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# Mechanism of cytotoxicity of human large granular lymphocytes: Relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement

(natural killer cells/liposomes/transmembrane channels/monoclonal antibodies/inhibition of cytotoxicity)

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ABSTRACT **A $M_r$ 70,000 protein was isolated from cytotoxic human large granular lymphocytes and shown to have cytotoxic activity. The protein was demonstrated to be immunochemically related to the ninth component (C9) of complement and was therefore designated C9-related protein (C9RP). This finding suggests that C9RP and C9 share homology in primary structure and have a common evolutionary ancestry. C9RP was isolated, by affinity chromatography employing anti-human C9-Sepharose, from either purified cytoplasmic granules or whole-cell lysates of cultured human large granular lymphocytes. The cells were isolated from healthy blood donors and maintained in interleukin-2-dependent cultures. The immunochemical crossreactivity of C9 with C9RP was 3–4%, using a murine anti-C9RP antiserum. Certain murine monoclonal antibodies to C9RP and to C9 inhibited killing of K562 cells by human large granular lymphocytes. Killed target cells, identified by propidium iodide staining and isolated by fluorescence-activated cell-sorting, exhibited clusters of circular membrane lesions that resembled poly(C9) in appearance. Polymerization of isolated C9RP in the presence of $Ca^{2+}$ resulted in the formation of two different circular structures, one having an inner diameter of ≈60 Å, and the other, of 125 Å. Polymerized C9RP could be incorporated into liposomes and, as such, gave rise to channels of two different sizes. The smaller channel had a functional diameter of 50–90 Å, and the bigger channel, a diameter >102 Å.**

The precise nature of the molecular mechanisms that cytotoxic lymphocytes utilize to kill their target cells is not known. It is known, however, that killing lymphocytes leave an electron-microscopically detectable lesion on the membranes of target cells (1) that resembles the polymerized ninth component of complement [poly(C9)] (2, 3). Further, killing requires effector–target cell contact (4), and after contact is achieved, the microtubule-organizing center and the Golgi apparatus of cytotoxic T lymphocytes and of natural killer cells are repositioned to face the contact area with the target cell (5, 6). This rearrangement might serve to direct the cytoplasmic granules toward the contact site and to allow their content to be secreted by exocytosis into the narrow space between effector and target cell. Under the influence of calcium, functional transmembrane channels are then formed in the target-cell membrane (7). Ultrastructural circular membrane lesions have been observed in cells killed by human (1), murine (8), and rat lymphocytes (9).

Because of the morphological resemblance of the lymphocyte-produced membrane lesion with the membrane-attack complex of complement and poly(C9), the question arose as to whether the channel-forming protein of killing lymphocytes is structurally related to C9 of complement. In a previous communication (10), initial evidence was reported from this laboratory indicating that the channel-forming protein of human cytotoxic large granular lymphocytes (LGLs) exhibits a low but definite degree of antigenic crossreactivity with human C9 (10). This antigenic relationship was detected by using antiserum directed to human C9.

In the following it will be shown that antibody produced to the isolated C9-related protein (C9RP) of cytotoxic human lymphocytes reacts with human C9 and that certain murine monoclonal antibodies to human C9RP or to human C9 efficiently inhibit target-cell killing by human LGLs. Suggestive evidence was obtained that C9RP is related also to C8. The size of the functional channel produced by polymerized C9RP was estimated by using liposomes with entrapped markers and was correlated with the electron microscopic appearance of the polymer.

## MATERIALS AND METHODS

**Isolation and Propagation of Human LGLs.** Blood from healthy human donors was obtained from the General Clinical Research Center of Scripps Clinic and Research Foundation and collected in solution containing 10 units of heparin per ml (final concentration). The peripheral blood lymphocytes were isolated by Ficoll-Isopaque gradient centrifugation (11) using lymphocyte separation medium (Litton Bionetics). Monocytes were removed by adherence to plastic flasks (60 min, 37°C). A discontinuous Percoll gradient (Pharmacia) (12), depletion of erythrocyte-rosette-forming T cells (13), and Ficoll-Isopaque gradient centrifugation were used for further purification of the LGLs. The cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid mixture, antibiotics (M. A. Bioproducts, Walkersville, MD) and 10% fetal bovine serum (HyClone, Logan, UT). The LGL culture was stimulated with 0.2% (vol/vol) phytohemagglutinin (PHA, M form, GIBCO, Chagrin Falls, OH), and subsequently, a lectin-free human interleukin 2 preparation (Cellular Products, Buffalo, NY) was repeatedly added at 10% (vol/vol).

