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Apparent direct cellular cytotoxicity mediated via cytophilic antibody

MULTIPLE Fc RECEPTOR BEARING EFFECTOR CELL POPULATIONS MEDIATING CYTOPHILIC ANTIBODY INDUCED CYTOTOXICITY

J. E. PARRILLO & A. S. FAUCI *Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland* 20014, *U.S.A.*

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Summary. Guinea-pigs immunized with chicken red blood cells (CRBC) developed cytotoxic effector cells in peripheral blood, spleen, lymph nodes, bone marrow and peritoneal exudate cells. Although it appeared that direct cytotoxicity was the mechanism of killing in this model, the true mechanism of cytotoxicity was in fact cytophilic antibody firmly bound to the effector cell rendering it specifically cytotoxic to the CRBC targets. Using multiple cell separation procedures, we demonstrated at least three distinct effector cell populations capable of mediating cytotoxicity in this model: a monocyte-macrophage, a non-phagocytic lymphocyte and a neutrophil, all bearing Fc receptors for Ig. Cell free eluates produced from immune effector cells were capable of rendering non-immune cells of all three Fc receptor bearing leucocyte classes cytotoxic.

It is noteworthy that several techniques commonly employed to deplete effector cell populations were shown also to remove cytophilic antibody from the surface of these effector cells. If this had not been recognized, the cytophilic antibody component of the system would have been overlooked and erroneous conclusions would have been made as to which cell populations were functioning as effectors.

Recent clinical studies have demonstrated a direct cytotoxicity by K lymphocytes—the usual effector cells in antibody dependent cellular cytotoxicity. The present study suggests that in at least some of these cases true direct cytotoxicity may not be the mechanism of killing and that K cells bearing cytophilic antibody may in fact be the effector cell operating by antibody dependent cellular cytotoxicity.

Correspondence: Dr J. E. Parrillo, Building 10, Room 11B06, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

INTRODUCTION

Two major types of cell mediated cytotoxicity are direct cytotoxicity and antibody dependent cellular cytotoxicity (ADCC). Although direct cytotoxicity, generally mediated by a T-cell (Cerottini & Brunner, 1974), has been demonstrated in certain models of allograft rejection and tumour immunity, it is difficult in many systems, particularly in humans, to demonstrate true direct cytotoxicity independent of antibody. On the other hand, in animals and humans, ADCC can be readily demonstrated (Perlmann & Holm, 1969) and is mediated by multiple effector cells depending on the target cell employed (MacDonald, Bonnard, Sardat & Zawodnik, 1975). In most systems, the antibody is reacted with the target cell and cytotoxicity is mediated via the Fc receptor on the effector cell (Perlmann & Holm, 1969). It has also been demonstrated, however, that effector cells

can be 'armed' with specific antibody (Perlmann & Perlmann, 1970; Perlmann, Perlmann & Biberfeld, 1972; Saksela, Imir & Makela, 1975; Temple, Loewi, Davies & Howard, 1973). In ADCC with human red blood cell targets the effector cells have been shown to be the monocyte-macrophage and the neutrophil (Holm, 1972); nucleated non-erythroid targets are killed by a non-phagocytic, non-T lymphocyte bearing a membrane Fc receptor (a K cell); and chicken red blood cells (CRBC) are somewhat unique in being killed by at least three separate effectors—the monocyte-macrophage, neutrophil and K cell (MacDonald *et al.*, 1975).

Recently, clinical reports have appeared describing 'direct' cell mediated cytotoxicity mediated by a K cell (O'Toole, Perlmann, Wigzell, Unsgaard & Zetterlund, 1973; Cochrane, Maussouros, Thompson, Eddelson & Williams, 1976)—the usual effector cell in ADCC. Since many such systems are characterized by high levels of *in vivo* antibodies directed against the target cell employed, we questioned whether in some cases this in reality could be ADCC mediated by an effector cell armed with cytophilic antibody.

The purpose of the present study was to establish the relative contributions of true direct cellular cytotoxicity versus cytotoxicity mediated by effector cells armed with cytophilic antibody in a model in which high levels of circulating target cell specific antibodies are present, and to delineate the effector cell or cells mediating this killing.

METHODS

Immunization

Strain 13 guinea-pigs were used throughout the study. Guinea-pigs were immunized with 5×10^8 CRBC in 1 ml of Freund's complete adjuvant (CFA). Animals were killed by CO_2 inhalation at 1–5 weeks post immunization and appropriate organs harvested.

Blood was obtained by intracardiac puncture, and mononuclear cells were obtained by Hypaque–Ficoll gradient centrifugation (Böyum, 1968). Mononuclear cell suspensions contained 70–85% lymphocytes, 15–30% monocytes and <2% neutrophils. Pure neutrophil suspensions (>98% neutrophils) were obtained by dextran sedimentation of the RBC-neutrophil button from the Hypaque–Ficoll centrifugation. Residual RBC were then removed by hypotonic lysis.

Spleen and lymph node (popliteal, femoral and scapular) mononuclear cells were obtained by mincing the tissues with scissors and then passing them through a wire mesh screen. The resultant cells were purified over Hypaque–Ficoll.

