IDENTIFICATION AND CHARACTERIZATION OF A PORE-FORMING PROTEIN OF HUMAN PERIPHERAL BLOOD NATURAL KILLER CELLS

By CHAU-CHING LIU, BICE PERUSSIA,* ZANVIL A. COHN, and JOHN DING-E YOUNG

*From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021; and *The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104*

How NK cells and CTL kill their targets has been extensively investigated in a number of laboratories (reviewed in references 1–7). These effector cells have been shown to assemble tubular lesions on target cell membranes (8–10). The cytolytic apparatus of CTL lines (CTLL)[1] and cloned NK-like cells appears to reside in the cytoplasmic granules. Granules isolated from these cells lyse target cells and assemble ringlike lesions on their membranes (11–18). Recently, the pore-forming protein (PFP, perforin) that mediates the formation of these transmembrane tubules has been isolated (19–23). The isolated polypeptide lyses tumor cells (23) and forms functional pores in lipid bilayers (20–23). It bears structural, functional, and immunologic homologies with the ninth component of the complement system (C9) (24, 25) and is actively secreted by murine CTLL that have been stimulated with calcium ionophore (25). To date, the biochemical analysis of PFP/perforin has been conducted only on murine cell lines and cloned cells. Although the use of cloned effector cells or cell lines has allowed the expansion of large numbers of these cells in culture, greatly facilitating studies on their lytic mechanisms, little is known about the lytic protein(s) of primary cytotoxic effector cells. Moreover, a role for similar cytolytic protein(s) in the killing mediated by human NK cells has not been established.

Resting mouse spleen (26) and human peripheral blood (27, 28) NK cells are known to respond directly to the lymphocyte growth factor IL-2 with enhanced spontaneous cytotoxic activity and, at later times, cell proliferation. The IL-2–induced NK cells maintain the characteristic phenotype of fresh NK cells, and no spontaneously cytotoxic cells are generated from other lymphocyte subsets in short-term cultures in the presence of IL-2 (3, 28). The activation of cytotoxic cells with IL-2 has recently generated wide interest in view of its potential application in tumor immunotherapy (29). Here, we report on the identification and isolation of a 70,000–72,000 M_r polypeptide from human peripheral blood

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[1] *Abbreviations used in this paper:* CTLL, cytotoxic T lymphocyte lines; DPBS, Dulbecco's PBS; LGL, large granular lymphocyte; PFP, pore-forming protein.

NK cells and a human NK cell line. The purified protein mediates the assembly of functional and structural pores and shares antigenic crossreactivity with C9. Our data suggest that this polypeptide may be generally involved in the cell-mediated killing and that the lytic event of both NK cells and CTL may develop in a similar fashion.

Materials and Methods

Cell Lines. The human NK cell line NK3.3, derived from PBL activated in primary mixed lymphocyte culture (30), was kindly provided by Dr. J. Kornbluth, University of Pennsylvania School of Medicine, Philadelphia. This IL-2–dependent cell line mediates both spontaneous (30) and antibody-dependent cytotoxicity against tumor targets (our unpublished data) and has surface phenotype of activated, proliferating NK cells. It bears the low-affinity Fc receptor (FcR) for immune-complexed IgG (CD16 antigen) detected by monoclonal antibodies B73.1 and 3G8 (unpublished), the 45-kD molecule corresponding to the sheep erythrocyte receptor on T cells and most NK cells (CD2 antigen), the 30–32-kD antigen CD8, present on cytotoxic T lymphocytes and on a variable proportion of NK cells, T10 and T200 antigens, and the 3A1 and HLA-DR activation antigens (30). None of the antigens present on the majority of T cells (i.e., the T cell receptor–associated antigen CD3, CD5, a 60-kD molecule on T cells, and CD4, T helper–associated antigen) is expressed on NK3.3 cells, which are also negative for CD11 (receptor for C3bi, or CR3) and Leu-7 (HNK-1) antigen (30). NK3.3 cells were maintained in culture in Iscove's medium (Gibco, Grand Island, NY), supplemented with 25 mM Hepes buffer, 10% heat-inactivated pooled human serum (Bio-Bee, Boston, MA), and 15% IL-2–containing leukocyte-conditioned medium (lymphocult-T, Biotest Serum Institute, Frankfurt, Federal Republic of Germany), as described (30).

The NK-sensitive human erythromyeloid leukemia cell line K562, derived from a patient with chronic myeloid leukemia in blast crisis (31) and the B lymphoblastoid cell line Daudi were maintained in culture in RPMI 1640 medium supplemented with 10% FCS. These lines are mycoplasma-free on repetitive testing.

Antibodies. Production and characterization of the anti-CD16 antibody B73.1 (IgG1), specific for the low-affinity FcR for aggregated IgG on NK cells and granulocytes (32–34), B36.1 (IgG2b, anti-CD2), and B33.1 (IgG2a, anti-HLA-DR nonpolymorphic determinant) have been previously reported (35). The anti-CD16 antibody 3G8 (IgG1, reference 36) was a generous gift of Dr. J. Unkeless (Mount Sinai Medical School, New York); and antibody N901 (IgG1) that reacted with NK cells, a subset of T cells, and some immature myeloid cells (37) was kindly provided by Dr. J. Griffin (Dana Farber Cancer Institute, Boston, MA). OKT3 (IgG2a, anti-CD3) (38) was produced in our laboratories from cells obtained from American Type Culture Collection (Rockville, MD). Specific polyclonal antibodies to reduced and alkylated human C9 were obtained as described (24, 25).

