CD59, AN LY-6-LIKE PROTEIN EXPRESSED IN HUMAN LYMPHOID CELLS, REGULATES THE ACTION OF THE COMPLEMENT MEMBRANE ATTACK COMPLEX ON HOMOLOGOUS CELLS

By ALEXANDRA DAVIES,* DAVID L. SIMMONS,‡ GEOFF HALE,§ RICHARD A. HARRISON,* HELEN TIGHE,§ PETER J. LACHMANN,* AND HERMAN WALDMANN§

*From the *MRC Molecular Immunopathology Unit, MRC Centre, Cambridge CB2 2QH; the ‡ICRF, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU; and the §Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom*

The lysis of cells by complement requires only the terminal components C5, C6, C7, C8 and C9 and is initiated by the cleavage of C5 to C5b. Sequential addition of C6, C7, C8, and C9 to C5b leads to the formation of the membrane attack complex (MAC)[1] which, when inserted into the lipid bilayer, can form transmembrane pores (1–5). It is well known that when complement of one species is activated on homologous erythrocytes, lysis is much less efficient than when it is activated on other species of cell, and even among different heterologous cell species the lytic efficiency may be very different. It has long been known that the basis of this variable lytic efficiency is found, at least in part, at the C8 and/or C9 step (6–9). More recently, specific membrane proteins have been described that appear to protect cells from homologous complement. The first of these to be described was the decay-accelerating factor (DAF), a membrane protein of ~70 kD molecular mass (10). This protein interferes with the assembly of the C3 converting enzymes both of the classical and alternative pathway (10, 11) and therefore it has only indirect effects on the cell lytic mechanism. A further membrane protein that does restrict homologous lysis, and that has been described both as the C8-binding protein (C8bp) (7, 12) and as homologous restriction factor (HRF) (13), has also been isolated. It seems likely that both these descriptions apply to a single protein of 65 kD molecular mass. In addition, a 55/65-kD MAC-inhibiting protein (MIP) with the capacity to bind C8 and C9 has been identified both on human erythrocyte membranes and in normal human serum (14). The relationship of this to HRF/C8bp is not yet clear. Both DAF and HRF/C8bp are bound on cell membranes by a glycolipid anchor (15, 16) and can be eluted from the cell membrane, at least in part, by phosphatidylinositol-specific phospholipase C. These proteins also have the capacity when they are isolated from

A. Davies was supported by an MRC research studentship during the course of this study. Address correspondence to H. Waldmann, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom.

membranes to be reinserted into lipid bilayers, and when HRF/C8bp (12, 17) is inserted in this way into sheep erythrocytes, this limits their lysis by human complement.

A mAb (YTH 53.1), raised against a lymphocyte surface antigen, has been demonstrated to potentiate lysis by homologous complement in a manner analogous to that of antibodies against HRF/C8bp. At the 4th Leucocyte Workshop this antibody, together with antibody MEM-43 (18), was placed in a cluster designated CD59 (19). Here we describe the isolation both from urine and from red cell membranes of the antigen recognized by YTH 53.1. It is distinct from HRF/C8bp in having a much smaller molecular mass (~20 kD), but appears to restrict homologous lysis in an analogous way. The cDNA encoding this protein has been isolated using transient expression in COS cells (20); the deduced amino acid sequence is in full agreement with the NH_2-terminal sequence of the purified protein and shows homology to murine LY-6 antigens. The functional data presented herein indicate that CD59 antigen is a novel lysis-restricting protein.

Materials and Methods

Antibodies and Reagents. The mAbs YTH 53.1 (rat IgG2b), YTH 89.1 (rat IgG2b anti-red cell glycophorin A), YTH 66.9 (rat IgM; CAMPATH-1), and YTH 3.2 (rat IgG2b CD7; CAMPATH-2) were obtained from the fusion of a DA rat spleen with the myeloma line Y3/Ag1.2.3 (21). The anti-blood group mAbs NB1/19.112.28 (mouse IgM anti-B) and A15/3D3.92.1 (mouse anti-A) were the kind gift of Dr. E. Lennox, Celltech, Slough, U.K. (22, 23). Polyclonal antiserum against MAC-inhibiting protein was the gift of M. J. Watts/ Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, UK. Phosphatidylinositol-specific phospholipase C was the gift of Dr. S. Udenfriend, Roche Institute, (Nutley, NJ). The mAb E11 against CR1 was the gift of Dr. N. Hogg (Imperial Cancer Research Fund, Lincoln's Inn Fields, London).

