

Immunity to Pathogenic Free-Living Amoebae: Role of Cell-Mediated Immunity

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The role of cell-mediated immunity in defense against pathogenic free-living amoebae was examined. Both the *in vitro* macrophage inhibition test and the *in vivo* delayed hypersensitivity test showed responses to both heterologous and homologous antigens, although homologous systems were the most efficient. It is suggested that exposure to nonpathogenic species of free-living amoebae can stimulate the immune system to be effective against pathogenic species. The significance of cell-mediated immunity as a defense against invasion by pathogenic free-living amoebae is discussed.

The role of the immune system in resisting infection by pathogenic free-living amoebae (PFLA) remains to be fully elucidated. Epidemiologically, the diseases caused by PFLA can be divided into the following three types: (i) an acute (incubation period, 10 days or less) meningo-encephalitis caused by *Naegleria fowleri* and usually contracted while swimming and a form caused by *Acanthamoeba* spp. (notably, *Acanthamoeba culbertsoni*), which is not contracted while swimming; these forms of infection are characterized by a massive acute inflammatory response; (ii) a chronic (incubation period, 10 days or more) infection caused by *Acanthamoeba* spp., which is not associated with swimming and is often associated with a granulomatous reaction; and (iii) hypersensitization to amoebic antigens present in humidifier systems of air conditioning units, resulting in an allergic alveolitis response.

Experimentally, animal challenge studies have demonstrated that primates are relatively resistant to either intranasal or intravenous challenge with *N. fowleri* or *A. culbertsoni* (2, 14, 15). Intrathecal or intracerebral challenge, however, routinely results in infection by these amoebae. The use of immunosuppressive drugs also predisposes primates to infection. Furthermore, mice previously immunized intranasally, intravenously, subcutaneously, or intraperitoneally with either nonpathogenic *Naegleria gruberi* or a sublethal infection of *N. fowleri* are subsequently more resistant to intranasal challenge (the proven portal of entry) with a lethal dose of *N. fowleri* (9, 12). These observations, combined with the sporadic and extremely low incidence of clinically diagnosed infections by PFLA, have prompted speculation on the importance of host-related susceptibility in resist-

ance to infection (1, 4, 8, 9). It has been proposed that unwitting exposure of humans to the more ubiquitous antigenically related nonpathogenic species of amoebae may immunize them against the more virulent pathogenic species. The relative effects of both heterologous and homologous antigens from pathogenic and nonpathogenic species on two indicators of cell-mediated immunity (CMI), namely, delayed hypersensitivity (DH) and macrophage inhibition by the lymphokine macrophage inhibition factor, were examined with this hypothesis in mind.

MATERIALS AND METHODS

The history and cultivation of *N. fowleri* strain MsT, *N. gruberi* strain P1200f, and *Naegleria jadini* strain 0400 are described in the accompanying paper (5).

White albino guinea pigs weighing between 0.6 and 0.8 kg were obtained from the Massey University Small Animal Production Unit.

For the preparation of antigens, axenic cultures of amoebae were centrifuged at $750 \times g$ for 10 min. The pellet containing the amoebae was then washed three times in sterile physiological saline and finally suspended in sterile physiological saline to give an approximate concentration of 10^8 cells per ml. The cells were then slowly freeze-thawed five times (-70 to 4°C) and injected according to the following schedule.

Guinea pigs were bled by cardiac puncture 1 week before the start of immunization to obtain preimmune sera. A 0.5-ml amount of an antigen-complete Freund adjuvant mixture (1:1, vol/vol) was injected intramuscularly into each hind leg. After 3 weeks, 0.2 ml of an antigen-incomplete Freund adjuvant mixture (1:1, vol/vol) was injected subcutaneously at weekly intervals. During week 6, 0.2 ml of antigen was injected subcutaneously, and 1.0 ml of antigen was injected intraperitoneally. After 7 weeks, 8.0 ml of blood was withdrawn aseptically by cardiac puncture.

In order to culture lymphocytes and to produce a

macrophage inhibition factor, sterile blood containing 20 U of heparin per ml was centrifuged, and the buffy coat cells were separated. The buffy coat cells were diluted 1:1 (vol/vol) with sterile 0.9% NaCl containing 20 U of heparin per ml and layered carefully onto a Ficoll-Hypaque gradient (ratio, 1:1, vol/vol). The gradients were then centrifuged at $1,250 \times g$ for 20 min. The lymphocytes were collected, washed twice in 10.0 ml of medium 199, and finally suspended in medium 199 containing 10% normal guinea pig serum, 100 U each of penicillin and streptomycin per ml, and 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer to a final concentration of 2×10^6 cells per ml. The lymphocytes were then tested for viability by the exclusion of trypan blue. After this, antigen (0.5 mg/ml) sterilized by membrane filtration (pore size, 0.22 μ m) was added, and the cells were incubated at 37°C overnight. After incubation, the cells were centrifuged at $1,500 \times g$, and the supernatant was filtered through a sterile 0.22- μ m filter and stored at -20°C.