Peripheral Blood Cells and Lymphocyte Subsets. Venous peripheral blood drawn from healthy donors was anticoagulated with preservative-free heparin, and mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (39). NK cells, NK cell–depleted subsets, and T cells were obtained from several different types of mononuclear cell preparations: (*a*) mononuclear cells depleted of the majority of monocytes by one or two cycles of adherence to plastic surfaces for 1 h at 37°C; (*b*) mononuclear cells purified as above from plateletphoresis bags obtained from New York Blood Center and depleted of B cells and monocytes by eluting the plastic nonadherent cells through a nylon wool column (40); (*c*) when larger numbers of NK cells were needed, these were purified from 10-d cultures of PBL with irradiated (50 Gy) Daudi cells in the absence of exogenously added IL-2. NK and T cells were purified from the above populations by indirect rosetting with $CrCl_3$-treated goat anti–mouse Ig–coated erythrocytes (41) after sensitization with the appropriate anti-NK (B73.1 or N901) or anti-T (B36.1, OKT3) and anti-B (B33.1) monoclonal antibodies as previously described (28, 32). To ensure maximal purification, the rosetting procedure was repeated twice. When the lymphocyte subsets were obtained

by positive selection, contaminating erythrocytes were lysed using hypotonic medium (28, 32). In all cases, after purification, the cells were maintained in culture (10^6 cells/ml of RPMI 1640 supplemented with 10% FCS) in a 5% CO_2 humidified atmosphere for 3 d with purified human recombinant IL-2 (Genzyme, Boston, MA; or TGP-3, Takada Chemical Industry, Inc., Osaka, Japan, a generous gift of Dr. T. Taguchi of Osaka University, Japan) added at a final concentration of 40–50 U/ml. Purity of the cell populations was tested by direct immunofluorescence using biotin-labeled monoclonal antibodies to leukocyte subsets, detected with FITC-avidin as previously described (28, 32). The proportion of cells positive with the different antibodies was scored by flow cytofluorometry on an Ortho Cytofluorograf 50H (Ortho Diagnostics, Westwood, MA) connected to a Data General MP200 microprocessor.

Spontaneous Cytotoxicity Assays. These were performed in round-bottom microtiter plates, using K562 cells as targets (10^4 cells/well) in a 3-h ^{51}Cr-release assay as previously described (32). Cytotoxic activity was quantitated from cytotoxicity curves obtained using various E/T ratios by calculating the number of LU per 10^7 cells at 45% specific lysis with the use of the linear regression to a modified von Krogh's equation (42). One LU is defined as the number of effector cells that lyse 45% of the target cells within the test time.

Biosynthetic Labeling of Cells with [^{35}S]Methionine. The lymphocyte subsets were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and incubated (10^6 cells/ml) for 18 h in methionine-free medium (using a kit obtained from Gibco), supplemented with 4% RPMI 1640 medium and [^{35}S]methionine (New England Nuclear, Boston, MA) (25 μCi/ml per 10^6 cells), containing 10% FBS and 15% IL-2–containing medium, both previously dialyzed against PBS to remove residual methionine.

Affinity Purification of NK Cell PFP. An anti–human C9 immunoadsorbent was used to purify PFP from cell lysates or from supernatants of cells treated with the calcium ionophore A23187. Both sources of PFP were adsorbed on 3G8-agarose (2.2 mg of 3G8/g of agarose, 80% coupling efficiency) before PFP purification, to deplete the cell preparations of FcR, and avoid its binding to the anti-C9 immunoadsorbent. To prepare this latter immunoadsorbent, IgG of the rabbit anti-C9 antiserum were fractionated by affinity chromatography using protein A–agarose (Boehringer Mannheim Biochemicals, Indianapolis, IN). After alternate washes with PBS and PBS containing 0.4 M NaCl (PBS/NaCl), the agarose-bound IgG were eluted with 0.1 M glycine, pH 2.5. The eluate was immediately neutralized with 10% fraction vol of 1 M Tris base and the eluted IgG were coupled to glutardialdehyde-activated agarose (Boehringer Mannheim Biochemicals) at 20 mg of protein per gram of agarose according to instructions provided by the supplier. Coupling efficiency ranged from 70 to 90%.

Two different cell sources of PFP were used. (*a*) Cells (10^7–10^8 PBL subsets and NK3.3 cells) were washed three times in DPBS at 4°C and resuspended to 10^7 cells/ml in ice-cold lysis buffer (1 M $NaKHPO_4$, 10 mM benzamidine, 1 mM PMSF, 1 mM EDTA, 0.5% NP-40 [Calbiochem-Behring Corp., La Jolla, CA], pH 7.4). After freezing and thawing the cell suspension twice and sedimenting the nuclei and unbroken cells by low-speed centrifugation (2,500 *g*, 5 min) the membrane-free lysate was obtained by centrifugation at 350,000 *g* in a table-top ultracentrifuge (model TL-100, Beckman Instruments, Inc., Palo Alto, CA) for 30 min at 4°C. (*b*) Cells were washed and resuspended to 10^7 cells/ml in DPBS containing 1 mM $CaCl_2$ at 37°C. Calcium ionophore A23187 (Calbiochem-Behring Corp.) was added to 0.5 μM final concentration, and the cell suspension was incubated at 37°C for 20 min. Cells were pelleted by centrifugation (2,500 *g*, 15 min, 4°C). The cell-free supernatants from either preparation were applied to a column of 1 g of agarose coupled to specific antibodies to human C9. The column was washed and the antigen was eluted as described above, with the exception of an additional wash with PBS/0.5% NP-40 before elution of the bound antigen.