Isolation of IgG from Serum and Ascites. IgG was prepared from serum or ascites by a method based upon that of Steinbuch and Audran (24). Firstly, the majority of unwanted plasma proteins were removed from the sample by precipitation with caprylic acid at a final concentration of 5%. The pH of the sample was first adjusted to 5.0 with 3 M acetic acid, and the caprylic acid was added dropwise during rapid stirring of the sample. After stirring for 30 min at room temperature, the protein precipitate was removed by centrifugation and IgG was purified from the resulting supernatant by precipitation with 40% ammonium sulphate. The precipitated IgG was dissolved in the minimum volume of distilled water and dialyzed against PBS. IgG preparations were checked for purity by immunoelectrophoresis.

Preparation of $F(ab')_2$ Fragments. A crude Ig fraction was prepared from ascitic fluid of YTH 53.1 by precipitation with 50% saturated ammonium sulphate. It was then dialyzed against 0.1 M sodium phosphate, pH 7.8, and adjusted to a protein concentration of 10 mg/ml. To 1 ml of this solution 0.3 mg of *Staphylococcus aureus* V8 protease was added, the mixture was incubated at 37°C for 4 h (25), then 1 ml of saturated ammonium sulphate was added to precipitate the $F(ab')_2$ fragments from the digest. The precipitate was redissolved in 0.2 ml of PBS and gel filtered on a column of Sepharose CL-6B (68 × 0.65 cm) equilibrated with PBS containing 0.02% sodium azide. Fractions were analyzed by SDS-PAGE and those containing pure $F(ab')_2$ fragments were pooled. No trace of intact heavy chain could be detected in the digest or the purified fractions.

Immunofluorescence and Flow Cytometry. Cells were incubated with YTH 53.1 followed by fluorescein-labeled goat anti-rat Ig as described previously (21). The cells were washed and resuspended in PBS containing 1% BSA and fixed by the addition of formaldehyde to a final concentration of 1%. The fluorescent cells were later analyzed using a Cytofluorograf model 50-H (Ortho Diagnostic Systems Inc., Westwood, MA) as described (21).

Complement-mediated Cell Lysis. Cells were labeled with ^{51}Cr and washed with culture medium (Iscove's modified Dulbecco's medium buffered with Hepes and containing 1% BSA

as described before (21). Aliquots (50 μl) of the cell suspension were added to microtiter plates containing 50 μl of antibody diluted in medium. After incubation for 10 min at room temperature, 100 μl of complement (autologous or AB serum diluted in medium) was added. When two antibodies were used (50 μl each) then 50 μl of double-strength complement was used so that the final volume was the same. The mixture was incubated for 45 min at 37°C, centrifuged at 100 *g* for 2 min and 100 μl of the supernatants was collected for measurement of released radioactivity. Specific release was calculated in the usual way as a fraction of the difference between a positive control (resuspended cells) and a negative control (no antibody).

Reactive Lysis. Reactive lysis of human or guinea pig erythrocytes was carried out in agarose plates as described by Harrison and Lachmann (26). Erythrocytes were washed three times in PBS and resuspended to a final concentration of 0.5% in agarose made to 1% in PBS/10 mM EDTA. Activated $\overline{C56}$ was prepared from acute-phase serum as described by the above (26) and normal human serum or rabbit serum were used as a source of C7-9. Reagents were added to 3-mm diameter wells cut in the agarose ~3 mm apart, and the plates were incubated at room temperature until lines of lysis developed between the wells (time specified in the text).

Affinity Purification of CD59 Antigen from Human Erythrocyte Membranes. Four units of blood (group A) were obtained from the Regional Blood Transfusion Centre, Cambridge. Approximately 1 liter of packed red cells that had been washed six times in PBS/10 mM azide were lysed in 10 liters of 5 mM sodium phosphate/1 mM EDTA/5 mM benzamidine/5 mM iodoacetamide, pH 7.8, by gentle stirring at 4°C. The erythrocyte ghosts were washed extensively in lysis buffer using a Pellicon cassette system (cassette PTGC 000 05; mol wt cutoff 1,000,000; Millipore [UK] Ltd., Watford, U.K.) and finally solubilized in lysis buffer containing 150 mM NaCl and 1% NP40. Insoluble material was removed by centrifugation and the supernatant was dialyzed against lysis buffer containing 150 mM NaCl and 0.2% NP-40. An affinity column was prepared by coupling 20 mg of YTH 53.1 IgG to 4 ml of CNBr-activated Sepharose CL-4B. To remove material that might adhere nonspecifically to the column of antibody-coupled Sepharose, two precolumns bearing nonimmune mouse IgG and E11 monoclonal IgG against CR1 were prepared. All columns were washed in PBS/10 mM azide and elution buffer before equilibration in lysis buffer containing 150 mM NaCl and 0.2% NP-40. The solubilized ghost extract was passed over the two precolumns, then once over the YTH 53.1/Sepharose column before being allowed to recirculate overnight. After washing in PBS/10 mM azide/0.2% NP-40 and 1 M NaCl/0.2% NP-40, the column was eluted with 0.1 M acetic acid, pH 3.0/0.2% NP-40. 0.5-ml fractions were collected into tubes containing 50 μl *N*-ethylmorpholine. The final yield of protein was ~700 μg.

