Mechanisms of Antibody-dependent Cytotoxicity of Rat Cells to Microfilariae:

Effects of Metabolic Inhibitors and Electron Microscopic Observations*

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SUMMARY A study was made concerning the effects of a variety of metabolic inhibitors and other agents on antibody-dependent cell-mediated cytotoxicity (ADCC) of rat neutrophils and macrophages to *Litomosoides carinii* and *Brugia pahangi* microfilariae. Cytochalasin B and N-ethylmaleimide inhibited ADCC by neutrophils. Cytochalasin B, colchicine, cycloheximide and azide all caused some inhibition of macrophage adherence. Oxygen scavengers and drugs stabilising lysosomes did not inhibit ADCC. Adherence and/or cytotoxicity were enhanced by 2-deoxyglucose, mannitol, ascorbate, azide, fluoride and cyanide, apparently by their effects on microfilariae.

Electron-microscopy showed close adherence of the effector cells to the microfilariae, with breakdown of the sheath followed by destruction of the microfilariae. Electron-dense material appeared to be deposited on the cuticle by macrophages but also by eosinophils, which were not cytotoxic.

ADCC to microfilariae appears to involve close adherence and to require normal microfilament function, but not to involve oxygen metabolites or lysosomes. Neutrophils and macrophages differ with regard to other metabolic requirements for ADCC.

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The termination of microfilaraemia in rats infected with the filarial parasite *Litomosoides carinii* is associated with the appearance of antibodies mediating cellular adhesion and cytotoxicity to the microfilariae[1] *in vitro*. Similarly, functioning antibodies to *Wuchereria bancrofti* microfilariae have been found in the sera of patients with elephantiasis, who may harbour the adult parasites but are usually amicrofilaraemic.[2] In rats, the antibodies are IgE and both neutrophils and macrophages are cytotoxic effector cells. In man, the antibodies are IgG and neutrophils are effector cells. In both species, eosinophils adhere to the microfilariae but apparently are not cytotoxic.[2-4] Because of the possible importance of these reactions and the widespread occurrence of antibody-dependent cellular reactions in the immune responses to helminths,[5,6] studies were made of their metabolic and ultrastructural basis. We describe here metabolic studies and ultrastructural observations on *L. carinii* and *Brugia pahangi* microfilariae.

MATERIALS AND METHODS

Filarial infections. Rats were infected with *L. carinii* by infected mites as described in detail by Bagai and Subrahmanyam.[7] *Brugia pahangi* infections in *Mastomys natalensis* were maintained as described by Sanger *et al.*[8]

Microfilariae. Methods for pur-

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ifying *L. carinii* microfilariae from blood by filtration and sedimentation were described by Subrahmanyam *et al.*[9] *B. pahangi* microfilariae were prepared by centrifugation of isosmotic Percoll in 0.25M sucrose using the method of Chandrashekar *et al.*[10]

Sera. Sera with antibodies to *L. carinii* were obtained from rats that had become amicrofilaraemic or from rats immunized with microfilarial sonicates in Freund's complete adjuvant (FCA).[4] Sera with antibodies to *B. pahangi* microfilariae were obtained from rats similarly immunised with *B. pahangi* microfilarial sonicates in FCA. Normal sera were obtained from rats not exposed to filarial infection. Foetal calf serum (FCS) was obtained from Australian Laboratory Services (Rockdale, N.S.W.) or Flow Laboratories(Sydney, N.S.W.).

Media. RPMI 1640 (buffered with 25mM Hepes) and Eagle's minimal essential medium (MEM) were obtained from Grand Island Biological Company. MEM/FCS/DNAse was made by supplementing MEM with FCS (10%) heated to 56°C for one hour and deoxyribonuclease (Sigma, 30 mg/l). Tyrode/gelatin/DNAse consisted of Tyrode's solution with 0.1% gelatin and DNAse, 30 mg/l. The RPMI 1640 and MEM contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

Cells. Neutrophils, macrophages and eosinophils were purified from peritoneal exudates induced by casein or oil, as described by Mehta *et al*[4] using the method of Vadas *et al.*[11] This involves centrifugation on a discontinuous density gradient of metrizamide (Sigma) in TGD and washing in MFD.