Current Measurements. Solvent-free planar bilayers were formed from monolayers of phospholipids according to Montal and Mueller (43), as described in detail elsewhere (44, 45). Acetone-extracted soybean phospholipids (46) were used to form the monolayers. Positive current was defined as that following from *cis* to *trans* compartments, with the

trans side defined as the virtual ground. Only bilayers with conductance that did not exceed 10 pS and capacitance of 0.69–0.75 $\mu F/cm^2$ were exposed to proteins. Proteins were always introduced through the aqueous phase of the *cis* compartment. All recordings were made at room temperature (22–24°C).

The cell proteins to be tested in bilayers were obtained by extracting plastic and nylon wool–nonadherent PBL with lysis buffer without NP-40 and the supernatants obtained after high-speed centrifugation were added directly to *cis* side. Affinity-purified PFP from NK cell populations were solubilized first with 0.1% Triton X-100 (Calbiochem-Behring Corp.) and added to bilayers in small volumes, resulting in a detergent dilution exceeding 400-fold. Addition of the same volumes of detergent solutions alone did not induce any conductance change in the bilayer.

Electron Microscopy. The eluate of anti-C9 affinity columns, containing PFP from NK cells and PBL populations, was incubated with 30 mM β-D-octylglucoside (Calbiochem-Behring Corp.) and 5 mM $CaCl_2$ for 2–4 h at 37°C and placed on Formvar-coated 400-mesh grids, stained with 2% phosphotungstic acid, pH 7, and examined under a JEOL 100 cx electron microscope (JEOL USA, Electron Optics Div., Peabody, MA). To incorporate PFP into liposomes, the eluate with detergent was sonicated with egg lecithin (type V-E, Sigma Chemical Co., St. Louis, MO) at 3.3 mg/ml and liposomes were formed by a one-step dilution protocol as outlined elsewhere (47). Proteoliposome suspensions were examined by negative contrast as described above.

Light Microscopy. Cytocentrifuge smears of the cell preparations were stained with May Grumwald-Giemsa at pH 8. Staining at this basic pH gave optimal preservation of the granule morphology.

SDS-PAGE, Immunoblotting, and Fluorography. To define the biochemical characteristics of the protein adsorbed on anti-C9 immunoadsorbent from [^{35}S]methionine-labeled cell lysates and from unlabeled cell lysates and supernatants, SDS-PAGE and immunoblotting were used respectively. 4–20% polyacrylamide gradient gels were prepared and developed according to Laemmli (48), using "mini-gels" (casted using model 360, Bio-Rad Laboratories, Richmond, CA). Gels were developed at constant current and the ones containing radiolabel were soaked in autoradiography enhancer (EN^{3}HANCE; New England Nuclear) for 1 h, washed, dried under vaccum, and subjected to autoradiography.

Immunoblots were developed as described (49). NK cells were ruptured by freezing and thawing, and the nucleus-free lysate was batch-adsorbed with 3G8-agarose (0.14 mg of 3G8 per 10^7 cells) for 2 h at 4°C to remove FcR. The nonadsorbed material was then electrophoresed under reducing conditions. Reduction and alkylation were performed with 50 mM DTT and 0.1 M iodoacetamide. All samples were boiled for 5 min before applying to gels. Protein transfer to nitrocellulose membranes (Millipore Corp., Bedford, MA) was performed at constant current of 400 mA for 2.5 h. Anti-C9 antisera and preimmune serum control were used at a 1:50 dilution and ^{125}I-labeled goat $F(ab')_2$ anti-rabbit IgG (New England Nuclear) was used at 1.2 μCi/ml as the secondary affinity label. All washes were done with 10 mM Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl, 2 mM EDTA, 0.25% NP-40, and 0.25% Tween 20 (Bio-Rad Laboratories). The autoradiography was always performed with intensifying screens.

For control, human C9 was also electrophoresed and immunoblotted with anti-C9 antibodies. C9 was purified from pooled human serum (New York Blood Center) according to a previously published procedure (50), with the exception that serum proteins were first eluted through a Sephacryl S-200 column before the hydroxyapatite column elution. The purity of the C9 preparation was ascertained by SDS-PAGE and silver staining.

Results

Characteristics of NK Cells Isolated by Indirect Rosetting. NK cell populations were purified by indirect rosetting using monoclonal antibodies directed against either NK- or T cell–specific surface antigens. Although NK cells could be isolated by either positive or negative selection, most experiments used the negative selection procedure in order to avoid possible triggering of cell degran-

