Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2426–2429, April 1987
Immunology

The cytolytic protein of human lymphocytes related to the ninth component (C9) of human complement: Isolation from anti-CD3-activated peripheral blood mononuclear cells

(cytotoxic T lymphocytes/cell activation/large granular lymphocytes/cell killing mechanisms)

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ABSTRACT **A 70-kDa channel-forming protein has recently been isolated from human large granular lymphocytes maintained in interleukin-2-dependent culture. The protein was shown to be immunochemically related to the ninth component of complement (C9) and was therefore designated C9-related protein (C9RP). Using the procedure that was developed for the isolation of C9RP from large granular lymphocytes—i.e., affinity chromatography employing anti-human C9 linked to Sepharose, a cytolytic protein has now been isolated from OKT3-activated human peripheral blood mononuclear cells. Nineteen to 40 μg of active protein was obtained from 1 × 10^9 human peripheral blood mononuclear cells after the cells were cultured for 3 days with OKT3 (monoclonal antibody to cell surface antigen T3). During this period, a marked increment occurred in the amount of the cytotoxic protein contained per cell, indicating that OKT3 induced *de novo* synthesis of the protein. By NaDodSO$_4$/PAGE the molecular mass was determined to be 70 kDa. By ELISA the isolated protein and C9RP of large granular lymphocytes reacted to the same extent with anti-C9RP. Using K-562 or M21 human melanoma cells as targets, the cytotoxic activity of the isolated protein, in the presence of 5 mM Ca^{2+}, was comparable to that of C9RP. The same cytolytic protein was isolated from peripheral blood mononuclear cells that were depleted of $CD16^+$ cells prior to OKT3 activation and that consisted primarily of $CD4^+$ and $CD8^+$ T lymphocytes. These results suggest that the cytolytic protein of OKT3-activated cytotoxic T lymphocytes is identical with C9RP of interleukin-2-stimulated large granular lymphocytes.**

The cytolytic protein of human large granular lymphocytes (LGL) has been isolated recently and characterized as a 70-kDa protein that is immunochemically related to the ninth component of complement (C9) (1, 2). The LGL were propagated and maintained in interleukin-2 (IL-2)-dependent culture. Isolation of the protein from LGL lysates was accomplished by affinity chromatography employing anti-human C9-Sepharose. In the presence of Ca^{2+}, the C9-related protein (C9RP) efficiently killed K-562 cells, human M21 melanoma cells and Raji cells (Burkitt lymphoma). Killed target cells identified by propidium iodide staining and isolated by fluorescence-activated cell sorting exhibited, upon examination by electron microscopy, clusters of circular membrane lesions that resembled morphologically the polymerized C9, poly(C9) (3). Exposure of isolated human C9RP to 5 mM Ca^{2+} at 37°C for 1 hr resulted in the formation of similar circular structures. Polymerized C9RP could be incorporated into liposomes and as such gave rise to functional transmembrane channels (2).

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Peripheral blood T lymphocytes can be stimulated by the anti-CD3 monoclonal antibody OKT3 to undergo proliferation (4–6). After 2–4 days of OKT3 stimulation these cells become potent killer cells (7). The cytotoxic potential of activated cytotoxic T lymphocytes (CTL) can be directed toward target cells by cell-bridging antibody conjugates with anti-target cell–OKT3 specificity (7–9). The question arose, therefore, whether OKT3-activated CTL contain C9RP and possibly use this protein as a mediator of their cytotoxicity.

Human peripheral blood mononuclear cells (PBMC) were depleted of $CD16^+$ cells by lysis employing anti-CD16 monoclonal antibody and rabbit complement. After treatment of the residual population, consisting largely of $CD4^+$ and $CD8^+$ cells, with OKT3 for 3 days, the cells were subjected to the procedure that was developed for the isolation of C9RP from LGL (1, 2). A cytolytically active protein was isolated that was indistinguishable from C9RP with respect to several properties examined.

MATERIALS AND METHODS

Monoclonal Anti-CD3 Antibody. The murine monoclonal antibody OKT3 was purified from the supernatant of OKT3-producing hybridomas obtained from American Type Culture Collection (Rockville, MD) using Sepharose-protein A (Pharmacia) affinity chromatography (10).

