Brief Definitive Report

THE PORE-FORMING PROTEIN (PERFORIN) OF CYTOLYTIC T LYMPHOCYTES IS IMMUNOLOGICALLY RELATED TO THE COMPONENTS OF MEMBRANE ATTACK COMPLEX OF COMPLEMENT THROUGH CYSTEINE-RICH DOMAINS

BY JOHN DING-E YOUNG, CHAU-CHING LIU, LAUREN G. LEONG, AND ZANVIL A. COHN

From The Rockefeller University, New York 10021

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022

Recent observations (1–3) made in several laboratories have implied structural and functional analogies between the cytolysis produced by complement and by lymphocytes. Cell damage produced in both instances appears to involve functional pore formation on the surface of targets. The membrane damage mediated by C involves the macromolecular assembly of the membrane attack complex (MAC), with association of the five terminal C components, namely C5b, C6, C7, C8, and C9 (4–6). C9 is the protein thought to be largely responsible for the formation of the ring-like lesions previously associated with the MAC (7). In the case of CTL and NK cells, a granule pore-forming protein (PFP, perforin or cytolysin) has been implicated in lymphocyte-mediated cell killing (1–3). The immunological similarity between C9 and PFP was recently assessed using specific antibodies (8–10). Human C9 is immunologically related to PFP isolated from mouse CTL (8), human peripheral blood large granular lymphocytes (9), and NK cells (10). We report here on immunological similarities between mouse lymphocyte PFP and other C components of MAC.

Materials and Methods

Purified C Components. C5b-6 (11), C7 (12), C8 (13), and C9 (14) were purified from outdated human serum (N.Y. Blood Center) according to published procedures. The homogeneity of purified C components was ascertained by SDS-gel electrophoresis (Fig. 1). In some experiments, commercially available C components (Calbiochem-Behring Corp., La Jolla, CA) were also used. The gel profile of the commercially obtained C components was comparable to that shown in Fig. 1.

Purified Lymphocyte PFP/Perforin. The mouse cytotoxic T cell line CTLL-R8 was maintained as previously described (15). Isolation of granules and PFP/perforin followed published procedure given elsewhere (16).

Polyclonal Antisera. Antisera against C5b-6, C7, C8, C9, and PFP/perforin were generated by multiple injections of antigens (10–30 μg, boiled in 1% SDS) into rabbits following the scheme outlined elsewhere (8, 17). Reduced and alkylated antigens were prepared as described (8).

Supported in part by grants from the Cancer Research Institute and the Lucille P. Markey Charitable Trust to J. D.-E Young, and by U.S. Public Health Service grants CA-30198 and AI-07012 to Z. A. Cohn. J. D.-E Young is a Lucille P. Markey Scholar.

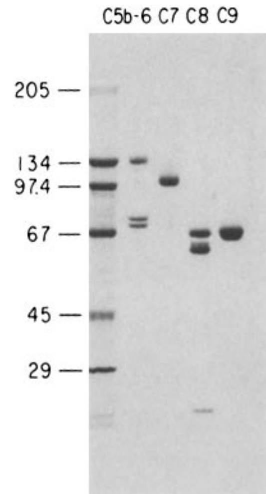

FIGURE 1. SDS-gel profile or purified components of human C. 10 μg of protein was applied per lane.

Gel Electrophoresis and Immunoblotting. Protein samples in 1% SDS were reduced and alkylated with 50 mM DTT and 100 mM iodoacetamide (8). After boiling for 5 min, the samples were applied to 20-cm gradient-gel slabs of 4–11% polyacrylamide, which were developed according to Laemmli (18), and stained with Coomassie Brilliant Blue.

Immunoblots were developed using mini-gels (Model 360, Bio-Rad Laboratories, Richmond, CA) with gradient polyacrylamide of 4–20%, followed by electrotransfer of proteins to nitrocellulose membranes at a constant 400 mA for 2.5 h (19). Membranes were blocked with 1% milk, 2% glycine in Tris-buffered saline for 5 h at 37°C. Antisera were used at a dilution of 1:50, and goat ^{125}I-F(ab′)$_2$ anti–rabbit IgG (5.6 μCi/μg; New England Nuclear, Boston, MA) was used as the secondary label at 0.12 μCi/ml. Membranes were washed with 20 mM Tris-buffer, pH 7.4, containing 0.4 M NaCl, 0.25% Tween-20 (Bio-Rad Laboratories), 0.25% NP-40 (Calbiochem-Behring Corp.), and 2 mM EDTA. Autoradiography was performed for 8–16 h with intensifying screens. The M_r standards used for regular gels were obtained from Sigma Chemical Co. (St. Louis, MO) and for the blots consisted of prestained protein markers obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Results