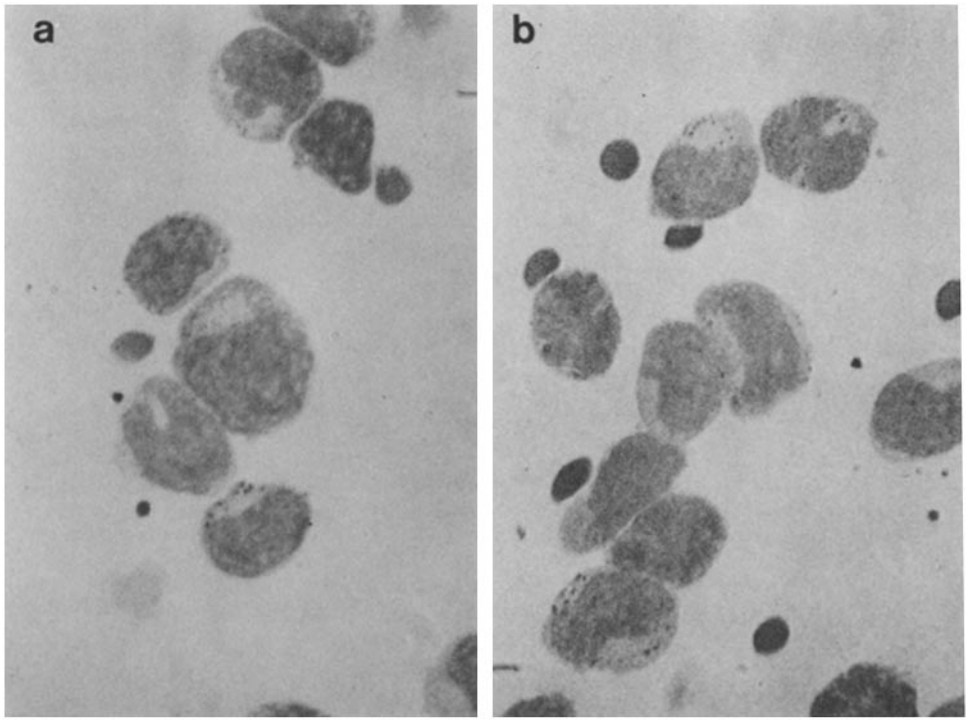

FIGURE 1. Morphology of OKT3$^-$ NK cells separated from 10-d cultures separated by indirect rosetting procedure (see Materials and Methods). *a* and *b* correspond to two separate experiments. Note the indented nucleus, the high cytoplasm/nucleus ratio, and the presence of numerous cytoplasmic granules. × 400.

ulation by the anti-NK cell antibodies. To obtain large numbers of cells for biochemical analysis, PBL were cultured for 10 d with irradiated Daudi cells before fractionation by negative selection using OKT3. OKT3$^-$ cells purified from these cultures have morphology of large granular lymphocytes (LGL) (51), with high cytoplasm/nucleus ratios and containing azurophilic granules in their cytoplasm (Fig. 1), like NK cells purified from fresh peripheral blood using similar procedures (not shown). The purity of these NK cell preparations typically exceeded 90%, as judged from their surface phenotypes (Fig. 2). The OKT3$^-$ cells express typical NK cell phenotype: they are CD16$^+$ (Fig. 2), CD2$^+$ and N901$^+$ (not shown) and do not express the T cell–associated markers CD5 (Fig. 2), CD4, and CD3 (not shown). Unlike freshly obtained NK cells, they express activation markers (HLA-DR, Tac antigen, and transferrin receptor) (not shown), albeit at lower levels than NK cells cultured under similar conditions and harvested on day 6 at the peak of their proliferation (52, 53). The OKT3$^+$ populations were largely depleted of NK cells, as assessed by surface markers and lack of significant levels of spontaneous (Table I, Fig. 3) and antibody-dependent cytotoxicity. All NK cell subsets purified from fresh PBL (Fig. 3*a*) and from PBL cultured for 10 d with Daudi cells (Fig. 3*b*) mediated spontaneous (Fig. 3) and antibody-dependent cytotoxicity (not shown), whereas the OKT3$^+$ subsets obtained from the same lymphocyte preparations and the NK cell–

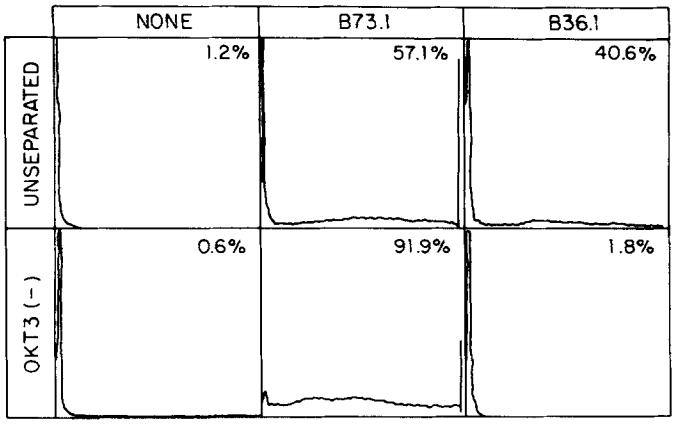

FIGURE 2. Surface phenotype of PBL cultured for 10 d with irradiated Daudi cells. PBL from one donor were cultured as described and NK cells were purified by negative selection using OKT3 and indirect rosetting as described in Materials and Methods. The proportion of cells positive for CD16 (B73.1) and CD2 (B36.1) antigens was evaluated by direct immunofluorescence (flow cytometry) using biotin-labeled B73.1 and B36.1 antibodies, respectively, detected with FITC-avidin. (*x* axis) intensity of fluorescence; (*y* axis) number of cells. *None* indicates the immunofluorescence of cells incubated with control irrelevant antibody and FITC-avidin. Figures reported in each histogram are the proportion of cells positive with the indicated antibodies.

TABLE I

Cytotoxic Activity and Surface Phenotype of NK Cells Expanded In Vitro

Lymphocyte subset*	Spontaneous cytotoxicity‡	Surface phenotype		
		B73.1	N901	B36.1
Total	135.5 ± 61.9§	48.4 ± 16.4‖	47.8 ± 21.0	47.8 ± 21.0
OKT3$^+$	20.8 ± 22.5	10.1 ± 5.5	ND	65.7 ± 8.5
OKT3$^-$	123.2 ± 91.0	88.9 ± 10.5	(85.4, 93.2)¶	5.5 ± 3.9

* PBL were cultured with irradiated Daudi cells for 10 d and the subsets were purified as described in Materials and Methods.

‡ Target cells were K562, in a 3-h ^{51}Cr-release assay.

