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A comparison of the cytotoxic activity of eosinophils and other cells by ^{51}chromium release and time lapse microcinematography

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Summary. Antibody dependent cytotoxicity of chicken erythrocytes by purified rat eosinophils, neutrophils, macrophages and K cells has been compared by ^{51}Cr release and time lapse microcinematography. Techniques have been developed for purifying these effector cell types. Both eosinophils and neutrophils cause rapid release of ^{51}Cr from erythrocytes. Time lapse observations indicated that this was the result of phagocytosis. Eosinophils show rapid membrane movement and repeatedly engulf and regurgitate the erythrocytes. On the other hand, neutrophils become quiescent after phagocytosing erythrocytes, and remain quiescent until the remains of the cell are expelled. Neutrophils presumably have a mechanism for the release of soluble material, as ^{51}Cr is released rapidly. Macrophages show a similar quiescence after phagocytosis, but in these cells there is apparently no rapid mechanism to expel material, as there is no significant ^{51}Cr release over 20 h. K cells appear to damage chicken erythrocytes more slowly than they destroy tumour cells. Mast cells cause antibody-independent cytotoxicity which can be attributed to the release of toxic materials. None of these effector cells produced the type of lysis seen with antibody and complement.

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INTRODUCTION

The observations that eosinophils can kill the causative agents of two of the world's major tropical diseases, *Schistosoma mansoni* (Butterworth, David, Franks, Mahmoud, David, Sturrock & Houba, 1977) and *Trypanosoma cruzi* (Sanderson, Lopez & Bunn Moreno, 1977), has stimulated new interest in the cytotoxic activity of eosinophils.

Experiments in which one cell type is apparently more cytotoxic than another can be criticized on the grounds that the negative effects of one cell type may be trivial, due for example, to inactivation during the purification procedure, and a positive control system is needed to overcome this problem. Further there has been a need to bring together cytotoxicity and phagocytosis in a single study, comparing cytotoxicity as measured by release of ^{51}Cr with morphological changes. In this paper we show that eosinophils as well as neutrophils (Greenberg, Shen & Medley, 1975) and K cells (Sanderson, Clark & Taylor, 1975) are cytotoxic towards antibody coated chicken red blood cells (CRBC). Thus these studies indicate that CRBC can be used as a positive control system especially when comparing the activity of eosinophils and neutrophils. In this paper we study the activity of rat eosinophils, neutrophils, macrophages, mast cells and K cells on CRBC, by comparing the release of ^{51}Cr with

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morphological changes seen in fixed preparations and in living preparations using time lapse microcinematography. To facilitate this study we have developed purification techniques for rat granulocytes.

MATERIALS AND METHODS

The procedures used for preparing granulocytes and macrophages, have been developed after a considerable amount of preliminary work, and will be described in some detail. All the other techniques have been widely used, and will be described only briefly.

Cell purification

For the purification of granulocytes and macrophages we have relied entirely on isopycnic sedimentation techniques. It should be noted that in our hands techniques based on adherence phenomena (carbonyl iron or nylon wool) provide relatively pure preparations of eosinophils (which are not adherent) or neutrophils (which are adherent) but the cytotoxic activity of the cells varied greatly from day to day. Also we have noted that non-isotonic gradients can produce purified eosinophils which have no cytotoxic activity. These cells appear to be more sensitive to variation in tonicity than neutrophils. Further it should be noted that whereas human eosinophils are of higher density than neutrophils, in the rat the reverse is the case and neutrophils are the more dense.

The density cut off points for the different cell types were determined by running the cells in continuous gradients of isotonic metrizamide (Nyegaard & Co. A/S, Oslo). Metrizamide was made up as a 35·3% solution in water (this is isotonic). The required concentrations were made by diluting in isotonic phosphate buffered saline (PBS) and adding foetal calf serum (2%). A linear gradient across an appropriate concentration range was prepared in a gradient former (total volume 5 ml in a 16 mm diameter tube). The cells were layered on top and centrifuged at 1000 r.c.f. (bottom of the tube) for 15 min. Fractions (0·5 ml) were collected after puncturing the bottom of the tube. Each fraction was monitored for metrizamide concentration with a refractometer, the cells were counted in a Coulter counter for total counts, and slides prepared for staining by Leishman's stain, with a cytocentrifuge. The profile of each cell type was plotted and the cut off concentrations of metrizamide determined. Routinely, cells were purified using a two step gradient, consisting of 1 ml of a high concentration of metrizamide on the bottom, 2 ml of a lower concentration layered over this, and finally 2 ml of cells suspended in medium (a maximum of 2×10^7 cells per 16 mm tube). The tube was centrifuged and the fractions collected as above.

