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IgE-dependent cellular adhesion and cytotoxicity to *Litomosoides carinii* microfilariae—nature of effector cells

K. MEHTA,* R. K. SINDHU, D. SUBRAHMANYAM, K. HOPPER & D. S. NELSON
Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh, India and Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, N.S.W., Australia

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SUMMARY

Albino rats immunized with sonicated microfilarial antigen incorporated in Freund's complete adjuvant, produce antibodies that promote cell-mediated adhesion and killing of *Litomosoides carinii* microfilariae *in vitro*. Using highly purified cell populations, it has been shown that macrophages and neutrophils are most active in this phenomenon. Eosinophils, while adhering readily to parasites in the presence of the antibody, did not affect the viability of the parasites when observed after 24 hr incubation.

INTRODUCTION

Antibody-dependent cell-mediated effector mechanisms have been recognized to exert deleterious effects on helminth parasites (Dean, Wistar & Murrell, 1974; Butterworth *et al.*, 1975; Capron *et al.*, 1975; Leventhal & Soulsby, 1976; Subrahmanyam *et al.*, 1976; Weiss & Tanner, 1979). Detailed studies in filarial infection of the rat implicate such mechanisms in the disposal of microfilariae leading to latency of the infection (Subrahmanyam *et al.*, 1976). The antibody promoting cellular adherence and cytotoxicity of *Litomosoides carinii* microfilariae was found to be of the IgE class (Mehta *et al.*, 1980). The present communication describes the nature of the effector cells.

MATERIALS AND METHODS

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

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

Preparation of sera. Sera from rats were prepared from blood collected by cardiac puncture or by tail bleeding. Immune serum was prepared either from blood of rats that had become amicrofilaremic (latent rat serum, LRS) or from rats immunized with sonicated microfilariae (actively immunized rat serum AIRS). For this purpose, 1 month old rats were given three intramuscular injections, 10–15 days apart, of microfilarial sonicates emulsified in Freund's complete adjuvant (FCA). Each rat received $1{\cdot}8 \times 10^5$ to $2{\cdot}5 \times 10^5$ sonicated microfilariae in three

* Present address: Department of Pharmacology, University of Texas, Houston, Texas 77025, USA.
Correspondence: Dr D. Subrahmanyam, CIBA-GEIGY Research Centre, Goregaon East, Bombay 400063, India.

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equal doses. Two to three weeks after the last immunization, the animals were bled and the sera collected.

Media. RPMI 1640 medium (GIBCO, New York, USA) was buffered with HEPES (25 mM). MEM, Eagle's minimal essential medium (GIBCO). MEM/FCS/DNAase, MEM was supplemented with 10% fetal calf serum (FCS) inactivated at 56°C for 1 hr. To this was added DNAase (Sigma), 30 mg/l. These media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Tyrode/gel/DNAase, Tyrode's solution contained 0·1% gelatin and 30 mg/l of DNAase.

Preparation of cells

Peripheral blood leucocytes (PBL). Ten millilitres of heparinized blood (10 units/ml) was sedimented with 2 ml of 6% Macrodex in normal saline (Macrodex-Dextran 70, Pharmacia Laboratories) for 60 min at 37°C. The leucocyte rich supernatant (buffy coat) was washed twice in MEM/FCS/DNAase by centrifugation at 400 *g* for 10 min and finally suspended in medium. The cells were counted in a haemocytometer.

Lymphocytes. Lymphocytes were prepared from PBL. The buffy coat cells taken in 2 ml of MEM/FCS/DNAase were subjected to separation on metrizamide discontinuous density gradients according to the method of Vadas *et al.* (1979). Briefly a stock solution of 30% metrizamide was prepared in Tyrode/gel/DNAase and was diluted with the medium to yield solutions of 18, 20, 22, 24 and 26% metrizamide. Gradients were prepared by carefully layering 2 ml volumes of decreasing densities of metrizamide solutions into a 15 ml conical polystyrene centrifuge tube. About $10–15 \times 10^7$ buffy coat cells were layered on top of the gradient and the tube was centrifuged at 1200 *g* for 45 min at 20°C. Cells from each interface were then collected into separate tubes, washed with the medium and total and differential cell counts were made. The topmost fraction contained lymphocytes of 95% purity (Table 1).