Affinity Purification of CD59 Antigen from Human Urine. The same affinity column that was used to purify CD59 antigen from human erythrocyte membranes was used to extract the protein from human urine. Urine was collected from normal donors in 8-liter batches over a period 5 d, during which time it was either stored at 4°C for immediate use, or frozen at −30°C for up to 3 wk, then filtered and concentrated to 300 ml using a Pellicon cassette system (cassette PSVP 000 05, molecular weight cutoff 10,000; Millipore (UK) Ltd.). Conditions for running and eluting the column were as before. Several preparations were made from different batches of urine, and the yield ranged from 20 to 100 μg per liter.

SDS-PAGE. SDS-PAGE was performed using the buffer system of Laemmli (27). Protein samples were prepared for electrophoresis under both reducing and nonreducing conditions, and gels were stained with Coomassie Brilliant Blue or silver nitrate. Molecular weight standards were purchased from BDH, Poole, U.K.

Raising Polyclonal Antiserum. A rabbit polyclonal antiserum was raised against the urine form of CD59 antigen. For the first injection, 80 μg of protein was emulsified in CFA. A second injection of 80 μg with Pertussis was given 2 wk later, followed by booster injections containing 50 μg alum-precipitated protein.

Protein Sequence Analysis. NH_2-terminal sequence analysis using 100–300-pmol samples of protein was carried out by Dr. L. Packman at the Protein Sequencing Facility of the Department of Biochemistry, University of Cambridge, using an Applied Biosystems (Foster City, CA) gas phase sequencer. One sample of the membrane-derived protein was reduced and alkylated before sequencing.

Protein Reduction and Alkylation. 0.5 mg of membrane-derived CD59 antigen in 0.5 ml Tris-HCl, pH 8.2, was reduced and alkylated using the following procedure. Urea at a final concentration of 9.6 M and dithiothreitol at a final concentration of 10 mM were added, and the reaction mixture was flushed with nitrogen before incubation in a sealed container for 20 min at room temperature. 0.1 ml of 0.2 M iodoacetic acid adjusted to pH 8.0 was then added, the container was flushed again with nitrogen and resealed, then left again at room temperature for 1 h in the dark. The reaction was terminated by the addition of two drops of 2-ME, and the sample was dialyzed extensively against distilled deionized water.

Construction of cDNA Libraries. RNA from an HTLV I-positive adult T cell leukemia (YT) was the gift of Tadasuki Taniguchi, Osaka Medical School, Japan, to Brian Seed. cDNA libraries were made as previously described (28) by a method based upon that of Gubler and Hoffman (29). The libraries were constructed in the vector CDM8 (30) and the ligated DNA was transformed into *Escherichia coli* MC1061/p3. Libraries were constructed and screened in the laboratory of Brian Seed, Department of Molecular Medicine, Massachusetts General Hospital, Boston, MA.

Recovery and Characterization of cDNA Clones. Screening for positive clones by transient expression and rescue was carried out as described (20). After three rounds of expression and panning in COS cells, on retransformation of *E. coli*, DNA was prepared from single colonies and retransfected into COS cells. Methods for transfection, immunoprecipitation, and RNA and DNA blot hybridization were all as previously described (20), except that probes were made by random priming on purified inserts. DNA sequencing was carried out by the supercoil method (31).

Treatment of COS Cells with Phosphatidylinositol-specific Phospholipase C (PI-PLC). COS cells (10^7) transfected with a cDNA encoding CD59 antigen (YTH 53.1/1) were taken into PBS/1 mM EDTA and iodinated with ^{125}I and Enzymobeads (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. The cell suspension in isotonic sucrose was then divided into two equal aliquots of 50 μl, and incubated for 30 min at 37°C with or without 10 U of PI-PLC. (*Bacillus thuringiensis*). Cells and supernatant were separated by centrifugation, and the cells were lysed in 100 mM Tris, pH 8.0/200 mM NaCl/1 mM EDTA/1% NP-40. Each fraction was incubated with 1 μg YTH 53.1 IgG overnight at 4°C, after which time 50 μl goat anti-rat Ig coupled to agarose (Sigma Chemical Co.) was added, the samples rotated at 4°C for 2 h, then washed by spinning through 30% sucrose in lysis buffer. Antigen was eluted from the agarose by boiling the beads for 2 min in 20 μl reducing buffer, then analyzed by SDS-PAGE followed by autoradiography.