Eosinophils and mast cells were obtained as a mixture (also containing macrophages) from normal August rats (Source, NIMR, Mill Hill) by peritoneal lavage. These rats have relatively high numbers of peritoneal eosinophils and mast cells. The cells were layered over a two step gradient made up of 17·5 and 16·5% metrizamide. After centrifugation, cells at the top interface were mostly macrophages. The second interface was eosinophils contaminated with macrophages and mast cells, and mast cells were recovered from the pellet. The second interface cells were re-run on a similar gradient, which considerably improved the purification of eosinophils.

Macrophages and neutrophils were obtained as a mixture from Agus rats (source, Bantin and Kingman Ltd., Yorks., U.K.) after an intraperitoneal injection of 5 ml of 3·5% dextran (mol. wt $5–40 \times 10^6$ as used by Lake, Sabbadini & Sehon, 1974). These rats have very few peritoneal mast cells or eosinophils. The cells were separated first on Ficoll-Hypaque. The cells at the interface were used as a source of macrophages. The cells in the pellet were centrifuged through a two step gradient of 20 and 17·5% metrizamide. The middle interface was used as the source of neutrophils.

K cells from Agus rat spleen were enriched by taking the cells remaining at the interface of a Ficoll-Hypaque separation, and passing these through nylon wool to remove adherent cells (Sanderson & Thomas, 1977a).

Cytotoxicity

Chicken erythrocytes were labelled with ^{51}chromium and isotope release was determined as described previously (Sanderson *et al.*, 1975), except that the carrier erythrocytes previously added to minimize the spontaneous release of chromium were not included. The tubes were centrifuged at 400 r.c.f. for 1 min, and then incubated for 4 h. The medium was RPMI-1640 containing HEPES buffer and 10% inactivated foetal calf serum.

Antiserum

Rat antiserum was obtained 18 days after a single

Table 1. Differential cell counts on purified cell preparations

Preparation	Morphological appearance				
	Neutrophil	Eosinophil	Mast cell	Lymphoid	Macrophage
Neutrophil	97*	1	0	0	2
Eosinophil	0	94	4	0	2
Mast cell	0	8	90	0	2
Macrophage	1	1	0	3	95
K cell	0	0	0	100	0

* Values given in percentage, rounded to nearest whole number. Zero represents no cells seen in a scan of 500 cells (i.e. <0·2%).

intraperitoneal injection of 10^9 CRBC. Mouse antiserum was obtained 14 days after a single injection of 10^8 CRBC. Rabbit antiserum was obtained after a long course of multiple injections of CRBC.

Time lapse microcinematography

Time lapse (TL) films were made as described (Sanderson, 1976; Sanderson & Thomas, 1977a), except that Nomarski differential interference-contrast optics with a ×40 objective was used (Carl Zeiss (Oberkochen) Ltd). Filming rate was 1 frame every 5 s, with an exposure of 0·2 s.

RESULTS

Purified cell populations were prepared as described in the Methods. Table 1 shows the differential counts from a typical experiment. It was possible to prepare; neutrophils with a 95% purity or higher, contaminated with macrophages and lymphocytes; eosinophils to 95% purity or higher, contaminated mostly with macrophages and mast cells; macrophages to 95%, contaminated with lymphocytes and a few eosinophils and neutrophils; mast cells to 90% contaminated mostly by eosinophils.