Bone marrow mononuclear cells were purified by a previously described method (Fauci, 1975). Briefly, 2 femurs from each guinea-pig were flushed out with RPMI-1640 media; the resultant cell suspension was applied to a sucrose gradient (15–35%) and centrifuged at 100 *g* for 7 min. The top 1/2 of the gradient contained 80–90% mononuclear cells: 65–75% lymphocytes and 15–25% monocytes.

Peritoneal exudate cells (PEC) were obtained by a previously described method (Balow & Rosenthal, 1973) in which cells were harvested 4 days after an intraperitoneal injection of oil. These PEC's consisted of 50–70% macrophages, 20–40% lymphocytes and 10–20% neutrophils.

All the cell suspensions were then washed in 50 ml of RPMI-1640 at least 4 times before being resuspended in RPMI-1640 supplemented with 10% heat inactivated foetal calf serum (FCS, 56° for 1 h) for the cytotoxicity assay.

Cytotoxicity assay

Cytotoxicity was performed in $1 \times 7{\cdot}5$ cm plastic tubes (Falcon Plastics, Oxnard, Calif.) in a total volume of 1 ml. All effector cells were used at 8×10^6, 4×10^6, 4×10^5 and 4×10^4 cells/ml in RPMI-1640 with 10% FCS when multiple effector : target ratios were done. 0·5 ml of the cell suspension was pipetted into duplicate tubes.

CRBC targets (Flow Laboratories, Rockville, Md.) were labelled with radioactive chromium (^{51}Cr) as previously described (Fauci, Balow & Pratt, 1976) and brought up to a final concentration of 2×10^5 cells/ml. Three other targets were employed to establish the specificity of the system. Human red blood cell targets obtained from an AB+ donor and sheep RBC were labelled as with CRBC. Chang liver cells were grown in 125 cm² Falcon flasks to confluence, trypsinized gently (0·05% trypsin in 0·02% EDTA) to a single cell suspension, labelled with ^{51}Cr as above and brought to 2×10^5/ml in RPMI-1640 with 10% FCS. With all targets, 0·1 ml of target cell suspension was pipetted into duplicate tubes containing 0·5 ml of effector cells at various concentrations. 0·4 ml of RPMI-1640 with 10% FCS was then pipetted into each tube to achieve a final volume of 1 ml.

Cultures were incubated at 37° in 5% CO_2 in air and 100% humidity for 18 h. Percent isotope release was determined as previously described in detail (Fauci *et al.*, 1976). The percent ^{51}Cr release of CRBC and HRBC in the presence of non-immune or non-armed cells was always <8% after 18 h of incubation. Chang targets showed substantial isotope release (30–40%) in the presence of non-immune cells and this was subtracted from immune effector release to give specific cytotoxicity.

Arming of effector cell populations
Serum from immune guinea-pigs (3 weeks post immunization with CRBC) was heat inactivated, diluted 1:5 in RPMI-1640 and incubated with non-immune effector populations for 1 h at 37°. The cells were then repeatedly washed (four times or more) with 50 ml of RPMI-1640 containing 10% FCS and used in the cytotoxicity assay.

Enumeration of surface receptors
T lymphocytes were determined by their ability to form rosettes with rabbit RBC as previously described (Fauci, 1975). B lymphocytes were identified by two methods: first, by the presence of surface immunoglobulin (sIg) demonstrated by fluoresceinated goat polyvalent anti-guinea-pig Ig and second, by their ability to bind SRBC (E) coated with IgM antibody (A) and complement (C) to form EAC rosettes. These two methods have been previously described (Fauci, 1975; Fauci *et al.*, 1976).

Surface Fc receptors were demonstrated by their ability to form rosettes with SRBC coated with rabbit anti-SRBC IgG, which were a gift from Dr Michael M. Frank and prepared as previously described (Frank & Gaither, 1970; Kedar, de Landazuri & Fahey, 1974).

Single and double rosetting assays
10^6 immune cells or armed non-immune cells were incubated for 5 min with 10^7 CRBC on a rotator. The cell mixture was then centrifuged at 100 *g* for 5 min and placed at 37° for 30 min. The mixture was gently resuspended and a cytocentrifuge preparation (Shandon-Elliott Cytospin) made. Cells were stained with Wright-Giemsa and 200 cells were counted by the same observer throughout the study. All cells with 3 or more adherent CRBC were considered rosettes.

Double rosetting was performed by allowing effector cell populations to rosette with both CRBC and SRBC coated with IgG antibody (EA). The technique used was a modification of that previously described above. 10^6 effector cells were mixed with 10^7 uncoated CRBC and $2{\cdot}5 \times 10^7$ SRBC coated with IgG antibody in a volume of 0·75 ml. For controls, SRBC without antibody or coated with 19S antibody were used. In addition, non-immune effector cells were assayed in parallel. Cells rosetting with 3 or more CRBC and 3 or more SRBC were considered positive. CRBC were easily distinguishable from SRBC because they are larger and nucleated.

