Clin. exp. Immunol. (1976) **23**, 91–97.

Characteristics of the effector cells mediating cytotoxicity against antibody-coated target cells*

III. ULTRASTRUCTURAL STUDIES

P. L. PENFOLD, A. H. GREENBERG & I. M. ROITT *Department of Immunology, Middlesex Hospital Medical School, London*

(*Received* 3 *June* 1975)

SUMMARY

On the basis of electron microscopic observations, four types of cells apparently cytotoxic for antibody-coated chicken erythrocytes were identified in non-immune mouse spleen; monocytes, polymorphonuclear leucocytes, immature granulocytes and a type of lymphoid cell. Both phagocytosis and extracellular lysis of target cells were observed. Monocytes and polymorphonuclears were able to interact with the target cell by both mechanisms while the intermediary granulocytes and lymphoid cells were only capable of extracellular lysis. It is argued that these observations provide a morphological basis for the previous classification of antibody-dependent cytotoxic cells into myeloid and lymphoid cells (Greenberg *et al.*, 1973b, 1975).

INTRODUCTION

Earlier studies on the cells in mouse spleen which are capable of killing antibody-coated chicken erythrocytes suggested that the majority were of myeloid origin (Greenberg *et al.*, 1973b; 1975). It is clear, however, that some of the effector cells are distinguishable by their relatively poor surface adherence properties and Mg^{2+}-independent complement receptors, suggesting that they may be of lymphoid origin (Greenberg *et al.*, 1975). In a previous morphological study on the effector cells in human peripheral blood it was noted that lymphocyte-like cells were found in association with lysed chicken erythrocytes (Biberfield & Perlmann, 1970). We have attempted to identify the morphology of murine cytotoxic cells, and to investigate the nature of their cytotoxic attack by observing the interaction of mouse spleen cells and antibody-coated CRBC under the electron microscope.

MATERIALS AND METHODS

Spleen cell preparations. Spleens were taken from BALB/c mice bred in our colony and the cells prepared as described previously (Greenberg *et al.*, 1973a, 1975). Fractionation of cells using carbonyl iron and glass bead columns has been described in detail in earlier papers (Greenberg *et al.*, 1975). Briefly, 3×10^6/ml washed cells were incubated with 4 mg/ml carbonyl iron (S.F., G.A.F. (U.K.) Ltd) in Eagle's minimal essential medium (MEM) containing 10% foetal calf serum for 1 hr at 37°C followed by removal of the iron on a powerful magnet. The cells were then washed once and resuspended in 3 ml of 50% FCS in MEM. They were then warmed for 10 min at 37°C and allowed to run into an 11 ml glass bead column (mesh 60, gas chromatography beads, BDH Ltd) which had previously been incubated with 50% FCS in MEM for 30 min at 37°C. After 20 min the column was slowly flushed with 25% FCS in MEM and the first 5 ml of the effluent collected.

* This work was supported by the Medical Research Council of Great Britain and the Medical Research Council of Canada.

Correspondence: Mr P. L. Penfold, Department of Immunology, Middlesex Hospital Medical School, London.

Incubation of spleen cells and chicken red blood cells (CRBC). Spleen cell preparations were adjusted to $2{\cdot}5 \times 10^8$/ml or 10^7/ml and a 200 μl sample incubated with equal volumes of 1/10,000 rabbit anti-CRBC serum and 10^7/ml CRBC at 37°C in a 5% CO_2 atmosphere. At the end of the incubation period the cells had sedimented into a pellet and the supernatant was carefully removed. Without disturbing the cells, a 1·7% solution of glutaraldehyde in 0·1 M phosphate buffer, pH 7·4, was added and left for 30 min at room temperature. The cells were then washed twice and suspended in phosphate buffered saline (PBS) pH 7·2. Controls incubated with normal rabbit serum in place of rabbit anti-CRBC serum were prepared in the same way.

Cytochemistry. The cells were treated with Graham and Karnovsky's medium (Graham & Karnovsky, 1966) for endogenous peroxidase activity using the cytochemical substrate 3,3′-diaminobenzidine (D.A.B., free base; Sigma Chemical Co.). After 15 min at room temperature in this medium they were washed again in PBS then post fixed in 1% Millonig's osmium for 30 min. The cells were then dehydrated in acetone and embedded in araldite.