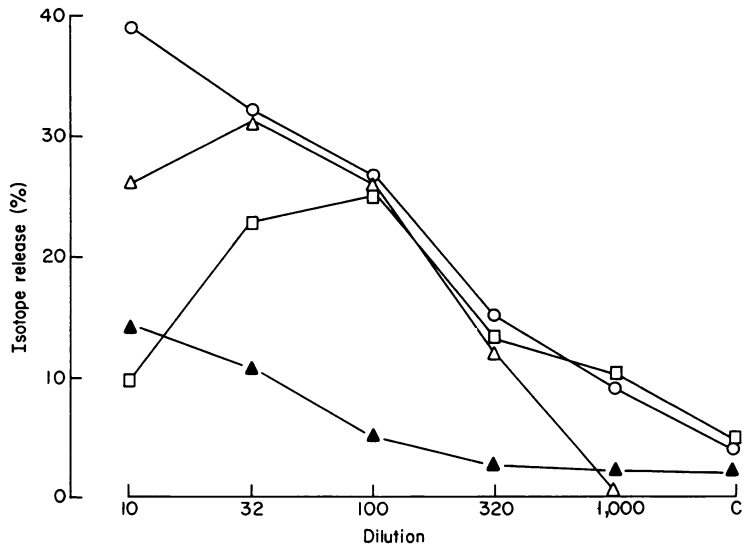


Figure 1. Titration of rat anti CRBC serum with eosinophils, ratio 1 : 1 (□); neutrophils, ratio 1 : 1 (△); macrophages, ratio 5 : 1 (▲) and K cells, ratio 50 : 1 (○). C = Control without antiserum and was not significantly different to normal rat serum dilutions. Incubation period 4 h.

The K cell preparations were 100% lymphoid cells.

The relative antibody requirements were tested by carrying out a serum titration, and testing with each of the cytotoxic effector cells (Fig. 1). With rat antiserum, the end of the plateau is approximately the same for both neutrophils and eosinophils indicating that the quantitative requirements for antibody are similar for both effector cell types. Macrophages on the other hand give very little isotope release. K cells show increasing isotope release with increasing serum concentration, which does not reach a plateau. This suggests that K cells require relatively more antibody molecules for maximum killing. In other experiments it was found that mouse antiserum showed similar characteristics to the rat antiserum, in that the relative cytotoxic activity of the different effectors was similar. However with a hyperimmune rabbit antiserum the end of the plateaux indicated that neutrophils and K cells require approximately 10 fold less antibody than eosinophils. Thus a dilution of 1 : 10,000 of the rabbit antiserum gave plateau levels of cytotoxicity with neutrophils (50% isotope release) whereas the eosinophils show virtually no cytotoxicity (15% isotope release compared with a control value of 13%). In all the other experiments rat antiserum was used, at a dilution of 1 : 100.

Relative cytotoxic activity

Different ratios of the different purified effectors were tested against CRBC in the presence and absence of antibody (Fig. 2). K cell activity was low compared to neutrophils and eosinophils but it must be remembered that this is an enriched preparation with an unknown proportion of K cells. Macrophages (and also mast cells, data not shown) gave very little chromium release over the 4 h incubation period. Macrophages added back to eosinophils and neutrophils showed marked inhibition of ^{51}Cr release, for example at a ratio of 5 : 1, neutrophils gave 50·6% release but this was decreased to 11·8% when 10% macrophages were added (control value 3·1%). It was more difficult to prepare eosinophils free of macrophages and in an experiment where 12% macrophages remained in the eosinophil preparation, 29·5% release was obtained at a ratio of 5 : 1 and 33·5% at a ratio of 0·6 : 1. This negative effect of increasing the effector : CRBC ratio can be attributed to inhibition of ^{51}Cr release by macrophages at the higher ratios.

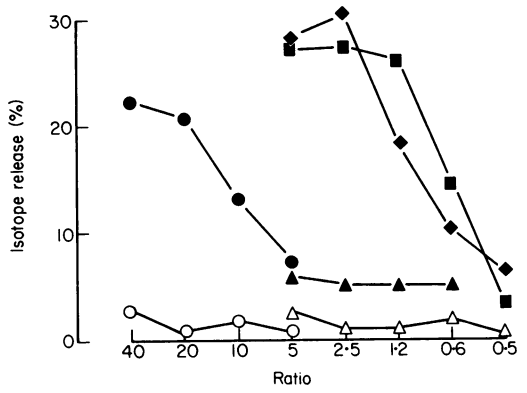


Figure 2. Percentage ^{51}Cr release at different effector: CRBC ratios. Effector cells in the presence of antibody: eosinophils (■), neutrophils (◆), macrophages (▲). Controls without antibody for all these cell types plotted on the same points and so for clarity only one symbol is shown (△). K cells with (●) and without antibody (○). Values above 3% release are significantly different from the control values (5% level of significance).

