Antibody-dependent macrophage-mediated cytotoxicity against *Entamoeba histolytica*

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Summary. Interactions between trophozoites of *Entamoeba histolytica* and peritoneal exudate macrophages from unsensitised and antigen-sensitised animals were studied *in vitro*. Normal macrophages killed trophozoites to some extent. This killing capacity was enhanced by prior sensitisation of the animals with specific antigen. Incorporation of anti-amoebic antiserum in the amoeba-macrophage mixture greatly enhanced the killing capacity of macrophages. Fraction one (F-I) of a crude amoebic extract was most effective in enhancing the cytotoxicity of macrophages by prior sensitisation and anti-F-I serum was the most effective antiserum. The cytotoxicity-inducing capacity of the immune serum resided in the IgG but not in the IgM fraction.

Introduction

Cellular immunity plays an important role in amoebic infections (Savanat *et al.*, 1973; Ortiz-Ortiz *et al.*, 1975; Harris and Bray, 1976; Jain *et al.*, 1980; Vinayak *et al.*, 1980), whereas humoral immune responses have little influence on the outcome of the disease (Vinayak *et al.*, 1981). Although macrophages are important in host defence against various bacterial infections (Mackaness, 1962; Ruskin, *et al.*, 1969; Behin *et al.*, 1979), their role in amoebic infection has not been well documented. Recently, Ghadirian and Meerovitch (1982a, 1983) suggested that macrophages may be important in host defence against hepatic amoebiasis. We have already reported on the cytotoxic effect of lymphocytes against *Entamoeba histolytica* in association with anti-amoebic serum (Vinayak *et al.*, 1984). Because macrophages also participate in antibody-dependent cellular cytotoxicity against various parasites (Capron *et al.*, 1982), we studied the ability of macrophages to destroy *E. histolytica* in the absence or presence of anti-amoebic serum.

Materials and methods

Trophozoites

E. histolytica (NIH:200) was grown in TPS-I medium (Diamond, 1968) under axenic conditions.

Antigens

Crude amoebic extract (CAE) was prepared by sonicating washed trophozoites from 48-h cultures for 5 min at 23 K Hz in the cold. Sonicated material was centrifuged at 10 000 *g* for 30 min and the clear supernate labelled CAE.

Fractionation of CAE

CAE was fractionated as described by Sawhney *et al.* (1980). The highest mol.-wt fraction (F-I; mol.-wt 650 000) was used for further study.

Immunisation

Three- to four-week-old guinea pigs were immunised with CAE and F-I as described previously (Vinayak *et al.*, 1984). Blood samples were collected on completion of the immunisation schedule just before killing the animals. The anti-amoebic antibody titres were determined by the method of Prakash *et al.* (1970).

Peritoneal exudate macrophages

Sixty ml of Iscove's MEM (Gibco, USA) were injected into the peritoneal cavities of guinea pigs. The abdomen of each animal was gently massaged and fluid withdrawn with a siliconised syringe. The peritoneal cells were harvested by centrifugation at 350 *g* for 10 min and resuspended in a small volume of MEM containing 10% normal heat-inactivated guinea pig serum. Cells (5×10^5) were seeded in each well of specially designed chambers prepared by fixing coverslips to one side of a hole (1 cm diameter) in a microscope slide. The well was completely filled with the medium and sealed with another coverslip. The chambers were incubated in air plus CO₂ 5% at 37°C for 1 h. Non-adherent cells were removed by washing

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with MEM and the chambers re-incubated for 3 h. The macrophages formed a monolayer on the lower cover-slips.

Cytotoxicity assays

The cytotoxic potential of sensitised and unsensitised macrophages was studied by incubating trophozoites of *E. histolytica* with macrophages in a fixed ratio (1:50), with or without anti-amoebic serum (Anti-CAE or Anti-F-I), for 4 h at 37°C in air plus CO₂ 5%. Trophozoites alone (with and without serum) were used as controls. After incubation the percentage of viable trophozoites was determined by a trypan blue dye exclusion method and phase-contrast microscopy (Guerrero *et al.*, 1976); 20 microscope fields ($\times 20$) were counted at random.

Effect of 2-mercaptoethanol

To determine the nature of the immunoglobulin involved in the cytotoxicity reaction, 50 μ l of 0.2 M 2-mercaptoethanol was mixed with 50 μ l of immune serum and added to the amoeba-macrophage mixture to give a final concentration of 0.03 M.

Isolation of IgG and IgM

IgM was isolated from anti-CAE and anti-F-I by chromatography on Sephadex G-200 (Lamon *et al.*, 1975). The first half of the first fraction was pooled and the protein precipitated with 45% ammonium sulphate. The ammonium sulphate was removed with Sephadex G-25. IgG was isolated by DEAE-cellulose ion-exchange chromatography (Hudson and Hay, 1980), followed by ammonium sulphate precipitation. Both IgM and IgG were checked for purity and activity and their cytotoxicity-inducing capacity determined.

Results

Cytotoxicity of macrophages

The table shows that $23.9 \pm 2.5\%$ of trophozoites were killed by unstimulated macrophages. Stimulation of macrophages *in vivo* with specific antigens resulted in enhanced killing capacity. CAE-stimulated macrophages killed $40.37 \pm 2.0\%$ amoebae and F-I-stimulated macrophages killed $61.0 \pm 3.5\%$ amoebae (table). The cytotoxic potential of macrophages was significantly enhanced when anti-amoebic antiserum was added to the amoeba-macrophage mixture. Almost all trophozoites ($99.08 \pm 1.82\%$) were killed by the combination of unstimulated macrophages and anti-F-I serum. Similarly, F-I stimulated macrophages killed $100 \pm 1.5\%$ of amoebae in the presence of anti-F-I serum (table). Inactivation of antiserum did not

Table. Killing of trophozoites by unstimulated and antigen-stimulated macrophages in the absence or presence of anti-amoebic serum

Effector cells	Percentage ($\pm$ s.d.) of trophozoites killed in the presence of		
	NGPS	Anti-CAE serum	Anti-F-I serum
Unstimulated macrophages	23.9 ± 2.5	46.33 ± 1.10	99.08 ± 1.82
CAE-stimulated macrophages	40.37 ± 2.0	70.12 ± 1.25	N.D.
F-I-stimulated macrophages	61.0 ± 3.5	N.D.	100 ± 1.5

N.D. = Not Done.

