

RODENT EOSINOPHILS AND NEUTROPHILS: MEMBRANE RECEPTORS,
EFFECTOR FUNCTIONS AND ANTIGEN DIFFERENCES

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ABSTRACTRODENT EOSINOPHILS AND NEUTROPHILS: MEMBRANE RECEPTORS, EFFECTOR FUNCTIONS AND ANTIGEN DIFFERENCES

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Purification techniques were developed for mouse eosinophils and neutrophils. Membrane receptors on mouse eosinophils and neutrophils were studied by means of rosette formation, phagocytosis and ^{51}Cr release assays using mouse complement and monoclonal IgM, IgG1 and IgG2b antibody-coated sheep erythrocytes. Eosinophils as well as neutrophils were found to possess complement and IgG receptors, with eosinophils showing higher complement and antibody requirements than neutrophils. Both cell types reacted more strongly with IgG2b than with the IgG1 used, but it is unclear whether this is a subclass effect or a reflection of a different antibody density on the sheep red cell membrane.

The possibility that lysis of trypanosomes in the acute phase of Trypanosoma cruzi infection may result in T.cruzi antigen coating of host cells, thus rendering them susceptible to the host effector mechanisms, was investigated. An in vitro model was used, in which mouse eosinophils and neutrophils were found to be cytotoxic against mouse cell lines coated with T.cruzi antigen in the presence of anti-T.cruzi antibody.

The finding that eosinophils could kill mammalian cells led to experiments in which rat eosinophils, neutrophils and K cells were tested against antibody-coated mouse cell line cells. All three effector cells were found to be active against cells of lymphoid origin. Cytotoxicity by granulocytes was shown to be specific for the antibody-coated target cells and to depend on the type and concentration of the antibody preparation used.

The production of a monospecific anti-eosinophil serum that would allow studies on the role of the eosinophil in experimental protozoan infections was attempted by hyperimmunizing rabbits with highly purified preparations of mouse eosinophils. The antisera thus obtained were found to cross-react with other leucocytes and, even after absorption with a range of mouse cells, no specificity for eosinophils was achieved. Monoclonal antibody techniques were then tried, and rat-mouse and rat-rat fusions produced several hybridomas secreting anti-eosinophil antibodies. One of these has been shown to be highly specific in vitro and to selectively deplete eosinophils in vivo.

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ABBREVIATIONS

A	Antibody
C	Complement
CFA	Complete Freund's Adjuvant
E	Sheep erythrocytes
F.A.C.S.	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
i.p.	Intraperitoneal(ly)
i.v.	Intravenous(ly)
HEPES	N-2-Hydroxyethyl piperazin-N'-2-ethanesulphonic acid
NIMR	National Institute for Medical Research, Mill Hill, London
PEG	Polyethylene glycol

CHAPTER 1

INTRODUCTION

Neutrophils have long been recognized as constituting a defensive barrier against invading microorganisms. They accomplish this by reaching the site of infection as a response to a chemotactic stimulus, and then ingesting and killing the pathogens.

In contrast to neutrophils, the role of eosinophils has only recently begun to be understood. Despite its frequent association with parasitic and allergic diseases, no specific function had been ascribed to the eosinophil until recently, and it had been suggested that these cells may be important in removing antigen-antibody complexes.

The observation that eosinophils are less active than neutrophils in killing Escherichia coli and Staphylococcus aureus, probably as a result of differences in their peroxidases (De Chatelet, Migler, Shirley, Muss, Szejda & Bass, 1978), suggest that neutrophils are the more active in controlling infection by these microorganisms.

Eosinophils, on the other hand, are selectively recruited by the mast cell tetrapeptide "eosinophil chemotactic factor of anaphylaxis" and contain in their granules an array of enzymes with antagonistic activity to the mast cell products. For example, both eosinophils and neutrophils can inactivate histaminase by means of histaminase; eosinophils and probably also neutrophils can produce prostaglandins as a response to membrane stimulation. Eosinophils have a high content of arylsulphatase B which inactivates the "slow reacting substance of anaphylaxis". Eosinophils also have phospholipase D which inactivates platelet activating factors, and a lysophospholipase which may protect the eosinophil from its own lysophospholipids. All these factors suggest that eosinophils could play a major role in controlling hypersensitivity reactions.

Although there is a well documented association between eosinophilia and some parasitic infections, the reason for this has remained elusive for many years. The demonstration that eosinophils could mediate antibody-dependent killing of the trematode Schistosoma mansoni (Butterworth, Sturrock, Houba, Mahmoud, Sher & Rees, 1975) produced evidence that eosinophils could function as effector cells against this parasite. Since then, eosinophils have also been shown to mediate antibody-dependent killing of the nematodes Litomosoides carinii (Subrahmayam, Rao, Mehta & Nelson, 1976), Trichinella spiralis (Kazura & Grove, 1978) and Nippostrongylus brasiliensis (Mackenzie, Jungery, Taylor & Ogilvie, 1980), and the protozoans Trypanosoma cruzi (López, Bunn Moreno & Sanderson, 1978) and Trypanosoma dionisii (Thorne, Glauert, Svenssen & Franks, 1979). Eosinophils have also been shown to phagocytose malaria parasite-infected red cells in the presence of antibody (Tosta & Wedderburn, 1980).

Neutrophils have also been found to be active against the protozoan T. cruzi (López et al., 1978) and T. dionisii (Thorne et al., 1979), however they do not appear to induce damage to the large parasites N. brasiliensis and T. spiralis (Mackenzie, Jungery, Taylor & Ogilvie, 1981). While some workers have found neutrophils to induce damage to antibody or complement-coated S. mansoni (Anwar, Smithers & Kay, 1979) others have shown eosinophils but not neutrophils to be the active cells (Vadas, David, Butterworth, Pisani & Siongok, 1979).

As eosinophils are often compared to neutrophils when tested for cytotoxic activity against parasites, some interesting differences are beginning to emerge. For example, the high ability of eosinophils to inflict damage to large parasites as compared to neutrophils could be explained at least in part by the presence in the eosinophil of a unique

constituent, the major basic protein (Gleich, Loegering & Maldonado, 1973) which has cytotoxic properties (Butterworth, Wassom, Gleich, Loegering & David, 1979a) and can promote a firm attachment of the eosinophil to the parasite membrane (Butterworth, Vadas, Wassom, Dessein, Hogan, Sherry, Gleich & David, 1979c).

It should be noted that while granulocytes represent a system that can induce damage to invading parasites, other cell types have been shown to lack this property. Thus, K cells which are highly active against antibody-coated cell line cells do not kill T.cruzi (Sanderson, Lopez & Bunn Moreno, 1977) T cells, although recognizing host antigens on the surface of schistosomula of S.mansoni fail to induce cytotoxicity (Butterworth, Vadas, Martz & Sher, 1979b). Macrophages, while being able to induce damage to S.mansoni (Capron, Dessaint, Joseph, Rousseaux, Capron & Bazin, 1977) appear to lack cytotoxic activity against T.cruzi and can even support its growth (Sanderson & de Souza, 1979).

Some of the difficulties in comparing eosinophils and neutrophils is derived from the need to work with highly purified preparations. This has prompted workers to develop purification techniques which have produced highly purified preparations of eosinophils and neutrophils obtained from the rat (Sanderson & Thomas, 1978) and human species (Vadas et al., 1979). In this thesis, purification techniques are described for mouse eosinophils and neutrophils (Chapter 3) which provide the basis for studies on their membrane receptors (Chapter 4), effector functions (Chapter 5) and antigenic differences (Chapter 7). Furthermore, by developing purification techniques for mouse granulocytes it is hoped that it will facilitate studies of these cells in an extensively used experimental model as the mouse, particularly regarding parasitic infections and immediate hypersensitivity.

Eosinophils and neutrophils carry out their effector functions by means of specific membrane receptors. These include receptors for the C3b-C4b fragments of complement (CR1 receptors), for the C3bi fragment (CR3 receptor) and for the Fc portion of IgG (Anwar & Kay, 1977a). Granulocytes appear to lack receptors for the C3d portion of C3 (CR2 receptor) (Ross, Tack & Rabellino, 1978; Tai & Spry, 1980) and for IgM. After some conflicting reports (Sullivan, Grimley, Metzger, 1971; Hubscher, 1975) eosinophils do seem to have receptors for IgE which are different to those for IgG (Capron & Capron, 1980). Furthermore, eosinophils have been shown to kill IgE-coated S.mansoni (Capron & Capron, 1980). Data on eosinophil and neutrophil binding to IgG subclasses is, however, scanty. When tested in a homologous system rat eosinophils appear to have receptors for IgG2a (Capron, Capron, Torpier, Bazin, Bout & Joseph, 1978), and guinea pig eosinophils bind sheep and ox erythrocytes coated with IgG1 and IgG2 antibodies (Butterworth, Coombs, Gurner & Wilson, 1976).

It is important to investigate the Ig isotype to which granulocytes can bind, and which effect results from this interaction, because differences in cytotoxicity by different cell types with different antisera may be explained on the basis of the particular subclass composition of each antiserum (Clark & Klebanoff, 1977; Chapter 6). Furthermore, the concept of "masking antibodies" to parasite antigens (Rickard, 1974) or tumour antigens may be directly related to the inability of the host effector cells to bind a specific isotype. That differences in the eosinophil and neutrophil binding to different IgG subclasses may exist is suggested also by inhibition experiments in which myeloma proteins of some subclasses were inhibitory in the rosette assay, while others were not (Messmer & Jelinek, 1970; Tai & Spry, 1976).

The advent of monoclonal antibodies offers the opportunity to study these differences. Monoclonal antibodies to sheep erythrocytes (E) have already proved useful tools to study macrophage binding to different IgG subclasses. Thus, mouse macrophages have been shown to phagocytose and lyse E coated with mouse IgG1, IgG2a, IgG2b and IgG3 monoclonal antibodies, and appear to be more active when E are opsonized with IgG2a or IgG2b (Ralph, Nakoink, Diamond & Yelton, 1980). Furthermore, these antibodies have allowed studies that show that macrophages have three different Fc receptors: one shared by IgG1 and IgG2b (Diamond & Scharff, 1980), another for IgG2a (Diamond & Scharff, 1980) and a third for IgG3 (Diamond & Yelton, 1981).

Although in previous studies mouse eosinophils appeared to be different to eosinophils from other species in that complement receptors could not be detected on their surface (Rabellino & Metcalf, 1975; Rabellino, Ross, Trang, Williams & Metcalf, 1978; Hoghart, Cruise, McKenzie & Mitchell, 1980), it is shown in Chapter 4 that they do possess complement receptors but that eosinophils have a higher complement requirement than neutrophils. Eosinophils are also shown to have a higher requirement for antibody molecules than neutrophils and studies have been carried out to investigate the binding of granulocytes to IgG1 and IgG2b monoclonal antibodies (see Chapter 4).

Despite the fact that eosinophils can kill large parasites coated with antibody and complement, the possibility that these cells could also mediate damage to mammalian cells in a similar fashion has received little attention. Results of Parrillo & Fauci, (1978) suggest that eosinophils may have low activity against antibody-coated nucleated mammalian cells. Thus, eosinophils appeared to be less effective than neutrophils which are very active against certain tumour cells (Gale & Zighelboim, 1975;

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Clark & Klebanoff, 1977). In this thesis, the cytotoxic activity of eosinophils and neutrophils is investigated in detail (Chapters 5 and 6) and it is shown that both types of granulocyte can induce similar levels of cytotoxicity from cell line cells coated with antibody. Through this effector function it is possible that eosinophils and neutrophils could play a central role in certain diseases (Chapters 5 and 6).

Previous work has indicated that eosinophils could play a protective role against some parasitic infections in vivo (James & Colley, 1976). The experiments described in this thesis suggest a possible role for granulocytes in T.cruzi infections (Chapter 5). Furthermore, their ability to kill tumour cells in vitro (Chapters 5 and 6) deserves further investigation in vivo. With this objective in mind, the production of monoclonal antibodies to mouse eosinophils and neutrophils was sought with the aim of obtaining a specific reagent with which to ablate these cells in vivo. The development of a monoclonal antibody highly specific for mouse eosinophils in vitro and which selectively ablates these cells in vivo has been achieved (Chapter 7). This approach is shown to offer the best possibilities of success to study the role of granulocytes in vivo.

CHAPTER 2

MATERIALS AND METHODS

2.01. Media

2.01.1. Media for general purposes

Phosphate buffer saline: NaCl 8 gm; KCl 0.2 gm; anhydrous Na₂HPO₄ 1.15 gm; KH₂PO₄ 0.2 gm; made up to 1 litre with distilled water.

The foetal calf serum (FCS) and RPMI 1640 media (Cat. No.10-601-22; powder preparation with glutamine, without sodium bicarbonate) were obtained from Flow Laboratories, Irvine, Scotland. The FCS was used after heat inactivated 30' at 56°C. The RPMI 1640 stock solutions were made up and sterilised by filtration by the Media Supply Department of the Clinical Research Centre, Harrow.

PBS-FCS

PBS containing 2% foetal calf serum

RPMI 1640 (X10; stock solution)

RPMI-H (X1; stock solution)

RPMI-1640 containing 20 mM HEPES (Flow Laboratories) buffer

RPMI-Bicarb (X1; stock solution)

RPMI-1640 containing 0.22% sodium bicarbonate (BDH Chemicals Ltd.,
Poole, England)

RPMI-H-F 10%

A stock solution containing RPMI-H and 10% Ficoll (Pharmacia Fine Chemicals AB, Upsala, Sweden).

When other concentrations were required, this stock solution was diluted in RPMI-H.

RPMI-H-FCS

RPMI-H containing 10% foetal calf serum, 100 IU/ml Penicillin-streptomycin (Glaxo Laboratories Ltd., Greenford, England) and 2 mM L-Glutamine (BDH Chemicals Ltd.).

RPMI-Bicarb-FCS

RPMI-Bicarb containing 10% foetal calf serum, 10 mM HEPES buffer

100 IU/ml Penicillin-streptomycin and 2 mM L-Glutamine.

RPMI-Bicarb-FCS-P

RPMI-Bicarb-FCS containing 100 mM sodium pyruvate (Flow Laboratories).

2.01.2. Hybridoma reagents and media

PEG

35% polyethylenglycol (Mol.wt. 1500; BDH Chemicals Ltd.) in RPMI-H. It was dissolved at 37°C and sterilised by filtration.

HT stock solution (100x)

H ₂ O	90 mls	} SIGMA London Chemical Co., Poole, England
Hypoxantine	136.1 mg	
Thymidine	38.7 mg	

dissolved at 44°C for 60'. Sterilised by filtration.

HAT stock solution (100x)

This was obtained by mixing 90 ml of HAT stock solution with with 10 ml of Solution A.

Solution A: 0.1 M NaOH 5 ml

Aminopterin 17.6 mg (ICN Pharmaceutical Inc. Cat. No.100623, Cleveland, USA).

Dissolved. 100 ml H₂O added. Sterilised by filtration.

Cloning medium

RPMI-Bicarb-FCS containing HT or HAT stock solutions in different final concentrations.

Agar stock solution

1.1% Agar (Difco-Bacto-Agar, Difco Laboratories, Detroit, USA) in deionised distilled water.

Agar medium

For 100 ml:	RPMI 1640 (10x)	16 ml
	NaHCO ₃ 8% w/v	2.5 ml
	Penicillin/streptomycin 100 IU/ml	5 ml
	Glutamine 2 mM	1 ml
	Sodium pyruvate	2 ml
	Foetal calf serum	40 ml
	H ₂ O	36 ml

Agar base layer

Equal volumes of agar stock solution and agar medium were mixed to give an agar concentration of 0.55%.

Agar top layer

Equal volumes of cloning medium and agar base layer were mixed to give an agar concentration of 0.275%.

2.01.3. Medium for freezing down cells

RPMI-H-FCS-DMSO

RPMI-H-FCS containing 10% dimethylsulphoxide.

2.02. Cell line cells

The cell line designation is followed, in brackets by the animal strain and the tissue of origin. The American Type Culture Collection (ATCC) and Certified Cell Line (CLL) number of each cell line is given when known.

2.02.1. Cell line cells of mouse origin

BW 5147 (AKR, thymic lymphosarcoma used for the production of T cell hybridomas) was a gift from Dr. E. Simpson (Clinical Research Centre, Harrow).

S2 (BALB/c, muscle-derived fibrosarcoma) was maintained in vitro by Dr. Ribeiro dos Santos (St. George's Hospital Medical School, Tooting).

Neuro-2a (BALB/c, neuroblastoma) was obtained from Dr. Ribeiro dos Santos. ATCC CCL 131.

P815 (DBA/2 mastocytoma) was maintained by Dr. C.J. Sanderson.

EL4 (C57 BL, thymic lymphosarcoma) was a gift from Dr. E. Simpson.

P3-NSI-1 Ag4-1 (BALB/c, myeloma) was a gift from Dr. E. Simpson.

This cell line, commonly referred to as NSI, is widely used for the production of monoclonal antibodies (Köhler, Howe and Milstein, 1976).

Although it does not secrete Ig, it synthesises K chain.

P3-X63- Ag8.653 (BALB/c myeloma) was a gift from Dr. B. Turner (NIMR).

This cell line, used for the production of monoclonal antibodies, was derived from NSI and does not express Ig heavy or light chain (Kearney, Radbruch, Liesegang and Rajewsky, 1979).

2.02.2. Cell line cells of rat origin

Y3-Ag 1.2.3 (LOU rat myeloma) was obtained from Dr. C. Milstein (MRC Laboratory of Molecular Biology, Cambridge). This cell line is used for the production of monoclonal antibodies (Galfre, Milstein & Wright, 1979). It is commonly referred to as Y3.

2.02.3. Cell line cells of human origin

Daudi (Burkitt lymphoma-derived) was a gift from Dr. S. Patterson (Clinical Research Centre).

MRC5 (Human embryonic lung; diploid); ATCC CCL 171, was supplied by the tissue culture service of the Clinical Research Centre.

All cell line cells, except Y3 were grown in RPMI-Bicarb-FCS. Y3 cells were grown in RPMI-Bicarb-FCS-P.

All these cell lines were used when in exponential growth.

2.03. Laboratory animal strains

2.03.1. Mouse

BALB/c, BALB/c nu/nu, A, DBA/2, C57BL/6, C57BL/10, SJL, TO, ATL, B10, AQR, CBA and AKR were originally obtained from OLAC Ltd., Bicester, England.

C57 BL/6-Ly-1^a was originally obtained from the Sloane-Kettering Institute for Cancer Research, New York, U.S.A. NH was obtained from the National Institute of Health, Bethesda, U.S.A.

All these strains were maintained in the special Pathogen Free Unit of the Clinical Research Centre.

2.03.2. Rat

AGUS rats were obtained from Bantin and Kingman Ltd., Hull, England.

August rats were obtained from NIMR.

LOU rats were obtained from OLAC Ltd.

2.03.3 Rabbit

New Zealand white rabbits were obtained from Ranch Rabbits Ltd., Crawley Down, England.

2.04 Antibody preparations

2.04.1. Monoclonal antibodies

Mouse anti-sheep erythrocytes (E): IgM from hybridoma supernatant (MAS 012b), IgG1 from hybridoma supernatant (MAS 013b) and serum/ascites (MAS 013c) and IgG2b from hybridoma supernatant (MAS 014b) and serum/ascites (MAS 014c) were all purchased from Sera-Lab Ltd. (Crawley Down, England). IgM 83/SCC1 was a gift from Dr. P. Lydyard (Middlesex Hospital Medical School).

Mouse H-2^d anti-H-2^k (antibody 27.R9; Lemke, Hämmerling, Höhmann & Rajewsky, 1978) was a gift from Dr. A. Müllbacher (Clinical Research Centre).

2.04.2. Antisera

Mouse anti-E: Laboratory stock, obtained after multiple injections of F. This is known as "Mouse anti-SRBC (B/C)ex AJM".

Mouse anti-Trypanosoma cruzi: this was obtained by Dr. Ribeiro dos Santos from BALB/c mice chronically infected with T.cruzi. The antiserum was absorbed with S2 and Neuro-2a cell line cells.

Mouse H-2^d anti-H-2^k (Searle Diagnostic, High Wycombe, England).

Rat anti-mouse cell lines: three antisera were raised in three individual AGUS rats: antiserum A and B were produced in a similar fashion in two rats that received weekly i.p. injections of 3×10^7 P815 cells over a two month period. Antiserum C was produced in another AGUS rat which received a single i.p. injection of 3×10^7 P815 cells followed by weekly i.p. injections of 3×10^7 Neuro-2a cells over a two month period.

Rat anti-human MRC 5: this was produced by weekly i.p. injections of MRC 5 cell line cells into an AGUS rat over a two month period.

Rabbit anti-T.cruzi: this was obtained by Dr. Ribeiro dos Santos from rabbits chronically infected with T.cruzi. The antiserum was absorbed

with S2 and Neuro-2a cell line cells.

Rabbit anti-mouse IgG: Laboratory stock. The rabbit IgG fraction was a gift from Dr. W. Thomas (Clinical Research Centre) and the anti-mouse IgG purified by affinity chromatography on a mouse IgG coupled-Sepharose column by Dr. C. Sanderson.

Rabbit anti-mouse eosinophil: three individual antisera were raised, AE₁, AE₂ and AE₃ by weekly injections of 5×10^7 BALB/c eosinophils in Complete Freund's Adjuvant (CFA, Difco Laboratories) in three New Zealand white rabbits over a four week period. AE₁ absorbed with 10^8 P815 cells is called AE_{1a}.

Rabbit anti-mouse neutrophil: this was produced by weekly injections of 3×10^7 BALB/c neutrophils in CFA in a New Zealand white rabbit over a four week period.

Goat anti-rat IgG: Laboratory stock. The IgG fraction was a gift from Dr. J. Howard (ARC, Babraham), and the anti-rat IgG purified by affinity chromatography on a rat IgG coupled-Sepharose by Dr. C. Sanderson.

Goat anti-rabbit IgG-FITC: (Nordic Immunology, London).

2.05. Complement

2.05.1. Mouse complement.

This was obtained from strain A(C5 deficient) mice. Mouse blood was pooled, allowed to clot 15' at 22°C and then at 4°C for 2 to 4 hrs. The serum thus obtained was twice absorbed with E (1 volume packed E: 5 volumes mouse serum) and kept at 4°C. It was used within two hours.

2.05.2. Guinea pig complement

Fresh guinea pig serum was absorbed with mouse spleen cells (1 volume packed splenocytes: 10 volumes guinea pig serum) for 1 h at 4°C. It was stored in 200 µl aliquots at -70°C until use. This complement was used in the cytotoxicity assays (Chapter 7) with mouse eosinophils, lymphocytes and macrophages at a dilution of 1:10 in RPMI-H-FCS. This dilution gave maximum cytotoxicity in a preliminary titration with a rat anti-mouse cell serum (antiserum A).

2.05.3. Rabbit complement

This was used in the cytotoxicity assay with mouse neutrophils (Chapter 7) since guinea pig complement gave very low levels of cytotoxicity with a rat anti-mouse cell serum (antiserum A) and hybrid M1/21.A9.H8.G5 (Table 2.1). Complement from six rabbits were then tested and the complement giving the highest levels of cytotoxicity and the lowest level of toxicity (antibody-independent activity) absorbed with different numbers of mouse spleen cells. Absorption of 100 µl of complement with 5×10^7 cells was found to give high levels of cytotoxicity with a low toxic effect and used at a dilution of 1:10 in RPMI-H-FCS.

2.05.4. Human complement

Laboratory stock, obtained from the serum of a normal individual and stored at -70°C.

Table 2.1. Cytotoxicity of mouse neutrophils by antibody and complement from different sources.

Complement at 1:10	Antibody preparations							
	Antiserum A		Monoclonal Ab*		Antiserum A		Mon.Ab*	
	+	-	+	-	+	-	+	-
guinea pig	25 [†]	4	3	0	20	3	5	0
rabbit	89	72	98	88	100	76	96	93
rabbit absorbed	77	14	59	9	NT	NT	NT	NT
Human	2	1	9	2	15	6	7	6
	without sodium azide				with 10 mM sodium azide			

* Monoclonal antibody M1/21 A9.H8.G5 (see Chapter 7)

† Percentage specific ⁵¹Cr release

NT = not tested

Increasing the complement concentration did not increase the specific ⁵¹Cr release.

Azide was used to inhibit an antibody modulation effect.

2.06. Cell suspensions

2.06.1. Mouse cells

Peritoneal exudates containing eosinophils and neutrophils, were collected from the peritoneal cavity in RPMI-H-FCS, washed (centrifugation at 400 r.c.f. for 5'), counted and resuspended to the required concentration.

Macrophages were obtained from the peritoneal cavity of mice three days after an injection of dextran (Mol.wt. $5-40 \times 10^6$, Koch-Light Laboratories Ltd., Colnbrook, England) and collected in RPMI-H-FCS.

Lymphocytes were obtained from normal spleens. The spleens were collected in RPMI-H-FCS, disrupted with a silicone rubber bung and the resulting cell suspension allowed to stand for 5' to let aggregates sediment. The single cell suspension was then transferred to another tube, washed and centrifuged on an interface of Metrizamide (Nyegaard & Co., Oslo) 14.5% at 1200 r.c.f. for 15'. The interface consisting of 100% mononuclear cells was the source of lymphocytes.

Mouse peripheral blood was obtained by cardiac puncture with a 1 ml syringe containing 40 I.U. of heparin (preservative free; Paines & Byrne Ltd., Greenford, England). The cells were then washed and resuspended in RPMI-H-FCS.

2.06.2. Rat cells

Eosinophils, neutrophils and K cells were obtained as previously described (Sanderson & Thomas, 1978). Briefly, eosinophils obtained from normal August rats in RPMI-H-FCS were centrifuged on a two step gradient of Metrizamide 17.5% and 16.5%. The second interface containing the eosinophils was centrifuged again on a second gradient of Metrizamide using the same concentrations. This produced a preparation consisting of

about 90% eosinophils.

Rat neutrophils were obtained in RPMI-H-FCS from AGUS rats after an i.p. injection of dextran. The cells were first centrifuged on Ficoll-Paque, and the resulting pellet centrifuged on a two step gradient of Metrizamide 20% and 17.5%. The second interface, containing about 95% neutrophils, was used.

K cells were obtained from the spleen of normal AGUS rats. This was disrupted (see Section 2.06.1.) and the cells in RPMI-H-FCS centrifuged on Ficoll-Paque. The interface, consisting of 100% lymphoid cells was used as the source of K cells.

2.07. Cell counting

This was done with a Coulter counter Model CBl (Coulter Electronics Ltd., Luton, England). This apparatus counts particles passing through a small aperture in an electrolyte solution. A fixed volume (usually 0.5 ml) of particles is made to pass through the aperture in which an electrical current has been established. As each particle passes through the orifice, the resistance is changed and registered as a pulse that is proportional to the particle volume. Coincidence corrections (when two cells pass through the orifice simultaneously) is made electronically. Using the electrical controls it is possible to select the volume range. Thus, with the instrument properly calibrated for a particular cell type only viable cells are counted as dead cells show a different electrical resistance and give a pulse height equivalent to a much smaller cell.

This instrument was used in conjunction with a Model P64 size distribution analyzer (Coulter Electronics Ltd.) which plotted size

distribution graphically.

When mouse peripheral blood and spleen cells were counted, Zaponin (Coulter Electronics Ltd.) was added to lyse the red cells.

2.08. Cell viability

This was tested by mixing equal volumes of filtered trypan blue 0.2% in PBS and the cell suspension. After 5' at 22°C the cells were examined in the microscope under phase contrast and those cells stained, scored as dead cells.

2.09. Staining

Cells were first sedimented onto a microscope slide with a Cyto-centrifuge (Shandon Scientific Company Ltd., London) and fixed in methanol for 4'-5'. The slides were then stained for two minutes with filtered Giemsa stain (Code No.35014, 2K, BDH) freshly diluted 1:5 in Sorensen's buffer (Mercia Brocades Ltd., West Byfleet, England).

2.10. Parasites used to induce peritoneal eosinophilia

2.10.1. Trichinella spiralis (gift from Dr. G. Lee, NIMR)

The infective larvae of this parasite was obtained from mice or rats carrying at least a six week old infection. The parasite could be obtained by digesting the wall of the cysts present in the skeletal muscle

of these animals with a solution of 1 N HCl 1% and pepsin (BDH) 1%.

2.10.2. Taenia crassiceps (Toi strain, gift from Dr. J. Chernin, North East Surrey College of Technology)

This parasite, whose definite host is the fox and the intermediate host a rodent (Freeman, 1962) was maintained by serial i.p. passage in mice. This parasite reproduces in the peritoneal cavity of mice by asexual exogenous budding.

2.10.3. Mesocestoides corti (gift from Professor Eckert, Institut für Parasitologie der Universität, Zürich)

This parasite appears to need two intermediate hosts, a mite and a small vertebrate before completing its life cycle in a bird or a mammal (Eckert, von Brand & Voge, 1969). In natural infections the larvae of M.corti passes from the gut to the peritoneal cavity of mice, and from here to the liver by direct penetration (Specht & Widmer, 1972). During asexual multiplication in the liver, few parasites are found in the peritoneal cavity, but after day 25 after infection many parasites are observed again in the peritoneum. In laboratory i.p. infections the larvae penetrates into the liver where it multiplies asexually and migrates back into the peritoneum. This parasite was maintained by serial i.p. passage in mice.

2.11. Cell separation techniques

2.11.1. Nylon wool column

This technique was used to separate adherent from non-adherent cells essentially as described by Julius, Simpson & Herzenberg (1973).

