0022-1767/89/14211-3913$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 142, 3913–3916, No. 11, June 1, 1989
Printed in U.S.A.

DISTINCT RESTRICTION OF COMPLEMENT- AND CELL-MEDIATED LYSIS[1]

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Complement- and cell-mediated killing utilize related effector proteins (C8/C9 and perforin, respectively), suggesting that proteins which protect cells against complement- and cell-mediated attack may also be similar. In homologous complement-mediated killing two protective proteins, which are anchored to the cell membrane by phosphatidylinositol glycan (PIG) tails, are known. To study whether similar PIG-tailed proteins protect against lymphocyte-mediated killing, nucleated cell lines with a mutation in the biosynthesis of the PIG anchor were used. It was found that PIG-tailed membrane proteins restrict homologous complement-mediated lysis but not three different types of cell-mediated killing or lysis by purified perforin. Furthermore, E from patients with an acquired defect in PIG tail biosynthesis did not differ from normal E in sensitivity to antibody-dependent cell-mediated cytotoxicity, in spite of their increased sensitivity to human C8 and C9.

The granule pore-forming protein perforin (cytolysin), which shares transmembrane channel-forming properties with the C component C9, has been implicated in lymphocyte-mediated cell killing (1, 2). Although little is known about restriction factors in cell-mediated killing, which limit damage to the target or killer cell, homologous complement-mediated killing is regulated by DAF[3] which restricts assembly of the C3/C5 convertases (3, 4), and HRF (C8/C9 binding protein) which inhibits transmembrane channel formation by C5b-9 (5–7). DAF and HRF are anchored to the cell membrane by PIG tails (8, 9). The homology between the lytic effector proteins of C (C9) and lymphocytes (perforin) suggested that proteins which protect cells against complement- and cell-mediated lysis may also be related, an idea which has recently received some experimental support (10). Evidence that HRF restricts both complement- and cell-mediated killing (10) came from studies on E from patients with PNH, an acquired defect resulting in selective deficiency in PIG-tailed proteins and susceptibility to lysis by homologous C (8, 9, 11–13).

To study the role of PIG-tailed proteins in protection of nucleated cells, the physiologically relevant targets of cell-mediating killing, we selected mutants of the human EBV-transformed JY cell line, which have a defect in the biosynthesis of the PIG anchor and do not express PIG-anchored proteins (14). These mutant cells as well as E from PNH patients were tested for susceptibility to complement-mediated and cell-mediated lysis. The results demonstrate differences in restriction of complement- and cell-mediated lysis. On both E and nucleated cells, PIG-anchored proteins restrict homologous complement-mediated lysis but not cell-mediated lysis.

MATERIALS AND METHODS

Cells. Wild-type and mutant JY cells and CTL were maintained in culture as described elsewhere (14).

Complement-mediated lysis. ^{51}Cr-labeled target cells were sensitized by incubation for 1 h at 4°C with W6/32 anti-HLA mAb and three washes. The cells were then incubated with fresh human serum for 1 h at 37°C. Supernatants were collected for radioactivity counting. For the acidified serum test ^{51}Cr-labeled cells were incubated for 1 h at 37°C with fresh human serum supplemented with 5 mM $MgCl_2$ and acidified to pH 6.6 with HCl. In some experiments cytotoxicity was quantitated by LDH release (15).

Cell-mediated lysis. CTL-mediated lysis was determined in a 4-h ^{51}Cr-release assay as described elsewhere (14). ADCC and natural killing were carried out with LGL as effector cells. LGL were isolated from human blood by Ficoll-Hypaque gradient centrifugation, nylon wool columns, and Percoll gradient centrifugation (16). They were incubated for 4 h with: 1) ^{51}Cr-labeled JY target cells either untreated (for natural killing assay) or sensitized with W6/32 anti-HLA mAb (for ADCC assay). 2) ^{51}Cr-labeled human E sensitized with the IgG fraction of rabbit anti-human E serum (Cooper Biomedical, Malvern, PA). Cytolysis was determined as described elsewhere (14).

Perforin-mediated lysis. Human perforin partially purified from IL-2-stimulated T cells was a gift of Dr. J.D.-E. Young (Rockefeller University, New York). Cytotoxicity was determined in a 3-h LDH release assay (15).