NGPS = Normal Guinea Pig Serum.

Fig. 1. Capacity of isolated immunoglobulins from anti-CAE to induce cytotoxicity by peritoneal exudate macrophages: normal guinea-pig serum (□), anti-CAE (▨), 2-mercaptoethanol-treated anti-CAE (■), IgG (▩) and IgM (░).

alter the ability of anti-amoebic serum to induce cytotoxicity of macrophages.

Nature of antibodies

2-Mercaptoethanol had no effect on the capacity of anti-amoebic serum to induce cytotoxicity (figs 1

Fig. 2. Capacity of isolated immunoglobulins from anti-F-I to induce cytotoxicity by peritoneal exudate macrophages: NGPS (□), anti-F-I (▨), 2-mercaptoethanol-treated anti-F-I (■), IgG (▨) and IgM (●).

Fig. 3. Immunoelectrophoresis of isolated IgG and IgM from anti-CAE against R/A/GPS. (A) IgG, (B) IgM, (C) NGPS.

and 2). Reasonably pure IgG and IgM was isolated from anti-CAE and anti-F-I (fig. 3). The ability of anti-amoebic serum to induce cytotoxicity was contained in the IgG fraction; killing in the presence of IgG was similar to that in the presence of whole serum ($44.77 \pm 1.27\%$ for IgG anti-CAE and $99.62 \pm 0.76\%$ for IgG anti-F-I). Figs 1 and 2 show

Fig. 4. Interaction between macrophages and *E. histolytica* in the presence of anti-F-I. Many macrophages can be seen attached to the trophozoite ($\times 1500$).

that IgM did not induce cytotoxicity of macrophages.

Physical contact between macrophages and trophozoites was required to induce killing by the former (fig. 4) and more than one macrophage attached to a trophozoite in the presence of anti-F-I serum.

Discussion

High levels of anti-amoebic antibodies have been reported in cases of hepatic amoebiasis (Kessel *et al.*, 1965; Prakash *et al.*, 1970; Vinayak, 1975). However, anti-amoebic antibody titres showed little or no correlation either with virulence indices or with severity of the disease (Vinayak *et al.*, 1981) indicating that humoral immunity alone had little or no role to play. On the other hand, cellular immunity is depressed during amoebic infection (Ortiz-Ortiz *et al.*, 1975; Jain *et al.*, 1980) and experimental immunosuppression with steroids and anti-lymphocytic serum resulted in more severe lesions in animals (Vinayak *et al.*, 1982). Because a high degree of protection is achieved by immunisation with partially purified antigens (Krupp, 1974; Vinayak *et al.*, 1980), and vaccination not only augments antibody production but also primes the effectors of cellular immunity, there may be an association between the two arms of the host defence system. We found that unstimulated macrophages killed amoebic trophozoites *in vitro*. The killing capacity of the resident macrophages was greatly enhanced by antigen sensitisation or by addition of anti-amoebic serum. The F-I fraction of CAE was the most effective inducer of macrophage cytotoxicity by prior sensitisation and by anti-F-I serum. This *in-vitro* cytotoxic potential correlated well with protection studies with F-I (Krupp, 1974; Vinayak *et al.*, 1980).

Peritoneal exudate cells of vaccinated animals are not phagocytosed by amoebae but kill them (Ghadirian and Meerovitch, 1982b). Similar result were obtained in our experimental system. However, we have shown that macrophage cytotoxicity could also be induced by anti-amoebic serum. Furthermore, this capacity of the immune serum resided in the IgG fraction—2-mercaptoethanol treatment did not alter it and purified IgG was equally effective. Involvement of IgG in antibody-dependent cellular cytotoxicity against parasites has been shown previously (Capron *et al.*, 1982).

The existence of Fc receptors of IgG on macrophages has been well documented (Unanue, 1981). It is conceivable that the IgG of an immune serum binds to the surface of the trophozoite by its Fab portion and to the macrophage by its Fc portion, thus signalling the macrophage to kill the trophozoite. It seems that attachment of macrophages to the trophozoite is necessary to induce cytotoxicity. It was observed that in the presence of anti-F-I serum a large number of macrophages attached to trophozoites, often completely surrounding them,

and that the absolute number of trophozoites (dead and alive) was significantly decreased ($p < 0.001$) after incubation for 4 h with macrophages in the presence of anti-F-I serum. Trophozoites were disintegrated, sometimes beyond recognition, by macrophages and it is thought that macrophages break down trophozoites before engulfing them.

We believe that specific anti-F-I antibodies may be important, in association with effector cells of the immune system, in combating invading trophozoites of *E. histolytica*. Specific cell-mediated immune responses are depressed after amoebic infection (Jain *et al.*, 1980) and it is probable that a deficiency in one arm of the immune system is responsible for the effectiveness of *E. histolytica* as a parasite.

It has been shown that patients with hepatic amoebiasis have circulating immune complexes in their serum (Pillai and Mohimen, 1982). These may bind to the Fc receptors on the macrophages, rendering them unavailable for binding to IgG of immune serum. This is being studied in our laboratory.

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