**Cell-Killing Assay.** Cytotoxic activity of LGLs was determined in a 4-hr $^{51}Cr$-release assay using human K562 cells as targets (14). The cytolytic activity of isolated C9RP was tested using identical conditions without effector cells.

**Isolation of Cytoplasmic Granules.** Cells ($10^9$) were pelleted, resuspended in 20 ml of relaxation buffer [100 mM KCl/3.5 mM $MgCl_2$/1 mM ATP/10 mM 1,4-piperazinebis-(ethanesulfonate) (Pipes) pH 6.8/1.25 mM EGTA] (15) and subjected to $N_2$ cavitation. Nuclei were removed by centrifugation at 1000 rpm for 10 min. The supernatant was subjected to a Percoll gradient centrifugation (16), and the

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Abbreviations: LGL, large granular lymphocyte; C9, ninth component of complement; C9RP, C9-related protein of killer lymphocytes.

fractions of density from 1.076 to 1.141 g/ml were collected. Percoll was removed by centrifugation in an SW41 rotor (Beckman) at 35,000 rpm for 16 hr at 4°C. Whole granules were lysed in 2 M NaCl with 2 mM EGTA at 4°C for 16 hr and centrifuged at 30,000 rpm in a Beckman SW50.1 rotor for 4 hr to remove membranes and residual Percoll (17). The soluble proteins were then dialyzed against 60 mM NaCl/10 mM Tris·HCl, pH 7.4.

**Isolation of C9RP by Immunoadsorption.** Starting material was either the soluble contents of the cytoplasmic granules or whole-cell lysates. LGLs were lysed in 2 M NaCl/20 mM Tris·HCl , pH 7.4/10 mM benzamidine/5 mM EDTA/5 mM EGTA/2 mM phenylmethylsulfonyl fluoride. The mixture was sonicated for 10 min, kept at 4°C overnight, and then centrifuged at 43,000 × *g* for 1 hr. The supernatant was dialyzed against 50 mM NaCl/20 mM Tris·HCl, pH 7.4/1 mM EGTA (starting buffer). The retentate was first passed over a rabbit IgG-Sepharose column to remove immunoglobulin-binding proteins. It was then applied to a rabbit anti-human C9-Sepharose column that was equilibrated and then washed with starting buffer. After a 0.5 M NaCl wash, the protein was eluted with 3 M NaCl/20 mM Tris·HCl, pH 7.4/1 mM EGTA.

**Monoclonal and Polyclonal Antibodies.** Murine monoclonal antibodies to human C9 and to human C9RP were produced according to established procedures (18). Monoclonal antibodies to Leu-7 and Leu-11 were purchased from Becton Dickinson. The anti-C9 serum used for C9RP affinity chromatography was produced in rabbits (R-2653). The mouse anti-C9RP was obtained from the animal sacrificed to produce hybridomas.

**ELISA.** A microtiter plate was coated with antigen, and mouse antiserum to human C9RP was used at different dilutions. Peroxidase-conjugated anti-mouse IgG antibody (Tago, Burlingame, CA) was used at a 1:1000 dilution. The enzyme substrate was 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim).

**Electron Microscopy.** Carbon-coated grids (400 mesh) were used for staining of either isolated polymerized C9RP or membrane fragments of killed K562 cells with 1% uranyl formate. The killed cells were isolated from reaction mixtures of target cells and LGLs by fluorescence-activated cell-sorting (FACS) after staining with propidium iodide.

**Marker-Retention Assay.** The marker proteins were used in $^{125}$I-labeled form. The liposomes were made of egg phosphatidylcholine and dicetyl phosphate. Five micrograms of polymerized C9RP was incorporated into liposomes containing 2 μmol of egg phosphatidylcholine. After the liposomes with entrapped markers were formed, they were passed over Sepharose 4B columns and the amount of radioactivity associated with liposomes was determined.

**Peptide Synthesis.** The 11-residue peptide Asp-Asn-Asp-Cys-Gly-Asp-Phe-Ser-Asp-Glu-Asp, corresponding to the sequence of human C9 residues 101–111 (19, 20), was synthesized by V. S. Ganu (Research Institute of Scripps Clinic), employing the solid-phase method of Merrifield (21) and a Biosearch (San Rafael, CA) peptide synthesizer.

