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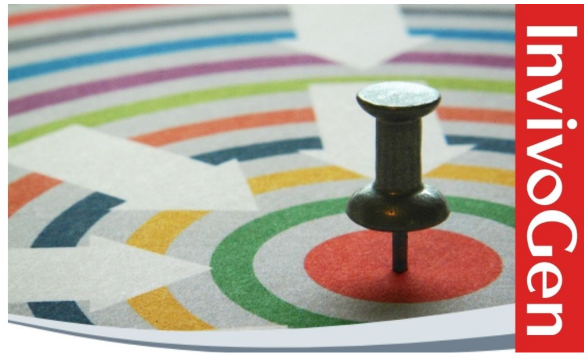

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PERMEABILITY CHANGES INDUCED IN ERTHROCYTE GHOST TARGETS BY ANTIBODY-DEPENDENT CYTOTOXIC EFFECTOR CELLS: EVIDENCE FOR MEMBRANE PORES

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Resealed human erythrocyte ghosts were used as model target cells to study membrane damage induced by effector cells in antibody-dependent cell-mediated cytotoxicity (ADCC). The release of the water soluble dye carboxyfluorescein (CF) from ghosts was shown to parallel the lysis of intact cell targets with regard to requirements for effector cells, antibody binding, and divalent ions. The release of soluble fluorescent markers resealed inside ghost targets was measured in a series of double label experiments with CF and rhodamine-labeled macromolecules. A sharp size dependence of marker release was observed: human lymphocyte effector cells caused the release of all protein and dextran markers below 500,000 daltons to the same extent as CF. Both protein and dextran markers above this size, however, were not released. With neutrophils as ADCC effectors, protein markers of the same size were released, whereas monocytes gave a slightly smaller m.w. cutoff. Lymphocyte effectors did not mediate CF release from smaller vesicle targets derived from whole ghosts. Treatment of whole ghost targets by antibody and complement also showed a sharp size cutoff for release of markers, but at about 10× lower m.w. for both proteins and dextrans. The results strongly suggest that ADCC effector cells inflict lethal damage to target cells by causing the formation of aqueous pores in the target membrane, and that such pores are larger than those formed by complement.

Cytotoxic lymphocytes are a well established arm of the efferent immune response and appear to play an important physiologic role in graft rejection, resistance to viral infection, and probably the rejection of tumors (1, 2). The mechanism by which lymphocytes specifically kill foreign cells has been studied by a number of groups who have dissected the process into several steps. After a specific binding event in which the lymphocyte attaches to the target cell, a cytolytic process is triggered that results in the subsequent death of the target cell (3–5). This description appears to be applicable to cytolysis by killer T lymphocytes that differentiate in response to alloantigen *in vitro* or *in vivo* and also to the antibody-dependent cytolysis mediated by lymphocytes and other cells (6).

Several lines of evidence suggest that cytotoxic lymphocytes inflict lethal injury to target cells by damaging their plasma membranes. Cytotoxic lymphocytes can lyse many different types of target cells, including such simple cells as human erythrocytes (7); lysis occurs within a few hours of killer-target binding (8). Colloid osmotic lysis of the target cell after damage to the target cell membrane is suggested by the kinetics of release of different sized markers from target cells (9), but this line of evidence has been questioned (10, 11). Support for this concept derives from experiments in which the release of macromolecules from target cells damaged by killer lymphocytes is inhibited by high concentrations of extracellular macromolecules (12, 13), but these experiments are controversial (14).

One approach to studying possible membrane damage of target cells by cytotoxic cells has been the use of simple and defined lipid membranes as targets. Antibody-dependent lymphocyte-mediated electrical conductance increases in black lipid membranes showed that large increases in permeability to small ions occur under conditions in which lysis of target cells would be expected. The formation of aqueous channels by killer cells in the target membrane was suggested as the most likely mechanism (15). In other studies, antibody-dependent lymphocyte-mediated release of markers from liposomes was reported by some investigators (16–18), but not by others (19, and unpublished observations by Henkart and Neels). In the present study, the action of antibody-dependent cytotoxic cells on resealed erythrocyte ghosts was investigated. These target membranes have the advantage of being equal in size and composition to the comparable erythrocyte targets, but they are unaffected by colloid osmotic forces after the ionic permeability is increased by lytic mechanisms. It was recently reported that small, but not large, proteins resealed inside such ghosts were released by complement (C), supporting the concept that C creates a defined aqueous pore in the membrane (20). The present study confirms these results by using different techniques and demonstrates that larger channels are created by cytotoxic lymphocytes.

MATERIALS AND METHODS

Fluorescent markers. Carboxyfluorescein was purchased from Eastman (Rochester, N. Y.) and recrystallized from ethanol/water (21). Rhodamine-labeled dextrans were prepared by reaction of rhodamine isothiocyanate (Research Organics, Cleveland, Ohio) with the aminoethylcarboxymethyl derivative of dextrans; the latter were prepared from sized dextran fractions (Dextran B512, Pharmacia, Piscataway, N. J.) by the method of Inman (22) and contained one amino group per eight monosaccharide residues. One hundred milligrams of dextran derivative were dissolved in 50 ml 0.05 M borate, pH 9.0, and 25 mg of rhodamine isothiocyanate in 2.5 ml *N,N*-dimethyl

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formamide (DMF)[1] were added and mixed. After several hours at room temperature, 0.5 ml acetic anhydride was added and the mixture was dialyzed against phosphate-buffered saline (PBS), pH 7.4, containing anion exchange resin AGl-X2 for 5 to 8 days at 5°C, changing the dialysis fluid two times per day. Proteins at 10 mg/ml were rhodamine-modified in 0.05 M borate, pH 9.0, by addition of rhodamine isothiocyanate (dissolved in DMF at 10 mg/ml) to a weight ratio of 1/20. After reacting for several hours at room temperature, the modified proteins were dialyzed against PBS for 1 week and lyophilized, then reconstituted in BSS-HEPES (see Abbreviations) at 10 mg/ml.

The proteins used, their m.w. (23), and sources are as follows: 1) α-lactalbumin, 17,500 daltons, from bovine milk (grade II, Sigma Chemical Co., St. Louis, Mo.); 2) carbonic anhydrase A, 30,000 daltons, from human erythrocytes (Sigma Chemical Co.); 3) pepsin A, 33,500 daltons, from hog stomach mucosa (Sigma Chemical Co.); 4) carboxypeptidase A, 34,600 daltons (Worthington Biochemical Corp., Freehold, N. J.); 5) pepsinogen, 40,000 daltons (Worthington Biochemical Corp.); 6) ovalbumin, 43,000 daltons (grade V, Sigma Chemical Co.); 7) bovine serum albumin, 68,000 daltons (Sigma Chemical Co.); 8) bovine γ-globulin BGG, 150,000 daltons (Miles Laboratories, Inc., Kankakee, Ill.); 9) catalase, 250,000 daltons, from beef liver (2× crystallized, Sigma Chemical Co.); 10) apoferritin, 480,000 daltons, from horse spleen (Sigma Chemical Co.); 11) thyroglobulin, 650,000 daltons, from pig (type II, Sigma Chemical Co.); and 12) hemocyanin, ∾800,000 daltons, from the keyhole limpet (Calbiochem-Behring Corp., Gaithersburg, Md.).