RESULTS AND DISCUSSION

The sensitivity of PIG-anchor mutant clones to the antibody-mediated classical C pathway was significantly higher than that of wild-type cells (Fig. 1*A*). In addition, when subjected to the acidified serum test, which predominantly determines sensitivity to reactive lysis (17) but also sensitivity to the alternative C pathway (18), mutant but not wild-type clones demonstrated susceptibility to homologous C (Fig. 1*B*). DAF deficiency of mutant cells has been clearly demonstrated by immunofluorescence staining (14) (*insert*, Fig. 1*B*). Hence, the abnormal sensitivity of mutant JY cells to C could be attributed, at least partially, to DAF absence. To assay for restriction

Received for publication December 21, 1988.
Accepted for publication February 24, 1989.
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[1] This work was supported by the Dorot Foundation, an American Cancer Society Faculty Award, and National Institutes of Health Grant CA-31798.
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[3] Abbreviations used in this paper: DAF, decay accelerating factor; HRF, homologous restriction factor; PIG, phosphatidylinositol glycan; PNH, paroxysmal nocturnal hemoglobinuria; LDH, lactate dehydrogenase; ADCC, antibody-dependent cell-mediated cytotoxicity; LGL, large granular lymphocytes.

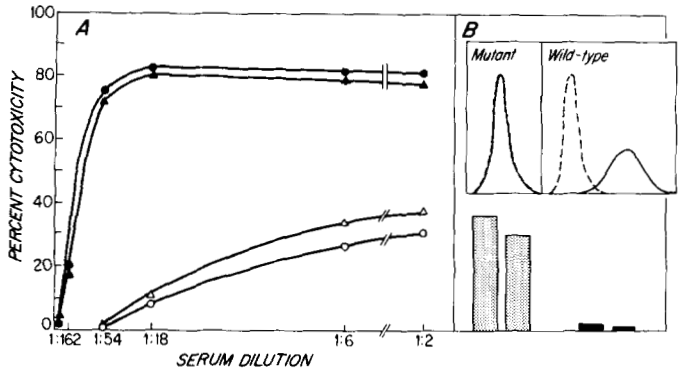

Figure 1. Mutant and wild-type JY cells differ in susceptibility to C. (*A*) Two different mutant clones (*closed triangles* and *circles*) and two different wild-type clones (*open triangles* and *circles*) were tested for susceptibility to lysis by antibodies and human C (classical pathway). (*B*) Two different mutant clones (*dotted bars*) and two different wild-type clones (*black bars*) were tested for susceptibility to lysis by acidified human serum (reactive lysis). A histogram of DAF expression in mutant and wild-type cells is inserted. The cells were stained with anti-DAF mAb (*solid line*) or with control Ig (*broken line*) and analyzed by flow cytometry. The abscissa represents fluorescence intensity and the ordinate represents cell number.

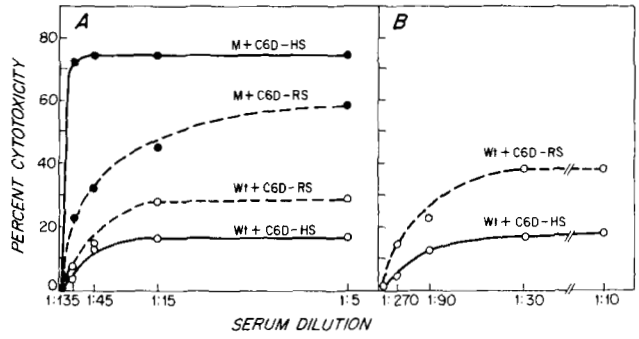

Figure 2. Lysis of C7-carrying JY cells by human or rabbit C8, C9. Wild-type (Wt) cells (*open circles*) or mutant (M) cells (*closed circles*) sensitized with anti HLA antibodies were incubated for 15 min at 30°C with C8D-HS (human serum from a patient with congenitally deficient C8 function) diluted 1/10 (in *A*), or 1:5 (in *B*). The C7-carrying cells were then washed and incubated for 60 min at 37°C with C6 deficient human serum-HS (*solid line*) or C6 deficient rabbit serum-RS (*broken line*) as a source of human or rabbit C8, C9. The cells were centrifuged and LDH release was determined. It should be emphasized that the mutant cells treated with either C6D-HS or C6H-RS (——, - - - - -) carried identical C5b-7 sites, and that wild-type cells (——, - - - - -) also carried identical C5b-7 sites.