Analysis of samples. Thin sections (60 nm) were cut on a Huxley Ultramicrotome and mounted on unsupported grids. They were lightly stained with uranyl acetate and lead citrate. On some sections the uranyl acetate was omitted so that the endogenous peroxidase reaction could be more easily recognized. Photomicrographs were taken with an A.E.I., E.M. 6B electron microscope, using an accelerating voltage of 50 kV.

A cytotoxic attack was inferred when a mouse cell in contact with a CRBC target was associated with one of the following features: (1) extensive vacuolation within the CRBC cytoplasm; (2) extensive distortion of surface and nuclear membrane of the CRBC; (3) phagocytosis of whole CRBC, or in some cases, only the nucleus; (4) the presence of CRBC 'ghosts' attached to the cell membrane.

Mouse spleen cells were classified on the basis of nuclear and cytoplasmic structures and endogenous peroxidase content according to Hirsch & Fedorko (1970).

For estimation of the percentages of the different cytotoxic cell types, a total of 350 interactions pooled from three separate experiments were analysed. These figures will of course be dependent on the differences in the time of effector cell–target cell contact as well as the absolute number of effector cells.

RESULTS

Whole spleen cells (10^7/ml) were incubated with CRBC at a 1 : 1 ratio in the presence of rabbit anti-CRBC antibody (1/10,000) for 90 min, then fixed with glutaraldehyde and stained as described under incubation of spleen cells, and cytochemistry. Four effector cell types were identified under the electron microscope in association with damaged target cells. They were classified as follows.

(1) Neutrophils, which made up approximately 15% of the total effector cell population, were characterized by their multi-lobed nucleus and densely granular cytoplasm (Figs 1 and 2); 50% of the cytoplasmic granules were peroxidase positive. Neutrophils were capable of phagocytosing entire target cells or inducing extracellular lysis associated with moderate interdigitation of the opposing cell membrane, the latter mechanism predominating.

(2) Thirty-five per cent of the effector cells had a bi-lobed or occasionally tri-lobed nucleus (Figs 3, 4 and 5). All the cytoplasmic granules (10–20/cell section on average) stained positively for peroxidase and these cells were classified as intermediary granulocytes. The characteristic features of attack by these cells were severe disruption of the target cell membrane, with complex interdigitation of the cells at the interface, apparently leading to extracellular lysis of the CRBC (Fig. 4). The attack usually involved only a single target cell.

(3) Twenty per cent of the effector cells had a bi-lobed nucleus with between one and ten positive peroxidase granules per cell section (Figs 6 and 7). These were classified as monocytes. The attack was extracellular and frequently involved three or four target cells with relatively mild interdigitation of the opposing cell surfaces. An additional 20% were mature phagocytic monocytes with only one or two peroxidase-positive cytoplasmic granules (Figs 8 and 9). The attack usually involved more than one target cell, and sometimes resulted in a complex containing numerous damaged CRBC. These cells were also capable of internalizing the nucleus of the CRBC, while excluding the cell membrane and cytoplasm (Fig. 9), a process recalling the formation of LE cells by PMNs (Robineaux, 1958).

(4) The final 10% of the effector cells were identified as lymphoid cells which always displayed distinct morphological features (Fig. 10). They had an ovoid, eccentric nucleus with dense peripheralized chromatin, no obvious nucleolus and a low nuclear–cytoplasmic

ratio. The cytoplasm displayed numerous mitochondria, moderately developed Golgi vesicles, sparse rough-surfaced endoplasmic reticulum but no peroxidase-positive lysosomes. The attack was non-phagocytic and usually involved one target cell. At the interface the membranes were closely associated but without complex interdigitations. Target cell destruction was apparently preceded by the appearance of zones of lysis within the target cell cytoplasm.

Two types of non-cytotoxic lymphocyte were identifiable in the spleen cell preparations: (1) a large cell with prominent nucleolus and dispersed nuclear chromatin which was similar in size to the cytotoxic lymphoid cell but with fewer cytoplasmic orangelles—this cell was never seen in association with damaged target cells (Fig. 11); and (2) a small lymphocyte with a high nuclear–cytoplasmic ratio and a small nucleolus which was occasionally seen ($<1\%$) in both experimental and control samples, involved in rosette formation but, again, with no indication of target cell damage (Figs 11 and 12).

Eosinophils and basophils did not appear to be cytotoxic. No cytotoxic interactions were observed in the control specimens containing normal rabbit serum.