Adherence and cytotoxicity assays. *L. carinii* (300 microfilariae in 50 μl) were mixed with rat cells (500-2,000 cells per microfilaria, in 50μl), 50 μl of undiluted normal rat serum, 50 μl of 1:4 dilution of immune rat serum and 50 μl of inhibitor in flat-bottomed 96-well Microtest II plates (Flacon). The inhibitors were dissolved in normal saline. All other suspensions or dilutions were dissolved in RPMI 1640. The plates were incubated for 6 or 16 hours at 37°C. Samples obtained from each well after resuspension were examined microscopically for (a) adherence of the cells to the microfilariae[1,2] and (b) death of the microfilariae revealed by nuclear staining with trypan blue.[3]

Reagents used in inhibitor studies. The following reagents were obtained from Sigma: colchicine, cytochalasin B, cycloheximide, 2-deoxyglucose, mannitol, superoxide dismutase, N-ethylmaleimide (NEM) and trypsin. L-Ascrobic acid and potassium cyanide were obtained from Ajax Chemicals Ltd., Sydney, catalase (from *Aspergillus niger*) and neuraminidase (from *Vibrio cholerae*), from Calbiochem-Behring Corporation (La Jolla, California and Sydney); sodium azide, from BDH Ltd. (Poole); chloroquin sulphate and sodium fluoride, from May and Baker Ltd. (Dagenham); and hydrocortisone acetate, from Merck, Sharp and Dohme (Sydney).

Enzyme pretreatment of effector cells. Suspensions of the purified

Table 1. Effect of metabolic inhibitors on antibody-dependent adherence and cytotoxicity of neutrophils and macrophages for *L. Carinii* microfilariae.

Reagent	Concentration (M)	Percentage of microfilariae reacting with			
		Neutrophils		Macrophages	
		Adherence	Cytotoxicity	Adherence	Cytotoxicity
None		57 ± 2	17 ± 5	54 ± 1	6 ± 4
Colchicine	10^{-4}	57 ± 4	21 ± 5	19 ± 7	0
	10^{-5}	64 ± 4	25 ± 2	27 ± 13	0
	10^{-6}			34 ± 3	0
Cytochalasin B	10^{-6}	2 ± 1	0	27 ± 7	3 ± 2
	10^{-7}	13 ± 4	0	22 ± 4	0
Cycloheximide	10^{-4}	52 ± 4	14 ± 1	35 ± 4	0
	10^{-5}	57 ± 3	32 ± 2	48 ± 8	0
	10^{-6}			46 ± 4	0
2-deoxyglucose	10^{-1}	62 ± 8	39 ± 2	66 ± 1	0
	10^{-2}	62 ± 3	24 ± 2	68 ± 9	0
Mannitol	10^{-2}	57 ± 3	27 ± 2	62 ± 1	0
	10^{-3}	57 ± 5	35 ± 4	43 ± 17	0
	10^{-4}			49 ± 11	0
Ascorbate	10^{-2}	85 ± 2	64 ± 6	46 ± 9	13 ± 3
	10^{-3}	72 ± 5	40 ± 5	49 ± 2	12 ± 2
Azide	10^{-2}	59 ± 3	33 ± 2	34 ± 10	5 ± 5
	10^{-3}	73 ± 5	66 ± 4	40 ± 15	3 ± 3
	10^{-4}			55 ± 5	15 ± 4
NEM	10^{-4}	17 ± 3	11 ± 3	ND	
	10^{-5}	44 ± 7	23 ± 4	ND	
Fluoride	10^{-3}	67 ± 6	38 ± 3	55 ± 3	15 ± 7
	10^{-4}	66 ± 3	35 ± 3	47 ± 3	13 ± 1
Cyanide	10^{-4}	86 ± 3	57 ± 3	68 ± 3	27 ± 3
	10^{-5}	81 ± 4	43 ± 4	53 ± 2	14 ± 2

Mean ± S.E. of four observations. ND, not done

cells (2 x 10^6/ml in RPMI 1640) were incubated at 37°C with 10 μg of trypsin for 30 minutes or with 10 units of neuraminidase for 60 minutes. They were washed three times and resuspended in RPMI, then counted again.

Preparation of mixtures for electron microscopy. Microfilariae, serum (a 1:4 dilution) and effector cells, at effector : target ratios of 20:1 to 500:1, were incubated as described under "Adherence and cytotoxicity assays" in polypropylene tubes for 6 or 16 hours. The tubes were then centrifuged at 200 x g for 20 minutes. The cell pellets were fixed in glutaraldehyde and processed as described by McLaren *et al*[12] with the exception that those for transmission electron-microscopy were placed in 2% agar after fixation.

RESULTS

Metabolic inhibitors

Table 1 shows the effects of a variety of agents on adherence and cytotoxicity of rat neutrophils and macrophages to *L. carinii* microfilariae. With the aim of maximising any effects, suboptimal doses of antibody were used.