Antibodies and complement. Affinity purified IgG rabbit and sheep anti-TNP, and $F(ab')_2$ fragments derived from these preparations by pepsin digestion were used. Affinity-purified rabbit anti-TNP IgG was used at a final concentration of 20 μg/well to mediate lymphocyte and neutrophil ADCC (see Abbreviations) and for C experiments. Human anti-blood group B antibody was used at a 1:100 dilution of whole serum to mediate monocyte-specific ADCC (24, 25). Lyophilized guinea pig C (Grand Island Biological Co., Grand Island, N. Y.) at 20 λ per well was used with 20 μg rabbit anti-TNP to induce marker release.

Preparation of resealed ghosts. A modification of the procedure of Schwoch and Passow was used (26). Fresh human blood was obtained by venipuncture, and the red cells were washed three times with PBS, removing the buffy coat. The red cells were TNP modified by reaction with 1.0 mM TNBS in PBS at pH 7.4 for 15 min at 37°C. After being washed three times with PBS, the packed cells were resuspended with an equal volume of 0.1 M HEPES-buffered BSS. One volume of this cell suspension was added to 10 volumes (20 ml total) of ice cold $MgSO_4$ buffer (4.0 mM $MgSO_4$, 3.5 mM acetic acid adjusted with NaOH to pH 6.2) maintained at 0°C and pH 6.2 with 1 M acetic acid. To this were added 10 μl of 200 mM carboxyfluorescein (CF) alone or with 15 mg of rhodamine-labeled macromolecules. After 5 min, 1.2 volumes of 10× stock of BSS divalent ions, and 1.2 volumes of 10× stock of BSS monovalent ions plus HEPES were added, thus raising the pH to 7.4. The hemolysate was incubated at 37°C for 1 hr to reseal the ghosts. The ghosts were washed three to five times with BSS-HEPES by using a Sorvall centrifuge rotor SS34 at 15,000 rpm for 10 min at 0°C.

Preparation of right-side-out vesicles. Unsealed ghosts were prepared from TNP-modified human erythrocytes (27). CF containing right-side-out vesicles from these ghosts were prepared by a modification of the procedure of Steck (28). Unsealed ghosts from 2-ml packed red cells resuspended in 50 ml of 10^{-7} M $MgSO_4$ were added and mixed. Membranes were then pelleted by centrifugation at 100,000 × G for 30 min. The pellet was resuspended in 2.0 ml of 0.5 mM phosphate, 0.1 mM $MgSO_4$, 0.1 mM CF, pH 8.0, and passed through a 27-gauge needle three times. The resulting vesicles were separated on an 8% dextran T-70 gradient, and the resealed vesicles were collected from the interface and washed (28).

Effector cells. All ADCC effector cells used in these experiments were of human origin. Lymphocytes were prepared as follows: 120 ml of blood were obtained by venipuncture and were anti-coagulated with 40 mg heparin (Sigma, Grade I from porcine intestinal mucosa). Ten milliliters of Plasmagel (Roger Bellon Laboratories, France) were mixed with 10 ml of anti-coagulated blood in a 14 × 150 mm tube and incubated in a 37°C water bath for 30 min. Supernatants from two tubes were added to a 50-ml plastic conical tube, diluted to 45 ml with Eagle's MEM containing 0.01 M HEPES, pH 7.4, and then 1.0 g of carbonyl iron (GAF Corp., Grade mix number 182) was added. These tubes were gently agitated for 30 min at 37°C. A magnet was used to clear the iron particles to the bottom of the tube. Ten milliliters of this supernatant were transferred to a new 50-ml tube, and 10 ml Ficoll-Hypaque (LSM solution, Bionetics) were gently layered underneath. These tubes were centifuged at 220 × G for 30 min. The cells at the interface were washed three times at 250 × G for 10 min and counted with a hemocytometer. Preparation of granulocytes and mononuclear cells were performed according to the procedure of Boyum (29). Human blood (7.5 ml) anti-coagulated with EDTA was mixed with 1.5 ml of 6% dextran T-500 (Pharmacia) and incubated for 30 min at 37°C. The supernatant was placed in a 15-ml conical tube, and 2.5 ml of Ficoll-Hypaque were layered under it. Centrifuging for 30 min at 220 × G yielded mononuclear cells at the interface and granulocytes and red cells in the pellet. Both cell types were washed three times and counted.

Release of fluorescent markers from ghosts: a) *by fluorometry.* Reactions were carried out on ghosts adherent to the bottom surface of flat-bottom microtiter wells, and the fluorescence of released markers was measured by fluorometry. Microtiter wells were treated with 25 μl of sheep anti-TNP $F(ab')_2$ (1 mg/ml in PBS) at room temperature and washed with PBS. One hundred microliters of a 5% suspension of resealed ghosts were added to each well, and the plate was centrifuged at 450 × G, 0°C for 30 min. Each well was washed by gentle filling and emptying 7 to 10 times; phase microscopy revealed an even, dense array of ghosts on the surface, with each well containing ∾7×10^5 ghosts. The wells were all refilled with BSS-HEPES and the desired reaction components (effector cells, antibody, C) were added to a final volume of 200 μl. The plate was centrifuged at 250 × G for 10 min and incubated at 37°C. In the case of C, this incubation was for 60 min, whereas for cell-mediated marker release a 3 to 5 hr incubation was used. One hundred-microliter aliquots were then withdrawn from each well and their fluorescence was determined on an Aminco-Bowman Spectrophoto-fluorometer (American Instrument, Co., Silver Spring, Md.). The wave lengths of excitation and emission used were 470 nm and 520 nm, respectively, for car-

[1] Abbreviations used in this paper: ADCC, antibody dependent cell-mediated cytotoxicity; BSS, balanced salt solution; FcR, Fc receptor; PBL, peripheral blood lymphocytes; Rh, tetramethyl rhodamine. DMF, *N,N*-dimethyl formamide; CF, carboxyfluorescein; EDTA; ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; KLH, keyhole limpet hemocyanin; BGG, bovine γ-globulin.

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boxyfluorescein, and 540 nm and 570 nm for rhodamine. In all experiments, the total releasable fluorescence was determined by the fluorescence released by 1% Triton X-100. In all experiments, samples were run in duplicate. Agreement between the two duplicates was within 15%.

b) *By fluorescence microscopy.* A monolayer of resealed TNP-ghosts was prepared on glass slides containing printed Teflon rings with an inner diameter of 8 mm (Roboz Surgical Instrument Co., Inc.). Five microliters of sheep anti-TNP $F(ab')_2$ (0.5 mg/ml in PBS) were used to coat the glass inside of the rings for 15 min at 25°C. This then was washed, and 50 μl of a 5% ghost suspension were added and allowed to settle at 0 to 4°C for 20 to 30 min. After washing the excess ghosts away, an evenly dense array of ghosts was seen with phase microscopy. The Teflon ring will accommodate 50 μl of fluid, so that desired reaction components were added to the monolayer pre-mixed to final volume of 50 μl. The slides were placed in a humidified chamber at 37°C for the desired times (see above). After the incubation, the slides were gently washed with BSS-HEPES. By using a Zeiss fluorescence microscope with a HBO W/3 mercury lamp with standard rhodamine and fluorescein filters, 200 ghosts were counted for each sample; ghosts were first identified with phase optics, then scored individually as containing CF or not and finally scored for the presence of the rhodamine macromolecule. In some experiments, the nonadherent lymphocytes (Fc receptor negative) were washed away from the ghosts monolayer after the lymphocyte preparation settled onto the target monolayer for 15 min at 25°C. For experiments in which marker release by effector cells was observed in progress, a coverslip was sealed over the monolayer, and the slide was placed on the microscope stage and heated to 37°C with an air stream incubator. For experiments in which marker release was scored by fluorescence microscopy, the slide was incubated at 37°C for 3 to 5 hr in a humidified chamber, the effector cells gently washed away, and a coverslip was sealed over the ghost monolayer before examination in the microscope.