Lymphocytes were also obtained by teasing the lymph nodes of animals into the medium. The cells were washed and counted.

Neutrophils. Peritoneal exudates were induced by injecting 5 ml of 12·5% casein intraperitoneally in rats. Sixteen hours after the injection the rats were killed and the peritoneal exudates were washed out with 20 ml MEM containing heparin, 10 units/ml. The cells were washed with MEM/FCS/DNAase and finally suspended in 2 ml of the same medium and subjected to metrizamide gradient separation. Neutrophils of 95–100% purity were obtained by this procedure at the 22/24% metrizamide interface. Neutrophil-rich populations (>90%) were also obtained by fractionation of PBL on the gradient.

Macrophages. When 48 hr casein-induced exudates were separated on metrizamide gradients

Table 1. Separation of rat cells on discontinuous gradients of metrizamide

Metrizamide interface	Source of cells	% differential count*				
		L	M	N	E	Mast
0/18%	PBL	95	4	1	0	0
18/22%	Casein-induced PEC (24 hr)	2	93	5	0	0
22/24%	Casein-induced PEC (48 hr)	0	1	99	0	0
24/26%	Oil-induced PEC	0	0	2	98	0
Pellet	Resident peritoneal cells	0	0	1	4	95

PBL = peripheral blood leucocytes; PEC = peritoneal exudate cells; L = lymphocytes; M = macrophages; N = neutrophils; E = eosinophils; Mast = mast cells.

* Means of all cell separations used in these experiments.

macrophages were mainly found at the 18/20% metrizamide interface. Macrophages were also obtained by culturing normal resident peritoneal cells, washed out as above in ring-and-slide chambers (More *et al.*, 1973). The non-adherent cells were washed off after incubation at 37°C for 2 hr or more leaving a macrophage monolayer.

Eosinophils. Eosinophils were induced in rats by injection of 5 ml of paraffin oil intraperitoneally. Forty-eight to seventy-two hours later, the peritoneal washout contained 30–60% eosinophils. The cellular exudate was washed with heparinized MEM and suspended in MEM/FCS/DNAase and layered over a metrizamide gradient. The 24/26% metrizamide interface contained eosinophils of 90–100% purity.

Mast cells. Resident peritoneal cells from normal rats were found to be a rich source of mast cells. After separation on metrizamide gradients the pellet contained mast cells of 90–100% purity.

State of cells from metrizamide gradients. The viability of the cells separated on metrizamide was more than 90% as judged by trypan blue exclusion and the cells were morphologically intact as seen by light microscopy after staining with May-Grünwald-Giemsa. Table 1 shows the average composition of the fractions separated on metrizamide.

Normal rat spleen cells. These were obtained by teasing the spleens in RPMI 1640 medium. They were washed with the medium twice before use.

Macrophage or monocyte depletion. Spleen cells were depleted of macrophages by means of glass adherence as described by Mosier (1967). PBL were depleted of monocytes by treatment with carbonyl iron as described by Butterworth *et al.* (1976).

Antibody-dependent cellular adherence. For adhesion experiments, the reaction mixture contained cells and microfilariae at ratios from 25:1 to 2000:1 and LRS, AIRS or normal rat serum at a final concentration of 25% 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 cytotoxicity.

Cytotoxicity assay. Trypan blue in saline was found to stain dead and damaged microfilariae when incubated for 5 min at 37°C. Live microfilariae failed to stain on incubation with trypan blue at 37°C for more than 6 hr. For this purpose 50 μl of the mixture of microfilariae cells and serum after 16 hr incubation, was mixed with 50 μl of 0·5% trypan blue in saline 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.

Fractionation of antiserum. Rat sera were separated on DEAE-cellulose (Whatman DE-52) and immunosorbent columns as described by Mehta *et al.* (1980).