Kinetics of ^{51}Cr release

The kinetics of ^{51}Cr release were followed with each effector cell type. Fig. 3 shows the release by eosinophils and neutrophils at a ratio of 5 : 1. In this experiment neutrophils showed considerably more activity than eosinophils which contained 12% macrophages. However in another experiment (also shown in Fig. 3) at a ratio of 1 : 1 the eosinophils with only 1% macrophages were more active than neutrophils.

Macrophages at a ratio of 5 : 1 (Fig. 2) showed no significant release over 20 h. K cells caused a linear release over the 20 h period (Fig. 4) and mast cells at a ratio of 5 : 1 showed antibody independent release after a lag phase of about 4 h (this varied in different experiments). In experiments where eosinophils were contaminated by mast cells there was an increase in the rate of release in control tubes which can be attributed to the mast cells.

Morphological changes

Stained preparations (Fig. 5) prepared after 1 h incubation at a ratio of 1 : 1 effectors to targets showed a considerable amount of phagocytosis of CRBC by neutrophils and eosinophils (Table 2). This only occurred in the presence of antibody. Consistently a slightly smaller proportion of eosino-

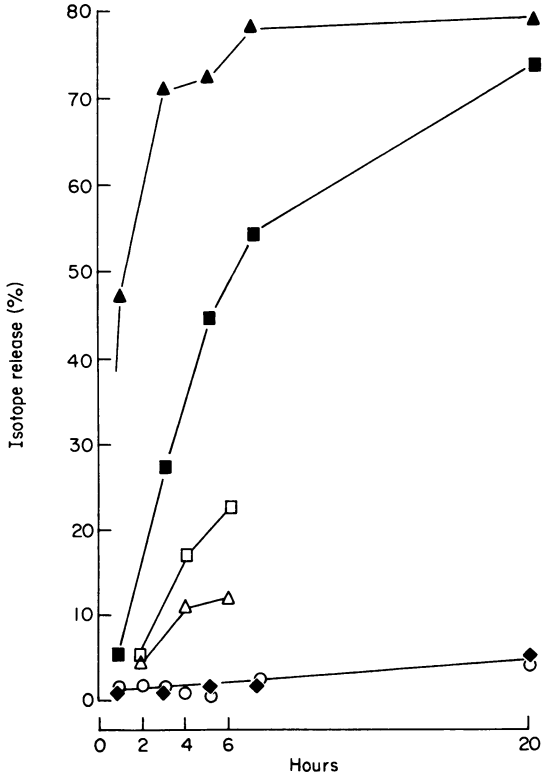


Figure 3. Kinetics of release of ^{51}Cr from CRBC by eosinophils, ratio 5 : 1 (■); ratio 1 : 1 (□); neutrophils, ratio 5 : 1 (▲); ratio 1 : 1 (△); macrophages, ratio 5 : 1 (◆). Controls in the absence of antibody were the same for all cell types and not significantly different from medium (○). These data are combined from two experiments (see test).

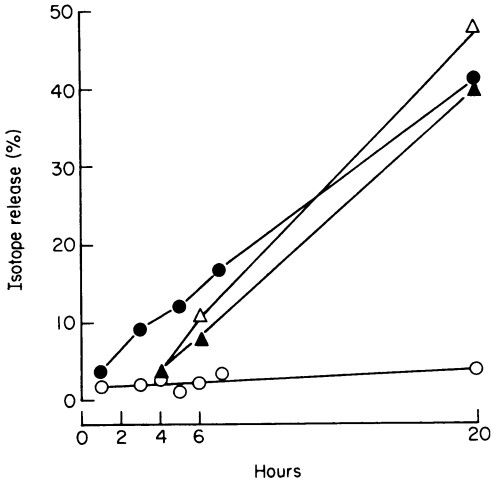


Figure 4. Kinetics of release of ^{51}Cr from CRBC by K cells, ratio 50 : 1 (●, ○) and mast cells ratio 5 : 1 (▲, △). Filled symbols are in the presence of antibody, empty symbols are in the absence of antibody.