Calculations of marker release. Marker release could be measured quantitatively by either of the above two methods by using analogous calculations. With measurements by fluorometry, calculations were expressed relative to the level of marker release by 1% (1.6 mM) Triton X-100. Specific release was defined as the percent marker released by antibody plus cells or C minus the percent marker released by C alone. In the case of fluorescence microscopy, specific release was defined as the percent of ghosts that lost the marker when exposed to antibody plus cells or C minus the percent of ghosts that lost the marker when exposed to cells or C alone. With both types of measurements, the relative release of macromolecules was defined as the specific release of the macromolecule divided by the specific release of CF.

^{51}Cr release from erythrocyte target cells. Fresh human erythrocytes were washed three times with PBS. Then 20 μl of packed cells were washed with Alsevers solution and resuspended in 0.8 ml of this buffer. After addition of $Na^{51}CrO$ (0.4 ml of 1 mCi/ml, 200–400 Ci/g, New England Nuclear), this mixture was incubated at 37°C for 1 h or more and then washed three times with PBS. Experiments in which erythrocyte target cell lysis was measured by ^{51}Cr release were carried out as described under fluorometry except that samples were counted in a γ-counter.

RESULTS

Erythrocyte ghosts as model targets for ADCC. The use of resealed erythrocyte ghosts to study membrane permeability damage by lytic agents was extended to antibody-dependent cell-mediated cytotoxicity (ADCC). As seen in Figure 1, it was found that use of a monolayer of red blood cell targets allowed an efficient ADCC with human peripheral blood lymphocytes, since close to 100% ^{51}Cr release could be obtained with a effector to target ratio of less than 3:1 in a 3 to 5 hr assay. It was also found that these lymphocytes caused an antibody-dependent release of CF from resealed red cell ghosts in this geometry, and that the levels of CF release from ghosts were similar to the levels of ^{51}Cr release from intact cells at each effector cell concentration. As can be seen in Figure 1, $F(ab')_2$ antibody did not increase ^{51}Cr release from red cells or CF release from ghosts in the presence of 2×10^6 effector cells per well, as is typical in ADCC.

An experiment demonstrating additional parallels between marker release from ghosts and lysis of target cells by ADCC is shown in Table I. In this experiment, ghosts were resealed with CF and rhodamine-dextran (m.w. 250,000). Although reasonable marker release is observed by using the complete system of lymphocytes and intact anti-TNP, omitting either of these or using $F(ab')_2$ anti-TNP causes a marked reduction in marker

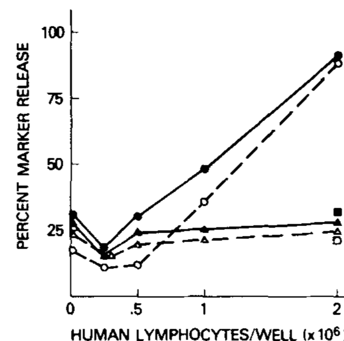

Figure 1. Antibody-dependent lymphocyte-mediated lysis of TNP-RBC and CF release from TNP-ghosts. Monolayers of ^{51}Cr RBC- or CF-filled ghosts were prepared in parallel on the bottom of microtiter wells as described in *Materials and Methods.* The indicated numbers of lymphocytes were added to each well in the presence or absence of 25 μg of anti-TNP. Marker release was determined by supernatant analysis after 3 hr at 37°C. Release with anti-TNP plus lymphocytes: CF, ●—●; ^{51}Cr, ○- -○. Release with lymphocytes only: CF, ▲—▲; ^{51}Cr, △- -△. Release with $F(ab')_2$ anti-TNP plus lymphocytes: CF, ■; ^{51}Cr, □.

TABLE I

Lymphocyte-mediated release of markers from resealed TNP-ghosts[a]

Effectors	Antibody	Medium	Specific % CF Release	Specific % Rh-Dex-250 Release
2×10^6 PBL	5 μg α-TNP	BSS	64	89
2×10^6 PBL	0	BSS	5	11
0	5 μg α-TNP	BSS	8	−5
2×10^6 FcR negative PBL	5 μg α-TNP	BSS	12	17
2×10^6 PBL	5 μg α-TNP $(Fab')_2$	BSS	6	9
2×10^6 PBL	5 μg α-TNP	10 mM EDTA in BSS	5	8

[a] Monolayers of TNP ghosts filled with CF and Rh-dextran (250,000 m.w.) were prepared as described in *Materials and Methods*, and the indicated effector cells, antibody, and medium added in a total volume of 200 μl. After 3 hr at 37°C, the marker release was determined by fluorometric analysis of the supernatant.

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release, similar to the data in Figure 1. In addition, when Fc receptor-positive lymphocytes were depleted, little marker release was observed, paralleling the previously demonstrated reduction in the ability of such populations to lyse antibody-coated target cells (30). Similarly, addition of EDTA to a level sufficient to chelate all divalent cations, which inhibits both ADCC and T cell killing (30), likewise results in an abrogation of marker release from ghosts. The experiments in Figure 1 and Table I thus argue strongly that the release of fluorescent markers from resealed ghosts by peripheral blood lymphocytes and antibody is qualitatively and quantitatively dependent on the same conditions as the lysis of intact target cells. A similar parallel between lysis of ^{51}Cr-erythrocytes and release of CF was demonstrated as a function of C concentration (C. Simone and P. Henkart, manuscript in preparation).

Microscopic observations. The process of lymphocyte-mediated release of fluorescent markers from resealed ghosts could be followed by phase and fluorescence microscopy. The lymphocytes were allowed to settle on a monolayer of antibody-coated ghosts containing CF and rhodamine catalase. In every experiment, greater than 95% of the ghosts in this monolayer contained CF and rhodamine catalase before addition of effectors and antibody. If the nonadherent lymphocytes were washed away after settling on the target monolayer for 15 min at 25°C, most of the remaining adherent lymphocytes could be seen to have established phase dark adhesions to the underlying ghosts (Fig. 2). Adhesion was largely to the antibody-coated ghosts, as was most strikingly seen by the absence of adherent lymphocytes in small areas devoid of ghosts. If the nonadherent lymphocytes were not washed away, a minority of the lymphocyte population showed a similar adhesion to the antibody-coated ghosts within 2 min of contact. On some occasions, individual lymphocytes were observed to have established adhesive contacts with two to four ghost targets at the same time. In many instances, vigorous motion of the membrane extensions of the adherent lymphocyte was observed.