Results

Cell Distribution of YTH 53.1. The reactivity of YTH 53.1 with a variety of fresh cells and lines was measured by immunofluorescence. The antigen is broadly distributed and most target cells were positive to some degree (Table I). This experiment was part of a much larger study in which 80 other mAbs were included, reacting with a variety of defined and novel cell surface antigens (32). Cluster analysis within the panel revealed no other similar antibodies, and comparison with data from the 3rd Leucocyte Workshop (33) likewise did not reveal any obvious candidates that might identify the same target. However, YTH 53.1 was included in the nonlineage panel of the 4th Leucocyte Workshop (antibody number NO36) and binding experiments on purified antigen indicated that it is similar to MEM-43 (antibody number N705), (Hořejší, V., personal communication).

Further experiments on cell distribution were carried out by measuring cell lysis induced with YTH 53.1 and human complement (Table I). Relatively few mAbs are lytic for human cells with homologous complement, a property that is as much dependent on the target antigen as on the antibody isotype (34) and it was this property that first led us to study the antibody.

TABLE I

Cell Distribution of YTH.53.1 by Immunofluorescence and Complement-mediated Lysis

Cell type	Immunofluorescence	Complement lysis
Lymphocytes	Strong	Weak
Granulocytes	Strong	
Platelets	Weak	
Erythrocytes	Weak	Strong
Activated T cells (3 d)	Weak	
Activated T cells (7 d)	Weak	
T cell lines		
MOLT4	Strong	Weak
HPBALL	Strong	
1301		Weak
551		Weak
JURKAT		Strong
Karpas45		Strong
B cell lines		
DAUDI	Weak	Negative
CLA4		Negative
NALM-1	Negative	Negative
NALM-6		Negative
EBV lymphoblastoid	Strong	
Myeloid cell lines		
U937	Variable*	Negative
HL60	Strong	Strong
Erythroid cell line		
K562		Weak
Epithelial cell line		
ESH92	Strong	
Bladder carcinoma line		
T24		Weak

Reactivity with the cell lines is defined as negative (<5% positive cells above negative control by immunofluorescence or <5% ^{51}Cr release above control), weak (20–70% positive cells by immunofluorescence, not completely discriminated from control or 5–10% ^{51}Cr release above control), or strong (>80% positive cells, clearly discriminated from negative control or >50% ^{51}Cr release).

* Reactivity with the cell line U937 varied from strong to negative, depending upon the particular subline subjected to analysis.

Enhancement of Complement-mediated Lysis. During experiments designated to test for synergistic lysis by pairs of antibodies against different epitopes on one antigen (compare with reference 35) it was noted that YTH 53.1 gave significantly enhanced lysis when combined with several other different specificities. This phenomenon was first demonstrated with intact Ig at sublytic or weakly lytic concentrations (data not shown), but similar results could be obtained using $F(ab')_2$ fragments. The $F(ab')_2$ fragments alone were never lytic, even at a saturating concentration, which simplifies subsequent interpretation. To illustrate the effect, different cell types have been used (erythrocytes and lymphocytes), as well as a variety of partner antibodies (anti-A, anti-B, anti-glycophorin, CAMPATH-1, CD7). We have not seen lysis when the partner

antibody itself was nonlytic (CD7, data not shown), but when it was lytic the degree of lysis was enhanced in the presence of YTH 53.1, i.e., the apparent titer of the partner antibody was increased (Fig. 1).

