Clin. exp. Immunol. (1975) **21**, 216–225.

OBSERVATIONS ON THE ANTIBODY-DEPENDENT CYTOTOXIC CELL BY SCANNING ELECTRON MICROSCOPY

J. R. INGLIS, W. J. PENHALE, ANNE FARMER, W. J. IRVINE AND A. E. WILLIAMS

Immunology Laboratories, 2 Forrest Road; Endocrine Department and University Department of Therapeutics, The Royal Infirmary; and Teaching and Research Centre, Western General Hospital, Edinburgh

(*Received* 24 *February* 1975)

SUMMARY

The cytotoxic effect of human peripheral blood leucocytes on antibody-coated sheep erythrocyte monolayers has been investigated using scanning electron microscopy.

Only a small proportion of leucocytes were found to adhere to the monolayers. A progressive destruction was observed beginning as small plaque-like areas of erythrocyte clearing which later became confluent.

Three distinct cell types were found to be associated with the areas of lysis.

No destruction was observed in control monolayers incubated for a similar period in the absence of either antibody or leucocytes.

Surface changes in the erythrocytes adjacent to the leucocytes suggest that mechanical factors may be involved in erythrocyte lysis in this system.

It is concluded that more than one leucocyte type may damage antibody-coated erythrocytes, possibly by a mechanism involving attachment to and mechanical disruption of the red cell membrane.

INTRODUCTION

A variety of experimental techniques are now available to demonstrate cytotoxic activities of cells *in vitro* (Perlmann & Holm, 1969; Cerottini & Brunner, 1974). These techniques usually involve the assessment of damage at a functional level either by measurement of the release of isotopically labelled components or by direct cell counting. These assays provide only limited information on the cellular events involved and hence a method of visualizing at high resolution the interaction between effector and target cells would contribute to the understanding of cytotoxic mechanisms.

We describe here a procedure for demonstrating cytotoxic activity by individual leuco-

Correspondence: Dr W. J. Irvine, Clinical Immunology Laboratories, Department of Therapeutics, The Royal Infirmary, Edinburgh EH1 2QW.

cytes using scanning electron microscopy and present preliminary observations on the mechanism of antibody-dependent cell-mediated cytotoxicity (K-cell cytotoxicity).

MATERIALS AND METHODS

Preparation of leucocytes

Human leucocytes were obtained from healthy volunteers by centrifugation of heparinized blood through a Ficoll–Triosil solution (Perper, Zee & Mickelson, 1968). The cell suspension (1×10^6/ml) in Eagle's basal medium (EBM) (Wellcome) supplemented with 10% foetal calf serum (FCS) (Wellcome) was incubated in sterile glass bottles in a horizontal position for 15 hr at 37°C. Cells remaining unattached to the glass were collected, counted and the suspension diluted to $0{\cdot}5 \times 10^6$ viable cells per millilitre for use.

Preparation of erythrocyte monolayers

The method of Kennedy & Axelrad (1971) was used with modifications. Sheep blood was collected in Alsever's solution, stored at 4°C and used within 7 days. Washed red cells were resuspended to a 2% v/v suspension in phosphate-buffered saline (PBS), incubated for 30 min at 37°C with rabbit anti-sheep red cell serum (Wellcome) (1/50 final dilution, heated to inactivate complement) and washed three times in PBS. Uncoated red cells were used as controls.

Monolayers were formed on polystyrene slips 25 mm^2 in area which were cut from 50×10 mm Petri dishes (Falcon Plastics) to fit the specimen stub on the scanning electron microscope (SEM). Each slip was immersed in a 50 μg/ml solution of poly-L-lysine (Sigma Chemical Company) (mol. wt 70,000) in PBS at room temperature for 1–2 hr. They were then rinsed with PBS and 1 ml of a 2% suspension of the antibody-coated or uncoated erythrocytes added. Sedimentation of these suspensions at room temperature overnight allowed erythrocytes to attach to the treated surfaces and unattached cells were removed by repeated rinsing in warm EBM + 10% FCS.

Cell cultures

The medium above the monolayer was replaced by 1 ml of the leucocyte suspension and sedimentation at 37°C allowed to take place. Controls of three types were set up in parallel: I, monolayers of uncoated red cells plus leucocytes; II, antibody-coated red cell monolayers without leucocytes; III, uncoated red cell monolayers without leucocytes.

Incubation was terminated at various time intervals by gentle pipetting and removal of the culture fluid, rinsing with warm unsupplemented medium and addition of 2–3 ml fixation fluid (2·5% glutaraldehyde in sodium cacodylate-buffered sucrose (Sabatini, Bensch & Barrnett, 1963). After fixation at room temperature for 60 min, the specimens were dehydrated in a graded series of ethanols and critical point dried from absolute ethanol before being mounted and vacuum coated with 200 Å of gold and examined in a Stereoscan IIA scanning electron microscope (Cambridge Scientific Instruments Ltd).