## RESULTS

**Isolation of the Cytotoxic C9RP from Human LGLs.** The LGLs were purified from the blood of healthy human donors by a multistep procedure that included removal of adhering monocytes and of erythrocyte-rosetting T lymphocytes. The cells were placed in culture and stimulated first with phytohemagglutinin and subsequently by repeated additions of interleukin 2. In most cases, the cells proliferated within 2–4 weeks from $10^7$ to 1–2 × $10^9$, as exemplified by the growth curve of an individual cell preparation (Fig. 1). To date, 51 different LGL preparations have been obtained, most of which exhibited strong proliferation in culture. The harvested

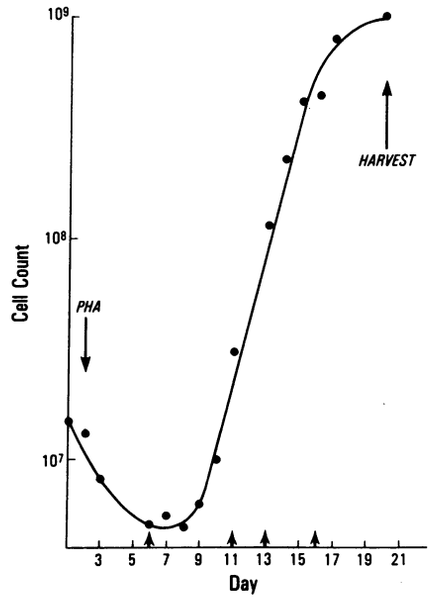


FIG. 1. Growth curve of human LGLs in culture. Isolated LGLs (2 × $10^7$) from 150 ml of blood from a healthy donor were placed in culture and stimulated with phytohemagglutinin (PHA). Interleukin 2 was added repeatedly, as indicated by arrows on the abscissa.

cells contained cytoplasmic granules that were azurophilic on staining according to Giemsa. By and large, 50–70% of the cells reacted positively with anti-Leu-7 and anti-Leu-11. With K562 cells as target cells, the cytotoxic activity varied widely between the different cell batches. Whereas some batches were inactive toward K562 cells, many killed up to 60% of the target cells within 4 hr at an effector/target cell ratio ≤5 (see below).

Starting material for the isolation of the cytotoxic C9RP was 1–5 × $10^9$ cultured LGLs. The cells were subjected to $N_2$ cavitation, the liberated cytoplasmic granules were isolated and lysed in 2 M NaCl containing 2 mM EGTA, and the membranes were removed by centrifugation. After dialysis of the soluble protein against 10 mM Tris buffer containing 60 mM NaCl and 1 mM EGTA, the C9RP was purified by affinity chromatography on an anti-human C9-Sepharose column. The column was washed with starting buffer and 0.5 M NaCl, and bound material was eluted with 3 M NaCl/10 mM Tris·HCl, pH 7.4. Alternatively, LGLs were lysed with 2 M NaCl at 4°C for 16 hr in the presence of EGTA and proteinase inhibitors, after which the particulate material was removed by centrifugation. The supernatant of this whole-cell lysate was dialyzed as above and directly applied to the immunoadsorbent column. The latter procedure afforded higher yields of C9RP than the former (50–100 μg of C9RP per $10^9$ cells).

**Molecular Weight and Cytotoxic Activity of the Isolated Human C9RP.** To date, 10 preparations of C9RP have been produced, 4 from isolated cytoplasmic granules and 6 from whole-cell lysates. On examination by NaDodSO$_4$/polyacrylamide gel electrophoresis, all preparations contained a major component of $M_r \approx 70{,}000$ (Fig. 2*A*).

The isolated C9RP was cytolytically active and caused $^{51}$Cr release from K562 cells in a dose-dependent manner (Fig. 3). Killing occurred in the presence of 5 mM $Ca^{2+}$. In contrast, C9RP completely lacked C9 hemolytic activity; i.e., it caused no C9-dependent lysis of EAC1-8 cells.

**Immunochemical Relationship Between C9RP and C9.** By immunoblot analysis, isolated C9RP gave a weak but reproducible reaction with polyclonal anti-human C9. By ELISA, anti-C9 binding to C9RP was 1% of that observed with C9.