Cytotoxic assays using ^{51}Cr release (Greenberg *et al.*, 1975) run in parallel with the morphological studies showed that at 90 min the cytotoxicity was 5% and by 18 hr it was 15%.

FIG. 1. A mature neutrophil from an unfractionated spleen engaging a CRBC in the presence of a rabbit anti-CRBC antiserum. Some distortion of the CRBC membrane is apparent and vacuolation has developed within the target cell. The nucleus has also become distorted. (Magnification × 7500.)

FIG. 2. A mature neutrophil which has phagocytosed a whole target cell (from the same preparation as Fig. 1). (Magnification × 5000.)

FIG. 3. An intermediary granulocyte engaging an as yet undamaged target cell with a lysed target cell (arrow) adhering to its surface. (Magnification × 7500.)

FIG. 4. An intermediary granulocyte exhibiting vigorous interdigitation with the target cell membrane, and the remains of a lysed target cell (arrow) at its surface. Pseudopodia from the intermediary granulocyte are visible bordering on the nuclear membrane of the target cell. (Magnification × 7500.)

FIG. 5. An intermediary granulocyte from the same preparation as Fig. 1. This section has not been stained with uranyl acetate and the peroxidase positive granules are readily visible. Note the vacuolation within the target cell and the distorted nucleus. (Magnification × 5000.)

FIG. 6. A mature monocyte with a lysed target cell, the nucleus (arrow) and membranous fragments of which are still visible. (Magnification × 7500.)

FIG. 7. A second example of a monocyte attack. Note the target cell nucleus (arrow) with membranous remains, and a second target cell engaged by the monocyte with mild interdigitations. (Magnification × 7500.)

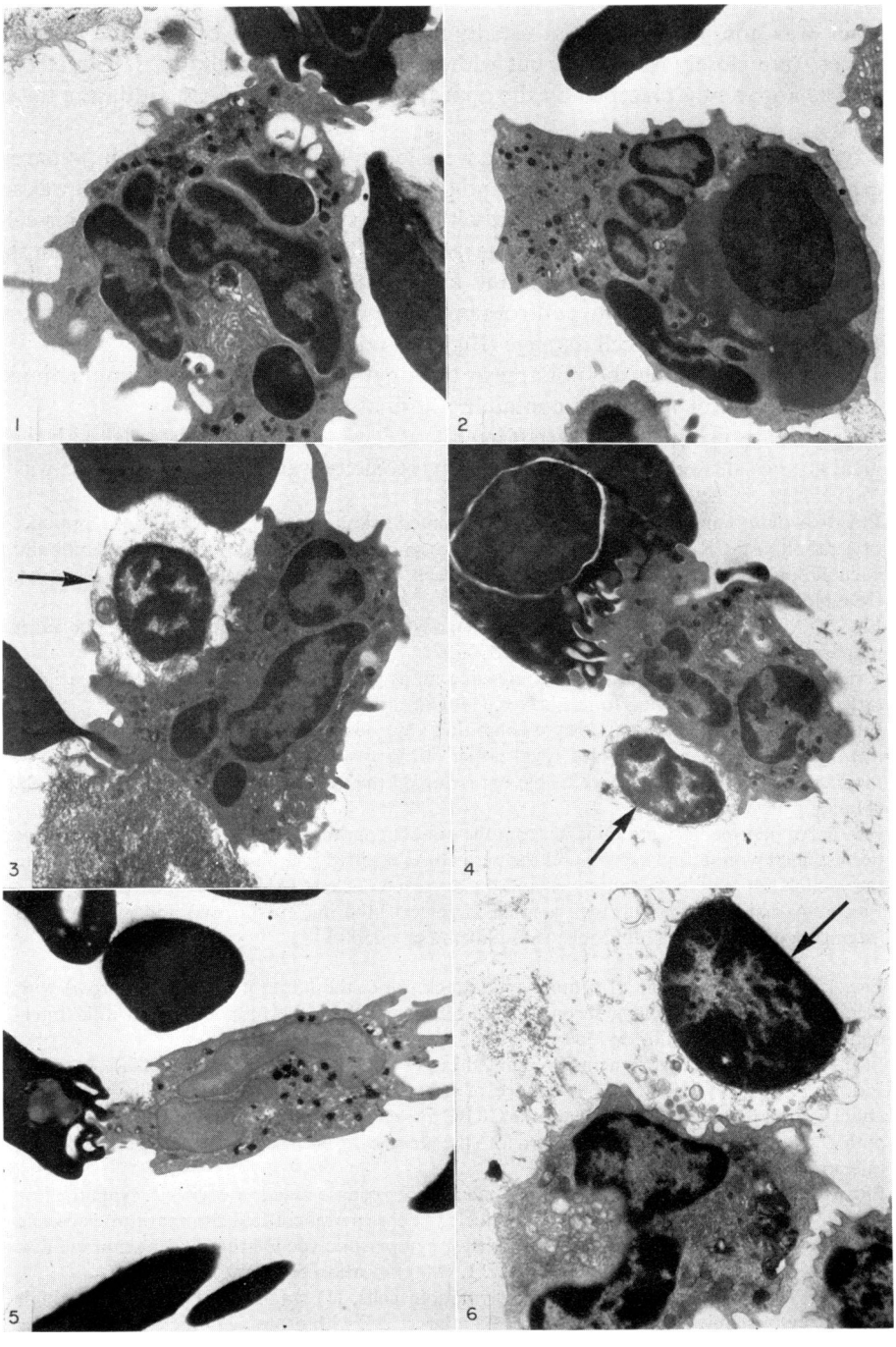
FIG. 8. A mature monocyte with an engulfed target cell. (Magnification × 10,000.)