Immunological Crossreactivity between PFP/Perforin and C Components. The immunological similarities between lymphocyte PFP/perforin and various components of human C MAC were assessed by immunoblot, using polyclonal antibodies specific for each component. Purified mouse lymphocyte PFP/perforin reacted on immunoblots with each of the antibodies raised against C5b-6, C7, C8, or C9 (Fig. 2*A*). We observed crossreactivity only when PFP/perforin was reduced and when anti-C antibodies were raised against reduced and alkylated antigens. Human C components that were boiled in 1% SDS but that were not reduced did not elicit crossreactive antibodies when used as immunogens (Fig. 2*A*).

Immunological Crossreactivity between the Different C Components. As expected, antisera raised against C5b-6, C7, and C8 also reacted with C9 (Fig. 2*B*). Conversely, anti-C9 antibodies reacted with C5b-6, C7, and C8α (Fig. 3). In

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022

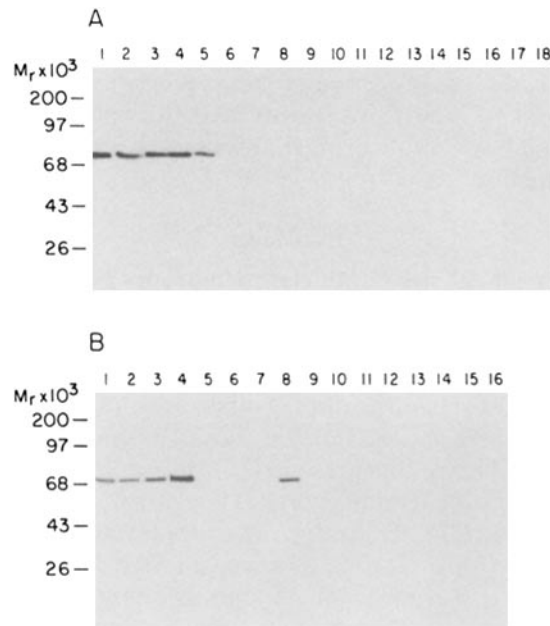

FIGURE 2. Immunoblot of mouse PFP/perforin and C9 using anti-C and anti-PFP antibodies. Reduced and alkylated forms of purified PFP/perforin (*A*) and C9 (*B*) were applied at 0.1 μg per lane. Immunoblots were developed with antisera specific for the nonreduced form of PFP (*A*, lane *1*), and the reduced forms of C5b-6 (*A, 2* and *B, 1*), C7 (*A, 3* and *B, 2*), C8 (*A, 4* and *B, 3*), C9 (*A, 5* and *B, 4*). (*A*) lanes *6–9* and (*B*) lanes *5–8* were reacted with antisera specific for nonreduced forms of C5b-6 (*A, 6* and *B, 5*), C7 (*A, 7* and *B, 6*), C8 (*A, 8* and *B, 7*), and C5b-6 (*A, 9* and *B, 8*). (*A*) lanes *10–18* (*B*) lanes *9–16* correspond to immunoblots developed under similar conditions with the respective preimmune sera in the sequence mentioned above. Autoradiography was performed for 16 h.

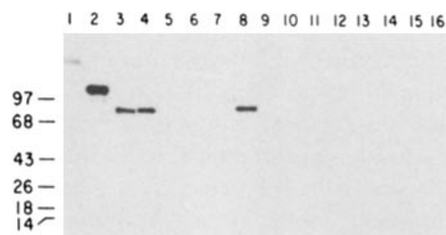

FIGURE 3. Immunological crossreactivities between human C components of MAC. C5b-6 (lanes 1, 5, 9, and 13), C7 (lanes *2, 6, 10,* and *14*), and C8 (lanes *3, 7, 11,* and *15*) were applied at 2 μg per lane. 0.5 μg of C9 was applied to lanes *4, 8, 12,* and *16*. Lanes *1–4* were developed with antisera specific for reduced and alkylated C9. Lanes *5–8* were reacted with antisera raised against the nonreduced form of C9 boiled in 1% SDS. Lanes *9–16* correspond to reaction with respective preimmune antisera in the sequence mentioned above. The x-ray film was exposed for 16 h. Lanes *1* and *9* were exposed for 48 h.

experiments using up to 2 μg of C5b-6 per lane and exposure of the autoradiogram for 48 h, only C6 reacted with anti-C9 antibodies (Fig. 3). However, on longer exposures (7 d), anti-C9 antibodies also reacted weakly with C5b (75-kD) (not shown). Immunoblots developed under similar conditions did not react with the preimmune serum. The crossreactivity observed here between C com-

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022

ponents was also only observed when the reactive antigens and the immunogens used to elicit antibodies were reduced and alkylated (Figs. 2*B* and 3). These results indicate that the crossreactivity observed between the different components of C MAC and PFP/perforin is restricted to cysteine-rich domains which are normally masked by disulfide bridges and become antigenically exposed only upon chemical reduction.