With neutrophils, only cytochalasin B caused marked inhibition of adherence and cytotoxicity. At concentrations down to 10^{-3} M, N-ethylmaleimide was toxic to the microfilariae but at 10^{-4} M, adherence was inhibited without any visible detrimental effect on the cells or microfilariae. None of the other reagents had any apparent toxic effect on either the cells or microfilariae when incubated with them separately. In separate experiments, chloroquine and hydrocortisone at 10^{-6} M and 10^{-7} M, catalase at 10 μg/ml or 1 μg/ml and superoxide dismutase at 10 μg/ml and 100 μg/ml had no effect on either reaction (data not shown). Mannitol, 2-deoxyglucose, ascorbate, azide, fluoride or cyanide included in the culture promoted adhesion and/or cytotoxicity. All agents tested had similar effects on the binding and killing of *B. pahangi* (data not shown). If the microfilariae were pre-incubated separately with the aforementioned reagents and then washed, promotion of adherence (as seen in Table 2) with *B. pahangi* also occurred; this was not observed when the cells were pretreated. In all cases, changes in adherence were accompanied by similar changes in cytotoxicity.

Colchicine, cytochalasin B, cycloheximide and azide, all caused some inhibition of the adherence of macrophages to *L. carinii* microfilariae. Cytotoxicity in the control mixtures was so low that significant inhibition could not be detected. Adherence was marginally enhanced by 2-deoxyglucose, mannitol and cyanide. Cytotoxicity was enhanced by ascorbate, fluoride and cyanide.

Enzyme treatment of cells

Trypsin treatment virtually abolished the capacity of rat neutrophils, macrophages or eosinophils to adhere to and to damage *L. carinii* microfilariae, whereas neuraminidase had no effect on the reaction (Table 3).

Electron microscopy

The appearance of *L. carinii* or *B. pahangi* was generally similar. Close apposition between the microfilariae and neutrophils was apparent on both scanning (Fig. 1) and transmission electron-microscopy (Figs. 2 to 6). After 6 hours, the adherent cells appeared to envelop the parasites with long extensions and intermittent points of close contact (Fig. 2). Higher magnification (Fig. 3) showed intimate contact between the neutrophils and the surface of microfilariae, sometimes with an almost complete absence of the sheath. There appeared to be no displacement of neutrophil organelles relative to the points of contact with the microfilariae. After 24 hours, only fragments of the microfilariae remain-

Table 2. Effect of reagents on antibody-dependent neutrophil-mediated cytotoxicity (ADCC) with *B. pahangi* microfilariae

Reagent*	ADCC (Fold stimulation over control)		
		Preincubation	
		Microfilariae	Cells
Ascorbic acid	2	4.5	1
KCN	5	6	1
NaN_3	5	6	1
NaF	2	1	1

*Concentration, 10^{-3}M; in pre-incubation experiments, 10^{-2}M

Table 3. Effect of trypsin and neuraminidase on antibody-dependent adhesion of cells to *L. carinii* microfilariae

	Per cent of microfilariae with bound cells		
	Untreated	Trypsin	Neuraminidase
Neutrophils	22	3	24
Macrophages	34	4	30
Eosinophils	12	0	18

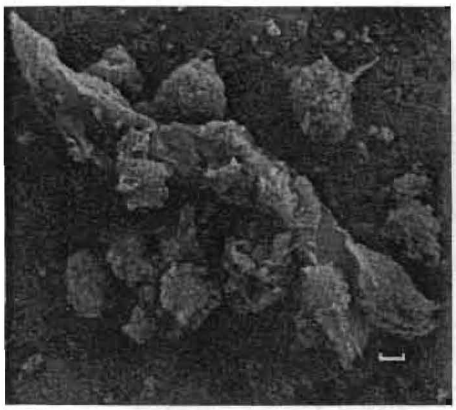

Fig. 1 Scanning electron-micrograph of rat neutrophils attached to a *B. pahangi* microfilaria after 6 hours in the presence of immune serum. The bar represents 2 μm (μm).