Purification of $CD16^+$-Depleted, OKT3-Activated Human PBMC. PBMC were isolated from heparinized normal human blood provided by the General Clinical Research Center of Scripps Clinic and Research Foundation, using Ficoll/Hypaque (Pharmacia) density-gradient centrifugation and kept in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine, 50 μg of gentamicin per ml and 10% heat-inactivated fetal calf serum. From each donor 1.5–2 × 10^8 cells were obtained. Anti-Leu 11b (Becton Dickinson), which is a monoclonal IgM with anti-CD16 specificity, was added at a concentration of 10 μg/ml, and the mixture was placed on ice for 30 min. After the cells had been washed twice, normal rabbit serum was added at a final dilution of 1:3 for incubation at 37°C for 45 min. After being washed 3 times in RPMI 1640 medium, the cells were resuspended to 5 × 10^5 per ml, and 200 ng of OKT3 per ml was added for subsequent 3-day culture in a humidified atmosphere containing 5% CO_2. The first two PBMC preparations were used without $CD16^+$ depletion.

Phenotypic Analysis of Treated PBMC. At various times in the treatment of PBMC, preparations were phenotyped using phycoerythrin or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: OKT4 (anti-CD4), OKT8 (anti-

Abbreviations: LGL, large granular lymphocytes; IL-2, interleukin-2; C9RP, C9-related protein of killer lymphocytes; PBMC, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocytes; FITC, fluorescein isothiocyanate; C9, ninth component of complement.

CD8), OKT11 (anti-CD2) (Ortho Diagnostics); IL-2R1 (anti-interleukin-2 receptor) (Coulter Immunology), and anti-Leu 11a (anti-CD16) (Becton Dickinson). OKT3 (anti-CD3) was used as a primary antibody at 10 μg/ml for 30 min on ice, followed by FITC-conjugated goat antimouse IgG (Tago, Burlingame, CA) at 1:30 final dilution. Single or two-color fluorescent measurements were performed on a FACS IV (Becton Dickinson) flow cytometer. Isotype, color-matched controls were used to determine the percentage of positive cells.

Target Cells. M21 cells, a human melanoma cell line originally provided by D. L. Morton (Univ. of California at Los Angeles), and K-562 cells (11) were maintained in RPMI 1640 medium supplemented as described above.

Cytotoxic Assays. K-562 cells (10^6/ml), obtained from the American Type Culture Collection, were labeled with ^{51}Cr (200 μCi/ml; 1 Ci = 37 GBq) for 90 min, washed twice, and plated onto a 96-well microtiter plate (6.25×10^4 cells per well in 200 μl). After the indicated amounts of C9RP were added, Ca^{2+} was added to a final concentration of 5 mM. Cells were incubated for 4 hr at 37°C and centrifuged; the supernatants were harvested for radioactivity determination. Cytotoxicity was calculated using the standard formula:

$$\% \text{ specific } {}^{51}Cr \text{ release} = \frac{(\text{cpm, experimental}) - (\text{cpm, background})}{(\text{cpm, 100\% lysis}) - (\text{cpm, background})} \times 100$$

where 100% lysis was obtained by adding 50 μl of 0.2% Nonidet P-40 to the target cells. M21 melanoma cell cytotoxicity assays were performed as above, except that the number of cells was 1×10^4 per well in a volume of 100 μl.

Isolation of the Cytolytic Lymphocyte Protein. OKT3-activated human PBMC (5×10^8 or 8×10^8) were disintegrated in 2 M NaCl/5 mM EGTA/5 mM EDTA/10 mM benzamidine/2 mM phenylmethylsulfonyl fluoride/20 mM Tris·HCl, pH 7.4, by sonication for 10 min and stirring overnight at 4°C. Insoluble material was removed by centrifugation at 10,000 rpm in an SS-34 rotor (Sorvall) for 1 hr. The supernatant was dialyzed against 0.05 M NaCl/5 mM EGTA/20 mM Tris·HCl, pH 7.4 (starting buffer). This material was applied to a Sepharose antihuman C9 column (14 × 25 cm) equilibrated with starting buffer. The column was washed with the same buffer and then with 0.3 M NaCl/5 mM EGTA/20 mM Tris·HCl, pH 7.4. The retained protein was eluted with 3 M NaCl, dialyzed against starting buffer, and concentrated to 1 ml.

A third preparation was started with 1×10^9 CD16-depleted OKT3-activated PBMC. The isolation procedure was as described above, except that anti-C9RP instead of anti-C9 was used for immune adsorption.

Other Methods. PAGE was performed according to Laemmli (12) using an acrylamide gradient of 1.7–17%.

ELISA. To determine the amount of C9RP present in PBMC, the cells were disintegrated by freezing and thawing, and the insoluble material was removed by centrifugation. Microtiter plates were coated with the cell lysate, and the protein A-purified rabbit anti-human C9RP was used at different dilutions. Peroxidase-conjugated $F(ab')_2$ anti-rabbit IgG (Tago) was used at 1:5000 dilution.

