Clin. exp. Immunol. (1982) **48,** 641–648.

Rat eosinophil-mediated antibody-dependent cellular cytotoxicity: investigations of the mechanisms of target cell lysis and inhibition by glucocorticoids

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(Accepted for publication 15 January 1982)

SUMMARY

Purified eosinophils from the peritoneal washings of *N. brasiliensis* infected rats demonstrated antibody-dependent cellular cytotoxicity (ADCC) for ^{51}Cr-labelled chicken erythrocytes. The F(ab′)$_2$ fragment of the antibody did not support cytotoxicity thereby demonstrating the importance of the eosinophil Fc receptor to this activity. Bystander lysis of erythrocytes did not occur, indicating that the eosinophil does not release lytic agents free into the medium. Cytochalasin B (1·25–5 μg/ml) colchicine (10^{-5}–10^{-3}M) and chloroquine (10^{-4}–10^{-3}M) inhibited eosinophil ADCC. Inhibition was also demonstrated by methylprednisolone, 10^{-7}–10^{-3}M and this inhibition was blocked by the protein synthesis inhibitor, cycloheximide (25 μg/ml). Cycloheximide alone had no effect. This block of steroid inhibition by cycloheximide suggests that the steroid effect on this system may be mediated by a newly synthesized protein and implies that the eosinophil may possess a glucocorticoid receptor.

INTRODUCTION

The role of eosinophil accumulations at sites of Type I hypersensitivity reactions is not understood. The potential of this cell for amelioration of such reactions has been extensively discussed (Austen & Orange, 1975; Hubscher, 1977). However the eosinophil also has the potential for cell damage as shown by its interaction with, and destruction of, many parasites (Butterworth *et al.*, 1975; Capron *et al.*, 1978; Butterworth *et al.*, 1979b).

Antibody-dependent cellular cytotoxicity (ADCC) against chicken erythrocytes by rat eosinophils (Sanderson & Thomas, 1978) may provide a useful *in vitro* system for the study of the potential of the eosinophil for cell damage. Other workers (Parillo & Fauci, 1978) have shown ADCC by human eosinophils against nucleated mammalian target cells. The mechanism by which eosinophils cause lysis is not understood. It could involve one or a combination of the following: phagocytosis, secretion of granule contents, oxidative activity i.e. production of superoxide and hydrogen peroxide, and peroxidative activity. The possible relevance of eosinophil ADCC to *in vivo* eosinophil activities cannot at present be assessed. However the assay provides a useful test system for the study of these potentially tissue destructive aspects of eosinophil activity and also for the investigation of the control of those activities.

In the control of many conditions which have an associated eosinophilia, glucocortocoids are of major importance, and the eosinopenia produced by steroids is well documented (for review see Beeson & Bass, 1977). However the effects of glucocorticoids on eosinophil activities rather than on

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distribution and production have been little studied. Eosinophils isolated from individuals dosed orally with glucocorticoids show reduced chemotaxis *in vitro* (Clarke, Gallin & Fauci, 1979) and also reduced adherence (Clarke *et al.*, 1979; Altman *et al.*, 1981). However, *in vitro* treatment of eosinophils with glucocorticoids has given conflicting results in chemotaxis experiments. Hydrocortisone, at concentrations up to 10^{-4}–10^{-3}M, showed no effect on chemotaxis (Jones & Kay, 1974) and inhibited chemotaxis (Gauderer & Gleich, 1978).

This report examines the effect of 10^{-7}–10^{-3}M methylprednisolone on rat eosinophil ADCC. In addition the effects of cytochalasin B, chloroquine and colchicine were also investigated.

MATERIALS AND METHODS

Preparation of eosinophils. Female Sprague-Dawley rats, weighing approximately 200 g on day 0, were each infected by the subcutaneous injection of 2000 *Nippostronglyus brasiliensis* stage 3 larvae. Sixteen days later the animals were injected intraperitoneally with 3 ml of 10% proteose peptone (Difco). Cells were removed on day 20 by peritoneal lavage with 20 ml Hanks balanced salt solution (HBSS, Oxoid) containing 5 units Heparin per ml (containing 0·15% chlorocresol, Evans Ltd). These lavages routinely contained 30% eosinophils and 65–70% mononuclear cells.

