Immunoprotective behaviour of plasma-membrane-associated antigens of axenic *Entamoeba histolytica*

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Summary. Immunisation of golden hamsters with plasma-membrane-associated antigens of a virulent subline of axenic *Entamoeba histolytica* strain NIH 200 V, entrapped in multilamellar phosphatidylcholine liposomes (MPL) or Freund's complete adjuvant (FCA), afforded protection against intrahepatic challenge with axenic amoebic trophozoites of the same strain. Amoebic liver abscess developed in 86% and 80% of the animals that received empty liposomes or buffer emulsified in FCA but in none of the animals that received plasma-membrane-antigen vaccines. All the immunised animals had significantly higher levels ($p < 0.001$) of antibodies to plasma-membrane components and significantly higher levels ($p < 0.001$) of cellular sensitisation. Antibody-dependent macrophage-mediated cytotoxicity against trophozoites was also found to be significantly greater ($p < 0.001$) in immunised animals. Liposome-entrapped antigens stimulated the immune system of the host as well as, or better than, antigens administered with FCA.

Introduction

It has been estimated that 480 million people carry *Entamoeba histolytica* in their intestinal tracts (World Health Organization, 1985). Untreated amoebic liver abscess or fulminating colitis may be fatal and there is no acceptable chemoprophylaxis for amoebiasis. However, rodents can be immunised against intrahepatic challenge with pathogenic isolates of *E. histolytica* (Ghadirian *et al.*, 1980; Gold *et al.*, 1982; Sharma, A. *et al.*, 1984; Purnima *et al.*, 1986). A crude extract of *E. histolytica* and a partially purified fraction, F-I, can protect against intestinal challenge with amoebae (Krupp, 1974; Vinayak *et al.*, 1980b; Sharma, A. *et al.*, 1984). Other trophozoites fractions, notably RNA (Sharma, G. L. *et al.*, 1984; Vinayak *et al.*, 1984b) and ribosomal and lysosomal fractions of *E. histolytica* (Arroyo-Begovich, 1978), also induce protective immunity.

Surface membrane antigens of *E. histolytica* have been demonstrated by immobilisation of trophozoites by immune serum (Cole and Kent, 1953), by surface binding of fluorescence-labelled immune sera (Biagi *et al.*, 1966) and by antibody-mediated lysis of trophozoites by complement (Ortiz-Ortiz *et al.*, 1978, Huldtt *et al.*, 1979). Twelve major proteins have been identified in the plasma membrane by

sodium dodecyl sulphate polyacrylamide gel electrophoresis (Aley *et al.*, 1980, Aust Kettis *et al.*, 1983). Plasma membrane antigens are important because they are the first to interact with the host immune system, but their potential to protect against amoebic disease has not been evaluated. In the present study we have investigated the ability of membrane-associated antigens of a virulent subline of axenic *E. histolytica* to protect hamsters against experimental hepatic challenge with amoebae.

Materials and methods

Parasite

Axenic *E. histolytica*, strain NIH200, was grown in TPS-I medium (Diamond, 1968). A subline of this culture which was subcultured more than 100 times in cholesterol incorporated TPS-I medium (Bos and Van de Griend, 1977) was designated *E. histolytica* NIH200 V. This subline exhibited enhanced virulence and produced infection in >70% of hamsters upon intrahepatic challenge (Purnima *et al.*, in press).

Preparation of plasma membrane

Membranes were prepared essentially as described by Aley *et al.* (1980). Briefly, trophozoites of *E. histolytica* NIH 200 V, harvested from 48-h cultures, were washed three times with physiological saline and were exposed

to an equal volume of concanavalin A (Con A) 1 mg/ml. The trophozoites were broken up with a hand-held glass homogeniser to provide a crude amoebic extract. The crude extract was layered on a discontinuous gradient consisting of 9% mannitol-25% sucrose and centrifuged at 250 *g* for 30 min at 4°C. The pellet containing the membrane sheets was treated with 1M α -methyl mannose to remove Con A and was subsequently re-homogenised by hand. The resulting vesiculated plasma membrane was concentrated by centrifugation in 25% sucrose. The purity of the membrane fraction was assayed by estimation of membrane-bound Ca^{++} -dependent ATPase activity (Kurebe, 1979) as modified by McLaughlin and Müller (1979). The protein content was estimated by a modification of Lowry's method (Lees and Paxman, 1972).