Kinetic Analysis of OKT3-Stimulation of PBMC. Five flasks, each containing 15×10^6 PBMC at 5×10^5 cells per ml in culture medium as described above, were kept at 37°C and 6% CO_2 in a humidified incubator. OKT3 (200 ng/ml final concentration) was added consecutively to four flasks in 24-hr intervals. OKT3 was omitted from the fifth flask, and these cells, which were maintained in OKT3-free culture for 4 days, were defined as day-0 cells in the kinetic experiment. They showed no evidence of proliferation or cell death during this period of time. At day-4 the cells of all flasks were harvested, washed, and 1×10^7 cells of each batch were processed for C9RP and total protein quantitation by ELISA and Bio-Rad protein assay (Bio-Rad), respectively.

RESULTS

Isolation of C9RP from Human PBMC. Two different preparations of human PBMC were exposed to OKT3 (200 ng/ml) for 3 days in culture. The cells were cytotoxic in a 4-hr assay using M21 human melanoma cells coated with anti-target cell–OKT3 conjugates. The cells were lysed in the presence of 5 mM EGTA and proteinase inhibitors as described. The solubilized material was dialyzed against 0.05 M NaCl containing 5 mM EGTA and passed over a Sepharose anti-human C9 column. After the column was washed, the adsorbed protein was eluted with 3 M NaCl. By $NaDodSO_4$/PAGE a major protein band was detected corresponding to a M_r of ≈70,000. The yield of protein was 13 μg from 5×10^8 OKT3-activated PBMC and 15 μg from 8×10^8 cells.

The third preparation was obtained from PBMC that were first depleted of the $CD16^+$ population, which represents natural killer cells and a large portion of LGL. After treatment of the PBMC with the IgM type monoclonal antibody anti-Leu 11b and rabbit complement, the cells were washed and activated in culture with OKT3 for 3 days. The phenotypic analysis of the purified PBMC, the $CD16^+$-depleted PBMC, and the depleted cell population cultured for 3 days with OKT3 is presented in Table 1. The percentage of $CD16^+$ cells was reduced by anti-Leu 11b and complement treatment from 10.4 to 0.9. The cytolytic protein of the $CD16^+$-depleted cells was isolated by immune adsorption using anti-C9RP (LGL). The yield was ≈40 μg of protein from 10^9 cells. The $NaDodSO_4$/PAGE pattern resembled that of the previous two preparations and was identical to the pattern of LGL-derived C9RP (Fig. 1).

Comparison of the PBMC-Derived Protein with C9RP of LGL. The two protein preparations obtained from PBMC and the one from $CD16^+$-depleted PBMC by anti-C9 or anti-C9RP immune adsorption were compared with C9RP of human LGL by ELISA and in cytotoxic assays. Both preparations of the protein isolated from OKT3-activated PBMC undepleted of $CD16^+$ cells were highly active in the killing of M21 human melanoma cells and human K-562 cells; 50% killing of 1×10^4 M21 cells and of 6.25×10^4 K-562 cells was

Table 1. Phenotype of CD16-depleted,* OKT3-stimulated PBMC†

PBMC	Mean percent positive cells ± SD‡					
	CD2	CD3	CD4	CD8	CD16	IL-2R
Untreated	90.2 ± 4.9	78.5 ± 6.4	43.3 ± 8.1	31.1 ± 9.2	10.4 ± 4.7	3.5 ± 1.7
CD16-depleted	92.4 ± 5.1	84.3 ± 7.2	46.1 ± 6.8	33.2 ± 8.1	0.7 ± 0.5	4.3 ± 1.3
CD16-depleted, 3-day OKT3	94.9 ± 6.7	88.9 ± 6.1	46.3 ± 10.1	37.1 ± 8.2	0.9 ± 0.4	68.7 ± 5.4

*PBMC were treated with anti-Leu 11b and rabbit complement.
†OKT3 was used at a concentration of 100 ng/ml.
‡Data is based on blood from four donors.

