Alterations of humoral, cell mediated and antibody dependent cell mediated cytotoxic responses during the course of amoebic infection in guinea pigs

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SUMMARY The cellular and antibody dependent cellular cytotoxic (ADCC) responses of splenic lymphocytes and peritoneal macrophages obtained from animals at variable intervals after inoculation were studied against trophozoites of axenic *E histolytica* (NIH:200). Cytotoxic responses of effector cells from infected animals were compared with those of effector cells from vaccine stimulated and unstimulated uninfected control animals. Cellular and antibody-dependent cellular cytotoxic responses of the effector cells from animals during the establishment and acute phase of infection were significantly suppressed, compared with unstimulated uninfected and vaccinated (FI amoebic proteins stimulated) effector cells. The effector cells from animals recovered from infection showed enhanced cytotoxic responses against trophozoites of *E histolytica*. The suppressed cytotoxic response was accompanied by impairment of cytotoxic cell activities and lack, or very low level of anti-FI antibodies in the sera of animals during the establishment phase of infection. With the rise in anti-FI antibodies in the sera of animals ADCC could be induced effectively against trophozoites of *E histolytica*, which seem to result in clearance of amoebic infection.

Amoebiasis is a disease caused by a protozoan parasite *Entamoeba histolytica*. It is world wide in distribution. According to a recent estimate,¹ 480 million people carried *E histolytica* in their intestinal tract. Of these, 48 million people suffered from acute amoebic disease. Amoebiasis thus is an important human parasitic infections specially in tropical and subtropical countries. Mass examination followed by selective chemotherapy can not be considered a solution to this problem. Immunological intervention, therefore, seems logical. A WHO expert group² in an informal meeting on 'Strategies for controls of amoebiasis' emphasised the urgency to elucidate cellular and humoral immune responses in relation to immunoprotection against amoebiasis.

Efforts have been made during last two decades to develop a suitable vaccine against *E histolytica*. Varying degree of success has been reported using crude amoebic extracts^{3,4} and a partially purified fraction (FI) of crude amoebic extract⁵⁻⁶ as vaccines in experimental animals. It is well known that an intense humoral immune response is mounted against amoebic antigens during hepatic infection⁷⁻¹⁰ whereas cellular immune responses have been shown to be suppressed.^{8,10,11} The importance of cell mediated immune responses is also evident from the results of experiments involving experimental immunosuppression of rats, guinea pigs and hamsters leading to exaggeration of amoebic lesions.¹²⁻¹⁴ Ghadirian and Meerovitch¹⁵ showed that immunity could be passively transferred with immune spleen cells and peritoneal exudate cells to normal hamsters. It is not yet clear, however, whether humoral or, cellular immune responses or a combina-

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Received for publication 19 March 1987.

tion of both would be required in controlling amoebic infection. Our recent investigation on antibody dependent cellular cytotoxicity (ADCC) against *E histolytica* *in vitro* have indicated that both humoral as well as cellular immune responses may be important in controlling amoebic infection.¹⁶⁻¹⁹ Antibody dependent cellular cytotoxicity, a manifestation of both cellular and humoral immune responses, has been shown to be operative against other parasites also.²⁰ Although the study of ADCC has provided new insights into immune defence against parasites, direct evidence of *in vivo* role of this mechanism is largely lacking. In order to assess the role of cellular and antibody dependent cellular cytotoxicity in development and control of amoebic infection, we studied the alteration of these effector mechanisms during the course of experimental amoebic infection in guinea pigs.

Methods

PARASITES

An axenic isolate *Entamoeba histolytica* (NIH:200) was used for preparation of antigens. The trophozoites of *E histolytica* (NIH:200) were used as target cells in the cytotoxicity assays. A polyaxenic, moderately virulent isolate of *E histolytica* (SB) was used for infecting animals.

ANTIGENS

Crude amoebic extract (CAE) of axenic *E histolytica* (NIH:200) was fractionated as described previously.²¹ The highest molecular weight (>650 000 d) fraction (FI) was used for immunisation of guinea pigs.