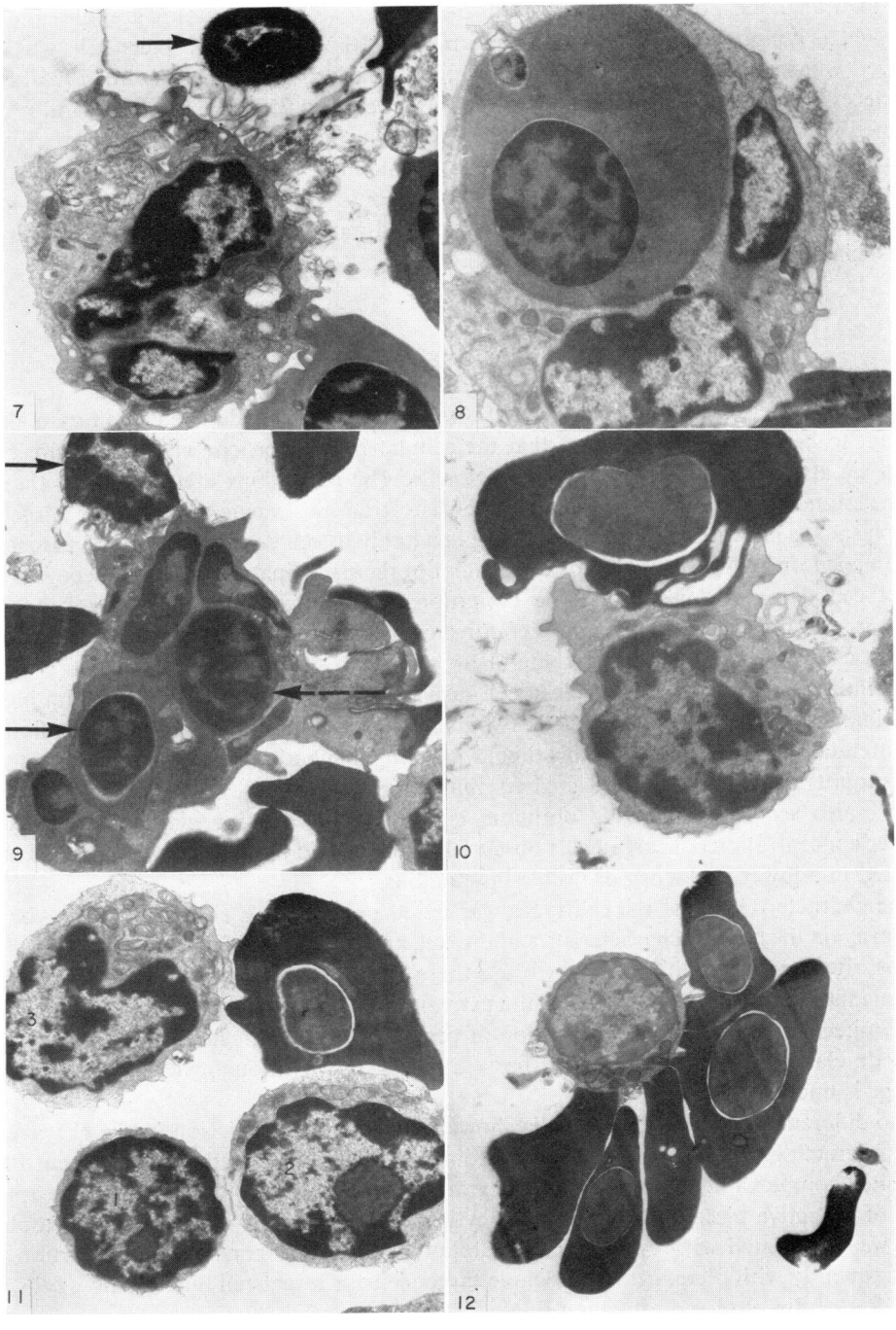
FIG. 9. A phagocytic complex. Evidence of both extracellular and intracellular target cell attack is apparent. Solid arrows mark the CRBC nuclei. The dotted arrow indicates the possible path of a target cell nucleus being drawn into the phagocyte, with the cytoplasm excluded at the cell surface. (Magnification × 7500.)