A column was made in a 10 ml syringe, filled with RPMI-H-FCS and incubated at 37°C for 1 hour. The column was then rinsed with fresh medium and the cells in RPMI-H-FCS added and incubated at 37°C. After 15' the non-adherent cells were obtained by eluting the column with 25 ml of RPMI-H-FCS. The adherent cells were recovered by forcing medium through the column with the syringe plunger.

2.11.2. Isopycnic centrifugation

This technique separates cells by centrifuging them at relatively high gravitational forces to their equilibrium position (isopycnic point) in a density gradient. The isopycnic point for each cell type was firstly determined by centrifuging the cells on a continuous linear gradient. Once this was determined, an interface, or a two step gradient was used which allowed the separation of a particular cell type from the other cells in a single centrifugation step.

Metrizamide (Nyegaard & Co. A S. Oslo) was chosen over Ficoll-sodium diatrizoate solutions as a gradient material because of its greater flexibility in covering a wide range of densities without changes in osmolarity and only minor changes in viscosity. A Metrizamide stock solution 35.3% w/v was made and filtered through a 0.22 µm Millipore filter. This stock solution has an osmolarity of 282 m Osm which is isotonic for the rat and slightly hypotonic for mouse cells.

Metrizamide of different concentrations were made by diluting the

stock solution in PBS and adding 2% FCS. Each concentration was then checked with a refractometer (Abbe refractometer M.46, Rank Precision Ltd., London) and adjusted if necessary (Table 2.2). It was stored at -20°C away from light.

A continuous linear 10-20% Metrizamide gradient was formed by separately dispensing these solutions into the chambers of a gradient former, the highest density solution into the chamber nearest the outlet. The tubing from the outlet was connected to a penitaitic pump (Varioperpex II, LKB Produkter Ltd., Sweden) which in turn was connected to the probe of an Auto Densi Flow (Searle, New Jersey, U.S.A.) apparatus. This instrument delivers the gradient into a centrifuge tube, and as the fluid enters the tube, the probe is automatically raised so that the fluid is delivered at the surface of the gradient in a continuous and non-turbulent manner. The cells were then placed on top of the gradient with the same apparatus and centrifuged for 15' at 1200 r.c.f. After centrifugation the sample layers were collected from the top of the tube in 0.5 ml fractions using the Auto Densi Flow in reverse. For each fraction the refractive index was determined as a measure of the Metrizamide concentration, the cells counted and a cytocentrifuge preparation made. In this way it was possible to establish the total number of each cell type that sedimented in each fraction.

Separation on a density interface

5 ml of the chosen Metrizamide concentration was placed underneath 5 ml of cells (up to 10^8 per 24 mm tube) in RPMI-H-FCS with a syringe.

Separation on a two step gradient

This was formed by layering 1 ml of the highest Metrizamide concentration at the bottom of a 10 ml plastic tube followed by 2 ml of the lowest Metrizamide concentration on top, and the cells in 1 or 2 ml

Table 2.2. Refractive index and density of a range of Metrizamide concentrations at 22°C.

Percent. Metrizamide	Refractive index	Density (gm/cm ³)
10	1.3483	1.0512
11	1.3500	1.0567
12	1.3516	1.0622
13	1.3532	1.0677
14	1.3548	1.0732
15	1.3564	1.0787
16	1.3580	1.0842
17	1.3597	1.0897
18	1.3613	1.0952
19	1.3630	1.1027
20	1.3646	1.1062
30	1.3809	1.1612
35.3	1.3895	1.1903

Ficoll-Paque has a density of 1.0077gm/cm³ which corresponds to a Metrizamide concentration of 14.8%.

(up to 2×10^7 cells per 16 mm tube) in RPMI-H-FCS on top of the gradient. Delivery of the Metrizamide concentrations and the cells in RPMI-H-FCS was done with the Auto Densi Flow.

For both types of separation the cells were centrifuged for 15' at 1200 r.c.f. The interfaces were individually recovered with a pasteur pipette taking care to avoid contamination with cells in the lower layer. Cells obtained from the interface or pellet were then resuspended in 20 ml RPMI-H-FCS and washed.

2.11.3. Velocity sedimentation at unit gravity

This technique is based on the principle that the velocity of a sphere sedimenting through a fluid under the influence of gravity is given by Stoke's law:

$$s = \frac{2}{9} \frac{(f-f')}{\eta} gr^2$$

where s = sedimentation velocity, f and f' the densities of the cells and fluid medium respectively, g is the sedimentation force, r the radius of the particle and η = viscosity.

In practical terms, provided $(f-f')$ and η remain constant, the cells sediment at rates largely determined by their volume according to the equation

$$s = \frac{r^2}{4} \quad (\text{Miller \& Phillips, 1969})$$

where s is expressed in mm/h and r in microns.

This technique was used essentially as described by Denman & Pelton (1973). The total sedimentation time used was of about 3.5 hrs. The sedimentation velocity for each cell type was calculated by correlating

the geometry of the sedimentation chamber to the volumes collected and the length of time given to the cells to sediment.

The sedimentation apparatus is illustrated in Figure 2.1. The technique was performed in the following way: to the sedimentation chamber (D) containing 50 ml of RPMI-H, up to 4×10^8 cells in 20 mls of RPMI-H-FCS 0.16% - F 0.16% were loaded under gravity. When the cell loading was completed the flow regulators F_1 , F_2 and F_3 were opened, thereby allowing the communication between A and B and the formation of a shallow 0.16% - 2% Ficoll gradient. As the gradient was formed, it entered the sedimentation chamber where a perforated porcelain disc and steel ring (H) were placed at the inlet to minimize turbulence. The cells were pushed upwards by the influence of gravity while the gradient was formed over a 40' period. The rate was controlled manually with flow regulator F_1 . Once the gradient entered the sedimentation chamber the cells were allowed to sediment for another two hours. Collection of the sedimented cells was done over 40'-50' either from the chamber inlet or from the top of the chamber by upward displacement with 40% sucrose. (The sucrose was passed by connecting the vessel E to the sedimentation chamber and opening F_4 .) As the cells reached the lower or upper cone of the chamber, according to the type of collection used, the flow rate was slowed down with flow regulator F_1 to minimize cell layer mixing. The cells were then collected in 10 ml fractions, counted and the percentage of each cell type examined in a cytocentrifuge preparation. Thus, it was possible to calculate the total number of each cell type per fraction.

2.12. Indirect heamagglutination (Coomb's test)

This assay was used in preliminary experiments designed to find the

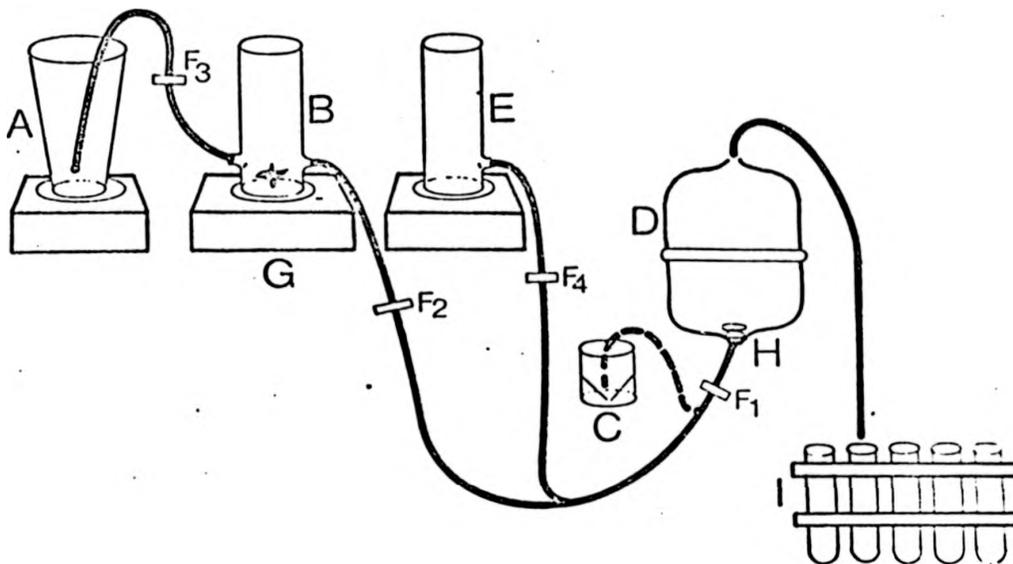


Figure 2.1. Sedimentation apparatus

- A) Chamber containing 300 ml RPMI-H-FCS2%-F2%
- B) Chamber containing 100 ml RPMI-H-FCS1%-F1%
- C) Universal tube containing cells in RPMI-H-FCS 0.16%-F 0.16%
- D) Sedimentation chamber. Diameter = 10 cm
- E) Vessel for applying sucrose 40%
- F) Flow regulators
- G) Magnetic stirrer
- H) Baffle
- I) 10 ml tubes for sample collection

sheep giving the highest titre with mouse monoclonal IgG1 and IgG2b anti-E antibodies. Sheep No.61 (Tissue Culture Services, Slough England) gave the best result out of ten sheep tested and was used throughout these experiments.

The assay was performed in a haemagglutination plate (V bottom, Cooke Microtitre System, Sterilin, Teddington, England) in which equal volumes (50 μ l) of washed E in RPMI-H (10^8 /ml) and antibody dilution (also in RPMI-H) were mixed. After 40' at 22°C the E were washed three times in the plates and the rabbit anti-mouse IgG at 1:200 added (this dilution gave optimum indirect haemagglutination with the mouse anti-E serum). After 3 h at 22°C, the agglutination titre was recorded.

Since in preliminary experiments the mouse monoclonal IgG1 and IgG2b anti-E antibodies obtained in the form of culture supernatant had given a low number of rosettes with both eosinophils and neutrophils, a comparison was made with these antibodies obtained in the form of serum/ascites. A higher titre was recorded with antibodies from the serum/ascites preparation (Table 2.3) and these preparations were therefore used in all the experiments described in Chapter 4. These antibodies did not show direct agglutination of E.

2.13. Complement fixation assay

This assay was used to select the mouse monoclonal IgM anti-E with the highest titre. IgM antibodies, IgM 83/SCC1 and IgM (Sera Lab) had been previously found not to produce direct agglutination of E, although IgM 83/SCC1 can be agglutinating when hybridoma supernatants are concentrated or when obtained in the form of serum/ascites (Dr. Lydyard,

Table 2.3. Indirect haemagglutination (Coomb's test) with monoclonal IgG1 and IgG2b anti-E antibodies

	Reciprocal of Antibody dilution														
	10	20	40	80	160	320	640	1280	2560	5120	10240	20480	4000	8000	16000
IgG1*	+++	+++	+++	+	±	-	-	-	-	-	-	-	-	-	-
IgG2b*	+++	+++	+++	+++	++	±	-	-	-	-	-	-	-	-	-
IgG1†	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-	-	-	-
IgG2b†	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	±	-

* Antibody from hybridoma supernatants

† Antibody from serum/ascites

personal communication).

Equal volumes (50 μ l) of washed E (10^8 /ml), antibody dilution and guinea pig complement (at a 1:10 dilution) were mixed in LP2 tubes (Luckham, Burgess Hill, England) and incubated 1 h at 37°C. At the end of the incubation period the tubes were centrifuged for 5' at 400 r.c.f. and the end point of haemolysis determined visually. The control pellet was classified as (+++) and no visible pellet (indicating 100% haemolysis as (-)).

IgM 83/SCC1 gave the highest titre (Table 2.4) and was therefore selected for the experiments described in Chapter 4. IgG1 and IgG2b were included as a comparison.

2.14. EA complexes

Washed E (10^8 /ml) were incubated for 40' at 22°C with different concentrations of IgM 83/SCC1, IgG1 and IgG2b anti-E antibodies. The complexes were then washed twice and resuspended in RPMI-H.

2.15. EAC complexes

a) Formation of EAC. EAC3b was prepared essentially as described by McConnell & Hurd (1976); 0.5 ml of EA_{IgM} (2×10^8 /ml) were incubated in mouse complement (see Section 2.05.1) diluted 1:2 in RPMI-H. After two minutes at 37°C the reaction was stopped by adding sodium suramin (Antrypol, Bayer 205, Bayer, U.K. Ltd., Haywards Heath, England, gift from Dr. McConnell.)

Table 2.4. Haemolysis of E by different antibody preparations and guinea pig complement

	Reciprocal of antibody dilution											
	5	10	20	40	80	160	320	640	1280	2560	5120	None
IgM 83/SCC1	+++	+++	+++	+++	+++	++	+	+	-	-	-	-
IgM(Sera Lab)	+++	+++	++	+	-	-	-	-	-	-	-	-
IgG1*	-	-	-	-	-	-	-	-	-	-	-	-
IgG2b*	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-

* obtained in the form of serum ascites

For the preparation of EAC3bi, incubation of EA_{IgM} in mouse complement was for 30' at 37°C; the cells were then washed and incubated for a further 1 h at 37°C in undiluted heat inactivated mouse serum as a source of Factor H and Factor I to ensure total cleavage of C3b into C3bi.

Both preparations, EAC3b and EAC3bi were then resuspended and diluted in RPMI-H-F 2%. Granulocytes were also suspended in RPMI-H-F 2% when tested in the rosette, phagocytosis and ⁵¹Cr release assays with these complexes. Ficoll 2% was used to replace FCS in these assays to avoid degradation on the complexes formed by Factor I present in the FCS. Control experiments with EA_{IgG2b} showed granulocytes to be equally active in the presence of FCS or Ficoll in a 4 hs cytotoxicity assay.

b) Testing with Daudi cells: Daudi cell line cells form rosettes with E coated with human C3bi and C3d but not with human C3b (Ross, Tack & Rabellino, 1978). They were used as a control indicator system for mouse complement-coated E. Between 20 to 55% of Daudi cells were found to form rosettes with EAC3b complexes, whereas more than 80% Daudi cells formed rosettes with mouse EAC3bi.

2.16. Rosette formation

For EA rosettes, 100 μ l of effector cells in RPMI-H-FCS (2×10^6 /ml) were mixed with 100 μ l of EA complexes in RPMI-H-FCS (10^8 /ml) and centrifuged at 100 g for 4' at 4°C as described by Tai & Spry (1980). For EAC rosettes, the mixtures in RPMI-H-F 2% were centrifuged at 22°C and then incubated for 2'-5' at 37°C. The pellets were gently resuspended, sedimented onto a microscope slide with a Cytocentrifuge and stained with Giemsa.

Rosette assays were done in triplicate, counting a minimum of 200 cells; three or more adhering cells were regarded as positive.

2.17. Phagocytosis assay

Effector cells and EA or EAC complexes were mixed as in the rosette assay (2.16) and incubated at 37°C. At the end of the incubation period, the mixtures were centrifuged for 1' at 100 g. Cytocentrifuge preparations were then made, and the percentage of cells with one or more ingested E was determined.

2.18. Trypanosoma cruzi antigen

This was prepared by Dr. Ribeiro dos Santos from cultured epimastigotes. The parasites were suspended in distilled water and disrupted by freezing and thawing twice (Ribeiro dos Santos & Hudson, 1980b). The lysate was made isotonic by adding 1.4 M sodium chloride solution and debris removed by centrifugation at 30,000 r.c.f. for 1 h at 4°C. The supernatant was adjusted to a protein concentration of 200 µg/ml.

2.19. Hybridization technique

This was used to raise the rat monoclonal antibodies to mouse eosinophils described in Chapter 7.

2.19.1. Immunization

Purified mouse eosinophils were injected into male rats in 1 ml of

PBS containing 2% heat inactivated normal rat serum. The rat giving the highest titre with mouse eosinophils was selected.

In fusion 1 (rat-mouse fusion) an AGUS rat was primed i.p. with 2×10^7 CBA eosinophils and three weeks later boosted i.v. with 3×10^7 CBA eosinophils. Spleen cells were fused with NS1 myeloma cells three days after the boost.

In fusion 2 (rat-mouse fusion) an AGUS rat was primed i.p. with 2×10^7 CBA eosinophils and three weeks later boosted i.v. with 6×10^7 CBA eosinophils. The spleen cells were passed through a nylon wool column (see Section 2.11.1.) and the adherent cells recovered and fused with NS1.

In fusion 3 (rat-rat fusion) a LOU rat received a single i.v. injection of 8×10^7 BALB/c eosinophils. The spleen cells were fused four days later to the rat myeloma Y3.

2.19.2. Fusion protocol

A single cell suspension of the rat spleen cells was prepared as with mouse spleen cells (see Section 2.06.1.). The spleen cells were then washed twice in RPMI-H, the second time together with the myeloma cells in a round bottom plastic tube. In the mixture, the spleen cell:myeloma cell ratio was of 10:1. The supernatant was aspirated and the pellet overlaid with 0.2 ml of PEG. After thoroughly mixing by flicking the bottom of the tube, the cells were centrifuged at 200 r.c.f. for 5'. RPMI-H was then carefully added without disturbing the pellet. After one minute, the pellet was resuspended in the supernatant over 3-4 minutes. The cells were then centrifuged at 250 r.c.f. for 10', the supernatant removed and the cells overlaid with RPMI-H-FCS without disturbing the pellet. After letting it stand for 5' this was resuspended and transferred

to a 50 ml tube where the volume was brought up to 48 ml. After mixing, the cells were distributed in aliquots of 1 ml into each well of a 24 well Linbro plate (Cat. No. 76-033-05, Flow Laboratories) and incubated at 37°C in an atmosphere of 5% CO₂. Two days later 1 ml of cloning medium containing double strength HAT was added to each well. Every 3 to 4 days, 1 ml of each well was replaced with 1 ml of cloning medium containing 1X HAT and a month later this was replaced by cloning medium containing 1X HT. Two months after the fusion, cloning medium without HT was used. In each fusion, myeloma cells alone were plated as a control for HAT solutions. After about 1 week all myeloma cells were dead.

2.19.3. Cloning

This was done in two ways: a) Limiting dilution; cells in cloning medium were placed in a microtitre plate (96 wells, flat bottom, Nunclon Delta Tissue Culture Plates, Catalogue No. N1480, Nunc U.K., division of Gibco Bio-Cult Ltd.) at approximately 1 cell/well. After about two weeks at 37°C in an atmosphere of 5% CO₂, colonies showed active growth and the supernatant was removed for testing. b) Cloning in agar; 15 ml of agar base layer were dispensed into a petri dish (90 mm diameter) and allowed to solidify at 22°C. Equal volumes (1-2 ml) of 5×10^3 cells in cloning medium at 37°C and 0.55% agar at 40°C were mixed and quickly spread over the base layer to give the top layer of 0.275% agar. 5×10^3 cells were used because this number of cells showed active growth with no overcrowding of the petri dish. Colonies appeared after about 12 days at 37°C in 5% CO₂. These were removed individually with a pasteur pipette and the cells placed in a small volume (1-2 ml) of cloning medium. After 4-5 days, the supernatants were removed for testing.

2.20. Indirect immunofluorescence (IIF)

2.20.1. Visual examination of methanol-fixed cells

Methanol was chosen as a fixative because preliminary experiments showed a better membrane fluorescence than with cells fixed with acetone, 0.1% glutaraldehyde, or 1% formaldehyde.

10^4 cells in 5 μ l were placed in each well of a tissue typing slide (tissue typing slides type H, Catalogue No. 999/801/140, G.D. Searle & Co. Ltd., High Wycombe, England), dried quickly at 60°C and fixed in methanol. The plates were then stored at 4°C in a sealed box containing CaCl₂. Prior to use each plate was washed three times in PBS and rinsed in distilled water. 5 μ l of diluted antiserum was added to each well and incubated for 30' at 22°C in a humidified chamber. The plates were then washed with PBS (three changes of PBS over a 30' period) in a Coplin container with a magnetic stirrer. 5 μ l of goat anti-rabbit IgG-FITC at a dilution of 1:20 (this was chosen as a preliminary checker board titration showed good membrane fluorescence with a positive control and no fluorescence when cells were incubated with normal rabbit serum or goat anti-rabbit IgG-FITC alone) were then added and the samples examined under phase contrast and fluorescence with a Carl Zeiss microscope with a IV F1 epi-fluorescence condenser (Carl Zeiss, Oberkochen, West Germany) and a X40 objective. Samples were scored from (-) to (+++) according to the intensity of the membrane fluorescence observed.

2.20.2. Examination of live cells on the fluorescence activated cell sorter (FACS)

25 μ l of cells (2×10^7 /ml) in RPMI-H-FCS were mixed with 25 μ l of rabbit antiserum dilutions in 1 ml conical plastic tubes. After 30' at 22°C, the cells were washed three times in RPMI-H-FCS and resuspended in

25 μ l of goat anti-rabbit IgG-FITC at 1:8 (this dilution was determined by a chequer board titration as described in 2.20.1). After an incubation period of 20' at 22°C, the cells were washed twice and resuspended in RPMI-H-FCS, ready to be examined with the FACS.

2.21. Radioisotopes

The following isotopes were obtained from the Radiochemical Centre Amersham.

2.21.1. Na¹²⁵I carrier free (Code No. IMS.30) was supplied in a 10 μ l volume or less with an activity of 1 mCi.

2.21.2. Na₂⁵¹CrO₄ was supplied as a sterile isotonic solution with an initial activity of about 5m Ci/ml (Code No. CJS.4).

2.22. Iodination of goat anti-rat IgG

Affinity purified goat anti-rat IgG (see Section 2.04.2) was iodinated by the Chloramin T method.

To 10 μ l of Na¹²⁵I (1 m Ci), 10 μ l of 0.5 M phosphate buffer pH 7.5 were added followed by 10 μ l of the IgG antibody at an approximate concentration of 1 mg/ml. The reaction was then initiated by adding 10 μ l of freshly prepared chloramin T (0.5 mg/ml). The reaction was stopped within 30 seconds with 450 μ l of a saturated tyrosine solution; 50 μ l of FCS were then added as a carrier protein. The whole mixture was

then placed on a prepacked Sephadex G-25M column (PD 10 Pharmacia Fine Chemicals Ltd.) The iodinated protein was collected and 1% FCS and 0.1% sodium azide added. It was stored at 4°C.

2.23. Binding assay

Preliminary experiments showed that cells fixed with 0.125% glutaraldehyde gave similar results to unfixed cells. Furthermore, by adhering the cells to the bottom of the wells of a microtitre plate (flexible polyvinyl chloride, 96 round "U" wells, Cat. No. 1-220-24, Dinotech Laboratories Incorporated, Alexandria, Virginia, USA) the washing was facilitated as centrifugation became unnecessary. Thus, each wash was carried out by filling the wells with the washing solution, and then flicking the plate to empty the wells.

The wells of a microtitre plate were treated with 200 μ l of polybrene (Cat. No. P-4515, SIGMA) at 0.05 mg/ml for 40' at 22°C and then washed twice with PBS. The cell suspension was washed twice with PBS to remove protein and 50 μ l of cells (2×10^6 /ml) were added to each well. The cells were then either allowed to settle for 1 h or centrifuged gently to allow cell attachment. After this, the cells were washed in PBS and each well covered with 200 μ l of PBS-FCS for 30'. The cells were then washed twice and checked under the microscope for attachment to the plastic. Antibody samples (50 μ l/wells) were added and incubated 40' at 22°C before washing three times in PBS-FCS. The 125 I labelled goat anti-rat IgG was then added, incubated 45' at 22°C before washing three times in PBS-FCS. After the last wash, the wells were filled with molten paraffin which was allowed to solidify before cutting out the wells.

These were finally counted in a gamma counter (Neon 2000, Nuclear Enterprises, Edinburgh, Scotland).

2.24. Cytotoxicity assay by ^{51}Cr release

In this assay, target cells in RPMI-H-FCS were labelled with about 100 μCi of ^{51}Cr for 1 h at 37°C. The cells were then washed, counted and diluted. The labelled target cells were then mixed with antibody and effector cells or complement, and incubated at 37°C.

The ^{51}Cr release was determined in the following ways:

2.24.1. Non-adherent target cells in LP2 tubes.

The cell mixtures were resuspended with a vortex mixer (Vortex Rotamixer deluxe, Hook and Tucker Instruments Ltd., Croydon, England) at the end of the incubation period and centrifuged 1' at 240 r.c.f.

From each sample (tested in duplicate or triplicate) half the supernatant was removed and dispensed into another LP2 tube (TUBE 2). In the original tube (TUBE 1) remained the residual cells and the other half of the supernatant. Both tubes were then counted in a gamma counter (Wallac, LKB Instruments Ltd., South Croydon, England).

The gamma counter data were analysed by computer using a program (COL 2) written by Dr. C.J. Sanderson. The program output gave total isotope for each sample (as a control on the number of target cells used), percent isotope release, the log of this value for statistical analyses, and mean of replicates. This program calculated percentage ^{51}Cr release from counts in the two tubes (after subtracting machine background) as:

$$\frac{\text{TUBE 2} \times \text{CF}}{\text{TUBE 1} + \text{TUBE 2}} \times 100$$

where CF (conversion factor) is the number obtained by dividing the total volume of the original sample by the volume removed for TUBE 2.

Percentage specific ^{51}Cr release was calculated from percent release:

$$\frac{\text{Test-control}}{\text{Total-control}} \times 100,$$

where "control" is the isotope released in the absence of effector cells or antibody. "Total" releasable was determined in a different way for each case.

This type of assay was used in the following studies:

- a) For the detection of mouse granulocyte receptors for IgG (Chapter 4):
100 μl of ^{51}Cr labelled E ($10^6/\text{ml}$) were mixed with an equal volume of antibody and effector cells ($5 \times 10^6/\text{ml}$) in LP2 tubes when the mouse anti-E was used. When IgG1 and IgG2b were used, E were firstly incubated with these antibodies as in 2.14. The formed EA_{IgG_1} or $\text{EA}_{\text{IgG}_2\text{b}}$ were then labelled with ^{51}Cr , washed, and 100 μl ($10^6 \text{ EA}/\text{ml}$) mixed with an equal volume of effector cells ($5 \times 10^6/\text{ml}$) in LP2 tubes. The mixtures were then incubated for 4 hs at 37°C . For the calculation of specific ^{51}Cr release, "Total" was the release of ^{51}Cr when E were treated with IgM 83/SCC1 and rabbit complement.
- b) For the detection of mouse granulocyte complement receptors (Chapter 4):
 EA_{IgM} was first formed as in 2.14 and then labelled with ^{51}Cr . Coating with complement was as in 2.15. The same experimental conditions as with IgG1 and IgG2b were then used. For the calculation of specific ^{51}Cr release, "Total" was also obtained in the same way.
- c) For the study of rat granulocytes and K cell cytotoxic activity against mouse cells (Chapter 6):
Equal volumes (100 μl) of ^{51}Cr labelled target cells ($10^5/\text{ml}$), antibody and effector cells, all in RPMI-H-FCS were mixed in LP2 tubes and

incubated at 37°C. For the calculation of specific ⁵¹Cr release, "Total" was considered as 100.

2.24.2. Adherent target cells in Linbro plates

This was done in studies of granulocyte cytotoxic activity against cell line cells coated with T.cruzi antigen (Chapter 5).

S2 or Neuro-2a target cells (10^5) in RPMI-H-FCS were placed in each of a 24 well Linbro plate and allowed to settle and adhere to the plastic by incubation at 37°C for 4 hs. The ⁵¹Cr was then added (about 12 μ Ci/well). After 1 h at 37°C the cells were washed and incubated with T.cruzi antigen (200 μ g/well) for 1 h. After washing off the antigen, 0.2 ml of rabbit or mouse anti-T.cruzi serum and 0.3 ml of effector cells in RPMI-H-FCS were added and incubated with the target cells at 37°C. At the end of the incubator period, 0.5 ml were removed from each well and dispensed in TUBE 2; 0.5 ml of 5% Triton X-100 (BDH Chemicals Ltd.) were added to the cells, and after about 10 hs dispensed in TUBE 1. Both tubes were counted in a gamma counter.

Percentage ⁵¹Cr release was determined using the COL 2 program (see Section 2.24.1). The conversion factor in this case was 1.

2.24.3. Complement cytotoxicity in microplates

This assay was used for testing complement-mediated lysis of mouse cells by different antibody preparations (Chapter 7).

Target cells, antibody and complement, each in a 10 μ l volume, were used. For each target cell used, six wells containing 10 μ l of target cells and 20 μ l of 5% Triton X-100, and six wells containing 10 μ l of target cells, 10 μ l of RPMI-H-FCS and 10 μ l of complement were set up to obtain the total isotope release and the control isotope release respectively.

10 μ l of ^{51}Cr labelled target cells ($10^6/\text{ml}$) were mixed with equal volumes of antibody dilution and complement dilution (all in RPMI-H-FCS) in a microtitre plate (U bottom, 96 wells, Linbro, Flow Laboratories) and incubated 1h and 15' at 37°C . At the end of the incubation period, 150 μ l of PBS-FCS were added to each well, the mixtures centrifuged 1' at 240 r.c.f. and 100 μ l taken from each well and counted in a gamma counter.

The gamma counter data were analysed by means of a computer program (SAHA) written by Dr. Sanderson. The program output, which has machine background subtracted, gave percentage isotope release, percentage specific ^{51}Cr release and the mean of a group of replicates. Percentage specific ^{51}Cr release was calculated from percentage ^{51}Cr release:

$$\text{Percentage specific } ^{51}\text{Cr release: } \frac{\text{Test-control}}{\text{Total-control}} \times 100,$$

where "Total" is the isotope released in the presence of Triton and control is the isotope released in the presence of complement without antibody.

2.25. Statistical methods

Rosette and phagocytosis data were arc sin transformed (Tai & Spry, 1980) and the standard deviation calculated.

Percentage ^{51}Cr release data were analysed by analyses of variance by means of a program (BMD 02V-Health Service Computing Facility, UCLA) modified by Dr. Franks (University of Cambridge). Individual points were tested by Duncan's multiple range test (Duncan, 1955) using the standard error obtained from the analysis of variance and the log value from the percentage ^{51}Cr release data.