RESULTS

Low power SEM observations (× 340) revealed that progressive destruction of the antibody-coated monolayer occurred in the presence of leucocytes. While little damage was apparent

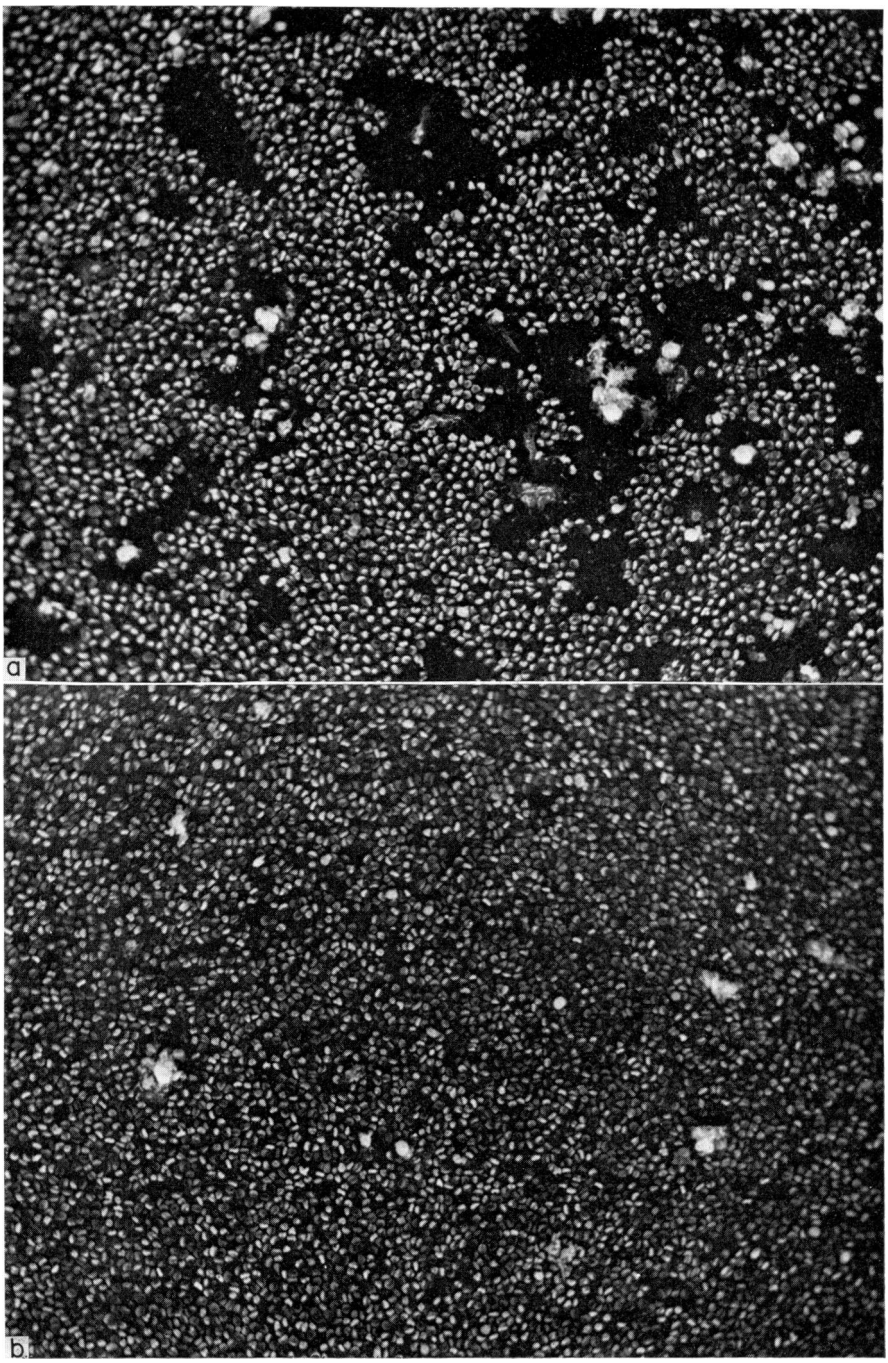

FIG. 1. (a) Low magnification scanning electron micrograph of antibody-coated erythrocytes incubated in the presence of leucocytes for 4 hr. Note the formation of plaque-like areas usually containing leucocytes. (Magnification ×340.) (b) Uncoated monolayer incubated for 4 hr with leucocytes. Note the uniform appearance of the monolayer. (Magnification ×310.)

