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Mouse immunoglobulin isotypes mediating cytotoxicity of target cells by eosinophils and neutrophils

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Summary. The cytotoxic activity of mouse eosinophils and neutrophils in the presence of antibodies of different isotypes has been studied. Mouse monoclonal anti-hapten antibodies of all the known mouse immunoglobulin isotypes have been used to coat hapten-coupled, ^{51}Cr-labelled target cells. Two different target cells have been used, sheep red cells, as a model for intracellular killing, and BW cell line cells, as a model for extracellular killing. It is shown that both eosinophils and neutrophils lyse sheep red cells coated with IgG1, IgG2a and IgG2b and to a lesser extent IgG3. No killing is detected when sheep red cells are coated with IgM, IgA or IgD. Neutrophils, but not eosinophils are shown to lyse IgE-coated sheep red cells. When tested against BW cells, neutrophils have been found to induce high levels of ^{51}Cr release in the presence of IgG1, IgG2a, IgG2b and IgE, but not when IgG3, IgM, IgA or IgD were used. In contrast, no killing of BW cells by eosinophils could be detected with any of the different antibody isotypes tested. However, it is shown that eosinophils are able to kill IgG-coated BW cells when hapten coupling is increased to maximum levels or when complement is added into the system, emphasizing our previous results showing that eosinophils require much higher levels of ligands than neutrophils to be effective. To test the possibility that eosinophils have a weak IgE receptor, complement was added to IgE-coated BW cells by means of a monoclonal IgM anti-Thy-1 antibody but no cytotoxicity was detected. It cannot be completely excluded that eosinophils have IgE blocking a putative IgE receptor.

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INTRODUCTION

The main function of the Fc receptor on granulocytes is to allow the attachment of these cells to antibody-coated particles. If the particles are small enough, granulocytes phagocytose them, while if they are too large to ingest, specific extra cellular degranulation on the surface of the antibody-coated particles occurs. Thus, by interacting with the antibody bound to the target cells, granulocytes can carry out their effector function.

Little is known about the immunoglobulin isotypes that are able to promote attachment and cytotoxicity of target cells by granulocytes, particularly eosinophils. In the few experiments carried out, rat eosinophils have been shown to adhere to schistosomula of *Schistosoma mansoni* mainly in the presence of IgG1 (Ramalho-Pinto, De Rossi & Smithers, 1979). Similarly, rat eosinophils appear to kill the same parasite in the presence of IgG2a (Capron, Capron, Torpier, Bazin, Bout & Joseph, 1978) and IgE (Capron, Bazin, Joseph & Capron, 1981). However, these experiments have relied upon the use of fractionated antiserum and

immunoadsorbants where some contamination with other isotypes cannot be ruled out. The advent of monoclonal anti-target cell antibodies of different isotypes appeared to offer a better alternative than conventional antisera. However, previous attempts using monoclonal anti-sheep red cell antibodies (López, Strath & Sanderson, 1981) have shown that because monoclonal antibodies are directed towards different antigens, comparable antibody density on the target cells cannot be achieved, and so no conclusions can be drawn from the differences observed between isotypes.

In the present study we have used monoclonal anti-hapten antibodies against hapten-coupled target cells to test the cytotoxic activity of mouse eosinophils and neutrophils in the presence of all the known mouse immunoglobulin isotypes. Since granulocytes can kill phagocytosable targets like micro-organisms and protozoa, and extracellular targets like tumour cells and multicellular parasites, two different systems have been studied: cytotoxicity of sheep red cells as a model for intracellular killing and cytotoxicity of BW cell line cells as a model for extracellular killing.

MATERIALS AND METHODS

Effector cells

Mouse eosinophils and neutrophils were obtained and purified as previously described (López *et al.*, 1981). Briefly, eosinophils were harvested from the peritoneal cavity of *Mesocestoides corti*-infected BALB/c mice and separated from lymphocytes and macrophages by velocity sedimentation at 1 *g*. The eosinophil-rich fractions were then pooled and centrifuged on an interface of 16% Metrizamide (Nyegaard & Co. A/S, Oslo). The pellet fraction consistently gave preparations of >95% pure eosinophils. Neutrophils, harvested from the peritoneal cavity of BALB/c mice which had been injected with 3·5% dextran, were purified by centrifugation on Ficoll-Paque (Pharmacia), followed by centrifugation on a two-step Metrizamide gradient of 15·5% and 17·5%.

