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Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity

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SUMMARY

To test the hypothesis that complement-mediated cell lysis and cell-mediated cytotoxicity operate by analogous mechanisms, cell membranes from two antibody-dependent cytotoxicity systems were examined by electron microscopy after negative staining. Ring-shaped membrane lesions generally similar to, but larger than, those previously described for complement lysis were observed. These findings are in agreement with recent measurements of larger functional pores for ADCC than complement.

INTRODUCTION

A considerable body of information has accumulated to suggest that the mechanism of damage caused by cell-mediated cytotoxicity is similar to the mechanism of complement damage in that a channel is formed in the membrane of the target cell leading to osmotic lysis (Mayer, 1977).

In studies on complement and antibody-dependent cellular cytotoxicity (ADCC), Simone & Henkart (1980) have estimated the size of the membrane channel by examining the cell-mediated release of labelled macromolecules incorporated in resealed human erythrocyte ghosts. In the case of release caused by ADCC, the maximum size of markers that were released was of the order of 12 nm in diameter; in the case of complement damage, the maximum size of the markers released was 5 nm in diameter.

In this study, we report the finding by electron microscopy of structural lesions in target cell membranes in ADCC that were comparable to those described for complement (Humphrey & Dourmashkin, 1969). Two series of experiments are described, the first in which human cell lines are used as targets, and the second in which the targets were resealed human erythrocyte ghosts.

MATERIALS AND METHODS

Experiments using human cell lines as targets

Mononuclear cells. Peripheral mononuclear leucocytes were prepared from healthy donors' buffy coats (Edgware Blood Transfusion Centre). A mixture of buffy coat diluted 1:1 with 1% dextran 500 (Sigma Chemical Co., London) dissolved in heparinized (10 iu/ml) phosphate-buffered

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saline (PBS) was sedimented at 1 *g* at room temperature for 45–60 min. The supernatant was centrifuged through Ficoll–Hypaque (Ficoll–Paque, Pharmacia) at a specific gravity of 1·077 for 30 min at 400 *g* at room temperature. The interphase, containing just the mononuclear fraction of cells, was washed three times in RPMI 1640 culture medium with added 5% foetal calf serum, penicillin, streptomycin and glutamine.

Rabbit antisera. Rabbit antisera against CLA4 and K562 cells were raised in adult albino rabbits by intravenous injection of 1×10^9 cells at days 1 and 14. Partial and complete bleeding of animals was performed at days 21 and 28 respectively. Antisera were pooled and frozen in aliquots. Both the calf serum and the rabbit antisera were heat-inactivated for 1 hr at 56°C to completely inactivate complement, and tested for complement haemolytic activity before they were aliquoted.

Cell lines. A myeloid cell line, K562, had been derived from a patient with myeloid leukaemia. A B cell lymphoblastoid cell line, CLA4, was derived from a patient with infectious mononucleosis (Steel, 1972). The cell lines were maintained as a suspension in RPMI 1640 medium with added 10% heat-inactivated foetal calf serum, antibiotics and glutamine in a CO_2 incubator.

^{51}Cr labelling. Five million cells in 1 ml RPMI 1640 medium were incubated with 0·1 mCi (^{51}Cr) sodium chromate (The Radiochemical Centre, Amersham) for 1 hr at 37°C. They were then washed four times in the same medium.

Experimental procedure. Mononuclear cells and target cells were mixed in ratios of 20:1 or 40:1 using 1×10^5 antibody-coated target cells per tube. The cells were suspended in 1 ml RPMI 1640 bicarbonate-buffered medium with 10% foetal calf serum, glutamine and antibiotics and placed in 5-ml flat-bottomed tubes, 8 mm in diameter. They were centrifuged at 125 *g* for 1 min and incubated for periods of 4–10 hr at 37°C. Controls examined were effector cells, sensitized target cells incubated in a final concentration of 0·01 M EDTA, and sensitized target cells alone incubated in the usual medium. Additional controls were carried out by incubating suspensions of twice-freeze-thawed target cells in the same medium as used for the cytotoxicity experiments for a period of 10 hr at 37°C. The purpose of this experiment was to test whether lesions on membranes were formed as the result of enzymes released from the target cells on lysis. Cytotoxicity was measured by the specific release of ^{51}Cr (Table 1). Six tubes were used for each experiment; after incubation, the cells were resuspended in a Whirlimix, pooled and centrifuged at 50 *g* for 10 min. The supernatants were then collected and centrifuged at 2,000 *g* for 20 min. The pellets were carefully collected and resuspended in approximately 0·05 ml of medium. This membrane suspension was concentrated on EM grids by placing a drop on a 2% agar block, overlaying the grid on the drop and allowing the fluid to be absorbed by the agar. The grids were floated on 2% sodium phosphotungstate, pH 6·5, and allowed to dry. This procedure concentrated the cell membranes released during cytotoxicity but did not distinguish between those derived from lysed target cells and those from damaged mononuclear cells.