Enhancement of Reactive Lysis Using YTH 53.1. In a reactive lysis system (1) where sensitizing antibody is not used, human red cells are not easily lysed by human complement in the fluid phase. However, human red cells are more easily lysed by human $C5\overline{6}$ and normal human serum, used as a source of C7-9, when they are incorporated into agarose. It is not clear why lysis of human red cells by homologous complement occurs more readily in solid phase than in fluid phase; however, it would appear that the phenomenon of homologous restriction is still operative in this system since lysis proceeded more quickly if normal human serum was replaced with normal rabbit serum (our unpublished observation). Therefore, this system was particu-

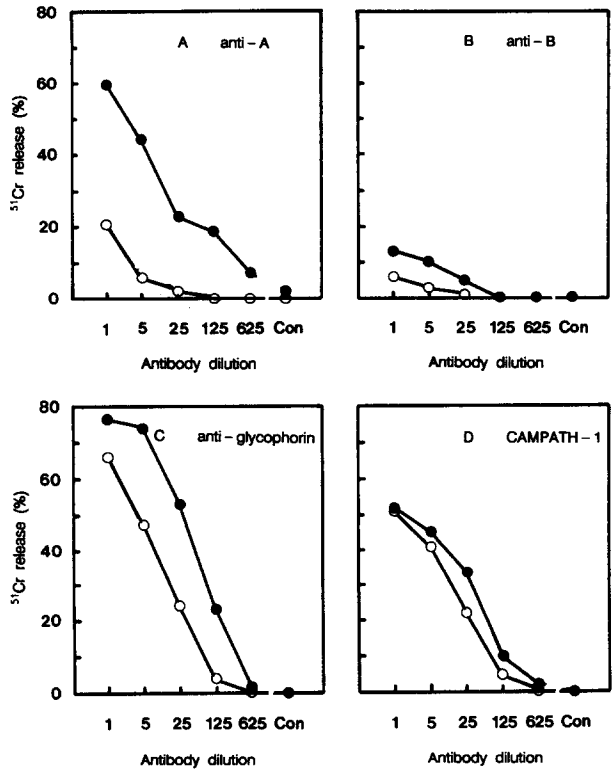

FIGURE 1. Enhancement of complement-mediated cell lysis by F(ab')$_2$ fragments of YTH 53.1. Each incubation contained 50 μl of F(ab')$_2$ fragments (50 μg/ml) (●) or medium (○), 50 μl of partner antibody or medium (control), 50 μl of ^{51}Cr-labeled cells, and 50 μl of complement.

Antibody	Cells	Complement
(*A*) Anti-A	Group A erythrocytes	A serum, diluted 1:4
(*B*) Anti-B	Group B erythrocytes	B serum, diluted 1:4
(*C*) Antiglycophorin	Group O erythrocytes	A serum, diluted 1:4
(*D*) CAMPATH-1	Blood lymphocytes	A serum, diluted 1:2.5

larly suitable for testing the effects of the mAb YTH 53.1 upon reactive lysis of human red cells.

YTH 53.1 IgG in a well adjacent to the line of lysis caused an enhancement of the lysis of human red cells by human $C\overline{56}$ and normal human serum (Fig. 2 *a*), whereas no enhancement was observed when guinea pig red cells were used in place of human red cells (Fig. 2 *b*). Plates were poured in PBS/10 mM EDTA in order to inhibit activation of early complement components in the whole serum used, and rat monoclonal anti-glycophorin A IgG, used as a control, had no effect upon the lysis of human red cells by homologous complement (Fig. 2 *a*). This antibody is of the same subclass as YTH 53.1 and in normally lytic for human red cells. The ability of YTH 53.1 to enhance reactive lysis of human red cells was also evident when sheep $C\overline{56}$ was used instead of human $C\overline{56}$, but was not observed when normal human serum was replaced with normal rabbit serum (Fig. 3). In Fig. 3, the marked difference between the degree of lysis of human red cells caused by rabbit serum and by human serum is due not only to the phenomenon of homologous restriction, but also to some extent to the fact that sheep $C\overline{56}$ reacts more readily with rabbit complement than it does with human complement. These data collectively indicate that YTH 53.1 reacts with and neutralizes the activity of a molecule that restricts lysis by homologous complement at a stage subsequent to the generation of $C\overline{56}$.

Purification of CD59 Antigen. The protein recognized by YTH 53.1 was purified from solubilized human erythrocyte membranes by affinity chromatography, as described. Material eluted from the column was run on a 10–20% gradient gel, and silver staining revealed a single band of approximate molecular mass of 19 kD under both reducing and nonreducing conditions (Fig. 4). When the same column was