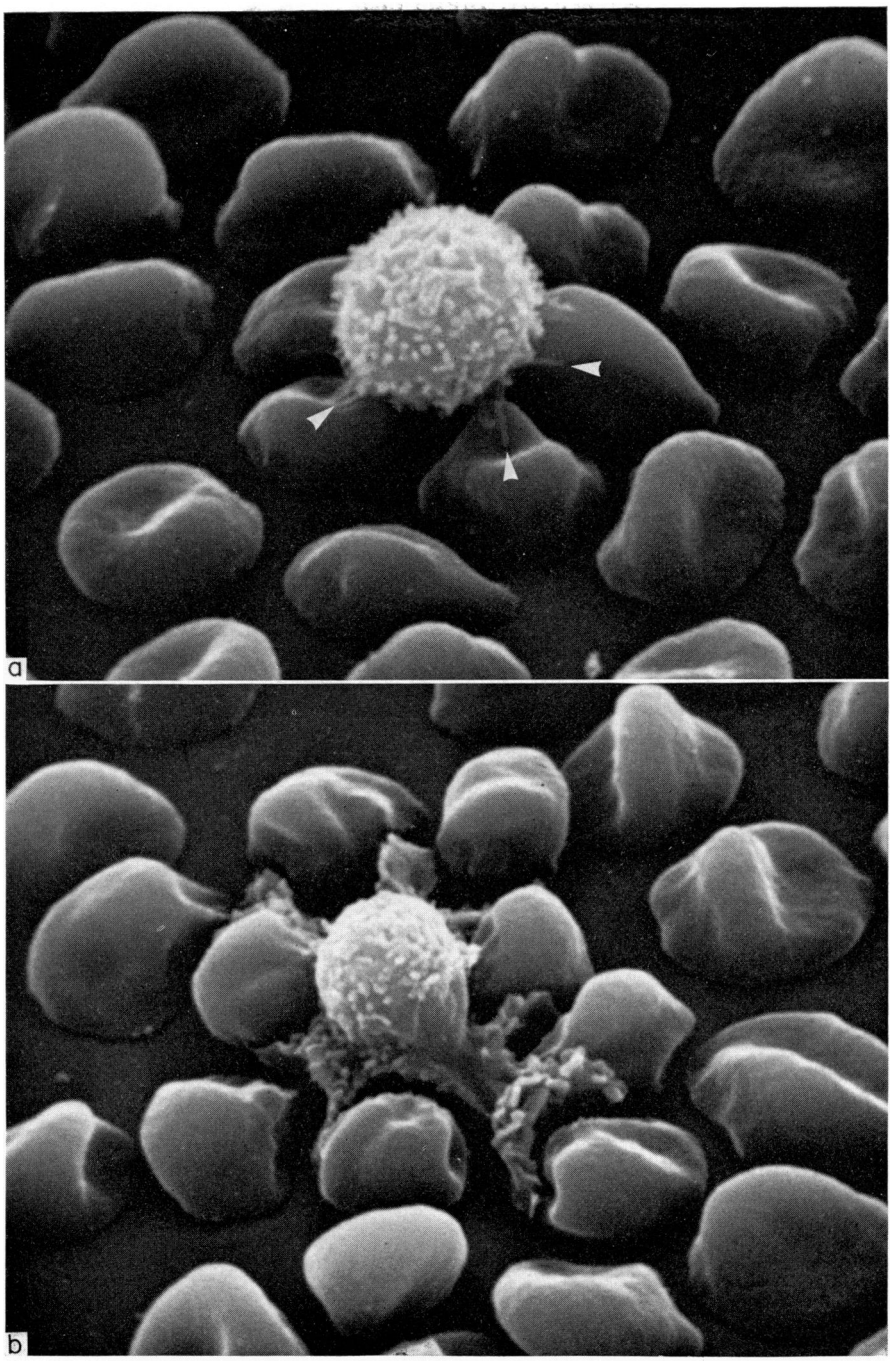

FIG. 2. (a) Type I cell after 30 min incubation on an antibody-coated monolayer, showing stub-like microvilli and the formation of filopodia (arrowed) linking the cell to undamaged erythrocytes. (Magnification ×6600.) (b) Type I cell after 30 min incubation on an antibody-coated monolayer, in a more flattened form. There is little sign of erythrocyte damage. (Magnification ×6600.)