Experiments using human resealed erythrocyte ghosts as targets

Human peripheral blood lymphocytes (PBL). One hundred and twenty millilitres of blood was heparinized while collecting with 40 mg of Sigma heparin. Ten-millilitre aliquots of blood were diluted with 10 ml of Plasmagel (Roger Bellom Labs). After the settling of erythrocytes the plasma portion was diluted 1:1 with buffered saline solution–HEPES (BSS) and approximately 1 g of carbonyl iron powder was added to 45 ml of diluted suspension. This was rotated for 25 min at 37°C and the iron was then drawn down to the bottom of the tube with a magnet. Twenty millilitres of suspension were layered on 10 ml of Ficoll–Hypaque separation medium in each tube and centrifuged at 400 *g* for 30 min. The lymphocyte layer was decanted and the cells washed three times with BSS.

Rabbit anti-TNP antibody. This was prepared from the serum of rabbits hyperimmunized to TNP-KLH. Specific antibody was bound to a column of DNP-lysine-Sepharose and eluted with 0·1 M acetic acid (Simone & Henkart, 1980).

Resealed human erythrocyte ghosts. Three millilitres of washed packed human RBC from the same donor as for the PBL was diluted with 10·5 ml of phosphate-buffered saline (PBS) and 1·5 ml of 10 mM trinitrobenzene sulphonate at pH 7·4 was added. After incubation at 37°C for 15 min the cells were washed with PBS. The packed cell pellet was diluted 1:1 with BSS, and 2 ml of this cell

suspension was diluted with 18 ml of a solution of 4 mM $MgCl_2$ and 3·5 mM acetic acid at pH 3·5 at 0°C. Fifty microlitres of 200 mM carboxyfluorescein was added as a fluorescent marker and the suspension was adjusted to pH 6·2 for 5 min. Then 2·4 ml of 10× stock of BSS divalent ions ($CaCl_2.2H_2O$ = 1·9 g/l; $MgSO_4.7H_2O$ = 2·0 g/l) and 2·4 ml of a 10× stock of BSS monovalent ions + HEPES (HEPES = 23·8 g/l; glucose = 18·0 g/l; NaCl = 80 g/l; KCl = 4 g/l; K_2HPO_4 = 0·68 g/l; $Na_2HPO_4.H_2O$ = 0·9 g/l) were added. The mixture was incubated at 37°C for 45–60 min and the resealed ghosts washed with BSS at 10,000 *g* for 10 min at 0°C. The resealed ghosts were examined in a fluorescence microscope as to the presence of the marker.

Experimental procedure. Four hundred-mesh gold EM grids were coated with a parlodion film strengthened with carbon and positively charged by floating on a fresh 1% solution of alcian blue in distilled water for 3 min at room temperature. They were then washed three times in distilled water, washed once in BSS and then immersed face up in a petri dish containing a thick suspension of resealed ghosts labelled with TNP. After 5 min the grids were washed in BSS. Examination by fluorescent microscopy or electron microscopy revealed a stable confluent monolayer of resealed ghosts. The grids were then placed in wells made by an appropriately sized hole punched in a Parafilm strip and placed on a glass slide. Antibiotics (penicillin, streptomycin and gentamicin) were added to all buffers used, the Parafilm-coated slides were sterilized in alcohol before use, and care was exercised to keep the buffers free of contamination.