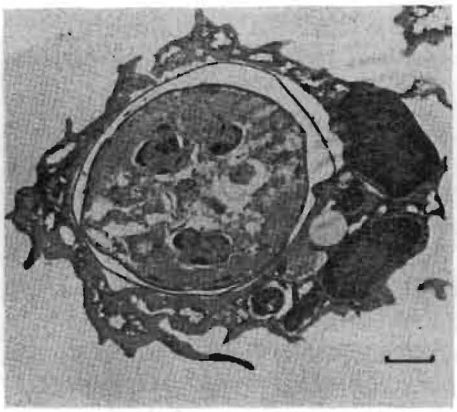

Fig. 2 Transmission electron-micrograph of rat neutrophils attached to a *B. pahangi* microfilaria after 6 hours in the presence of immune serum. The bar represents 1 μm.

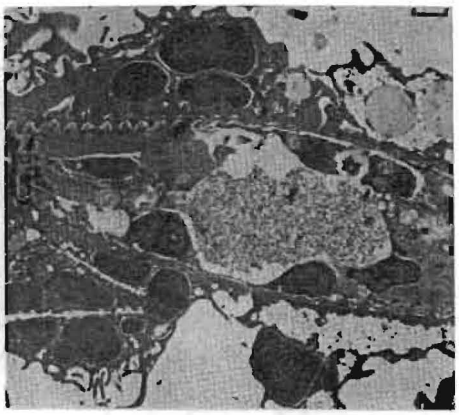

Fig. 3 Rat neutrophils attached to a *B. pahangi* microfilaria, as in Figure 2. The bar represents 1 μm.

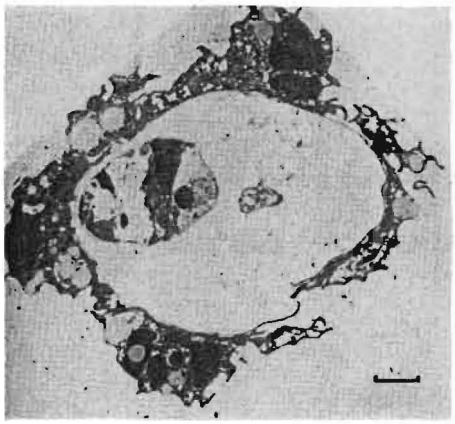

Fig. 4 Rat neutrophils and a *L. carinii* microfilaria: ADCC after 16 hours, with rupture and breakdown within the microfilaria. The bar represents 2 μm.

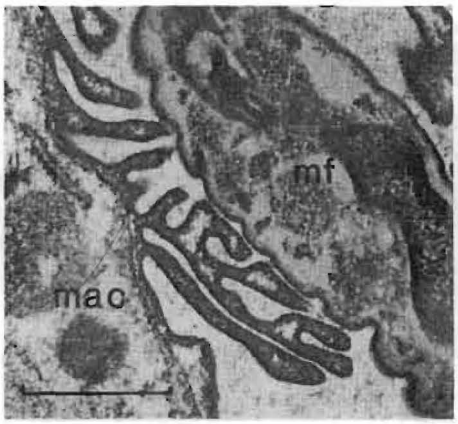

Fig. 5 Rat macrophage (MAC) attached to *L. carinii* microfilaria (mf) after 6 hours in the presence of immune serum. Electron-dense material is present between the macrophage and the microfilaria. The bar represents 1 μm.

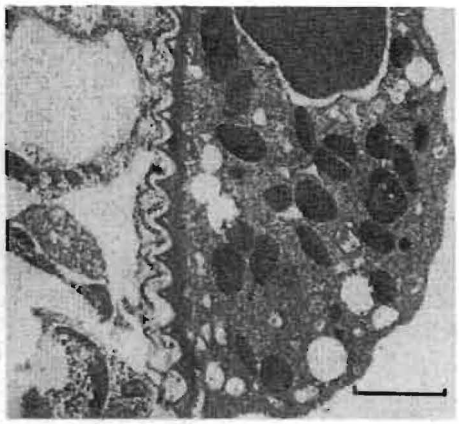

Fig. 6 Rat eosinophil attached to *L. carinii* microfilaria after 6 hours in the presence of immune serum. There is electron-dense material between the microfilaria and the closely approsed cell (right). The bar represents 1 μm.

ed. After 16 hours, rupture and cellular breakdown within the microfilariae had occurred (Fig. 4). The adherence of macrophages (Fig. 5) and eosinophils (Fig. 6) to the microfilariae in the presence of antibodies was also close and both were accompanied by the deposition of electron-dense material between the cell and the microfilarial sheath. Binding of macrophages ultimately resulted in the breakage of the sheath and invasion of the space between the sheath and the cuticle by the electron-dense material. This was followed by disintegration of the microfilariae. Eosinophils also adhered tightly, with deposition of electron-dense material on the sheath. Although damage to the sheath occurred, the extensive destruction seen with neutrophils and macrophages after 16 hours did not result from eosinophil binding.