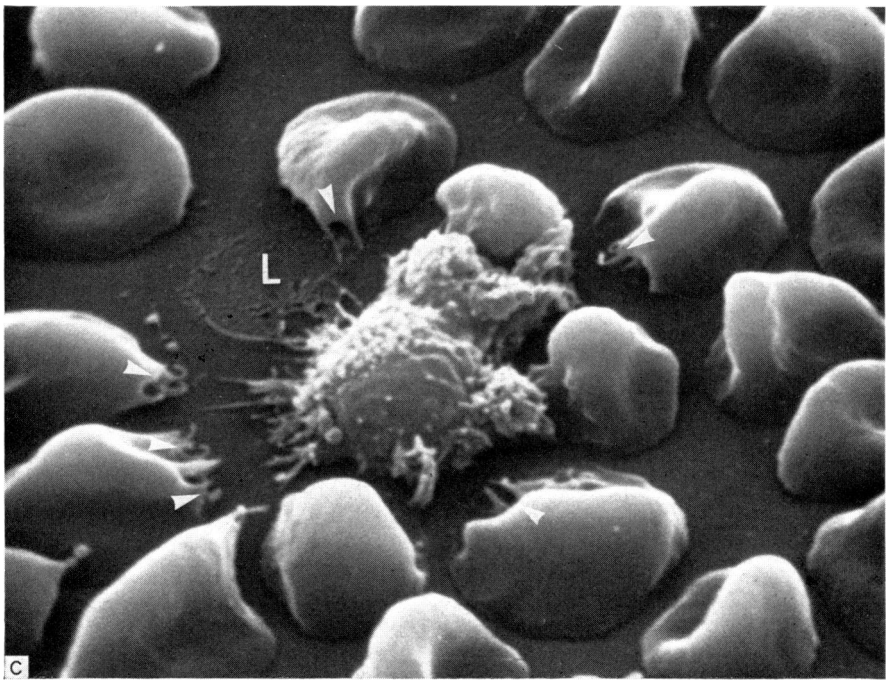

FIG. 2. (c) Type I cell after 60 min incubation on an antibody-coated monolayer, showing a flattened configuration and many filopodia, some of which have broken.

Note the characteristic appearance of the damaged membrane on adjacent erythrocytes, showing tendril-like projections (arrowed), possibly caused by contraction of previously attached filopodia. These erythrocytes show partial collapse or lysis (L). (Magnification $\times 6900$.)

before 60 min incubation, small areas of lysis could be detected after 90 min. After 4 hr, large irregular plaques had appeared with which leucocytes were usually associated (Fig. 1a). In contrast, antibody-coated red cells in the absence of leucocytes remained intact and monolayers of uncoated cells incubated with leucocytes were found after 4 hr to have only small and isolated areas of damage (Fig. 1b).

At higher magnification ($\times 6600$), three types of leucocytes clearly distinguishable by surface morphology were found in association with antibody-coated red cells: Type I, cells with short stub-like microvilli found initially in a rounded form (Fig. 2a) and subsequently in a more flattened form (Fig. 2b and c); Type II, cells with a highly irregular surface of plate or leaf-like structures (Fig. 3a and b); Type III, thin, flattened cells with a relatively smooth surface (Fig. 4).

A characteristic feature of Type I and Type II cells was the formation of fine extensions of the cell membrane (filopodia), varying in length and thickness and found all over the cell with the exception of the upper region. Filopodia attached the cells to both the plastic surface and to the surrounding antibody-coated red cells (Figs 2a and 3b). Membrane extensions of a somewhat similar appearance were also seen on Type III cells but in this instance they were found only in the plane of the plastic surface. This restriction in the

