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CHARACTERIZATION OF THE ANTIBODY-DEPENDENT CYTOTOXIC CELL
A NON-PHAGOCYTIC MONOCYTE?

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SUMMARY

Death of antibody-coated chicken erythrocytes (CRBC) mediated by non-phagocytic cells was studied. The distribution of effector cells in mouse spleen lymphoid populations after fractionation by velocity sedimentation on Ficoll gradients, isopycnic centrifugation on BSA gradients, and affinity chromatography on Sephadex-linked anti-Fab columns was followed. Cytotoxicity corresponded closely with the distribution of the non-phagocytic monocytes. The cytotoxic effector cell showed similar characteristics to monocytes with respect to size, surface adherence properties and binding affinity for immunoglobulin subclasses.

INTRODUCTION

In a previous paper (Greenberg *et al.*, 1973) we proposed that the non-phagocytic cell death of antibody coated chicken erythrocytes (CRBC) by normal mouse spleen cells was caused by a 'null' lymphoid cell, that is, a cell with neither B nor T surface markers. The present study reports experiments which suggest that the cytotoxic cell is a non-phagocytic monocyte and describes some of its functional characteristics.

MATERIALS AND METHODS

Preparation of lymphoid cells

Normal BALB/c spleens were gently teased apart with forceps and the debris allowed to settle for 15 min at 4°C. The cells were then washed three times in Eagle's minimum essential medium (MEM) and treated with iron powder to remove phagocytes (Greenberg, 1973). Spleens used for velocity sedimentation were prepared in phosphate buffered saline (PBS) made up in triple distilled water.

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Velocity sedimentation on linear Ficoll gradients

Velocity sedimentation was carried out as previously described (Greenberg, 1973). Briefly, a linear Ficoll gradient of 4–10% was generated by an LKB Ultrograd gradient generator in 50 ml polycarbonate tubes giving a gradient height of 9·5 cm. A sample of 2×10^8 spleen cells, free of phagocytes, were carefully layered on the surface of the gradient and centrifuged at 100 ***g*** for 15 min. The gradient was then fractionated by an Isco density gradient fractionator into 2 ml samples. The cells were washed twice and counted in haemocytometer chambers. Cell recovery was 65–72%.

BSA density fractionation

One millilitre of phagocyte-free spleen cell suspension (3×10^7/ml) was layered on 3 ml of 30% BSA in Tyrode's buffer then spun at 1000 ***g*** for 15 min. Cells remaining on the surface of the BSA and those that had pelleted were removed with a pipette and washed three times in Eagle's MEM.

Sephadex-linked sheep anti-mouse Fab columns

Details of this procedure have been given previously (Schlossman & Hudson, 1973; Greenberg *et al.*, 1973). Sheep anti-mouse Fab or normal sheep Ig (NSIg) were linked covalently to Sephadex with cyanogen bromide. Spleen cells were passed through the anti-Fab or NSIg columns and the effluent assessed for Ig-bearing cells by indirect immunofluorescence. The effluent from the anti-Fab columns usually contained $<3\%$ Ig-bearing cells compared to 30–45% in the NSIg column effluents.

Cell morphology

Cell samples were spun onto glass slides by a cytocentrifuge and fixed in methanol. Morphology was assessed independently by two observers after staining with May–Grünwald and Giemsa.

Cytotoxicity assay

Cytotoxicity was assayed by the method of Perlmann & Perlmann (1970). Rabbit anti-chicken erythrocyte (CRBC) antibody was raised by injecting 2 ml of 10% CRBC intravenously every week for 4 weeks. Rabbits were bled 7 days later and the serum heat inactivated. Optimal titres for use in the cytotoxic assay were determined.

The cytotoxic assay was set up in a 600 μl incubation mixture consisting of 200 μl rabbit anti-CRBC (1:10,000), 200 μl of lymphoid cells (5×10^5/ml to $2{\cdot}5 \times 10^6$/ml), 100 μl ^{51}Cr-labelled CRBC (2×10^5/ml) and 100 μl sheep erythrocytes (2×10^7/ml). Sheep erythrocytes were added to the assay to reduce the spontaneous release of ^{51}Cr from the CRBC (van Boxel *et al.*, 1972). All samples were assayed in triplicate. After 18 hours incubation at 37°C in a 5% CO_2 atmosphere, 800 μl of Eagle's MEM was added and the incubation mixture centrifuged at 2000 rev/min for 5 min. A portion of the cell free supernatant was separated and the two fractions counted. Cytotoxicity was calculated according to the following formula:

$$\frac{\%\,^{51}\text{Cr released in the sample} - \%\,^{51}\text{Cr released in the control}}{\%\,^{51}\text{Cr released in distilled water} - \%\,^{51}\text{Cr released in the control}} \times 100.$$

The coefficient of variation on a given sample was usually < 8% when assessed at 50% of maximum cytotoxicity.