Table 2. Promotion of cellular adherence and cytotoxicity by sera from animals immunized with sonicated *L. carinii* microfilariae in adjuvant

Expt	Antiserum	Mf with cells* adherent (%)	Mf with cells adherent, stained with trypan blue (%)
1	Whole serum	89	75
2	Serum fractionated on anti-rat IgE-CNBr sepharose		
	Fraction 1 (unbound)	4	0
	Fraction 2 (bound and eluted)	93	84
3	Heated serum		
	56°C, 30 min	4	—
	56°C, 30 min + NRS†	24	—
	56°C, 3 hr	0	—
	56°C, 3 hr + NRS†	0	—

* Cells from peritoneal exudates induced by casein injected i.p. 16 hr earlier. † NRS = normal rat serum.

RESULTS

Antibody-dependent cellular adherence with sera of actively immunized rats (AIRS)

The AIRS promoted intense adhesion of speen cells to *L. carinii* microfilariae, with a cytotoxic effect on the parasites as was found using latent rat serum (LRS). The serum of rats injected with FCA alone did not possess this activity. The heat lability and affinity chromatography characteristics on anti-IgE immunosorbent columns of AIRS (Table 2) were also identical with those of LRS (Mehta *et al.*, 1980).

Nature of the effector cells

Role of monocytes and macrophages. Peripheral blood leucocytes readily adhered to and were cytotoxic to microfilariae in the presence of antibody (Table 3). On separation of the cells over Ficoll-Hypaque (1·077 g/ml density) (Böyum, 1968), the cells in the pellet were approximately twice as active as those recovered from the interface. The sedimented cells contained 70–80% polymorphonuclear leucocytes (neutrophils, eosinophils, basophils), and 15–30% mononuclear cells (lymphocytes and monocytes), whereas the interphase comprised 10–20% polymorphs and 80–90% mononuclear cells. Depletion of monocytes from the interface preparation, either by repeated cycles of incubation with carbonyl iron and magnetic separation or by adherence to glass petri dishes at 37°C, caused progressive loss of adherence and cytotoxic activity. The activity could be recovered to some extent in the cells removed with carbonyl iron (Table 3).

That macrophages were highly active in inducing adhesion and cytotoxicity to microfilariae was shown when peritoneal cell populations were used with antibody. Macrophages prepared from PEC purified on metrizamide (Table 4 and Fig. 1a) and monolayers of normal resident macrophages in ring-and-slide chambers were active in this respect. An effector:target ratio of 1000:1 was optimum in adhesion and killing of the parasite although a significant effect could be seen even at a ratio of 100:1 (Table 4).

Role of neutrophils. The pellet obtained on sedimentation of PBL over F/H gradient consisted of neutrophils, eosinophils and basophils and was active in the adhesion reaction. Neutrophil-rich populations (95–100% purity) prepared from casein-induced PEC on metrizamide gradients (Table 4) were most active in adhering to (Fig. 1b) and killing the microfilariae. At similar effector:target ratios, neutrophils were more effective than macrophages in this reaction (Table 4). Neutrophil-rich populations of PBL separated on metrizamide gradients were also active.

Table 3. Effector cells in antibody-dependent damage to *L. carinii* microfilariae

Cells*	Antibody	Mf with cells adherent (%)	MF with cells adherent, stained with trypan blue (%)
Spleen	+	100	86
	−	3	0
Spleen cells depleted of glass adherent cells	+	20	14
PBL	+	99	79
F/H interface†	+	26	17
F/H pellet	+	48	42
F/H interface depleted of monocytes (carbonyl iron treated)	+	5	1
Monocytes (carbonyl iron separated)	+	50	41

* Experiments were carried out at effector:target ratio of 2000:1

† PBL were separated into interface and pellet after centrifugation over Ficoll-Hypaque (F/H).