Target cells

Sheep erythrocytes (SRBC) were kept in Alsever's solution at 4°. BW 5147 cell line cells (a mouse thymoma from AKR strain that expresses Thy-1 antigen) were maintained in RPMI 1640 medium containing 10 mM HEPES, antibiotics and 10% foetal calf serum.

Preparation of target cells

TNP coupling. This was done essentially as described by Dresser (1978) for SRBC and by Schmitt-Verhulst & Shearer (1975) for BW cells. Briefly, 1 ml of 10% SRBC was mixed with 7 ml of 2, 4, 6,-trinitrobenzene sulphonate (TNBS; Sigma) for 20 min at 20°, while 0·5 ml of BW cells (10^7/ml) were incubated with 0·1 ml of TNBS for 10 min at 37°. TNBS was always freshly made at 10 mg/ml in PBS and adjusted to pH 7·2.

NP coupling. The azide derivative of 4-hydroxy-3-nitrophenacetyl (NP) was prepared as described by Brownstone, Mitchison & Pitt-Rivers (1966) and kept as a 5 mg/ml solution in dimethyl-sulphoxide at −70°. 10 μl of NP-azide were mixed with 0·5 ml of SRBC (2×10^8/ml) or BW cells (2×10^7/ml) for 10 min at 37°.

SRBC and BW cells were washed twice in PBS before incubation with TNBS or NP-azide. At the end of the incubation period the cells were resuspended in medium, washed once and incubated with 100 μCi of Na_2 [^{51}Cr]O_4 (Radiochemical Centre, Amersham) for 1 hr at 37° and then washed further three times in medium.

Monoclonal antibodies of different isotypes and complement

Mouse monoclonal anti-TNP and anti-NP antibodies were produced by the following: the IgM (K7), IgG2a (K3), and IgG2b (K1) anti-DNP by Dr M. Kennedy; the IgG1 (Hy 5·19) and IgG2a (Hy 1·2) anti-DNP by Dr G. Köhler; the IgG1 anti-DNP (F42/8/1) by Dr B. Askonas; the IgG3 (S24) and IgD (B1-8 IgD) anti-NP by Dr K. Rajewsky (Reth, Hämmerling & Rajewsky, 1978); the IgE anti-TNP (IGELa1 and IGELa2) by Dr M. Wabl (Rudolph, Burrows & Wabl, 1981), and the rat IgM anti-mouse Thy-1 (NIMR3) by Dr A. Chayen (Chayen & Parkhouse, 1982). All these monoclonal antibodies were used in the form of ascites. The isotype of the IgM and IgG anti-hapten monoclonal antibodies was confirmed in an antiglobulin test using anti-mouse isotype specific antisera (gift from Dr D. Dresser). Neither IgA, nor IgE, nor IgD were reactive with these antisera. The presence of IgA was confirmed by precipitation with an anti-IgA (Meloy, Springfield, Va) specific antiserum. IgE was confirmed by inducing a passive cutaneous anaphylaxis (PCA) reaction in rats. Fresh A strain mouse serum (C5 deficient) was used as the source of complement.

Direct haemagglutination

This was done by mixing 5 ml of hapten-coupled SRBC (10^6/ml) and 5 ml of antibody dilution. After 1

hr at room temperature the cells were centrifuged and the pellets resuspended in 1 ml of medium. The last dilution still showing aggluttinated SRBC was then recorded (Table 1).

Radioimmunoassay

SRBC (10^5/well) were made to adhere to the wells of a microtitre plate (flexible polyvinyl chloride, 96 'U' wells, Dynatech Laboratory Inc., Va) by pretreatment with 0·05 mg/ml of Polybrene (Sigma). Serial dilutions of monoclonal anti-hapten antibodies were then incubated with the cells for 40 min. The wells were then washed in phosphate-buffered saline (PBS) containing 0·1% gelatin and 0·02% Tween 20. ^{125}I-labelled goat anti-mouse κ chain (gift from Dr R.M.E. Parkhouse) or rabbit anti-mouse IgG was then added for a 40 min period, at the end of which the wells were washed as before.