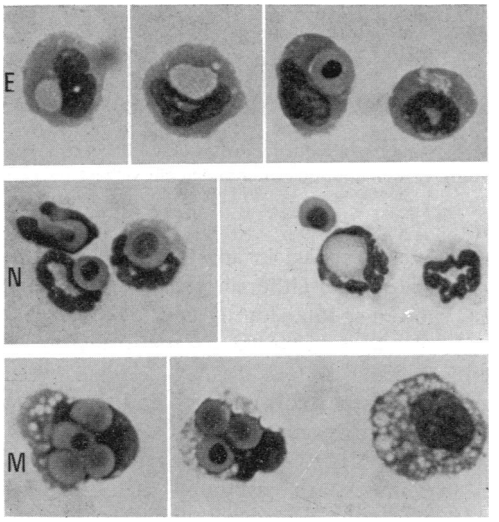


Figure 5. Stained preparation made after 1 h incubation, of CRBC inside eosinophils (E), neutrophils (N) and macrophages (M). The CRBC can be seen to be in different stages of degeneration, and in some the nucleus has disappeared (× 700).

phils appeared to be phagocytic than neutrophils. Macrophages in the absence of antibody showed some phagocytosis, and this was increased in the presence of antibody. In all cases phagocytosed CRBC showed signs of degeneration (staining less intense).

Mast cells on the other hand showed no signs of phagocytosis. There was considerable evidence of degranulation and a number of CRBC showed signs of degeneration. K cell preparations showed no sign of phagocytosis or CRBC degeneration after 1 h.

Morphological changes by time lapse microcinematography

Firstly an examination of lysis by antibody and complement, shows the CRBC become spherical although without obvious swelling, and within 3 min becoming a flattened disc with a central bulge showing the remains of the nucleus (Fig. 6). This type of

Table 2. Proportion of different cell types that phagocytose CRBC

Preparation	Antibody	% Phagocytosing CRBC	No. of CRBC in phagocytosing cells
Neutrophils	+	21	1·05
	−	0	0
Eosinophils	+	14	1·05
	−	0	0
Mast cells	+	0	0
	−	0	0
Macrophages	+	62	1·75
	−	14	1·3
K cells	+	0	0
	−	0	0

Incubation period 1 h. Zero represents no cells seen in a scan of at least 500 cells.

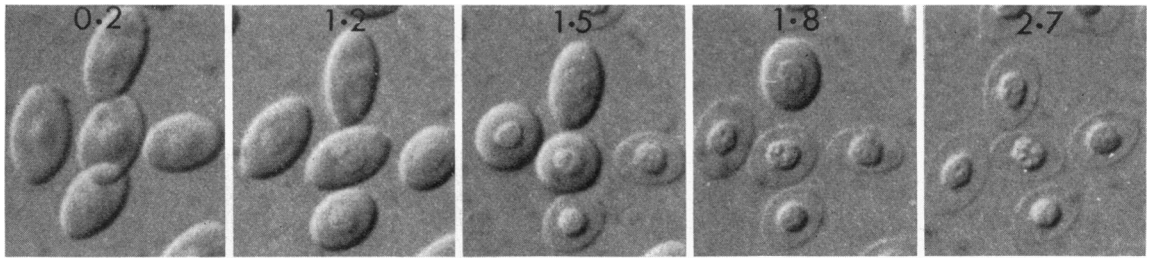


Figure 6. Time lapse sequence from the addition of specific antibody and complement (time zero) to complete lysis of CRBC (2·7 min). Lower dilutions of complement slowed the reaction but the morphological changes were similar (× 1000).