of C8 and C9 function, cells were sensitized with antibody and C8-deficient human serum to generate C7 sites, and then treated with C6-deficient homologous (human) or heterologous (rabbit) serum as a source of C8 and C9. Mutant C7-cells were twofold more sensitive than wild-type C7-cells to rabbit C8,C9 (Fig. 2*A*). Since lysis by heterologous C8,C9 was not expected to be restricted (5) the increased lysis of mutant cells was attributed to increased C7 sites due to DAF deficiency. C7-carrying mutant cells were fourfold more sensitive than wild-type C7-cells to human C8,C9 (Fig. 2*A*). Thus, in addition to quantitative difference in C7 sites, homologous species restriction was operative at the stage of C8,C9 in wild-type but not mutant cells. Moreover, mutant cells carrying identical numbers of C7 sites revealed a degree of human C8,C9-mediated lysis, which was higher than that mediated by rabbit C8,C9, in contrast to C7-carrying wild-type cells which manifested higher sensitivity to rabbit C8,C9 than to human C8,C9 (Fig. 2*A*). These findings indicate that mutant cells lack homologous restriction at the lytic phase involving C8,C9. The relative small difference between C8,C9 restricted and nonrestricted lysis of wild-type JY cells as compared to larger differences observed for E is attributed to the ability of nucleated cells to eliminate C5b-9 channels from the cell surface (15). Repeated titrations with cells carrying more C7 sites still revealed twofold increase with rabbit C8,C9 over human C8,C9 (Fig. 2*B*), consistent with values usually observed for nucleated cell killing by multimeric versus monomeric C9 containing channels (15).

These results clearly show that PIG-anchored proteins restrict complement-mediated lysis of nucleated cells. We explored whether PIG-anchored proteins similarly restrict cell-mediated lysis. Mutant cells were used as targets for cell-mediated killing by CTL (Fig. 3, *A* and *B*), LGL-mediated ADCC (Fig. 3*C*), LGL-mediated natural killing (Fig. 3*D*), and perforin-mediated lysis (Table I). Mutant and wild-type cells were lysed to the same extent in these diverse types of killing assays using purified perforin and both CTL and LGL effectors, indicating that PIG-anchored regulatory proteins are not involved in regulation of cell-mediated damage.

Because killing of nucleated cells may be more complicated than the simple channel insertion proposed for E membranes, the possibility arose that in spite of its inactivity in nucleated cells, HRF might affect cell-mediated E killing. In support of this idea, it has been reported that HRF protects E from ADCC (10). We therefore compared the sensitivity of normal and HRF-deficient type III PNH E (9, 11–13) to homologous LGL-me-

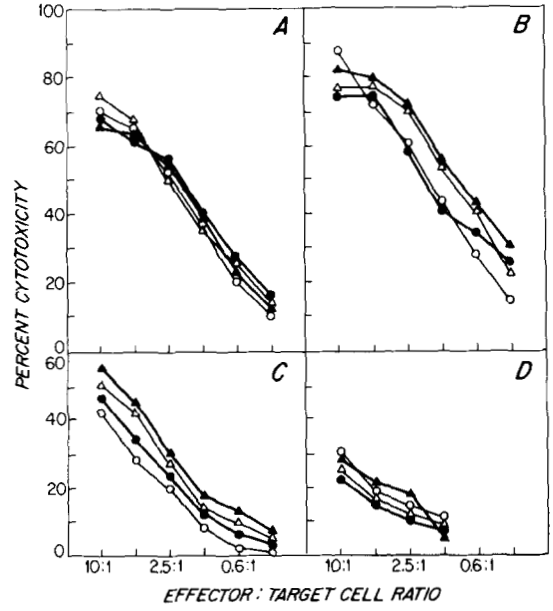

Figure 3. Mutant and wild-type JY cells do not differ in susceptibility to cell-mediated lysis. Mutant (*closed symbols*) and wild-type (*open symbols*) clones were tested for their susceptibility to HLA-DPw2-specific human CTL (in *A*), to HLA-A2-specific human CTL (in *B*), to ADCC by human LGL (in C), and to natural killing by human LGL (in *D*).