Continuous observation of the process of CF release from a single field of ghosts is not possible because CF is rapidly bleached by the mercury lamp. Therefore the lymphocyte-mediated marker release was followed with phase optics occasionally switching to fluorescence illumination for several seconds. When a motile lymphocyte adhered to a ghost in the monolayer, one of two outcomes was observed: 1) no marker was lost from the ghost during the time of observation (about 10 min); 2) a loss of CF fluorescence was observed to begin within 5 min. The observable loss of CF fluorescence from a single ghost occurred over the course of 5 min. If a ghost target did not lose its CF within 5 min after the initial adhesion by the lymphocyte, there was no loss subsequently. As can be seen in Figure 2, an effector lymphocyte is sometimes in contact with as many as five to six ghosts at one time; in such cases we did not observe marker loss in all targets contacted. After 20 to 30 min, rectilinear tracts of ghosts devoid of CF made by a lymphocyte were sometimes observed. Individual adherent lymphocytes were observed to cause marker release in 0 to 15 ghosts in 1 hr. New adhesions between effector cells and ghosts appear to form more slowly after 1 hr of incubation [probably because of Fc receptor modulation (31)]. In several instances, ghosts with adherent lymphocytes appeared greatly elongated. Both CF and rhodamine catalase were initially distributed homogeneously in the distorted ghost. CF was released from the still distorted ghost within 3 min of attachment. Several minutes later, the lymphocyte detached and the ghost assumed its normal round shape. Rhodamine catalase was still (0 to 12 min) distributed homogeneously in the ghost devoid of CF. This distortion of the ghost targets was an occasional observation and not observed in every experiment.

Size dependence of marker release. A series of double label

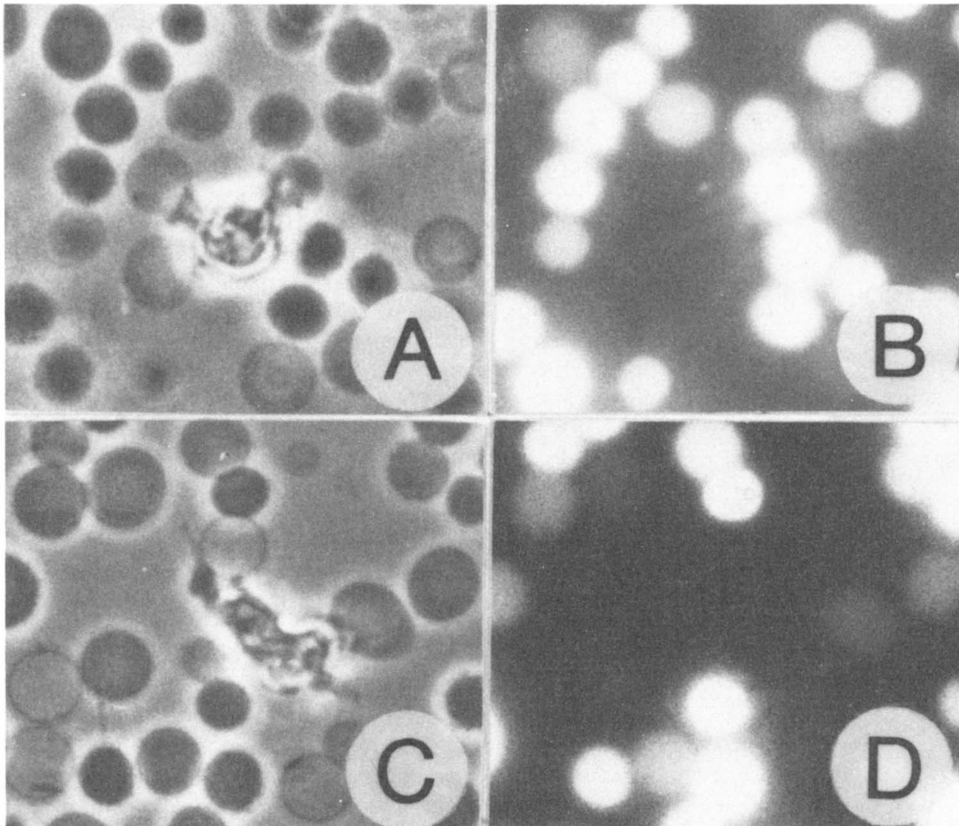

Figure 2. Antibody-dependent release of CF from ghosts by lymphocytes. A monolayer of resealed TNP-erythrocyte ghosts containing CF was formed on a glass slide. Anti-TNP and lymphocytes were added, a cover slip was sealed on the slide, and the action of lymphocytes on the ghosts was observed at 37°C. Initially greater than 95% of the ghosts contained CF. *A*, 25 min later a lymphocyte is seen in contact with three ghosts. *B*, fluorescence illumination shows that two of these ghosts (to the left of the lymphocyte) have lost their CF. *C*, similarly, 60 min after lymphocytes were added, an elongated lymphocyte is seen in contact with two to six ghosts. *D*, fluorescence illumination shows that some of these ghosts have completely lost their CF, some show partial release of their CF, whereas others retain their original fluorescence.

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experiments were carried out to study the size dependence of lymphocyte-mediated marker release from resealed ghosts. Marker release was followed both by fluorometry of released markers in the supernatant and by fluorescence microscopy. Excellent agreement between these two means of analysis was obtained as shown by typical results from five individual experiments with different proteins in Table II. It can be seen that most of the rhodamine-labeled macromolecules behave very similarly to CF with regard to level of background release and requirement for a complete ADCC system to obtain release. However, very large proteins, such as thyroglobulin and hemocyanin, were exceptions to this behavior. They were not released from the ghosts, although CF resealed inside the same ghosts was released. Fluorescence microscopy of the ghost monolayer after the 3 hr incubation with lymphocytes showed that individual ghosts that lost their CF retained the large proteins in a diffuse distribution inside the ghost.

As shown in Figure 3, when data for 3-5 lymphocyte ADCC experiments were averaged and plotted as relative release, i.e., specific release of protein divided by the specific release of CF, a striking size dependence was demonstrated. No exceptions to this m.w. dependence were observed. The largest proteins released from resealed ghosts by cytotoxic lymphocytes are much larger (greater than 10 times in m.w.) than the maximal proteins released by C. The same protein markers were used in both studies. A similar size dependence for relative marker release was observed in the presence of 1×10^6 effector lymphocytes per well. Specific CF release ranged from 27 to 58% with this number of effector lymphocytes.