RESULTS

Fractionation of cytotoxic lymphoid cells

A. *Velocity sedimentation*, Fig. 1. illustrates the distribution of cytotoxic cells in fractionated spleens and compares them to the total population of lymphoid cells and the percentage of Ig-bearing B cells in each fraction. Cytotoxicity was assessed at a constant lymphoid to target cell ratio of 10 : 1.

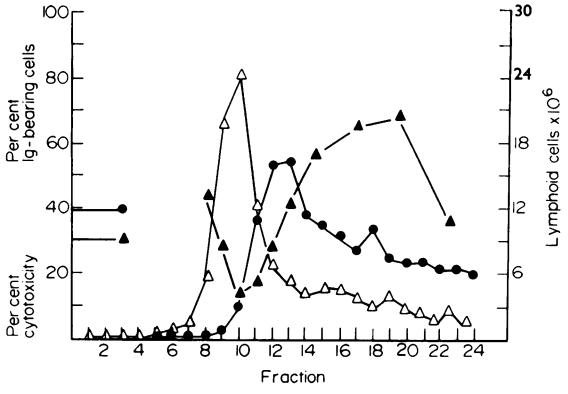

FIG. 1. Fractionation of normal mouse spleen by velocity sedimentation. The distribution of cytotoxic cells (●) is compared to the total population of lymphoid cells (△) and the percentage of Ig-bearing B cells (▲) in each fraction. Cytotoxicity is assessed at a lymphoid to target cell ratio of 10:1. The percentage of Ig-bearing B cells and cytotoxicity in unfractionated spleen is illustrated on the left ordinate.

Fractionation by velocity sedimentation, which depends primarily upon cell diameter, results in larger cells moving a greater distance through the gradient. The cytotoxic cells, having their maximal concentration in fractions 12 and 13, are therefore distinctly larger than most splenic lymphoid cells which were found in earlier fractions. Although 45% of the recovered cells were in fractions 8, 9 and 10, virtually no cytotoxic cells could be detected.

Ig-bearings B cells were identified by indirect immunofluorescence using purified sheep anti-mouse Fab and fluorescent rabbit anti-sheep Ig. In Fig. 1 their distribution is expressed as the percentage of the total lymphoid cell population in each fraction. It is clear that the distribution of cytotoxicity bears no relationship to the percentage of Ig-bearing B cells. Fractions 8 and 9 which contain 44 and 28% Ig-bearing cells respectively have no demonstrable cytotoxic cells. Similarly, as the percentage of Ig-bearing cells increases further down the gradient the cytotoxicity in corresponding fractions is decreasing.

The percentage of Ig bearing B cells shows an apparent decrease in fractions coinciding with the peak of lymphoid cell distribution. This could represent a separation of B from other lymphoid cells, or alternatively, that the expression of the Ig receptor of the B cell is varying with cell size. The latter possibility would be similar to the observation of Buell &

Fahey (1969) who showed that Ig receptor expression varied during different stages of the cell cycle in a synchronized human lymphoma.

Fig. 2 compares the distribution of cytotoxicity to morphologically identifiable spleen subpopulations. Cell distribution is expressed as the mean of two independent observations. No correlation could be demonstrated between the distribution of cytotoxic cells and either small lymphocytes or lymphoblasts (Fig. 2a). However, both monocytes and polymorphonuclear leukocytes (PMN) coincided closely with cytotoxicity (Fig. 2b and c).