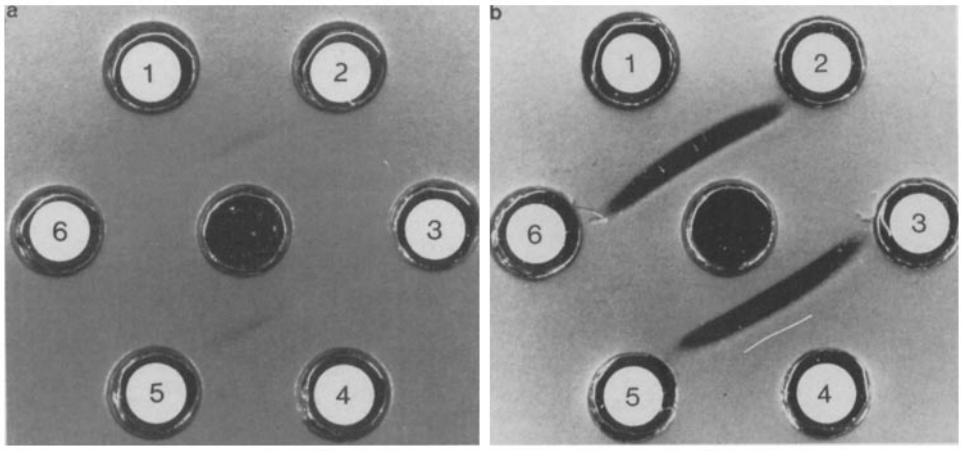

FIGURE 2. Enhancement of reactive lysis by YTH.53.1. Reactive lysis plates were prepared as described in Materials and Methods. (*a*) Human erythrocytes incubated for 22 h at room temperature. (*b*) Negative control. Guinea pig erythrocytes incubated for 5 h at room temperature. The center wells contained 10 μl of human $C\overline{56}$ (2.2 mg/ml). The contents of the outer wells (10 μl) were as follows: (*1*) normal human serum; (*2*) YTH 53.1 IgG prepared from ascites (4 mg/ml); (*3*) YTH 89.1 (anti-glycophorin) IgG prepared from ascites (1 mg/ml); (*4*) normal human serum; (*5*) YTH 53.1 IgG prepared from ascites (1 mg/ml); (*6*) YTH 89.1 (anti-glycophorin) IgG prepared from ascites (4 mg/ml).

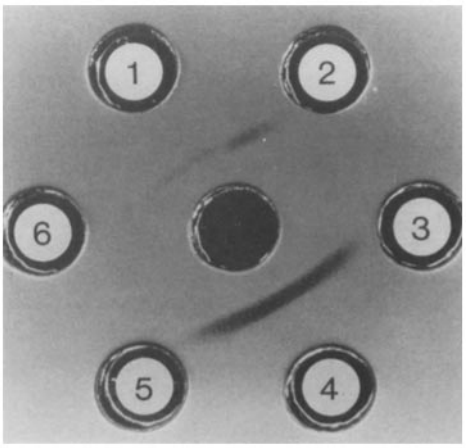

FIGURE 3. Comparison of the effects of YTH 53.1 upon lysis mediated by sera from different species. The reactive lysis plate contained human erythrocytes and was incubated at room temperature for 5 h. The center well contained 10 μl sheep C56. The contents of the outer wells (10 μl) were as follows: (*1*) normal human serum; (*2*) YTH 53.1 IgG prepared from ascites (4 mg/ml); (*3*) YTH 89.1 (anti-glycophorin) IgG prepared from ascites (1 mg/ml); (*4*) normal rabbit serum; (*5*) YTH 53.1 IgG prepared from ascites (1 mg/ml); (*6*) YTH 89.1 IgG (anti-glycophorin) IgG prepared from ascites (4 mg/ml).

used to extract protein from concentrated human urine, a slightly larger band of ~20 kD was observed by SDS PAGE (Fig. 4), with a second, lightly stained band at ~45 kD occurring in some preparations (Fig. 5). On two occasions, a larger protein with approximate molecular mass of 80 kD under reducing conditions was noticed, once in the absence of the usual 20-kD protein; the significance of this component and its relationship to the 20-kD protein are currently under investigation.

Effects of a Polyclonal Antiserum against CD59 Antigen on Reactive Lysis. A polyclonal antiserum raised against urine-derived CD59 antigen detected CD59 antigen in urine in Ouchterlony tests. IgG prepared from this antiserum also enhanced the reactive lysis of human red cells by human complement (Fig. 6). IgG prepared from preinoculation serum taken from the same animal was without effect.

Relationship of CD59 Antigen to MIP. MIP is a 55/65-kD protein found in human serum and on human red cell membranes, with the capacity to bind C8 and C9 (14). In these respects, it is highly similar to HRF/C8bp (7, 13). The relationship

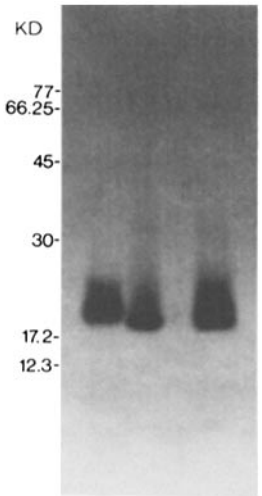

FIGURE 4. SDS-PAGE of urine- and membrane-derived CD59 antigen. 20 μl samples of pooled fractions eluted from the YTH 53.1 affinity column were heated at 100°C in 20 μl of reducing buffer before loading onto a 10–20% acrylamide gel. Protein bands were detected by silver staining. (Lane *1*) Urine-derived CD59 antigen; (lane *2*) membrane-derived CD59 antigen; (lane *3*) urine- and membrane-derived CD59 antigens.