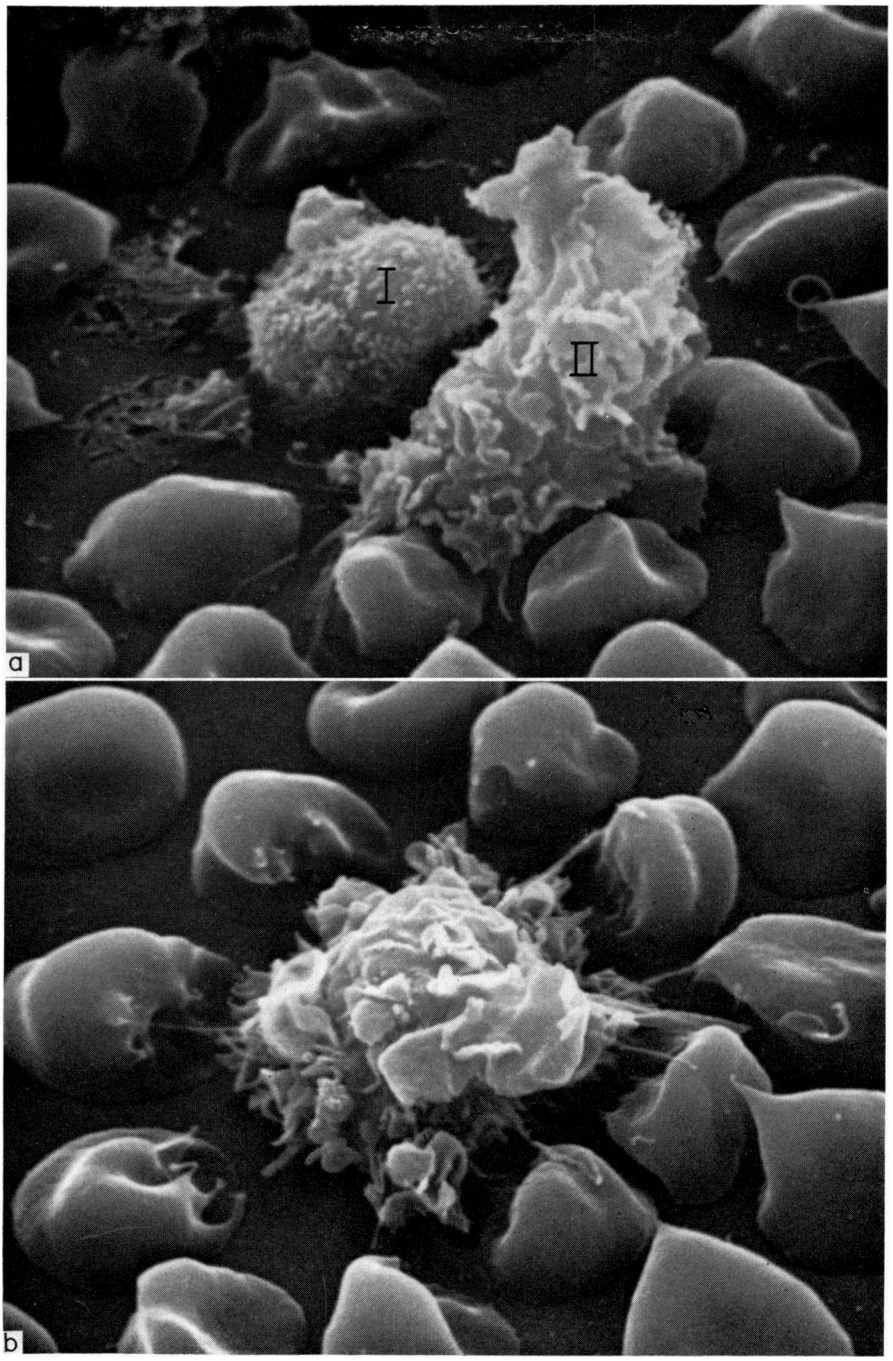

FIG. 3. (a) Type II cell (II) after 30 min incubation on an antibody-coated monolayer. Note the leaf-like surface structures and the formation of filopodia linking the cells to the substrate and adjacent erythrocytes. A Type I cell is also present (I). (Magnification × 6600.) (b) Type II cell after 60 min incubation on an antibody-coated monolayer showing extensive filopodia formation. Surrounding erythrocytes have changes similar to those shown in Fig. 2c. (Magnification × 6900.)

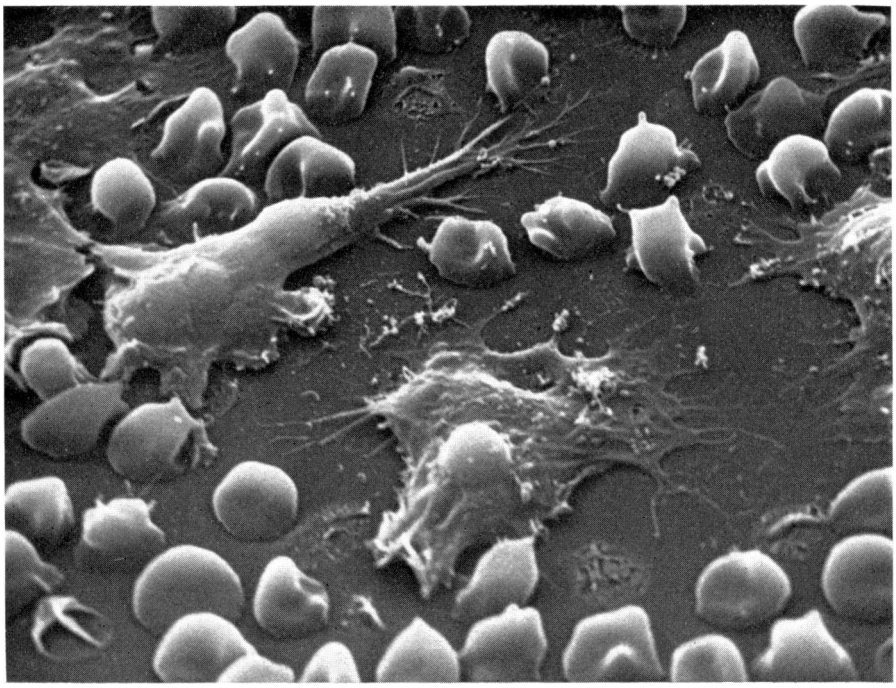

FIG. 4. Type III cells after 90 min incubation on an antibody-coated monolayer, attached to the substrate and probably phagocytosing erythrocytes or debris from previously damaged cells. Note their extremely flattened appearance and relatively smooth surface. (Magnification ×3000.)