Cell separation techniques
Monocytes were removed from peripheral blood mononuclear cell suspensions by three different techniques:

(1) The plate adherence method as described by Mosier (Mosier, 1976). After 3 h of plating at 37°, adherent cells were removed either with a rubber policeman or by incubation with 12 mM xylocaine in RPMI-1640. Adherent cells reproducibly contained >80% monocytes as judged by morphology and latex bead ingestion. Supernatant cells contained <4% monocytes. Cell recovery was 80–90% of the pre-incubation number.

(2) The carbonyl iron-magnet method as previously described (Broder, Humphrey, Durm, Blakman, Mead, Goldman, Strober & Waldman, 1975). Resulting cell preparations contained <1% monocytes as judged by morphology and latex bead ingestion. Cell recovery was 50–70% of the pre-incubation number.

(3) Passage over G-10 Sephadex columns as previously described (Fauci *et al.*, 1976). This procedure resulted in no change in T-cell or B-cell percentage but depleted monocytes to <2% of the cell suspension. Eluted cell recovery was 60–80% of the precolumn number.

Enrichment of lymphocyte populations
T cell enriched and T cell depleted populations were obtained by a modification of a previously described technique (Dean, Silva, McCoy, Leonard, Cannon & Heberman, 1975). T cells are identified in the guinea-pig by rosetting with rabbit RBC (Fauci, 1975). 12 ml of blood mononuclear cells at 4×10^6/ml were mixed with 18 ml of rabbit RBC at 10^8/ml and 6 ml of foetal calf serum in a 50 ml conical tube. The mixture was incubated on a rotator at 37° for 5 min, spun at 200 *g* for 5 min and then incubated at 4° for

2 h. The mixture was then gently resuspended and 12 ml of Hypaque–Ficoll was injected under the mixture which was then spun at 400 *g* for 40 min at 20°. The cells rosetting with rabbit RBC were spun into a button and non-rosetting cells remained at the interface. Using this technique the usual percentage of T cells in blood mononuclear cell preparations (55–60%) are enriched to 85–95% in the rosetting fraction and depleted to <6% in the non-rosetting interface cells. The percentage of B cells as measured by the presence of a complement receptor or by surface Ig (usually 20–30%) were decreased to 5–15% in the pelleted cells and enriched to 45–70% in the interface cells.

RESULTS

Cytotoxicity of immune cells

Fig. 1 shows the cytotoxicity of blood mononuclear cells from guinea-pigs immunized with CRBC. Cytotoxicity reached a maximum at 3 weeks and then levelled off. Serum antibody as determined by haemagglutination shows titres rising to a maximum at 10–14 days and then plateauing. All subsequent experiments were performed 3 weeks post immunization.

Fig. 2 shows cytotoxicity of immune blood mononuclear cells at different effector: target ratios. Cytotoxicity reached a maximum at 100:1 ratio.

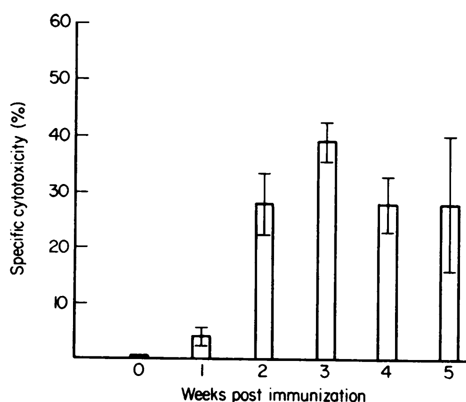

Figure 1. Cytotoxicity of blood mononuclear cells from guinea-pigs immunized with 5×10^8 CRBC in CFA at 0–5 weeks post immunization. Bars represent percent specific cytotoxicity (±s.e.m.) of four separate experiments.

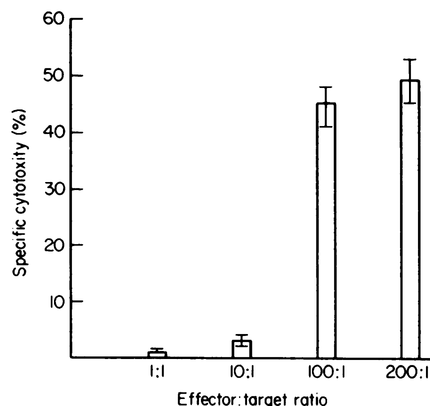

Figure 2. Cytotoxicity of immune blood mononuclear cells at different effector : target ratios. Bars represent percent specific cytotoxicity (±s.e.m.) of four separate experiments.

All subsequent experiments were performed at 100:1 E/T ratio.

Fig. 3 shows the cytotoxicity of mononuclear cells from different organs of immune guinea-pigs. Blood, spleen, lymph node and bone marrow mononuclear cells all show comparable levels of cytotoxicity. Peritoneal exudate cells showed significantly greater levels of cytotoxicity than the other organs.