FIG. 10. A lymphoid effector cell. No endogenous peroxidase reaction is visible. Note the low nuclear-cytoplasmic ratio, ovoid eccentric nucleus, dense peripheralized chromatin and absence of a nucleolus. The target cell shows extensive cytoplasmic vacuolation and membrane distortion. From a similar preparation to Fig. 1. (Magnification × 10,000.)

FIG. 11. Three morphologically distinct lymphoid cells, (1) small lymphocyte with a high nuclear:cytoplasmic ratio and nucleolus, (2) a larger cell with prominent nucleolus and lower nuclear:cytoplasmic ratio. Neither of these cell types have been identified in association with damaged target cells. (3) Intermediate sized lymphoid cell with lower nuclear: cytoplasmic ratio, absent nucleolus and abundant cytoplasmic organelles similar to the type seen as a cytotoxic cell in Fig. 10. This cell is also differentiated from the lymphoblasts by its dense peripheral chromatin pattern and absent nucleolus. (Magnification × 7500.)

FIG. 12. A small lymphocyte of high nuclear:cytoplasmic ratio forming a rosette in which no damage has occurred to the CRBC. The section has not been stained with uranyl acetate. (Magnification × 5000.)


1
2
3
4
5
6


7
8
9
10
3
2
1
11
12

Spleen cells fractionated using carbonyl iron and glass bead adherence columns were also examined (see Materials and Methods). Although occasional monocytes and granulocytes were identified, 99% of the remaining cells had no peroxidase-containing granules and were classified as lymphoid cells. The cytotoxic capacity of the fractionated spleen cells was much reduced when compared to the whole spleen preparations (Greenberg *et al.*, 1975), and it was necessary to increase the ratio of spleen: target cells to 25:1 ($2{\cdot}5 \times 10^8$/ml) and prolong the incubation period to 18 hr. With these conditions cytotoxic interactions were frequent enough to allow detailed observation. The cell type involved in the attack in these spleen preparations was identical to the cytotoxic lymphoid-type cell seen in the unfractionated spleen samples (Fig. 10). The few granulocytes and monocytes remaining were never seen in association with antibody-coated CRBC.

DISCUSSION

The nature of electron microscopic studies using glutaraldehyde-fixed sections makes it difficult to be completely confident that the cellular interaction one visualizes represents a cytotoxic attack on antibody-coated target cells. The limitations are obvious in that one must assume that the observed membrane distortion and vacuolation of the target cell cytoplasm are indicative of changes leading inevitably to cell death and that the association of damaged or lysed cells with leucocytes implies the participation of that leucocyte in the target cell damage. Accepting these limitations and realizing the need for supporting cinematographic studies to come to definitive conclusions, one can still make useful interpretations of the observations presented here.

Preliminary studies on whole spleen cell suspensions indicated that the optimum incubation time with antibody and CRBC was 90 min. A maximum number of effector cell: target cell interactions was observed at this time, while long incubation periods resulted in increasing amounts of cell debris and decreased numbers of target cells. A spleen: target cell ratio of 1:1 also seemed to provide optimum conditions for viewing cytotoxic interactions. Higher concentrations of target cells obscured the cytotoxic cells, and lower concentrations resulted in a lower frequency of visible interactions.

The characterization of the cell types was based on Hirsch & Fedorko (1970) who rely primarily on the nuclear characteristics of the cell, and the nature of its cytoplasmic granules. It was often very difficult, however, to use these criteria to distinguish mature PMN from intermediary granulocytes because of the extreme variability in their nuclear characteristics and limited differences in the proportion of peroxidase-staining cytoplasmic granules. The separate classification of the intermediary granulocyte was thought necessary because its lineage is uncertain.