A series of rhodamine-labeled dextrans were also used as markers resealed in ghost targets for lymphocyte ADCC. Figure 4 depicts the size dependence of the release of these dextran markers relative to CF. As in the case of proteins, a strong size dependence was observed, with smaller dextrans up to 250,000

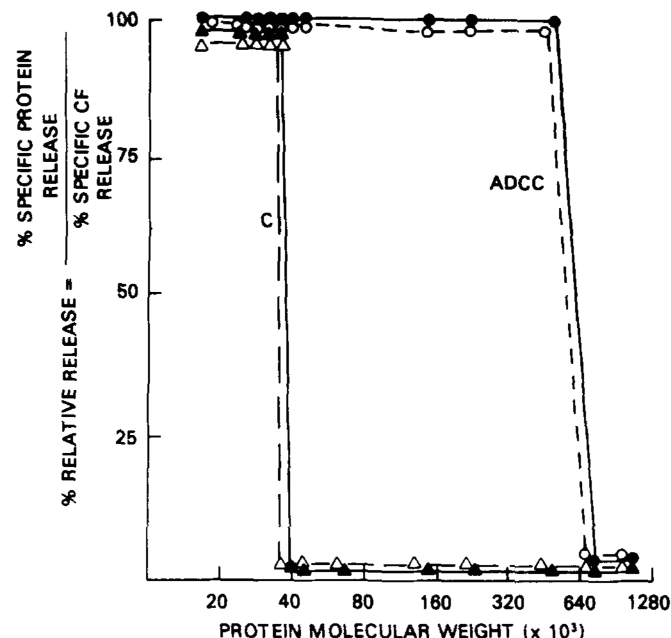

Figure 3. Size dependence of protein release from resealed ghosts by C and lymphocytes. A series of double label experiments (three to five experiments for each protein) was carried out to study the size dependence of marker release from resealed ghosts containing CF and rhodamine-protein. Circles indicate results obtained when 2×10^6 lymphocytes plus 25 μg anti-TNP were added to microtiter wells containing a monolayer 0.5 to 1.0×10^6 of ghosts and incubated for 3 hr at 37°C. Specific CF release ranged from 74 to 95% in these experiments (mean = 89%). *Triangles*, results for C-mediated release of the same markers; *open symbols*, results analyzed by fluorescence microscopy; *closed symbols*, results analyzed by fluorometric analysis of the supernatants.

daltons being released to the same degree as CF, whereas the largest dextran, 2,000,000 daltons, was not released. In contrast to all other markers tested with the various lytic agents, the dextran fraction with an average m.w. of 500,000 daltons showed a partial release relative to CF. This was observed most strikingly by fluorometry of the supernatants. When the ghost targets remaining after ADCC were examined in the fluorescence microscope and carefully compared with control ghosts, it could be seen that most targets retained a weak rhodamine fluorescence, i.e., in this case a partial release from individual targets was observed. Size heterogeneity of the 500,000 dalton dextran marker appears to be a reasonable explanation for this finding. C-induced dextran release also showed a strong size dependence, but the m.w. cutoff was about 10-fold less than the lymphocyte mediated cutoff (Fig. 4).

Kinetics of marker release. The time course of lymphocyte-mediated release of CF and catalase from resealed ghosts as measured by fluorometry is shown in Figure 5. Generally similar rates of release of the two markers were seen, in spite of the large difference in their m.w. In both cases, no significant further increase was seen after 3 hr; this plateau of marker release was also observed for the lysis of ^{51}Cr TNP-red cells (data not shown) and has been attributed to Fc receptor modulation (31).

Observation of the process of marker release by fluorescence microscopy showed that a distinct difference in rates of release of these two markers from individual ghost targets did occur. As previously described, CF release from ghosts occurred within 5 min after lymphocyte adhesion; when the ghost also contained a protein such as catalase, its release from the ghost target did not noticeably begin until 15 min later, and was not complete

TABLE II

Lymphocyte mediated release of proteins from resealed ghosts[a]

Rh-Protein (m.w. × 10³)	Supernatant Analysis			
	% CF release		% Rh-protein release	
	Lymphocytes	Lymph + α−TNP	Lymphocytes	Lymph + α−TNP
Pepsin (33.5)	19	87	27	88
BGG (150)	22	88	21	90
Catalase (250)	17	92	23	100
Apoferritin (480)	7	94	10	90
Thyroglobulin (650)	17	93	14	14
KLH (800)	18	94	14	13
	Fluorescence Microscopy: % Ghosts that Have Lost Marker			
	CF		Rh-protein	
	Lymphocytes	Lymph + α−TNP	Lymphocytes	Lymph + α−TNP
Pepsin (33.5)	1	99	2	100
BGG (150)	1	99	1	100
Catalase (250)	1	100	2	98
Apoferritin (480)	1	99	1	99
Thyroglobulin (650)	2	100	3	5
KLH (800)	1	100	1	2

[a] The data shown are a series of typical individual double label experiments with different protein markers performed as described. The results, obtained after 3 hr incubation at 37°C with 2×10^6 lymphocytes/well, were analyzed by fluorometric measurement of the released markers in the medium and by fluorescence microscopy of the ghosts.

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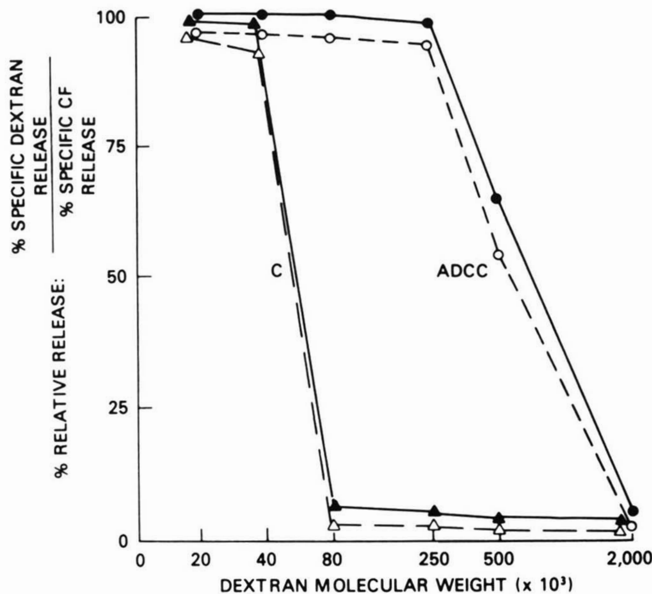

Figure 4. Size dependence of dextran release from resealed ghosts. A series of double label experiments similar to those shown in Figure 3 was carried out with rhodamine-labeled dextrans instead of proteins. *Circles*, results of lymphocyte-mediated marker release, *triangles*, results of C-mediated marker release; *open symbols*, results measured by fluorescence microscopy; *closed symbols*, results measured by fluorometric analysis of the supernatants.

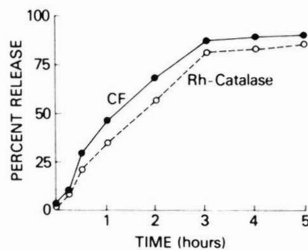

Figure 5. Kinetics of lymphocyte mediated marker release from resealed ghosts. TNP modified ghosts were resealed with CF and rhodamine-catalase and a monolayer of these ghosts was made on the bottom of microtiter wells as previously described in *Materials and Methods.* To each well were added 2×10^6 lymphocytes plus 25 μg anti-TNP at 37°C. At varying times supernatants were removed from different wells and marker release was measured by fluorometry. ●——●, CF; ○- - - -○, rhodamine-catalase.

until 50 to 75 min after lymphocyte adhesion. The reason this time lag and slower rate of release of the macromolecule were not detected by analysis of released marker in the supernatant (above) is not entirely clear, but the effective lymphocyte:ghost ratio was considerably smaller in the experiments done for microscopic observation. It is likely that the rate limiting step in experiments such as that shown in Figure 5 is the formation of pores in the target membrane and not diffusion of the markers.