Since studies had suggested only the monocyte–macrophage was an effector in this system, in the present study monocytes from immune blood mononuclear cells were depleted by passage over a G-10 Sephadex column (Table 1). Three passages over the column very effectively removed monocytes

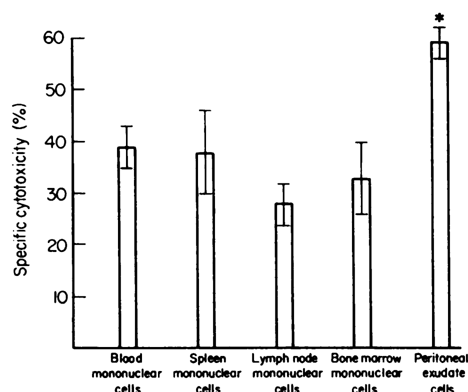

Figure 3. Cytotoxicity of mononuclear cells from different organs of immune guinea-pigs at 100 : 1 effector : target ratio. Bars represent percent specific cytotoxicity (±s.e.m.) of four separate experiments. * $P < 0.01$.

Table 1. Removal of cytophilic antibody from blood mononuclear cells by G-10 Sephadex column

	% Cytotoxicity	% Monocytes*
Before column	22	19
After passage over column × 1	9	4
After passage over column × 2	4	2
After passage over column × 3	1	1
Addition of immune sera and wash × 4	28	1

* Monocytes determined by morphology and latex bead ingestion.

and decreased cytotoxicity. The question arose whether the column passage removed cytophilic antibody from the surface of the effector cells rather than removed the effector cell itself. If the latter were true, it should be possible to re-arm these effectors with immune serum. The monocyte-depleted eluted cells which had lost their cytotoxic capabilities were thus armed with a 1 : 5 dilution of immune sera. As a result, all the cytotoxicity returned, proving that effector cells were still present in the monocyte depleted population and that in addition to the depletion of monocytes, the column passage had removed cytophilic antibody from the eluted cells. This experiment was also performed using non-immune cells which were subsequently armed *in vitro* and then passed over a G-10 column. The column removed the cytotoxicity of these armed cells and the eluted cells could then be re-armed with immune sera and the cytotoxicity returned.

The plate adherence technique was then used to obtain monocyte enriched and monocyte depleted populations. It was found that the monocyte enriched fractions that had been removed from the plate with 12 mM xylocaine showed decreased cytotoxicity. However, these monocyte enriched fractions regained all their cytotoxicity when they were re-armed with immune sera. This suggested that the 12 mM xylocaine had removed cytophilic antibody from the effector cell surface. If this were not appreciated, erroneous conclusions would have been made as to which effector cells could mediate cytotoxicity.

To take this one step further, subsequent experiments were performed by first doing separation procedures on non-immune blood mononuclear cells and then arming the cells *in vitro* with immune sera. Such arming consistently conferred substantial cytotoxic capability on Fc receptor bearing leucocyte populations.

Fig. 4a shows the results of plate adherence separation of mononuclear effectors which were separated and subsequently armed. Monocyte enriched and monocyte depleted populations obtained by plate adherence had the same cytotoxicity as unfractionated mononuclear cells. Also, as shown in Fig. 4b, carbonyl iron depletion of monocytes did not change the level of armed mononuclear cell cytotoxicity.

Fig. 4c shows the effect of monocyte depletion by G-10 Sephadex column on cytotoxicity by effectors which were first depleted of monocytes and then armed. Although the number of monocytes are dramatically reduced (<2%), there is no change in cytotoxicity.

Thus, using three different cell separation techniques, it has been demonstrated that both monocyte enriched as well as monocyte depleted (i.e. >99% lymphocytes) mononuclear subpopulations function as highly efficient effector cells in this cytotoxicity system. Furthermore, Fig. 5 shows the effect of T cell enrichment and T cell depletion on mononuclear populations which were first fractionated and then armed. The T cell enriched fraction had only half the cytotoxicity of unfractionated cells. However, the T cell depleted fraction had almost 2× the cytotoxicity of unfractionated cells. This experiment of T cell enrichment and T cell depletion was also performed on mononuclear cells depleted of monocytes by carbonyl iron (Table 2). Again the T cell enriched population showed decreased cytotoxicity whereas the T cell depleted population demonstrated increased killing. The killing in T cell enriched could be due to the 10% Fc+ cells which may be non-T or T cells with Fc receptors. This experiment confirms that a non-T lymphocyte can function as an effector in this system. Moreover, cells bearing a membrane receptor for the Fc portion of immunoglobulin were increased in the T cell

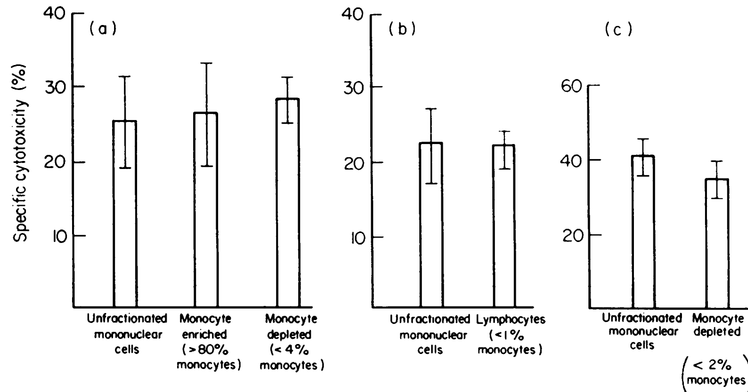

Figure 4. (a) The effect of plate adherence fractionation of cells on the cytotoxicity of armed mononuclear effector cells. Non-immune guinea-pig mononuclear cells were fractionated by adherence to a plastic plate. The unfractionated, monocyte enriched and monocyte depleted mononuclear cells were then armed and run in the cytotoxicity assay against CRBC at 100 : 1 effector : target ratio.