CHAPTER 3

PURIFICATION OF MOUSE EOSINOPHILS AND NEUTROPHILS

INTRODUCTION

Before any experiments with mouse eosinophils and neutrophils could be attempted, the methods for obtaining these cells with a high degree of purity, good yield and maintaining function had to be developed. This is necessary as the use of highly purified cell preparations constitutes a necessary prerequisite in cytotoxic studies (Chapters 4, 5 and 6), so that activity and inhibition effects by other cell types can be ruled out. Similarly, separation from macrophages, which actively bind and phagocytose E has been found to increase the sensitivity of the rosette and phagocytosis assays (Chapter 4). Finally, the production of antibody preparations specific for mouse eosinophils and neutrophils requires highly purified cells for the immunization of animals and for the screening of antibody specificity (Chapter 7).

One of the problems encountered when working with mouse eosinophils and neutrophils is their difficult accessibility: August and AM-2 (Instituto Biomedico, Universidade Federal Fluminense, Niteroi, Brazil) rats have a high number of resident peritoneal eosinophils. However, these appear to be special cases as none of the other readily available rat strains have comparable numbers of peritoneal eosinophils. The peritoneal cavity of normal mice is virtually devoid of granulocytes, and their numbers in the peripheral blood are relatively low.

Advantage is usually taken of the fact that a peritoneal eosinophilia occurs in mice carrying certain parasitic infections. Thus, high numbers of eosinophils obtained from S. mansoni-infected mice were used to raise an anti-eosinophil serum (Mahmoud, Warren & Boros, 1973). Similarly, eosinophils obtained from M.corti-infected mice allowed studies on their surface markers (Hoghart, Cruise, McKenzie & Mitchell, 1980). In the case of neutrophils, they can be recovered from the peritoneal

cavity after stimulation with a variety of agents (Watt, Burgess & Metcalf, 1979). This has paved the way for workers to attempt the purification of these cells by different techniques, among which, isopycnic centrifugation has been the most extensively tested (James, Leid & Sher, 1979; Watt et al., 1979; Burgess, Cruise, Mitchell & Watt, 1980).

Usually a compromise has to be made between the number of recovered cells and the purity, since the more the purification steps, the lower the yield. The effect of a separation technique on cell function is an important consideration. For example, carbonyl iron treatment to remove adherent cells has been shown to greatly impair the cytotoxic ability of non adherent granulocytes (López, Bunn, Moreno & Sanderson, 1978).

In this chapter, procedures are compared for the induction and purification of mouse eosinophils and neutrophils. Subsequent chapters will show that the functions of the purified cells have been retained.

RESULTS

3.1. Search for resident peritoneal eosinophils in strains of mice

Fourteen strains (BALB/c, BALB/c nu/nu, A, DBA/2, C57BL/6, C57/BL/10, ATL, AQR, C57BL/6-Ly-1^a, NH, SJL, TO, CBA and AKR) were examined in the hope of finding a strain with a high number of peritoneal eosinophils. However, as none of these strains showed more than 10^5 eosinophils per mouse (less than 5% of the total peritoneal cell population), experiments were carried out with different parasites to find a satisfactory method for inducing eosinophilia in mice.

3.2. Induction of mouse eosinophils

Three different parasites were tested:

3.2.1. Trichinella spiralis

BALB/c mice were given 1000 larvae in 0.5 ml of PBS directly into the oesophagus with a syringe and the peritoneal cavity examined at different intervals, from day 14 to day 77 after infection. No significant changes in the number of peritoneal cells or the percentage of eosinophils were seen in this period: the total number of cells was about $0.5 - 2 \times 10^7$ per mouse with less than 5% of these cells being eosinophils. Following reinfection with 1000 larvae per mouse, a slight increase in the total number of peritoneal cells occurred while the percentage of eosinophils rose to about 20% by day 12 after reinfection.

3.2.2. Taenia crassiceps (Toi strain).

Packed cysticerci of T. crassiceps, obtained from the peritoneal cavity of heavily infected mice, were injected i.p. into BALB/c mice and the peritoneal exudate thus induced examined at different times. The total number of peritoneal cells per mouse was found to increase from about 3×10^7 on day 9 to 8×10^7 on day 15 after infection, remaining at this level up to day 33. The percentage of eosinophils present in the peritoneal exudate was about 25% throughout this period. No observations were carried out beyond this time.

3.2.3. Mesocestoides corti.

Experiments with this parasite were carried out following the observation that a prominent eosinophilic response takes place in the peritoneal cavity of BALB/c mice (Mitchell, personal communication and Johnson, Nicholas, McKenzie & Mitchell, 1979).

Approximately 150 μ l of packed larvae of M. corti in PBS, obtained from the peritoneal cavity of animals with long standing infections, were injected i.p. in each mouse. At different times the peritoneal exudates were examined and the total number of each cell type determined. Eosinophils could be seen in greatest numbers and percentages by the fourth week after infection (Figure 3.1). At this time, about 8×10^7 cells could be recovered from each mouse and about 50% of these cells were eosinophils. After the fourth week the total number and percentage of eosinophils were found to decrease while macrophages became the predominant cell type in the exudate. The number of lymphocytes and neutrophils remained low throughout the infection, although some individual animals have shown high numbers of neutrophils.

Although a detailed comparison was not carried out, M. corti appeared to induce fewer cells with a lower percentage of eosinophils in CBA mice than that observed in BALB/c mice.

Since M. corti was found to be superior to T. spiralis and T. crassiceps in inducing large numbers of eosinophils in mice (summarized in Table 3.1), it was used throughout these studies and the peritoneal cells collected in the fourth week after infection.

As the exudate obtained in this way consisted of about 50% eosinophils, techniques were compared for the removal of the other cell types present in this preparation. In all the fractionation experiments described below, the total cell number and the differential counts per fraction were determined, so that the absolute number and yield of each cell type could be calculated.

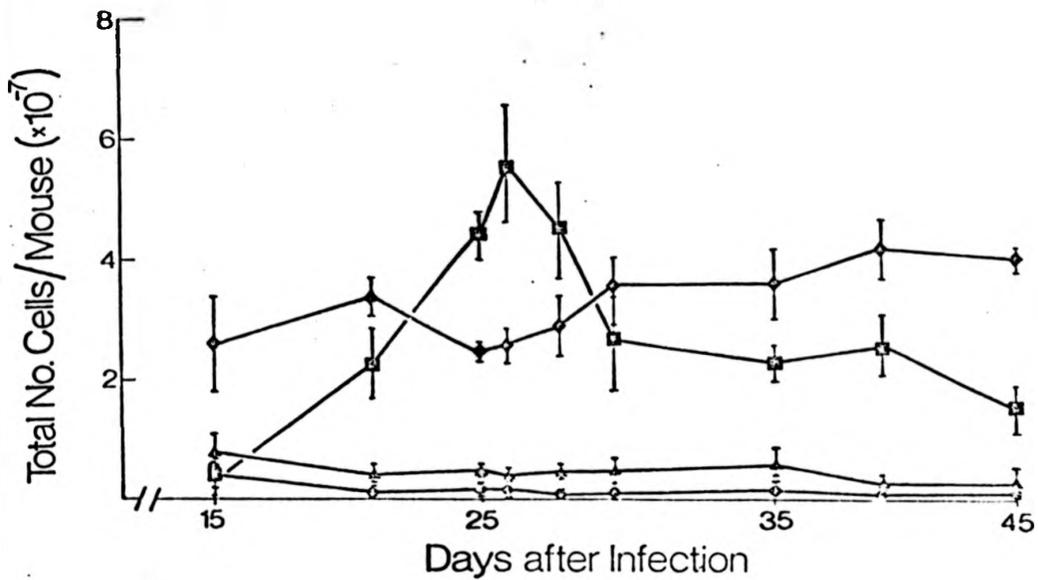


Figure 3.1. Cellular response in the peritoneal cavity of BALB/c mice injected with M.Corti. ■ = eosinophils; ◆ = macrophages; ▲ = lymphocytes; ● = neutrophils. Each point between day 25 and 50 is a mean of 12 animals. Other points are a mean of at least 3 animals. Bars represent the standard deviation for each point.

Table 3.1. Induction of eosinophils in the peritoneal cavity of
BALB/c mice by different parasites

Parasites	Total number of cells/mouse	% Eosino- phils	Total number, eosinophils/mouse
<u>T. spiralis</u>	0.5-2.5 x 10 ⁷ *	5-20	0.5-5 x 10 ⁶
<u>T. crassiceps</u>	3-9 x 10 ⁷	10-30	0.5-2 x 10 ⁷
<u>M. corti</u>	4-17 x 10 ⁷	40-75	2-9 x 10 ⁷

* Ranges at the time of maximal eosinophilic response
for each parasite.

3.3. Comparison of different techniques for the purification of mouse eosinophils

3.3.1. Adherence to nylon wool column

This technique was used because previous experiments with rat eosinophils (López *et al.*, 1978) had shown these cells to have a loose adherence to the nylon wool which could be used to separate them from the more adherent neutrophils and macrophages.

With this technique, only about a third of the original eosinophil population could be recovered with a purity of approximately 70% in the non-adherent fraction (Table 3.2). However, an eosinophil enrichment could be seen in both adherent and non-adherent fractions probably due to the irreversible binding of macrophages and neutrophils to the nylon wool.

3.3.2. Isopycnic centrifugation

The isopycnic points of each cell type in the peritoneal exudate were established by centrifuging these cells on a continuous gradient of Metrizamide 10-20%. It can be seen (Figure 3.2) that eosinophils were very heterogeneous in density, with their peak at a Metrizamide concentration of 15.5% (density of 1.0814 gm/cm^3). Macrophages overlapped with eosinophils over a wide range of densities and only high density eosinophils could be recovered with a purity of about 80-90%. The eosinophils recovered at Metrizamide 15.5% appeared to be smaller and to possess brighter granules than those eosinophils obtained at Metrizamide 17.5%, suggesting that a selection of a subpopulation was taking place.

Other attempts were made by centrifuging the cells on a two step Metrizamide gradient using different combinations, but none gave satisfactory results.

Table 3.2. Separation of M.corti-induced mouse peritoneal cells
by filtration through nylon wool column

Fractions	Cell types				Total
	Macrophages	Lymphocytes	Neutrophils	Eosinophils	
Unseparated	9×10^6 (36) *	3×10^6 (12)	5×10^5 (2)	1.2×10^7 (50)	2.5×10^7
Non-adherent	10^6 (14)	1.1×10^6 (15)	7×10^4 (1)	5×10^6 (70)	7.2×10^6
Adherent	3.4×10^6 (34)	9×10^5 (9)	0 ⁺	5.7×10^6 (57)	10^7

* Total number of each cell type followed by the percentage between brackets.

+ Zero represents no neutrophils seen in a scan of 200 cells (i.e. less than 0.5%).

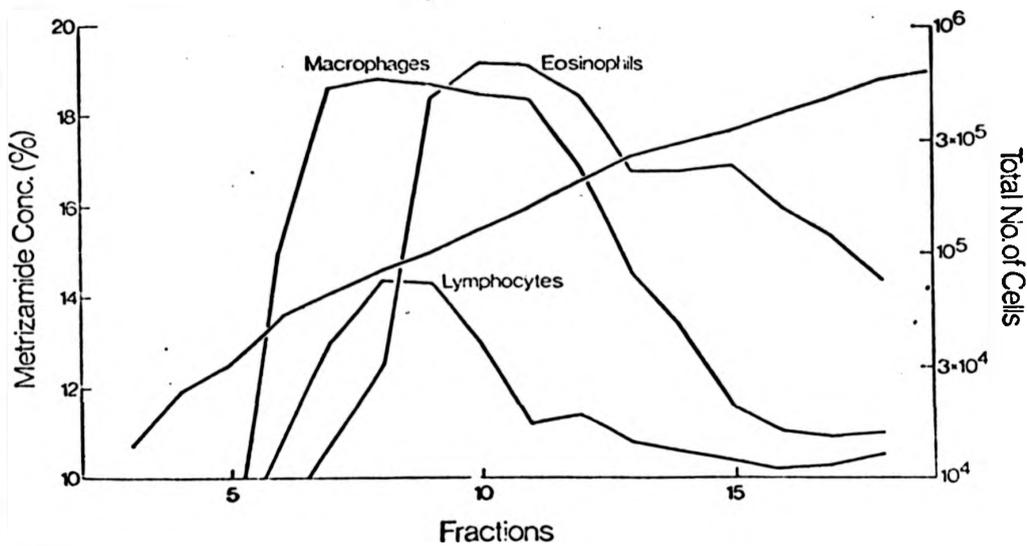


Figure 3.2. Distribution of *M. corti*-induced peritoneal cells on a continuous Metrizamide gradient. Cell numbers expressed in a log scale.

Pretreatment of peritoneal cells by nylon wool filtration, followed by centrifugation of the non-adherent cells on a continuous or on a two step Metrizamide gradient did not appreciably improve the eosinophil separation.

3.3.3. Velocity sedimentation at unit gravity

Experiments in which sedimented cells were collected from the bottom of the chamber (Figure 3.3) indicated that eosinophils could be separated from macrophages and lymphocytes to about 80-85% purity. A Coulter counter plot of this type of collection showed (Figure 3.4) that a separation of cells according to their size had been achieved. A clear difference appeared between fraction 12 containing mainly macrophages and fraction 17 which was the peak for eosinophils. However, some overlapping between eosinophils and macrophages occurred as shown in fraction 14. Lymphocytes also overlapped with eosinophils, with their peak in fraction 19, but these were too few to be distinguished in the Coulter counter plot.

An improvement in the resolution of the sedimenting cells was achieved by collecting the cells from the top of the sedimentation chamber by upward displacement with 40% sucrose. The sedimentation profile of the cells sedimented in this way (Figure 3.5) showed a better eosinophil separation from macrophages, although some overlapping with eosinophils still persisted. With this type of collection about 85-95% eosinophils could be obtained after a total sedimentation time of 3.5 hs. Eosinophils were found to sediment at about 5 mm/h, lymphocytes at about 4 mm/h and macrophages at about 6 mm/h and faster.

To further improve the purification, a second step using a 14.8% Metrizamide interface was carried out. This concentration was chosen as

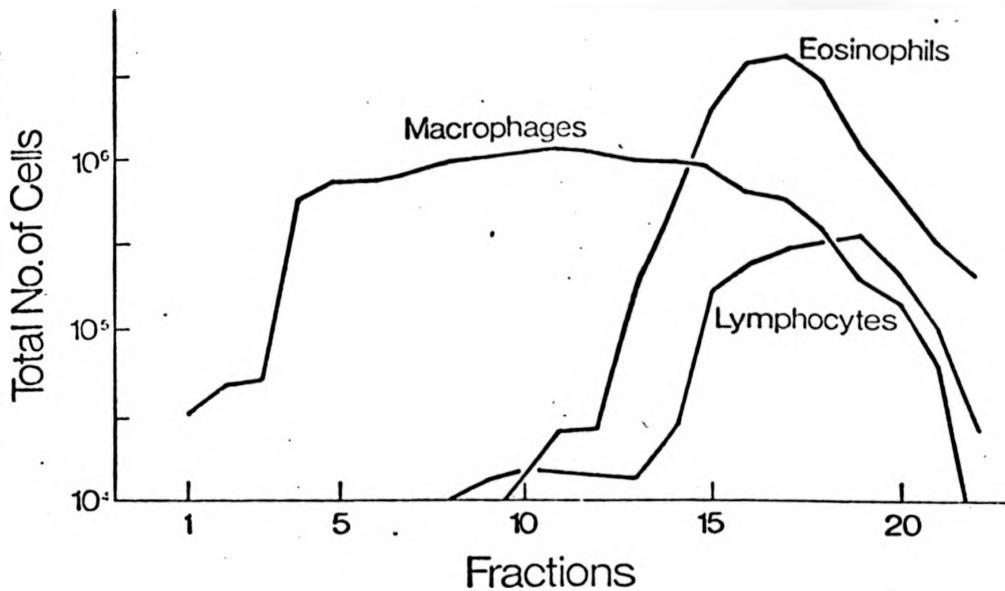


Figure 3.5. Fractionation of *M. corti*-induced peritoneal cells under unit gravity. The fractions were collected from the bottom of the sedimentation chamber. Cell numbers expressed in a log scale.

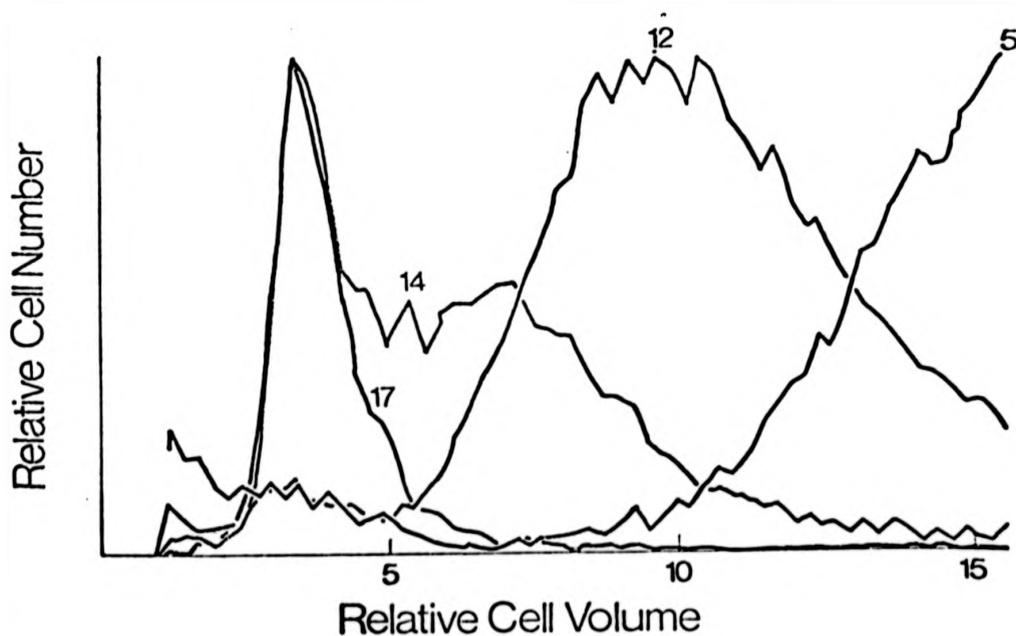


Figure 3.4. Coulter counter plot of *M. corti*-induced peritoneal cells fractionated under unit gravity. The numbers on each peak correspond to the fractions shown in Figure 3.3. The same settings were used for all four fractions.

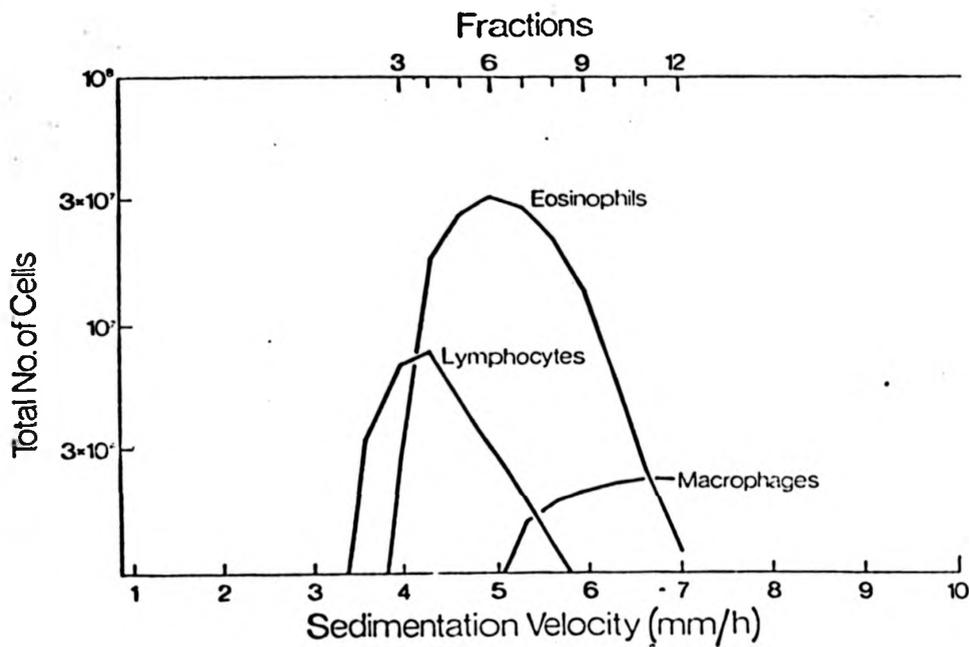


Figure 3.5. Sedimentation profile of *M. corti*-induced peritoneal cells separated under unit gravity. The cells were collected from the top of the sedimentation chamber. Cell numbers expressed in a log scale.

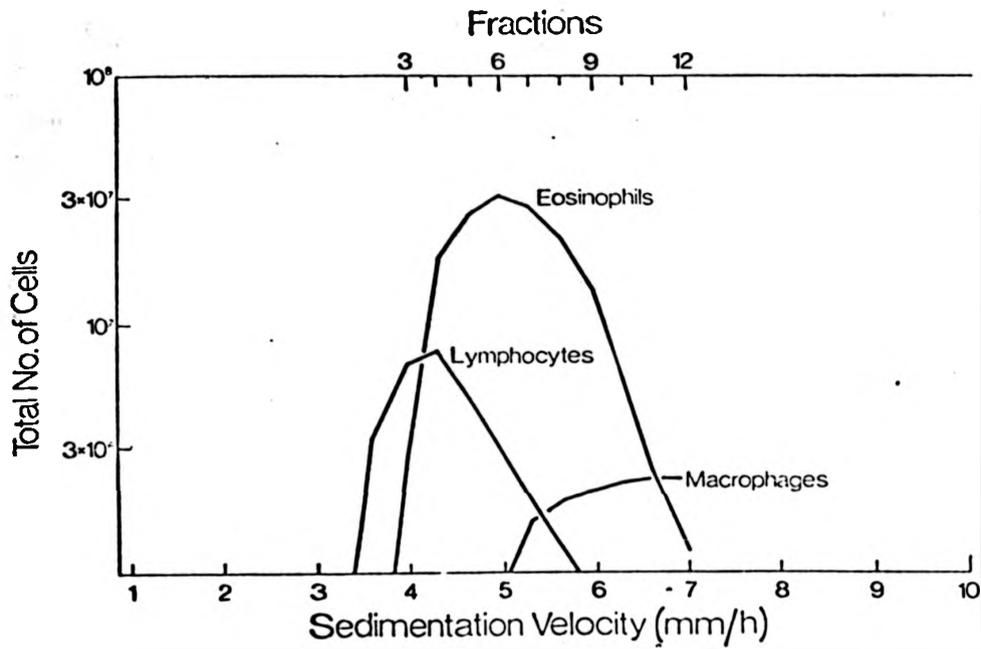


Figure 3.5. Sedimentation profile of *M. corti*-induced peritoneal cells separated under unit gravity. The cells were collected from the top of the sedimentation chamber. Cell numbers expressed in a log scale.

the cut off point for eosinophils based on a continuous Metrizamide gradient (Figure 3.2). Thus, the fractions (obtained as shown in Figure 3.5) containing the highest number of eosinophils were pooled and centrifuged on the Metrizamide interface. In this way, while most mononuclear cells and some eosinophils were retained in the interface, the resulting pellet contained more eosinophils with a higher degree of purity than could be expected from any individual fraction (Table 3.3). Figure 3.6 illustrates this eosinophil preparation.

A combination of velocity sedimentation at unit gravity and isopycnic centrifugation was then found to give the best results from all the combinations tested (summarized in Table 3.4), and all eosinophil preparations used throughout these studies were obtained in this way.

3.4. Induction of mouse neutrophils

Preliminary experiments in which an i.p. injection of 3.5% dextran (Mol.wt. $5-40 \times 10^6$; Koch-Light Lab.) and an i.p. injection of 1% glycogen (Glycogen II, SIGMA) were compared, showed that dextran induced a higher number and percentage of peritoneal neutrophils.

Experiments were then carried out to determine the best conditions for inducing neutrophils with dextran. An i.p. injection of 2 ml and a collection of the peritoneal exudate 15 hs later was found to give the highest yield of neutrophils. Cells could also be collected 4 hs after an injection of dextran with a similar percentage of neutrophils in the exudate, although their total numbers were lower.

3.5. Purification of mouse neutrophils

Velocity sedimentation at unit gravity was not effective as neutrophils showed a very heterogeneous size distribution. Isopycnic centrifugation

Table 3.3. Purification of mouse eosinophils by velocity sedimentation at unit gravity, followed by isopycnic centrifugation

Cell sample	Total no. of cells	% Eos.	Total no. of Eos.	Eos. yield
Unfractionated	3.5×10^8	55	1.9×10^8	100%
Fraction 7	2.6×10^7	73	1.9×10^7	10%
Fraction 8	2.8×10^7	94	2.6×10^7	14%
Fraction 9	2.5×10^7	89	2.2×10^7	12%
Fraction 10	1.9×10^7	77	1.4×10^7	7%
Fractions 8 & 9 pooled	5.3×10^7	90	4.8×10^7	25%
Fractions 7,8 & 9 & 10 pooled(P)	9.8×10^7	83	8.1×10^7	43%
Pellet from P centrifuged on 14.8% Metrizamide	4×10^7	97	3.9×10^7	21%

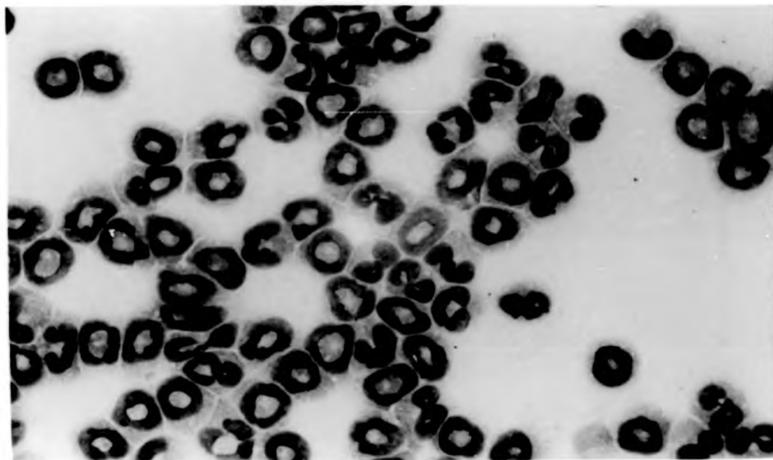


Figure 3.6. Giemsa stained cytocentrifuge preparation of purified mouse eosinophils

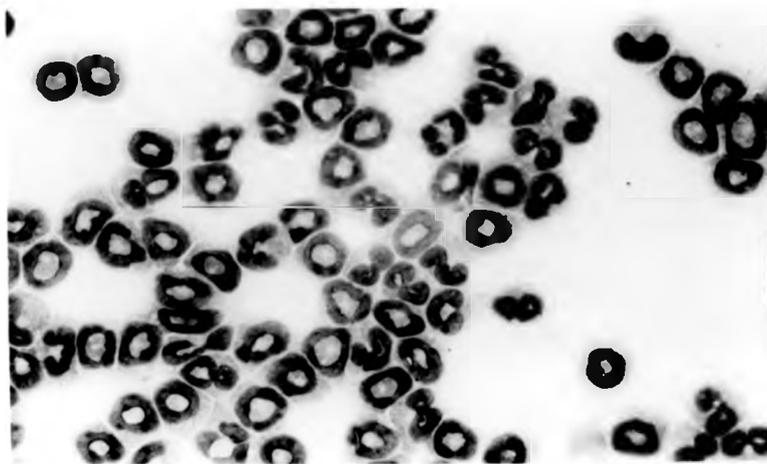


Figure 3.6. Giemsa stained cytocentrifuge preparation of purified mouse eosinophils

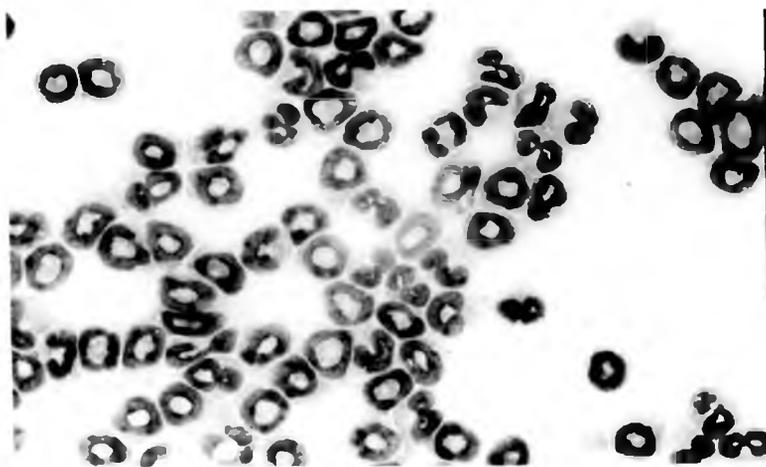


Figure 3.6. Giemsa stained cytocentrifuge preparation of purified mouse eosinophils

Table 3.4. Summary of the different techniques tested for the separation of mouse eosinophils

Method	Eosinophil* purity	Yield*
Nylon wool column	60-75	35-45
Nylon wool column + isopycnic centrifugation (continuous gradient)	80-90	5-10
Isopycnic centrifugation (continuous gradient)	80-90	10-20
Two step isopycnic centrifugation	60-75	40-60
Velocity sedimentation at unit gravity (collection from the bottom of the chamber). Peak fraction	80-85	10-20
Velocity sedimentation at unit gravity (collection from the top of the chamber). Peak fraction	85-95	10-20
Velocity sedimentation at unit gravity (collection from the top of the chamber) Pool of eosinophil-rich fractions + isopycnic centrifugation on interface of Metizamide.	95-98	20-40

* Range in percentage

was more satisfactory as the density of neutrophils was sufficiently high to achieve a good separation from other cells.