Table 4. Antibody-dependent adherence and cytotoxicity of cells separated on metrizamide gradients

Cells	Cells per microfilaria	Antibody (AIRS)	Mf with cells adherent (%)	Mf with cells stained with trypan blue (%)
Macrophages	25	+	21	14
(95–99%)	100	+	45	39
	500	+	91	76
	1000	+	100	92
	1000	−	0	0
Neutrophils	25	+	21	14
(92–100%)	100	+	62	56
	500	+	92	85
	1000	+	100	98
	1000	−	4	1
Eosinophils	50	+	42	3
(95–100%)	200	+	69	6
	1000	+	100	2
	1000	−	9	0
Lymphocytes	1000	+	0	0
(90–95%)	1000	−	0	0
Mast cells	1000	+	0	0
(100%)	1000	−	0	0

Each value was an average of four observations.

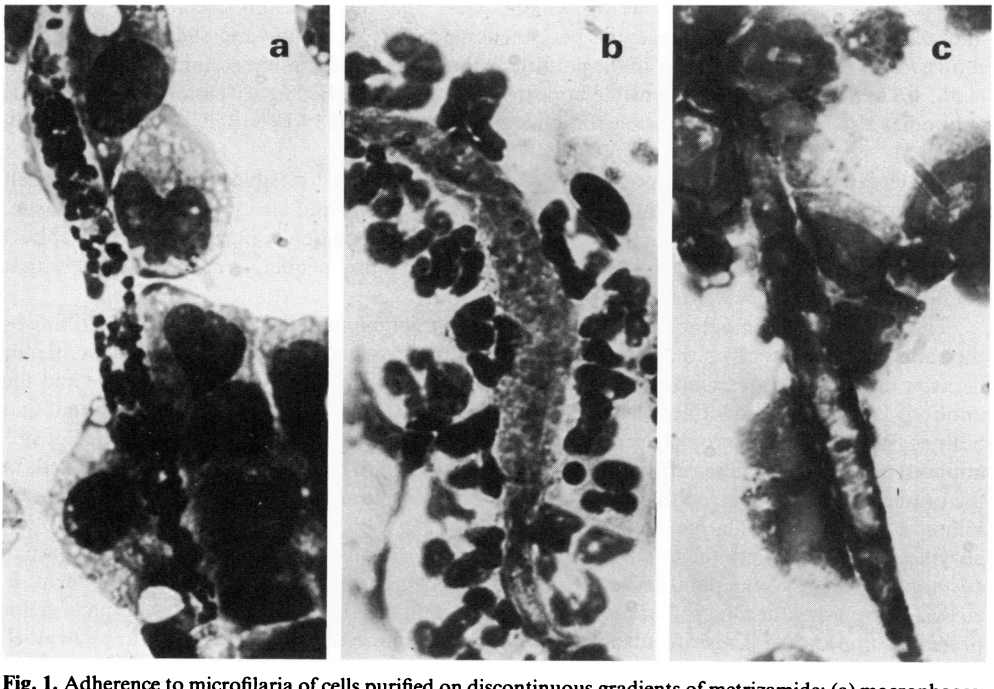

Fig. 1. Adherence to microfilaria of cells purified on discontinuous gradients of metrizamide: (a) macrophages; (b) neutrophils; (c) eosinophils, some in the process of degranulation. May-Grünwald-Giemsa stain. (Original magnification × 1,000).

Eosinophils as effectors. Eosinophil rich populations (>90% purity) obtained by separating the oil-induced PEC by metrizamide gradient readily adhered to microfilariae in the presence of antibody (Table 4 and Fig. 1c). The adhesion did not, however, result in damage to the parasite on incubation for 24 hr as revealed by the trypan blue staining technique (Table 4). On the other hand, the eosinophils spread on the parasite surface degranulated and became stainable with trypan blue.

Mast cells and lymphocytes. Mast cells of 100% purity obtained by sedimenting normal resident peritoneal cells on metrizamide gradients did not adhere or show cytotoxicity to microfilariae in the presence of antibody at an effector:target ratio of 1000:1. Similarly, lymphocytes of 95% purity were not able to adhere or induce cytotoxicity to microfilariae in the presence of immune serum (Table 4). Lymph node lymphocytes were also inactive.

Effect of accessory cells on eosinophil-mediated adhesion. It was found that eosinophils did not affect the parasite survival even when supplemented with 10% lymphocytes, neutrophils, macrophages or mast cells. Similarly, basophils degranulated in the presence of 10 mM sodium fluoride did not induce eosinophils to become cytotoxic to the parasite.