(b) The effect of carbonyl iron depletion of monocytes on armed mononuclear cell cytotoxicity. Non-immune guinea-pig mononuclear cells were depleted of monocytes by carbonyl iron method then armed with immune sera and run in the cytotoxicity assay against CRBC. All assays are at 100 : 1 effector : target ratio.

(c) The effect of G-10 Sephadex column depletion of monocytes on armed mononuclear cell cytotoxicity. Non-immune guinea-pig mononuclear cells were depleted of monocytes by passage over a G-10 Sephadex column 3 times. The cells were then armed with immune sera and run in cytotoxicity assay against CRBC at 100 : 1 effector : target ratio. For Fig. 4a–c, bars represent mean specific cytotoxicity (± s.e.m.) of four separate experiments.

depleted fraction which showed greater cytotoxicity, and Fc receptor bearing cells were decreased in the T cell enriched fraction which showed decreased cytotoxicity. This is consistent with the concept that the Fc receptor bearing lymphocytes and monocytes are the cells most capable of binding cytophilic antibody.

Neutrophil cytotoxicity

Since the neutrophil also bears a membrane Fc receptor and since it is active in antibody dependent cellular cytotoxicity against CRBC, the question arose whether the neutrophil was also active in this cytophilic antibody system. Fig. 6 shows cytotoxicity of blood mononuclear cells and blood neutrophils from immune guinea-pigs. Neutrophils from immune animals showed significant cytotoxicity against CRBC while neutrophils from non-immune animals showed < 5 % cytotoxicity.

We next investigated whether non-immune neutrophils could be armed. Fig. 7 shows neutrophil

Table 2. The effect of carbonyl iron depletion of monocytes and subsequent T cell enrichment and depletion on armed mononuclear cell cytotoxicity

	% Cytotoxicity	% Monocytes	% E rosettes	% Fc-Receptor bearing cells	% Complement receptor bearing cells	% sIg bearing cells	Cell yield No.	Cell yield %
Unfractionated mononuclear cells	35	18	62	29	18	26	210×10^6	100
After carbonyl iron fractionation	34	< 1	70	19	14	24	138×10^6	66
T cell enriched population	13	< 1	91	8	2	3	62×10^6	25 } 39
T cell depleted population	68	1	5	55	69	43	29×10^6	14 }

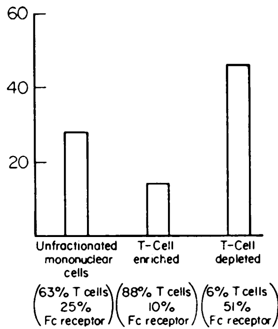

Figure 5. Effect of T-cell enrichment and T-cell depletion on armed mononuclear cell cytotoxicity. Non-immune mononuclear cells were rosetted with rabbit RBC and spun on Hypaque–Ficoll to separate a T-cell enriched from a T-cell depleted population. The cells were then armed with immune sera and run in the cytotoxicity assay at 100 : 1 effector : target ratios for all populations. Bar represents percent specific cytotoxicity of a representative experiment. Experiment was repeated 3 times with similar results. The percent E and EA rosettes in the populations are noted in parentheses.

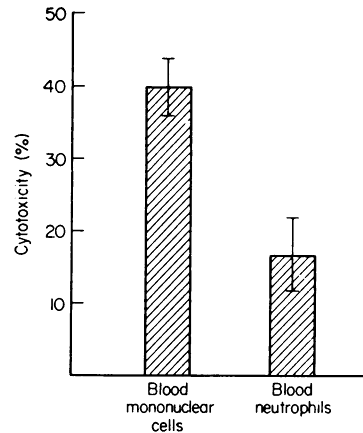

Figure 7. Neutrophil mediated cytotoxicity by armed neutrophils. Non-immune blood neutrophils were armed with immune sera and run in the cytotoxicity assay at 100 : 1 effector : target ratio. Armed mononuclear cells are included for comparison. Bars represent mean specific cytotoxicity (± s.e.m.) for 4 separate experiments.