Peritoneal cells from 10 rats were pooled, washed once with HBSS and the washed cells taken up with 8 ml HBSS. Two millilitre aliquots of cells were applied to 10 ml Triosil (Nyegaard & Co., Oslo), density 1·131–1·132 at room temperature, and centrifuged at 400 ***g*** for 20 min at 4°C. The eosinophil pellets were pooled, washed three times with HBSS and twice with minimal essential medium buffered with bicarbonate and containing 10% fetal calf serum (MEM-FCS, Flow Labs) and finally suspended at 2×10^6/ml in MEM-FCS. These preparations normally contained 75–85% eosinophils, 2–10% mast cells and the remainder were large mononuclear cells.

Rat neutrophils were collected from uninfected animals 18 hr after the intraperitoneal injection of proteose peptone. Neutrophils were separated from mononuclear cells by centrifugation on Ficoll-Hypaque, density 1·08 at 400 ***g*** for 40 min at 4°C. After washing in MEM-FCS, the neutrophils (from the Ficoll-Hypaque pellet) were suspended at 2×10^6/ml in MEM-FCS. These preparations normally contained around 90% neutrophils.

Target cells. Chicken red blood cells (CRBC) were washed three times with phosphate-buffered saline (PBS) and labelled with ^{51}Cr ($NaCrO_4$, Radiochemical Centre, Amersham) by incubating 0·1 ml of 1/10 dilution of packed washed CRBC with 200 μCi ^{51}Cr at 37°C for 60 min. The erythrocytes were washed three times with MEM 5% FCS to remove loosely bound ^{51}Cr, and finally washed and resuspended in MEM-FCS at 1×10^6 cells/ml.

Antibodies. For the majority of experiments antibody to CRBC raised in rats was used. On days 0 and 21 rats received 10^8–10^9 chicken erythrocytes i.v. and on day 28 the animals were bled and serum prepared. A mixed immunoglobulin fraction was isolated by salt precipitation with 37% saturated ammonium sulphate. This preparation containing mainly IgG plus IgM was routinely used at a range of dilutions between 1/1024 and 1/128 to give 10% to 50% ^{51}Cr release.

Where indicated in the results, an IgG preparation of rabbit antiserum against chicken erythrocytes was used. This was prepared according to the method of Stevenson & Dorrington (1970), from antiserum raised by the injection of 10^9–10^{10} erythrocytes on days 0 and 21. Rabbits were bled one week later. The $F(ab')_2$ fragment of the IgG fraction was prepared by the method of Porter (1959).

Cytotoxicity assay. Conditions were adapted from Sanderson & Thomas (1978). In a total volume of 0·4 ml of MEM-FCS, 2×10^5 eosinophils plus 10^5 ^{51}Cr-radiolabelled CRBC and various dilutions of antibody were incubated for 4 hr at 37°C in a 5% CO_2 atmosphere. After incubation 0·6 ml ice cold MEM-FCS was added, the cells removed by centrifugation at 200 ***g*** for 10 min, and 0·8 ml of supernatant counted in an automatic gamma counter (Wilj) to measure released ^{51}Cr.

All incubations were carried out in triplicate and the mean counts per second (c.p.s.) used for further calculation.

$$\% \, ^{51}\text{Cr released} = \frac{\text{E}-\text{C}}{\text{T}-\text{C}} \times 100$$

Where E equals the counts released in the experimental group; C, the counts released in the control group in the absence of eosinophils; and T, the total releasable counts as determined by freezing and thawing of 10^5 target cells in distilled water. Counts released in the control group were normally less than 10% of the total releasable counts. An analysis of variance was carried out on pooled steroid inhibition data followed by Duncans multiple range test to compare specific means (Duncan, 1955).

The following compounds, when present, were added at the beginning of the 4 hr incubation, methylprednisolone sodium succinate (Upjohn, Solumedrone), cytochalasin B (Sigma), chloroquine (Winthrop, Aralen), colchicine (Sigma), cycloheximide (Sigma), actinomycin D (Sigma) and puromycin (Sigma). At the concentrations used (see results) methylprednisolone sodium succinate, cytochalasin B and colchicine did not cause a significant decrease (< 10%) in eosinophil viability as measured by trypan blue exclusion in the 4 hr incubation period. Chloroquine however at 10^{-4} and 10^{-3}M decreased viability to 80% and 70% respectively. None of the agents caused a significant increase in background ^{51}Cr loss from the target cells.

RESULTS

Fc dependence

Preliminary experiments confirmed the findings of Sanderson & Thomas (1978) in that isotope release from target cells was not mediated by eosinophils alone but required the presence of specific antibody for CRBC. To investigate the role of the Fc receptor, experiments were carried out comparing isotope release in the presence of the IgG fraction of rabbit anti-CRBC serum with release in the presence of its F(ab')2 fragment. As shown in Table 1, in the presence of intact IgG anti-CRBC, eosinophils caused marked target cell lysis over the concentration range 0·6–600 μg/ml whereas in the presence of the F(ab')2 fragment target cell lysis did not occur.