Cells were centrifuged on a continuous gradient of Metrizamide 10-20%. It can be seen (Figure 3.7) that the neutrophil peak occurred at a Metrizamide concentration of about 16% (density of 1.0842 gm/cm³) overlapping high density macrophages and lymphocytes. The cut-off points were then determined and a two step Metrizamide gradient of 17.5% and 15.5% was found to give the best separation. Mononuclear cells were retained in the first interface while about 85% neutrophils could be obtained in the second interface. However, if the cells were first separated on an interface of Metrizamide 15.5% or Ficoll-Paque (Pharmacia) to remove a high proportion of macrophages, and the pellet then centrifuged on the two step Metrizamide gradient, a preparation containing more than 90% neutrophils could be routinely obtained (Table 3.5). This neutrophil preparation is illustrated in Figure 3.8.

DISCUSSION

The best method for purifying eosinophils was found to be a combination of velocity sedimentation at unit gravity and isopycnic centrifugation on an interface of Metrizamide 14.8%. In this way, preparations consisting of more than 95% eosinophils with a yield between 20-40% can be routinely obtained. Previous attempts to purify mouse eosinophils have relied mainly on isopycnic centrifugation. Thus, centrifugation of cells (with a starting eosinophil percentage similar to the one described above) on sodium diatrizoate (Mahmoud *et al.*, 1973

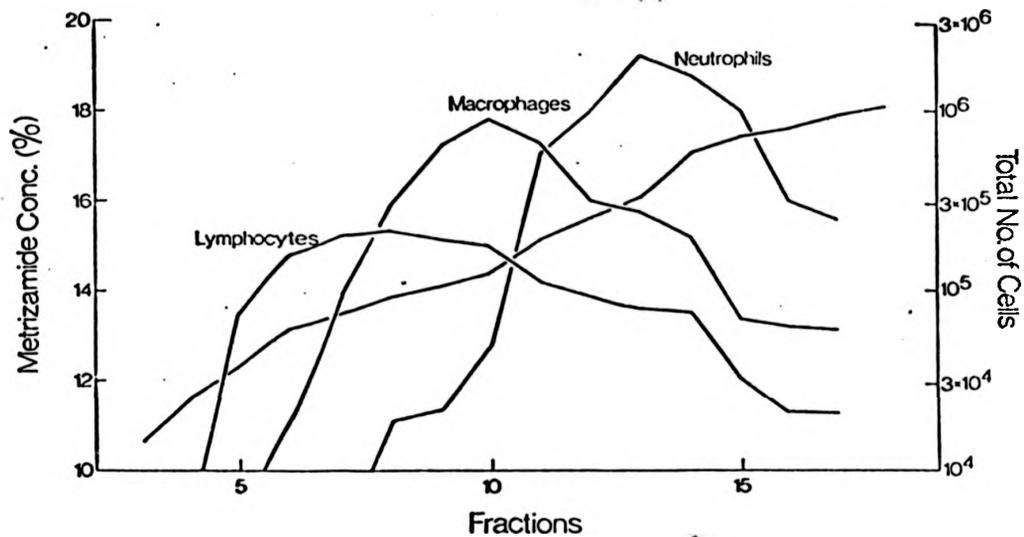


Figure 3.7. Distribution of dextran-induced peritoneal cells on a continuous Metrizamide gradient. Cell numbers expressed in a log scale.

Table 3.5. Purification of mouse neutrophils

Purification step	Total no. of cells/mouse	Differential counts				Total no. neutr.	Yield
		Macr.	Lymph.	Neutr.	Eos.		
Unseparated	2.5×10^7	27	6	65	2	1.6×10^7	100%
After Ficoll-Paque	1.1×10^7	10	2	87	1	9.5×10^6	60%
After Metrizamide	4×10^6	4	1	94	1	3.7×10^6	23%

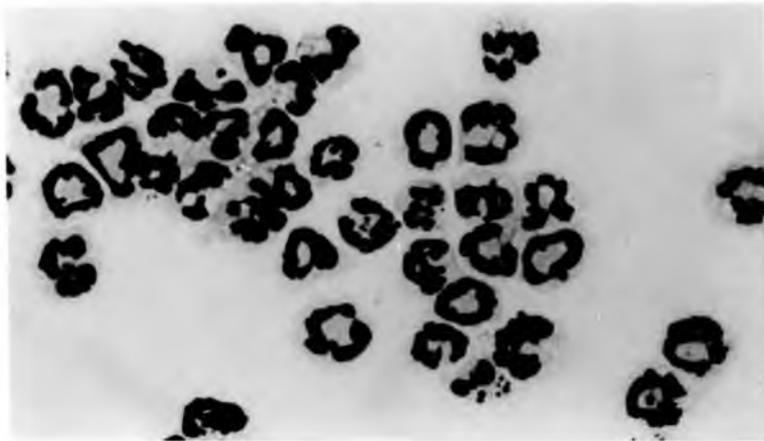


Figure 3.8. Giemsa stained cytocentrifuge preparation of purified mouse neutrophils



Figure 3.8. Giemsa stained cytocentrifuge preparation of purified mouse neutrophils



Figure 3.8. Giemsa stained cytocentrifuge preparation of purified mouse neutrophils

or hyper tonic metrizamide gradients (James et al., 1979) produced preparations of about 90% eosinophils with variable results. Centrifugation on slightly hypotonic Percoll gradients (Burgess et al., 1980) has been shown to produce preparations of about 77% eosinophils. This was improved to about 93% when the initial eosinophil percentage was increased. This could be achieved by irradiating the mice 24 hrs before harvesting and selecting mice with less than 25% macrophages.

Of the parasites tested, M.corti induced the highest peritoneal eosinophilia with a total of about 5×10^7 eosinophils/mouse. The number of eosinophils induced by each of these parasites was found to be consistent with the observations of Johnson et al. (1979) for M. corti, Coker (1956) for T.spiralis and Freeman (1964) for T.crassiceps. S.mansoni is also known to induce a peritoneal eosinophilia although the percentage of eosinophils appears to vary in different laboratories, (Mahmoud et al., 1973; James et al., 1979). Recent reports indicate that Toxocara canis could induce a higher proportion as well as total numbers of eosinophils (Sugane & Oshima, 1980) than these results with M.corti. These authors infected mice with 500 eggs of T.canis, followed, two weeks later, by an i.p. injection of between 1 and 10 mg of protein from T.canis. Two days after this, each mouse gave about 1.2×10^8 peritoneal cells, 80% of which were eosinophils.

Velocity sedimentation at unit gravity has proved to be simple and reproducible. It is important not to overload the chamber with cells as this will produce "streaming", a phenomenon characterized by the excessively fast sedimentation rate of the cells which results in the distortion of the interface between the cell bands and the underlying medium (Miller & Phillips, 1969). Streaming can be identified either visually, or by analysis of the sedimenting profile of the cells. In the

experiments described above no streaming was observed by loading up to 4×10^8 cells on a chamber with a 10 cm diameter. On the contrary, three clear bands were visible after approximately 2.5 hs of sedimentation, presumably representing from top to bottom lymphocytes, eosinophils and macrophages.

Because neutrophils could not be separated from eosinophils, peritoneal exudates from M.corti-infected mice were examined prior to loading in the sedimentation chamber, and those exudates containing neutrophils discarded.

The use of a buffered step Ficoll gradient plays only a minor role in the cell separation but stabilizes the fluid in the chamber. Furthermore, its use has been shown in practice (Miller & Phillips, 1969) and by theoretical considerations (Mason, 1976), to increase about four-fold the number of cells that can be loaded when compared to a sheer step gradient. A minor disadvantage is the low filling rate at which it must be used if disturbance of the cell layers is to be avoided.

Collection of cell fractions from the top of the sedimentation chamber gave a better resolution than collection from the inlet. Using a tilting procedure, Bont, de Vries, Geel, van Dongen & Loos (1979) have claimed a more rapid separation by increasing the surface area in which the cells sediment. This may also increase the capacity of cell separation at unit gravity. An apparatus of this type may therefore, be an improvement over the stationary chamber.

Separation of eosinophils by isopycnic centrifugation was found to be relatively unsatisfactory as these cells sedimented over a wide range of densities. This is probably a consequence of differences in the stages of maturation of these cells. Neutrophils also show some heterogeneity in their isopycnic sedimentation but the fact that they are of

higher density than eosinophils means that they separate more completely from lymphocytes and macrophages. Better results have been claimed using calcium caseinate to induce neutrophils, followed by purification on Percoll (Watt *et al.*, 1979). This is probably not due to the use of Percoll since this does not appear to offer any advantages over Metrizamide (in fact Metrizamide is easier to use and its concentrations can be checked by refractive index), but to the fact that in this case the starting peritoneal exudate contained $\geq 82\%$ neutrophils.

It is interesting to note that there are species differences in the relative densities of eosinophils and neutrophils. Thus, while in mouse (Figures 3.2 and 3.7) and rat (Sanderson & Thomas, 1978), neutrophils are of higher density than eosinophils, in humans the reverse is the case (Vadas, David, Butterworth, Pisani & Siongok, 1979). The apparent differences in density between rat and mouse granulocytes may be a result of the osmolarity of the Metrizamide solutions used. It has been shown that rat eosinophils sediment at higher densities than 1.0869 gm/cm^3 (Metrizamide 16.5%) and rat neutrophils at higher densities than 1.0924 gm/cm^3 (metrizamide 17.5%) (Sanderson & Thomas, 1978), while mouse eosinophils sediment at a density of 1.0814 gm/cm^3 (Metrizamide 15.5%) and mouse neutrophils at a density of 1.0842 gm/cm^3 (Metrizamide 16%). Thus, it is likely that the use of Metrizamide (35.3% stock solution) at 282 m Osm which is isotonic for the rat (rat serum = 282 m Osm) but slightly hypertonic for the mouse (mouse serum = 308 m Osm) may have induced a shift of mouse cells to lower densities as a result of cell swelling, an effect previously observed with mouse lymphocytes (Williams, Kraft & Shortman, 1972).

SUMMARY

The development of purification techniques for mouse eosinophils and neutrophils is shown. Eosinophils are induced in largest numbers to the peritoneal cavity of mice 25-28 days after an i.p. injection of Mesocestoides corti. By combining velocity sedimentation at unit gravity and isopycnic centrifugation on a Metrizamide interface a good yield of highly purified eosinophils is obtained. Neither adherence to nylon wool nor isopycnic centrifugation by itself have provided a satisfactory separation of eosinophils from other cell types. Neutrophils are shown to be induced to the peritoneal cavity of mice by an i.p. injection of dextran. An effective separation is achieved by using isopycnic centrifugation.

CHAPTER 4

**IgG AND COMPLEMENT RECEPTORS ON MOUSE
EOSINOPHILS AND NEUTROPHILS**

INTRODUCTION

Complement receptors have been recognized on human (Gupta, Ross, Good & Siegal, 1976; Anwar & Kay, 1977a) rat (Ramalho-Pinto, McLaren & Smithers, 1978; Incani & McLaren, 1981) and guinea pig (Henson, 1969) eosinophils and neutrophils. Complement receptors are also present on mouse neutrophils. However, a number of studies have failed to demonstrate such receptors on mouse eosinophils (Rabellino & Metcalf, 1975; Rabellino, Ross, Trang, Williams & Metcalf, 1978; Hoghart *et al.*, 1980), and it was suggested that murine eosinophils might be different to eosinophils from other species (Rabellino *et al.*, 1978). Fc receptors, on the other hand, have long been detected on eosinophils and neutrophils from a number of species, and their presence enables granulocytes to make contact and kill antibody-coated tumour cells (see Chapters 5 and 6), parasites (López *et al.*, 1978) and red cells *in vitro* (Archer & Hirsch, 1963; Sanderson & Thomas, 1978). It has also been shown that different antisera can preferentially react with eosinophils or neutrophils, probably due to the IgG subclass composition of each antibody preparation (see Chapter 6). The availability of mouse monoclonal anti-E antibodies of IgG1 and IgG2b subclasses offers the possibility of comparing eosinophil and neutrophil binding to these two subclasses. These subclasses are of particular interest as they have been found associated with Schistosoma mansoni (Ramalho-Pinto, De Rossi & Smithers, 1979) and Mesocestoides corti (Mitchell, Marchalonis, Smith, Nicholas & Warner, 1977) infections in mice. In this chapter, the ability of mouse eosinophils and neutrophils to bind, phagocytose and lyse (as judged by ⁵¹Cr release) E coated with mouse complement, IgG1 and IgG2b has been investigated. Their relative antibody and complement requirements, and the advantages and limitations of using monoclonal reagents are also considered.

RESULTS

4.1. Detection of complement receptors

Mouse eosinophils and neutrophils formed high numbers of rosettes with EAC3b complexes (Table 4.1). Figure 4.1 and Figure 4.2 show eosinophils and neutrophils respectively, completely surrounded by strongly adhering EAC3b, some of which have been phagocytosed. ^{51}Cr release was low in relation to the number of rosettes and phagocytosis observed. There was a marked difference between eosinophils and neutrophils when the amount of IgM (and presumably the amount of complement) was varied. Thus, while an IgM dilution of 1:10 induced similar numbers of rosettes on both cell types, IgM used at 1:50 showed a decrease in the number of eosinophils forming rosettes although the proportion of neutrophil rosettes remained essentially the same. The values obtained with IgM at 1:10 were not increased by using the IgM or complement undiluted.

These granulocytes were also found to react with EAC3bi complexes, although to a lesser extent than with EAC3b. Control preparations of EA_{IgM} alone or after incubation with heat inactivated mouse serum were found to be unreactive.

4.2. Detection of IgG receptors

Mouse eosinophils and neutrophils were found to form high numbers of rosettes with IgG_{2b} coated E and to phagocytose this complex very actively (Table 4.2). More than 70% of eosinophils and 90% of neutrophils formed rosettes when E were coated with IgG_{2b} at 1:10 and 1:100. However, when IgG_{2b} was used at 1:1000, 22% eosinophils formed rosettes compared to 66% neutrophils. Thus, eosinophil binding is decreased more than neutrophil binding at high antibody dilution. A similar effect was observed in the phagocytosis assay.

Table 4.1. Effect of two different concentrations of IgM on the detection of complement receptors on mouse eosinophils and neutrophils

Indicator cells	IgM dilution	Effectors	% Rosettes	% Phagocytosis	% ⁵¹ Cr release
EAC 3b	1:10	Eosinophils	89 ± 6.0*	34 ± 5.1*	9.4
		Neutrophils	91 ± 4.2	51 ± 10.6	12.5
EAC 3b	1:50	Eosinophils	25 ± 3.1	13 ± 2.5	0 ⁺
		Neutrophils	90 ± 4.0	55 ± 4.6	14.5
EAC3bi	1:10	Eosinophils	60 ± 9.5	23 ± 8.0	9
		Neutrophils	65 ± 12.2	37 ± 6.8	14.5
EAC3bi	1:50	Eosinophils	20 ± 6.2 *	10 ± 3.8	0
		Neutrophils	45 ± 8.5	29 ± 8.5	7.1

* Mean values ± 1 standard deviation of a representative experiment from a total of six experiments.

Phagocytosis was measured after 30' of incubation without centrifugation.

+ Zero = not significantly different from controls at the 5% level.

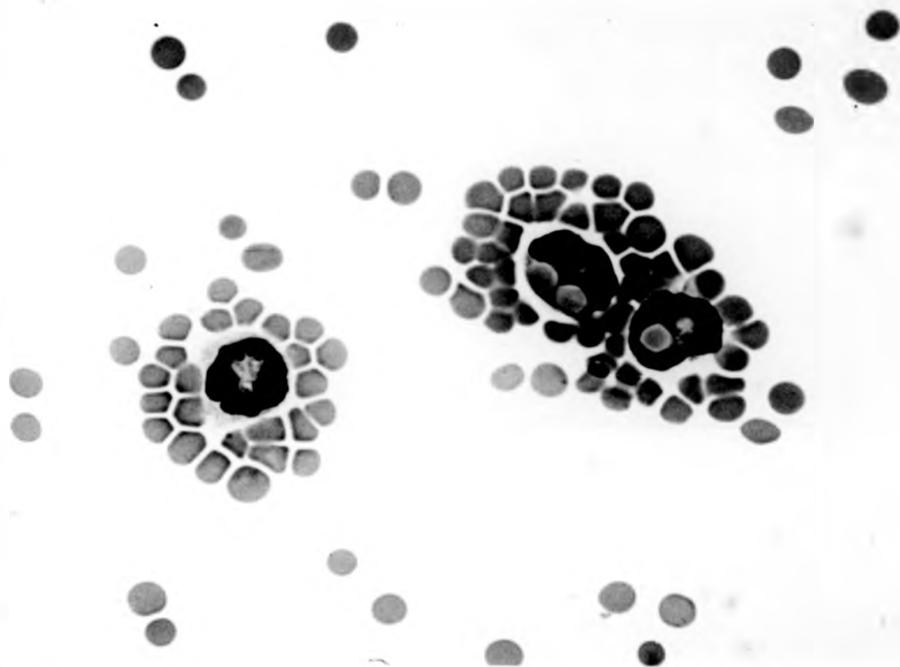


Figure 4.1. Giemsa stained preparation of eosinophils forming rosettes and showing phagocytosis with EAC3b.

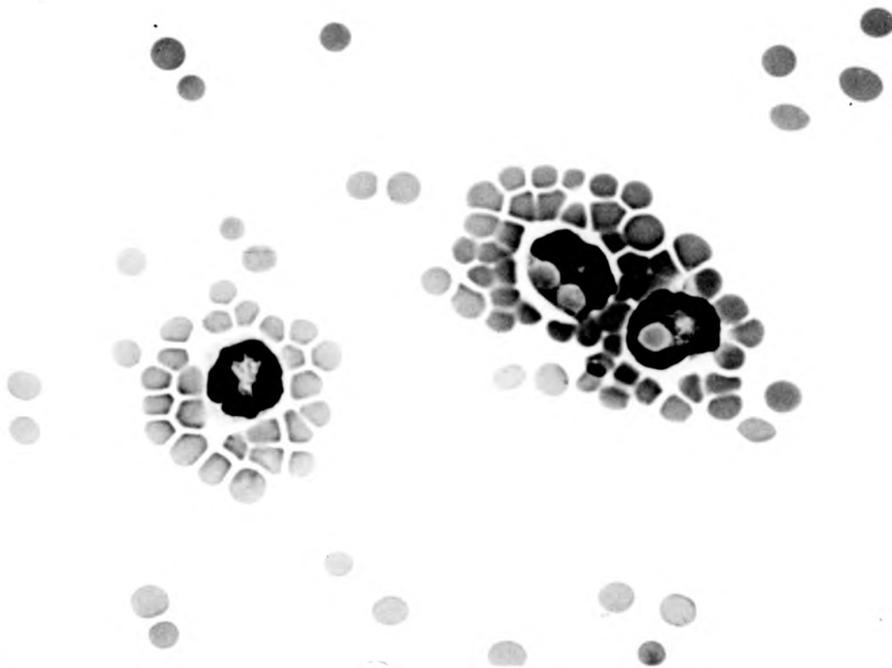


Figure 4.1. Giemsa stained preparation of eosinophils forming rosettes and showing phagocytosis with EAC3b.

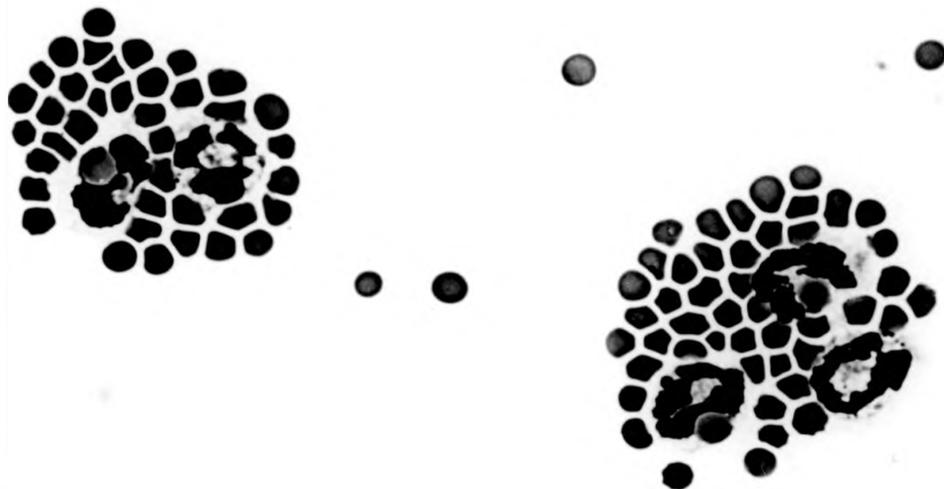


Figure 4.2. Giemsa-stained preparation of neutrophils forming rosettes and showing phagocytosis with EAC 3b.

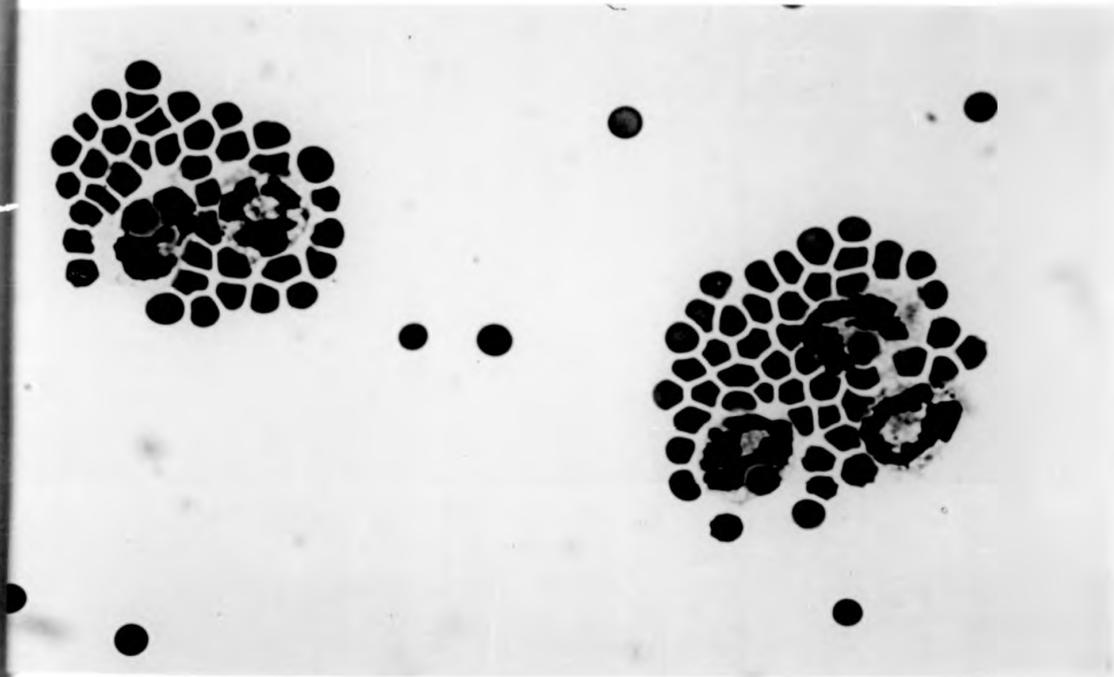


Figure 4.2. Giemsa-stained preparation of neutrophils forming rosettes and showing phagocytosis with EAC 3b.

Table 4.2. Rosette formation and phagocytosis of EA_{IgG2b}⁻ and EA_{IgG1} by eosinophils and neutrophils

Indicator cells	Effectors	% Rosettes	% Phagocytosis
EA(IgG2b 1:10)	Eosinophils	73 ± 7.8*	13 ± 3.1
	Neutrophils	93 ± 4.2	41 ± 6.7
EA(IgG2b 1:100)	Eosinophils	78 ± 4.6	11 ± 2.5
	Neutrophils	94 ± 3.2	49 ± 4.9
EA(IgG2b 1:1000)	Eosinophils	22 ± 9.1	4.5 ± 4.5
	Neutrophils	66 ± 12.8	31 ± 8.2
EA(IgG1 1:10)	Eosinophils	14 ± 4.5	2 ± 1.5
	Neutrophils	44 ± 8.7	32 ± 6.8
EA(IgG1 1:100)	Eosinophils	13 ± 3.5	2.5 ± 1.2
	Neutrophils	57 ± 7.1	29 ± 4.6
EA(IgG1 1:1000)	Eosinophils	10 ± 6.1	2 ± 1.2
	Neutrophils	43 ± 15.2	23 ± 8.0

* Mean values ± 1 standard deviation of a representative experiment from a total of five similar experiments.

Phagocytosis was measured after 1 h of incubation without centrifugation.

Rosette formation and phagocytosis of E coated with IgG1 was found to be lower than with IgG2b for both eosinophils and neutrophils. Approximately 12% eosinophils formed rosettes with EA-IgG1 and phagocytosis was virtually negative, while about 50% neutrophils formed rosettes and 30% showed phagocytosis of this complex. Rosette and phagocytosis values for both types of granulocyte were not essentially changed by diluting the IgG1 up to 1:1000.

The ^{51}Cr release assay showed IgG2b to induce about 40% cytotoxicity by both eosinophils and neutrophils (Figure 4.3). In correlation with the rosette and phagocytosis assays, IgG1 induced lower levels of ^{51}Cr release by neutrophils, and almost undetectable levels by eosinophils. In contrast, the mouse anti-E serum induced very high levels of cytotoxicity by both cell types. It was consistently found that eosinophil activity decreased more than neutrophil activity following dilution of IgG2b or the mouse anti-E serum. Thus, it appears that comparisons between eosinophil and neutrophil activities can be critically determined by the dilution of the antibody preparation used.

4.3. Effect of the incubation time on the phagocytosis assay

Effector cells and E coated with optimum concentrations of IgG1, IgG2b and C3b were mixed, centrifuged and incubated at 37°C . At three different time intervals, the number of eosinophils and neutrophils showing phagocytosis was recorded. It was found (Table 4.3) that in each case, the highest values were obtained after 5 minutes of incubation. Longer incubation periods resulted in a marked reduction in the number of cells showing phagocytosis.

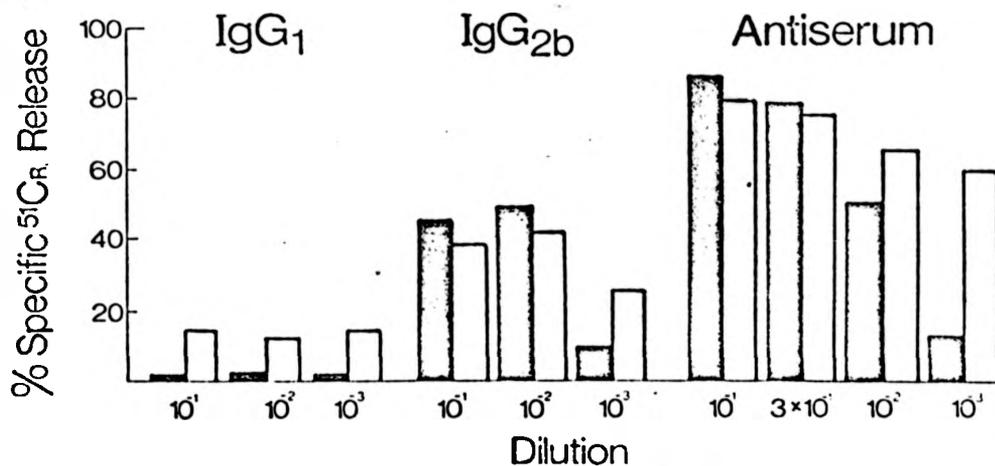


Figure 4.3. Titration of IgG₁, IgG_{2b} and mouse anti-E serum with mouse eosinophils (filled blocks) and neutrophils (empty blocks). Eosinophil values are not significantly different (5% level) from neutrophil values at IgG_{2b} dilutions of 10⁻¹ and 10⁻², and at antiserum dilutions of 10⁻¹ and 3 × 10⁻¹. All other values are significantly different from control and between both effector cells at the 5% level or greater.

Table 4.3. Percentage of effector cells showing phagocytosis at different times after incubation

Indicator cells	Effector cells	Incubation time (minutes)		
		5	30	60
EAIgG1	Eosinophils	5 ± 2.3*	2 ± 1.8	3 ± 2.0
	Neutrophils	57 ± 3.5	28 ± 6.2	29 ± 4.6
EAIgG2b	Eosinophils	57 ± 5.9	20 ± 2.1	15 ± 3.3
	Neutrophils	74 ± 9.2	43 ± 3.0	25 ± 7.8
EAC3b	Eosinophils	51 ± 6.0	20 ± 1.8	12 ± 1.8
	Neutrophils	NT	NT	NT
E	Eosinophils	0	0	0
	Neutrophils	2 ± 1.1	1 ± 1.5	2 ± 1.5

* Mean values ± 1 standard deviation

NT = not tested.