Some grids were sensitized with a 1:20 dilution of rabbit anti-TNP IgG, and were overlaid with a suspension of 1×10^6 PBL in BSS. Some grids were prepared as effector–target cell combinations without antibody and others as target cells alone. After preparing the experiments, the glass slides were placed in damp sealed boxes and incubated at 37°C for 3 hr. Subsequently, the grids were rinsed gently in BSS, floated on a solution of 0·25% trypsin in BSS for 15 min at room temperature and then floated on 1% formalin (freshly made from paraformaldehyde) in H_2O for 15 min at room temperature. They were then rinsed in distilled water, negatively stained with 1% sodium phosphotungstate, and examined in a Philips 300 electron microscope. Most of the resealed ghosts remained on the grids throughout this procedure but the PBL were almost completely removed.

RESULTS

Experiments using human cell lines

In each trial (Table 1) careful examination by electron microscopy of membrane fragments revealed a few membranes showing circular ring structures that were similar to but larger than the lesions caused by complement (Figs 1 and 2). The structures associated with ADCC consisted of clusters of rings with an average internal diameter of 16 nm. Seen in profile, they were raised from the surface of the membrane as cylinders. They were also found separate from the rest of the membrane and some appeared to be breaking down to smaller units. Approximately one in 200 membrane fragments examined showed these structures. Exhaustive examination of membranes obtained in the same manner from antibody-sensitized targets alone, or from freeze-thawed targets incubated in

Table 1. Per cent specific ^{51}Cr-release and experimental details

Experiment	Incubation (hr)	Per cent specific release*		Target cell line	Ratio effectors/targets
		In RPMI	In RPMI + EDTA		
1	4	33	n.d.	CLA4	20:1
2	9	64	6	K562	20:1
3	10	93	35	K562	40:1

* Per cent specific release $= \frac{(\text{experimental } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release})}{(\text{maximum } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release})} \times 100.$