The different classes of effector cells showed quite marked variation in the character of their interactions with target cells. Intermediary granulocytes and to a lesser extent PMN, exhibited complex interdigitations with the target cell membranes almost giving the appearance of an active piecemeal 'micro-phagocytic dissection' of the chick red cell cytoplasm. This was associated with cytoplasmic vacuolation and nuclear membrane distortion, and less frequently with phagocytosis of whole target cells or target cell nuclei. These cells were most frequently observed attacking single CRBC.

The interfacial membranes of monocytic and lymphoid effector cells were less complex with minimal pseudopod formation associated with vacuolation and lysis of the target cells. On morphological grounds, it seems likely that the cytotoxic mechanism is different from that involved in killing by granulocytes. Unlike the other effector cells, monocytes were often seen in association with three or four target erythrocytes possibly reflecting a higher density or avidity of Fc receptors or perhaps a longer period of contact before lysis occurred. In

contrast to the observations of Biberfield & Perlmann (1970) in human peripheral blood, the mouse lymphoid effector cells never exhibited phagocytosis.

Only the lymphoid type effector cell was seen to be active in purified spleen populations which had been freed of phagocytic and adherent cells. Although possessing the attributes of lymphoid cells with respect to overall morphology, surface adherence and a high proportion of Mg^{2+}-independent C3 receptors (Greenberg *et al.*, 1975), this cell was unusual, differing from the small lymphocytes observed in non-cytotoxic rosette formation (cf. Fig. 12) in its somewhat larger size, low nuclear : cytoplasmic ratio and abundance of cytoplasmic organelles, and from other lymphocytes of comparable size by its apparent lack of nucleoli. This cell is also differentiated from the lymphoblast by its dense peripheral chromatin pattern and absent nucleolus.

The present study implies that many cell types may be capable of killing antibody-coated targets and there may well be others which we have missed, whether by confining our attention to the spleen, or through our experimental design which minimizes the chance of detecting cells like the cytotoxic T lymphocyte (Able, Lee & Rosewall, 1970) which require only a brief contact with the target cell to initiate the events leading to its destruction.

These observations do, however, allow us to classify the observed cytotoxic cells into two general groups based on their morphology: (1) cells of myeloid origin, monocytes, PMN and immature granulocytes; and (2) cells of apparent lymphoid morphology.

REFERENCES

ABLE, M.E., LEE, J. & ROSEWALL, W. (1970) Lymphocyte-target cell interaction *in vitro*. Ultrastructural and cinematographic studies. *Amer. J. Path.* **60,** 421.

BIBERFIELD, P. & PERLMANN, P. (1970) Morphological observations on the cytotoxicity of human lymphocytes for antibody-coated chicken erythrocytes. *Exp. Cell. Res.* **62,** 433.

GRAHAM, R.C., JR. & KARNOVSKY, M.J. (1966) The early stages of absorption of injected horseradish peroxidase into the proximal tubules of mouse kidney. *J. Histochem. Cytochem.* **14,** 291.

GREENBERG, A.H., HUDSON, L., SHEN, L. & ROITT, I.M. (1973a) Antibody-dependent cell-mediated cytotoxicity due to a 'null' lymphoid cell. *Nature: New Biology*, **242,** 111.

GREENBERG, A.H., SHEN, L. & ROITT, I.M. (1973b) Characterization of the antibody-dependent cytotoxic cell, a non-phagocytic monocyte? *Clin. exp. Immunol.* **15,** 251.

GREENBERG, A.H., SHEN, L., WALKER, L., ARNAIZ-VILLENA, A. & ROITT, I.M. (1975) Characteristics of the effector cells mediating cytotoxicity against antibody-coated target cells. II. The mouse non-adherent K cell. *Europ. J. Immunol.* (In press).

HIRSCH, J.G. & FEDORKO, M.E. (1970) *Mononuclear phagocytes* (ed. by R. Van Furth), p. 7. F. A. Davis Co., Philadelphia.

ROBINEAUX, R. (1958) Etude microcinematographique en contraste de phase du mechanisme du phenomene. LE *18th Int. Symposium Immunopathology* (ed. by P. Grabar and P. Miescher), p. 416. Schwabe and Co., Basel.