^{51}Cr release assay

Equal volumes (100 μl) of target cells (SRBC at 10^6/ml; BW cells at 10^5/ml), antibody dilution and effector cells were mixed and incubated at 37° for 4 hr. Effector:target ratios were 5:1 for SRBC and 20:1 for BW cells. In experiments where complement was added to the assay, BW cells were incubated first with excess rat IgM monoclonal antibody anti-mouse Thy-1 antigen. To 100 μl of the BW-IgM complex were then added 50 μl of antibody dilution, 50 μl of complement dilution and 100 μl of effector cells.

Percentage specific ^{51}Cr release was calculated from the percentage isotope release: [(test − control)/(total − control)] × 100, where 'control' is the isotope released in the absence of antibody, complement or effector cells, and 'total' is the isotope released by treating the target cells with Triton X-100 (90% for SRBC, and 80% for BW cells). Data was analysed by analysis of variance and Duncan's multiple range test.

RESULTS

Lysis of SRBC coated with antibodies of different isotypes

Table 1 summarizes the properties and binding activity of the monoclonal anti-hapten antibodies used. Although more complete data could not be obtained due to the lack of suitable reagents (an anti-λ chain serum, for example), the combination of a binding assay with an anti-IgG and an anti-κ chain sera, with a haemagglutination assay, clearly shows that all the monoclonals have high antibody activity. IgD is the exception to this and could not be fully tested. This IgD showed low haemagglutinating activity but so did the other two monoclonals anti-NP, the IgG3 and an IgG with mixed γ1 and γ3 heavy chains despite showing high levels of binding.

In each case a full titration experiment was carried out to compare the agglutinating, binding and cell-mediated cytotoxic activity of the monoclonals. A

Table 1. Monoclonal anti-hapten antibodies used

Isotype	Name	Hapten	Light chain	Radioimmunoassay		HA titre
				Anti-mouse IgG	Anti-mouse κ	
IgM	K7	DNP	λ	ND	ND	10^{-6}
IgG1	Hy5.19	DNP	κ	$24{\cdot}2(10^{-3})$*	$18{\cdot}7(10^{-3})$	10^{-5}
IgG1	F42/8/1	DNP	κ	ND	ND	3×10^{-5}
IgG2a	K3	DNP	κ	$73{\cdot}0(10^{-3})$	$27{\cdot}0(10^{-3})$	10^{-5}
IgG2a	Hy1.2	DNP	κ	ND	ND	3×10^{-5}
IgG2b	K1	DNP	κ	$13{\cdot}3(10^{-4})$	$10{\cdot}0(10^{-4})$	3×10^{-5}
IgG3	S24	NP	λ	$27{\cdot}6(10^{-2})$	$11{\cdot}6(10^{-2})$	10^{-2}
IgA	MOPC315	DNP	λ	ND	$4{\cdot}5(10^{-1})$	3×10^{-7}
IgE	IGELa 1	TNP	κ	$5{\cdot}8(10^{-2})$	$17{\cdot}6(10^{-2})$	10^{-6}
IgE	IGELa 2	TNP	κ	$9{\cdot}6(10^{-2})$	$20{\cdot}4(10^{-3})$	3×10^{-6}
IgD	B1-8.IgD	NP	λ	$2{\cdot}9(10^{-2})$	$3{\cdot}6(10^{-2})$	3×10^{-2}
Ig(γ1γ3)	S19	NP	κ	$55{\cdot}0(10^{-3})$	$45{\cdot}7(10^{-3})$	10^{-3}

* Counts per second after subtracting background. The highest dilution of the monoclonal giving this level of binding is shown in parenthesis.

ND, not done.