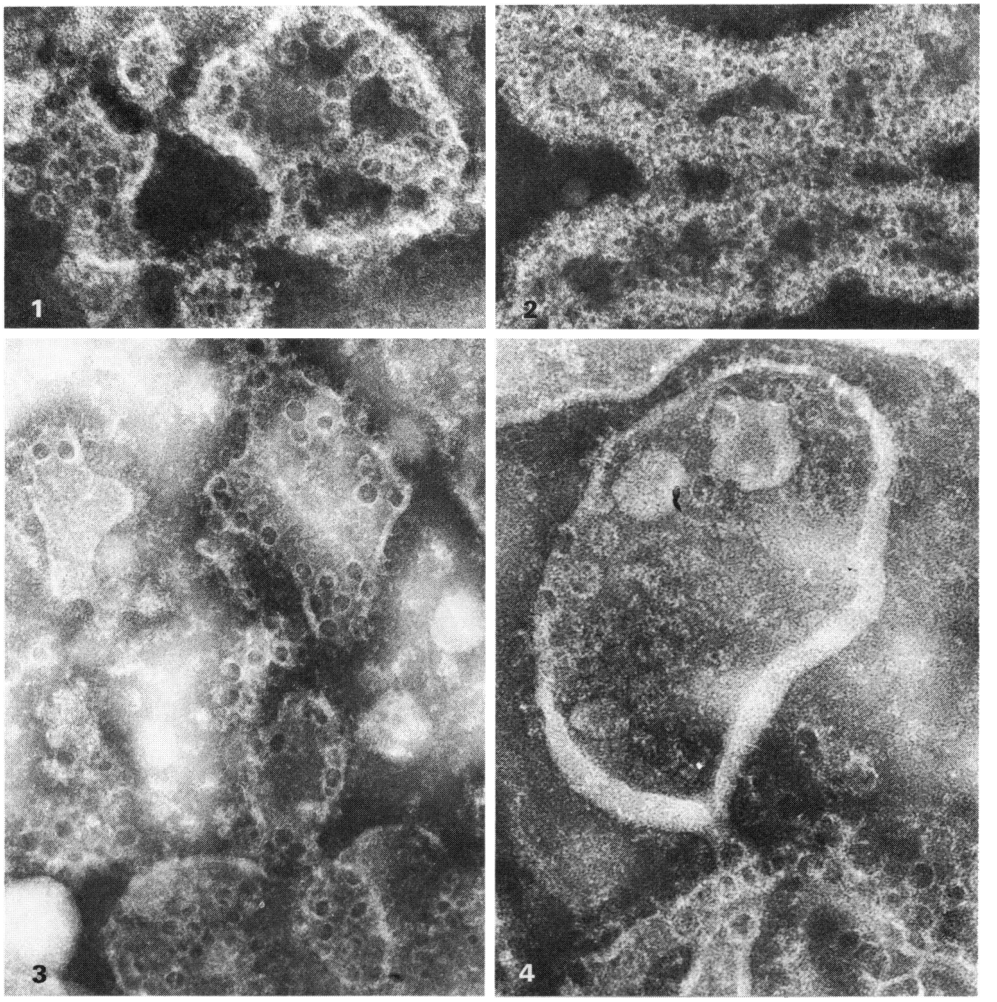

Fig. 1. Membrane lesions from an ADCC experiment using K562 cell line as targets. (× 140,000.)

Fig. 2. Complement lesions in membranes from K562 cells treated with antibody and human complement. Note that the complement lesions are smaller than those resulting from ADCC in Fig. 1. (× 140,000.)

Fig. 3. Lesions in erythrocyte membranes from an ADCC experiment using TNP-labelled human erythrocytes as targets. (× 140,000.)

Fig. 4. The same as in Fig. 3. Lesions can be seen in silhouette as cylinders projecting from the membrane surface. (× 220,000.)

medium alone, or effector–target cell mixtures incubated with EDTA, did not show any such structures. However, the limitations of this experimental system made it impossible to distinguish membranes from target cells or effector cells, nor the interior endoplasmic reticulum membranes from the cell membranes. In addition it was also not possible to have a control in which antibody was omitted from the effector–target cell mixture as natural killers were effective in this system. However, these findings suggested that the ring structures represent the lesions caused by ADCC.

Experiments using human resealed erythrocyte ghosts

It was easily possible to distinguish between the ghost membranes, which were smooth, and the

membranes of those lymphocytes which remained in the sample to which mitochondria were adherent. The centres of lymphocytes were extremely dense due to the nuclei. Four independent experiments were performed with ghost targets and purified lymphocyte effector cells using conditions generally similar to those used previously for measurement of marker release from ghosts (Simone & Henkart, 1980). In each case, circular ring structures were found in ghost membranes after incubation with anti-TNP and effector cells. These structures were similar to those seen in the experiments using human cell lines. They were usually seen in clusters, often breaking off from the cell membrane, and appearing as individual rings (Fig. 3). In two experiments the ring structures were seen only rarely, but in a third, lesions were seen in about one in five ghost membranes. Because of the frequency of the lesions, it was possible to characterize them with greater accuracy. Their internal diameters varied greatly, much more so than those caused by complement. Whereas the human complement lesion has an internal diameter of 10 nm (Humphrey & Dourmashkin, 1969), the ADCC lesion had a diameter whose mode was 15·5 nm and whose range was from 7·9 to 20·2 nm (Fig. 5). In the case of lesions observed in profile (Fig. 4), the height of the cylinders thus shown was 10·8 nm from the membrane surface. The thickness of the external ring was difficult to measure; the mean was 2·5 nm. The external ring appeared to be thickest at the top, as in complement. In each of the three experiments described, ghosts incubated with effector cells in the absence of antibody showed no lesions. Similarly, treatment of the ghosts with anti-TNP as above in the absence of effector cells did not give rise to any ring structures. In one experiment with lymphocyte effector cells, generally similar ghost lesions were seen with equal frequency in the presence or absence of antibody. This experiment was found to be contaminated by bacteria, which are known to be capable of causing such ring structures (Dourmashkin, unpublished observations). In this case the structures differ from those described above in that they are extremely regular in diameter.