Red cell ghost vesicles as targets. When resealed right-side-out vesicles filled with 0.1 mM CF were prepared from TNP-red cells, their appearance under the fluorescence microscope was that of a "starry night," which was converted into a diffuse green fluorescence upon addition of detergent. Such vesicles specifically adhered to the surface of microtiter wells via $F(ab')_2$ anti-TNP antibodies (in an analogous fashion to the ghosts) as detected by fluorometry of detergent-treated wells. The appearance of such vesicles is shown in Figure 6, which shows the average vesicles to be somewhat flattened and several hundred nanometers in diameter. CF release from such vesicles was mediated by rabbit anti-TNP and C in an analogous experiment to those of ghosts; a typical experiment is shown in Table III. However, attempts to use peripheral blood lymphocytes to induce CF release from such vesicles were uniformly unsuccessful. A typical experiment of this sort is also shown in Table III. The repeated failure of peripheral blood lymphocytes to cause antibody-dependent CF release from vesicle targets (including some experiments simultaneously run with intact ghost targets that showed the usual good CF release) led us to question whether or not lymphocyte Fc receptors were recognizing the anti-antibody complexes on target vesicles. Two lines of evidence suggested that such recognition did occur: 1) when lymphocytes were incubated with vesicle suspensions in the presence or absence of anti-TNP and then washed and examined in the fluorescence microscope, about 20% of the lymphocytes showed fluorescent vesicles bound to their surface if anti-TNP had been used, whereas none were stained without anti-TNP [such incubations were analogous to the binding of soluble antigen-antibody complexes to the Fc receptor (32, 33)] b) phase microscopy of peripheral blood lymphocytes settled onto surfaces coated with vesicles as described showed that the shape change characteristic of the binding of human Fc receptor-positive lymphocytes to immobilized antigen-antibody complexes (34) occurred in the presence but not in the absence of rabbit anti-TNP. It thus appears that the size or curvature of the target membrane can determine the success of CF release by lymphocytes in this system.

Neutrophil and monocyte-mediated ADCC. Since cells other than lymphocytes have been shown to be mediators of ADCC, we have tested two other human white blood cells for their ability to release markers from resealed ghosts. Neutrophil effector cells were about twice as efficient as the lymphocytes in Figure 1 in causing ^{51}Cr release from TNP-red cells in this

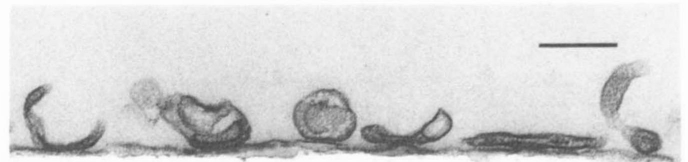

Figure 6. Right-side-out erythrocyte membrane vesicles adherent to plastic. Right-side-out vesicles were prepared from TNP-modified erythrocytes, filled with CF and adhered to plastic with anti-TNP as described in *Materials and Methods.* The appearance of such vesicle monolayers in the electron microscope is shown here. The continuous dark line at the bottom is the protein layer formerly adsorbed to the polystyrene dish, which was eroded away after polymerization of the Epon. The bar represents 0.25 μ.

TABLE III

Erythrocyte membrane vesicles as immunologic targets

Expt. No.	Anti-TNP[a]	Effector	% CF Release[b]
I	0	0	8.0 ± 1.8
	+	0	6.1 ± 0.4
	0	1:20 GP C	18.4 ± 3.1
	+	1:20 GP C	66.9 ± 5.5
II	0	0	5.0 ± 0.4
	+	0	4.4 ± 0.3
	0	2×10^6 PBL	5.7 ± 0.1
	+	2×10^6 PBL	5.2 ± 0.2

[a] Fifty micrograms per milliliter.

[b] Measured after 1 hr at 37°C for C, or 3 hr at 37°C for lymphocytes, by fluorometric analysis of the supernatant.

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geometry. As can be seen in Table IV, CF release from resealed ghosts by neutrophils is strongly antibody dependent. Fluorescence microscopy showed that the CF or rhodamine protein that was released from ghosts was not detectably taken up by the neutrophils, and there was no evidence for an intracellular or phagocytic pathway of marker release in these experiments. The size dependence of marker release from resealed ghosts was again determined by a series of double label experiments with CF and rhodamine-labeled proteins. By using the same series of markers, neutrophils gave an identical size dependence of marker release as did lymphocytes (Table V).

In contrast to the anti-TNP ADCC system described above, if anti-blood group antibodies are used, human red cells are lysed efficiently by monocytes but not by lymphocytes (24, 25). This preferential recognition by monocytes was also observed with regard to CF release from ghosts as shown in Table IV. If human anti-B antibodies were used with the purified lymphocyte preparations, little or no CF was released. On the other hand, when peripheral blood mononuclear cells (containing roughly 20% monocytes in addition to lymphocytes) were used, efficient marker release was observed. The size dependence of this monocyte-mediated protein release was determined as previously carried out for lymphocytes and neutrophils, and the results are shown in Figure 7. Again, a sharp size dependence was observed. However, in this case, apoferritin (m.w. 480,000) was consistently retained in the ghost, whereas with neutrophil and lymphocyte-mediated marker release from ghosts, this protein was released. Thus, a subtle difference in maximal size of released markers is apparent in monocyte-mediated ADCC compared to the other effector cells. Fluorescence microscopy again confirmed the results obtained with fluorometry; it was additionally noted that the majority of monocytes took up the released rhodamine-protein but not CF within 45 min of attachment to the ghosts.

TABLE V

Antibody-dependent neutrophil-mediated release of proteins from resealed red cell ghosts[a]

Protein	% Relative Release: Supernatant analysis	% Relative Release: Fluorescence microscopy
Carbonic anhydrase	100	100
Carboxypeptidase A	96	100
BGG	100	100
Catalase	100	100
Apoferritin	100	100
Thyroglobulin	4	0
KLH	0	0

[a] 1×10^6 Neutrophils and 25 μg/well rabbit anti-TNP were added to a microtiter well containing a monolayer of ghosts resealed with CF and the indicated protein for 3 hr at 37°C. The relative release shown represents an average of three to five experiments.

TABLE IV

CF release from ghosts by neutrophils and monocytes

Effector Cells (Cells/Well)	% CF Release without Antibody[a]	Antibody	% CF Release with Antibody[a]
None	12	Anti-TNP[b]	13
Neutrophils (0.5×10^6)	15	Anti-TNP	70
Neutrophils (1.0×10^6)	16	Anti-TNP	90
None	13	Anti-B[c]	18
Lymphocytes (2×10^6)	17	Anti-B	18
Monocytes (0.5×10^6)	17	Anti-B	59
Monocytes (1.0×10^6)	19	Anti-B	81

[a] Measured by fluorometry of the supernatant after 3 hr incubation at 37°C.

[b] Twenty-five micrograms of anti-TNP/well.

[c] Human anti-blood group B antibody used at 1:100 dilution of whole serum.