§ LU (45%) per 10^7 cells, mean ± SD of eight experiments.

‖ Proportion of cells positive (direct immunofluorescence, flow cytometry), with the indicated biotin-labeled antibodies. Mean ± SD of eight experiments.

¶ Results from two separate experiments.

depleted subsets were virtually depleted of cells with spontaneous (Fig. 3 and Table I) and antibody-dependent cytotoxicity (not shown).

Identification of the PFP in NK Cells. Previous reports (24, 25) have shown that the mouse lymphocyte PFP/perforin and the human C9 are immunologically related. The shared antigenic epitopes of these proteins are limited to cysteine-rich domains that are exposed antigenically only when these proteins are chemically reduced (24, 25). Here, we have used polyclonal antibodies derived against human C9 to identify a C9-related polypeptide produced by human NK cells (Fig. 4). The anti-C9 antibodies react with a unique polypeptide, contained in peripheral blood NK cells but not T cells from the same PBL preparations, that migrates with an M_r of 70,000–72,000 under reducing conditions (Fig. 4, lanes *4–6*). The blots developed with preimmune serum did not react with the NK cell

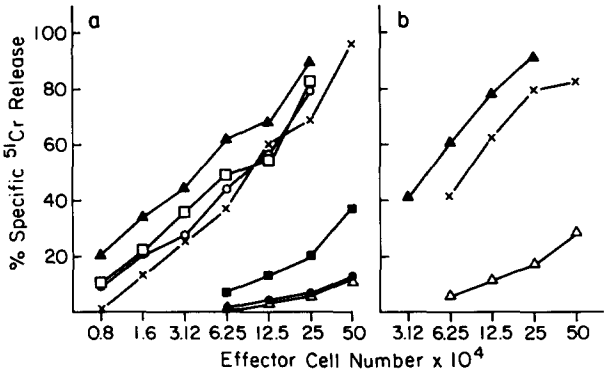

FIGURE 3. Spontaneous cytotoxic activity of PBL subsets. Different lymphocyte subsets were purified by indirect rosetting from (*a*) freshly obtained PBL or (*b*) PBL cultured for 10 d with irradiated Daudi cells. Each PBL subset was tested for spontaneous cytotoxicity in a 3-h ^{51}Cr-release assay against K562 targets. (×) total unseparated lymphocyte population; (△) OKT3$^+$ cells; (▲) OKT3$^-$ cells; (○) B73.1$^+$ cells; (●) B73.1$^-$ cells; (□) N901$^+$ cells; (■) N901$^-$ cells.

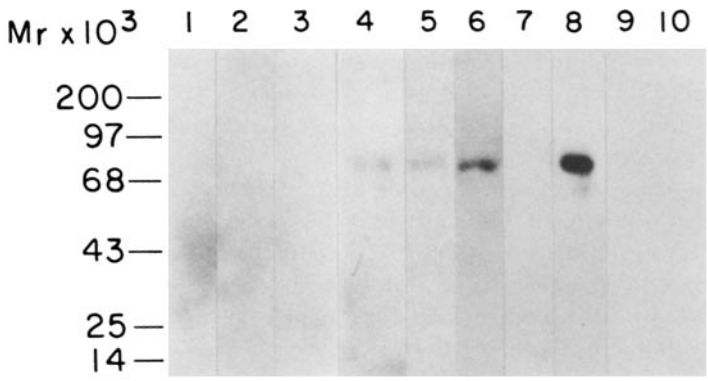

FIGURE 4. Immunoblots of NK cell lysate proteins with anti-C9 antiserum. Cell lysates were prepared, as described in Materials and Methods, from several different PBL preparations. Lanes *1* and *4*: monocyte-depleted PBL. Lanes *2* and *5*: B73.1$^+$ cells purified from cultured PBL. Lanes *3* and *6*: N901$^+$ cells purified from fresh cells. Lanes *7* and *8*: C9, at 0.29 μg per lane. Lane *9*: B73.1$^-$ cells purified from cultured PBL. Lane *10*: N901$^-$ cells purified from fresh cells. Cell lysates were prepared and applied at 1.5×10^5 cell-equivalents per lane, except for lanes *2* and *5*, which contained lysate from 9×10^4 cells. Gels were developed under reducing conditions. The autoradiography was performed overnight for all the samples.

lysate (lanes *1–3*). The M_r of this protein resembled that of purified C9 immunoblotted under the same conditions (Fig. 4, lane *8*). The same protein was not found in OKT3$^+$ and NK cell–depleted populations (Fig. 4, lanes *9* and *10*), pointing to the specificity of the immunologic crossreactivity between C9 and NK PFP. The polypeptide identified on the immunoblot was not related to the NK cell FcR (which could have bound to the agarose-linked immunoglobulins) because all cell lysates had been preadsorbed with anti-FcR monoclonal 3G8–agarose before gel electrophoresis of cell protein samples.