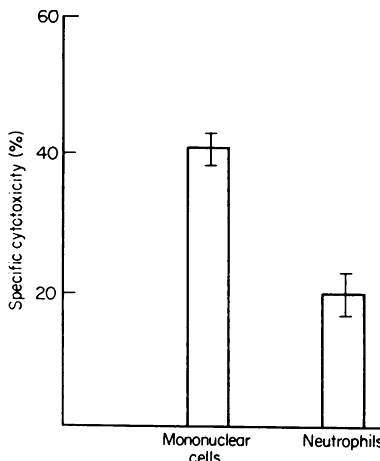

Figure 6. Cytotoxicity of blood neutrophils from immune guinea-pigs at 100 : 1 effector : target ratio (3 weeks post immunization). Immune blood mononuclear cells are included for comparison. Bars represent mean specific cytotoxicity (± s.e.m.) of 4 experiments.

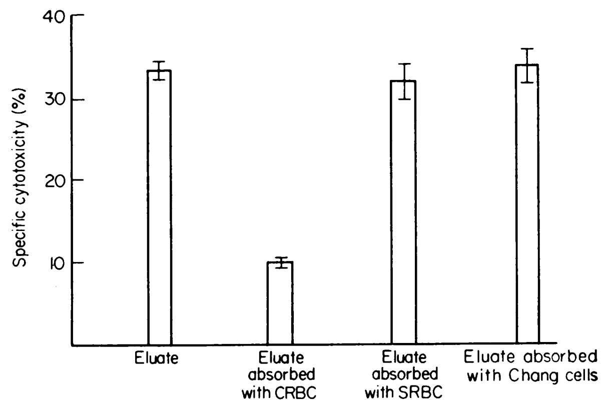

Figure 8. Eluates from immune cells can confer cytotoxicity on non-immune effectors and the eluate activity can be specifically absorbed with CRBC. Immune mononuclear cells at 30 × 10^6 cells/ml were heated 56° for 30 min to elute cytophilic antibody. The immune eluate unabsorbed and after absorption with CRBC, SRBC and Chang cells was then placed over non-immune blood mononuclear cells and CRBC targets added at effector : target ratio of 100 : 1. Bars represent mean specific cytotoxicity of 3 separate experiments.

mediated cytotoxicity induced by arming with immune sera. Neutrophils from immune animals as well as armed neutrophils from non-immune animals manifested substantial cytotoxic capabilities though of a lesser degree than blood mononuclear cells.

Mechanism of cytotoxicity

In order to determine the target cell specificity of this cytotoxicity, immune blood mononuclear cells were also assayed against sheep and human RBC

Table 3. Ability of purified IgG from immune guinea-pigs to arm non-immune mononuclear cells and neutrophils

Effector	Arming fraction	% Cytotoxicity
Mononuclear cells	Non-immune whole sera	0
	Immune sera	17
	Immune IgG	17
Neutrophils	Non-immune whole sera	0
	Immune sera	14
	Immune IgG	15

IgG fraction was obtained from immune sera by caprylic acid precipitation and DEAE-S2 batch purification. The fraction was pure IgG as determined by immunoelectrophoresis.

targets. No cytotoxicity was noted against these targets.

To confirm previous reports that cytophilic antibody is the mechanism of cytotoxicity, eluates were obtained from immune mononuclear cells as described below. Eluates from non-immune mononuclear cells served as controls. Blood mononuclear cells were brought to 30×10^6 cells/ml in RPMI-1640, heated at 56° for 30 min to release cytophilic antibody and the dead cells centrifuged and discarded. These eluates were then placed on 2×10^6 non-immune blood mononuclear cells and 2×10^4 labelled CRBC targets added. Fig. 8 shows that eluates from immune blood mononuclear cells conferred cytotoxicity upon non-immune mononuclear cells while eluates from non-immune cells conferred no cytotoxic activity. When these eluates were absorbed with CRBC, the cytotoxicity was markedly reduced. However if the eluates were absorbed with SRBC or Chang cells, the cytotoxicity was unchanged.

Immunoglobulin nature of the cytophilic antibody

Pooled immune guinea-pig sera were purified by precipitation of non-gammaglobulins with caprylic acid and subsequent DEAE52 batch purification (Steinburch & Audron, 1969). This resulted in a preparation containing only IgG as determined by immunoelectrophoresis using antisera against guinea-pig immunoglobulin and specific antisera against guinea-pig IgG. Both the unfractionated immune sera and the purified IgG fraction of immune sera were used to arm non-immune guinea-pig mononuclear cells and neutrophils (Table 3). All of the arming capability of immune sera was present in the IgG fraction. This strongly suggests that IgG is the cytophilic arming factor though mediation by a trace protein cannot be excluded.

Blockade of Fc receptor binding with aggregated gamma globulin

Mononuclear cells were incubated for 30 min at 37° with 5 mg/ml of heat aggregated (63° for 15 min) gamma globulin (AGG) and then armed with immune sera as outlined in the Methods. Mononuclear cells not pre-incubated with AGG were run concomitantly as controls. Similar experiments were run for neutrophils. Incubation of mononuclear cells or neutrophils in AGG significantly decreased their ability to arm resulting in decreased cytotoxicity (Table 4).

