A C9 related channel forming protein in the cytoplasmic granules of human large granular lymphocytes

Leora S. ZALMAN, Mary A. BROTHERS and Hans J. MÜLLER-EBERHARD

Division of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037, U.S.A.

(Received 28 October 1985)

Large granular lymphocytes (LGL) from human blood maintained in culture for 2 to 6 weeks with IL-2 were found positive in the K562 cell killing assay. The cytoplasmic granules of the LGL were isolated, lysed and the soluble proteins were passed over a Sepharose-anti-C9 column. The retained protein was eluted with NaCl and found to consist by SDS polyacrylamide gel electrophoresis of essentially one component with a molecular weight of approximately 70,000. The protein did not give a positive precipitation test with anti-human C9 by Ouchterlony analysis, but it reacted reproducibly with anti-human C9 by Western blot analysis. By ELISA the cross reaction with human C9 was less than 1%. The C9 related lymphocyte protein lacked C9 hemolytic activity, but it formed functional pores in liposomes in presence of Ca++. These results suggest that the cytoplasmic granules of human LGL that are capable of killing NK target cells contain C9 related protein which is involved in the cellular cytotoxicity reaction.

Killing lymphocytes are capable of elaborating a tubular structure for insertion into target membranes that morphologically resembles the membrane attack complex (MAC) of complement (1). This tubular structure is currently regarded as the principal candidate for the cytolytic mechanism of cytotoxic T lymphocytes (CTL), natural killer (NK) cells and killer (K) cells (2) and has been observed in cytolytic reactions mediated by human (1), rat (3) and mouse (4) lymphocytes. The precursor protein of the cylindrical structure is contained in the cytoplasmic granules of killing lymphocytes (5–7) which have been isolated and found, in the presence of calcium ions, to lyse sheep erythrocytes or certain nucleated cells (5, 7) and to cause marker release from lipid vesicles (3). A soluble protein (mol.wt. approximately 66,000) has recently been isolated from cytoplasmic granules of the mouse CTL line B6.1 that was reported to have the potential of forming tubular structures on lipid vesicles (8).

To explore the possible relationship between the MAC of complement (9) and the cytolytic apparatus of killing lymphocytes, we have employed...
large granular lymphocytes (LGL) from human blood which have antibody
dependent cellular cytotoxicity (ADCC) and NK activity (2,10). The
purified cells were maintained in culture for several weeks with regular
addition of interleukin-2 (IL-2) before the cytoplasmic granules were
isolated and the granular proteins harvested. We wish to report the identification of a pore-forming protein that is immunochemically related to the

Materials and Methods

Preparation and culturing of human LGL. Blood from normal human
donors was obtained from the General Clinical Research Center of Scripps
Clinic and Research Foundation and collected in 10 U/ml of heparin. The
peripheral blood lymphocytes were isolated by Ficoll-Isopaque gradient
centrifugation (11) using lymphocyte separation medium (Litton Bionetics,
Kensington, MD). The monocytes were removed by adherence to plastic
flasks (60 min, 37°C). A discontinuous Percoll gradient (Pharmacia Fine
Chemicals, Piscataway, NJ) was used to separate the LGL of relatively low
density from the higher density cells (12). The LGL were further purified by
depletion of E-rosette forming T cells (13). The non-rosette forming LGL
were collected from the interface of a Ficoll-Isopaque gradient. The cells
were cultured in RPMI-1640 containing 2mM L-glutamine, 1 mM sodium
pyruvate, 0.1 mM non-essential amino acid mixture, antibiotics (M.A. Bio-
products, Walkersville, MD) and 10% fetal calf serum (Hyclone, Logan, UT).
Cell smears were prepared by centrifuging 2 × 10⁵ cells in a cytocentrifuge
for 7 min, fixing in methanol and staining with Giemsa. The LGL culture
was stimulated with 0.2% (v/v) phytohemagglutinin (PHA) (M form, GIBCO,
Chagrin Falls, OH) to initiate cell proliferation. After three days, human
IL-2 (delectinated) was added at 10% (v/v) (Cellular Products Inc., Buffalo,
NY).

Monoclonal antibodies to lymphocyte antigens. The surface phenotypes
of the LGL culture were determined using monoclonal antibodies from
Becton Dickinson (Mountain View, CA). The fluorescein isothiocyanate or
phycoerythrin conjugated monoclonal antibodies Leu-3a, Leu-4, Leu-7 and
Leu-11a were used.

Anti-human C9. Two antisera were employed, both produced in this
laboratory with isolated human C9 (14). G-318 is a low titer, monospecific
goat antiserum and R-7176 is a high titer rabbit antiserum that recognizes
monomeric C9 and the neoantigen specific for poly C9 (15).

Cell killing assay. Cytotoxic activity of LGL was determined by an 18 h
⁵¹Cr release assay using human K562 cells as targets (16).

ELISA. A standard enzyme linked immunosorbent assay (ELISA) was
used to determine the antigenicity of the C9 related protein. A microtiter
plate was coated with dilutions of C9 related protein (8 ng to 1 μg). Rabbit
antiserum to human C9 was used at a dilution to 1:1000. Peroxidase conjugated
goat anti-rabbit IgG antibody (Tago Inc., Burlingame, CA) was used at a
1:1000 dilution. The enzyme substrate was ABTS (2,2'-azino-di-[3-
ethylbenzthiazoline 6-sulfonic acid]) (Boehringer Mannheim, Indianapolis,