distribution of these extensions on this particular cell type may be due to their extreme thinness, or may reflect differences in function.

Leucocyte involvement in erythrocyte cytotoxicity was first apparent after 60 min incubation. Traces of cells which had lysed completely were found as ghosts or membrane fragments on the plastic surface (Fig. 2c) but damage was more frequently found as deformation due to partial collapse of the erythrocyte and the formation of short tendrils on the normally smooth membrane (Fig. 2c, arrowed). It was particularly noticeable that damage occurred only on erythrocytes adjacent to leucocytes of Types I and II and that filopodia linked each leucocyte to some of the surrounding damaged cells. Furthermore, the position and appearance of the tendrils on these red cells strongly suggest that they were formed by the breaking of previously attached filopodia.

The association of Type III cells with damaged erythrocytes and areas of lysis were noticeable after 90 min incubation but in this case filopodia were rarely found to link these cells to erythrocytes. It could not therefore be firmly established whether Type III cells interacted with antibody-coated cells in the same way as Type I and Type II cells (Fig. 4). However, Type III but not Types I or II were found to phagocytose red cells.

In control cultures, where monolayers were not coated with antibody, cells of Types I, II and III also became adherent, but in much fewer numbers. High magnification showed that

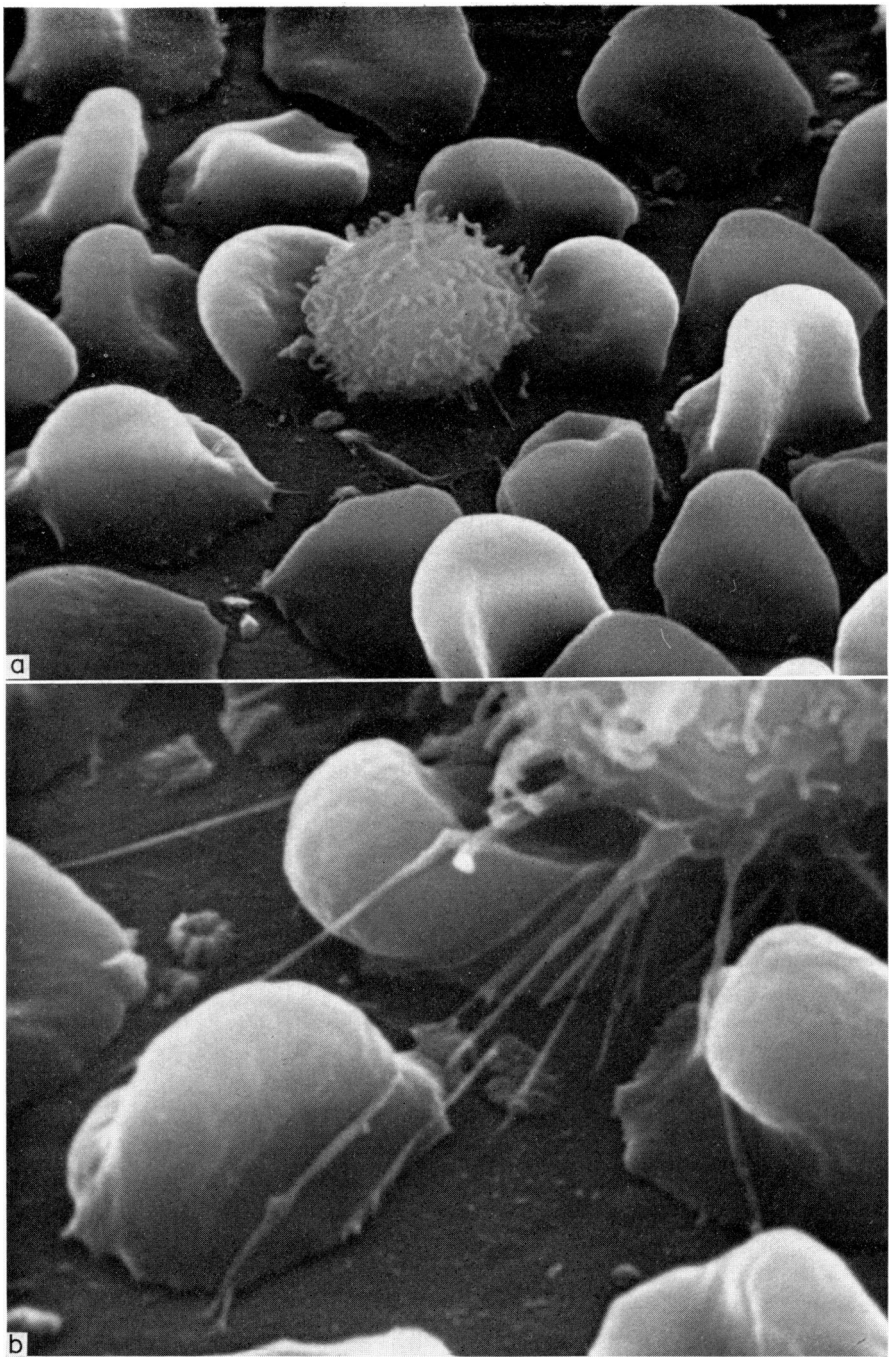

FIG. 5. (a) Control culture. Type I cell after 4 hr incubation on an uncoated monolayer. Note that the filopodia are attached only to the plastic surface and that surrounding erythrocytes are undamaged. (Magnification × 6900.) (b) Control culture. High magnification of a leucocyte (probably Type II) after 4 hr incubation on an uncoated monolayer. Note that the numerous filopodia are not attached to the surface of surrounding erythrocytes. (Magnification × 14000.)

attachment was due to filopodia of similar appearance to those previously described (Fig. 5a). However, in these cultures filopodia were found to attach only to the plastic surface (Fig. 5b) and to show no interaction with uncoated red cells. Furthermore, damage was not observed in red cells adjacent to adherent leucocytes.

DISCUSSION

The effect of normal leucocytes on target cells in the presence of specific antibody is currently the subject of much interest (Perlmann, Perlmann & Wigzell, 1972; MacLennan, 1972; Forman & Möller, 1973) and there is at present no general agreement on the cells responsible for lysis in this sytem. Several cell types have been implicated on the basis of functional studies on leucocytes depleted of various subpopulations of cells *in vitro* (Greenberg, Shen & Roitt, 1973; Dennert & Lennox, 1973; Möller & Svehag, 1972; Calder *et al.*, 1974a) and *in vivo* (Harding *et al.*, 1971; Calder *et al.*, 1974b). The technique described in this paper has the advantage that cytotoxic cells may be identified directly and information on the mechanism of lysis obtained from direct observations of cell–cell interactions. It may also be adaptable to direct quantification.