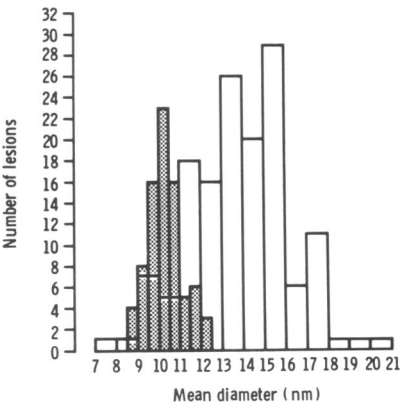

Fig. 5. Histogram showing distribution of sizes of complement lesions and ADCC lesions. The diameters are the mean of two measurements taken at right angles of the internal 'dark' portion of the ring. ADCC lesions are enumerated as clear bars, 1 nm in width, complement lesions are shaded bars, 0·5 nm in width. Some of the bars representing numbers of ADCC lesions fall below the height of those repesenting the complement lesions.

DISCUSSION

In these experiments, we have described the observation of cylindrical ring structures occurring in membranes after ADCC lysis. In the experiments utilizing resealed ghosts, we have been able to recognize the membranes as the targets both by their morphology and by the means with which they were prepared for electron microscopy. Omission of antibody abrogated ADCC and ghost marker release in this system (Simone & Henkart, 1980) and prevented the appearance of the lesions.

The variable size of the ADCC lesions appears to contrast markedly with the narrow range of

size in complement lesions. It is tempting to suggest that the cylindrical ring structures represent channels analagous to those caused by complement, possibly utilizing some of the proteins in the complement cascade. Although whole complement is not required for either T cell-mediated cytotoxicity or ADCC, there is evidence that C8 may be involved (Perlmann, Perlmann & Lachmann, 1974); it is conceivable that complement or other proteins may be secreted by the lymphocyte membrane on contact with the membrane of the target cell (Sundsmo & Müller-Eberhard, 1979).

Giavedoni, Chow & Dalmasso (1979) and Simone & Henkart (1980) found that the functional pore for complement is 5 nm in diameter, which is smaller than the 10 nm observed in the electron microscope. Similarly the functional pore for ADCC was found to be 12–16·5 nm (Simone & Henkart, 1980), which is somewhat smaller than the maximal diameter observed in these experiments (Fig. 5). One explanation for this size difference is that there may be an occlusion in the pore which is structurally small but which limits the size of passing molecules.

The concept that cell-mediated cytotoxicity is caused by a membrane lesion is supported by data based on physiological and morphological studies, encompassing both T cell-mediated cytotoxicity and ADCC (K cell killing). Henney (1973) suggested that T cell-mediated destruction of target cells is caused by colloid osmotic lysis following the establishment of a lesion in the target cell membrane. This was supported by two sorts of evidence: one that the dose–response curve of cell killing corresponded to a one-hit mechanism, and the other, that the release of small molecular-weight markers preceded the release of cellular contents of large molecular weight. Ziegler & Henney (1975) subsequently demonstrated similar data for antibody-dependent cellular cytotoxicity. Studies employing extracellular macromolecules to inhibit T cell-mediated lysis (Henney, 1974; Ferluga & Allison, 1974) have indicated the presence of either an osmotic or diffusion-limiting protective mechanism. Burakoff, Martz & Bennacerraf (1975) found that the colloid osmotic phase of complement-lysed tumour cells is temperature-sensitive, suggesting that the temperature-dependency of T cell-mediated lysis is not inconsistent with osmotic lysis. Golstein & Smith (1977) suggested that T cell-mediated lysis does not require the presence of functional effector cells as the effector cell may leave the target cell before damage is observed (Koren, Ax & Freund-Moelbert, 1973); Ferluga & Allison (1975) found that isolated plasma membranes from oxazolone-painted mice were cytotoxic towards cells. However, this report is at variance with others, suggesting that viable effector cells are required to effect lysis (Henney, 1977).

In contrast to the reports that cell-mediated cytotoxicity is caused by osmotic damage, Sanderson (1976a, b) in kinetic and morphological studies on T cell-mediated lysis found that target cell death is accompanied by the sudden release of cytoplasmic contents, and is characterized by an explosive reaction termed 'zeiosis'. The EM observations of Grimm, Price & Bonavida (1979) showed the presence of close junctions between effector and target cell membranes in T cell-mediated cytotoxicity. They found that the target cell cytoplasmic bridge was often fractured, presumably leading to loss of cytoplasmic contents and to zeiosis. The bulk of the evidence concerning the mechanism of cellular cytotoxicity supports the concept that membrane lesions are formed on the target cell, such as we have visualized by electron microscopy.

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