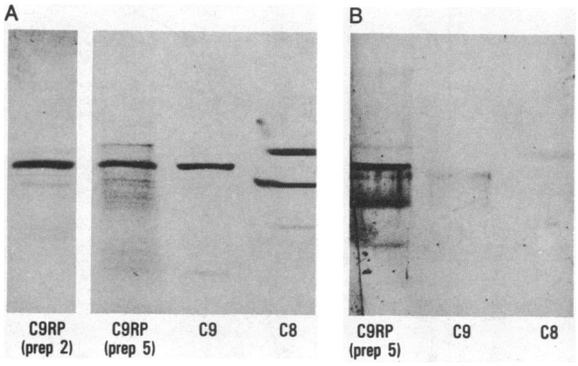


FIG. 2. Demonstration of human lymphocyte C9RP by $NaDodSO_4$/polyacrylamide gel electrophoresis (1.7–17% acrylamide gradient) followed by Coomassie blue staining (*A*) and blot analysis (*B*). For comparison, identical amounts of human C8 and C9 are shown. C9RP preparation 2 was obtained from isolated cytoplasmic granules of LGLs; C9RP preparation 5 was from a whole-cell lysate. C9RP and C9 were analyzed after reduction with 2-mercaptoethanol, whereas C8 was unreduced. The immunoblot was developed with rabbit anti-human C9RP.

Anti-C9RP was raised in a mouse and in rabbits. Immunoblots with rabbit anti-C9RP gave a weak reaction with C9 and the $\alpha$–$\gamma$ subunit of C8 (Fig. 2*B*). Positive reactions of the antiserum with human C8 and human C9 were also seen in dot blots. When the reactivity of mouse anti-C9RP with C9RP and with C9 was compared by ELISA, C9 exhibited 3–4% crossreactivity with C9RP (Fig. 4).

**Inhibition of LGL Cytotoxicity by Monoclonal Antibodies to C9 and C9RP.** Of 45 monoclonal antibodies to C9 tested, 20 reacted positively in dot blots with C9RP. The latter were tested for their ability to inhibit the killing of K562 cells by LGLs. Three monoclonal antibodies were clearly inhibitory, whereas the others were weakly or not at all inhibitory. Fig. 5 *Left* shows killing as a function of effector/target cell ratio. In a parallel assay, using the highest effector/target cell ratio, monoclonal antibody C9-2 exhibited inhibition of killing, whereas 9-5D5 was not inhibitory (Fig. 5, *Right*).

The mouse that produced the antiserum to C9RP which was used to demonstrate the immunochemical crossreaction with C9 (Fig. 4) was sacrificed and its spleen cells were fused with mouse myeloma cells for monoclonal antibody formation. In exploratory screening, a number of the hybridomas produced anti-C9RP that was strongly inhibitory in the cytotoxic assay (unpublished data), including monoclonal antibody C9RP 1-42.10.

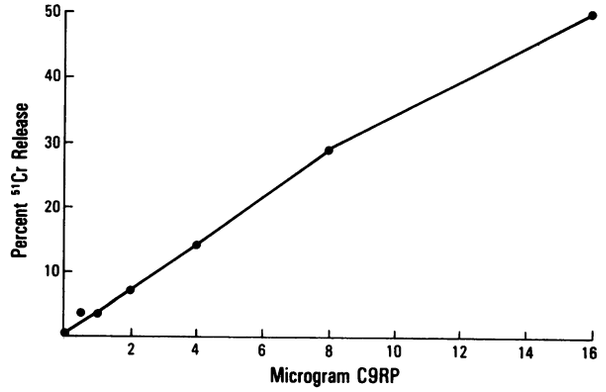


FIG. 3. Killing of K562 cells by C9RP isolated from human LGLs. $^{51}Cr$-labeled K562 cells ($6.25 \times 10^4$) were incubated at 37°C for 4 hr in a total volume of 200 $\mu$l with the indicated amounts of C9RP.

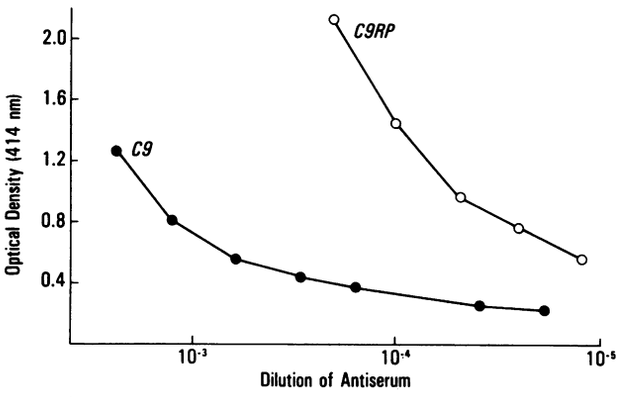


FIG. 4. Comparative analysis of human lymphocyte C9RP and C9 of complement by ELISA using mouse anti-C9RP.