Preparation of anti-membrane antiserum

Antiserum against the membrane antigens was raised in three hamsters. Each animal received four subcutaneous injections at intervals of 4 days of 0.4 mg (0.25 ml) of plasma membrane protein emulsified in an equal volume of Freund's complete adjuvant (FCA). Animals were bled to death 7 days after the last injection and sera were separated. The pooled sera had an anti-plasma membrane indirect haemagglutinating antibody titre of 2048.

Preparation of liposomes

Multilamellar phosphatidylcholine liposomes (MPL) were prepared by the method of Van Rooijen and Van Nieuwmegen (1980) as modified by Vinayak and Sharma (1986). Briefly, 150 mg of egg lecithin, i.e., phosphatidylcholine (Council of Scientific and Industrial Research Centre for Biochemicals, New Delhi, India), was dissolved in 25 ml of chloroform and evaporated in a round-bottomed flask by rotary vacuum evaporation at 37°C. The thin film on the walls of the flask was dispersed by gentle shaking for 10 min in 3.3mM phosphate-buffered saline, pH 7.2 (PBS 7.2) containing 10 mg of plasma membrane protein/ml. The suspension was kept at room temperature for 2 h and was then subjected to ultrasonic disintegration (M.S.E. Scientific Instruments, Crawley, Sussex), at 23 kHz for 45 s in ice. The liposomes were centrifuged at 100 000 *g* at 4°C and washed three times in PBS 7.2. The multilamellar character of the liposomes was confirmed by phase contrast microscopy. The amount of antigen entrapped in the liposomes was determined by a modification of the Lowry method in which the liposomes were solubilised with sodium dodecyl sulphate (Lees and Paxman 1972).

Immunisation of hamsters

Syrian golden hamsters (2–3 weeks old) (Disease-Free Small Animal House of Haryana Agricultural University, Hisar, India) were divided into 5 groups: group I (7 animals) received 0.2 ml of MPL containing 0.4 mg of plasma-membrane protein; group II (7 animals) received

0.2 ml of MPL prepared with PBS 7.2; group III (6 animals) received 0.4 mg (0.25 ml) of plasma-membrane protein emulsified in an equal volume of FCA; group IV (5 animals) received PBS emulsified in FCA; group V (4 animals) were unimmunised and uninfected controls. Groups I to IV each received three subcutaneous injections at 4-day intervals.

All the animals were bled from the retro-orbital plexus 7 days after completion of the immunisation schedule. All animals of groups I–IV were then challenged intrahepatically with 6×10^6 cholesterol-fed trophozoites of *E. histolytica* NIH200 V after laparotomy. Seven days later all animals were again bled by cardiac puncture and killed by ether narcosis. The livers were examined for amoebic lesions and portions of the tissue were fixed in formalin 10% for histological examination.

Immunological investigations

The functional aspect of peritoneal macrophages was studied by anti-membrane antibody-dependent macrophage-mediated cytotoxicity assay as described by Saxena *et al.* (1986).

Spleens were removed aseptically and the leukocyte migration inhibition (LMI) index was determined by the method of Maini *et al.* (1973) with plasma-membrane proteins as antigen and phytohaemagglutinin (PHA) as nonspecific mitogen. The anti-membrane antibody titres of serum samples collected after completion of immunisation (i.e., before challenge) and at the time the animals were killed were determined by a micro-haemagglutination test (Vinayak *et al.*, 1980a). The significance of the results was assessed by Student's *t*-test.