DISCUSSION

Mouse eosinophils are shown to possess complement receptors and to have higher antibody and presumably higher complement requirements than neutrophils. Thus, it has been shown, that a five-fold dilution of IgM results in a marked decrease in the number of eosinophils forming rosettes with EAC3b, while the proportion of neutrophils forming rosettes remains essentially unchanged. This high complement requirement by mouse eosinophils is likely to be responsible for the failure of Hoghart *et al.* (1979) to detect complement receptors on *M.corti*-induced eosinophils, as these authors used 25-fold less complement than in the experiments described in this chapter. Furthermore, the relatively long incubation period (30 minutes) of EA_{IgM} with mouse serum has probably been a contributing factor as this would lead to C3bi formation, and granulocyte reactivity with EAC3bi is not as strong as with EAC3b (Table 4.1). The apparent absence of complement receptors from normal eosinophils from blood and spleen (Rabellino *et al.*, 1978) and from colony eosinophils grown in soft agar cultures (Rabellino & Metcalf, 1975; Rabellino *et al.*, 1978) may also be due to the low levels of complement on the indicator cells. Human eosinophils have been previously shown to require a larger amount of C4 than neutrophils or lymphocytes. However, the proportion of eosinophils forming rosettes remained well below neutrophil values, even with the highest amount of C4 added (Gupta *et al.*, 1976).

Both eosinophils and neutrophils phagocytose EAC3b and EAC3bi very actively. Previous studies with human phagocytes (Ehlenberger & Nussenzweig, 1977) have indicated that complement itself does not induce phagocytosis but that it acts in a synergistic fashion with IgG. This synergistic effect could be mimicked by centrifugation or with a variety of non-immunological agents. In the experiments described in this chapter

however, it is unlikely that complement is having a synergistic effect with IgG since a monoclonal IgM was used to sensitize E, and the mouse serum was twice absorbed with E. Moreover, no phagocytosis was seen after centrifugation of effector cells with complexes prepared with heat inactivated complement. It is possible that complement mediated phagocytosis is a property of stimulated cells. Phagocytosis of EAC complexes by eosinophils has been previously observed in studies with cells from patients with Löffler's cardiomyopathy but not when the cells were obtained from normal donors (Spry & Tai, 1976). Similarly, normal and induced macrophages have been shown to bind to EAC complexes but phagocytosis has only been observed with the latter (Bianco, Griffin & Silverstein, 1975; Rabellino *et al.*, 1978).

Mouse complement has been used in this study, as a homologous system is more representative of the situation *in vivo* than heterologous combinations. Mouse cells react variably with complement of other species (Dierich, Pellegrino, Ferrone & Reisfeld, 1974), and have poor affinity for human complement in particular (Bianco, Patrick & Nussenzweig, 1970; Dierich *et al.*, 1974). The unavailability of purified mouse complement components has prompted several workers to attempt the production of different fragments of mouse C3 by varying the incubation conditions of EA_{IgM} with mouse serum. Thus, previous studies have used incubation times of 10 minutes (Griffin, Bianco & Silverstein, 1975) 8 minutes (Rabellino *et al.*, 1978), and 5 minutes (McConnell & Hurd, 1976) to coat EA_{IgM} with mouse C3b. A short time is necessary to avoid enzymatic degradation of C3b by Factor H and Factor I present in mouse serum. In these experiments, an incubation time of only 2 minutes was used to maximise the probabilities of having mainly C3b on the E surface. However, since a variable proportion of Daudi cells were found forming

rosettes with this complex (see Section 2.15b), it is conceivable that some conversion of C3b into C3bi has already taken place; alternatively this could be due to cross-reactivity of human C3d receptors with mouse C3b. A long incubation of EA_{IgM} with mouse serum had been previously shown to produce the C3d fragment of C3 (Rabellino et al., 1978b; Griffin et al., 1975). However, recent reports (Ross & Rabellino, 1979) suggest that the complex thus obtained, was not C3d but C3b - cleaved C3bi, and that C3d is produced by treating C3bi with plasmin or trypsin. Furthermore, C3d appears to be unreactive with granulocytes (Tai & Spry, 1980) and macrophages (Ross & Rabellino, 1979). The complex produced in the present study should therefore be regarded primarily as EAC3bi, although some C3d may have also been formed by the action of trypsin-like proteases present in the mouse serum. This complex (C3bi) reacted more strongly with Daudi cells than with mouse granulocytes; the reverse was seen with EAC3b. Thus, although these two indicator systems cannot be proven to represent pure components, granulocytes show a preference for C5b. Whether the interaction of granulocytes with this complex occurs via their receptor for C3b or via a receptor analogous to the one recently shown on Raji cells (Okuda & Tachibana, 1980) for Factor H-modified C3b, remains to be seen.

Mouse granulocytes are shown to react with mouse IgG2b and IgG1 monoclonal anti-E antibodies. Monoclonal antibodies offer the possibility of studying receptors for a particular IgG subclass without the problem of contamination with other subclasses. However, the main disadvantage lies in the variation in the number of epitopes to which each monoclonal antibody binds. Thus, although eosinophils and neutrophils react more strongly with IgG2b than with IgG1, this could be attributed to differences in antibody density rather than to a subclass effect, as seven times more

of the IgG2b binds to E than the monoclonal IgG1 antibody (Galfre, Milstein & Wright, 1979), and IgG2b gives a higher titre by indirect haemagglutination (Table 2.3). Using the same monoclonal antibodies, Kerbel (1980) found that lymphoid cells showed a similar pattern of reactivity as the one shown in Table 4.2 with granulocytes. Clearly, therefore, further studies on granulocyte reactivity with different IgG subclasses must await the availability of other monoclonal antibodies.

There was found to be a correlation between rosette formation, phagocytosis and cytotoxicity for both effector cell types, reacting with either EA_{IgG2b} or EA_{IgG1}. Neutrophils form more rosettes and are more active than eosinophils when EA_{IgG1} is used. This difference may be related to the low numbers of IgG1 molecules coating E, as eosinophils show a higher antibody requirement than neutrophils. This can be seen with IgG2b in all three assays and with whole anti-E serum in the ⁵¹Cr release assay. Thus, while eosinophils and neutrophils induce similar levels of ⁵¹Cr release at high antibody concentrations, neutrophils are clearly the more active at high antibody dilutions.

For this reason, previous reports indicating higher EA binding by neutrophils than by eosinophils (Gupta *et al.*, 1976; Anwar & Kay, 1977; Tai & Spry, 1980) may be due to the limiting antibody dilution used. The ⁵¹Cr release assay circumvents this problem, allowing the use of anti-serum at agglutinating concentrations, and has the further advantages of accuracy, objectivity and the small amount of reagents used. The ⁵¹Cr release assay also shows that granulocyte activity is much higher in the presence of a whole antiserum than when IgG2b or IgG1 monoclonal antibodies are used, suggesting the need for a higher antibody density on the E membrane than can be provided by these monoclonal antibodies, or the involvement of other IgG subclasses.

In contrast to the results with IgG, eosinophils and neutrophils

consistently gave very little ^{51}Cr chromium release from complement-coated E, despite the fact that high numbers of rosettes and phagocytosis were observed. The reason for this is not clear. It may be that different stimuli induce a differential enzymatic release from the effector cell granules (Spry, 1978).

Cinematography studies have shown that phagocytosis of opsonized red cells by granulocytes is very rapid (Archer & Hirsch, 1963; Sanderson & Thomas, 1978). However, phagocytosis of red cells by granulocytes has been frequently measured after relatively long periods of incubation (Spry & Tai, 1976; Gupta *et al.*, 1976; Sanderson & Thomas, 1978; Tai & Spry, 1980). In the present study, the highest levels of phagocytosis were observed 5 minutes after centrifugation.

It is clear from these experiments that eosinophils have different IgG and complement requirements from neutrophils, which is probably a reflection of the lower number or affinity of the eosinophil membrane receptors. Thus, comparisons between eosinophil and neutrophil activities cannot be properly evaluated unless the optimal conditions for each cell type are fulfilled.

SUMMARY

Mouse eosinophil and neutrophil receptors for IgG and complement have been examined by means of rosette formation, phagocytosis and ^{51}Cr release assays, using mouse monoclonal antibodies and complement-coated sheep erythrocytes. Mouse eosinophils and neutrophils form a high number of rosettes in the presence of mouse complement but eosinophils show a higher requirement for complement molecules. Both types of granulocyte phagocytose complement-coated sheep erythrocytes very actively although

low levels of ^{51}Cr release are obtained. Eosinophils and neutrophils show higher activity in the presence of IgG2b than in the presence of IgG1, and while both cell types are similarly active when the former antibody is used, neutrophils are the more active when IgG1 is used. However, it remains uncertain whether this is a result of the higher binding obtained with the IgG2b monoclonal. Both cell types behave similarly at high antibody concentrations but neutrophils are the more active at high antibody dilutions. The ^{51}Cr release assay is shown to be superior to the rosette assay as it allows comparisons between eosinophils and neutrophils at high antibody concentrations. A time course study indicates that highest values of phagocytosis of opsonized red cells are obtained after 5 minutes rather than the half to one hour incubation periods normally used.

CHAPTER 5

ANTIBODY-DEPENDENT CYTOTOXICITY OF TRYPANOSOMA CRUZI ANTIGEN-COATED
MURINE CELL LINE CELLS BY MOUSE EOSINOPHILS AND NEUTROPHILS

INTRODUCTION

American trypanosomiasis (Chagas' disease) has been estimated to affect more than 10 million people in South and Central America, and another 30 million are believed to be exposed to the infection. The causative agent is Trypanosoma cruzi, a parasite protozoan that has, as vectors, species of reduviid bugs of the family Triatominae and, as definite hosts, a wide range of vertebrates including many mammals.

The bugs ingest T.cruzi by taking the blood of infected vertebrates. The ingested blood trypomastigotes transform into amastigotes which divide in the foregut of the bug. The amastigotes transform into epimastigotes in the midgut of the bug and then into metacyclic trypomastigotes in the hindgut. These metacyclic trypomastigotes are the non-dividing infective form of the parasite which the bugs deposit with their faeces at the site of the bite after the blood sucking act. The trypomastigote then enters into the bloodstream of the vertebrate through the bite wound or a mucous surface like the conjunctiva, and actively parasitizes the tissue cells of the host. The tissue distribution apparently varies with the strain of T.cruzi. Within the host cell, the trypomastigote changes into a round amastigote which is the dividing form. After a few days, the cell is filled with amastigotes forming a pseudocyst. Shortly before the pseudocyst ruptures, the amastigotes transform into trypomastigotes which are subsequently released into the extracellular space and bloodstream perpetuating the cycle.

In American trypanosomiasis, two periods can be distinguished, the acute phase and the chronic phase which differ in their clinical manifestations and pathological findings.

The acute phase resembles many acute septicaemic conditions by its local inflammatory reaction at the site of parasite penetration and a

high parasitaemia. Moderate fever may be present together with weakness, tachicardia, dysphagia or diarrhoea. Antibodies to T.cruzi appear shortly after the onset of the symptoms, sometimes accompanied by an increase in serum IgM or IgG levels.

The acute phase subsides as the result of the immune response of the host (apparently regardless of the strain of T.cruzi), and the patient enters into the chronic phase of the disease. The number of trypanosomes and antibody levels in the peripheral blood decrease sometimes to become undetectable. It is generally accepted that there is no cure for the infection and in immunosuppressive states the parasite can be shown again in the circulation.

This chronic phase is largely asymptomatic but after a variable period of time the so-called "Chagas' syndromes" may appear. The chagasic cardiopathy is one of these and by far the most frequent. Other syndromes are the result of the dilation and alterations in the motility of other organs mainly of the digestive tract. However, many patients remain asymptomatic for life, death usually occurring suddenly, presumably due to cardiac failure.

The pathology of the acute phase of American trypanosomiasis shows that although the parasite can be found in most tissues, it has a preference for muscle cells and an intense parasitism can be seen in the heart, all hollow muscular organs and the skeletal muscle. It is of interest to note that it is quite common to find parasitized cells without any inflammatory reaction in their surroundings (Vianna, 1911; Torres, 1941; Köberle, 1968). A cellular infiltrate is however found in the vicinity of a ruptured pseudocyst where parasites can be seen to disintegrate in the extracellular space, due either to their incomplete differentiation into trypomastigotes or to the host effector mechanisms. Köberle (1968) has found this cellular infiltrate to consist mainly of

eosinophils and neutrophils and this observation has been confirmed by other authors (Tafuri, 1970; Deutschländer, Volhertun & Hungerer, 1978). Eosinophils and neutrophils have also been described with astrocytes and histiocytes in brain granulomas of a new-born child and in experimental animals carrying T.cruzi infections (Dominguez & Gavaller, 1962).

Perhaps the most significant pathological finding in the acute phase of T.cruzi infections is central and peripheral denervation. In humans, quantitative studies done on autopsies of patients who died of acute chagasic myocarditis have shown the number of heart neurons to be reduced to about a third of that found in humans who died of unrelated diseases (Becker, 1975; Lopes, Tafuri, Bogliolo, Almeida, Chapadeiro & Raso, 1977). A great reduction in heart neurons has also been observed during the acute phase of T.cruzi infections in mice (Tafuri, 1970; Ribeiro dos Santos & Hudson, 1981) and rats (de Alcantara, 1959). It has been suggested, therefore, that the lesions in the autonomic nervous system are responsible for the anatomical and functional disorders occurring in the heart, esophagus and colon which lead to the cardiopathy and enteropathy encountered in the chronic phase.

The chronic phase of the disease in humans has occasionally shown inflammatory foci consisting mainly of mononuclear cells (Köberle, 1968). A diminution in the number of neuron cells in the central and peripheral nervous system and attributed to damage in the acute phase of the disease has also been observed. In studies of chronically infected rabbits the presence of lymphocyte infiltrates in the heart has been described (Teixeira, Teixeira & Santos-Buch, 1974).

While the pathogenesis of the disease remains largely unknown, different mechanisms have been postulated: Torres (1941), observing that the

inflammatory lesions are not initiated around the parasite or parasitized cells but at the level of the small vessels in the tissues suffering the most intensive colonization by T.cruzi, suggested the involvement of a T.cruzi toxin. Later Köberle (1968) proposed a similar explanation in which a "neurotoxic substance" liberated by the degenerated parasite would be responsible for the degenerative lesions and lysis of the uninfected ganglion cells in the vicinity. However, such a substance could not be isolated from preparations of T.cruzi grown in tissue culture (Jörg, 1964). A direct effect by the parasite does not seem to be an important factor since neurons are very rarely parasitized in T.cruzi infections (Köberle & Alcantara, 1960).

Some of the pathological changes may be due to an autoimmune phenomenon. Some patients have antibodies to endocardium, vascular and interstium structures (Cossio, Laguens, Diez, Szarfman, Segal & Arana, 1974) or antibodies against neuronal tissue (Ribeiro dos Santos, Marquez, Von Gal Furtado, Ramos de Oliveira, Martins & Köberle, 1979). It should be noted that in other circumstances (rheumatic fever, chronic heart disease, idiopathic cardiopathy, after cardiotomy, and after myocardial infarction) antibodies to heart tissue have also been found (reviewed by Laufer, 1975). No correlation has been clearly established, however, between the presence of anti-heart antibodies and myocarditis in any of these cases or in T.cruzi infections.

Lymphocytes from infected mice have been shown to be cytotoxic against T.cruzi infected mouse fibroblasts (Kuhn & Murnane, 1977) or against adherent mouse cells coated with T.cruzi antigen (Ribeiro dos Santos & Hudson, 1980b). Lymphocytes from chronically infected rabbits (Santos-Busch & Teixeira, 1974) and mice (Ribeiro dos Santos & Hudson, 1980b) have been found to lyse non-parasitized cells and it has been suggested

(Santos Busch & Teixeira, 1974) that this is the result of cross-reactivity between host and parasite antigens. However, these findings have been reported using animals with chronic T.cruzi infections when the reduction in heart neurones has already taken place (Ribeiro dos Santos & Hudson, 1981), suggesting that while a host auto-immune response may perpetuate the disease the damage to the nervous system has occurred in advance of it.

An effector mechanism that could be responsible for many of the pathological findings but has so far received little attention is granulocyte cytotoxicity. Eosinophils and neutrophils have been shown to phagocytose and lyse T.cruzi in vitro as judged by ³H-uridine release (López et al., 1978) and morphological criteria (Sanderson & de Souza, 1979). This probably also occurs in vivo, as histological studies have shown parasites within granulocytes (Vianna, 1941). Furthermore, in several reports granulocytes have been described near the ruptured pseudocyst in the acute phase of T.cruzi infections and associated with the degenerative lesions of the non-infected cells in the vicinity (reviewed by Köberle, 1968).

Recent work (Ribeiro dos Santos & Hudson, 1980a) has shown that parasite antigen can be adsorbed to the membrane of cells in vitro. This observation raised the possibility that if antibody is bound to the adsorbed antigen, this may promote the attachment of granulocytes by means of their Fc receptors, a situation potentially damaging for the host cells.

This hypothesis was tested by using two murine cell line cells, S2 (muscle-derived) and Neuro-2a (neuroblastoma) as models for the tissues where the most significant damage in T.cruzi infections is observed. A parasite antigen derived from cultured epimastigotes (see Section 2.18)

was used to coat the cell line cells, which were subsequently incubated with antibody to T.cruzi and granulocytes. It is shown that both eosinophils and neutrophils are cytotoxic to syngeneic cell line cells coated with T.cruzi antigen in the presence of anti T.cruzi antibody.

RESULTS

5.1. Comparison of two different incubation periods to detect cytotoxicity by granulocytes.

Purified mouse eosinophils and neutrophils were tested for cytotoxic activity against ^{51}Cr labelled S2 cell line cells coated with T.cruzi antigen and anti-T.cruzi antibody. It was found (Figure 5.1) that both types of granulocyte induced significant ^{51}Cr release from T.cruzi antigen-coated cells in the presence of rabbit anti-T.cruzi serum. Neutrophils showed a higher cytotoxic activity than eosinophils although they were tested at a lower effector to target ratio than eosinophils. The results show that 5 hr was an adequate time to detect cytotoxicity. After 18 hr incubation, the isotope released in the presence of antigen, antibody and effector cells increased but so did the control values. In all subsequent experiments ^{51}Cr release was measured after 5 hrs of incubation.

5.2. Relative antibody requirement

An antiserum titration using S2 cells coated with T.cruzi antigen showed that the relative antibody requirement was similar for both types of granulocyte (Figure 5.2). This experiment also showed that when a mouse anti-T.cruzi serum was used, eosinophil and neutrophil activities were very

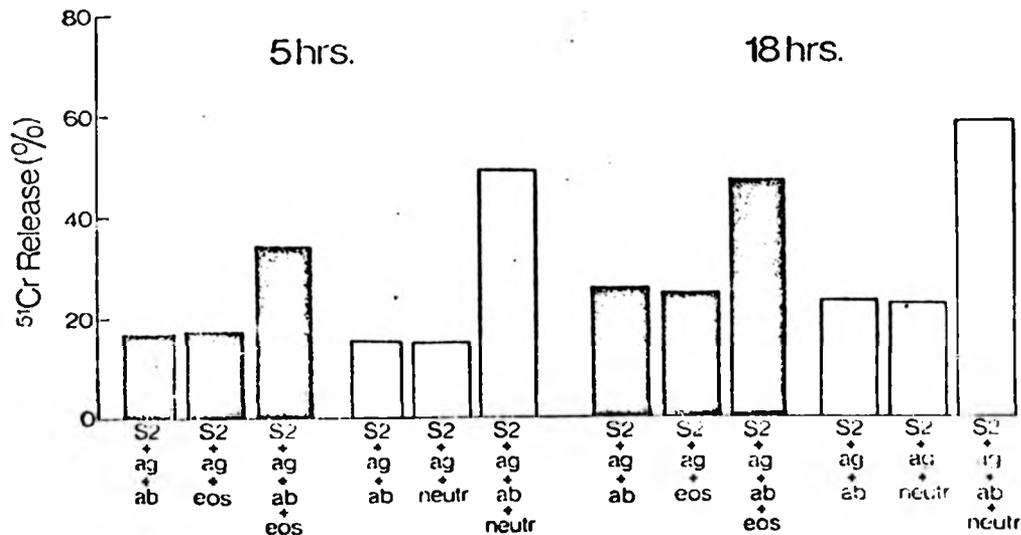


Figure 5.1. Antibody-dependent killing of S2 cells coated with T.cruzi antigen by eosinophils and neutrophils at two different incubation periods.
 Eosinophils (filled columns) to target ratio of 30:1
 Neutrophils (empty columns) to target ratio of 20:1
 Rabbit anti-T.cruzi used at 1:100
 Values obtained in the presence of anti-T.cruzi antibody are significantly different from values obtained without antibody or effector cells at the 5% level or greater. Neutrophil activity is significantly different from eosinophil activity in both incubation periods.

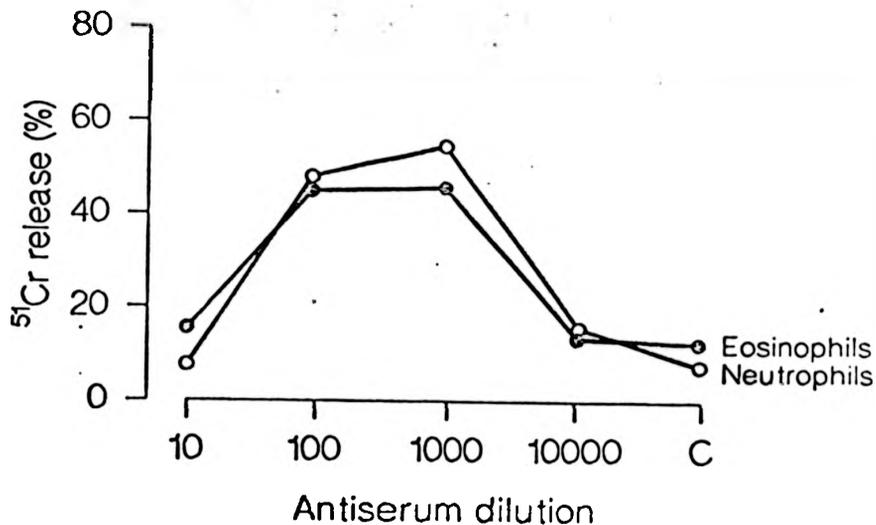


Figure 5.2. Antibody titration of mouse anti-T.cruzi serum with eosinophils (filled symbols) and neutrophils (open symbols) using S2 cells coated with T.cruzi antigen as target cells. Effector to target ratio of 20:1

C = control with no antiserum.

Other controls, with the percentage ^{51}Cr release between brackets included: S2 cells alone (8.2), S2 cells + antiserum at 1:100 + eosinophils (10.0) or neutrophils (9.6), and S2 cells + T.cruzi antigen + normal mouse serum at 1:100 + eosinophils (9.2) or neutrophils (8.0).

Values obtained with antiserum at 1:100 and 1:1000 are significantly different from control values at the 5% level or greater. Values obtained with antiserum at 1:10,000 are not significantly different from control.

Eosinophil and neutrophil activity are significantly different from each other at antibody dilutions of 1:10 and 1:1000.

similar. Maximum ^{51}Cr release was observed when T.cruzi antigen-coated cells were incubated with the mouse anti-T.cruzi serum at 1:100 or 1:1000 dilutions and effector cells. A higher concentration of antibody (1:10) completely inhibited cytotoxicity by granulocytes. No significant isotope release was observed when the antiserum was diluted to 1:10,000, or when either T.cruzi antigen, antibody or effector cells were omitted.

Essentially the same results were obtained when Neuro-2a cells coated with T.cruzi antigen were used as target cells (Figure 5.3).

5.3. Relative eosinophil activity

Different numbers of eosinophils were tested against S2 and Neuro-2a cells coated with T.cruzi antigen in the presence of the mouse anti-T.cruzi serum (Figure 5.4). Eosinophils induced significant ^{51}Cr release at an effector to target ratio of 5:1 and appeared to be reaching a plateau when used at a ratio of 10:1. As in previous experiments, no antibody independent activity was observed, and no cytotoxicity was detected in the absence of T.cruzi antigen.

DISCUSSION

Granulocytes could be involved in the pathogenesis of American trypanosomiasis in two ways. Firstly by being directly cytotoxic to the parasite when this is coated with antibody. Secondly, granulocytes can lyse host cells coated with T.cruzi antigen and antibody.

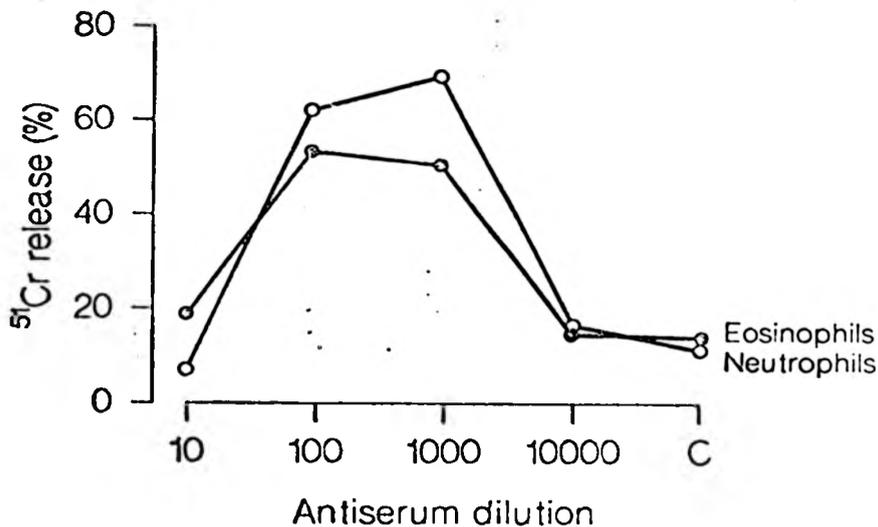


Figure 5.3. Antibody titration of mouse anti-T.cruzi serum with eosinophils (filled symbols) and neutrophils (empty symbols) using N2a cells coated with T.cruzi antigen as target cells. Effector to target ratio of 20:1.

C = control with no antiserum.

Other controls, with the percentage ⁵¹Cr release between brackets, included: N2a cells alone (12.8), N2a + eosinophils (12.5) or neutrophils (10.8), N2a + normal mouse serum at 1:100 + eosinophils (11.5) or neutrophils (12.0), and N2a + anti-T.cruzi serum at 1:100 + eosinophils (15.7) or neutrophils (14.2).

Values obtained with antibody at 1:100 and 1:1000 are significantly different from control at the 5% level or greater.

Values obtained with antibody at 1:10 and 1:10000 are not significantly different from control. Eosinophil values are significantly different from neutrophil values at antibody dilutions of 1:10, 1:100 and 1:1000.

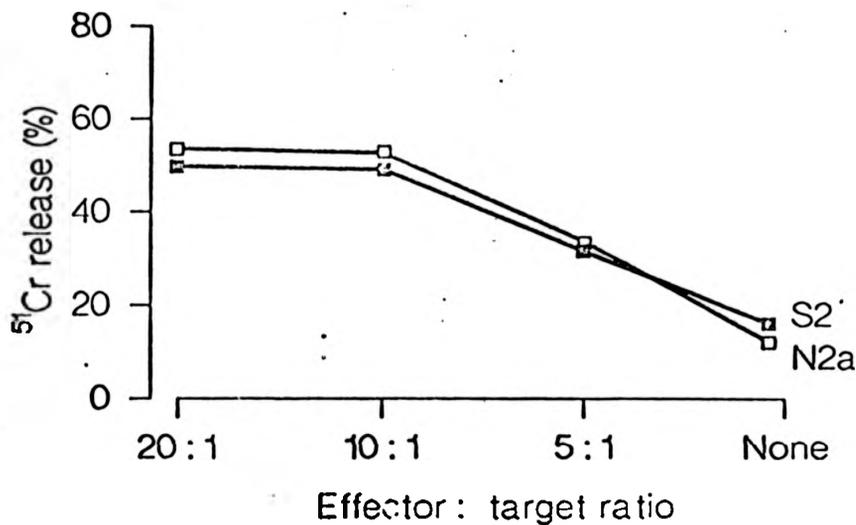


Figure 5.4. Antibody-dependent killing of *T.cruzi* antigen-coated S2 (filled symbols) and N2a (empty symbols) cells by eosinophils at different effector:target ratios. Mouse anti-*T.cruzi* used at 1:100. Controls, with the percentage ^{51}Cr release between brackets, included: S2 (14.5) or N2a (17.5) alone, and S2 (16.5) or N2a (16.0) + *T.cruzi* antigen. Eosinophil activity is significantly different from control at all effector:target ratios at the 5% level or greater. Values obtained at eosinophil:target ratios of 20:1 and 10:1 are not significantly different from each other. Values obtained using S2 as target cells were not significantly different from values obtained using Neuro-2a as target cells.

In this chapter, mouse eosinophils and neutrophils have been shown to be cytotoxic against two syngeneic cell line cells, S2 and Neuro-2a coated with T.cruzi antigen in the presence of anti-T.cruzi antibody. An incubation time of 5 hs showed to be an adequate time to test granulocyte activity. No advantage resulted by using a longer incubation period. Both effector cell types were similarly cytotoxic in the presence of mouse anti-T.cruzi serum. However, when a rabbit anti-T.cruzi serum was used, neutrophils were the more active.

Titration of the mouse anti-T.cruzi serum showed maximum activity at 1:100 and 1:1000 dilutions. Interestingly, no significant activity was detected when the antiserum was used at 1:10. This inhibition of granulocyte activity at high antiserum concentrations has been previously seen using chicken red cells as targets (Sanderson & Thomas, 1978). This effect may be due to an excessive amount of antibody competing for antigenic sites resulting in a low binding of antibody molecules which does not allow a close contact between granulocytes and target cells, or to the subclass composition of the antiserum (i.e. a subclass that does not bind or does it weakly to granulocyte Fc receptors may have an inhibitory effect at high concentrations).

The mechanism by which the epimastigote antigen used in this experiment is adsorbed on the membrane of S2 and Neuro-2a cells has not yet been elucidated. Another antigen preparation obtained from the amastigote form of the parasite has also been shown to adsorb to S2 and Neuro-2a cells and it has been suggested that the antigen binds to the fibronectin network of adherent cells, as non-adherent lymphocytes and red cells fail to bind the antigen (Ribeiro dos Santos & Hudson, 1980a).