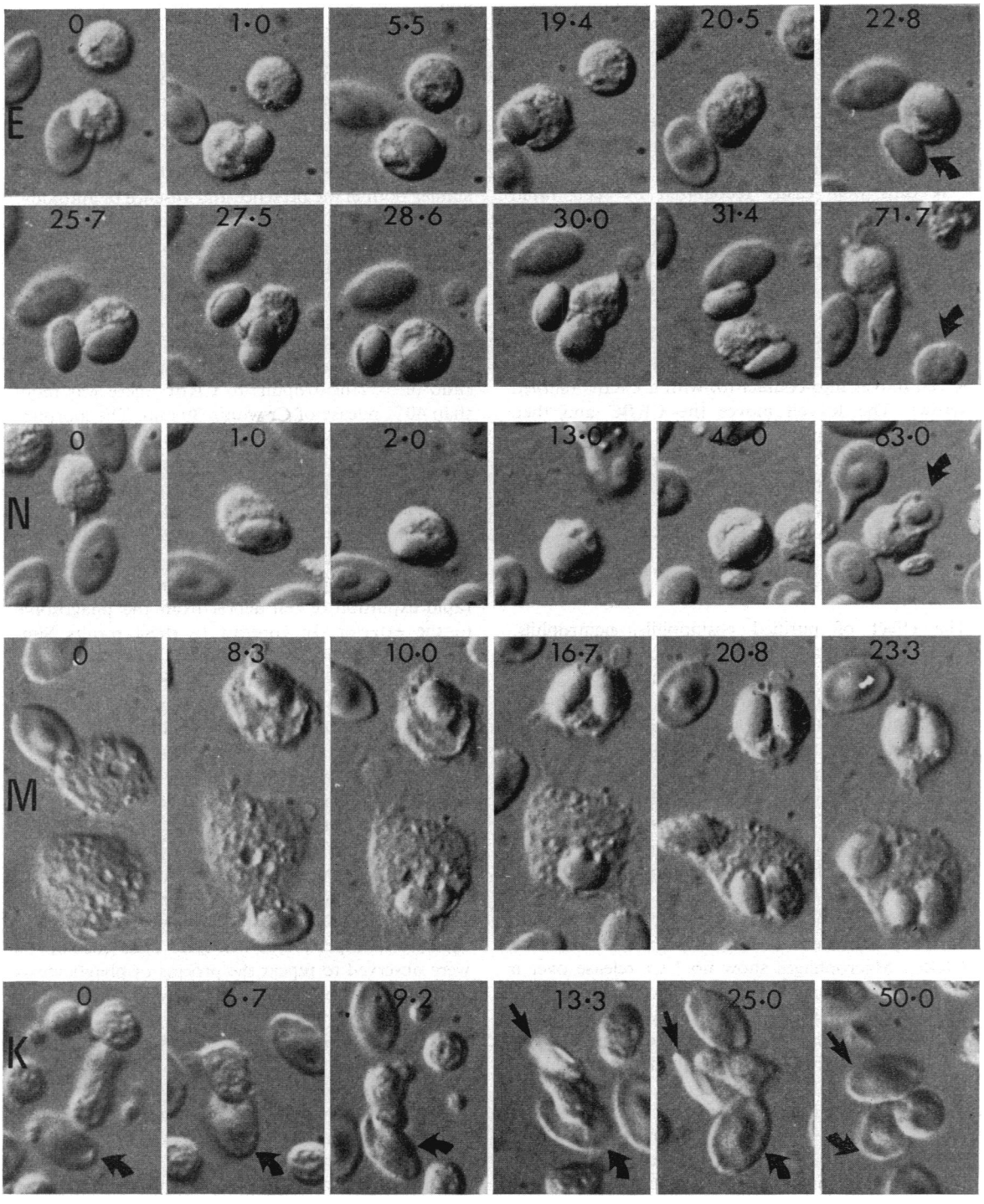
lysis was not observed in any of the cell mediated cytotoxic reactions, although some preparations of CRBC showed spontaneous lysis of this type. These experiments were regarded as unsatisfactory and were discarded.

Eosinophils repeatedly engulfed and then regurgitated the antibody coated CRBC. An example is shown in Fig. 7 (E). The numbers refer to the frame times in minutes. An eosinophil engulfs a CRBC (0) and after 19·4 min expels and then engulfs it again (20·5). The eosinophil then starts to engulf another CRBC and at the same time expels (22·8) the first (curved arrow). It starts to expel the second CRBC (27·5), engulfs it again (28·6), expels it (30·0) and starts to engulf it again (31·4). By 71·7 the second CRBC is still inside the eosinophil when it starts to engulf a third (arrow). The remains of the first CRBC are shown (curved arrow).

Neutrophils showed rapid phagocytosis, of up to three or four CRBC, which simply remained inside the cell, with no obvious regurgitation. The erythrocytes could be clearly seen inside the neutrophils several hours after phagocytosis. In one case (Fig. 7N) the remains of a CRBC was expelled 63 min after phagocytosis (curved arrow).