Results

Purity of isolated plasma membranes

The specific enzyme activity (Ca^{++} -dependent ATP-ase activity) of the crude extract of *E. histolytica* exhibited a mean value of 0.03 Units ($\mu\text{M P}_i$ released/min) in four experiments (range 0.01–0.07 U); the corresponding mean value for the plasma-membrane preparation was 0.13 U (range 0.07–0.24 U).

Protection in hamsters

Immunisation of hamsters with plasma-membrane antigens entrapped in MPL (group I) or given with FCA (group III) completely protected the animals against subsequent intrahepatic challenge with *E. histolytica* (fig. 1). Histologically, amoebae could not be demonstrated in the liver of any of these animals. However, six (86%) of the seven animals that received empty liposomes (group II) and four (80%) of the five animals given FCA without plasma-membrane antigens (group IV) developed amoebic liver abscess. Macroscopic lesions were evident in the livers of these animals

Fig. 1. Percentage protection against intrahepatic challenge with *E. histolytica* in animals immunised with: plasma membrane MPL (group I) empty MPL (group II); plasma membrane emulsified in FCA (group III); and buffer emulsified in FCA (group IV); group V were unimmunised and uninfected control animals.

and amoebae were also demonstrated histologically. The observed protection obtained in the hamsters that had received plasma-membrane antigens (i.e., in excess of the nonspecific protection observed in control groups II and IV) was 86% (group I) and 80% (group III) (fig. 1).

Immune responses

The mean $\log_2$ anti-membrane indirect haemagglutinating antibody titres of animals immunised with plasma membrane entrapped MPL was 7.6 (SD 0.9) at time of challenge with *E. histolytica* rising to 8.4 (SD 0.5) at the time the animals were killed. Similarly, the mean $\log_2$ titre in animals immunised with plasma-membrane antigen emulsified in FCA was 6.3 (SD 0.7) rising to 8.2 (SD 1.2) after challenge. The animals of controls groups II and IV had mean $\log_2$ anti-membrane antibody titres of 6.2 (SD 0.9) and 5.7 (SD 1.5) respectively, when the animals were killed (table I). These values were significantly lower ($p < 0.001$) than antibody levels in groups I and III.

The animals immunised with membrane antigens entrapped in MPL also had significantly higher ($p < 0.001$) levels of cellular sensitisation than control animals, as judged by inhibition of leukocyte migration. However, there was no significant difference ($p > 0.05$) between the levels of cellular sensitisation in animals immunised with membrane antigens entrapped in MPL or emulsified in FCA (fig. 2). Furthermore, the response to PHA was significantly depressed ($p < 0.001$) in control groups II and IV compared to unimmunised and uninfected controls (group V). Peritoneal macrophages obtained from infected animals of groups II and IV were also found to have a significantly reduced ($p < 0.001$) capacity to kill amoebic trophozoites *in vitro* either in the absence or in the presence of anti-membrane antiserum (fig. 3). In contrast, macrophages obtained from group-I animals killed 71.3 (SD 5.1)% and 77.3 (SD 4.4)% of axenic amoebic trophozoites in the presence of homologous serum obtained from the animals or anti-membrane hyperimmune serum, respectively.

Table I. Indirect haemagglutinating antibody titres in the sera of immunised and control animals

Group	Treatment	Titre after immunisation							Titre when animals were killed						
		<64	64	128	256	512	1024	Mean (log ₂)	<64	64	128	256	512	1024	Mean (log ₂)
I (n=7)	PM entrapped in MPL	0	1	2	3	1	0	7.6 (0.9)	0	0	0	4	3	0	8.4* (0.5)
II (n=6)	Empty MPL	6	0	0	0	0	0	0	2	1	3	0	0	0	6.2 (0.9)
III (n=6)	PM emulsified in FCA	0	0	2	1	3	0	6.3 (0.7)	0	0	1	2	2	1	8.2* (1.2)
IV (n=5)	PBS emulsified in FCA	5	0	0	0	0	0	0	2	1	0	1	0	0	5.7 (1.5)
V (n=4)	Unimmunised and uninfected	4	0	0	0	0	0	0	0	0	0	0	0	0	0

* $p < 0.001$ compared with groups II and IV.