**Inhibition of the Cytotoxic Activity of Isolated C9RP by Monoclonal Antibody to C9RP and Polyclonal Antibody to Peptide 101–111 of C9.** In reaction mixtures of K562 cells ($6.25 \times 10^4$) and 6 $\mu$g of isolated C9RP, the addition of monoclonal antibody C9RP 1-42.10 caused marked inhibition of cytotoxicity (Fig. 6). The polyclonal rabbit antibody to the synthetic C9 peptide 101–111 showed likewise pronounced inhibition of the LGL cytotoxic reaction (Fig. 6).

**Electron Microscopic and Functional Analyses of the LGL-Produced Membrane Channel.** K562 cells killed by LGL were identified by propidium iodide staining and isolated by fluorescence-activated cell-sorting. Viewed by electron microscopy, their membranes exhibited clusters of circular lesions with inner and outer diameters of approximately 60 and 125 Å, respectively (Fig. 7, *Right*). Upon exposure to 5 mM $Ca^{2+}$, isolated C9RP polymerized to form circular structures indistinguishable from those evoked by LGL on killed target cells (Fig. 7, *Left*). Fig. 8 shows individual images of larger circular structures also observed in fields of polymers obtained from isolated C9RP. The inner diameter of these rings was 130–140 Å.

To prove the channel function and channel size of polymerized C9RP, isolated C9RP, polymerized in the presence of $Ca^{2+}$, was incorporated into liposomes together with entrapped radiolabeled protein markers. After passage of the liposomes through Sepharose 4B, the retention of marker was measured. Fig. 9 shows that polymerized C9RP caused formation of functional pores of two different diameters, one between 50 and 90 Å and the other greater than 102 Å.

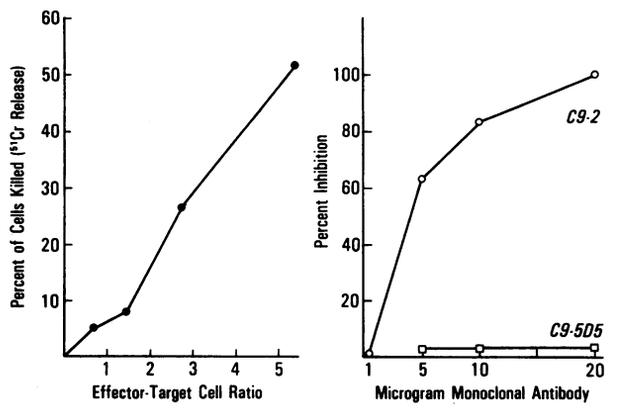


FIG. 5. (*Left*) Killing of K562 cells by human LGLs. (*Right*) Inhibition of killing (at 5:1 effector/target ratio) by monoclonal antibody (C9-2) to C9 (○). Monoclonal antibody C9-5D5 had no effect (□). Both antibodies were purified from culture fluids by protein A chromatography.

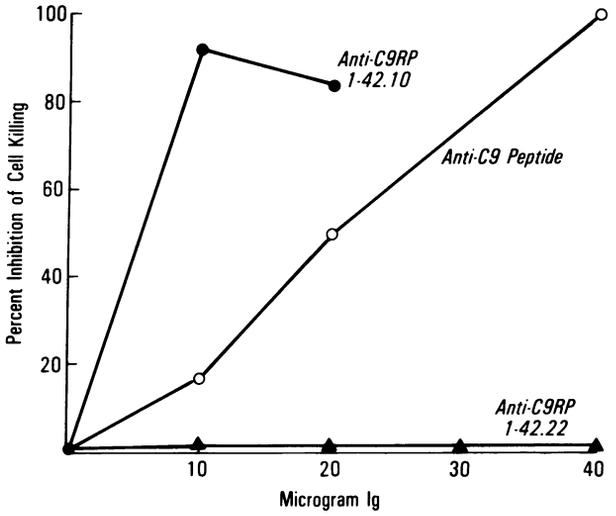


FIG. 6. Killing of K562 cells by isolated C9RP of human LGL: Inhibition by murine monoclonal anti-C9RP (1-42.10) and rabbit polyclonal anti-synthetic C9 peptide 101–111. The amount of C9RP was 6 $\mu$g per 6.25 $\times$ $10^4$ cells. The total volume was 200 $\mu$l. Monoclonal antibody C9RP-1-42.22 was ineffective.