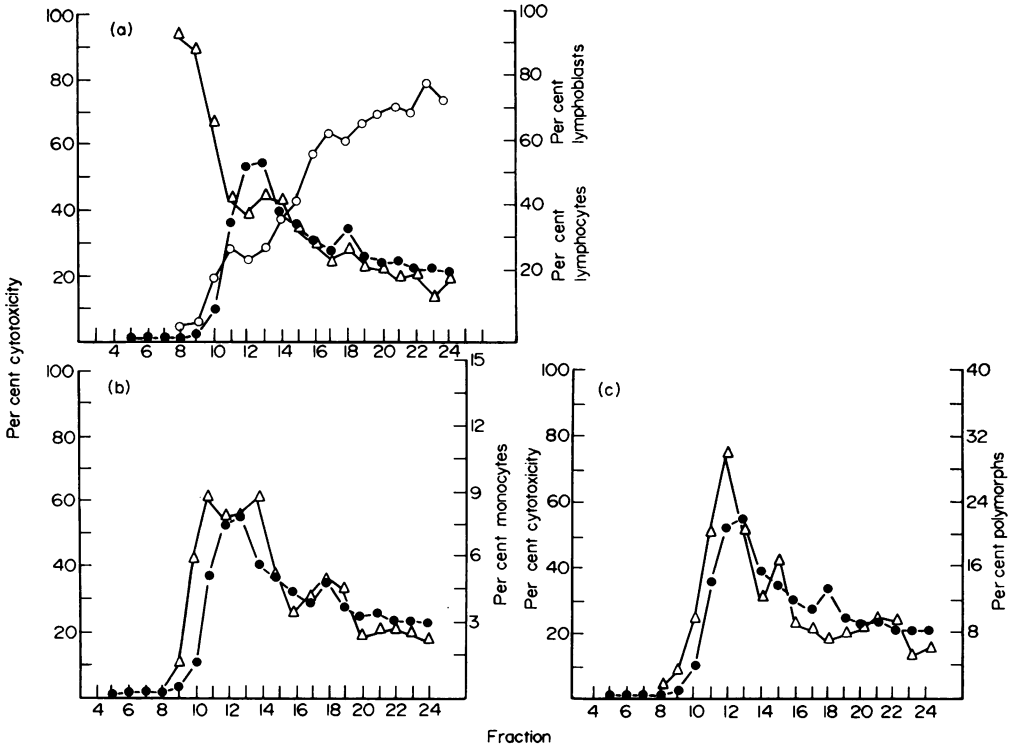

FIG. 2. Fractionation of normal spleen cells by velocity sedimentation. The distribution of cytotoxic cells (●) compared to (a) small lymphocytes (△) and lymphoblasts (○), (b) monocytes, (△) and (c) polymorphonuclear leucocytes (△).

B. *BSA density fractionation.* Spleen cells freed of phagocytes were fractionated into monocyte-enriched PMN-depleted (low density) and monocyte-depleted PMN-enriched (high density) populations by sedimentation through 30% BSA. Fig. 3 compares the cytotoxicity of the high and low density fractions to whole spleen and to the percentages of the various lymphoid subpopulations. Monocyte-enriched fractions show an increase in cytotoxicity relative to unfractionated spleen, and conversely monocyte-depleted fractions exhibit greatly reduced cytotoxicity. The distribution of PMN bears an inverse relationship to that of the cytotoxic cell.

The effect of anti-Fab on anti-Fab column fractionated spleen cells

In previous experiments (Greenberg *et al.*, 1973) it was demonstrated that spleens deprived of Ig-bearing B cells by passage through an anti-Fab column were enriched in cytotoxic cells relative to cells passed through a normal Ig column. The depletion of Ig-bearing cells was demonstrated by the loss of surface staining with fluorescein-labelled purified rabbit anti-mouse Fab. To further demonstrate that the few Ig-bearing cells which pass through

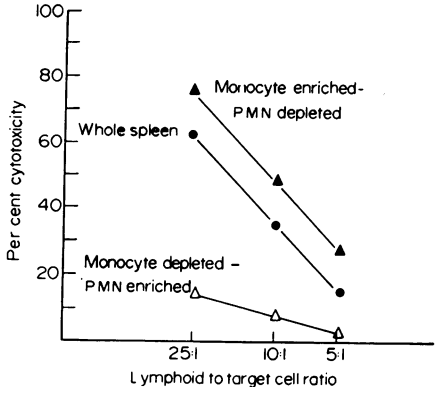

FIG. 3. Fractionation of normal spleen cells by centrifugation through 30% BSA in Tyrode's buffer. Cytotoxicity in the (▲) low density and (△) high density fractions is compared to (●) whole spleen. Morphological assessment of the fractions was made with May–Grünwald Giemsa:

	% lymphocytes	% monocytes	% PMN
Unfractionated spleen	75·2	12·3	12·5
Low density fraction	58·5	34·5	7·0
High density fraction	68·4	3·4	28·2

TABLE. 1 The effect of sheep anti-mouse Fab on the cytotoxicity of anti-Fab column effluent

Lymphoid* cells		Lymphoid to target cell ratio	% cytotoxicity		% reduction of cytotoxicity
Source	% Ig bearing		Anti-Fab	NSIgG	
Anti-Fab column effluent	2·8	12:1	23·8	22·6	0
Whole spleen	36·5	15:1	27·9	35·0	20·3

* In this and all subsequent experiments, lymphoid cells were freed of phagocytes before testing or fractionating unless otherwise indicated.

the column have no cytotoxic potential, the following experiment was performed. 10^7 anti-Fab column passed cells and normal spleen cells were treated with 100 μg of purified sheep anti-mouse Fab or normal sheep Ig for 30 min at 4°C and washed three times. The lymphoid

cells were then placed in the cytotoxic assay. Table 1 shows the lack of effect of the anti-Fab on the cytotoxic cells present in the anti-Fab column effluent. The inhibition of cytotoxicity in the whole spleen population is similar to that described earlier (Greenberg *et al.*, 1973).

