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IgE-dependent adherence and cytotoxicity of rat spleen and peritoneal cells to *Litomosoides carinii* microfilariae

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SUMMARY

Serum taken after the termination of microfilaraemia from rats infected with the filarial parasite *Litomosoides carinii* brought about adherence and cytotoxicity of normal rat spleen and peritoneal cells to microfilariae. The activity could be absorbed to, and eluted from, anti-rat IgE, but not anti-rat IgG, immunosorbent columns. Immune serum heated to 56°C for 3 hr did not cause cellular adherence or cytotoxicity; the addition of fresh normal rat serum failed to restore activity. Fresh rat serum did, however, restore activity to immune serum which was inactive after being heated to 56°C for 30 min. EDTA, EGTA and diethylcarbamazine inhibited adherence. It is concluded that IgE antibodies are responsible for cellular adherence and cytotoxicity and that complement may play a part, as yet undefined, in these reactions.

INTRODUCTION

Antibody-dependent cell-mediated effector mechanisms have been shown to play an important role in the rejection of tumours and allografts (Grant *et al.*, 1971; Allison, 1972; MacLennan, 1972). There has been increasing evidence that such mechanisms operate in various parasitic diseases. Recent studies in schistosomiasis have provided evidence for the interaction of IgG antibodies with schistosomulae and a variety of cells, including neutrophils (Dean, Wistar & Murrell, 1974), eosinophils (Butterworth *et al.*, 1975; Capron *et al.*, 1978b) and macrophages (Perez & Smithers, 1977). The participation of IgE antibodies in such reactions involving mononuclear phagocytes has also been reported (Capron *et al.*, 1975, 1976; Joseph *et al.*, 1978). These reactions have been considered to have a role in the acquisition of resistance to schistosomal infection.

Evidence for similar effector mechanisms has emerged from work on *Nippostrongylus brasiliensis* in rats (Ogilvie & Love, 1974), *Trichinella spiralis* in rats (Mackenzie, Preston & Ogilvie, 1978) and filarial parasites in rats and man (Bagai & Subrahmanyam, 1970; Higashi & Chowdhury, 1970; Subrahmanyam *et al.*, 1976, 1978; Tanner & Weiss, 1978). Bagai & Subrahmanyam (1970) reported that the termination of microfilaraemia in albino rats infected with *L. carinii* was associated with adherence of macrophages, lymphocytes and polymorphonuclear cells *in vivo* to microfilariae. Subsequently it was observed that serum obtained at the onset of latency promoted

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intense adherence of spleen cells and consequent cytotoxicity to microfilariae *in vitro* (Subrahmanyam *et al.*, 1976). We report here experiments which demonstrate that the antibody in latent serum responsible for promoting cellular adhesion is of the IgE class.

MATERIALS AND METHODS

Filarial infection. Albino rats were infected with *L. carinii* by infected mites, *Bdellonyssus bacoti.* Methods for maintenance of the infection and monitoring it by means of microfilarial counts in peripheral blood smears were described by Bagai, Subrahmanyam & Singh (1968).

Isolation of microfilariae. Microfilariae (Mf) were separated from the infected blood of high-count rats by sedimentation on Ficoll–Hypaque of density 1·05 (Subrahmanyam *et al.*, 1978).

Preparation of sera and cells. The IgG fractions were prepared from: rabbit anti-rat IgG (Cappell Laboratories, USA); rabbit anti-rat IgM (Miles Laboratories, USA); goat anti-rat IgA (Miles Laboratories, USA); and rabbit anti-rat IgE (kindly supplied by Dr Graham Mayrhofer, Clinical Immunology Research Unit, Princess Margaret Children's Medical Research Foundation, Perth, Western Australia). Latent rat serum (LRS) was prepared from blood collected 1 week after rats became amicrofilaraemic, either by bleeding the animals from the tail or by cardiac puncture. Serum from normal (NRS) or infected rats (IRS) was similarly collected. The serum was stored at −20°C in 1-ml lots.