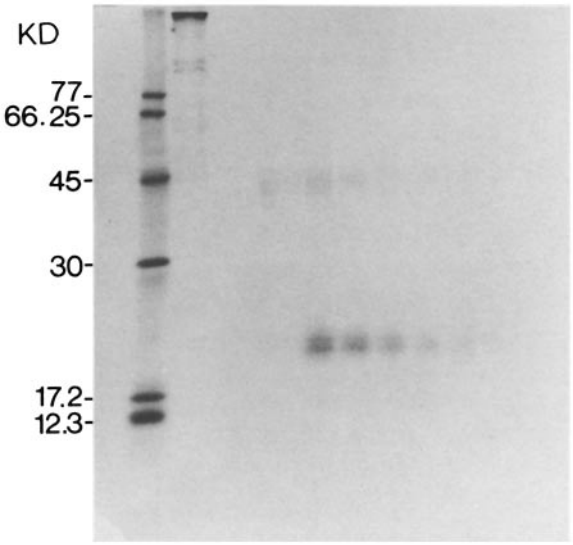

FIGURE 5. SDS-PAGE of fractions eluted from the YTH 53.1 affinity column. 20-μl samples from 0.5-ml fractions were heated at 100°C in 20 μl nonreducing buffer before loading onto a 10–20% gradient gel. Bands were visualized by Coomassie stain. (Lane *1*) Molecular weight standards. (Lane *2*) 10 μg YTH 53.1 IgG used to prepare the column. (Lanes *3–8*) Samples from a series of alternate fractions collected after commencement of elution with 0.1 M acetic acid, pH 3.0/0.2% NP40.

of CD59 antigen to MIP was investigated in order to rule out the possibility that CD59 antigen might be a truncated version of this larger protein. Comparative double diffusion on Ouchterlony plates using polyclonal antisera against CD59 antigen and against MIP, with concentrated urine as an antigen source, failed to demonstrate a relationship between CD59 antigen and MIP, as the precipitation lines found cross and therefore show nonidentity (data not shown).

Isolation of cDNA Clones Encoding Antigenic Determinants Recognized by the mAb YTH 53.1. cDNA libraries prepared as described were introduced into COS cells by spheroplast fusion. COS cells expressing cDNAs encoding the protein of interest were detected by panning (20) using YTH 53.1. Hirt supernatant DNA prepared from the positive cells was transformed into *E. coli*, and plasmid DNA isolated from eight of the colonies was transfected into COS cells for analysis by immunofluores-

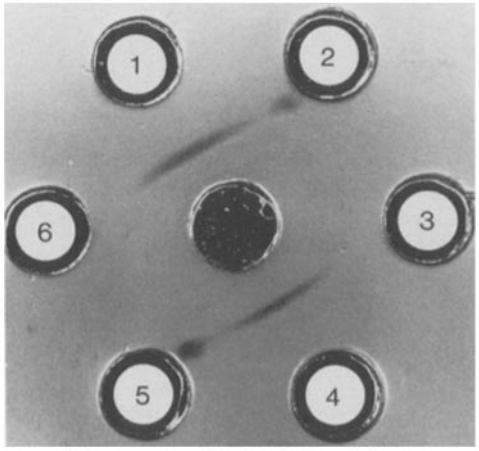

FIGURE 6. Enhancement of reactive lysis of human erythrocytes by rabbit polyclonal antibody against CD59 antigen. The plate was prepared as described in Materials and Methods, and incubated at room temperature for 5 h. The center well contained 10 μl of human $C5\overline{6}$ (2.2 mg/ml). The contents of the outer wells (10 μl) were as follows: (*1*) normal human serum; (*2*) polyclonal anti-CD59 antigen IgG prepared from serum (3 mg/ml); (*3*) nonimmune IgG prepared from preinoculation serum (1 mg/ml); (*4*) normal human serum; (*5*) polyclonal anti-CD59 antigen IgG prepared from serum (1 mg/ml); (*6*) nonimmune IgG prepared from preincubation serum (3 mg/ml).