DISCUSSION

The observations reported here suggest that ADCC of rat neutrophils and macrophages for *L. carinii* and *B. pahangi* microfilariae requires intact microfilament function and involves close attachment and destruction of the sheath. In neither case did oxygen metabolites or lysosomes appear to be important. There were some morphological and metabolic differences between ADCC of neutrophils and macrophages. For example, macrophages appeared to cause more rapid and extensive damage to the sheath and to deposit electron-dense material. Interpretation of the effects of various agents on the

cytotoxicity of macrophages was more difficult because cytotoxicity in the controls was very slight.

Cytochalasin B strongly inhibited the reactions of neutrophils and macrophages, the former to a greater extent than the latter. This drug is a potent inhibitor of most forms of antibody-dependent cellular cytotoxicity, including that of human eosinophils for schistosomulae[12] and rat eosinophils for chicken erythrocytes,[13] and of human[14] and rabbit K cells[15] for nucleated target cells. It also inhibits specific cytolysis of immune T cells[14,16] and the nonspecific but selective cytotoxic effect of activated macrophages on tumour cells.[17,18] It thus seems that microfilament function may be essential for cytotoxicity in general.

Colchicine inhibited the adherence of macrophages quite strongly but had no effect on ADCC of neutrophils. In other cytotoxicity reactions, there also was considerable variability in the requirement for microtubule function. Colchicine did not inhibit the cytotoxicity of rabbit K cells to sensitised chicken erythrocytes[15] but did inhibit activated macrophage cytotoxicity to tumour cells.[17,18] Antibody-dependent cytotoxicity of schistosomulae by human eosinophils was inhibited by colchicine[12] whereas that of chicken erythrocytes by rat eosinophils was not.[13] Inhibitors of protein synthesis also had no effect on antibody-dependent adherence and cytotoxicity of human eosinophils to schistosomulae[12] and rat eosinophils to chicken erythrocytes.[13] They did inhibit the nonspecific cytotoxicity of tumour cells by activated macrophages from guinea pigs[18] but not mice.[19]

There was less pronounced inhibition with some other agents: cycloheximide in the case of macrophages, but not neutrophils; and N-ethylmaleimide in the case of neutrophils. This suggests some requirement for protein synthesis on the part of macrophages and the possible involvement of a serine esterase. The increase in cytotoxicity of neutrophils in the presence of 10^{-5}M cycloheximide was not investigated further.

Catalase, superoxide dismutase, mannitol and ascorbate were not inhibitory, suggesting that reactive oxygen metabolites play no role in ADCC to microfilariae. In other systems, oxygen metabolites may[20] or may not[21] be involved in eosinophil-mediated cytotoxicity to schistosomulae. They appear to be important in antibody-dependent cytotoxicity of macrophages to tumour cells but less so in the nonspecific cytotoxicity of activated macrophages to tumour cells.[22-24]

Some agents enhanced ADCC: 2-deoxyglucose, mannitol, ascorbate, azide, fluoride and cyanide in the case of neutrophils; and ascorbate, azide, fluoride and cyanide in the case of macrophages. Pre-incubation of the microfilariae (but not the cells) with ascorbate, fluoride or cyanide also enhanced ADCC. Although the mechanism of action is unknown, it is conceivable that they interfered with microfilarial activity which antagonised ADCC.

Trypsin treatment of the cells abolished their activity. This could be due to destruction of a receptor for IgE or for complement, which appears to be necessary in these reactions.[1] The electron micrographs showed that the eosinophils were bound as closely as neutrophils or macrophages and that they could destroy the sheath; however, further destruction was limited in the time allowed. This is compatible with our earlier observations that eosinophils could bind but not kill the microfilariae.[4] Binding was accompanied by the deposition of electron-dense material but there appeared to be no selective migration of basic granules towards the interface with the microfilariae. The most extensive observations on effector cell-parasite interactions have been made with eosinophils and schistosomulae,[12,25] *Trichinella spiralis* and *Nippostrongylus brasiliensis*.[12] Prominent in those reactions were degranulation and the deposition of peroxidase and other eosinophil products on the surface of the parasites. These and other observations have suggested that lysosomes may have an important general role in antibody-dependent cellular damage of parasites.[26] Our observation that eosinophils adhered to but did not damage microfilariae, although there was both an electron-dense deposit and the lack of effect of chloroquin and hydrocortisone, suggests that some caution is necessary with regard to interpretation.

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