ANIMALS

Three to four weeks old guinea pigs free from natural infection were divided into six groups four of which were infected intracaecally after laparotomy with $80\,000 \pm 20\,000$ amoebic trophozoites of isolate of SB.²²

Group 1 (six animals): Uninfected and unimmunised control animals; group 2 (six animals): Immunised with FI emulsified with complete Freund's adjuvant; group 3 (six animals): Infected and killed three days postinoculation; group 4 (six animals): Infected and killed seven days postinoculation; group 5 (six animals): Infected and killed 14 days postinoculation; group 6 (six animals): Infected and killed 21 days postinoculation.

On the specific postinoculation day animals were bled by cardiac puncture and killed by ether narcosis.

IMMUNOLOGICAL INVESTIGATIONS

Anti-CAE and anti-FI-antiamoebic antibody titres

were determined using CAE and FI amoebic proteins as antigens respectively at various days postinoculation by the micro indirect haemagglutination (IHA) method.⁴ All antisera were heat inactivated at 56°C for 30 minutes. Leucocyte migration inhibition (LMI) responses to amoebic proteins (FI) and phytohaemagglutinin (PHA) was studied essentially as described by Jain *et al.*²³

COLLECTION OF EFFECTOR CELLS

Spleen cells were layered on Ficoll-paque (Pharmacia, Sweden) and centrifuged at 2000 rpm for 20 minutes. Mononuclear cells (MNC) were collected from the interface of gradient and the medium and washed twice with cold MEM. Cell concentration was adjusted to 10^6 /ml.

Resident macrophages were collected by injecting cold MEM into the peritoneum of guinea pigs and macrophage monolayers were prepared in specially designed chambers.¹⁹ The macrophages were characterised by giemsa staining, Fc receptor expression and acid phosphatase activity and found to be 98% pure.

CYTOTOXICITY ASSAY

The cytotoxicity assay was carried out by mixing effector cells – that is, splenic MNC or peritoneal resident macrophages and trophozoites of *E histolytica* as described earlier^{18,19} using trypan blue dye exclusion method. The cytotoxic potentialities of both the types of effector cells – that is, splenic MNC and peritoneal macrophages obtained from unimmunised uninfected, infected and FI stimulated animals were studied with normal guinea pig serum (NGPS), heat inactivated hyperimmune anti-FI antiserum and serum samples obtained from guinea pigs at various days post inoculation. Each assay was done in duplicate and the data were analysed by Student's *t* test.

PARASITOLOGICAL EXAMINATION

At the time of death, the caecum of each animal was examined for the presence of macroscopic lesions and the presence of trophozoites in the caecal contents. Caecal tissues with lesions were fixed in 10% formal saline, processed and stained both by H & E and PAS methods.

Results

COURSE OF AMOEBIC INFECTION

The animals infected with moderately virulent polyaxenic isolate of *E histolytica* were killed at days 3, 7, 14, and 21 postinoculation (PI). The parasitological examination revealed that the trophozoites were present in the ceecal content at day 3 PI in all the

animals. Histopathological examination of caecal tissues confirmed the presence of parasite which were found just invading the villi (Fig. 1a). Minimum to no invasion was observed at this stage. Animals killed at day 7 PI, however, not only had trophozoites in the caecal contents, the parasite had also invaded the gut epithelium (Fig. 1b) and the caecum showed typical amoebic lesions (Fig. 1c). At day 14 PI although the trophozoites were still present in the caecal content, minimum (35% of animals) to no invasion was observed. Trophozoites were absent from the caecal content as well as caecal tissues by day 21 PI (Fig. 1d) indicating the clearance of infection.