Macrophages also showed rapid phagocytosis of

Figure 7. Time lapse sequences of antibody coated CRBC cytotoxicity by different effector cells. The numbers give the time interval in minutes after an arbitrary time zero (× 1000). (E) eosinophil showing sequential engulfing and regurgitation of two CRBCs. The remains of the first CRBC (curved arrow) can be seen in frame 71·7. (N) neutrophil. The cell became quiescent after phagocytosing the CRBC. After 63·0 min the neutrophil expels the remains of the CRBC and commences normal movement again. (M) macrophages. These cells became quiescent after phagocytosing 2 or 3 CRBC and remain motionless over the full course of the film (3 h). (K) lymphoid K cells. This K cell made contact (time 0) with a CRBC (curved arrow) and then a second CRBC (13·3). After 50 min the first CRBC is distorted.


E
0
1·0
5·5
19·4
20·5
22·8
25·7
27·5
28·6
30·0
31·4
71·7
N
0
1·0
2·0
13·0
46·0
63·0
M
0
8·3
10·0
16·7
20·8
23·3
K
0
6·7
9·2
13·3
25·0
50·0

several CRBC (Fig. 7M). They then became rounded up and remained virtually stationary for several hours. Mast cells on the other hand showed no phagocytosis or movement (photomicrographs not shown). Some erythrocytes became slightly flattened as the number of free granules increased in the field (presumably as a result of degranulation, although this was not observed).

K cells were easily recognized by their rapid motility, and ability to actively move one or more CRBC across the field, and out of the plane of focus. The CRBC became distorted which made the nucleus more prominent. There was no sign of lysis as seen with complement, nor was there any sign of zeiosis as described when tumour cells were killed by K cells (Sanderson & Thomas, 1977a). In Fig. 7K a K cell moves into contact (0) with a CRBC (curved arrow). The K cell moves the CRBC and then (13·3) makes contact with a second CRBC (arrow). The two CRBCs are moved about. There is no obvious lytic step but after 50 min the first CRBC can be seen to be distorted.

DISCUSSION

The effect of purified eosinophils, neutrophils, macrophages, mast cells and enriched K cells on CRBC has been studied. These erythrocytes can be killed by K cells (Sanderson *et al.*, 1975) whereas mammalian erythrocytes appear to be resistant to K cell activity (Sanderson, unpublished). Tumour cells which are susceptible to K cell activity, are not susceptible to granulocyte activity (Sanderson *et al.*, 1975; Sanderson *et al.*, 1977). Thus CRBC provide a convenient system for the study of cell-mediated cytotoxicity by the wide variety of effectors studied here.

Eosinophils, neutrophils and K cells all cause antibody-dependent release of ^{51}Cr from labelled CRBC. Macrophages show no ^{51}Cr release over a 20 h incubation period. Stained preparations made after 1 h incubation showed many of the phagocytosed CRBC undergoing degenerative changes. This was not completely antibody-dependent as preparations without antibody showed significant phagocytosis. Time lapse showed that after rapid phagocytosis of CRBC, the macrophage became rounded up and quiescent. These experiments indicate that while macrophages can phagocytose and damage CRBC, they do not release significant amounts of cell debris (represented by ^{51}Cr) over a 20 h period. Other reports have shown that macrophages eliminate bacterial degradation products within a few hours of phagocytosis (Cohn, 1963; Thomas, Holt & Keast, 1974). The reason for these differences are not clear.

Mast cells caused a significant release of ^{51}Cr after a lag phase of approximately 4 h. This was similar both in the presence and absence of antibody. Morphological studies showed no evidence of either contact or phagocytosis. It seems likely therefore that this damage results from a nonspecific release of toxic materials as the mast cells degranulate *in vitro*.

Neutrophils caused a very rapid release of ^{51}Cr from antibody coated CRBC. For example, at a ratio of 5 : 1 neutrophils to CRBC there was more than 40% release of Cr within 30 min. The morphological studies indicated that this was by phagocytosis of the CRBC. Stained preparations showed many degenerating CRBC inside neutrophils after 1 h. Time lapse studies produced no evidence of extracellular lysis. The CRBC were rapidly phagocytosed and retained within the cell cytoplasm. Presumably the neutrophil has a mechanism for the rapid expulsion of cell debris from the phagosome to the exterior. In contrast to these results Simchowitz & Schur (1976) described lectin dependent cytotoxicity of CRBC by human neutrophils in which they were unable to demonstrate phagocytosis. It is unclear whether this difference is due to the effect of lectin or simply a species difference.