Isolation of the C9-related NK Cell PFP/Perforin. The C9-related NK cell PFP was isolated by affinity chromatography using polyclonal anti-C9 antibodies as the immunoadsorbent (see Materials and Methods). Peripheral blood NK cells,

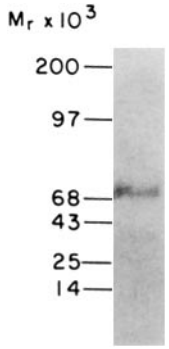

FIGURE 5. SDS-polyacrylamide gel profile of NK cell PFP purified by affinity chromatography. OKT3$^-$ NK cells (8×10^7) were purified from 10-d cultures of PBL and labeled with [^{35}S]methionine as described in Materials and Methods. Labeled cells were stimulated with A23187 and the cell-free supernatant was applied to an anti-C9 IgG-agarose column. 15 μl of the peak radioactivity fraction eluted from the column was applied to the lane. The autoradiography was performed for 2 wk.

biosynthetically labeled with [^{35}S]methionine, were stimulated with A23187 in the presence of Ca^{2+} and the cell-free supernatant was applied to the anti-C9 affinity column. As expected, the antigen bound to and eluted from the affinity column migrated with an M_r of 70,000–72,000 as analyzed by SDS-PAGE (Fig. 5). The eluted radioactivity accounted for 3% of the total label found in the cells. The C9-related polypeptide could also be isolated directly from cell lysates of biosynthetically labeled NK cells by affinity chromatography. The isolated polypeptide showed identical SDS-PAGE profile as that obtained from ionophore-stimulated cell supernatant and a similar 70,000–72,000 M_r polypeptide was identified also in the NK 3.3 cell line which has been maintained in serum-free medium (not shown).

Ultrastructural Analysis of Tubular Lesions. The protein eluted from the anti-C9 affinity column was incubated with β-D-octylglucoside at 37°C for 2–4 h in the presence of Ca^{2+} and examined by negative-contrast staining under electron microscopy. Tubular lesions of various sizes (ranging 50–180 Å) were observed but the more frequently observed tubules had an internal diameter averaging between 150 and 170 Å (Fig. 6*a*). In addition to complete tubules, partially assembled polymers were also seen. The protein/detergent mixture that had been reconstituted into proteoliposomes (see Materials and Methods) gave similar morphology of lesions (Fig. 6, *b–f*). Similar tubules were assembled by affinity-purified PFP of human peripheral blood NK cells isolated by several different protocols (Fig. 6, *a–e*). Tubules were also formed by the PFP that had been affinity-purified from NK3.3 cells (Fig. 6*f*). Thus, the lesions formed by affinity-purified PFP of NK cells resemble the previously reported lesions associated with whole peripheral blood cells (8).

Functional Channels Formed by Putative NK PFP/Perforin. The polypeptide isolated from the supernatants of A23187-stimulated OKT3$^-$, N901$^+$ and B73.1$^+$ cells lacked hemolytic activity and was not capable of inserting spontaneously into planar bilayers to form ion channels. Similar results were obtained previously with mouse CTLL (25), and were attributed to the rapid polymerization and

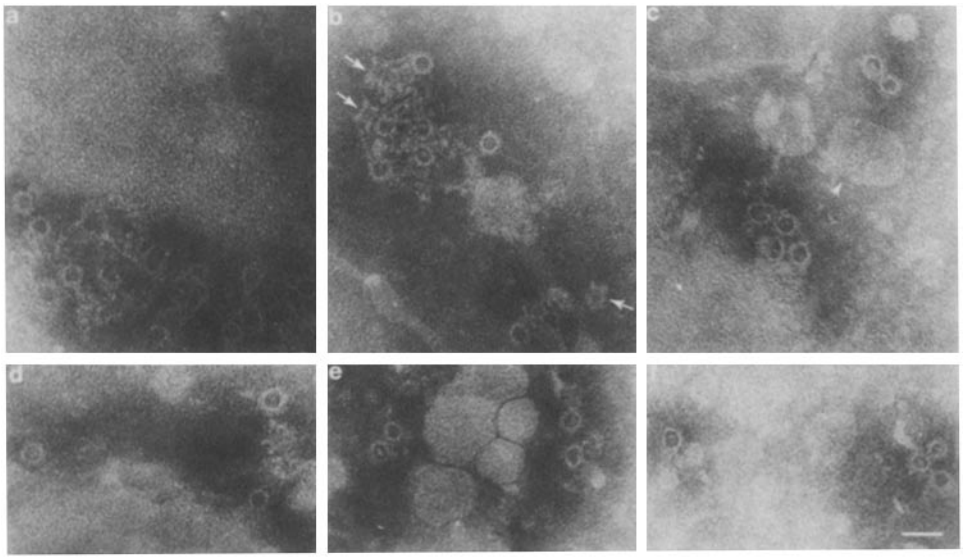

FIGURE 6. Morphology of ringlike lesions produced by the human NK cell PFP. Selected images of affinity purified NK cell PFP assembled in the (*a*) absence or (*b–f*) presence of lipid vesicles and examined by negative contrast (see Materials and Methods). The PFP was purified from NK cells positively selected using (*a* and *b*) N901 and (*c*) B73.1 or negatively selected using (*d* and *e*) OKT3 and (*f*) from NK3.3 cells. All NK cell subsets (*a–c*), with the exception of $OKT3^-$ (*d* and *e*), were obtained from fresh PBL. $OKT3^-$-selected cells were obtained from 10-d cultures of PBL. Arrows point to longitudinal views of the tubules. The arrowhead in *c* depicts a tubule inserted into the membrane of the lipid vesicle. Scale bar: 460 Å.

inactivation of the cell released PFP in the extracellular medium, which contains Ca^{2+}. The affinity-purified PFP, however, could be incorporated into planar lipid bilayers provided that the protein was solubilized first with detergent. Because of their high impedance, planar bilayers are capable of resolving very small current fluctuations associated with individual channel molecules. The addition of affinity-purified protein (solubilized with 0.1% Triton X-100) resulted in a progressive increase of membrane current, which occurred as a summation of discrete current steps (Fig. 7*a*). This behavior is indicative of incorporation of individual or groups of channels into the lipid bilayer (54). Discrete steps ranging 0.3–1 nS were observed. The heterogeneity of channel sizes was also exemplified by traces of single-channel fluctuations, which showed large and heterogeneous unit conductances (Fig. 7, *b* and *c*). The ionic channels formed by this protein are highly resistant to closing by increasing voltages. To induce channel closing, voltages exceeding 100 mV had to be applied (Fig. 7, *b* and *c*). This behavior resembles that observed for the mouse PFP/perforin incorporated into the planar bilayer (18, 21, 23). Ion channels of similar properties were formed by the polypeptides purified from NK3.3 cell line and peripheral blood NK cell populations.