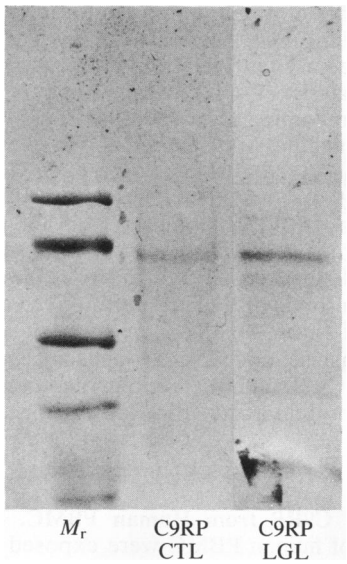

FIG. 1. Comparison of C9RP isolated from OKT3-stimulated human PBMC (C9RP-CTL) and C9RP isolated from human LGL (C9RP-LGL) by $NaDodSO_4$/PAGE (1.7–17%). The PBMC were depleted of $CD16^+$ cells before OKT3 activation and consisted primarily of $CD4^+$ and $CD8^+$ T lymphocytes. Apparent M_r for both proteins was ≈70,000. *Left*, M_r standards from 94,000 (upper) to 14,300 (lower). Stain, Coomassie blue.

achieved with 0.1 μg and 1.2 μg of protein, respectively. The cytotoxic activity of LGL-derived C9RP was very similar. The third preparation obtained from $CD16^+$-depleted, OKT3-activated PBMC was of similar activity and comparable to C9RP of LGL: 50% killing of 6.25×10^4 K-562 cells was caused by 1.2 μg and 0.95 μg of protein, respectively (Fig. 2). Both dose–response curves are sigmoidal. Comparing the cytolytic protein of the depleted and activated PBMC with LGL-derived C9RP by ELISA revealed virtually identical reactivity with rabbit anti-C9RP (LGL) (Fig. 3). These results together with the M_r data suggest that the cytotoxic protein of PBMC is identical with C9RP of LGL.

Increase of C9RP Content of PBMC During Activation of the

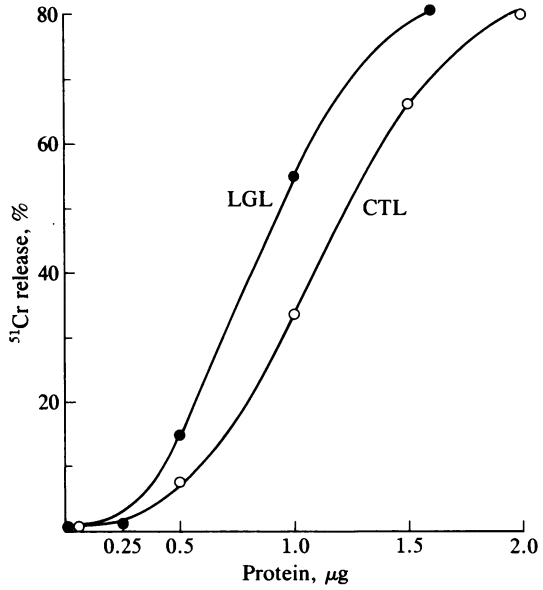

FIG. 2. Comparative cytolytic activity of C9RP isolated from human CTL and human LGL. Target cells used were K-562 cells, the number of cells was 6.25×10^4, and time was 4 hr.

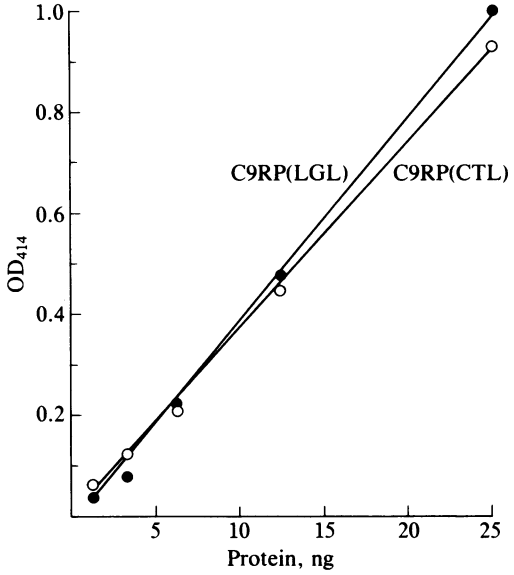

FIG. 3. Reactivity of C9RP (CTL) and C9RP (LGL) with anti-C9RP (LGL) by ELISA.

Cells with OKT3. Aliquots of a PBMC preparation were cultured with 200 ng/ml of OKT3 for 0, 1, 2, 3, or 4 days. The cells were disintegrated as described above to liberate C9RP, and the protein was quantitated in the cell lysates by ELISA, using anti-C9RP. Fig. 4 shows that the C9RP content was very low in unactivated PBMC, increased ≈20-fold by day-2 of OKT3 stimulation, and fell markedly after day-3. Using isolated C9RP form LGL as standard, the average C9RP content on day-2 was calculated to be $\approx 6 \times 10^6$ molecules per cell.