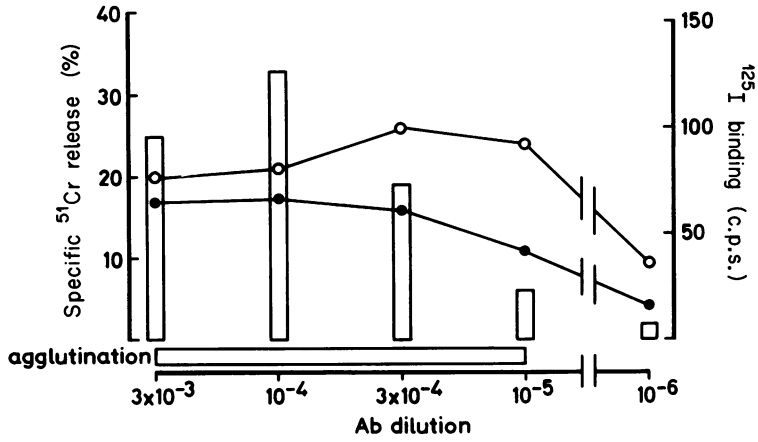

Figure 1. Titration of IgG2a (K3) anti-DNP onto TNP-coupled SRBC by a ^{51}Cr release assay with eosinophils (●) and neutrophils (○), and by direct haemagglutination (horizontal column) and radioimmunoassay (vertical columns).

typical titration experiment with IgG2a (Fig. 1) showed that maximum cytotoxic activity by eosinophils and neutrophils occurred at high antibody concentration, as judged by the high binding obtained and the fact that the antibody was still at agglutinating concentration. A prozone effect in the radioimmunoassay was always noticeable. This is probably due to the fact that in the radioimmunoassay the cells are washed several times and excess antibody is removed, in contrast to the ^{51}Cr release assay in which cells and antibody remain in the mixture throughout.

The levels of cytotoxicity induced by eosinophils and neutrophils when SRBC were coated with the different antibody isotypes are shown in Table 2. Similar levels of cytotoxicity were obtained with each of the isotypes IgG1, IgG2a and IgG2b, although neutrophils were more active than eosinophils. Mixing these subclasses together did not result in increased cytotoxicity by eosinophils and neutrophils. When IgG3 was used, low levels of cytotoxicity were obtained with both effector cells. It was possible to test different monoclonals of the same isotype and in each case essentially similar results were obtained. Similarly, if purified antibody was used instead of ascites, no significant changes in the levels of killing were seen.

Although IgA by itself was unable to induce cytotoxicity by neutrophils, it was used in mixtures with IgG2a to test whether it could have a synergistic effect with IgG in promoting killing by neutrophils as previously suggested (see discussion). It was found, however, that while neutrophils induced good levels of cytotoxicity (30·9%) in the presence of IgG1 (Hy 5.19) at 10^{-2} dilution in the absence of IgA, this was decreased to 28·6%, 20·1% and 9·5% when IgA was added at dilutions of 10^{-5}, 10^{-4} and 10^{-3}, respectively. The same degree of inhibition was observed when different concentrations of IgA were mixed with IgG2a (K3) at 10^{-3}.

Killing of BW cells coated with different isotypes

Neutrophils but not eosinophils were found to induce high levels of cytotoxicity from hapten-coupled BW

Table 2. Cytotoxicity of hapten-coupled SRBC by eosinophils and neutrophils in the presence of antibodies of different isotypes

	Effector cells	
Isotypes	Eosinophils	Neutrophils
IgM (K7)	0·1* NS	1·7 NS
IgG1 (Hy5.19)	15·1	29·8
IgG2a (K3)	17·4	26·3
IgG2b (K1)	17·0	28·8
IgG3 (S24)	7·5	3·9
IgA (MOPC315)	0·1 NS	0·6 NS
IgE (IGELa2)	0·5 NS	24·8
IgD (B1-8.IgD)	1·2 NS	0·5 NS

* Percentage specific ^{51}Cr release.

NS, not significantly different from control (absence of antibody or effector cells) at the 5% level. All other values are significantly different at the 5% level or greater.