The fact that coating of muscle-derived and neurone-like cells with T.cruzi antigen renders them susceptible to the cytotoxic activity of granulocytes in vitro may provide an explanation for the tissue destruction

observed in the acute phase of T.cruzi infections, although, clearly, other effector mechanisms such as T cells, K cells, or complement may also be involved.

It seems possible that the rupture of a pseudocyst with amastigote disintegration (Köberle, 1968), the lysis of parasites by antibody and complement (Krettli, 1978) or the release of degenerated parasites from granulocyte phagosomes (Sanderson & Thomas, 1979) may result in non-specific adsorption of parasite antigen to the host cells. Since in humans antibodies to T.cruzi have been detected early in the infection, (Schmunis, Szarfman, Coarasa & Vainstock, 1978), and in experimental animals all trypomastigotes recovered by day eight after infection have been shown to be coated by antibody (Krettli, 1978), it is possible that antibody and probably also complement may bind to the parasite antigen adsorbed to the surrounding cells. Indeed, T.cruzi antigen, antibody and complement have been detected on the surface of myocardial fibres of mice 10 days after T.cruzi infection (Ribeiro dos Santos & Hudson, 1981). This can lead to granulocyte attachment and lysis of host cells as shown in this chapter. T cells have also been shown to be cytotoxic to T.cruzi antigen-coated host cells (Ribeiro dos Santos & Hudson, 1980b). The destruction of host cells in the acute phase of the disease may then induce the production of auto-reactive T cells and autoantibodies.

The demonstration that granulocytes can lyse T.cruzi antigen and antibody-coated syngeneic nucleated cells raises the possibility that granulocytes may play an effector role in American trypanosomiasis by lysing parasitized cells. These appear to express an antigen (parasite or modified-self) that is recognised by T cells from infected animals (Kuhn & Murnane, 1977). The possibility remains to be investigated whether

antibody binding to this antigen, enables granulocytes to lyse T.cruzi infected cells in an analogous situation to that described using granulocytes and antibody-coated virus-infected cells (Siebens, Tavethia and Babior, 1979).

In contrast to the numerous observations of granulocyte infiltration in the tissues, there are few reports concerning their level in the peripheral blood. Thus, Torres (1941) quotes E. Dias in 1912 as noting an increase in the relative number of eosinophils and neutrophils as the patients enter the chronic phase; this eosinophilia shows a variable number of immature eosinophils and is accompanied by a discrete basophilia. Mazza (1938) describes eosinophilia in one detailed case while emphasizing that this is a frequent finding. The relevance of these observations remains to be seen as these cases may have been carrying unrelated parasites which are responsible for the eosinophilia. However, the level of eosinophils and neutrophils in the peripheral blood in T.cruzi infections does not itself indicate whether these cells play an effector role. The main activity of granulocytes probably takes place in the tissues where the parasite divides, a cellular response takes place and where the degenerative lesions of the host cells is observed. Thus, granulocytes may play a role in the pathogenesis of the disease in the absence of gross eosinophilia or neutrophilia.

On current evidence, granulocytes may be important in the pathogenesis of the disease as a result of their ability to kill the parasite and to lyse syngeneic cells coated with T.cruzi antigen and antibody in vitro. However, it is not until eosinophils and neutrophils can be selectively depleted in vivo that their role in the pathogenesis of American trypanosomiasis will be unravelled.

SUMMARY

Eosinophils and neutrophils are shown to be cytotoxic against two syngeneic murine cell line cells when these are coated with T.cruzi antigen and anti-T.cruzi antibody. Activity is detected after 5 hs of incubation. Highest levels of cytotoxicity are obtained at antibody dilutions of 1:100 and 1:1000, while antiserum at 1:10 is shown to be inhibitory. Eosinophils show significant activity at an effector to target ratio of 5:1. No cytotoxicity occurs in the absence of either antigen, antibody or effector cells. This phenomenon may be a model for the tissue destruction in acute T.cruzi infection, where the lysis of trypanosomes may lead to antigen coating of host cells, followed by antibody-dependent granulocyte-mediated cytotoxicity of the host cells.

CHAPTER 6

ANTIBODY-DEPENDENT CELL MEDIATED CYTOTOXICITY OF NUCLEATED
MAMMALIAN CELLS BY RAT EOSINOPHILS AND NEUTROPHILS

INTRODUCTION

Eosinophils are known to share several morphological and functional characteristics with neutrophils. Although more is known about neutrophils, mainly because of their ready availability, some comparative studies of their activities have been carried out. For example, eosinophils have been reported to be less microbicidal and phagocytic than neutrophils (Baehner & Johnston, 1971; Mickenberg, Root & Wolf, 1972); they apparently have similar cytotoxic activity against Trypanosoma cruzi (López et al., 1978) and their relative activity against Schistosoma mansoni is still controversial (Vadas et al., 1979). Recent reports indicate that neutrophils can be active against antibody-coated tumour cells (Gale & Zigelboim, 1975; Clark & Klebanoff, 1977). On the other hand, although the major basic protein (Butterworth, Wassom, Gleich, Loegering & David, 1979) and the peroxidase system (Jong & Klebanoff, 1980) of the eosinophil have been shown to be toxic to tumour cells in vitro, in the few studies of antibody-dependent eosinophil cytotoxicity carried out, only low activity was observed against antibody-coated tumour cells (Parrillo & Fauci, 1978).

In this chapter, rat eosinophils, neutrophils and K cells have been compared for their ability to kill mammalian cells in a homologous antibody-dependent system. The implications of the type and concentration of the antiserum used to mediate the reaction have also been examined.

RESULTS

6.1. Cytotoxicity of target cells

A range of different target cells were tested for susceptibility to eosinophil, neutrophil and K cell cytotoxicity in the presence of antiserum A. Table 6.1 summarises this data giving the level of cytotoxicity at the optimum antiserum dilution in each case. It can be seen that whereas all cells tested were susceptible to K cell killing, there were considerable differences in susceptibility to granulocyte killing. In general, tumour cells of lymphoid origin were more susceptible to granulocyte activity. Lymphoma cells (BW) were the most susceptible of the mouse cell lines tested. Human lymphoma cells (Daudi) were also highly susceptible when coated with anti-human antibodies (data shown in Table 6.3). Macrophages were not significantly killed by either eosinophils or neutrophils, and P815 cells which were used in the immunization scheme showed only low levels of cytotoxicity by granulocytes. However, K cells were very active against both macrophages and P815 target cells.

6.2. Antibody requirement

Since BW cells were susceptible to granulocyte and K cell activity, they were used in experiments designed to compare the cytotoxic activity of each effector in the presence of three different antisera.

A titration of antiserum A (Fig.6.1) showed that while granulocytes were more active against BW cells at a high antiserum concentration, K cells induced maximum ^{51}Cr release at an antiserum dilution of 1:1000 and were still active at a dilution of 1:10,000. However, the effector cell activity was not only dependent on the amount of antibody present but also on the type of antiserum used. When all three effector cells

Table 6.1. Summary of antibody dependent cytotoxicity of different mouse cell types

Targets	Eosinophils (ratio 20:1)	Effectors Neutrophils (ratio 20:1)	K cells (Ratio 40:1)
BW	48.8*	72.1	30.7
EL4	8.9	19.9	NT+
NS-1	31.2	52.7	NT
X63-Ag8	NT	41.6	NT
P815	3.6 (ratio 5:1)	10.0	48.1
Neuro-2a	NT	0	37.4
BALB/c macrophages	0	0	53.7
BALB/c eosinophils	26.9	48.5	37.2
BALB/c neutrophils	0	33.2	22.2

* Percentage specific ^{51}Cr release

Values shown as zero were not significantly different from controls at the 5% level. NT = not tested. Ratio expressed as effector:target cells.

+ Not tested but known from other experiments to be susceptible.

The data for each target cell type comes from a single representative experiment.

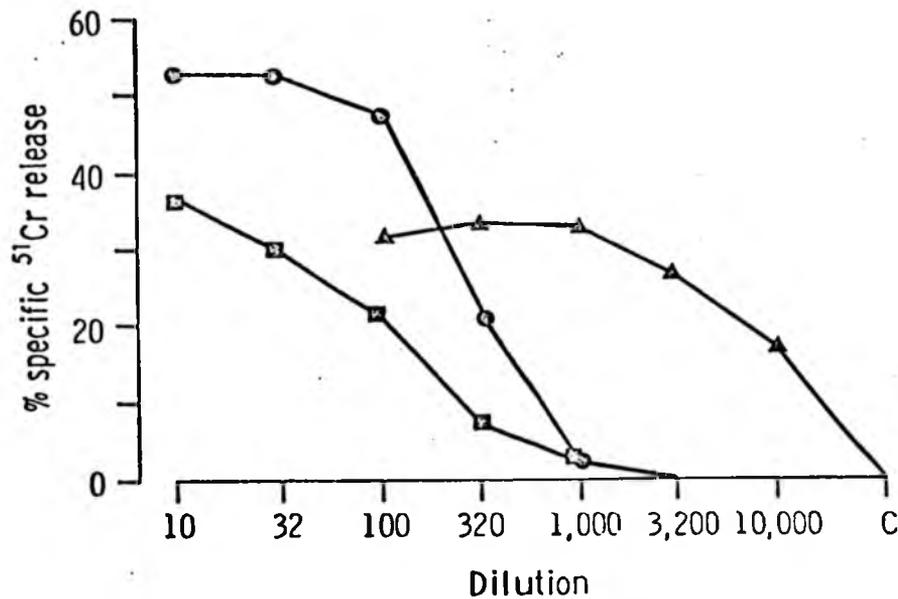


Figure 6.1. Titration of antiserum A with eosinophils, ratio 20:1 (■); neutrophils, ratio 20:1 (●) and K cells, ratio 50:1 (▲). All values are significantly different from control at the 5% level or greater. The eosinophil preparation consisted of 88% eosinophils, 5% mast cells, 5% macrophages and 2% lymphocytes. The neutrophil preparation contained 97% neutrophils, 2% eosinophils and 1% macrophages. The K cell preparation contained 100% lymphocytes.

were tested against BW cells in the presence of three different antisera (Table 6.2), each effector was shown to react better with a particular antiserum, which in turn was different for eosinophils, neutrophils and K cells.

6.3. Characteristics of the reaction

Eosinophils and neutrophils showed similar levels of activity at different effector : target ratios (Fig.6.2), with significant activity detectable at a ratio of 2.5:1. Both types of granulocyte were also found to behave very similarly in kinetic studies (Fig.6.3). Both cells induced a rapid ^{51}Cr release, showing significant activity after only 15' of incubation.

The possibility that granulocytes interacting specifically with antibody-coated target cells might lead to non-specific killing of non-antibody-coated bystanders was investigated. It was found (Table 6.3) that while both BW and Daudi cells were susceptible to eosinophil and neutrophil cytotoxicity when coated with their specific antiserum, no cytotoxicity was observed when the same cells were used as bystanders, indicating the need for immunological contact.

6.4. Inhibition by macrophages

The fact that neutrophils and macrophages were the main cells recovered from the peritoneal cavity of AGUS rats injected with dextran, facilitated studies on the effect of varying numbers of macrophages on the cytotoxic activity of the effector cell preparation. By testing the effector cells obtained after each step of the purification procedure against BW cells (Table 6.4), it was found that a decrease in the number of macrophages was accompanied by an increase in the ^{51}Cr release.

Table 6.2. Cytotoxicity of BW cells by eosinophils, neutrophils and K cells in the presence of different antisera

	Antiserum C	Antiserum A	Antiserum B
Eosinophils (ratio 10:1)	<u>12.9*</u>	<u>10.5</u>	22.8
Neutrophils (ratio 20:1)	4.6	47.7	5.5
K cells (ratio 40:1)	40.5	<u>30.7</u>	<u>30.4</u>

* Percentage specific ^{51}Cr release

All values shown are significantly different from controls (no antibody) at the 5% level or greater

Ratio expressed as effector:target cells

Values connected by bars are not significantly different at the 5% level.

Antisera used at a 1:100 dilution. The eosinophil preparation contained 95% eosinophils, 3% mast cells and 2% macrophages. The neutrophil preparation contained 98% neutrophils and 2% macrophages. The K cell preparation contained 100% lymphoid cells.

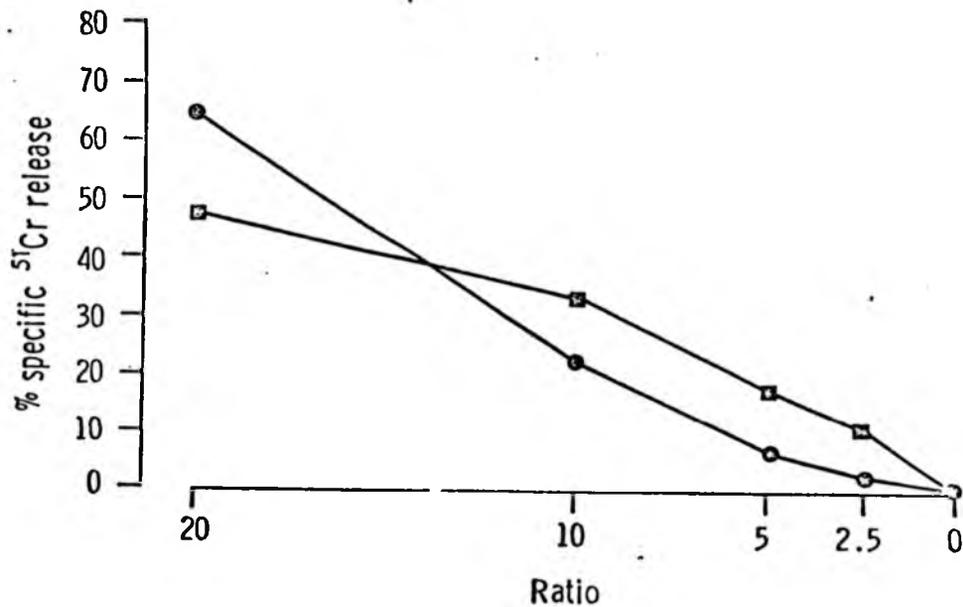


Figure 6.2. Cytotoxicity of BW cells at different effector:target ratio by eosinophils (■) and neutrophils (●) in the presence of antiserum A (dilution 1:50). All values are significant at the 5% level. The eosinophil preparation consisted of 87% eosinophils, 6% mast cells, 4% macrophages and 3% lymphocytes. The neutrophil preparation consisted of 95% neutrophils, 3% macrophages and 2% eosinophils.

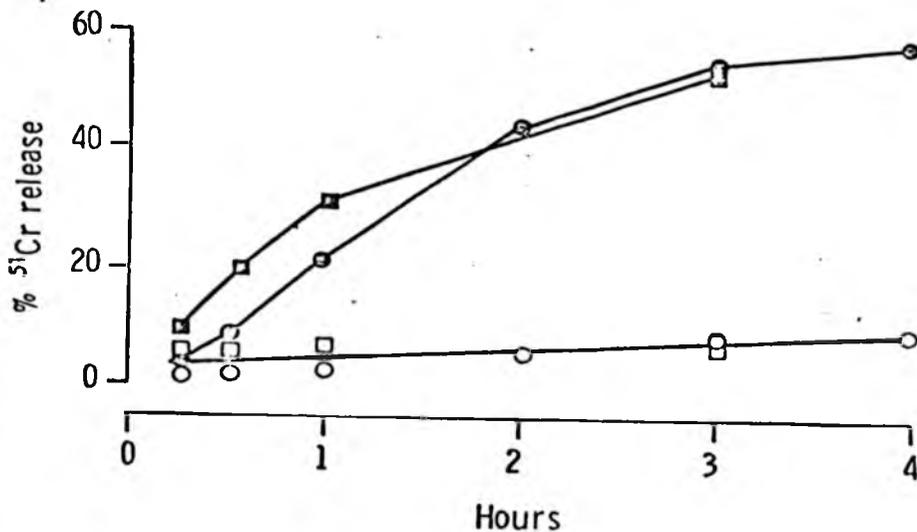


Figure 6.3. Kinetics of release of ⁵¹Cr from BW cells by eosinophils (■, □) and neutrophils (●, ○) at an effector to target ratio of 20:1. Empty symbols represent spontaneous ⁵¹Cr release. Filled symbols represent release in the presence of effectors and antibody. All eosinophil and neutrophil values are significantly different from their respective control values at the 5% level or greater. The eosinophil preparation consisted of 95% eosinophils, 3% mast cells, 1% macrophages and 1% lymphocytes. The neutrophil preparation consisted of 95% neutrophils, 3% Macrophages and 2% eosinophils.

Table 6.3. Cytotoxicity of antibody-coated tumour cells by eosinophils and neutrophils does not result in cytotoxicity of bystander cells

Antiserum	⁵¹ Cr-labelled targets	Unlabelled targets	Eosinophils	Neutrophils
anti-mouse(A)	BW	-	21.5*	72.1
anti-mouse(A)	Daudi	BW	0.3 (n.s)	1.36 (n.s)
anti-human	Daudi	-	68.1	82.6
anti-human	BW	Daudi	1.5 (n.s)	NT

* Percentage specific ⁵¹Cr release. The experiments with eosinophils and neutrophils were performed on different days. Control values in the absence of antibody (eosinophils and neutrophils respectively) were 9.4 and 13.4 for BW and 5.7 and 8.1 for Daudi. Antiserum A was used at a 1:50 dilution. The anti-human serum was used at 1:100. Ratio effector:target cells of 20:1. NT = not tested; n.s = not significant at the 5% level. The eosinophil preparation consisted of 92% eosinophils, 4% mast cells, 2% macrophages and 2% lymphocytes. The neutrophil preparation consisted of 97% neutrophils, 2% eosinophils and 1% macrophages.

Table 6.4. Antibody dependent cytotoxicity of BW cells by dextran-induced peritoneal cells at different stages of purification

Purification stage	Differential counts				% Specific ⁵¹ Cr release
	macroph.	lymph.	neutr.	oesin.	
Untreated	23	1	74	1	15.9
After Ficoll-Paque	6	2	89	3	31.5
After Metrizamide	2	0	97	1	42.3

Antiserum A, dilution 1:100 was used. All values are significantly different from each other, and from control values at the 5% level or greater. Effector to target ratio of 20:1.

This effect cannot simply be explained by a higher neutrophil:BW ratio, and rather suggests that macrophages are inhibiting neutrophil activity.

DISCUSSION

Eosinophils and neutrophils are shown to be active at low ratios in a homologous antibody-dependent system, against a range of mammalian cells, with cells of lymphoid origin being the more susceptible. P815 cells, although highly susceptible to K cell activity, and widely used as a target cell for T cell killing, appear to be relatively resistant to granulocytes. Similarly, Neuro-2a cells and mouse macrophages which bear the same H-2 antigens as P815 cells show low susceptibility to rat granulocytes, although high levels of ^{51}Cr release were induced by K cells. This difference could be due either to the membrane resistance of these targets to the action of granulocytes or to a failure of the antisera used to provide adequate contact between granulocytes and the target cells.

Similar activity has been reported for human neutrophils (Clark & Klebanoff, 1977). However, studies on eosinophils from patients with the hypereosinophilic syndrome (Parrillo & Fauci, 1978) had shown low levels of cytotoxicity even at a ratio of 100:1. It is not clear whether this low activity is due to the altered state of these eosinophils (Spry, 1978) or to the targets or the rabbit antiserum used. In general, experiments that use heterologous antisera to the effector cells are difficult to interpret since these appear to be less effective than homologous antisera (Scornik, Cosenza, Lee, Köhler & Rowley, 1974), possibly because of Fc differences between the species (Leslie & Nicmetz,

1979). The eosinophil in particular has shown large differences in its adherence to red cells coated with antibodies of different species (Butterworth, Coombs, Gurner & Wilson, 1976).

The type of antiserum used and its concentration is shown to play a central role in granulocyte-mediated cytotoxicity. Titration experiments (Fig.6.1) show that eosinophils and neutrophils need a large amount of antibody while K cells show maximum activity at high antisera dilution. In this context, the negative effect of neutrophils on a human lymphoblastoid cell line (Trinchieri, Bauman, de Marchi & Tökes, 1975) might have been due to the fact that an alloantiserum was used for the sensitization of the target cells, particularly since lymphoid cells used in these experiments and elsewhere (Gale & Zigelboim, 1975; Clark & Klebanoff, 1977) appear to be very susceptible to granulocyte activity.

The fact that each antiserum can react preferentially with one effector cell type constituted a surprising finding, since P815 cells were used for the immunization of the three rats and may reflect individual differences in the IgG subclass composition of each antiserum. It has been noted that the activity of effector cells involved in antibody-dependent cell-mediated cytotoxicity can vary according to the IgG subclass present in the system (Holm, Engwall, Hammarström & Natvig, 1974; Urbaniak & Ayoub Greiss, 1980), and inhibition studies with heat-aggregated IgG subclasses (Greenberg, Shen & Roitt, 1973; MacLennan, Howard, Gotch & Quie, 1973; Holm *et al.*, 1974) have further strengthened this view. Furthermore, Fc receptor differences also exist between eosinophils and neutrophils (see Chapter 4). These differences in antibody requirement would explain previous observations showing susceptibility of tumour cells to K cell activity, but not to granulocyte

activity (Sanderson, Clark & Taylor, 1975), although the low susceptibility of P815 cells to granulocytes might have also contributed to produce this effect.

Neutrophil phagocytosis of antigen-antibody complexes or their interaction with antigen-antibody complexes attached to the surface of non-phagocytosable particles have been shown to result in the secretion of granule contents to the outside of the cell (Henson, 1971). Similarly, eosinophils have shown intense degranulation into the extracellular space when exposed to antigen-antibody precipitates too large to ingest (Archer & Hirsch, 1963). The further findings that neutrophils (Clark & Klebanoff, 1975) and eosinophils (Jong & Klebanoff, 1980) could non-specifically kill mammalian cells upon stimulation with opsonized zymosan in the presence of halides, raised the possibility that intimate contact between granulocytes and tumour cells might not be essential. On the other hand, close contact was suggested by experiments in which hepatoma cells uncoated with antibody were not killed by neutrophils reacting with antibody-coated hepatoma cells (Hopkins & Dale, 1980). In the bystander experiment shown in Table 6.3, two different targets, BW and Daudi cells which were highly susceptible to granulocyte activity were used. These data show that eosinophil and neutrophil killing is specific for the antibody-coated target cells, and suggest that their granule contents may need to be concentrated at the site of contact with the target cells to prove effective.

The finding that macrophages can prevent the action of neutrophils on cell line cells extends previous studies where macrophages were found to inhibit granulocyte activity on chicken erythrocytes (Sanderson & Thomas, 1978). It seems possible that stimulated macrophages by virtue of the high number or avidity of their Fc receptors (Rhodes, 1975) may be cytophilically absorbing antibody molecules that neutrophils need in

a large number. Indeed, a similar effect has been previously described by Hersey & MacLennan (1973) who found that macrophages protected tumour cells from K cell killing.

The observation that individual antisera can preferentially react with a given effector cell in vitro, taken together with reports that a particular antigen like Rh can give rise to antisera consisting of IgG₁, IgG₃ (Holm et al., 1974), or IgG₄ (Frame, Mollison & Terry, 1970) subclasses in different individuals, suggest that the effector cell type active in vivo may vary in different individuals according to the subclass produced, even in a similar disease situation.

The fact that eosinophils and neutrophils can be cytotoxic against mammalian cells in vitro suggests a possible involvement in tumour rejection or autoimmune phenomenon in vivo. Eosinophilia and neutrophilia have been detected in patients with a variety of malignant diseases (Beeson & Bass, 1977), and since eosinophils have been found infiltrating lesions in Hodgkin's disease (Lukes, Butler & Hicks, 1966) and tumour produced eosinophilotactic factors have been described (Wasserman, Goetzl, Ellman & Austen, 1974) the possibility that granulocytes could play an effector role in tumour immunity deserves further investigation.

SUMMARY

Rat eosinophils, neutrophils and K cells have been compared for their ability to kill antibody-coated mammalian cells. Eosinophils are shown to have similar cytotoxic activity to neutrophils. Both cells are active at a low effector to target cell ratio and induce a rapid ⁵¹Cr release. Eosinophil and neutrophil cytotoxic activity differ from K cell activity in that granulocytes need a higher antiserum concentration. Furthermore, when different homologous antisera were compared each effector

cell reacted preferentially with a different antiserum. Cytotoxicity by eosinophils and neutrophils is shown to depend on specific contact with the target cells as susceptible bystander cells are not killed. Neutrophils appear to be inhibited by macrophages present in the effector cell population since higher levels of ^{51}Cr releases are obtained following their depletion by the purification techniques employed.

CHAPTER 7

PRODUCTION OF ANTIBODY PREPARATIONS SPECIFIC FOR
MOUSE EOSINOPHILS AND NEUTROPHILS

INTRODUCTION

Most of our knowledge on the biochemistry and function of eosinophils and neutrophils is derived from in vitro studies. Thus, eosinophils and neutrophils have been shown to kill bacteria, tumour cells and protozoans and metazoan parasites (see Chapter 6). However, very little is known about the role of these cells in vivo. Furthermore, the in vitro studies often show some conflicting results. For example, while some authors have described damage of S.mansoni by eosinophils (Butterworth et al., 1975; Ramalho Pinto, McLaren & Smithers, 1978) others have found eosinophils to be inefficient in this system (Dean, Wistar & Murrel, 1974). Similarly, while some workers have found both eosinophils and neutrophils to induce damage of S.mansoni (Anwar et al., 1979; Incani & McLaren, in press), others have found eosinophils but not neutrophils to be the active cells (Vadas et al., 1979).

Another problem is that there appears to be a lack of correlation between the in vitro data and the in vivo findings. Thus, eosinophils have been seen to be the most prominent cell in the inflammatory response in the skin following challenge of S.mansoni-infected mice, but they have been rarely observed in contact with the parasite (von Lichtenberg, Sher, Gibbons & Doughty, 1976; Savage & Colley, 1980). In addition, mice sensitized with a cercarial preparation and mounting a marked eosinophil response, were not protected from cercarial challenge (Colley, Savage & Lewis, 1977).

Whatever the in vitro activity of eosinophils against infective agents, the role of these cells in the pathogenesis of a disease can only be determined indirectly by means of selectively depleting them with a specific antiserum.

Since the observations by Metchnikoff (1899) that anti-leucocyte serum can be prepared by heteroimmunizations, attempts have been made to

produce antisera specific for different cell types. An antilymphocyte serum has been produced and shown to be immunosuppressive (reviewed by Lance, Medawar & Taub, 1973). An anti-neutrophil serum has allowed studies on the kinetics of bone marrow release of neutrophils (Lawrence, Caddock & Campbell, 1966). More recently, rabbit anti-eosinophil sera have been raised and shown to deplete eosinophils when injected in the appropriate species of animals (Mahmoud, Warren & Boros, 1973; Gleich, Loegering & Olson, 1975; Jones & Kay, 1976).

The use of the anti-mouse eosinophil serum in S. mansoni (Mahmoud, Warren & Peters, 1975) and T. spiralis (Grove, Mahmoud & Warren, 1977)-infected mice suggests that the eosinophil can play a role in vivo against these parasites. Similarly, experiments using anti-guinea pig eosinophil sera (Jones & Kay, 1976; Gleich, Olson & Loegering, 1979) indicate that eosinophils may be important in regulating immediate-type hypersensitivity reactions.

The use of heterogeneous antisera suffers, however, from some disadvantages. Firstly, they are non-specific reagents and must be extensively absorbed to remove cross-reacting antibodies. Secondly, even after absorption, specificity is difficult to achieve and the antiscrum titre may be markedly reduced. Thirdly, as individual animals produce antisera of different specificity, there is a limited supply which restricts its general use.

The development of a cell fusion technique between myeloma cells and antibody-producing cells with the production of cell lines (hybridomas) secreting antibodies to the immunizing antigen (Köhler & Milstein, 1975), has provided a new method for obtaining homogeneous (monoclonal) antibody preparations of exquisite specificity and virtually unlimited supply. By appropriate cloning and screening techniques, hybrids secreting the antibody of the desired specificity can be derived. With this technique,

monoclonal antibodies to a wide variety of antigens have already been produced, for example monoclonal antibodies for the major histocompatibility complex of the mouse (Lemke et al., 1978), for the Fc receptor of mouse macrophages and lymphocytes (Unkeless, 1979), and to malaria parasite (Yoshida, Nussenzweig, Potocnjak, Nussenzweig & Aikawa, 1980). Furthermore, the monoclonal antibody to malaria parasite has been shown to be effective in vivo conferring protection to mice (Potocnjak, Yoshida, Nussenzweig & Nussenzweig, 1980).

Once a hybridoma-secreting antibody of the desired specificity has been derived, it can be made to grow in culture as an immortal cell line or it can be injected into suitable animals where it will grow as a tumour. This second possibility offers the advantage of obtaining monoclonal antibodies from the serum and ascites of the tumour-bearing animals at a concentration 1,000 times higher than that obtained from tissue culture supernatants. However, tumour growth depends on both the antibody-producing cell and the myeloma parent used in the fusion, belonging to the same species.

Until the availability of a rat myeloma, antibodies to mouse antigens (apart from alloantigens) were produced by fusing rat spleen cells to a mouse myeloma. These would not grow as tumours and so antibody could only be obtained as culture supernatants.

Another problem derived from fusing rat spleen cells immunized with mouse eosinophils to mouse myeloma cells, is the presence in the rat spleen of cytotoxic T cells to mouse cells and K cells which could reduce the efficiency of the fusion.