DISCUSSION

Acquired resistance to filarial infection, as seen by the disappearance of microfilariae from the peripheral blood, occurs in albino rats about 6 months after initial infection. At this stage antibodies can be detected in their sera that promote cellular adhesion and cytotoxicity to the parasite *in vitro*. The present studies revealed that such an antibody response could be elicited in the animals in about 6 weeks by immunization of the animals with sonicated microfilariae in FCA. The data thus suggest that dead parasites are capable of inducing antibodies that promote adhesion of cells and cytotoxicity to the parasite.

Our earlier studies with the pleural exudates of rats with latent infection tentatively identified the cells surrounding the microfilariae as lymphocytes, macrophages and eosinophils (Bagai & Subrahmanyam, 1970). More recently, peritoneal macrophages in ring-and-slide chambers were shown *in vitro* to adhere intensely to the parasite in the presence of immune serum (Subrahmanyam *et al.*, 1976). The antibody responsible appeared to be of the IgE class (Mehta *et al.*, 1980). IgE antibodies also seem to be responsible for adherence induced by the AIRS used in the present study (Table 2).

The elegant technique developed by Vadas *et al.* (1979) made it feasible to isolate different cell types to a high degree of purity and to identify those that adhere and are cytotoxic to the parasite. The most active cells were macrophages and neutrophils. The degree of purity achieved (Table 1) and the effectiveness of the cells as low effector:target ratios (Table 4) make it unlikely that contaminating cell types were responsible.

The nature of immunoglobulin and effector cells participating in the adhesion to and killing of the parasites may depend on the host, the parasite species involved and their maturation. A role for hamster macrophages was shown by Weiss & Tanner (1979) with *D. viteae* microfilariae and the antibody involved was identified to belong to the IgM class. Haque *et al.* (1981) demonstrated that both eosinophils and macrophages of rat adhere to *D. viteae* microfilariae in the presence of IgE antibody although the killing effect on the parasite appeared to be more macrophage-mediated. In the present study, rat neutrophils were found to be somewhat more effective than macrophages in killing *L. carinii* microfilariae. This could be due to the high content of peroxidase and other enzymes in neutrophil granules (Yam, Li & Crosby, 1971), compared with macrophages. Although eosinophils adhere to the parasites in the present study, they appear ineffective in killing them. *L. carinii*, being a sheathed microfilariae, the role of sheath in the lack of damage by eosinophils in this process is not known. Experiments are underway to examine this phenomenon with exsheathed microfilariae. Furthermore, while macrophages (Capron *et al.*, 1977) and eosinophils (Capron *et al.*, 1981) have been shown to bind IgE, the mechanism by which neutrophils interact with the antibody is not clear. It is of interest to note the recent observation of Johnson *et al.* (1981) that the IgG dependent feline granulocyte-mediated adhesion to *B. pahangi* microfilariae was dependent on

the maturation of the parasites. This aspect has not been investigated in the present study. Eosinophils were also found to adhere to antibody coated *W. bancrofti* infective larvae without causing any apparent damage to them (Higashi & Chaudhury, 1970).

The adherence of different cell types in presence of an appropriate antibody was also shown in other parasite systems. Rat macrophages were shown by Capron *et al.* (1975) to bind to schistosomula in presence of IgE antibodies. Dean *et al.* (1974) and more recently Anwar, Smithers & Kay (1979) reported participation of neutrophils in causing antibody dependent damage to these parasites. Using metrizamide, Mackenzie *et al.* (1980) separated rat resident peritoneal cells into different cell types and studied their role in the adhesion reaction to *T. spiralis* and *N. brasiliensis* larvae. They found that macrophages, neutrophils and eosinophils adhered for long periods of time, though mast cells adhere only briefly. In our studies no adherence of mast cells was seen, nor did the addition of mast cells (or any other cells) promote eosinophil adherence and cytotoxicity as was observed by Capron *et al.* (1978) for schistosomula.

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