## DISCUSSION

The most pertinent contribution of this study to the elucidation of the molecular basis of human lymphocyte cytotoxicity is the finding that the lymphocyte cytotoxic protein is immunochemically related to C9 of complement. This fact was first shown in this laboratory by using polyclonal antibody to C9 (10). It is demonstrated in the present paper that antiserum to C9RP also crossreacts with C9. The crossreactivity between C9 and C9RP is fundamental to the understanding of immunologically mediated cell-killing mechanisms. It suggests that C9 of complement and C9RP of killing lymphocytes are structurally homologous proteins and have a common evolutionary ancestry.

C9 greatly enhances the rate and extent of cell killing by C5b–8 (22, 23). It accomplishes this function by undergoing oligomerization or by forming tubular C9 polymers. Poly(C9) is a cylindrical structure with an inner diameter of $\approx$100 Å and a height of 160 Å. Tubular polymerization of isolated human C9 is dependent on metal ions (22, 24). Spontaneously polymerizing isolated C9 does not, however, attack biological membranes. In contrast, polymerizing isolated C9RP is cytolytic for K562 cells (Fig. 3). Poly(C9RP) forms circular

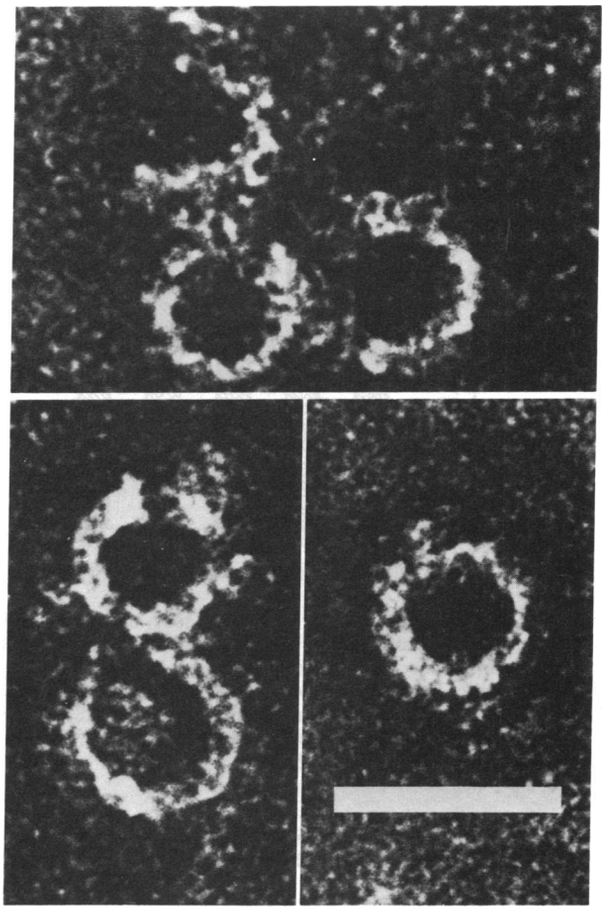

FIG. 8. Electron microscopic visualization of large circular polymers of C9RP. (Bar = 250 Å.)

structures with an inner diameter smaller (Fig. 7) or larger (Fig. 8) than that of poly(C9). The smaller circular structures have been visualized on K562 cells that were killed by human LGLs (Fig. 7). The occurrence of channel structures with different diameters has also been suggested by the marker-retention assay using liposomes with incorporated polymerized C9RP (Fig. 9). Circular structures have been shown to occur on target cells killed by lymphocytes of human (1), rat (7), and murine (8) origin, and the channel function of these structures has been demonstrated (25). The precursor ($M_r$