Rosetting

To demonstrate further that 3 different effector cells were capable of cytotoxicity in this system, non-immune effector cells (lymphocytes, monocyte–macrophages and neutrophils) were armed with immune sera, washed exhaustively as noted above, and then rosetted with CRBC as outlined in the Methods. Fig. 9a–c shows that all 3 cell types rosette with CRBC, clearly demonstrating they are capable of attaching antibody to their surface membrane. Non-armed effectors did not rosette with CRBC.

Using the double rosetting technique, the surface markers of the effectors rosetting with CRBC were investigated. Monocytes, lymphocytes and neutrophils rosetting with CRBC also rosetted with SRBC

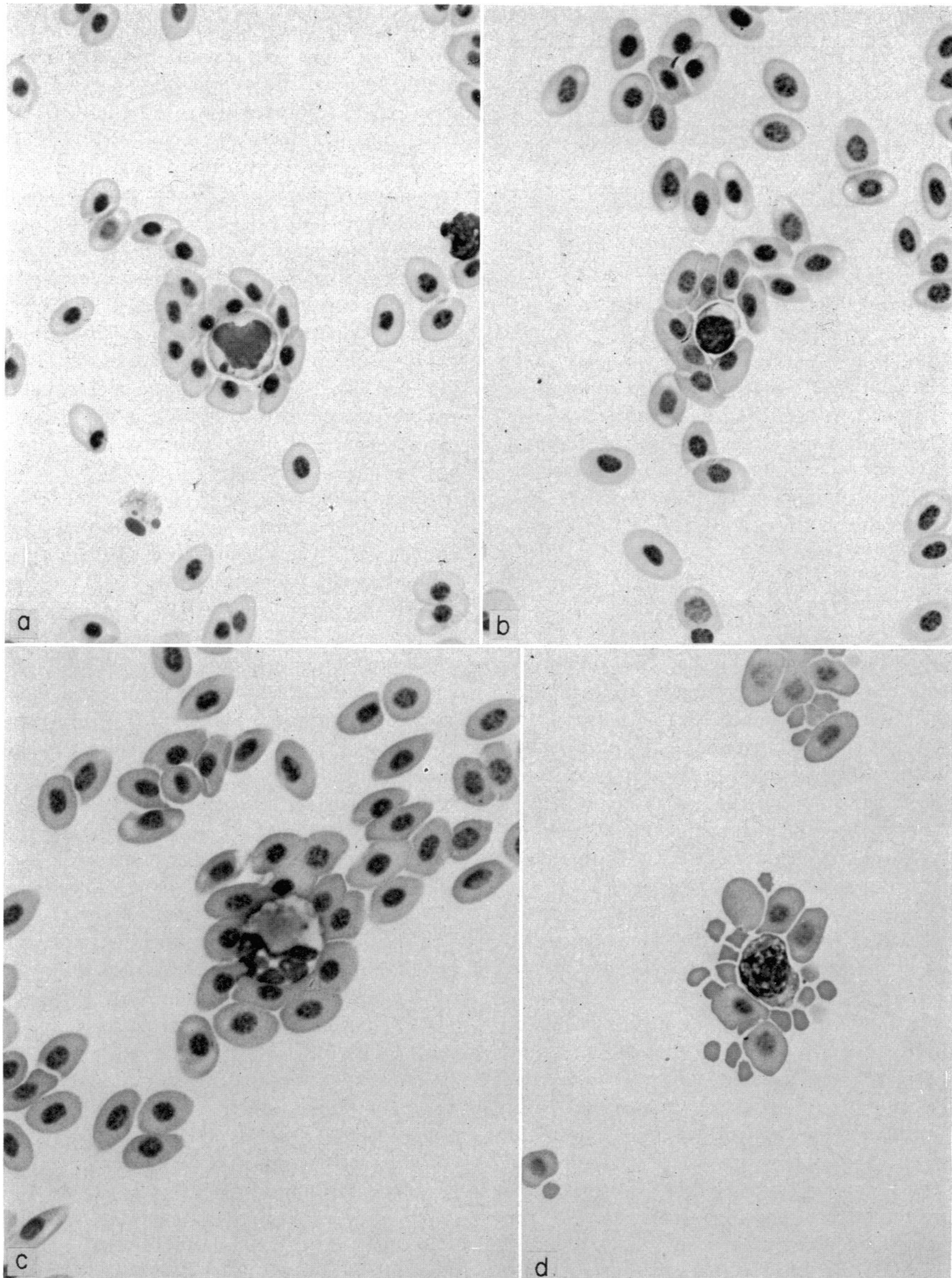

Figure 9. (a) Armed blood monocyte rosetting with CRBC. (b) Armed blood lymphocyte rosetting with CRBC. (c) Armed blood neutrophil rosetting with CRBC. (d) Armed blood lymphocyte double rosetting with CRBC and SRBC coated with IgG antibody.

SRBC are the small erythrocytes. CRBC are the large nucleated erythrocytes.