Ion selectivity ratios were obtained from the reversal potential necessary to null the current flow from a 10-fold higher salt concentration with the *cis* compartment made more concentrated. Permeability ratios of 1:0.8:0.4:0.2 were

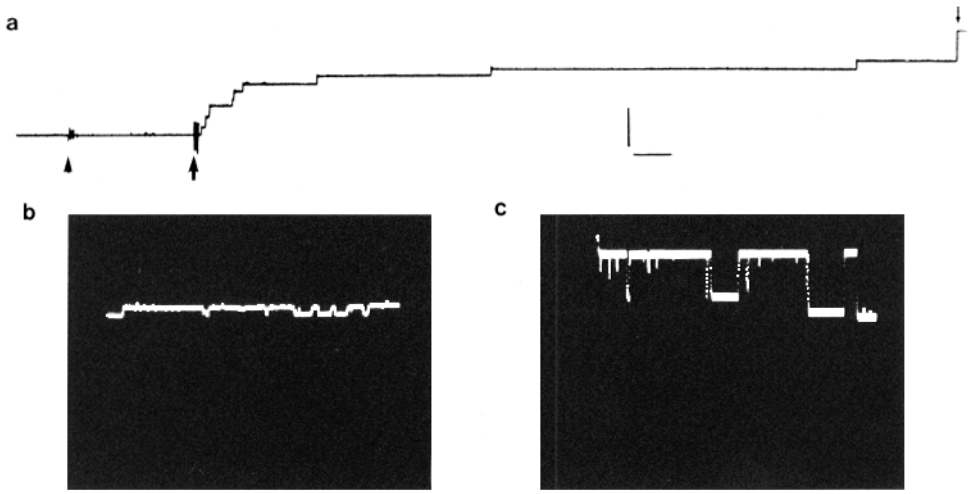

FIGURE 7. Effect of human NK cell PFP on planar lipid bilayers. Planar bilayers were formed in 0.1 M NaCl, 1 mM $CaCl_2$, 5 mM Pipes, pH 7.4. The bilayers were clamped at +30 mV in *a* and +120 mV in *b* and *c*. The record in *a* was obtained from a chart recorder and *b* and *c* were stored in a Nicolet 4094 digital oscilloscope. (*a*) NK cells were selected by using the monoclonal antibody B73.1. The $B73.1^+$ and $B73.1^-$ cells (from fresh PBL) were washed and resuspended in lysis buffer without NP-40 (see Materials and Methods) at 10^7 cells/ml and ruptured by freeze-thawing. 50 μl of the nucleus-free supernatant was applied to the aqueous phase. Arrowhead and arrow indicate addition of $B73.1^-$ and $B73.1^+$ cell supernatants, respectively. Note the immediate rise in current in steps after addition of proteins from $B73.1^+$ cells. The current increased progressively until the membrane broke (small arrow on the right). The noise in the recording is due to stirring of the membrane chamber following addition of proteins. (*b*) Affinity-purified NK cell PFP in 0.1% Triton X-100 (from 10^5 cell equivalents of $OKT3^-$ cells of 10-d cultures of PBL) was applied to the bilayer and the recording obtained 5 min later. The upward deflections indicate channel openings. (*c*) Same material as in *b* incubated in the bilayer at 37°C for 2 h in the absence of detergent. The material was then solubilized with 0.1% Triton X-100 and 10^5 cell-equivalents of material were applied to the bilayer. A selected trace is shown. Note the closing of the channel to an intermediate state prior to completely closing at the end of the record. Scale bars: (*a*) vertical, 60 pA, horizontal, 50 s; (*b*) vertical, 0.24 nA, horizontal, 20 s; (*c*) vertical, 80 pA, horizontal, 20 s.

found for $K^+/Na^+/Cl^-/Ca^{2+}$. The NK cell PFP–mediated channels were also permeable to Mg^{2+} and Zn^{2+}, indicative of a lack of ion selectivity.

Discussion

NK cells are bone marrow–derived lymphocytes functionally defined by their capability of lysing a wide variety of normal, virus-infected, and tumor-derived target cells without prior sensitization (1, 3). They bear a characteristic combination of surface differentiation antigens that serve to define them as a discrete and homogeneous leukocyte subset, distinct from B and T lymphocytes and myelomonocytic cells (3, 32). Monoclonal antibodies have become useful in the separation of this cell subset from other PBL (32–34, 36). Here we have separated NK cell populations from fresh and cultured human PBL using several different monoclonal antibodies in both positive and negative selection procedures.