At 6 days before the isolation of lymphocytes, an unsensitized guinea pig was injected intraperitoneally with 30.0 ml of sterile paraffin oil. After 7 days the guinea pig was exsanguinated by cardiac puncture, and the peritoneal exudate cells were collected in 150 ml of cold Hanks balanced salt solution containing 20 U of heparin per ml and 100 U each of penicillin and streptomycin per ml. The peritoneal exudate cells were centrifuged at $750 \times g$ and washed three times with sterile Eagle minimal essential media containing penicillin-streptomycin. After the final wash, the peritoneal exudate cells were suspended in Eagle minimal essential medium containing 15% normal guinea pig serum, 100 U each of penicillin and streptomycin per ml, and 30 mM HEPES and packed into capillary tubes. These tubes were placed in cell culture dishes and covered with 2.0 ml of minimal essential medium containing 15% normal guinea pig serum plus 0.5 to 1.0 ml of the macrophage inhibition factor lymphokine or antigen alone. The peritoneal exudate cells were then incubated at 37°C for 12 to 16 h in a desiccator containing 5.0% CO₂. The resulting migration was quantified by photographing the migration and relating the area of inhibition to the area moved by a control as a percentage. Each experiment was done three to five times, each time in duplicate.

The technique used for DH testing was as follows. A 0.1-ml amount of antigens (5 mg/ml) was inoculated intradermally into shaved skin on the flanks of infected and control guinea pigs, and lesions were measured after 48 h with a vernier caliper. The guinea pigs were then killed, and the lesions were excised, fixed in 10% Formol-saline, dehydrated, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for histological infiltration of mononuclear cells.

RESULTS

DH. Table 1 shows that guinea pigs sensitized to the three species of *Naegleria* all developed typical DH reactions, as judged by the time taken to form hard nodule lesions and the typical infiltration of mononuclear cells after infection

with either homologous or heterologous antigens. In most cases, the best results occurred with the homologous system. The results also indicate that *N. jadini* is more antigenically related to *N. gruberi* than to *N. fowleri*.

Macrophage inhibition. The response of macrophages to heterologous antigens was also reflected by the production of macrophage inhibition factor (Table 2). The data in Table 2 are mean percentages from individual experiments which were done in duplicate three to five times and once again show that the homologous system was more efficient. The variability shown within each batch of experiments as defined by sensitizing antigen used was within $\pm 20\%$ of the means shown in Table 2.

DISCUSSION

The results confirm that both in vitro macrophage inhibition factor and in vivo DH indicators of CMI were able to respond to both heterologous and homologous amoebic antigens after immunization with complete Freund adju-

TABLE 1. Cross-reactivity of homologous and heterologous antigens of *Naegleria* spp. as judged by DH

Sensitizing antigen	Test antigen	Diam of DH nodule after 48 h (mm)
<i>N. fowleri</i> MsT	MsT	9.0 \pm 0.1
	P1200f	8.0 \pm 0.1
	0400	6.7 \pm 0.1
	Saline	— ^a
<i>N. jadini</i> 0400	MsT	7.0 \pm 0.1
	P1200f	10.5 \pm 0.1
	0400	9.0 \pm 0.1
	Saline	—
<i>N. gruberi</i> P1200f	MsT	7.0 \pm 0.1
	P1200f	12.0 \pm 0.1
	0400	8.3 \pm 0.1
	Saline	—

^a —, No reaction.

TABLE 2. Cross-reactivity of homologous and heterologous antigens of *Naegleria* spp. as judged by inhibition of macrophages

Sensitizing antigen	Lymphocyte-stimulating antigen	% Inhibition of macrophages
<i>N. fowleri</i> MsT	MsT	52.38
	P1200f	36.83
	0400	25.2
<i>N. jadini</i> 0400	0400	67.62
	MsT	25.64
<i>N. gruberi</i> P1200f	P1200f	68.36
	Saline	0
	MsT	0
	P1200f	0
	0400	0
	None	0

vant and also confirm the closer antigenic similarity between *N. gruberi* and *N. jadini* than between *N. gruberi* and *N. fowleri* (13).