Table 3. Cytotoxicity of hapten-coupled BW cells by neutrophils in the presence of antibodies of different isotypes

Isotypes	Specific ^{51}Cr release (%)
IgM (K7)	2·7 NS
IgG1 (Hy5.19)	70·9
IgG2a (K3)	84·4
IgG2b (K1)	85·2
IgG3 (S24)	0·6 NS
IgA (MOPC315)	0·5 NS
IgE (IGELa2)	48·9
IgD (B1-8.IgD)	0·8 NS

NS, not significantly different from control (absence of antibody or effector cells) at the 5% level. All other values are significantly different from control at the 5% level or greater.

cells (Table 3). Similarly to the lysis of SRBC, neutrophil activity against BW cells was dependent on the presence of antibodies of the IgG1, IgG2a, IgG2b and IgE isotypes. IgM, IgA and IgD did not induce significant cytotoxicity by neutrophils nor did IgG3 in this system. Eosinophils, however, induced 20% cytotoxicity of IgG2a-coated BW cells when these were treated with three times as much TNBS for $1\frac{1}{2}$ hr at 20°. In this way the amount of ligands on BW cells could be almost doubled, as a radioimmunoassay with an anti-κ chain serum showed the levels of ^{125}I to have increased from 223·8 to 393·4 counts per second. It should be noted that although cytotoxicity of BW cells by eosinophils could be thus demonstrated, toxic effects on the target cells were becoming apparent as suggested by the fact that the uptake of ^{51}Cr by the target cells was reduced by half.

Table 4. Cytotoxic activity of eosinophils and neutrophils against IgE and IgG2a-coated BW cells in the presence of complement

	Effector cells	
TNP-BW coated with	Eosinophils	Neutrophils
IgM	2·4* NS	0·0 NS
IgM + C3	0·5 NS	23·0
IgG2a(10^{-3})	0·0 NS	81·3
IgG2a(10^{-3}) + C3	33·3	82·5
IgG2a(10^{-6})	0·2 NS	22·7
IgG2a(10^{-6}) + C3	ND	29·8
IgE(10^{-2})	0·0 NS	37·0
IgE(10^{-2}) + IgMαφ + C3	0·5 NS	36·1
IgE(3×10^{-3})	3·7 NS	4·9
IgE(3×10^{-3}) + IgMαφ + C3	ND	33·4

* Percentage specific ^{51}Cr release.

NS, not significantly different from control (absence of antibody or effector cells) at the 5% level. All other values are significantly different from control at the 5% level or greater.

ND, not done.

In order to increase the ligand density on BW cells in a non-toxic manner, complement was added to the system. It was found (Table 4) that while complement and IgG2a were unable to induce cytotoxicity by eosinophils when used separately, they showed a synergistic effect when added together. Neutrophils, on the other hand, were cytotoxic to BW cells in the presence of either complement or IgG2a.

To test the possibility that complement could show up a weak IgE receptor on eosinophils, complement was added to IgE-coated BW cells by means of a monoclonal IgM anti-Thy-1 antigen since IgE does not fix complement. It was found (Table 4) that while complement and IgE did not induce cytotoxicity of BW cells by eosinophils, complement enhanced cytotoxicity of IgE-coated BW cells by neutrophils from 4·9% to 33·4%.

DISCUSSION

Mouse eosinophils and neutrophils have been shown to carry out their cytotoxic activity through receptors for antibodies of the IgG1, IgG2a, IgG2b and to a lesser extent IgG3 isotypes. No cytotoxic activity was detected when IgM, IgA or IgD isotypes were used. IgE was found to induce cytotoxicity of SRBC and BW cells by neutrophils but not eosinophils. This was a surprising finding in view of the well known association between eosinophilia and IgE in allergic reactions and parasitic infections and previous reports (Capron *et al.*, 1981; Haque, Ouaissi, Joseph, Capron & Capron, 1981) suggesting an effector role for the eosinophil against IgE-coated parasites.

Eosinophils have been shown to induce similar levels of ^{51}Cr release from SRBC coated with IgG1, IgG2a or IgG2b. This observation makes it unlikely that these cells adhere better to targets coated with IgG1 rather than with IgG2a or IgG2b as previously suggested (Ramalho-Pinto *et al.*, 1979). The differences observed by these authors are probably related to the amount of anti-target antibody in each IgG subclass fraction. Similarly, neutrophils have been shown to be cytotoxic to SRBC or BW cells to a similar

extent whether IgG1, IgG2a or IgG2b are used. In all these experiments neutrophils have always been found to be more active than eosinophils.