HUMORAL IMMUNE RESPONSE

Figure 2 shows anti-CAE and anti-FI antibody titres by IHA during the course of amoebic infection. The anti-amoebic antibodies were not detectable by IHA at day 3 PI. The anti-CAE and anti-FI-geometric mean titre (GMT) at day 7 PI were 45 and 14

respectively which increased to 64 and 32 respectively by day 21 PI. The rise in anti-FI titres was accompanied by invasion and subsequent clearing of the parasite by the animals.

CELLULAR IMMUNE RESPONSE

Figure 2 also shows leucocyte migration inhibition (LMI) response to specific amoebic antigen (FI) and PHA during the course of amoebic infection. Response to FI amoebic proteins in terms of LMI indices increased gradually from $19.2 \pm 3.5\%$ at day 3 PI to $36.6 \pm 3.4\%$ at day 21 PI whereas the response to PHA decreased initially from $35.0 \pm 3.5\%$ at day 0 to $21.0 \pm 3.2\%$ at day 7 PI and then increased to $30.5 \pm 6.5\%$ at day 21 PI.

ALTERATION OF CELLULAR AND ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY

The Table shows that the induction of anti-FI antibody dependent MNC mediated cytotoxic response

Fig. 1 Course of intestinal amoebic infection (a) amoebic trophozoites (arrow) can be seen entering the mucosa at day 3 PI, (b) a typical flask shaped ulcer at day 7 PI, (c) a deep penetrating ulcer showing large number of trophozoites (arrows), (d) the infection is cleared at day 21 PI. (PAS stained.)

Fig. 2 Levels of antiamoebic antibodies and leucocyte migration inhibition during the course of amoebic infection. Geometric mean IHA titres, anti-CAE (○—○), anti-FI (●—●). Leucocyte migration inhibition using PHA (△—△) and FI amoebic proteins (▲—▲) as antigens. I=mean±SD.

at day 7 PI ($34.7 \pm 4.3\%$ amoebae killed) was significantly ($p < 0.001$) low as compared with that at day 0 ($48.0 \pm 2.0\%$ amoebae killed). The ADCC response, however, increased gradually thereafter. By day 21 PI, MNC killed $77.2 \pm 5.0\%$ trophozoites in the presence of anti FI antibodies (Table). Unstimulated macrophages killed $98.0 \pm 2.5\%$ trophozoites in the presence of anti-FI antibodies while only $40.7 \pm 3.6\%$ trophozoites were killed by day 7 PI macrophages in association with anti-FI antibodies. Killing capacity of macrophages also increased thereafter; $77.2 \pm 5.0\%$ trophozoites were found to be killed by day 21 PI macrophages. On the other hand, FI stimulated MNC and macrophages killed $78.0 \pm 2.4\%$ and 100% trophozoites in the presence of anti-FI antibodies (Table).

The ability of serum obtained from guinea pigs killed at various days postinoculation to induce cytotoxicity by MNC and macrophages is shown in Figure 3. Cytotoxic potentialities of MNC ($18.1 \pm 4.2\%$ amoebae killed) and macrophages ($23.3 \pm 2.9\%$ amoebae killed) at day 7 PI in the presence of serum

Table Percent trophozoites of *E histolytica* (NIH:200) killed by MNC or macrophages in the presence of anti-FI antibodies

Days PI	% Trophozoites killed by	
	MNC	Macrophages
0	48.0 ± 2.0	98.0 ± 2.5
3	$36.98 \pm 2.34^*$	75.47 ± 4.56
7	34.73 ± 4.33	$40.76 \pm 3.68^*$
14	60.11 ± 3.06	60.33 ± 4.12
21	77.23 ± 5.01	77.20 ± 5.01
FI stimulated	78.0 ± 2.5	100.0

* $p < 0.001$ as compared with day 0.