Previous work by Diffley et al. (6) suggested that CMI may play an important role in resistance to infection in guinea pigs. These authors observed that guinea pigs which survived a subcutaneous challenge by *N. fowleri* developed DH when tested with soluble antigens. Culbertson et al. (3) observed granuloma formation, which is an indicator of a CMI response, in guinea pigs infected subcutaneously with *N. fowleri*, and the common finding of a granulomatous response and distinctive pathological lesions in *Acanthamoeba* infections has been commented upon (2, 11). Further indirect support of CMI and of the hypothesis that constitutive phagocytes play a decisive role in resistance to infection by PFLA is demonstrated by the increased susceptibility to infection by PFLA exhibited by corticosteroid-treated animals (2, 7, 10, 14, 15).

In summary, it appears that both the humoral and CMI arms of the immune system participate in resistance to infection by PFLA. We hypothesize that unwitting exposure of humans to antigenically related nonpathogenic amoeba species may prime the immune system to respond to the pathogenic species. It is also possible that, like *Toxoplasma*, a background of inapparent infection exists and that clinical infections become apparent by invasion via the olfactory route.

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LITERATURE CITED

- Anderson, K. G., and A. Jamieson. 1972. Agglutination test for the investigation of the genus *Naegleria*. *Pathology* 4:273-278.
- Culbertson, C. G. 1971. The pathogenicity of soil amoebae. *Annu. Rev. Microbiol.* 25:231-254.
- Culbertson, C. G., P. W. Ensminger, and W. M. Overton. 1972. Amebic cellulocutaneous invasion by *Naegleria aerobia* with generalized visceral lesions after subcutaneous injections. *Am. J. Clin. Pathol.* 57:375-386.
- Cursons, R. T. M., and T. J. Brown. 1978. The use of cell cultures as an indicator of pathogenicity of free-living amoebae. *J. Clin. Pathol.* 31:1-11.
- Cursons, R. T. M., T. J. Brown, E. A. Keys, K. M. Moriarty, and D. Till. 1980. Immunity to pathogenic free-living amoebae: role of humoral antibody. *Infect. Immun.* 29:401-407.
- Diffley, P., M. R. Skeels, and F. Gogandanes-Bernal. 1976. Delayed type hypersensitivity in guinea-pigs infected subcutaneously with *Naegleria fowleri* (Carter). *Z. Parasitenkd.* 49:133-137.
- Fauci, A. S. 1978. Mechanisms of the immunosuppressive anti-inflammatory effects of glucocorticosteroids. *J. Immunopharm.* 1:1-25.
- Haggerty, R. M., and D. T. John. 1978. Innate resistance of mice to experimental infection with *Naegleria fowleri*. *Infect. Immunol.* 20:73-77.
- John, D. T., R. R. Weik, and A. C. Adams. 1977. Immunization of mice against *Naegleria fowleri* infection. *Infect. Immunol.* 16:817-820.
- Markowitz, S. M., T. Sobrieski, A. J. Martinez, and R. J. Duma. 1978. Experimental *Acanthamoeba* infections in mice pretreated with methylprednesolone or tetracycline. *Am. J. Pathol.* 92:733-744.
- Martinez, A. J., C. Sotelo-Avila, J. Garcia-Tamago, J. T. Moron, E. Willaert, and W. P. Stamm. 1977. Meningo-encephalitis due to *Acanthamoeba* sp. Pathogenesis and clinico-pathological study. *Acta Neuro-pathol.* 37:183-191.
- Thong, Y. H., C. Shepherd, A. Ferrante, and B. Rowan-Kelley. 1978. Protective immunity to *Naegleria fowleri* in experimental amebic meningo-encephalitis. *Am. J. Trop. Med. Hyg.* 27:238-240.
- Willaert, E., and J. B. Jadin. 1974. Analyse immunoelectrophoretique comparative de quelques especes du genre *Acanthamoeba*. Apparetement d'autres amibes libres. *J. Protozool.* 21:477.
- Wong, M. M., S. L. Karr, and W. B. Balamuth. 1975. Experimental infections with pathogenic free-living amoebae in laboratory primate hosts: I. (A) A study on susceptibility to *Naegleria fowleri*. *J. Parasitol.* 61:199-208.
- Wong, M. M., S. L. Karr, and W. B. Balamuth. 1975. Experimental infections with pathogenic free-living amoebae in laboratory primate hosts: I. (B) A study on susceptibility to *Acanthamoeba culbertsoni*. *J. Parasitol.* 61:682-690.