Table 4. The ability of aggregated gamma globulin (AGG) to block arming of mononuclear cells and neutrophils

	Control	Pre-incubated with AGG
Mononuclear cells	31 ± 2*	13 ± 1
Neutrophils	21 ± 2	7 ± 2

* Mean percent cytotoxicity (±s.e.m.) of 3 experiments.

coated with IgG antibody. Fig. 9d illustrates this typical double rosetting phenomenon in an armed lymphocyte. When double rosetting was performed with CRBC and SRBC coated with 19S antibody or when performed with CRBC and uncoated SRBC, no double rosettes were seen. Thus, Fc receptor bearing lymphocytes, monocytes and neutrophils are capable of binding cytophilic antibody and mediating specific cytotoxicity.

DISCUSSION

This study has demonstrated that *in vivo* 'direct' cytotoxicity may in fact be mediated by cytophilic antibody on the surface of the effector cell resulting in reality in ADCC. It has been clearly shown that at least three distinct cell types—the monocyte–macrophage, a non-T lymphocyte (and perhaps a T lymphocyte) and the neutrophil, all bearing Fc receptors for Ig—are efficient effector cells capable of binding cytophilic antibody *in vivo* or *in vitro* and mediating specific killing against CRBC targets. Previous studies (Temple *et al.*, 1973) had suggested that only a macrophage was the effector in this model.

In addition, effector cells from a variety of organs including spleen, lymph nodes, blood, PECs and bone marrow were capable of mediating this cytophilic antibody dependent cytotoxicity. Cell free eluates produced from immune effector cells could render non-immune cells capable of lysing targets, and the eluate activity could be largely absorbed out with target antigen. Upon purification, all the cytophilic arming activity was found in the purified IgG fraction. These findings suggest that antibody and not antigen–antibody complexes were largely responsible for the arming activity.

Perlmann & Perlmann (1970) recognized that antibody could be placed on the effector cell to render it specifically cytotoxic. They used rabbit anti-CRBC antibody and human leucocytes freed of phagocytic cells and found that 10^{-1} dilution of rabbit antisera could arm human lymphocytes to give 70–90% cytotoxicity against CRBC targets. In a later study Perlmann, Perlmann & Biberfeld (1972) using the same system found that antigen–antibody complexes were more efficient than antibody alone in absorbing to lymphocytes and rendering them cytotoxic. Greenberg & Shen (1973) used antigen–antibody complexes to arm mouse spleen cells which had been depleted of phagocytic cells. The effector cell was found to be a non-T, non-surface Ig bearing lymphocyte and arming was blocked by aggregated Ig. Their data suggested that the effector cell was an Fc receptor bearing lymphocyte but they did not report arming of either monocyte–macrophages or neutrophils.

Recently, Saksela, Imir & Makela (1975), using NIP-anti-NIP antigen–antibody complexes on mouse and human lymphocytes freed of phagocytic cells, demonstrated that antibody alone as well as complexes were capable of arming human and mouse lymphocytes. They also tested antibody and effector cells of different species and found that when the antibody and effector cell came from the same species, the arming was most efficient. This may explain why our system which employed antibody and effector cell from the same species was more efficient than studies using antibody and effectors from different species (Perlmann *et al.*, 1972).

Schirrmacher *et al.* have demonstrated that spleen cells from mice immunized against a certain antigen will be cytotoxic to CRBC coated with that antigen. The mechanism in this system also appears to be cytophilic antibody on the surface of the effector cell (Schirrmacher, Rubin, Pross & Wigzell, 1974). The effector cell in this system is felt to be a non-T cell (Golstein, Schirrmacher, Rubin & Wigzell, 1973) but it has not been further characterized.

Recent human studies of transitional cell carcinoma of the bladder (O'Toole *et al.*, 1973) and chronic active hepatitis (Cochrane *et al.*, 1976) have described direct cytotoxicity mediated by K cells—the effector cell commonly identified with antibody dependent cellular cytotoxicity. Although this may indeed have been the case in those studies, the present study as well as those cited above (Saksela & Makela, 1975; Perlmann *et al.*, 1972; Greenberg & Shen, 1973; Perlmann & Perlmann, 1970) have demonstrated that K cells bearing cytophilic antibody (or

antigen–antibody complexes) may be efficient cytotoxic effector cells in *in vitro* assays of cellular cytotoxicity in which direct cellular cytotoxicity appears to be the major mechanism of killing.

The present study demonstrates that certain commonly used cell separation techniques were capable of dissociating effector cell from cytophilic antibody. Both passage over G-10 Sephadex columns and treatment with 12 mM xylocaine reduced cytotoxicity of immune and armed populations by removing cytophilic antibody. This finding is of practical importance since these cell fractionation procedures are widely used and erroneous conclusions as to the types of effector cells would be made if the antibody dissociating potential of the technique is not realized. In the case of G-10 Sephadex depletion of monocytes, if this dissociation of antibody had not been appreciated, the monocyte would have been incorrectly considered as the only effector cell in the system. It is noteworthy that in a model similar to that used in the present study, it was felt that only macrophages bearing cytophilic antibody were the cytotoxic effector cells.

Hence, it is advisable whenever 'direct' cytotoxicity is demonstrated and especially in those instances where high levels of circulating antibody against target antigens can be demonstrated, that cytophilic antibody induced cytotoxicity be thoroughly investigated as the possible cytotoxic mechanism.

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