Fig. 3 Antibody dependent MNC mediated (A, B) and antibody dependent macrophage mediated (C, D) cytotoxicity of unstimulated (▨), FI stimulated (▤), and infected (□) animals in the presence of serum obtained at day 7 and day 21 postinoculation. I=mean±SD.

obtained from the same animals were significantly low as compared with MNC ($24.2 \pm 3.3\%$ amoebae killed, $p < 0.01$) and macrophages ($45.0 \pm 4.5\%$ amoebae killed, $p < 0.001$) obtained from uninfected animals. Cytotoxic potentialities of effector cells obtained from 7 day PI animals in the presence of homologous serum was also significantly low ($p < 0.001$) as compared with FI stimulated effector cells under similar conditions (Fig. 3a, c). By day 21, however, PI cytotoxic capacity of effector cells increased and was found to be comparable with normal effector cells (Fig. 3b, d) in the presence of day 21 PI serum.

Discussion

In the present investigation we have attempted to correlate cellular cytotoxicity and antibody dependent cellular cytotoxic responses against *E histolytica* *in vitro* with the diseased state and immunoprotected state of guinea pigs. It is well known that FI immunised animals are protected to a very high degree

against experimental challenge with highly virulent isolates of *E. histolytica*.^{4,6} Thus, FI stimulated animals represent protected state. The use of a moderate virulent challenge strain of *E. histolytica* enabled us to show three phases of infection – that is, establishment of infection (0 to 3 days PI), the acute phase (three to seven days PI) and clearing of infection by the animals (14–21 days PI). Histopathology showed that amoebae had broken through the mucosa and submucosa where a great number of amoebic trophozoites could be seen. The submucosa showed marked oedema and a collection of polymorphs, mononuclear histiocytes and lymphocytes. This histopathological picture is similar to that found in human intestinal lesions except that cellular infiltration is relatively less marked in the latter.

The immune responses were monitored in all phases. It was observed that the establishment of amoebic infection was accompanied by suppression of LMI response to PHA. At day 7 PI when the infection was in the acute phase, response to PHA was found to be lowest. On the other hand, LMI response to specific Sephadex G-200 chromatographed FI of crude amoebic extract increased gradually with the establishment of infection and so did the anti-amoebic antibody titres. The increasing sensitisation of host cells and the appearance of anti-amoebic anti-FI antibodies were accompanied by invasion of host tissues by the parasite. This increasing trend continued until day 14 PI and was stabilised thereafter. The response to PHA also showed an increasing trend after day 7 PI and reached normal levels by day 21 PI. These observations confirm those of others who showed a good response to antigen while suppressed response to PHA in acute amoebic infection.^{8,10,11} The suppression of the ability of effector cells to kill the parasite *in vitro* during the establishment phase parallels the lowered response to PHA. We have shown here that with clearing of amoebic infection, the cell mediated immune responses are gradually restored to normal. Cellular cytotoxicity and ADCC of both MNC and macrophages, a functional manifestation of CMI and HMI showed significant decline during the first seven days PI followed by increased ADCC activities which was accompanied by gradual clearing of amoebic infection. FI stimulated effector cells on the other hand showed significantly higher cytotoxic responses against *E. histolytica*. Therefore the ability of effector cells to kill trophozoites of *E. histolytica* correlates with the diseased state because these responses were suppressed in the acute phase of infection and also with the immunoprotected state of guinea pigs. Our results correlate with others who showed that effector cells obtained from vaccinated hamsters²⁴ and gerbils²⁵ could kill trophozoites of *E. histolytica* *in*

