antibody secreting clones were selected. One clone P1D11 (labeled as C0) that gave the highest ELISA OD value with affinity purified 58 KD antigen was selected for further study. This clone was expanded as ascites and the ascitic antibody had a titre of 1:64 in ELISA (OD > 0.5). The immunoglobulin isotyping of ascitic MoAb from clone C0 revealed the antibodies to be of IgG1 isotype (Fig.15) confirming the monoclonality of the ascitic antibody. The monoclonal antibody secreted by clone C0 recognized 58 KD amoebic polypeptide in western immunoblot of CAE. This MoAb also shared epitopes with 29 KD antigen (Fig. 16). The specificity of MoAb C0 in recognition of a 58 KD amoebic antigen was also indicated by failure of MoAb to react with crude sonicated extract of G. lamblia trophozoites or promastigotes of L. donovani in an ELISA (Fig. 17). The surface localization of 58 KD molecule recognised by MoAb C0 was evidenced by a strong surface fluorescence of live amoebic trophozoites. Permeabilisation of surface integrity of amoebic trophozoites with acectone fixation again revealed fluorescence in only
surface membrane (Fig. 18b). The absence of fluorescence in any cytoplasmic component (which in fact was stained red with counter stain, Evan’s blue), confirmed the distribution of 58 KD molecule on the surface membrane of the parasite. Inability of MoAb C6 to react with spent TYI-S medium or horse serum suggests that 58 KD antigen being recognised by MoAb C6, is an integral component of *E. histolytica* trophozoites and not a non-specific material or protein merely attached on to the surface of the parasite from the culture medium. Therefore 58 KD molecule is a surface localized amoebic material. Earlier studies by investigators have indeed identified several surface localized antigens of *E.histolytica*. These include a 220 KD protein (Meza *et al.*, 1987), 170 KD lectin (Petri *et al.*, 1987), 112 KD protein (Arroyo and Orozco, 1987), 96 KD protein (Torian *et al.*, 1987), 30 KD antigen (Tachibana *et al.*, 1990; Blakely *et al.*, 1990) and 29 KD antigen (Shandil and Vinayak, 1992). Most of these molecules have been shown to be immunogenic and have also been used in serological procedures. Some of these surface localized antigens like 220 KD, 170 KD, 112 KD, 96 KD, 30 KD and 29 KD have been shown to be playing crucial role in identification and subsequent invasion of target cells and are thus important in understanding pathogenesis of the disease.

The involvement of the 58 KD amoebic antigen in invasive amoebic disease process has been suggested in the Phase I of the present study. The presence of this antigen in circulation in ALA patients and development of humoral immune response by human host against this 58 KD antigen supports immunogenic nature of this molecule.

The data of the present study indicated recognition of 15 (88.2%) of the 17 cases of ALA by affinity purified 58 KD antigen. Lack of recognition of this antigen by the serum samples of 2 ALA patients may be attributed to a) the presence of comparatively lower undetectable levels of anti 58 KD antibodies in these subjects b) failure of these subjects to mount antibody response or c) because of complex mosaic of
amoebic antigens, there may be competitive inhibition of receptors of 58 KDa repertoires by the host.

The significance of detectable levels of anti 58 KD antibodies in an individual case as a diagnostic/confirmatory test remains unclear since the persistence of such antibodies following 'cure' of ALA cases has yet not been worked out. Moreover detection of anti 58 KD antibodies in 2 cases from non amoebic hepatic disorders cases casts reservations for considering presence of 58 KD antibodies as diagnostic markers. These subjects probably had an earlier exposure with amoebae leading to the development and persistence of anti 58 KD antibodies. Eight samples from clinically suspected ALA cases also had anti 58 KD antibodies. Six of the 8 patients responded well to the anti-amoebic chemotherapy, metronidazole, confirming that these (six cases) were indeed the cases of ALA. The explanation for detectable levels of anti 58 KD antibodies in the sera of other suspected ALA cases could again be a past amoebic exposure.

Circulating antibodies to the 58 KD neutral thiol proteinase in at least 80% of the patients with invasive amoebic disease has been documented (Reed et al., 1989). In another investigation, Joyce and Ravdin (1988) identified a 59 KD mannose containing glycoprotein in amoebic extracts that was recognised by immune human sera from 11 patients cured of amoebic liver abscess (Joyce and Ravdin, 1988). This means that, even after the cure of ALA, antibodies persisted. Thus, it is felt that mere detectability of anti 58 KD antibodies in the sera of amoebic patients may not be an index of an ongoing infection and it is inappropriate to use the same as a diagnostic marker.

Further investigations were carried out to assess the detectability of 58 KD antigen in the dissociated CIC from serum of amoebic patients and controls. Two types of direct antigen detection systems were developed. In the first, 58 KD monospecific antibodies raised in rabbits, and in the second, ascitic monoclonal antibody from clone C6.
were employed as "detecting antibody". In both the assay, 58 KD antigen in dissociated CIC from serum samples of confirmed ALA cases was detected. Failure to detect 58 KD antigen in serum samples from patients with non-amoebic hepatic disorders indicates the specific nature of either of these assay systems (specificity = 97.4%). The reason for the detection of 58 KD antigen in CIC of one of the healthy subjects is rather not clear. Although no explanation can be offered at the movement, the most likely reason for such an observation may be related to this subject having had onset of an early amoebic disease at the time when the sample was collected. It would, however, have been ideal to follow up this case. It has earlier been observed that amoebic specific CIC disappear or decline following treatment.

It has been immunoreactivity of the 58 KD molecule upon treatment with heat, trypsin, pronase and sodium metaperiodate oxidation suggested glycoproteinic nature of this molecule. Interestingly, the mannose rich 59 KD molecule identified by Joyce and Ravdin (1988) was also a glycoprotein. The clinical significance in terms of immunoreactivity of 58 KD molecule as reported in our work and 59 KD molecule of Joyce and Ravdin (1988) is clearly indicated because either or both of these molecules are recognized by cases of ALA. It is felt that the 58 KD molecule identified in the present study may be identical to 59 KD molecule of Joyce and Ravdin (1988). The significance of 58 KD/59 KD molecule in terms of diseases processes can be discussed as follows.

Investigations have indicated that the virulence, or the ability of the axenically cultured *E. histolytica* correlates with the presence of proteolytic enzymes found on the surface of amoebae, in secretions or in extracts of whole trophozoites (Gadasi and Kessler, 1983; Gadasi and Kobiler, 1983; Munoz *et al*., 1984; Keene and Mackerrow, 1984; Lushbaugh *et al*., 1984; Leshbaugh *et al*., 1985). Keene *et al*.

(1986) have identified
a 56 KD neutral proteinase that is secreted by *E. histolytica* trophozoites and could be identified in the soluble fraction of trophozoite lysate also. The proteinase mediated detachment of epithelial cells from the bowel mucosa may be an important pathogenic mechanism in amoebiasis (Keene *et al.*, 1986). In a subsequent study Keene *et al.* (1990) have shown 56 KD to be cysteine (thiol) proteinase and showed that expression and release of the cysteine proteinase is an important factor in producing cytopathic effect presumably by its degradation of cell anchoring proteins (Keene *et al.*, 1990). Another study has indicated that 56 KD protease is immunogenic in host and its expression correlates with invasive amoebiasis (Reed *et al.*, 1989).

Investigations relating to the proteolytic activity of molecule identified in the present study with molecular mass of 58 KD have not been carried out, but its involvement in the invasion of gut mucosa by amoebic trophozoites could be speculated.