The results of these preliminary studies suggest that human blood leucocytes obtained after Ficoll–Triosil separation and incubation on glass are a heterogeneous population and may contain at least three cell types distinguishable by surface morphology which can interact with antibody-coated erythrocytes. These cells are only a small proportion of the original leucocyte suspension since most did not adhere to the monolayer after incubation. Furthermore, functional studies suggest that the cells non-adherent to antibody-coated sheep red blood cells show no cytotoxic potential when subsequently cultured with antibody-coated chicken erythrocytes (J. R. Inglis, W. J. Penhale and W. J. Irvine, unpublished results).

Of the adherent cells, those classified as Type I have the surface microvilli reported to be characteristic of both human (Polliack *et al.*, 1973) and mouse lymphocytes (Linthicum *et al.*, 1974a) but since smooth blood leucocytes were found to develop pseudopodia and microvilli after adherence to glass (Linthicum *et al.*, 1974b), the possibility that these features appeared only after contact with the monolayer or its substrate cannot be excluded. The cells with leaf or plate-like surface structures (Type II) are similar in appearance to the macrophages described by Carr and co-workers (Carr, Clarke & Salsbury, 1969; Carr & Carr, 1970). The flattened cells with relatively smooth surfaces (Type III) seem to have the appearance in the light microscope of polymorphonuclear leucocytes (A. E. Williams, unpublished results).

The involvement of adherent cells in antibody-dependent erythrocyte lysis is suggested by their attachment by filopodia to red cells showing distortion and membrane disruption. The role of these filopodia in causing damage is at present unknown but the characteristic deformation of the membrane on damaged erythrocytes would suggest that lysis was induced mechanically, perhaps following filopodial contraction. The presence of contractile elements at the leucocyte surface is suggested by the recent demonstration of smooth muscle antigen in the microvilli of lymphoid cells (Fagreus, Lidman & Biberfeld, 1974). The absence of filopodial attachment to uncoated red cells in control monolayers suggests that antibody may facilitate linkage by providing a specific point of attachment. Alternatively, the attachment of filopodia to red cell surfaces and subsequent stretching of these bonds as the cell

moves away from the site of adhesion, may generate sufficient tension to rupture the erythrocyte membrane. The formation of fibres during the movement of polymorphonuclear leucocytes on glass surfaces has been reported by Ramsay (1972).