Table 1. The Fc dependence of rat eosinophil ADCC of chicken erythrocytes

*Antibody (protein μg/ml)	% ^{51}Cr released	
	IgG	F(ab')2
600	36·3	1·8
60	34·1	1·4
6	15·7	2·2
0·6	10·5	1·1
0·06	0	0

* Total protein in the IgG fraction of antibody raised in rabbits measured at 280 nm ($\varepsilon^{1\%} = 14{\cdot}3$).

%^{51}Cr release after 4 hr incubation of 2×10^5 rat eosinophils with 10^5 ^{51}Cr containing CRBC. Figures are means of triplicate determinations in one experiment.

Antibody to CRBC raised in rats was also shown to promote eosinophil mediated ADCC and unless otherwise indicated, was the antibody used for the experiments reported below. In the presence of this antibody at dilutions from 1/1024 to 1/128, eosinophils normally gave ^{51}Cr release over the range 10–50%. The relationship between antibody dilution and ^{51}Cr release was usually linear. The results from a typical experiment are shown in Fig. 1. Rat neutrophils were also capable of mediating ADCC and had similar requirements for antibody (see Fig. 2).

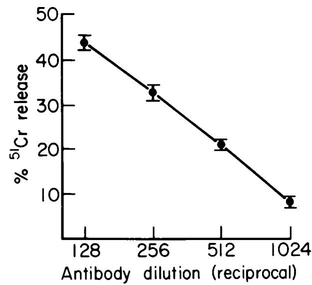

Fig. 1. ADCC of chicken erythrocytes by rat eosinophils. Data from a typical experiment to show % ^{51}Cr released after incubation for 4 hr of 2×10^5 eosinophils with 10^5 ^{51}Cr-CRBC in the presence of rat antibody as indicated. Points represent mean ± s.d. of triplicates from a single experiment.

Eosinophil-mediated ADCC was rapid with around 50% of the total ^{51}Cr released at 4 hr being released in the first 60 min. This fast phase was followed by slower release which plateaued between 3 and 4 hr.

Cell–cell interaction and ADCC: bystander lysis experiment

The restriction of lysis to those CRBC which have interacted with eosinophils via red cell bound antibody was demonstrated in the following manner. Unlabelled CRBC, which had been precoated with antibody, were mixed with uncoated CRBC containing ^{51}Cr and eosinophils. After 4 hr incubation, less than 10% ^{51}Cr release was observed. These results indicate that target cell lysis of those cells which have not interacted with eosinophils via antibody does not occur.

The effect of cytochalasin B, colchicine and chloroquine on eosinophil-mediated ADCC

In order to investigate the mechanisms by which eosinophils mediate antibody-dependent cytotoxicity, the effects of cytochalasin B, colchicine and chloroquine were tested.

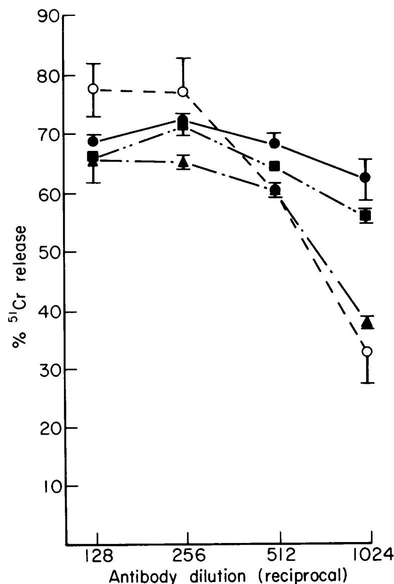

Fig. 2. ADCC of chicken erythrocytes by rat neutrophils and the effect of methylprednisolone. Data from a typical experiment to show %^{51}Cr released after incubation for 4 hr of 2×10^5 neutrophils with 10^5 ^{51}Cr-CRBC in the presence of zero (●); 10^{-3}M (○); 10^{-5}M (▲) and 10^{-7}M (■) methylprednisolone sodium succinate. Points represent mean ± s.d. of triplicate samples from a single experiment.

Table 2. The effect of cytochalasin B, colchicine and chloroquine on eosinophil ADCC.