Previous studies have characterized a cytolytic PFP (perforin) of mouse CTLL

and cell lines with NK-like activity (19–23). Here, we have identified a similar polypeptide with an M_r of 70,000–72,000 (by SDS-PAGE) in human primary NK cells and in a human NK cell line. Both freshly prepared human peripheral blood NK cells and NK cells that have been stimulated with IL-2 in short-term cultures contain PFP. This polypeptide, like the murine PFP, assembles functional and structural lesions in target membranes. It shares immunologic cross-reactivity with human C9 and has been purified by affinity chromatography using anti-C9 antibodies bound to agarose as the immunoadsorbent. PFP is not present in non-NK populations selected using monoclonal antibodies under the same conditions and from the same PBL populations from which NK cells were purified. PFP is not present in detectable amounts in fresh T cells, and its production is increased in NK but not in T lymphocytes upon IL-2 treatment. The T cells purified from the 10-d cultures of PBL bear markers of activated lymphocytes, but unlike CTLL, lack PFP. These observations indicate that, among lymphocytes, only NK cells are the predominant cells that constitutively produce PFP, and suggest the possibility that PFP production by T cells may require specific allogeneic stimulation.

The NK cell PFP is released from NK cells after direct stimulation with calcium ionophore, suggesting that, like the PFP of murine CTLL, the PFP of human primary NK cells may also be localized in secretory compartments (granules) of their cytoplasm. During cell killing, this protein may be actively released and concentrated in the intercellular space of contact between the NK cell and its target, assembling thereafter into transmembrane tubules on the target surface. Several observations support the contention that a secretory event may be involved in NK cell–mediated killing: the lymphocyte Golgi apparatus and microtubule organizing center become aligned toward the region of effector/target contact (55, 56), and the cell granules translocate to the site of cell contact (56, 57). The formation of large structural and functional pores by this protein and its voltage resistance to closing would ensure that once this protein is inserted into the target membrane, it would remain as an open transmembrane pathway, allowing the free leakage of water, ions, and macromolecules across the plasma membrane.

The human NK cell PFP is immunologically related to human C9 and has been purified using polyclonal C9-specific antibodies. The nonreduced human NK cell PFP reacts with anti-C9 antibodies, indicating that the shared antigenic domain between human C9 and human NK cell PFP is not limited to a cysteine-rich domain as in the case of human C9 and mouse PFP (24, 25). Several lines of evidence indicate that the purified protein is not C9: (*a*) the radioactivity of the purified protein was derived from biosynthetically labeled cells and therefore could not have been associated with contaminating C9 found in the serum; (*b*) C9 polymerizes only after prolonged incubation (48–64 h) at temperatures exceeding 37°C or in the presence of Zn^{2+} (24, 58, 59), in contrast to the rapid polymerization observed here for the NK cell protein; (*c*) the tubules formed by polymerized C9 have an internal diameter of 100 Å (24, 58, 59) vs. the more frequently observed diameter of 150–170 Å observed here (Fig. 6); (*d*) NK cell–depleted populations do not contain PFP/perforin (Fig. 4), reflecting the specificity of its cellular distribution. The purified polypeptide is also unrelated to the

NK cell low-affinity FcR as adsorption with anti-FcR monoclonal antibodies does not deplete the C9-related antigen from NK cell lysates.

The human NK cell PFP presents a number of biochemical, structural, and functional properties that closely resemble the mouse CTL and NK cell–like PFP/perforin (19–23). The observations suggest that a unique lytic polypeptide is operative during cell killing mediated by both human and murine CTL and NK cells. It is not clear whether other granule constituents may also play an important role during cell killing. Other lytic mediators, such as proteases (reviewed in reference 6) and NK cytolytic factors (60) have also been implied in NK cell– and CTL-mediated killing of targets. It is conceivable that a channel of the size described here could also function as a transmembrane conduit for other toxic molecules (proteases, nucleases, and other cytolytic factors) that might permeate through the target membrane. The permeation of a second toxic mediator would explain the recently observed DNA fragmentation and nucleus breakdown that appear to occur early during lymphocyte-mediated killing (61). In this regard, it is noteworthy to point out that granules of murine CTLL appear to contain tumor necrosis factor and lymphotoxin-like molecules (Young, Liu, and Cohn, unpublished observations).

Pore formation may represent a general step in the mechanism of cell-mediated cytotoxicity, and has also been described for eosinophils (62) and the protozoan parasite *Entamoeba histolytica* (44, 63–65). Interestingly both cell-derived (NK PFP) and humoral (at least C9) immune mediators seem to share structural and functional homologies. These observations indicate that these proteins may have been derived from a common ancestral protein but have diverged during evolution to become specialized for their respective effector roles in either cellular or humoral immunity.

Summary

We show here that human peripheral blood NK cells contain a pore-forming protein (PFP) with an M_r of 70,000–72,000 that assembles structural lesions (with an average internal diameter of 150–170 Å) and forms functional channels. The PFP was isolated by affinity chromatography from human NK cells, using a specific anti-C9 antiserum as the immunoadsorbent. The NK cells were isolated from PBL by positive or negative selection by indirect rosetting using a panel of monoclonal antibodies directed against different NK and T cell surface antigens. PFP was identified in NK cells freshly isolated and isolated from cultured PBL, both stimulated with interleukin 2, but not in NK cell–depleted lymphocytes. In planar bilayers, the channels formed by the NK cell–derived PFP are highly voltage resistant, with most channels persisting in the open state once they have inserted into the bilayer. The unit conductances of these channels range 0.3–1 nS in 0.1 M NaCl. The channels show poor selectivity for monovalent and divalent ions. The PFP is also released from human NK cells stimulated with the calcium ionophore A23187, suggesting that this protein, like the one produced by murine CTL lines, may be similarly secreted during cell-mediated killing. Its identification in primary human NK cell cultures indicates that this protein may play an active role in NK cell–mediated killing.

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Note added in proof: Zalman et al. (66) reported similar findings using unseparated LGL populations stimulated with PHA and maintained in culture for 10 d with IL-2.

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