The fact that similar levels of ^{51}Cr release are obtained with IgG1 or IgG2b anti-TNP-coated SRBC supports our previous speculation (López *et al.*, 1981) that the higher levels of ^{51}Cr release induced by granulocytes when SRBC were coated with IgG2b anti-SRBC as compared with IgG1 anti-SRBC was due to differences in the antibody density on the SRBC membrane. This emphasizes the advantage of a system that uses hapten-coupled target cells where the antigen concentration is kept constant and only the antibody affinity will influence the amount of antibody bound to the target cells. However, antibody binding affinity is not likely to be an important factor in the ^{51}Cr release assay where no washing of the antibody is involved and this can be tested at agglutinating concentrations.

IgG3 has been shown to be less effective than the other IgG subclasses in promoting lysis of SRBC by eosinophils and neutrophils. This does not seem to be the result of a lower number of molecules of IgG3 bound to NP-SRBC since a radioimmunoassay with an anti-mouse IgG showed similar binding values to those obtained with IgG1, IgG2a or IgG2b (Table 1). Furthermore, the low activity of IgG3 with mouse eosinophils and neutrophils has also been found with a monoclonal IgG3 anti-TNP antibody recently obtained from Dr J.B. Fleischman that had a haemagglutination titre of 10^{-5}. It remains to be seen whether granulocytes have a low number of Fc receptors for IgG3 or lack them completely, the low activity observed being due to IgG3 cross-reactivity with the Fc receptor for the other IgG subclasses.

In contrast to neutrophils, eosinophils have been shown not to be cytotoxic against IgE-coated SRBC or BW cells. IgE has been found unable to induce cytotoxicity of BW cells by eosinophils even when complement was added by means of a monoclonal IgM anti-Thy-1, a treatment shown to enhance killing of IgE-coated BW cells by neutrophils. Furthermore, the addition of complement has been shown to induce killing of BW cells by eosinophils if the targets are coated with IgG2a instead of IgE. Although the possibility that eosinophils obtained from *M. corti*-infected mice may have had host IgE blocking a putative IgE receptor cannot be completely excluded, a radioimmunoassay with an anti-mouse κ chain showed insignificant binding to the surface of these eosinophils. Thus, these results are in agreement with those of Mehta, Sindhu, Subrahmanyam, Hopper & Nelson (1982) who working with rat cells found that neutrophils but not eosinophils killed IgE-coated microfilariae. On the other hand, rat eosinophils have been claimed to kill schistosomula of *S. mansoni* (Capron *et al.*, 1981) and microfilariae (Haque *et al.*, 1981), although in these reports other contaminating cells may have played an effector role.

In contrast to the observation of Shen & Fanger (1981), we have found no synergistic effect between IgG and IgA in inducing cytotoxicity of target cells by neutrophils. Instead, IgA has been shown to inhibit the lysis of IgG-coated SRBC by neutrophils, presumably through competition with IgG for the same determinants. This is in agreement with a recent report showing that in rats, secretory IgA inhibits phagocytosis of IgG-coated bacteria by granulocytes (Magnusson & Stjernström, 1982). Thus, while IgA may be important in neutralizing micro-organisms in mucosal surfaces, it is unlikely that this will promote phagocytosis and elimination of the micro-organisms by granulocytes. Neither eosinophils nor neutrophils have been found to be cytotoxic against SRBC or BW cells coated with IgM or IgD. IgM may then be relevant to granulocytes by its ability to fix complement but has no direct interaction with these effector cells.

In view of the large body of evidence attributing an effector role to eosinophils against multicellular parasites, it is surprising to find that *in vitro* these cells are less effective than neutrophils. However, the requirement by eosinophils for high antibody density on the target cell should be borne in mind. Thus, it must be noted that although eosinophils are poorly cytotoxic against antibody-coated hapten-coupled BW cells, they show high cytotoxic activity when BW cells are coated with an anti-mouse serum (López & Sanderson, 1982). It is therefore possible that *in vivo* a higher antibody density than the one observed in the TNP or NP system is achieved. Furthermore, the presence of complement *in vivo* probably ensures that the target cell is coated with enough ligands to allow eosinophils to attach and carry out their cytotoxic activity.

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