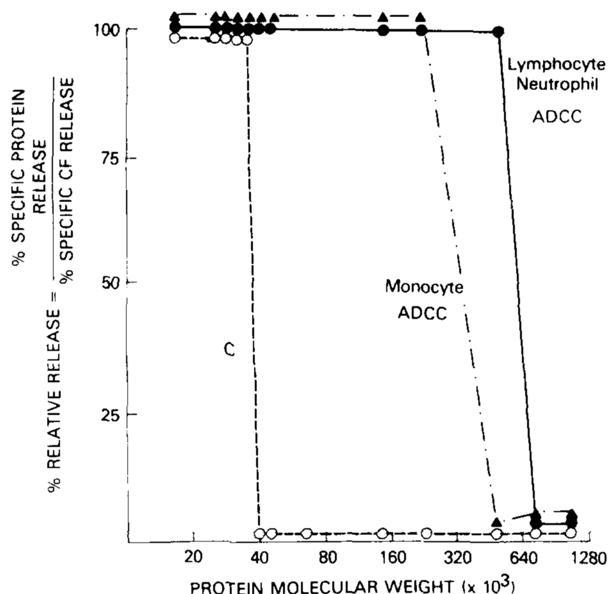

Figure 7. Comparison of size dependence of protein release from resealed ghosts by C and lymphocyte, neutrophil, and monocyte effector cells. Monocyte-mediated release was measured by using 10^6 mononuclear cells and 1/100 anti-B serum with the released markers measured by fluorometry after 3 hr of incubation at 37°C (▲–·–·▲). Data for C (○- - - -○) and for lymphocytic-mediated release (●——●) from Figure 3 for comparison.

DISCUSSION

The use of resealed erythrocyte ghosts as targets for antibody-dependent cytotoxic cells has allowed us to demonstrate a clear alteration in the permeability of natural cell membranes inflicted by the cytotoxic cells. The evidence suggesting that this membrane damage is associated with the killing mechanism is substantial: 1) the quantitative correlation of marker release from ghosts with the death of intact cells measured by ^{51}Cr release, as a function of effector cells (Fig. 1); 2) the antibody dependence of this effect and its requirement for an intact Fc portion (Fig. 1, Table I); 3) the dependence of marker release on lymphocytes bearing Fc receptors (34); and 4) the dependence of the effect on divalent cations in the medium. A particular technical advantage of using fluorescent markers in this study is the combination of quantitative estimates of released markers with fluorometry and the visual counting of individual target ghosts with the fluorescence microscope. The correlation of these two means of measuring marker release in experiments where about half of the maximal release occurred clearly indicates that loss of markers is an "all or none" phenomenon for an individual target (with the exception of 500,000 m.w. dextran previously discussed). The failure to observe transfer of fluorescent markers from ghosts to lymphocytes makes it doubtful that significant membrane fusion or gap junction formation occurs between the killer cell and the target (35).

The chief objective of our studies with erythrocyte ghosts was to learn more about the nature of membrane damage inflicted by lytic immunologic effector mechanisms. By using

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resealed erythrocyte ghosts which have lost over 95% of their hemoglobin, a membrane bag is created that can be used to study the permeability properties of the membrane. The permeability of such ghosts to small ions and glucose and other small molecules (18, 19) has been extensively studied and the ghost membrane behaves functionally like that of the intact cell. In the present case, in which drastic increases in membrane permeability are induced by lytic agents, the ghosts have the advantage of not undergoing the osmotic swelling and lysis that occurs in the intact cell after the initial permeability increase. The presence of additional external macromolecules to balance the residual colloid osmotic pressure does not alter the C-induced protein release cutoff, indicating that colloid osmotic forces are negligible (C. Simone and P. Henkart, manuscript in preparation).

We have considered three well defined possible mechanisms for the permeability increases induced by immunologic lytic mechanisms: 1) Formation of aqueous pores in the membrane, as is established in the case of gramicidin and polyene antibiotics (36, 37) and as proposed for C (38); 2) formation of ion carriers in the membrane, as is believed to be the case for peptide-like antibiotics such as valinomycin; and 3) a general rearrangement of the lipid molecules so that the bilayer character of the membrane breaks down over a substantial area of the cell. In addition to these well defined mechanisms, we are aware that other possibilities exist.

In testing the permeability of a series of molecules to ghost membranes treated with various lytic agents, several classes of results can be anticipated. First, concentrations of agents that cause lysis in intact red cells may not induce increases in the permeability of any markers tested. (In experiments to be reported elsewhere, we have observed this result in the present system with gramicidin and valinomycin.) This may mean that a) membrane permeability effects are not causing lysis or b) the induced permeability increases do not affect the markers used. Obviously, little can be inferred as to the mechanism of permeability increase with this type of result. Second, a nonselective membrane permeability increase may be observed at all concentrations lytic to cells. This result is obtained for some detergents such as lysolipids (C. Simone and P. Henkart, manuscript in preparation). This implies that there is either a pore larger than any of the markers used or a general breakdown in the structure of the lipid bilayer. Third, a selective permeability increase may be observed; this could be explained by either a carrier or pore mechanism. In some cases, such as the present results with C and lymphocytes, the pattern of selectivity can strongly implicate one of these mechanisms. Since the permeability is so strikingly size dependent, and since there are no known carriers capable of inducing permeability increases of such large and varied macromolecules, a channel mechanism is the only one of the above three well defined mechanisms compatible with our data. The sharp size cutoff for marker release can be used to estimate a maximal pore size (see below) but clearly does not in itself give any information regarding the shape, heterogeneity, or stability of the pore, or about the material responsible for creating it. Although less well defined mechanisms (e.g., localized lipid bilayer breakdown) may also be involved to explain the release of markers from ghosts, a pore-like property remains the most attractive explanation for the observed sieving property of marker release. In the case of lymphocyte ADCC, pores had previously been suggested as the most likely interpretation of the black lipid membrane experiments to explain the discrete step increases in the electrical conductances as well as the voltage dependence of the conductance increase (15).

It has been proposed that phospholipase A is involved in the process of lymphocyte-mediated killing (39) since its action on phospholipid generates lysophospholipids, which are detergents and hence lytic. Our data make such a mechanism unlikely since, as previously discussed, when lysolecithin or lysophosphatidyl ethanolamine was added to the medium containing ghosts, all contents of the ghosts were released. Hence, these detergents appear to act by a generalized lipid bilayer breakdown mechanism rather than by formation of pores. Since low concentrations of some other detergents do apparently form membrane pores in ghosts, we cannot rule out detergents entirely; however, such pores formed by all detergents tested thus far are similar in size to those induced by C (20); i.e., much smaller than the pores induced by ADCC effector cells (C. Simone and P. Henkart, manuscript in preparation). We therefore think that detergents are unlikely to be the source of the lethal membrane damage to the target cell, but if they are involved, they must be acting to form a pore (perhaps in conjunction with other molecules) rather than by breaking down the lipid bilayer over a substantial area of the membrane.