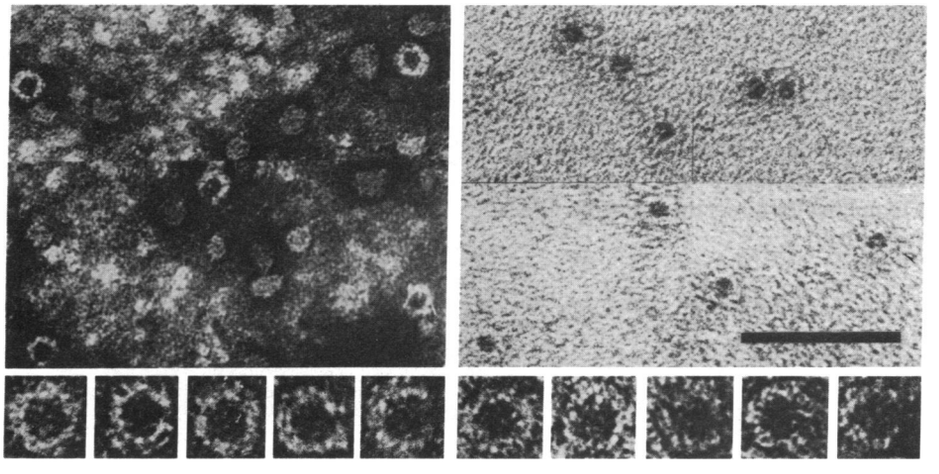

FIG. 7. Electron microscopic visualization of polymerized lymphocyte C9RP (*Left*) and of circular membrane lesions present on K562 cells killed by human LGL (*Right*). (Bar = 500 Å). The bottom row represents a 2-fold magnification of selected individual images.

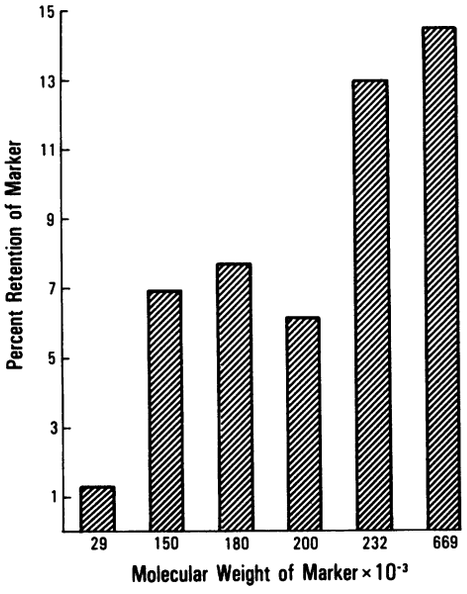


FIG. 9. Estimation, by marker-retention assay, of the size of the functional channel produced by polymerized lymphocyte C9RP incorporated into liposomes. Markers used were carbonic anhydrase [$M_r$ 29,000; Stokes radius ($R_S$) = 24.6 Å], alcohol dehydrogenase ($M_r$ 150,000; $R_S$ = 45 Å), complement component C3 ($M_r$ 180,000; $R_S$ = 51 Å), β-amylase ($M_r$ 200,000), catalase ($M_r$ 232,000), and thyroglobulin ($M_r$ 669,000; $R_S$ = 85 Å).

66,000) of the circular structure has been isolated from the cytoplasmic granules of mouse cytotoxic T-lymphocyte lines (17).

A relationship of C9RP to C8 is suggested by immunoblot analysis (Fig. 2), which shows that rabbit anti-C9RP weakly detects the α–γ subunit of C8. Further, antibody to an 11-residue peptide synthesized according to the known sequence of human C9 from position 101 to position 111 (19, 20) inhibited the cytolytic activity of isolated C9RP (Fig. 3). Antibody to this peptide has been shown to crossreact with the α chain of C8 (26).

Inhibition of LGL cytotoxicity by some monoclonal antibodies to C9 (Fig. 5) or to C9RP, but not others, indicates that C9RP plays a critical role in the killing of target cells by human lymphocytes. Recently, it has been reported (28) that monoclonal antibodies raised against LGL/K562 killing mixtures crossreacted with complement-killed targets and inhibited antibody-dependent cellular cytotoxicity. Three questions arise that can be answered in the foreseeable future. (*i*) Is the channel produced by C9RP the only cytolytic mechanism used by lymphocytes, or do the cytoplasmic granules (15, 17, 27) contain a cytotoxin that is injected through the channel into the target cell? (*ii*) Can target cells protect or defend themselves against lymphocyte attack? (*iii*) How do killing lymphocytes escape self-injury—i.e., what is the mechanism by which lymphocytes direct the cytotoxic effect entirely toward the target cell?

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