Dilution of antibody	% ^{51}Cr released				
			Cytochalasin B (μg/ml) in DMSO (0·5%)		
	Control	DMSO 0·5%	1·25	2·5	5·0
1/128	41·5	24·8	11·9	7·4	10·5
1/256	29·8	15·9	6·1	4·5	3·3
1/512	17·3	9·7	3·6	2·0	1·0
1/1024	8·2	4·4	1·1	1·0	1·0
		Colchicine			
	Control	10^{-5}M	10^{-3}M		
1/128	23·3	18·3	9·1		
1/512	18·8	16·2	7·9		
		Chloroquine			
	Control	10^{-5}M	10^{-4}M	10^{-3}M	
1/128	41·5	40·8	7·6	8·0	

Eosinophils (2×10^5), ^{51}Cr-CRBC (1×10^5), antibody and drug were incubated at 37°C for 4 hr. Figures represent the mean % ^{51}Cr released of triplicate samples in a single experiment.

Because of the poor water solubility of cytochalasin B experiments using this compound were carried out in the presence of 0·5% DMSO. DMSO itself caused substantial inhibition of eosinophil-mediated ADCC, but as shown in Table 2 this inhibition was significantly increased by the addition of cytochalasin B. In a series of experiments, cytochalasin B (5 μg/ml) gave between 57% and 100% inhibition. Colchicine, 10^{-5} and 10^{-3} M also inhibited eosinophil ADCC as did chloroquine, 10^{-4} and 10^{-3} M (Table 2).

The effect of glucocorticoids on eosinophil-mediated ADCC

Experiments were carried out using the water soluble steroid derivative, methylprednisolone sodium succinate, because preliminary experiments using DMSO and ethanol showed that these vehicles caused marked inhibition of ADCC.

Eosinophil ADCC was inhibited by methylprednisolone and was concentration dependent over the range 10^{-7}–10^{-3}M steroid. Pooled inhibition data from five such experiments are given in Table 3. In contrast to eosinophil-mediated ADCC, neutrophil ADCC was less sensitive to inhibition by methylprednisolone (Fig. 2).

The mechanism of glucocorticoid inhibition of eosinophil-mediated ADCC

The activity of glucocorticoids has been shown in a number of systems to be dependent on protein synthesis (Baxter & Harris, 1975, Tsurufuji, Sugio & Takemasa, 1979). Experiments were therefore carried out to determine whether a similar mechanism was involved in the corticosteroid inhibition of eosinophil-mediated ADCC.

As shown in Fig. 3, the protein synthesis inhibitor, cycloheximide (25 μg/ml) had no effect on eosinophil-mediated ADCC but completely blocked the inhibitory activity of 10^{-5}M and 10^{-3}M methylprednisolone. However this block of steroid inhibition was not evident when two other

Table 3. The inhibition of eosinophil-mediated ADCC by methylprednisolone

Dilution of antibody	% Inhibition of ^{51}Cr release		
	Methylprednisolone concentration		
	10^{-7}M	10^{-5}M	10^{-3}M
1/128	8·7*	16·5	32·3
1/256	12·0*	22·9	39·4
1/512	17·2	26·7	44·2
1/1024	15·2	27·9	42·3

* $P < 0.05$ significance, all the rest $P < 0.01$ significance. Pooled data from five experiments.

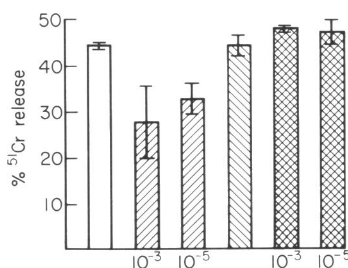

Fig. 3. Eosinophil ADCC of chicken erythrocytes, the effect of cycloheximide on steroid inhibition. ^{51}Cr release in controls (□), plus methylprednisolone (▨), plus cycloheximide, 25 μg/ml (▧) and plus methylprednisolone and cycloheximide (▩). Methylprednisolone was tested at 10^{-5}M and 10^{-3}M as indicated. Results represent mean ± s.d. of triplicate samples from a typical experiment.

inhibitors of protein synthesis, actinomycin D (25 μg/ml) and puromycin (62·5 μg/ml) were tested. In an attempt to understand the activity of cycloheximide additional properties of this compound were investigated. It has been demonstrated that cycloheximide and succinimide inhibit NADH linked dehydrogenases (Latuasan & Berends, 1958). Our experiments showed however, that succinimide did not block steroid inhibition of eosinophil ADCC. It seems unlikely therefore that dehydrogenase inhibition is important to the mechanism by which cycloheximide blocks steroid activity.