The creation of pores in the target membrane clearly requires an active participation by the effector cell, but how such pores are created must still be left to speculation. Mechanisms to account for lymphocyte killing must account for the exquisite specificity of the process: neither the killer cell nor nearby "innocent bystanders" appear to be damaged by the killing process (1, 40). Pores in membranes are thought to be created by the terminal C complex (an aggregate of large proteins (38)), by small polypeptides such as gramicidin (37), by polyene antibiotics (36), and by natural membrane proteins in gap junctions (41). Proteins that can be activated, such as the terminal C complex, present an attractive model for the effector cell molecules involved, but there is little possibility that serum components are involved—all the experiments presented were done in the absence of serum. It is possible that effector cells possess some or all of the C proteins on their membranes or in their cytoplasm (42). The different pore sizes seen in ADCC and C do not rule out the participation of C components in ADCC, but only indicate a different maximal pore size. The participation of target cell proteins in the formation of pores cannot be eliminated in the ghost experiments. It can be argued that a submembrane protein network such as the spectrin-actin complex may produce the seiving effects seen, but it is difficult to explain the variety of apparent pore sizes seen with lymphocytes, monocytes, C, and detergents. In experiments with artificial lipid bilayers (15), there are no target membrane proteins, so we favor a hypothesis for pore formation that does not involve them.

Since monocytes and neutrophils are phagocytic cells, there is a possibility that the release of fluorescent markers mediated by these cells involves phagocytosis and subsequent lysosomal breakdown of the ghosts. No such intracellular uptake of ghosts was observed with the fluorescence microscope. Furthermore, since the ghosts were attached to a surface in these experiments, it would require a prior release from this surface to allow phagocytosis. Experiments with unattached erythrocyte target cells make it clear that surface attachment of the target cell by the techniques used in these studies effectively prevents a phagocytosis requiring pathway for ^{51}Cr release from target cells (43).

The finding that smaller resealed vesicles of ghost membranes were not detectably attacked by lymphocytes under conditions in which whole ghosts showed complete marker release deserves comment. The vesicles used were prepared according to procedures demonstrated to yield right-side-out

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vesicles (27) so that the same surface molecules should be exposed in both the ghosts and the vesicles. Since TNBS modifies only the exterior proteins of the red cell (44) and since the vesicles we have prepared from TNBS-modified red cells adhere specifically to plastic coated with anti-TNP and have their CF efficiently released by anti-TNP and C, our own experiments confirm this sidedness. It thus appears that the size of such vesicles, their radius of curvature, or their loss of a membrane component prevents their successful attack by the effector cell. One possible explanation for this failure is that the killer cells release ionophoric material into the target membrane by a highly localized process, so that only one or two vesicles under a lymphocyte would be affected when a lymphocyte is triggered to kill. In this case, many more triggering events would be required to give a measurable percentage of marker release with vesicles than with ghosts. (There are over 10^8 vesicle targets per well, but fewer than 10^6 ghost targets per well.) Whatever the explanation for the loss of sensitivity to cell-mediated marker release with smaller size, we believe that a similar mechanism explains our failure and that of others (19, and unpublished observations) to observe marker release from liposome targets as models for ADCC. The liposome targets used have been the size of the ghost vesicles or smaller. It is interesting to note that liposomes bearing membrane proteins have recently been used successfully as targets for killer T lymphocytes (45).

An estimate of the size of the pores created in the target membrane can be obtained from the dimensions of the marker molecules on each side of the m.w. cutoff. Several reservations must be kept in mind in considering such size estimates. First, the pore size may be variable with time; if so, our size estimates may be larger than the time average, since the measurements are carried out over 3 hr. Over this time period, marker escape could occur from expanded pores even if they were only open maximally for a fraction of the time. Second, heterogeneity of pore sizes is not easily seen by this technique; escape of large markers may occur through the largest pore in a cell. We do not have any way of estimating the number of functional pores created per ghost. Table VI summarizes the data on the sizes of

TABLE VI

Molecular dimensions of markers used to estimate pore size

Marker	m.w. × 10^3 (39)	% Relative Release (Supernatants)		Stokes-Einstein Diameter (Å)[a]	X-ray/EM Shape and Size
		Lymphocyte ADCC	Monocyte ADCC		
Catalase	250	100	99	104	Sphere, 80 Å diameter (41)
Apoferritin	480	99	4	119	Sphere, 122 Å diameter (43)
Thyroglobulin	650	4	2	165	
KLH	800	3	3	390	Cylinder, 360 Å diameter Cylinder, 400 Å high (44)
Dextran	250	100	100	211	
	500	58	40	292	
	2000	2	1	556	

[a] Molecular diameter calculated from diffusion constant using the Stokes-Einstein equation (which assumes molecules are hard spheres). Diffusion constants are from Reference 39 for proteins and interpolated from data given in Reference 40 for dextrans.

the unmodified markers we have used to define the pores created by lymphocytes, monocytes, and C. It is obvious that a substantial discrepancy exists between the Stokes-Einstein diameters of proteins and dextrans used to define the pore size. Thus, this measure of molecular size does not give a consistent estimate of the pore diameter for both proteins and dextrans, although it does correlate with the ability of both these types of molecules to penetrate into cross-linked dextran beads during gel filtration (25) and with the permeability of cellulose dialysis membranes to these molecules, as predicted by the pore theory developed by Renkin (26). We believe that the dextran size estimate by the Stokes-Einstein equation is not meaningful for the present purpose since this calculation is based on the diffusion constant and assumes that the molecules are hard spheres. This is clearly not realistic for polysaccharides such as dextran, which assume an extended random coil conformation in aqueous medium. This open conformation causes them to diffuse more slowly than an equivalent m.w. protein (which is closer to a hard sphere). Nevertheless, it seems possible for a biologic membrane of 100-Å thickness that the flexible dextran molecules can "wiggle" through a pore considerably smaller than the calculated Stokes-Einstein diameter due to the rapidly changing conformation in solution.

As can be seen from Table VI, the maximal pores induced by lymphocytes (and neutrophils) have an estimated diameter of 119 to 165 Å, those induced by monocytes are 104 to 119 Å in diameter, whereas this method gives an estimate of about 50 Å for C-induced pores. Regardless of the accuracy of these estimates, the data from both protein and dextran markers indicate that ADCC-induced pores are substantially larger than those made by C. Figure 7 shows a direct comparison of the ability of C and ADCC mechanisms to release protein markers from resealed ghosts.

The discrete conductance increases observed in the BLM experiments with lymphocytes as an ADCC model (15) are smaller than would be expected from 120-Å diameter pores; this may indicate a heterogeneity of pore sizes exists in ADCC as it apparently does with C. Maximal membrane pores induced by ADCC are larger than any reported by other agents. They are large enough to allow for the leakage of many soluble cytoplasmic proteins, including hemoglobin from red cells. This implies that colloid osmotic lysis may not occur for red cell targets in ADCC; however, osmotic swelling and lysis could still occur if the rate of equilibration of small ions and water across the membrane containing these pores was rapid, although the rate of exit of hemoglobin was slow.

We believe that the evidence presented in these studies strongly suggests that formation of aqueous pores in the target membrane is the mechanism by which ADCC effector cells inflict lethal damage. We have no evidence whether or not a similar mechanism operates for cytolytic T lymphocytes since red cells and their ghosts do not seem to make good targets for such effectors. Our current efforts are devoted to extending the present results to more complex target membranes to generalize on these findings.

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