Inhibition of cytotoxicity by heat-aggregated Ig subclasses

Highly purified immunoglobulins of various subclasses (Torrigiani, 1973) were heat aggregated at 63°C for 10 min. Fig. 4 illustrates the inhibitory effect of some of these aggregated immunoglobulins on cytotoxicity, presumably mediated by competition for the Fc receptor of the cytoxic cell with the anti-CRBC antibody. No inhibition was detected with either the aggregated IgM, IgA or Fabγ. However, profound inhibition of cytotoxicity was noted with IgG2b. IgG2a and IgG1 showed significant but less marked inhibition with IgG1 the least effective of the subclasses.

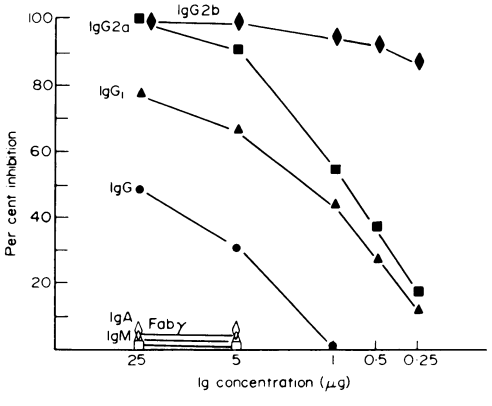

FIG. 4. Inhibition of cytotoxicity by heat aggregated immunoglobulin. Aggregated Ig subclasses were added directly to the lymphoid cells and incubated at 22°C for 15 min prior to the addition of anti-CRBC antiserum and CRBC target cells.

Surface adherence characteristics of the cytotoxic cell

Surface adherence to glass, polyacrylic beads and cotton wool was examined. 3×10^7 phagocyte-free spleen cells incubated for as little as 5 min at 37°C in a $5 \times 1{\cdot}5$ cm column of 60 mesh gas chromatography glass beads or 'Degalan' polyacrylic plastic beads, or a 400 mg column of cotton wool, resulted in considerable, if not complete, loss of cytotoxicity (Table 2). Incubations of 30 min or more totally removed all cytotoxic cells. Reducing the surface area available to the cells by using large (0·4 cm diameter) glass beads or plastic Petri dishes (Mosier, 1967) resulted in somewhat smaller but significant losses of cytotoxic cells than with the fine beads.

Cytotoxicity of phagocytic and non-phagocytic lymphoid cells

Comparison of whole spleen to iron powder-treated phagocyte-free spleen indicates that phagocytic cells account for only 15–20% of cytotoxicity observed in whole spleen (Table 3). It should be noted that only a small number (5–8%) of the remaining monocytes and PMN

found in iron treated spleens were phagocytic when subsequently tested by exposure to neutral red or zymosan at 37°C for 15 min. An occasional cell containing iron which had not been removed by the magnet could also be identified.

TABLE 2. Adherence characteristics of the cytotoxic cell

Treatment	% cytotoxicity lymphoid to target cell ratio			Morphology		
	25:1	10:1	5:1	% lymphocytes	% monocytes	% PMN
Whole spleen	54·1	34·8	19·9	84·0	6·8	9·2
Glass beads*	12·0	7·1	1·6	96·8	1·9	1·3
Cotton wool	30·0	12·4	0·1	93·1	3·9	3·0
Whole spleen	73·6	47·9	27·3	78·8	11·3	9·9
Plastic beads†	0	0	0	99·0	0·2	0·8

Cells were exposed to the various surfaces for 5 min at 37°C. All surfaces were pretreated with Eagle's MEM containing 10% FCS fo 30 min at 37°C.

* 60 mesh chromatography beads.

† Degalan polyacrylic beads.