In this chapter, attempts to produce conventional antibody preparations specific for mouse eosinophils and neutrophils are described. These were unsuccessful, but were not pursued because experiments that used the

hybridoma technique were more promising. The production and testing of the monoclonal antibodies are described.

Due to the unavailability of the rat myeloma Y3 at the time, most of the monoclonal antibodies to eosinophils described were produced by fusing rat spleen cells to the NS1 myeloma. This originated rat-mouse hybrids which could not be grown in vivo, limiting therefore the amount of antibody for experimental use.

To overcome the problem of having cytotoxic T cells and K cells in the rat spleen, two fusions used rat spleens that had been immunized against eosinophils carrying a different H-2 to the myeloma parent. Furthermore, in one fusion spleen cells were first filtered through a nylon wool column with the aim of depleting T cells and K cells, and enriching for B lymphocytes. In this context, it should be noted that it has not yet been clearly established which cell (B cells or blast cells) participates in the fusing event. What has been shown in fusions that used spleen cells immunized with soluble antigens, is that the specific efficiency (i.e. = number of antigen specific hybridoma clones/total number of hybridomas) of this type of fusion directly correlates with the number of stimulated large lymphocytes formed in the spleen (Stahli, Staehelin, Miggiano, Schmidt & Haring, 1980).

RESULTS

7.1. Rabbit antisera to mouse eosinophils and neutrophils

Antisera raised to eosinophils and neutrophils (see Section 2.04.2) were cross-tested for specificity by means of three different assays.

7.1.1. I.I.F.

The antisera were tested by I.I.F. for reactivity with eosinophils, neutrophils and P815 cells (Table 7.1). It was found that all of them reacted with the three cell types tested. One of these antisera (AE_{1a}) was absorbed with P815 cells to remove anti-mouse antibodies, however this lowered the fluorescence titre to all the cell types tested, indicating that very little antibody specific for eosinophils was present.

7.1.2. FACS analysis

Because I.I.F. can be a very subjective assay, and because the FACS is more sensitive, further studies were carried out using the FACS. Eosinophils and neutrophils were treated with each antiserum at a 1:100 dilution followed by the goat anti-rat IgG-FITC (see Section 2.20.2). When the cells were analysed with the FACS (Figure 7.1), a clear cross-reactivity of AE_1 and AN with eosinophils and neutrophils could be seen, while in the absorbed serum (AE_{1a}) the reactivity was reduced almost to that of the control.

7.1.3. ^{51}Cr release assay

A ^{51}Cr release assay using guinea pig complement with the rabbit antisera showed activity only against eosinophils (Figure 7.2). Because these antisera were shown to be cross-reactive by IIF and FACS analysis, it was thought this might represent lack of complement lysis rather than antibody specificity. For this reason, different sources of complement were tested (see Section 2.05.3) and rabbit serum absorbed with mouse spleen cells was found to be effective against neutrophils. This was confirmed by experiments in which CBA leucocytes were tested for the presence of H-2 antigen on their membrane (Figure 7.3). Eosinophils, lymphocytes and macrophages were lysed

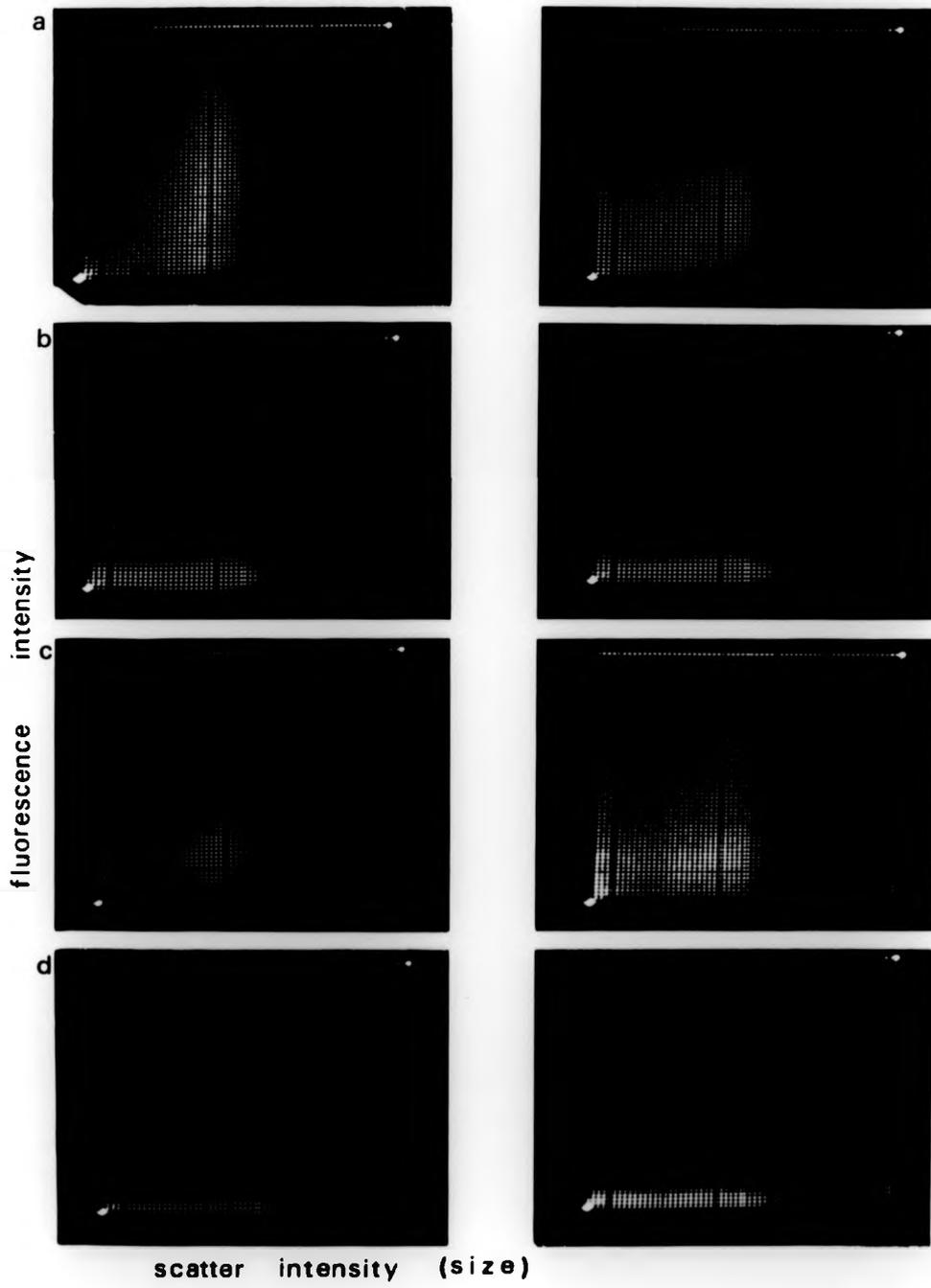
Table 7.1. Indirect immunofluorescence of eosinophils, neutrophils and P815 cells with different rabbit antisera

Anti-serum	Cell type	Antiserum dilution (reciprocal)									
		20	40	80	160	320	640	1280	2560	5120	None
AE ₁	Eosinophils	+++	+++	+++	+++	+++	+++	++	+	+	-
	Neutrophils	+++	+++	+++	+++	++	+	±	-	-	-
	P815	+++	+++	+++	+++	++	+	-	-	-	-
AE _{1a}	E	++	++	+	-	-	-	-	-	-	-
	N	+	±	-	-	-	-	-	-	-	-
	P	+	±	-	-	-	-	-	-	-	-
AE ₂	E	+++	+++	+++	++	++	+	+	-	-	-
	N	+++	+++	++	+	±	-	-	-	-	-
	P	+++	+++	+++	++	+	+	-	-	-	-
AE ₃	E	+++	++	++	+	±	-	-	-	-	-
	N	++	++	++	+	±	-	-	-	-	-
	P	++	+	±	-	-	-	-	-	-	-
AN	E	+++	+++	++	++	+	+	±	-	-	-
	N	+++	+++	+++	+++	++	+	+	-	-	-
	P	+++	++	++	+	+	±	-	-	-	-
Normal rabbit serum	E	-	-	-	-	-	-	-	-	-	-
	N	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-

Figure 7.1. FACS two dimensional maps of eosinophils and neutrophils labelled with AE_1 (a), AE_{1a} (b), AN (c) and normal rabbit sera (d).

EOSINOPHILS

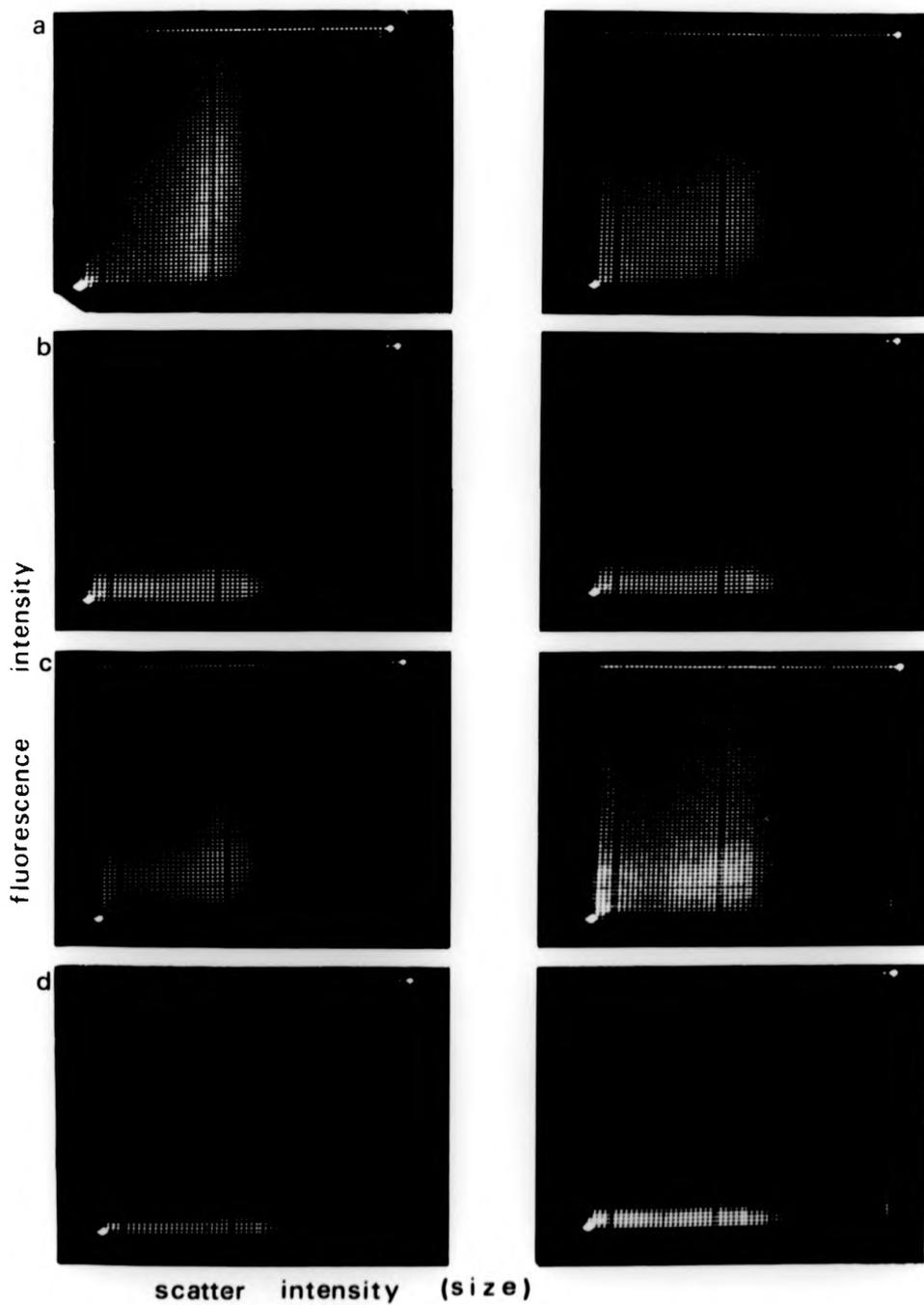
NEUTROPHILS



AN (c)

EOSINOPHILS

NEUTROPHILS



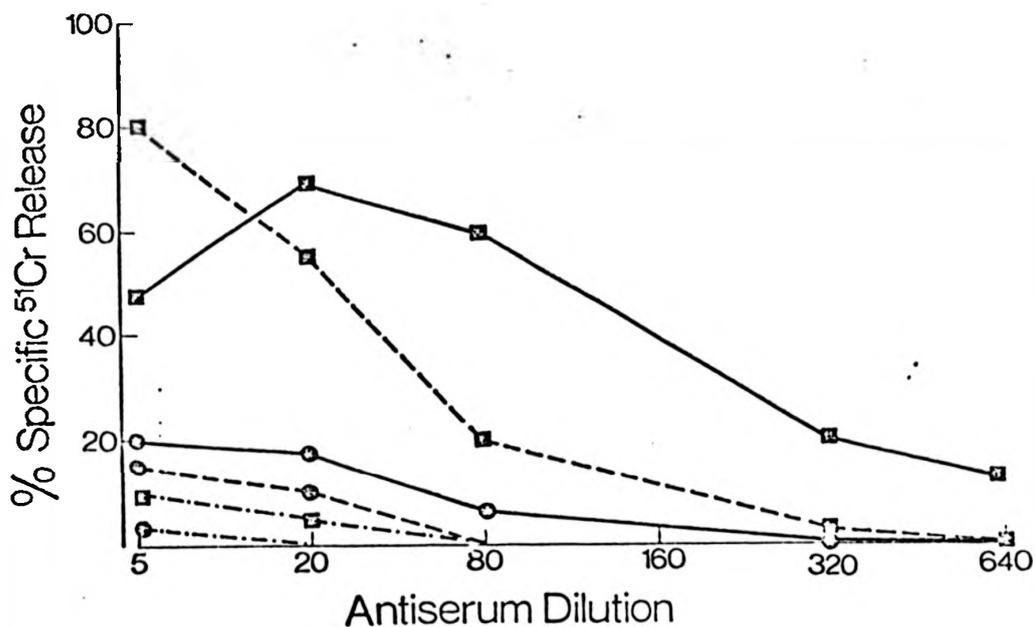


Figure 7.2. Cytotoxicity of eosinophils (■), and neutrophils (●), by AE₁ (—), AE_{1a} (---) and AN (· · ·) antisera.

Eosinophil values with each antiserum are significantly different at the 5% level from eosinophil values obtained with the other antisera.

Neutrophil values are significantly different from eosinophil values at the 5% level when AE₁ and AN were used but not when AE_{1a} was used.

Neutrophil values obtained with AE₁ and AN were significantly different from AE_{1a} values at the 5% level, but were not significantly different between each other.

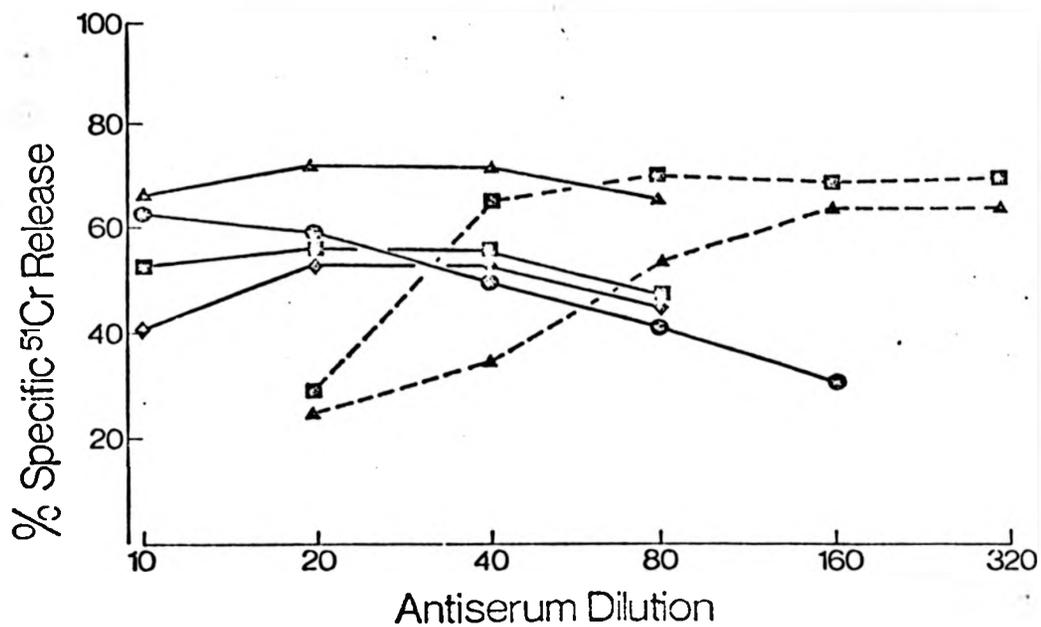


Figure 7.5. Cytotoxicity of eosinophils (■), neutrophils (●), lymphocytes (▲) and macrophages (◆) by H-2^d anti-H-2^k serum (unbroken line) and monoclonal antibody anti-H2^k 27 R9 (broken line)

by H-2^d anti-H-2^k serum and guinea pig complement, while neutrophils were lysed in the presence of rabbit complement. Therefore, in all subsequent experiments with mouse neutrophils as target cells, rabbit serum absorbed with spleen cells was used as the source of complement.

To provide a baseline of comparison to the rabbit anti-eosinophil sera described above, Dr. A. Mahmoud kindly supplied some of his rabbit anti-eosinophil serum. When this was tested for cytotoxicity against mouse leucocytes an extensive cross-reactivity with all the cell types tested could be seen (Figure 7.4).

7.2. Monoclonal antibodies to eosinophils

From six fusion experiments performed, one was lost due to excessive CO₂ in the 37°C incubator, two did not show growth, and three produced the monoclonal antibodies described in this section.

The two fusions in which growth was not observed used rat spleen cells sensitized with BALB/c eosinophils for fusion with NS1 cells. In the second of these fusions, a ⁵¹Cr release assay was set up in which the rat spleen cells were tested for cytotoxic activity against P815 cells (same H-2 as NS1). It was found that the rat spleen contained cells cytotoxic to P815 cells at an effector:target ratio of 100:1 in a 4 hrs cytotoxicity assay. This cytotoxic effect could be important in a long term culture and may be responsible for the lack of growth observed in the two fusions.

In order to avoid the induction of T cells cytotoxic to cells carrying H-2^d antigen, the rat used in the next fusion was immunized with eosinophils obtained from CBA mice (H-2^k). This produced the first successful fusion, in which the supernatants of 8 wells were found to react with CBA eosinophils (Table 7.2).

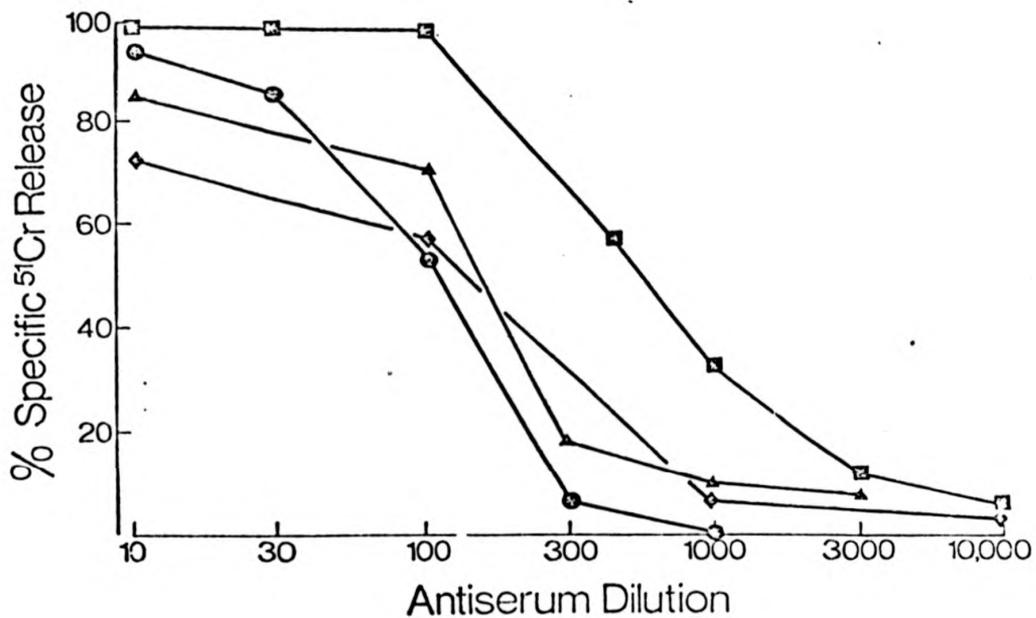


Figure 7.4. Cytotoxicity of eosinophils (■), neutrophils (●), lymphocytes (▲) and macrophages (◆), by rabbit antieosinophil serum obtained from Dr. A. Mahmoud.

Table 7.2.

Summary of the three successful anti-mouse eosinophils fusions performed

Fusion	Immunogen	Myeloma	Spleen cells	No. of spleen cells	No. of wells showing growth	No. of positive wells
1	CBA eosinophils	NS1	Whole spleen	10^8	13/48	8
2	CBA eosinophils	NS1	Non-adherent spleen cells	1.2×10^7	23/54	19
3	BALB/c eosinophils	Y3	Whole spleen	1.2×10^8	56/96	5

The ratio spleen cells: myeloma cells used was 10:1 in all three fusions.

Because it was possible that cytotoxic T cells to mouse cells unrestricted to H-2 were present in the rat spleen, another fusion was carried out with spleen cells that had been filtered through a nylon wool column. In addition to removing T cells, this technique also removes non-adherent K cells. This second successful fusion using only adherent cells produced 19 wells showing antibody activity against CBA eosinophils.

Although hybrids secreting anti-eosinophil antibodies with a high degree of specificity could be obtained in this way (see below) the fact that they were derived from rat-mouse fusions meant that they could not be easily grown in vivo. In fact, an attempt was made to grow one of these hybridomas (M2/43.G4.A4) in 5 BALB/c nu/nu mice by injecting up to 5×10^7 cells/mouse. However, the hybridoma did not grow. Thus, when the rat myeloma Y3 became available, another fusion was set up in which BALB/c eosinophils were used for the immunization and screening for specificity. This third successful fusion produced 5 wells showing antibody activity against BALB/c eosinophils.

In every case, hybridomas showing growth were screened for the production of antibodies to eosinophils by an antibody-binding assay, and tested for cytotoxic activity against a range of mouse cells. The antibodies produced showed different patterns of reactivity with each cell type (summarized in Table 7.3). 5 wells showing some specificity for eosinophils were selected and the hybrids cloned.

Clones were tested by both binding and cytotoxicity assays. An example is shown in Figure 7.5. where it can be seen that most isolated clones showed similar activity, while only one F2, showed no production of anti-eosinophil antibody. Clones with highest activity were grown up and stored frozen in liquid nitrogen.

Table 7.3. Summary of cytotoxic and binding activity of different monoclonal antibodies to mouse eosinophils on eosinophils and other leucocytes

	Cytotoxic titre				¹²⁵ I-anti IgG binding-cps		
	Eos.	Neutr.	Lymph.	Macr.	Eos.	Neut.	Lymph.
M1/1	650*	NT	80	NT	27 [†]	NT	21
M1/2.A4	15	<1	<1	<1	41	13	18
M1/2.A4 concentrated	300	<1	10	<1	NT	NT	NT
M1/9	4	NT	<1	NT	30	NT	18
M1/20.G1	100	50	25	<1	44	42	30
M1/21.A9.H8.G5	100	5	5	<1	47	37	29
M2/26.A2	<1	<1	<1	<1	17	58	34
M2/32	5	NT	2	NT	46	NT	35
M2/42	10	NT	<1	NT	27	NT	16
M2/43.G4.A4.	15	<1	<1	<1	55	9	11
R1/16	1050	20	<1	<1	56	55	31
R1/36	200	NT	<1	<1	20	24	16
R1/71	20	4	<1	<1	30	23	15
R1/84	1000	1200	3	<1	17	12	12

* Reciprocal of the antibody dilutions at which 50% specific ⁵¹Cr was obtained.

† After subtracting the background (supernatant of the myeloma parent), this always being between 8-22 cps.

NT = not tested.

<1 = 50% specific ⁵¹Cr release not reached with undiluted or diluted hybridoma supernatant.

For the designation of the hybridomas, the initial letter corresponding to the species of the spleen parent, is followed by the number of the fusion and the clone and subclone numbers.

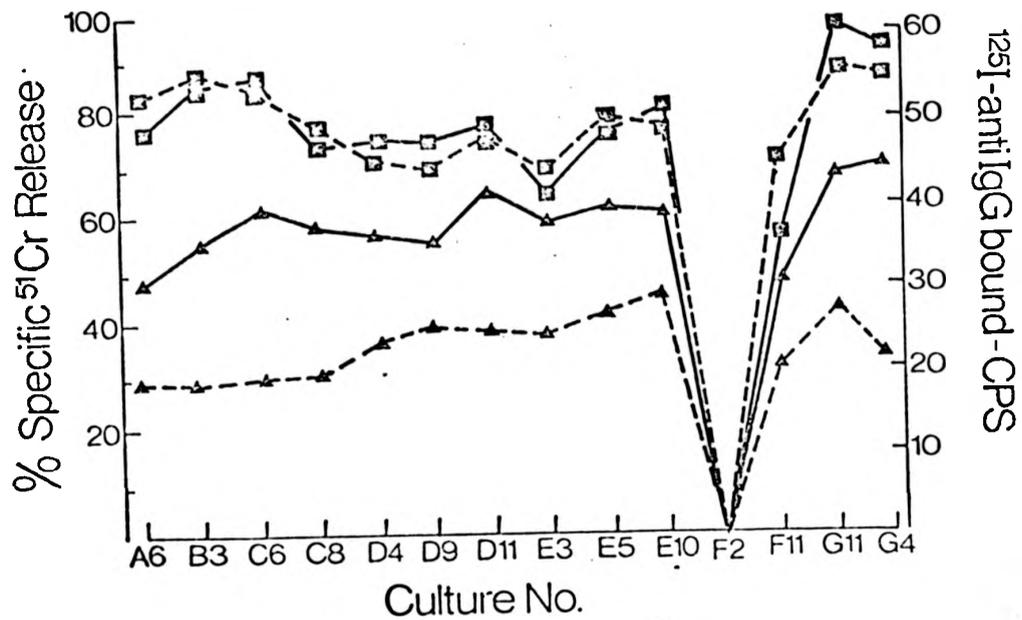


Figure 7.5. Cytotoxic (—) and binding activity (----) of different clones from hybridoma M1/20 on eosinophils (■) and lymphocytes (▲).

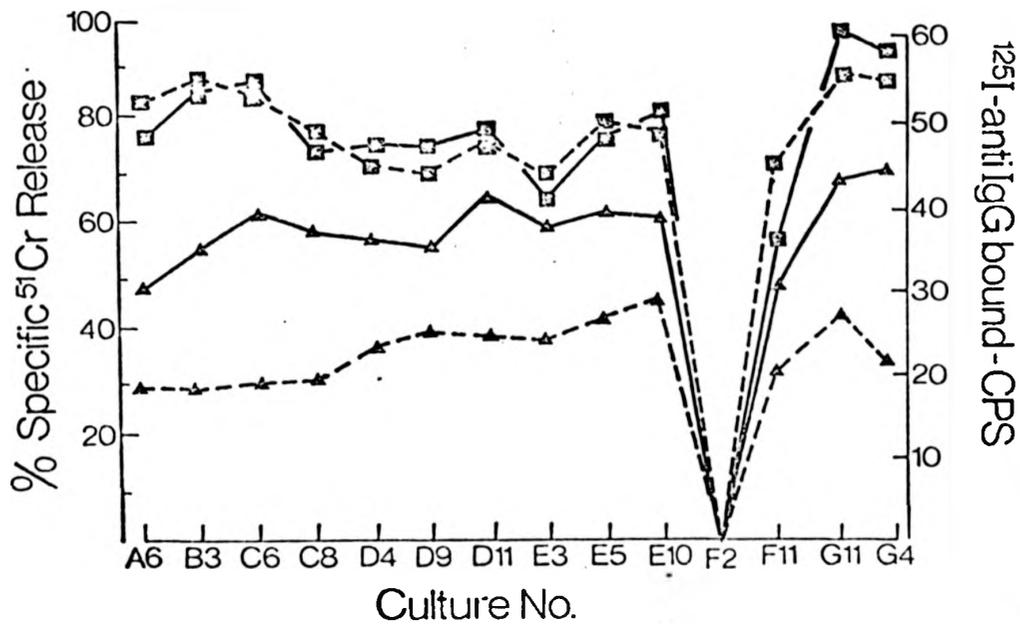


Figure 7.5. Cytotoxic (—) and binding activity (----) of different clones from hybridoma M1/20 on eosinophils (■) and lymphocytes (▲).

Those hybrids showing the highest degree of specificity for eosinophils (M2/43.G4.A4 and M1/2.A4) were grown in bulk cultures. The supernatant pool of each of them was concentrated about 40 times by molecular filtration with a PM30 membrane (Amicon Ltd., High Wycombe, England). These concentrated supernatants were then tested for cytotoxicity against mouse leucocytes. Hybrid M2/43.G4.A4 gave the highest degree of specificity for eosinophils (Figure 7.6). Cross-reactivity was higher with lymphocytes than with neutrophils or macrophages, but this reactivity was approximately 100-fold less than with eosinophils.

All these hybridomas were tested against leucocytes obtained from both CBA and BALB/c mice. Similar results were obtained, indicating that these antibodies were not reacting with alloantigens.