Discussion

Antibodies prepared against target cell membranes damaged by lymphocytes have previously been shown (20, 21) to crossreact with C proteins. However, the identity of the crossreactive proteins and the extent of the immunological crossreactivity were not determined in those studies. Recent results (8–10) have shown that C9 and PFP/perforin display structural, immunological, and functional similarities. Both species have been shown to undergo polymerization to form circular lesions. The results presented in this report extend these observations by showing that the lymphocyte PFP/perforin is also immunologically related to C5b-6, C7, and C8. The antigenic epitope(s) shared by mouse lymphocyte PFP and human MAC is restricted to cysteine-rich domain(s). These regions are thought to be exposed after major structural rearrangement of the proteins, which presumably occurs after membrane insertion and polymerization. It may be noteworthy to point out that the primary sequence of human C9, recently deduced by molecular cloning (22, 23), contains cysteine-rich domains that show extensive homologies to the low-density lipoprotein (LDL) receptor. It is possible that the same cysteine-rich region(s) are present in other components of MAC and in lymphocyte PFP/perforin. The function of these highly conserved domains remains unclear. In the case of LDL receptor they are thought to be involved in ligand binding (24). A similarly important function might be played by homologous domains of C components and lymphocyte PFP in the attachment of these proteins to lipid membranes and/or in their subsequent pore formation.

The results presented here further support and extend the notion of "complement supergenes" previously proposed for C6 and C7 (25, 26). On the basis of structural and functional similarities, a genetic relationship has also been proposed for several other complement proteins, including Clr and Cls; C3, C4, and C5; C2 and factor B (reviewed in reference 27). The observed immunological similarities between lymphocyte PFP/perforin and several C components provide a structural basis for the analogies observed between C and lymphocyte-mediated cell killing.

While this manuscript was in preparation, Tschopp et al. reported (28) that antibodies raised against a synthetic peptide prepared to mimic a region of homology between C9 and LDL receptor also react against other C components and lymphocyte PFP/perforin.

Summary

Structural, functional and immunological similarities between the ninth component of complement (C9) and the lymphocyte pore-forming protein (PFP, perforin) have recently been described (8–10). PFP is shown here to be immunologically related to all other components of the membrane attack complex

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022

(MAC) of human complement, namely, C5b-6, C7, C8, and C9. Polyclonal antibodies raised against purified human C5b-6, C7, C8, or C9 react with other components of the MAC and with mouse lymphocyte PFP. The antigenic epitopes shared by human complement proteins and mouse lymphocyte PFP are limited to cysteine-rich domains. Only complement proteins that have been reduced and alkylated elicit the production of crossreactive antibodies when used as immunogens. The nonreduced forms of complement components or lymphocyte PFP neither react with these antibodies nor give rise to crossreactive antibodies. The homologous domains of complement proteins and lymphocyte PFP may play related functions in their attachment to lipid membranes and assembly of membrane lesions.

We thank the excellent technical assistance provided by S. S. Ko, M. A. DiNome, and A. Damiano during different parts of this research. We also acknowledge initial collaborative studies done with Drs. E. R. Podack, C. F. Nathan, and M. A. Palladino.

Received for publication 30 September 1986.