TABLE 3. Cytotoxicity of phagocytic and non-phagocytic lymphoid cells

Lymphoid cells	% cytotoxicity lymphoid to target cell ratio		Morphology		
	25:1	10:1	% lymphocytes	% monocytes	% PMN
Whole spleen	63·0	39·6	63·3	13·2	22·5
Phagocyte-free spleen	54·1	34·8	84·0	6·8	9·2
Whole spleen	54·9	44·9	65·3	13·4	21·3
Phagocyte-free spleen	45·1	36·4	81·9	8·3	9·5

DISCUSSION

There is general agreement that a T cell-deprived lymphoid population, whether produced by thymectomy (Harding *et al.*, 1971; van Boxel *et al.*, 1972; Greenberg *et al.*, 1973), thoracic duct drainage (MacLennan & Harding, 1970a), treatment with anti-θ and complement (van Boxel *et al.*, 1972), or heterologous anti-T and complement (Greenberg *et al.*, 1973), is still capable of killing antibody-coated target cells. These observations led some investigators (Perlmann & Perlmann, 1970; van Boxel *et al.*, 1972; Möller & Svehag, 1972) to suggest that antibody-dependent cytotoxicity is due to a B cell. This was further supported by the observation that anti-κ serum pretreatment of mouse spleen cells blocks

cytotoxicity (van Boxel *et al.*, 1972). Considerable doubt about this hypothesis was raised, however, in our previous study in which Ig-bearing B cells were fractionated and recovered on digestible anti-Fab columns (Greenberg *et al.*, 1973). In these experiments the removal of Ig-bearing B cells from normal mouse spleen resulted in enrichment of cytotoxicity in the remaining cells while the B cells recovered from the anti-Fab column exhibited little or no cytotoxicity. In addition, it was suggested that the blocking effect of anti-Fab serum, similar to that demonstrated by van Boxel *et al.* (1972), was due to the release of complexes formed between antibody and cell surface Ig rather than to a direct effect on the cytotoxic cell. The present observation that sheep anti-mouse Fab does not inhibit cytotoxicity in a lymphoid population deprived of Ig-bearing B cells supports this hypothesis. Further evidence against the Ig-bearing B lymphocyte being the effector cell comes from experiments which demonstrate the separation of Ig-bearing B cells and cytotoxic cells on velocity sedimentation gradients. The observations that antibody-producing B cells are not effector cells (MacLennan & Harding, 1970b), and that neither antibody-producing cells nor the precursors of B cells have Fc receptors (Basten, Warner & Mandel, 1972) make it very unlikely that any B cell subpopulation is capable of killing antibody-coated target cells. The affinity for IgG subclasses is also quite different: whereas the B cell Fc receptors bind IgG1 the most strongly, IGg2b more weakly and IgG2a apparently not at all (Basten, Warner & Mandel, 1972), the cytotoxic cells were inhibited most effectively by aggregated IgG2b and least by IgG1.

Although these inhibition studies with aggregated Ig suffer from the limitation that only a single anti-CRBC serum was studied, it is of considerable interest that the general pattern of affinity of the Fc receptors for the various subclasses is very similar to that seen with monocytes (Hay & Torrigiani, 1973). Other data obtained in the present study point to further similarities between the cytotoxic cell and the monocyte. Using a morphological assessment of spleens fractionated by velocity sedimentation and isopycnic centrifugation, a correlation was seen only between the distribution of cytotoxicity and the monocytic cells. Like the monocyte, the cytotoxic cell is distinctly larger and less dense than the majority of lymphoid cells (Pretlow & Pushparej, 1972; Holm & Hammerström, 1972). In addition the cytotoxic cells adhered strongly to different surfaces in a way typical of monocytes (Cohn & Benson, 1965).

Considering the mechanism of cytolysis mediated by the monocyte, it is well known that phagocytosis is often an important factor (Holm & Hammerström, 1973). However, since all actively phagocytic cells were removed from the lymphoid populations we studied, it is likely that the cytotoxic cells were operating through an extracellular lytic mechanism, assuming of course that the monocytes which are non-phagocytic immediately after iron powder treatment remain so through the assay incubation. In fact extra cellular lysis of antibody-coated CRBC by lymphoid cells has already been clearly described by Biberfeld & Perlmann (1970). These observations, however, do not exclude the possibility that cells capable of phagocytosis may also kill by an extracellular lytic mechanism.

In summary then, while it has not yet proved possible to characterize the cytotoxic cell directly, the evidence argues firmly against its identification with any lymphocyte of the T or B series and in favour of its classification as a monocyte. The other view, that the killer cells belong to an as yet unrecognized subpopulation of lymphocytes, finds support only in the morphological observation that in the human at least, they resemble small lymphocytes (Biberfeld & Perlmann, 1970).

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