7.3. Effect of monoclonal antibody M2/43.G4.A4 *in vivo*

BALB/c mice infected with M.corti were given injections of concentrated M2/43.G4.A4 antibody to test its ability to ablate eosinophils in vivo. As a control, parent myeloma supernatants concentrated to the same degree were used. At different times after infection with M.corti, all animals or remaining animals were injected i.p. with 1 ml of antibody or NS1 supernatant. Animals were sacrificed 8 hs after each injection and the blood and peritoneal exudate collected. Total numbers of cells and differential counts were determined in each case so that the total number of each cell type could be calculated.

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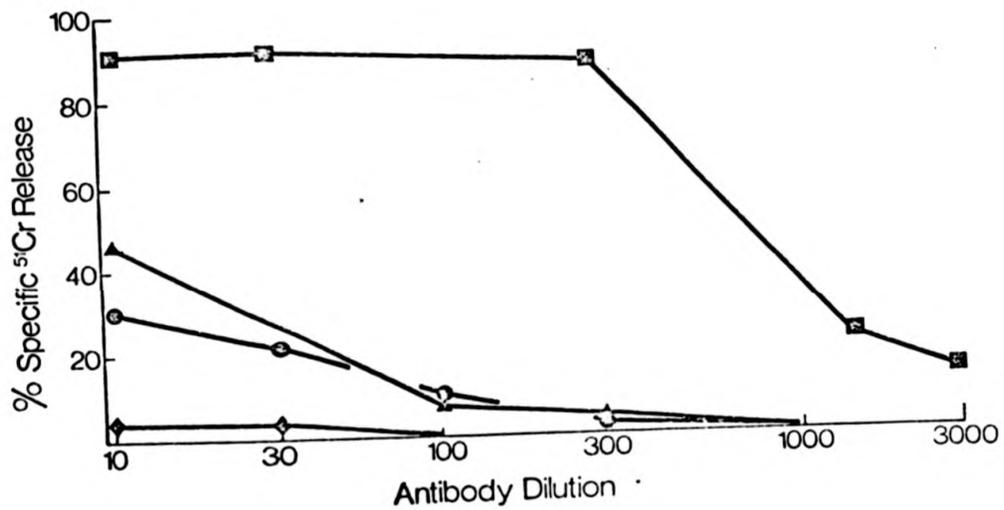


Figure 7.6. Titration of antibody M2/43.G4.A4 (concentrated) on eosinophils (■), neutrophils (●), lymphocytes (▲) and macrophages (◆).

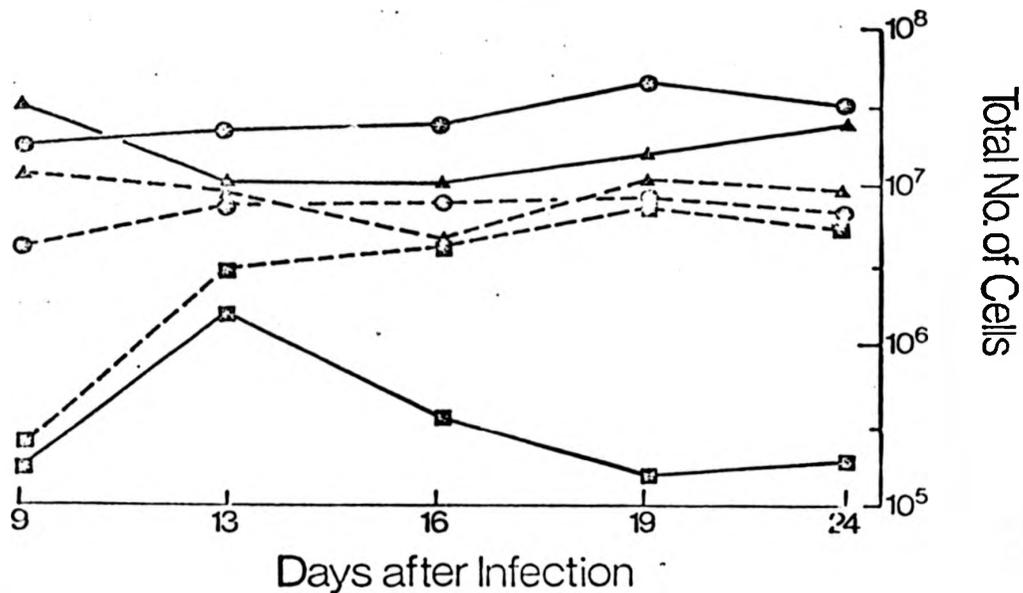


Figure 7.7. Effect of antibody 12/43, G4.A4 (—) compared to control (----) on *M.corti*-induced peritoneal eosinophils (■), neutrophils (●) and mononuclear cells (▲). Each point represents the mean of two animals and is calculated from differential counts and total cell number determined in each case. No detailed statistical analysis was done on these data, however, the difference between eosinophil percentages was well outside the normal counting error (see text).

Differential counts showed eosinophils to constitute 2% or less of the peritoneal exudate at days 16, 19 and 24 after M.corti infection as compared to control exudates in which 20-30% of the cells were eosinophils. There was found to be no decrease in the number of neutrophils and mononuclear cells. Macrophages were seen with cells, presumably eosinophils, in their interior at different stages of degeneration.

Peripheral blood eosinophils appeared to be reduced by the monoclonal antibody (Figure 7.8) but not as completely as the peritoneal eosinophils. Differential counts showed that at days 19 and 24 after M.corti infection 5-8% of leucocytes were eosinophils in antibody-treated animals while between 6-22% were found to be eosinophils in control animals. Again, no decrease in the number of neutrophils and mononuclear cells was evident.

DISCUSSION

Immunization of three rabbits with purified mouse eosinophils failed to produce an antiserum specific for eosinophils. The three anti-eosinophil sera cross-reacted with neutrophils and P815 cells. Absorption of one of these antisera with P815 cells did not improve specificity for eosinophils but resulted in a marked decrease in its reactivity with both eosinophils and neutrophils.

In contrast to the IIF assay and FACS analysis in which a cross-reactivity of these rabbit antisera with eosinophils and neutrophils was observed, a ⁵¹Cr release assay suggested specificity for eosinophils as these were lysed to a greater extent than neutrophils. However, the reason for this was found to be the apparent resistance of mouse neutrophils

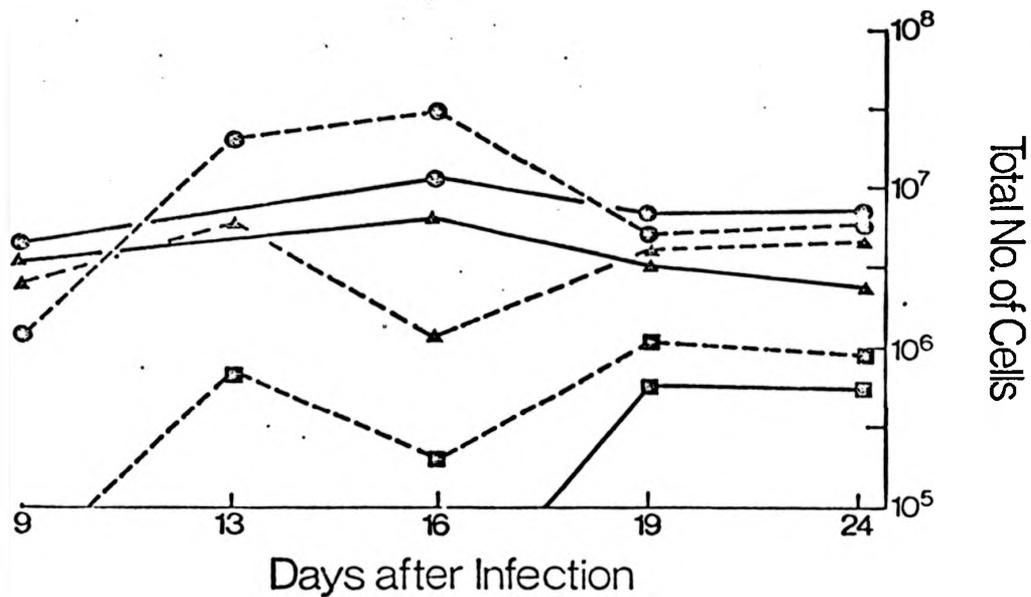


Figure 7.8. Effect of antibody M2/43.G4.A4 (—) compared to control (----) on *M.corti*-induced blood eosinophils (■), neutrophils (●) and mononuclear cells (▲). Each point represents the mean of two animals calculated as in Figure 7.7. The difference between the eosinophil percentages is probably not statistically significant.

to guinea pig complement as seen in experiments in which neutrophils were lysed by a monoclonal antibody and a rat anti-mouse cells serum (see Section 2.05.3) in the presence of rabbit complement but not when guinea pig complement was used.

The inability of guinea pig complement to lyse mouse neutrophils was first examined by treating these cells with 10 mM azide which would have inhibited antigen modulation and allowed lysis by antibody and complement (Gordon & Stevenson, 1981). However, this failed to induce cytotoxicity. Rabbit complement was then tested, and preliminary experiments showed high levels of toxicity (antibody-independent lysis). The possibility that this was the result of activation of the alternative pathway by neutrophils as it has been shown with thymocytes (Kierszenbaum & Budzko, 1977) was ruled out as incubation of complement at 56°C for 20', a technique that reportedly destroys the activity of Factor B (Kierszenbaum & Budzko, 1977) failed to remove toxicity. Absorption with rabbit complement with 5×10^7 mouse spleen cells (see Table 2.1), however, removed toxicity and produced a reagent able to lyse mouse neutrophils in the presence of monoclonal antibodies or heteroantisera.

Although Mahmoud's antiserum showed some specificity for eosinophils, extensive cross-reactivity with neutrophils, lymphocytes and macrophages was observed. The apparent resistance of mouse neutrophils to guinea pig complement may explain the apparent specificity for eosinophils by a rabbit anti-mouse eosinophil serum previously described (Mahmoud *et al.*, 1973).

Attempts to produce rabbit antisera specific for guinea pig eosinophils were unsuccessful (Gleich *et al.*, 1975; Jones & Kay, 1976). These antisera were found to lack specificity as their activity against eosinophils could be removed by absorption with different guinea pig cell types including neutrophils.

One laboratory, however, has apparently succeeded in producing rabbit

antisera specific for mature mouse eosinophils (Mahmoud et al., 1973) and to immature mouse eosinophils (Mahmoud & Warren, 1977). Specificity has also been claimed for two rabbit antisera to human mature eosinophils (Mahmoud, Kellermeyer & Warren, 1974). These observations have also been interpreted as an indication of stage specificity and suggest that eosinophils as they mature gain one antigen and lose another. However, in view of the clear cross-reactivity of one of these rabbit antisera to mouse eosinophils with other leucocytes (Figure 7.4), this interpretation seems unlikely.

The use of rabbit anti-eosinophil serum (Mahmoud et al., 1973) in S.mansoni (Mahmoud, Warren & Peters, 1975) and T. spiralis (Grove, Mahmoud & Warren, 1977)-infected mice, has indicated that eosinophils have a protective role in parasitic infections. Animals injected with anti-eosinophil serum show a higher worm burden and a decrease in their resistance to these parasites when compared to untreated infected animals. It should be noted, however, that the mode of action of this rabbit anti-eosinophil serum is still unknown. Cross-reactivity with lymphocytes may impair the host immune response. Furthermore, this work has not yet been confirmed in other laboratories probably due to difficulties in producing specific antisera.

The production of monoclonal antibodies to mouse eosinophils described in this chapter, represents an attempt to produce a monoclonal reagent of high specificity. One monoclonal antibody (M2/43.A4.G4) has shown remarkable specificity for mouse eosinophils. Its isotype has not been determined, but comparison of these data with work on rat myeloma proteins (Medgyesi, Füst, Gergeley & Bazin, 1978) suggests that this antibody belongs to the IgG2a or IgG2b subclass since it is bound by the goat anti-rat IgG, fixes complement and appears to be bound by macrophages. This antibody reacts with an antigen on the surface of the eosinophil which is present in only

small amounts on other cell types. Furthermore, antibodies to this antigen selectively ablate eosinophils in vivo as can be seen by the large decrease of eosinophil numbers in the peritoneal cavity of BALB/c mice. It should be noted that these in vivo results have been produced using only one dose regime. It may be that M.corti-infected mice need a higher antibody dose or more frequent injections to produce a total ablation of eosinophils. Clearly, therefore, although this antibody is shown to specifically deplete eosinophils in vivo, the best experimental conditions remain to be established.

The mechanism of eosinophil ablation is not known. Complement may have a direct lytic effect in vivo. Another possibility is that macrophages may phagocytose antibody or antibody and complement-coated eosinophils by means of their Fc and complement receptors. Injection of heterologous anti-neutrophil serum in guinea pigs (Simpson & Ross, 1971) has been shown to result in phagocytosis and digestion of neutrophils by liver and spleen macrophages. No ultrastructural evidence of neutrophil lysis in the blood was obtained suggesting that the antiserum was not cytotoxic in vivo but that it was effective in promoting opsonization and uptake of neutrophils by macrophages.

Although this antibody is of limited practical value as it can only be grown in tissue culture, these results indicate that a monoclonal antibody can be produced with specificity for eosinophils and in vivo activity. A monoclonal antibody of this type produced by a rat-rat fusion will be a real advance as it will constitute a standard reagent for worldwide use with which to establish the role of the eosinophil in vivo.

SUMMARY

Conventional antisera raised against mouse eosinophils are shown to lack specificity as they cross-react with other cell types. The rabbit antiserum to mouse eosinophils obtained from Dr. A. Mahmoud shows very little specificity for eosinophils and to extensively cross-react with other leucocytes. With a hybridoma technique for the production of monoclonal antibodies, hybrids secreting antibodies of higher specificity are obtained. One of these monoclonal antibodies shows a 100-fold difference between its reactivity with eosinophils and other leucocytes. This antibody selectively ablates eosinophils from the peritoneal cavity of M.corti-infected mice. The hybridoma technique appears, therefore, to be the method of choice for producing a highly specific antibody preparation for mouse eosinophils.

CHAPTER 8

GENERAL DISCUSSION

Rodent eosinophil and neutrophil membrane receptors and effector functions have been studied in vitro, and efforts have been directed towards an examination of the role of these cells in vivo by developing antibody preparations that could identify specific antigens on their surface.

Mouse eosinophils, induced to the peritoneal cavity of mice by Mesocestoides corti were purified using a combination of velocity sedimentation at unit gravity and isopycnic centrifugation (Chapter 3).

Since the mouse eosinophils were induced, it is possible that a proportion of these cells were in an "activated" state. Although the meaning of activation in this case has not been well defined, it has been used to indicate that the granulocyte has undergone a series of changes in response to a stimulus. Sometimes, however, it appears impossible to distinguish activation from simply maturation.

Functional, biochemical and morphological changes have been reported based on comparisons between eosinophils from normal donors and from patients with the hypereosinophilic syndrome. A functional change frequently reported has been the increased binding to EA complexes by eosinophils from patients with hypereosinophilia (Tai & Spry, 1976; Spry & Tai, 1976; Tai & Spry, 1980). It has also been shown that there is an increase in the number of eosinophils obtained from normal donors that bind to EA and EAC complexes after incubation with the ECF-A (Capron, Capron, Goetzl & Austen, 1981), however if the eosinophils are obtained from patients with hypereosinophilia, only the binding to EAC complexes is increased (Anwar & Kay, 1977a). Similarly, eosinophils binding to EA complexes can be increased by incubation with E.coli endotoxin and lipid A, only if the cells are obtained from normal donors (Tai & Spry,

1980). These results suggest that the eosinophils from hyper-eosinophilic patients cannot increase their binding to EA complexes in vitro because they have already responded to a stimulus to increase their Fc receptors in vivo.

Another functional change observed in eosinophils from patients with hypereosinophilia is their increased antibody-dependent adherence and cytotoxic activity to S.mansoni regardless of the cause of the eosinophilia (David, Vadas, Butterworth, Azevedo de Brito, Carvalho, David, Bina & Andrade, 1980). This suggests that high numbers of peripheral blood eosinophils and activation are concomitant phenomena probably induced by the same stimulus.

The biochemical changes observed in eosinophils from patients with hypereosinophilia include increased acid phosphatase activity, and higher membrane hexose transport and hexose monophosphate shunt activities (Bass, Grover, Lewis, Szejda, De Chatelet & McCall, 1980).

A morphological alteration seen in eosinophils from hypereosinophilic patients is their hypogranular appearance (Tai & Spry, 1976; Spry & Tai, 1976; Catovsky, Bernasconi, Verdonck, Postma, Hows, van der Does-van den Berg, Rees, Castelli, Morra & Galton, 1980). That this is a consequence of granule release in the circulation with damaging effects on the cardiovascular system remains an attractive possibility.

Mouse eosinophils have been found to be very heterogeneous in density (see Chapter 3) which may indicate different stages of maturation or activation. Recently, studies with human eosinophils showed that the low density eosinophils obtained from a patient with hypereosinophilia expressed some of the morphological and biochemical features of activation (Olsson, Olofsson, Venge & Winqvist, 1980).

Rat eosinophils were obtained from the peritoneal cavity of normal August rats and purified following previously developed isopycnic

centrifugation techniques (Sanderson & Thomas, 1978). Whether these eosinophils are also in an activated state is not known. However, it should be noted that eosinophils show changes in their granules and in the activity of their enzymes as they reach the tissue (Parmley & Spicer, 1975).

In this thesis neutrophils have been induced by high molecular weight dextran. Mouse neutrophils were purified (Chapter 3) by a slight modification of the isopycnic centrifugation previously described for rat neutrophils (Sanderson & Thomas, 1978). Although few comparisons have been carried out between normal and induced neutrophils, they show differences in their respiratory burst and O_2^- production (Badwey, Curnutte, Robinson, Lazdins, Briggs, Karnovsky & Karnovsky, 1980). Furthermore, human neutrophils exposed to chemotactic factors have shown enhanced chemiluminescence, an enhanced ability to produce superoxide anion and increased bactericidal activity (Van Epps & Garcia, 1980), suggesting a state of activation.

The demonstration of C3 receptors on mouse eosinophils deserves some comment as previous studies had not detected them (Rabellino & Metcalf, 1975; Rabellino *et al.*, 1978; Hoghart *et al.*, 1980). The fact that the cells used in the studies described in this thesis may have been activated, therefore probably expressing more receptors could be important. It is more likely, however, that in view of the high complement requirement by eosinophils, previous workers have used suboptimal levels.

Previous studies with induced mouse neutrophils (Mantovani, 1976) and normal human peripheral blood neutrophils (Scribner & Fahrney, 1976; Ehlenberger & Nussenzweig, 1977) had failed to detect phagocytosis of EAC complexes in the absence of IgG molecules. In this thesis, however, a direct phagocytosis of EAC complexes is observed by both eosinophils

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and neutrophils (Chapter 4). This indicates that whether there are differences or not in the expression of complement receptors between normal and induced granulocytes, these will not be detected nor their phagocytic role observed unless the conditions described in Chapter 4 are met.

The fact that eosinophils and neutrophils bind and phagocytose complexes formed with C3b and C3bi (Chapter 4) but not with C3d (Tai & Spry, 1980), probably restricts the action of these cells to sites where complement activation is taking place as C3b is very short lived. In this way they could have a beneficial role for the host by clearing recently opsonized bacteria or attaching to and killing parasites in primary parasitic infections, as some parasites are known to activate the complement system via the alternative pathway (Mackenzie, Jungery, Taylor & Ogilvie, 1980).

Binding and phagocytosis of E by granulocytes have been shown to occur when E were coated with either IgG or complement (Chapter 4), however, whereas IgG led to high levels of cytotoxicity, complement gave very little cytotoxicity. A possible explanation could be that as the EAC complex is internalized the C3 is broken down, so that although phagocytosis has occurred, ligand-receptor interaction no longer exists to induce degranulation.

Although different enzymes have been detected as eosinophils and neutrophils interact with IgG or complement-coated targets, there is no clear evidence that different enzymes are secreted as a consequence of different stimuli. It is important to determine cytochemically the enzymes released at the site of contact of granulocytes with C3 or IgG-coated non phagocytosable targets rather than in the supernatant, as the nature and concentration of the enzymes released locally are responsible for the lyses of nucleated mammalian cells (Chapter 6).

Eosinophils have been shown to have higher IgG and complement requirements than neutrophils (Chapter 4). This low affinity or paucity of eosinophil receptors may explain the need for coating parasites with both antibody and complement to induce high levels of cytotoxicity. Although some cytotoxicity can be shown with antibody alone, this is greatly enhanced by the addition of complement (Anwar et al., 1979; Mackenzie et al., 1980). Although it is controversial, eosinophils do not generally appear to be cytotoxic when parasites are coated with complement alone (Mackenzie et al., 1980). In only a few cases has eosinophil killing of S.mansoni in the presence of complement alone been reported (Ramalho-Pinto et al., 1978; Anwar et al., 1979). In the former case it can be argued that the damage to the parasite has been started by the late complement components as whole normal rat serum was used, and complement has been previously shown to be cytotoxic to S.mansoni by itself (Clegg & Smithers, 1972).

Mouse eosinophils and neutrophils have been shown to bind an antibody of the IgG2b subclass. The low binding with IgG1 was difficult to interpret because of the low number of antibody molecules bound to E. The availability of other monoclonal antibodies will certainly solve this problem allowing the characterization of granulocyte Fc receptors of all subclasses. This approach has already proved useful in studying macrophage Fc receptors (see Introduction).

Another advantage of using monoclonal antibodies to detect granulocyte receptors will be that standardized reagents could be used by different laboratories. At the present time the large variation in the reported numbers of eosinophils forming rosettes could be explained by the species in which the antiserum was raised as well as the subclass properties of each particular antiserum. For example, while in one laboratory a rat

antiserum induces the formation of more EA rosettes by human eosinophils than with a rabbit antiserum (Capron et al., 1981), in another laboratory highest values are obtained with a guinea pig antiserum followed by rat, rabbit and human antisera in that order (Tai & Spry, 1980).

The variability in the percentage of eosinophils forming rosettes according to the antiserum used is analogous to the observation that individual antisera raised in the same fashion can determine whether eosinophils, neutrophils or K cells will be the more active effector cell against antibody-coated tumour cells (see Chapter 6). This suggests that there may be differences among effector cell types in their binding to IgG subclasses and further emphasizes the need to design experiments to answer this question.

In contrast to previous reports (Parrillo & Fauci, 1978) eosinophils have been shown to induce high levels of cytotoxicity from antibody-coated nucleated mammalian cells (see Chapters 5 and 6). Both eosinophils and neutrophils showed similar activity and were effective at low effector to target ratio. It is interesting to note that while neutrophils reacting with antibody-coated non phagocytosable targets can induce cytotoxicity if the target is a tumour cell (Chapters 5 and 6; Gale & Zigelboim, 1975; Clark & Klebanoff, 1977), parasites such as S.mansoni, Nippostrongylus brasiliensis and Trichinella spiralis appear to be resistant to neutrophil activity despite the fact that adherence is accomplished (Vadas et al., 1979; Mackenzie et al., 1981). In the case of S.mansoni degranulation does not occur (Caulfield, Korman, Butterworth, Hogan & David, 1980), a phenomenon that may be related to the particular biology of this parasite. Eosinophils, on the other hand, adhere (McLaren, Mackenzie & Rumalho-Pinto, 1977) and kill these parasites (Butterworth et al., 1975; Mackenzie et al., 1980).

Granulocytes have been shown to require large numbers of antibody molecules to exert their cytotoxic activity in vitro whether this is carried out by internalization of the target (see Chapter 4) or by exocytosis (see Chapter 6). An exception to this was the inhibition at high antiserum concentration when granulocytes were tested against T.cruzi antigen-coated target cells (see discussion, Chapter 5). This situation is probably different in vivo, where the concomitant binding of complement can reduce the effective number of antibody molecules that granulocytes need (Ehlerberger & Nussenzweig, 1977).

Granulocytes have been shown to be very active cells which exert their phagocytic, microbicidal and cytotoxic activity very rapidly. Maximum phagocytosis of opsonized E has been observed at 5' of incubation (see Chapter 4) and significant isotope release from phagocytosed E can be detected within a few minutes of incubation (Sanderson & Thomas, 1978). Similarly, phagocytosis of Staphylococcus aureus by eosinophils and neutrophils has been shown to be complete by 10' and maximum microbicidal activity is achieved at 60' (De Chatelet et al., 1979). Phagocytosis and lysis of protozoa by granulocytes have also been found to be rapid events (Sanderson, Bunn Moreno & López, 1978; Thorne et al., 1979). Killing of non-phagocytosable targets can also be rapidly accomplished, and 15' after incubation of granulocytes with antibody-coated tumour cells, significant levels of cytotoxicity have been obtained (Chapter 6). At 4-5 hs this type of reaction appears to be reaching a plateau (see Chapters 5 and 6).

In this thesis, lysis of target cells has been detected by means of a ^{51}Cr release assay. This assay has been widely used with mammalian target cells and is generally accepted to be an accurate indication of cell death. Experiments using cytotoxic T cells as effector cells (Sanderson, 1976) showed that ^{51}Cr is released from mammalian cells at the

same rate as cell macromolecules, indicating that cytoplasmic rupture and ^{51}Cr release are causally related phenomena. Nevertheless, in order to provide evidence that the release of ^{51}Cr from tumour cells caused by granulocytes was a result of cell death, experiments were carried out with BW cells, in which RNA was labelled by preincubation with ^3H uridine and the release of RNA and ^{51}Cr compared. It was found that the release of ^{51}Cr was parallel to the release of cell RNA (data not shown), indicating that ^{51}Cr release was indeed a result of target cell death.

While cytotoxicity by granulocytes has been generally found to be antibody or complement-mediated, there are some reports of neutrophils causing direct lysis of cell line cells (Williams, Lyons & Brande, 1977) and phagocytosis and lysis of protozoa have been found to take place (López *et al.*, 1978; Thorne *et al.*, 1979) in the absence of antibody. The reason for this is not clear but in the case of protozoa it may be that neutrophils but not eosinophils have receptors that mediate internalization and lysis of these parasites. A remote possibility is that these protozoa can actively enter neutrophils but not eosinophils.

The fact that granulocytes show cytotoxic activity against a large variety of opsonized particles, indicate a wide ranging potential in vivo. Thus, not only microorganisms but also metazoan parasites, tumour cells and host cells can become granulocyte targets when they are coated with antibody and complement. Interestingly, though, if the in vitro situation in which only cells making specific contact with granulocytes are lysed (see Chapter 6) also occurs in vivo, then the liberated enzymes may be important in participating in a localized inflammatory process, without being directly detrimental to the surrounding host cells.

However, if the host cells themselves become coated with antibody and complement, a situation observed in some autoimmune phenomena, this

can lead to the destruction of host tissue cells by granulocytes. This may be the case in glomerulonephritis and polyarteritis nodosa. In human glomerulonephritis, for example, antibody and complement have been found deposited in the glomerular basement membrane and in acute exacerbations a neutrophil infiltrate can be found moving through endothelial cells to make contact with the basement membrane (reviewed by Cochrane, 1956). Furthermore, in experimental animals, only one neutrophil attack over a 12 hs period has been shown to induce gross damage to the glomerulus (Cochrane, 1954).

A similar situation can occur in American trypanosomiasis where the adsorption of parasite antigen to host cells and subsequent binding of antibody, may render these cells susceptible to granulocyte cytotoxic activity as observed in vitro (see Chapter 5). This phenomenon may provide an explanation for some of the tissue destruction observed in the pathogenesis of the disease.

The high cytotoxic activity of granulocytes towards tumour cells of lymphoid origin in vitro (see Chapter 6) suggests that granulocytes could play an effector role in tumour rejection. Recently, eosinophilia has been found directly associated with lymphoid malignancies (Catovsky et al., 1980). In some patients with tumours eosinophilia has appeared before or at the time of diagnosis of the tumour with peripheral eosinophil counts decreasing during remission (Catovsky, et al., 1980) or after removal of the tumour (Weiss, 1926) but rising again if a relapse occurs (Catovsky et al., 1980).

Eosinophils have also been found infiltrating lymphoid tumours and tumours of other origins (Lukes et al., 1966; Goetzl et al., 1978, and Catovsky et al., 1980). The reason why eosinophils can accumulate at the site of the tumour is not known. It has been suggested that eosinophils

may be induced by virtue of the T cell-like properties of some tumour cells (Catovsky et al., 1980) in an analogous situation to a human leukaemic T cell line with potent colony stimulating activity (Golde, Quan & Cline, 1978). Eosinophils could also be responding to a stimulus produced by T cells responding to tumour specific antigens (Spitzer & Garson, 1973). In one case a product similar to the eosinophi chemotactic factor of anaphylaxis has been shown to be produced by an anaplastic squamous cell carcinoma which is chemotactic for eosinophils and neutrophils but not for mononuclear cells (Goetzl et al., 1978). The significance of these observations remains to be seen in terms of diagnosis, prognosis or immunological state, but at least it has been shown in vitro that eosinophils constitute yet another potential mechanism active against tumour cells.

Although there is an ever increasing amount of information regarding eosinophil and neutrophil function in vitro, a definite role for these cells in the organism awaits the results of in vivo studies. Eosinophils and neutrophils are not easily accessible. Attempts to selectively deplete them with antiserum have been made; however, the specificity of these is doubtful.

The production of monoclonal antibodies to eosinophils and neutrophils has been shown (Chapter 7) to offer prospects of success in obtaining a reagent which will specifically ablate one cell type, and hence to establish its role in vivo. One of the monoclonal antibodies to mouse eosinophils developed has been shown to selectively ablate eosinophils in vivo. Moreover, monoclonal antibodies could be valuable in identifying cellular antigens expressed as a result of cell maturation or activation and to study qualitative and quantitative changes in granulocyte membrane receptors that may accompany these processes.

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