DISCUSSION

The cytolytic protein isolated from OKT3-activated PBMC has in common with C9RP of LGL (1, 2) the following properties: molecular mass of ≈70 kDa, binding to anti-human C9-Sepharose, reactivity with anti-C9RP by ELISA, and the ability to kill K-562 and M21 target cells in the presence of Ca^{2+}. Both dose–response curves are sigmoidal, suggesting a cooperative mechanism underlying pore formation.

A cytolytic protein has been isolated from a murine cytotoxic T cell line that has similar physicochemical characteristics (13). This protein was subsequently also obtained from cytoplasmic granules of the murine CTLL-2 cell line (14), and it was recently shown to cross-react immunochemically with human C9 (15). These observations suggest that the

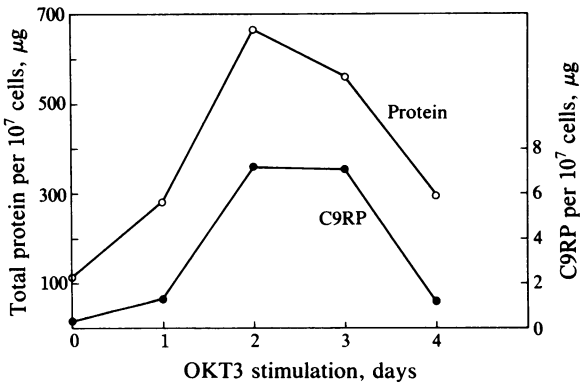

FIG. 4. Kinetic analysis of C9RP and total protein content of OKT3-stimulated $CD16^+$ cell-depleted PBMC.

murine lymphocyte protein named perforin (16), or cytolysin (17), is analogous to the cytolytic C9RP of human cytotoxic lymphocytes (1, 2).

Identification of C9RP as the cytolytic protein of human LGL raised the question as to whether human CTL also utilized this protein as a channel former. These two cell populations are considered relatively distinct (17–19). In this study, the LGL purified from peripheral human blood were largely positive to the antigens Leu-7- and Leu-11-(CD16) (1, 2). Their proliferation in culture was IL-2-dependent and frequently allowed expansion of 1×10^7 cells to 1×10^9 within 14–20 days. These cells not only behaved as effectors in the antibody-dependent cellular cytotoxicity reaction, but also were effectors in the antibody-independent killing of K-562 cells. In contrast, the PBMC are composed of primarily $CD4^+$ and $CD8^+$ cells, particularly after depletion of $CD16^+$ cells. The latter cells represent the majority of the LGL and natural killer cells in peripheral blood (20). $CD16^+$ cells were removed because OKT3 stimulation of PBMC is known to lead to IL-2 secretion, which may cause activation of $CD3^-$ cells. Under stimulation with OKT3, the residual $CD4^+$ and $CD8^+$ cells transformed to large lymphoblasts within 3 to 4 days and concomitantly acquired cytotoxic potential as evidenced by the killing of M21 melanoma cells mediated by anti-target cell–OKT3 antibody conjugates (7). PBMC not stimulated with OKT3 and maintained in culture for the same period exhibited little cytotoxicity under the same conditions. The proliferative response of these cells to OKT3 declined beyond day-3 of culture as did the acquired cytotoxic activity (unpublished data, G.J., D.E.M., H.J.M.-E.).

Acquisition of cytotoxicity by the PBMC upon stimulation with OKT3 (7) was associated with apparent C9RP synthesis, as shown in this study. Unstimulated PBMC, freshly isolated or held in culture for 4 days, contained little C9RP. In contrast, PBMC from the same donor, cultured in the presence of OKT3, exhibited a 20-fold rise in C9RP content by day-2 and 3 of culture and a subsequent decline.

The results indicate that the cytolytic protein of activated $CD3^+$ human PBMC is identical with C9RP of human LGL and that CD3–ligand interaction induces C9RP synthesis in cytotoxic T cells. Kinetic studies are in progress to examine the correlation between PBMC proliferation, acquisition of cytotoxicity, and C9RP synthesis.

The authors acknowledge the excellent technical assistance of Mary A. Brothers and Nancy Engel. This is publication 4511-IMM from the Department of Immunology, Research Institute of Scripps Clinic. The investigation was supported by United States Public Health Service Grants AI 17354, CA 27489, HL 16411, and RR 00833. L.S.Z. is supported by United States Public Health Service Training Grant HL 07195. D.E.M. is the recipient of a National Institutes of Health Physician-Scientist Award administered through the University of California at San Diego. G.J. is supported by Fellowship 300-402-516-5 from the Stiftung Deutsche Krebshilfe (Mildred Scheel Stipendium).

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