We conclude that more than one human leucocyte type may have the ability to lyse antibody-coated target cells and that the action of filopodia in linking effector and target cells may be important in the cytotoxic mechanism.

The work is supported by a grant from the M.R.C. J.R.I. is a recipient of a Vans Dunlop Scholarship from the University of Edinburgh. Mr J. Goodall of the Department of Engineering, University of Edinburgh, provided excellent technical assistance.

REFERENCES

CALDER, E.A., URBANIAK, S.J., PENHALE, W.J. & IRVINE, W.J. (1974a) Characterization of human lymphoid cell-mediated antibody-dependent cytotoxicity. *Clin. exp. Immunol.* **18,** 579.

CALDER, E.A., AITKEN, R.M., PENHALE, W.J., MACLEMAN, D. & IRVINE, W.J. (1974b) Lymphoid cell-mediated antibody-dependent cytotoxicity in untreated and bursectomized chickens. *Clin. exp. Immunol.* **16,** 137.

CARR, K. & CARR, I. (1970) How cells settle on glass: a study by light and electron microscopy of some properties of normal and stimulated macrophages. *Z. Zellforsch.* **105,** 234.

CARR, I., CLARKE, J.A. & SALSBURY, A.J. (1969) The surface structure of mouse peritoneal cells—a study with the scanning electron microscope. *J. Microsc.* **89,** 105.

CEROTTINI, J.-C. & BRUNNER, K.T. (1974) Cell-mediated cytotoxicity, allograft rejection and tumour immunity. *Advanc. Immunol.* **18,** 67.

DENNERT, G. & LENNOX, E.S. (1973) Phagocytic cells as effectors in a cell-mediated immunity system. *J. Immunol.* **111,** 1844.

FAGREUS, A., LIDMAN, K. & BIBERFELD, G. (1974) The reaction of human smooth muscle antibodies with human blood lymphocytes and lymphoid cell lines. *Nature* (*Lond.*), **252,** 246.

FORMAN, J. & MÖLLER, G. (1973) The effector cell in antibody-induced cell mediated immunity. *Transplant. Rev.* **17,** 108.

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

HARDING, B., PUDIFIN, D.J., GOTCH, F. & MACLENNAN, I.C.M. (1971) Cytotoxic lymphocytes from rats depleted of thymus processed cells. *Nature: New Biology*, **232,** 80.

KENNEDY, J.C. & AXELRAD, M.A. (1971) An improved assay for haemolytic plaque-forming cells. *Immunology*, **20,** 253.

LINTHICUM, D.S., SELL, S., WAGNER, R.M. & TREFTS, P. (1974a) Scanning immunoelectron microscopy of mouse B and T lymphocytes. *Nature* (*Lond.*), **252,** 173.

LINTHICUM, D.S., MUJAI, K., SELL, S. & WAGNER, R.M. (1974b) Lymphocyte surface architecture and topography of surface immunoglobulin as revealed by scanning immunoelectron microscopy. *Fed. Proc.* **33,** 765A.

MACLENNAN, I.C.M. (1972) Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transplant. Rev.* **13,** 67.

MÖLLER, G. & SVEHAG, S.E. (1972) Specificity of lymphocyte-mediated cytotoxicity induced by *in vitro* antibody-coated target cells. *Cell. Immunol.* **4,** 1.

PERLMANN, P. & HOLM, G. (1969) Cytotoxic effects of lymphoid cells *in vitro*. *Advanc. Immunol.* **11,** 117.

PERLMANN, P., PERLMANN, H. & WIGZELL, H. (1972) Lymphocyte-mediated cytotoxicity *in vitro*. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* **13,** 91.

PERPER, R.J., ZEE, T.W. & MICKELSON, M.M. (1968) Purification of lymphocytes and platelets by gradient centrifugation. *J. Lab. clin. Med.* **72,** 842.

POLLIACK, A., LAMPEN, N., CLARKSON, B.D., DEHARVEN, E., BENTWICH, Z., SIEGAL, F.P. & KUNKEL, H.G. (1973) Identification of human B and T lymphocytes by scanning electron microscopy. *J. exp. Med.* **138,** 607.

RAMSAY, W.S. (1972) Locomotion of human polymorphonuclear leucocytes. *Exp. Cell Res.* **72,** 489.

SABATINI, D.D., BENSCH, K. & BARRNETT, R.J. (1963) Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17,** 19.