vitro. As ADCC involved both effector cells and anti-amoebic antibodies, we sought to determine whether impairment of ADCC was caused by suppression of cells or was because of the inability of host antibodies to induce cytotoxicity or both. It was observed that both unstimulated lymphocytes and macrophages showed increasing capacity to kill trophozoites with increasing anti-FI anti-amoebic antibody titres obtained at different days postinoculation indicating that such antibodies from infected animals were capable of inducing cytotoxicity. The effector cells obtained at different days PI on the other hand, failed to kill a significant number of parasites *in vitro* in the presence of homologous and hyperimmune anti-FI antibodies until day 7 PI. Thereafter, both the effector cells – that is, splenic lymphocytes and peritoneal macrophages of infected animals, could kill sufficient number of trophozoites which was accompanied subsequently by clearing of infection by such animals. The cytotoxic potentialities of effector cells obtained from infection stimulated animals, however, were not comparable with those of FI stimulated animals probably because the levels of anti-FI antibodies achieved in immunised animals is much higher than that obtained during intestinal infection. It has been shown recently^{26,27} that in the patients after amoebic liver abscess, specific cell mediated immune mechanism develop that are effective *in vitro* against the parasite. It is also well known that patients successfully cured of amoebic liver abscess rarely develop a second infection^{28,6} indicating the development of immunity against this form of the disease. The question whether this immunity is a result of enhanced cellular and antibody dependent cellular cytotoxic responses is open to debate. The observations that experimental immunosuppression with ALS and corticosteroids exaggerated amoebic lesions in experimental animals favour this correlation. The exaggeration of amoebic lesion has also been reported in patients on corticosteroid therapy.^{29,30} Lack of cellular responses in patients suffering from amoebiasis, however, are against it.

We feel that establishment of amoebic infection is accompanied by suppression of cellular immune response as reflected by suppressed cellular cytotoxicity both of T lymphocytes and macrophages reaching their lowest level during the acute phase. The rising anti-amoebic antibodies particularly anti-FI antibodies and restoration of cytotoxic responses against trophozoites direct the infection to recede and eventually clear from the animals. During the establishment phase both absence or low level of anti-FI antibodies and suppressed cytotoxic responses make it possible for the trophozoites to invade and establish in the host.

The Indian Council of Medical Research is gratefully acknowledged for financial assistance.