The most interesting aspect of this study is the demonstration of cytotoxicity by eosinophils. Like neutrophils, these show rapid antibody-dependent release of ^{51}Cr from CRBC. The time lapse studies showed them to complete the phagocytosis of an erythrocyte in a similar time as neutrophils; they appeared to be more motile and to have a greater capacity to expel the erythrocyte. Thus eosinophils were observed to repeat the process of phagocytosis and regurgitation of an erythrocyte several times over a period of an hour.

K cells showed an antibody-dependent release of ^{51}Cr which was linear over a 20 h period, whereas with tumour cells (Sanderson & Thomas, 1977b) there is a rapid release over the first 15 min followed by a much slower rate of release. These results with tumour cells were shown to be a result of sudden explosive lysis of the tumour cell within 15 min of contact by a K cell. This was not observed in the

present studies. K cells could be identified in the time lapse studies by their rapid movement and ability to make contact and move the CRBC about the field. There was no spectacular lysis as was seen with antibody and complement, nor was there evidence of phagocytosis, the CRBC remained extracellular and became distorted. In comparison with earlier work (Sanderson & Thomas, 1977b) these results suggest that K cells do not damage CRBC as readily as they can tumour cells.

The lysis of CRBC by antibody and complement produced a flattened disc, with a prominent shrunken nuclear remnant. In none of the cell-mediated reactions were changes of this type produced. Eosinophils and neutrophils damaged the CRBC in such a way that the nucleus disappeared leaving an intact shell which was reduced in size compared to an intact erythrocyte.

Using homologous rat antiserum eosinophils and neutrophils showed similar requirements for antibody, as judged by titration curves. However with a high dilution of a rabbit antiserum neutrophils appeared to be more effective than eosinophils. This difference may be due to differences in the way the antisera were raised, rather than species differences, however a similar phenomenon has been observed with human eosinophils where homologous IgG reacts better than rabbit IgG (Tai & Spry, 1976). Moreover Penfold, Greenberg & Roitt (1976) failed to demonstrate eosinophil cytotoxicity with rabbit antiserum and mouse effector cells.

These results clarify some factors concerning cell mediated cytotoxicity of CRBC. Firstly we have previously criticized (Sanderson & Taylor, 1976) the assumption that carbonyl iron simply removes phagocytic cells. The technique (depending how it is applied) removes some adherent non-phagocytic cells and some phagocytic cells. Eosinophils are a clear example of phagocytic cells which are not removed by carbonyl iron under conditions which remove neutrophils and macrophages (Sanderson *et al.*, 1977). This assumption has tended to overestimate the role of macrophages in cytotoxicity as measured by chromium release. Although clearly cytotoxic, as shown in this study, and by Penfold *et al.* (1976), they do not appear to cause significant release of ^{51}Cr. In fact they cause marked inhibition of ^{51}Cr release. This has been demonstrated using pure macrophages (Fig. 3), as well as inactivation by silica (Sanderson & Taylor, 1976). Finally, the high activity of neutrophils could entirely account for the activity found in mouse spleen, and termed null cells (Greenberg, Hudson, Shen & Roitt, 1973). On current evidence there is no reason to propose more than four antibody-dependent rodent effectors active against CRBC: macrophages, which do not release ^{51}Cr; lymphoid K cells, which are present in very low numbers in mouse spleen; eosinophils, which function better with homologous antibody; and neutrophils.

Chicken erythrocytes are probably not a relevant model for tumour immunity, allograft rejection or autoimmunity because of the variety of effector cells which are active against CRBC but not against cell line cells. However, they provide a useful model for studying the mechanism of cytotoxicity. While neutrophils and eosinophils have similar activity against CRBC, evidence is emerging of interesting differences in their activity against different parasites (Butterworth *et al.*, 1977; Sanderson *et al.*, 1977). The reasons for this will no doubt become clearer in the future, as CRBC could be a useful positive control system to check that the differences do not lie in trivial effects of source, methods of purification or contamination by inhibitor cells.

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Note: Copies of the time lapse film can be made available for teaching or research purposes.

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