DISCUSSION

In agreement with Sanderson & Thomas (1978) it was found that lysis of CRBC occurs following the antibody-mediated interaction of targets and eosinophils and is dependent upon Fc recognition by the eosinophils. Studies with parasites have shown that antibody is also important for the attachment of eosinophils to parasites (Mackenzie *et al.*, 1977), and it has been demonstrated (Butterworth *et al.*, 1979a) that eosinophil binding to schistosomula is a two stage process and that the initial Fc dependent binding is readily reversible. Irreversible attachment subsequent to Fc-mediated binding was dependent upon degranulation onto the surface of the parasite. The importance of degranulation in CRBC ADCC by eosinophils is not known. The bystander lysis experiment (see Results) indicates that if degranulation does occur it is effective in causing lysis only of closely interacting target cells. The eosinophils did not release into the medium sufficient lytic products to lyse non-attached CRBC.

The experiments with cytochalasin B, colchicine and chloroquine were carried out to try to define the process(es) whereby target cell lysis is caused by eosinophils. The inhibition of eosinophil ADCC by cytochalasin B (5 μg/ml) is in accord with the effects of this compound on other eosinophil functions. Thus eosinophil damage to schistosomula (David *et al.*, 1977), release of granule enzymes (Zeiger & Coulten, 1977) and Fc rosette formation (Tai & Spry 1980) have all been shown to be inhibited by equivalent concentrations of cytochalasin B. In contrast neutrophil lysosomal enzyme release is enhanced rather than inhibited by similar concentrations of cytochalasin B (Zurier, Hoffstein & Weissman, 1973; Hawkins, 1973) and preliminary experiments have shown that neutrophil ADCC in the CRBC assay is not inhibited by this compound (unpublished results).

Colchicine inhibited eosinophil ADCC at 10^{-5}M and 10^{-3}M. Neutrophil ADCC has also been shown to be inhibited by 10^{-3}M colchicine (Katz *et al.*, 1980).

The lysosomotropic agent, chloroquine, (de Duve *et al.*, 1974) also inhibited eosinophil ADCC. This agent accumulates in lysosomes and interferes with lysosomal function. Neutrophil lysosomal enzyme release stimulated by immune complexes is inhibited by 10^{-4}–10^{-3}M chloroquine (Hawkins, 1974). In addition intracellular protein degradation by cathepsin B is inhibited by chloroquine (Wibo & Poole, 1974). Inhibition of either enzyme release or intracellular digestion could inhibit target cell lysis and hence explain the effect of chloroquine on eosinophil ADCC.

Target cell lysis was inhibited by glucocorticoids. Inhibition was concentration dependent in the range 10^{-7}–10^{-3}M. Other *in vitro* cell activities which are inhibited by similar levels of glucocorticoids include eosinophil chemotaxis (Gauderer & Gleich, 1978); neutrophil Fc receptor expression (Klempner & Gallin, 1978) and neutrophil lysosomal enzyme release (Hawkins, 1974). The steroid inhibition of eosinophil ADCC was blocked by cycloheximide. This suggests that the eosinophil may possess a glucocorticoid receptor and these steroid effects may result from a newly synthesized protein (Baxter & Harris, 1975). However, a similar block of steroid inhibition by other inhibitors of protein synthesis, actinomycin D and puromycin, has not been observed. Although it is possible that technical reasons were responsible for their lack of effect, e.g. cell permeability, time necessary for interaction, it is a cause for concern that these two inhibitors of protein synthesis did not block steroid effects in the same manner as cycloheximide. Possible alternative activities for cycloheximide have been sought. A report that cycloheximide and succinimide inhibit dehydrogenase enzymes in yeast (Latuasan & Berends, 1958) was followed up by investigating the effects of succinimide on steroid inhibition of ADCC. Succinimide did not block the steroid effect. This suggests that although cycloheximide may have inhibitory effects on dehydrogenases, this activity is not responsible for the blocking of steroid inhibition by cycloheximide.

Steroids could have many points of influence in a multicomponent system such as ADCC. Further information as to the mechanism of target cell lysis is needed before the point of action of steroids can be examined in more detail.

In conclusion, therefore, it has been shown that the mechanism of rat eosinophil ADCC involves Fc receptor activity and it is inhibited by cytochalasin B, colchicine and chloroquine. The observation that glucocorticoids can inhibit this eosinophil activity leads to the suggestion that steroid control of eosinophil activities in the tissues may be important in the mode of action of steroids *in vivo*.

The gifts of rabbit anti-CRBC IgG and $F(ab')_2$ fragment from Dr E.V. Elliott are gratefully acknowledged.

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