Normal rat spleen cells were obtained by teasing the spleens in HEPES-buffered RPMI 1640 tissue culture medium (Grand Island Biological Company) containing penicillin (100 u/ml) and streptomycin (100 μg/ml) (referred to hereafter as medium). They were washed with medium twice before use. Peritoneal cells were prepared by washing out the peritoneal cavities of normal rats with medium containing preservative-free heparin (20 u/ml).

Antibody-dependent cellular adherence. For adherence experiments the reaction mixture contained microfilariae and cells in a ratio of 1:200 to 1:2,000 and infected, latent or normal rat serum at a final concentration of 30% in a volume of 0·4 ml. The mixtures were incubated at 37°C for 16 hr with occasional shaking in capped, sterile flat-bottomed plastic vials and were examined microscopically at 6 and 16 hr for adhesion and cytoxicity.

Cytotoxicity assay. Trypan blue in saline was found to stain dead and damaged microfilariae when incubated for 30 min at 37°C. Live microfilariae failed to stain on incubation at 37°C even for more than 6 hr. For this purpose 50 μl of the mixture, after 16 hr of incubation, was mixed with 50 μl of 0·5% trypan blue in saline (Centron Research Laboratories, Bombay) and a drop of the mixture was observed under the microscope. In dead or damaged microfilariae nuclei were clearly stained, either locally, near adherent cells, or generally.

Ion exchange chromatography. The serum from latent rats (LRS) was fractionated on columns of diethylaminoethyl cellulose (DE-52, Whatman) as described earlier (Subrahmanyam *et al.*, 1978). Two fractions were eluted, one with 0·015 M sodium phosphate buffer, pH 8·2, and one with 0·3 M potassium phosphate buffer, pH 8·2.

Immunosorbents. Five millilitres of rabbit anti-rat IgE was precipitated with ammonium sulphate at 33% saturation. The sediment was washed twice with 40% ammonium sulphate. The final precipitate was resuspended in 0·1 M carbonate buffer, pH 8·2, containing 0·5 M sodium chloride and dialysed against the same buffer. Globulin yield was 30 mg per ml of the original antiserum. CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was swollen and washed with 0·001 M HCl. The immunoglobulin was tagged onto the absorbent gel by the procedure of Axén, Porath & Earnback (1967).

Two millilitres of the LRS was diluted to 8 ml with the carbonate buffer and was passed through a 1 × 14 cm column, containing 15 ml of the swollen gel equilibrated in the carbonate buffer with a flow rate of 8 ml/hr. One hundred and fifty millilitres of the buffer was passed through when the first protein peak was eluted, as determined by absorbance at 280 nm. The gel was subsequently eluted with 60 ml of 0·2 M glycine–HCl buffer, pH 2·0, containing 0·5 M NaCl with an elution rate of 75 ml/hr when the second fraction emerged. The fractions of the second peak were immediately adjusted to neutral pH with 0·2 M KOH. The fractions of both peaks were pooled separately,

dialysed against medium overnight at 4°C and concentrated to the original serum volume under vacuum in cellophane bags. Both fractions were then passed through Millipore filters (0·22 μm).

An anti-rat IgG immunosorbent was also tested as a control. A similar procedure was followed for tagging the IgG fraction of rabbit anti-rat IgG to CNBr-Sepharose and two fractions were isolated using carbonate buffer of pH 8·2 and glycine–HCl buffer of pH 2·0 respectively.

RESULTS

General features of the adherence reaction

The incubation of Mf with LRS and spleen cells caused intense cellular adherence to the parasites (Fig. 1). Adherence was apparent after 6 hr of incubation but was maximal after 16 hr. Damage to, and death of, Mf following adherence were much greater after 16 hr than at 6 hr, as seen by microscopic examination and by trypan blue exclusion (Table 1). The survival of Mf was not