Figures in brackets denote standard deviation.

Discussion

Earlier investigations have shown a high degree of protection by a partially-purified fraction (F-I) of *E. histolytica* (Krupp, 1974; Ghadirian *et al.*, 1980; Vinayak *et al.*, 1980b, Gold *et al.*, 1982; Purnima *et al.*, in press). Our data show that

plasma-membrane antigens of axenic *E. histolytica* NIH 200 V, entrapped in MPL, also afford a high degree of protection to hamsters.

Our data further revealed that anti-membrane antibody levels produced had a significant ($p < 0.001$) correlation with protection ($r = 0.98$). These anti-membrane antibodies appear to prevent the

Fig. 2. Correlation between inhibition of leukocyte migration and protection in the immunised and unimmunised animals, □ Percentage protection, ▨ percentage inhibition of leukocyte migration with plasma-membrane antigen, ■ percentage inhibition of leukocyte migration with PHA. Groups as in fig. 1. Bars indicate SEM.

Fig. 3. Correlation between anti-membrane antibody-dependent macrophage-mediated cytotoxicity and percentage protection in the immunised and unimmunised animals: □ percentage protection; ▨ percentage killing in the presence of normal hamster serum; ■ percentage killing in the presence of homologous serum; ▩ percentage killing in the presence of anti-plasma membrane hyperimmune serum. Bars indicate SEM.

establishment of infection in immunised animals. Such animals (groups I and III) developed high levels of anti-membrane antibodies after immunisation, but before challenge, while those that developed lesions (groups II and IV) had no anti-membrane antibodies at the time of challenge. Unimmunised animals did produce anti-membrane antibodies following the development of liver abscess, but the levels detected at the time the animals were killed were significantly lower ($p < 0.001$) than those observed in immunised animals. It is likely that levels of anti-membrane antibody that developed during the course of infection, together with other immune responses, notably cell-mediated immunity and antibody-dependent cellular cytotoxicity, may enable the host subsequently to clear the infection spontaneously.

Cell mediated immunity is known to play a major role in recovery from amoebic infection (Savanat *et al.*, 1973; Ortiz-Ortiz *et al.*, 1975; Harris and Bray, 1976; Vinayak *et al.*, 1980a). Observations in experimental models have indicated that cellular sensitisation stimulates the host to control the

infection (Vinayak *et al.*, 1980b; Sharma A *et al.*, 1984). Moreover, antibody-dependent lymphocyte-mediated cytotoxicity is effective in killing amoebic trophozoites *in vitro* (Vinayak *et al.*, 1984a). The present data indicate that immunisation with membrane antigens can stimulate high levels of cellular sensitisation, and show that macrophages obtained from immunised animals exhibit an enhanced capacity to kill amoebic trophozoites *in vitro*. Furthermore the depressed response to PHA amongst infected control animals correlated well with the failure of the macrophages to kill amoebic trophozoites *in vitro*. We therefore believe that specific antibody-dependent cell-mediated cytotoxicity, involving both the humoral and cellular arms of the immune response is likely to be the main mechanism for controlling infection.

Entrapment in liposomes effectively potentiated the immunogenicity of plasma-membrane proteins of *E. histolytica*; we have previously reported similar results with *Plasmodium yoelii* merozoite antigens (Vinayak and Sharma, 1986). Liposomes have an advantage over FCA in being biodegradable (Van

Rooijen and Van Nieuwmegen, 1980) and we observed no focal granulomas in animals given plasma membrane antigen entrapped in MPL. We thus feel that MPL can effectively replace FCA as an immunopotentiator.

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