TABLE I

Perforin-mediated lysis of mutant and wild-type JY cells

Perforin Concentration (μg protein/ml)	Percent Cytotoxicity[a]	
	Mutant target cells	Wild-type target cells
50	50 ± 3	50 ± 8
25	16 ± 5	31 ± 3
8	6 ± 2	12 ± 3

[a] Mean ± SD of triplicate samples.

diated ADCC. Type III PNH E were isolated by virtue of their deficiency of two other PIG-anchored proteins, acetylcholinesterase or DAF, and used as targets in ADCC (Fig. 4). For Figure 4*A*, cells were passed through an acetylcholinesterase immunoadsorbent column (19). Normal cells expressing acetylcholinesterase are retained on the immunoadsorbent column, whereas abnormal E are eluted. For Figure 4*B*, DAF-negative cells were sorted by flow cytometry after fluorescein labeling of PNH E with anti-DAF antibodies. Whichever method was used (Fig. 4, *A* and *B*), normal and HRF-deficient E were equally susceptible to LGL-mediated ADCC in all experiments, in distinction to the results of Zalman et al. (10). The contradiction may result from differences in the source of LGL and assay conditions used in the two studies: we used fresh LGL in multiple effector cell concentrations, the highest concentration being 5×10^6/ml, as opposed to Zalman et al. who used cultured LGL with a single, extremely high effector cell concentration (4×10^8/ml); packed cultured cells are approximately 10^9/ml.

The present data show that, in contrast to C regulation, PIG-anchored proteins do not restrict cell-mediated lysis of either E or nucleated cells. Two recent papers have similarly indicated the absence of homologous restriction in perforin-mediated lysis of RBC (20, 21) or nucleated cells (21). These two papers demonstrated that perforin, unlike C, lysed target cells across a variety of species, including the homologous one. We reached the same conclusion by using a different approach and methodology. Distinct regulatory pathways for complement-mediated and cell-mediated cytolysis thus exist. This appears logical in view of the different functions of complement- and cell-mediated lysis. The C system is critical for elimination of microorganisms. However, accidental attack of host cells by autologous C is undesirable and is prevented by C regulatory proteins present on the surface of host cells. Recognition is species-specific (homologous restriction). In contrast, cell-mediated cytotoxicity is critical for elimination of modified host cells. Resistance to autologous cell-mediated attack may therefore be disadvantageous. In this context it is intriguing that CTL clones unlike other cells are resistant to CTL-mediated lysis (22, 23). This resistance may be conferred by a restriction protein exclusively expressed on CTL. It has been reported that HRF protects activated T lymphocytes from CTL-derived perforin, suggesting that HRF spares CTL from their own lytic effect (24). However, since recent experiments have challanged the importance of lytic granules and perforin in CTL activity (25), resistance to perforin and to CTL-mediated killing may be unrelated phenomena. Lysis by LGL, though, appears to be mediated by perforin-granule constituents (26). We found no role for HRF or other PIG-anchored proteins in homologous killing mediated either by CTL, LGL, or perforin.

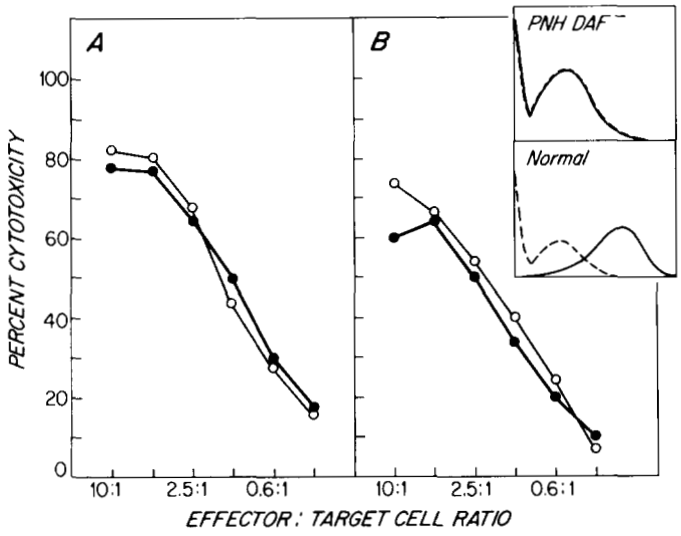

Figure 4. Abnormal type III PNH E and normal type I human E do not differ in susceptibility to ADCC. *Closed circles*: abnormal E. *Open circles*: normal E. Abnormal E were purified from type III PNH blood by an immunoadsorbent column of acetylcholinesterase-antibodies (in *A*) or by staining with DAF-antibodies and separating DAF-negative cells by flow cytometry (in *B*). A histogram of DAF expression in normal and abnormal sorted E is inserted (for details, see Fig. 1).

Acknowledgments. We wish to thank Dr. S. Burakoff and Dr. S. Shaw for the CTL lines, Dr. V. Nussenzweig for the DAF-antibodies, and Dr. J.D.-E. Young for the purified perforin.

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