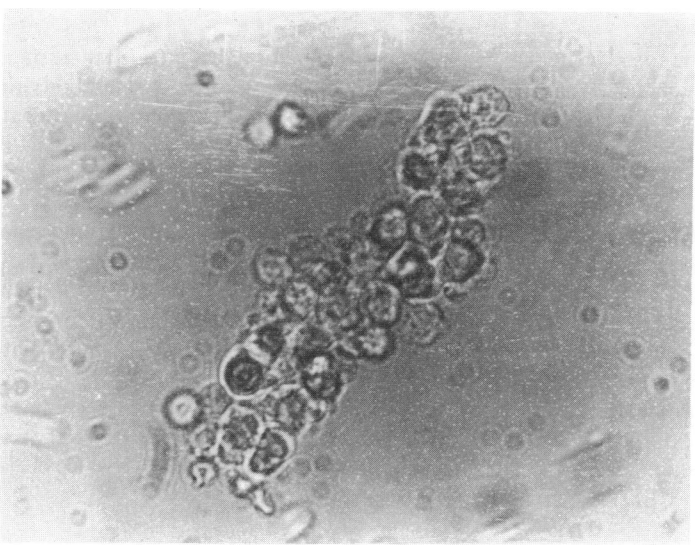

Fig. 1. Adherence of spleen cells to a microfilaria of *L. carinii* after 16 hr *in vitro* in the presence of latent rat serum.

Table 1. Antibody-dependent cellular adherence and cytotoxicity to *L. carinii* microfilariae

Incubation period (hr)	Serum source	Microfilariae with cells adherent (%)	Microfilariae stained with trypan blue (%)
6	Normal rat	6	2
	Infected rat	5	3
	Latent rat	78	32*
16	Normal rat	6	4
	Infected rat	7	4
	Latent rat	98	89*

Each value was an average of six observations

* Of these 80 to 88% were dead, i.e. non-motile on prolonged observation.

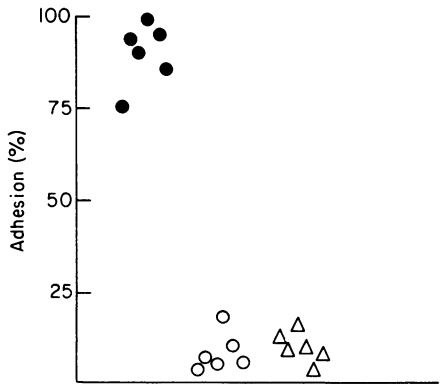

Fig. 2. Adherence of spleen cells to *L. carinii* microfilariae after 16 hr *in vitro* in the presence of latent (•), infected (○) or normal (△) rat serum.

affected when they were incubated with spleen cells and normal or infected rat serum and there was no significant adherence of the cells to the parasites under these conditions throughout the incubation period (Fig. 2). Spleen cells from normal rats and from rats with prepatent, patent or latent infection were equally active in adhering to the parasites in the presence of LRS.

Factors involved in adherence

Heat treatment of LRS (56°C, 30 min) completely prevented attachment and damage to Mf by spleen cells. The activity of heated LRS could be restored partially by addition of fresh NRS (Table 2). However, when LRS was heated at 56°C for 3 hr, it failed to promote cellular adherence and activity could not be restored by the addition of fresh NRS. These observations suggested that two thermolabile factors could be responsible for spleen cell adherence. The less labile material (56°C, 30 min), being present in fresh NRS, could be complement.

Ethylenediamine tetracetic acid (EDTA) at a final concentration of 0·002 M completely inhibited adherence and cytotoxicity suggesting again a requirement for complement in the reaction. Adherence was also inhibited by 0·002 M ethyleneglycol tetracetic acid (EGTA), which chelates calcium as effectively as does EDTA but chelates magnesium much less.

Table 2. Factors affecting adherence and cytotoxicity of rat spleen cells to *L. carinii* microfilariae

Treatment of latent rat serum	Microfilariae with cells adherent (%)	Microfilariae stained with trypan blue (%)
None	94	75
Heated (56°C, 30 min)	5	2
Heated (56°C, 30 min)+ NRS	47	41
Heated (56°C, 3 hr)	0	0
Heated (56°C, 3 hr)+ NRS	3	1
EDTA (2 mM)	0	n.d.
EGTA (2 mM)	0	n.d.
DEC (2 mg/ml)	4	n.d.