References

- Walsh JA. *Epidemiology and magnitude of the problem of amoebiasis. A magnitude of the problem (morbidity and mortality on a global scale)*. Workshop on the World Problem of Amoebiasis. Current Status Research Needs and Opportunities for advancement. Prospect Hill 16–18 June 1982, 14.
- World Health Organization. *Informal meeting on strategies for control of amoebiasis*. Geneva: WHO, 1984.
- Krupp IM. Protective immunity to amoebic infection demonstrated in guinea pigs. *Am J Trop Med Hyg* 1974; **23**: 355–60.
- Vinayak VK, Sawhney S, Jain P, Chugh S, Chakravarti RN. Protective effect of crude and chromatographic fraction of axenic *Entamoeba histolytica* in guinea pigs. *Trans R Soc Trop Med Hyg* 1980; **74**: 483–7.
- Ghadirian E, Meerovitch E, Hartman DP. Protection against amoebic liver abscess in hamsters by means of immunization with amoebic antigen and some of its fractions. *Am J Trop Med Hyg* 1980; **29**: 779–84.
- Sepulveda B, Diamond LS, eds. *Immunology of amoebiasis*. Mexico: Inst Mexicano del Seguro Social Mexico, 1975: 186–702.
- Kessel JF, Lewis WP, Pasquel CM, Turner JA. Indirect haemagglutination and complement fixation tests in amoebiasis. *Am J Trop Med Hyg* 1965; **14**: 540–50.
- Gold D, Norman LG, Maddison SE, Kagan IG. Immunologic studies on hamsters infected with *Entamoeba histolytica*. *J Parasitol* 1978; **64**: 866–73.
- Vinayak VK. The specificity of indirect hemagglutination test in the immunodiagnosis of amoebiasis. *Ind J Prev Soc Med* 1975; **6**: 271–6.
- Vinayak VK, Jain P, Gupta B, Kaushik SP, Sawhney S. Cellular and humoral immune responses in amoebic patients. *Trop Geogr Med* 1980; **32**: 298–302.
- Simjee AE, Gathiram V, Coovadia HM, et al. Cell mediated immunity in hepatic amoebiasis. *Trans Roy Soc Trop Med Hyg* 1985; **79**: 165–8.
- Vinayak VK, Chitkara NL, Chhuttani PN. Effect of corticosteroid and irradiation on caecal amoebic infection in rats. *Trans Roy Soc Trop Med Hyg* 1979; **73**: 266–8.
- Vinayak VK, Sawhney S, Jain P, Chakravarti RN. Immunosuppression and experimental amoebiasis in guinea pigs. *Trans Roy Soc Trop Med Hyg* 1982; **76**: 309–16.
- Tanimoto-weki M, Calderon P, De La Hoz R, Aguirre Galca J. Inoculation of *Entamoeba histolytica* trophozoites in hamsters on immunosuppressive therapy. *Arch Invest Med* 1974; **5**: 441–6.
- Ghadirian E, Meerovitch E. Passive transfer of immunity against hepatic amoebiasis in the hamsters by cells. *Parasit Immunol* 1983; **5**: 369–76.
- Vinayak VK, Chugh S, Saxena A, Sharma SP. Antibody dependent lymphocyte mediated cytotoxicity in amoebiasis. *Ind J Med Res* 1984; **80**: 421–7.
- Chugh S, Saxena A, Vinayak VK. Interactions between trophozoites of *Entamoeba histolytica* and cells of the immune system. *Aust J Exp Biol Med Sci* 1985; **63**: 1–8.
- Saxena A, Chugh S, Vinayak VK. Elucidation of cellular population and nature of anti-amoebic antibodies in cytotoxicity to *Entamoeba histolytica* (NIH:200). *J Parasitol* 1986; **72**: 434–8.
- Saxena A, Chugh S, Vinayak VK. Antibody dependent macrophage mediated cytotoxicity against *Entamoeba histolytica*. *J Med Microbiol* 1986; **22**: 17–21.
- Capron A, Dessaint JP, Haque A, Capron M. Antibody dependent cell mediated cytotoxicity against parasites. *Prog Allergy* 1982; **31**: 234–67.
- Sawhney S, Chakravarti RN, Jain P, Vinayak VK. Immunogenicity of axenic *Entamoeba histolytica* antigens and its fractions. *Trans R Soc Trop Med Hyg* 1980; **74**: 26–29.
- Vinayak VK, Naik SR, Sawhney S, Sehmi N, Chhuttani PN. Pathogenicity of *Entamoeba histolytica* virulence of strains of amoebae from symptomatic and asymptomatic cases of amoebiasis. *Ind J Med Res* 1977; **66**: 935–41.
- Jain P, Sawhney S, Vinayak VK. Experimental amoebic infection in guinea pigs immunized with low grade amoebic infection. *Trans R Soc Trop Med Hyg* 1980; **74**: 347–50.
- Ghadirian E, Meerovitch E. In vitro amoebicidal activity of immune cells. *Inf Immun* 1982; **36**: 243–46.
- Chadee K, Meerovitch E, Moreau F. In vitro and in vivo interaction between trophozoites of *Entamoeba histolytica* and Gerbil lymphoid cells. *Inf Immun* 1985; **49**: 228–32.
- Salata RA, Pearson RD, Ravdin JI. Interaction of human leukocyte and *Entamoeba histolytica*: killing of virulent amoebae by activated macrophages. *J Clin Invest* 1985; **76**: 491–9.
- Salata RA, Martinez-Palombo A, Murray HW, et al. Patients treated for amoebic liver abscess develop cell mediated immune responses effective in vitro against *Entamoeba histolytica*. *J Immunol* 1986; **136**: 2633–9.
- Krupp IM, Jung RC. Immunity of amoebic infection. In: Cohen S, Sadun EH, eds. *Immunology of parasitic infections*. Oxford: Blackwell Scientific Publications, 1976: 163–6.
- Villarejos SVM. Corticosteroid and experimental amoebiasis in rats. *J Parasitol* 1962; **48**: 194.
- El-Hannawy M, Abd-Rabbo H. Hazards of cortisone therapy in hepatic amoebiasis. *J Trop Med Hyg* 1978; **81**: 71–3.