References

1. Henkart, P. A. 1985. Mechanisms of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 3:31.
2. Podack, E. R. 1985. The molecular mechanism of lymphocyte-mediated tumor cell lysis. *Immunol. Today.* 6:21.
3. Young, J. D.-E., and Z. A. Cohn. 1986. Role of granule proteins in lymphocyte-mediated killing. *J. Cell. Biochem.* 32:151.
4. Muller-Eberhard, H. J. 1975. Complement. *Annu. Rev. Biochem.* 44:697.
5. Bhahdi, S., and J. Tranum-Jensen. 1983. Membrane damage by complement. *Biochim. Biophys. Acta.* 737:343.
6. Mayer, M. M., D. W. Michaels, L. E. Ramm, M. B. Whitlow, J. B. Willoughby, and M. L. Shin. 1981. Membrane damage by complement. *CRC Crit. Rev. Immunol.* 2:133.
7. Podack, E. R., and J. Tschopp. 1982. Polymerization of the ninth component of complement (C9): formation of poly(C9) with a tubular ultrastructure resembling the membrane attack complex of complement. *Proc. Natl. Acad. Sci. USA.* 79:574.
8. Young, J. D.-E., Z. A. Cohn, and E. R. Podack. 1986. The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities. *Science (Wash.).* 233:184.
9. Zalman, L. S., M. A. Brothers, F. J. Chiu, and H. J. Muller-Eberhard. 1986. Mechanism of cytotoxicity of human large granular lymphocytes: relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement. *Proc. Natl. Acad. Sci. USA.* 83:5262.
10. Liu, C.-C., B. Perussia, Z. A. Cohn, and J. D.-E Young. 1986. Identification of a pore-forming protein of human peripheral blood NK cells. *J. Exp. Med.* 164:2061.
11. Podack, E. R., and H. J. Muller-Eberhard. 1980. Limited proteolysis of C5b-6: functional stability of the degraded complex. *J. Immunol.* 124:332.
12. Podack, E. R., W. P. Kolb, and H. J. Muller-Eberhard. 1976. Purification of the sixth and seventh component of human complement without loss of hemolytic activity. *J. Immunol.* 116:263.
13. Kolb, W. P., and H. J. Muller-Eberhard. 1976. The membrane attack mechanism of complement: the three polypeptide chain structure of the eighth component (C8). *J. Exp. Med.* 143:1131.

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022

14. Biesecker, G., and H. J. Muller-Eberhard. 1980. The ninth component of human complement: purification and physicochemical characterization. *J. Immunol.* 124:1291.
15. Young, J. D.-E., C. F. Nathan, E. R. Podack, M. A. Palladino, and Z. A. Cohn. 1986. Functional channel formation associated with cytotoxic T cell granules. *Proc. Natl. Acad. Sci. USA.* 82:8629.
16. Young, J. D.-E, E. R. Podack, and Z. A. Cohn. 1986. Properties of a purified pore-forming protein (perforin 1) isolated from H-2-restricted cytotoxic T cell granules. *J. Exp. Med.* 164:144.
17. Young, J. D.-E, L. G. Leong, C.-C. Liu, and Z. A. Cohn. 1986. Extracellular release of lymphocyte pore-forming protein (perforin) after ionophore stimulation. *Proc. Natl. Acad. Sci. USA.* 83:5668.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
19. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
20. Sundsmo, J. S., and H. J. Muller-Eberhard. 1979. Neoantigen of the complement membrane attack complex on cytotoxic human peripheral blood lymphocytes. *J. Immunol.* 122:2371.
21. Ward, R. H. R., and P. J. Lachmann. 1985. Monoclonal antibodies which react with lymphocyte-lysed target cells and which cross-react with complement-lysed ghosts. *Immunology.* 56:179.
22. DiScipio, R. G., M. R. Gehring, E. R. Podack, C. C. Kan, T. E. Hugli, and G. H. Fey. 1984. Nucleotide sequence of cDNA and derived amino acid sequence of human complement component C9. *Proc. Natl. Acad. Sci. USA.* 81:7298.
23. Stanley, K. K., H. P. Kocher, J. P. Luzio, P. Jackson, and J. Tschopp. 1985. The sequence and topology of human complement component C9. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:375.
24. Yamamoto, T., C. G. Davis, M. S. Brown, W. J. Schneider, M. L. Casey, J. L. Goldstein, and D. W. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell.* 39:27.
25. Lachman, P. J., and M. J. Hobart. 1978. C6-C7: a further "complement supergene." *J. Immunol.* 120:1781.
26. Podack, E. R., W. P. Kolb, A. F. Esser, and H. J. Muller-Eberhard. 1979. Structural similarities between C6 and C7 of human complement. *J. Immunol.* 123:1071.
27. Campbell, R. D., M. C. Carroll, and R. R. Porter. 1986. The molecular genetics of components of complement. *Adv. Immunol.* 38:203.
28. Tschopp, J., D. Masson, and K. K. Stanley. 1986. Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. *Nature (Lond.).* 322:831.

Downloaded from http://rupress.org/jem/article-pdf/164/6/2077/1096552/2077.pdf by guest on 24 September 2022