Each value was an average of four observations after 16 hr incubation.

n.d. = Not done.

Of particular interest was the observation that diethylcarbamazine (DEC, UNICHEM, Bombay), the drug known to clear microfilaraemia *in vivo* in infected individuals, at a final concentration of 1–2 mg/ml inhibited the adherence of spleen cells to Mf (Table 2).

Spleen cells did not adhere to Mf preincubated with LRS at 37°C for 1 hr and washed once in medium. Similarly, spleen cells preincubated with LRS and washed did not adhere to Mf. The presence of spleen cells, Mf and LRS throughout the incubation period appeared to be essential for adherence.

Immunoglobulin involved in adherence

As the adherence-promoting activity of LRS was totally lost after heating to 56°C for 3 hr it seemed likely that the immunoglobulin involved could be IgE. LRS was therefore fractionated on DEAE-cellulose and on immunosorbent columns of anti-rat IgE and anti-rat IgG. As shown in Fig. 3, the first fraction from DEAE-cellulose, containing most of the IgG, had no activity, but activity was recovered at the beginning of the second fraction, which was eluted with 0·3 M buffer. Table 3 shows that adherence-promoting activity was bound to, and could be eluted from, an anti-rat IgE

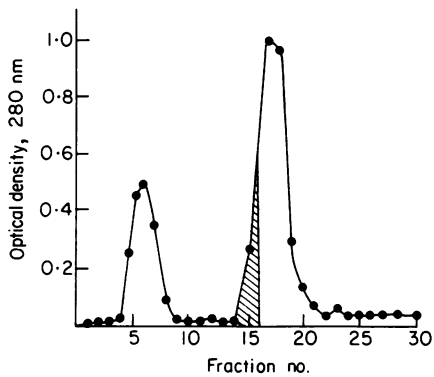

Fig. 3. Fractionation of latent rat serum on diethylaminoethyl cellulose: (▨) adherence-promoting activity.

Table 3. Adherence and cytotoxicity of cells to microfilariae in the presence of serum fractions from immunosorbent columns

Serum	Immunosorbent	Fraction	Cells	Microfilariae with cells adherent (%)	Microfilariae stained with trypan blue (%)
Normal rat	None	Whole serum	Spleen	5	3
			Peritoneal	5	n.d.
Latent rat	None	Whole serum	Spleen	75	70
			Peritoneal	100	91
Latent rat	Anti-rat IgE	Unbound	Spleen	0	0
			Peritoneal	2	1
		Bound and eluted	Spleen	91	88
			Peritoneal	98	93
Latent rat	Anti-rat IgG	Unbound	Spleen	66	52
			Peritoneal	70	n.d.
		Bound and eluted	Spleen	0	0
			Peritoneal	0	0

Each value was an average of three observations made after 16 hr incubation.
All reaction mixtures contained 25% normal rat serum.
n.d. = Not done.

immunosorbent column. The active material was not bound to an anti-IgG immunosorbent but was recovered in the unbound fraction of serum. The presence of IgE in the active fractions and of IgG in the inactive fractions was confirmed by immunodiffusion tests in agar with monospecific antisera (Ouchterlony, 1949). The activity in the IgE-containing fractions was destroyed by heating to 56°C for 3 hr. These fractions were not active unless fresh NRS was present. Fresh NRS would not, however, restore activity lost after heating to 56°C for 3 hr.

DISCUSSION

Our earlier observations suggested that both humoral and cellular factors were involved in the destruction of Mf leading to the amicrofilaraemic stage of *L. carinii* infection in albino rats (Bagai & Subrahmanyam, 1968, 1970; Subrahmanyam, Chaudhury & Jain, 1974; Subrahmanyam & Chaudhury, 1975; Subrahmanyam *et al.*, 1976). In those experiments spleen cells were found to adhere to Mf in the presence of whole LRS. The experiments described here provide additional information on the nature of the factors present in LRS responsible for cellular adherence and damage to Mf. A strong correlation was found between trypan blue exclusion and lethal damage to the microfilariae. Similar dye exclusion tests have been employed to measure cytotoxicity to schistosomulae, mediated by IgE and macrophages (Joseph, Dessaint & Capron, 1977) or by antibody, complement and eosinophils (Anwar *et al.*, 1979).

The results of the experiments described here suggest that two humoral factors are necessary for cellular adherence and cytotoxicity to *L. carinii* microfilariae. One of these factors is IgE antibody, as shown by its occurrence in LRS, but not in NRS or IRS, by its absorption onto an anti-IgE immunosorbent and by its heat lability. A similar involvement of IgE antibodies in cellular adherence and cytotoxicity has been found in *Schistosoma mansoni* infections in rats (Capron *et al.*, 1975), baboons and humans (Joseph *et al.*, 1978). Tanner & Weiss (1978) have, however, reported that IgM antibodies promote the adherence and cytotoxicity of macrophages to Mf of *Dipetalonema viteae*. In rat schistosomiasis IgG2a antibodies can promote the adherence of eosinophils to schistosomulae in the presence of mast cells (Capron *et al.*, 1978a). It seems unlikely that such a reaction could occur with *L. carinii* Mf, but be overlooked in the present experiments. IgG-rich fractions of serum prepared either by DEAE-cellulose chromatography or by means of an immunosorbent, failed to promote cellular adherence in the presence or absence of normal serum, even when peritoneal cells, with abundant eosinophils and mast cells, were used as effectors. Conceivably, however, such antibodies could be produced later in the course of the immune response.

A second humoral factor (or factors) was required for adherence and cytotoxicity of spleen cells. Both in the present experiments and in earlier ones (Subrahmanyam *et al.*, 1976) this factor(s) was lost after heating (56°C, 30 min) and could be partly or wholly replaced by fresh NRS. It was also lost after absorption with an immune complex (Subrahmanyam *et al.* 1976). It is therefore extremely tempting to identify the factor(s) as complement or some components thereof. This conclusion is consistent with the inhibitory effects of EDTA and EGTA on adherence, though the possibility cannot be excluded that divalent cations were required for some cellular activity, rather than for complement activation. Aggregated IgE has been reported to fix late components of complement by the alternative pathway rather than the classical pathway (Ishizaka, Soto & Ishizaka, 1972). In the case of human serum EGTA has been reported to inhibit classical, but not alternative, pathway activation, whereas EDTA inhibits both (Fine *et al.*, 1972). If these results are extrapolated to rat serum it seems that the reaction of IgE antibodies with microfilarial antigens leads to complement fixation by the classical pathway and that this is required for cellular adherence and cytotoxicity. This conclusion can only be accepted with considerable reservations, both because of the lack of precedent for it in studies of antigen–antibody systems and because of the lack of involvement of complement in otherwise rather similar systems involving antibodies, cells and schistosomulae (Capron *et al.*, 1975). Antibody-dependent destruction of schistosomulae does, however, require complement (Dean *et al.*, 1974, 1975).

DEC was a potent inhibitor of adherence, though it is not known whether it acted on the Mf, antibody, complement or the cells. The requirement for all components to be present simul-

taneously for adherence makes analysis difficult. DEC treatment has been found to inhibit immediate skin reactions in human filariasis patients (Katiyar *et al.*, 1974; Murthy *et al.*, 1978) and may inhibit some immune reactions in dogs infected with *D. immitis* (Desowitz *et al.*, 1978).

The present studies were not concerned with the nature of the cells active in adherence and cytotoxicity. It has been found that rat macrophages, neutrophils and eosinophils can all adhere to Mf in the presence of LRS (Nelson & Subrahmanyam, unpublished findings). As noted above, mixed populations were used in these studies. The total failure of adherence to occur in the absence of IgE or heat-labile co-factors suggests that IgE antibodies and, tentatively, complement are involved in the adherence of each type of cell. Studies currently in progress should show whether some cells are more potent cytotoxic effectors than others, and whether similar factors operate in human filariasis.

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