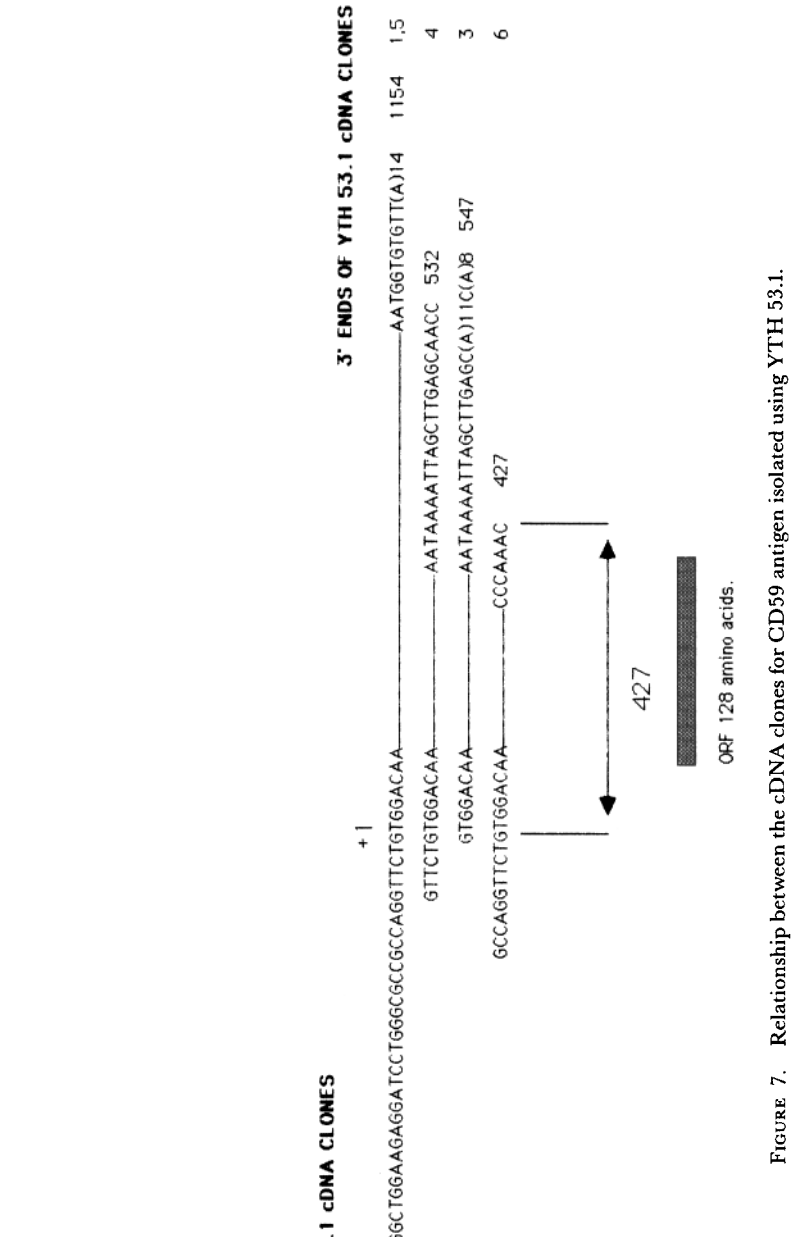

FIGURE 7. Relationship between the cDNA clones for CD59 antigen isolated using YTH 53.1.

cence. Six of these eight miniprep samples were positive; five of these six clones (YTH 53.1/1,3,4,5, and 6) were pursued further. Restriction enzyme analysis revealed that YTH 53.1/1 and YTH 53.1/5 are probably identical, as are YTH 53.1/3 and YTH 53.1/4.

cDNA Sequence Analysis. The clones YTH 53.1/1, 3, and 6 were sequenced as described. The sequence data show that YTH 53.1/1 and YTH 53.1/5 are ~1,154 bp in length and have poly(A) tails (A_{14}), YTH 53.1/6 is 427 bp, YTH 53.1/3 547 bp, and YTH 53.1/4 530 bp in length. YTH 53.1/3, which also has a poly(A) tail ($A_{11}CA_8$), utilizes a polyadenylation signal at 509 bp that is ignored in YTH 53.1/1 and YTH 53.1/5. Both YTH 53.1/4 and YTH 53.1/6 lack a poly(A) tail (Fig. 7).

NH_2-terminal Sequence Analysis of CD59 Antigen. Automated sequence analysis was carried out first on a sample of urine-derived CD59 antigen and subsequently on

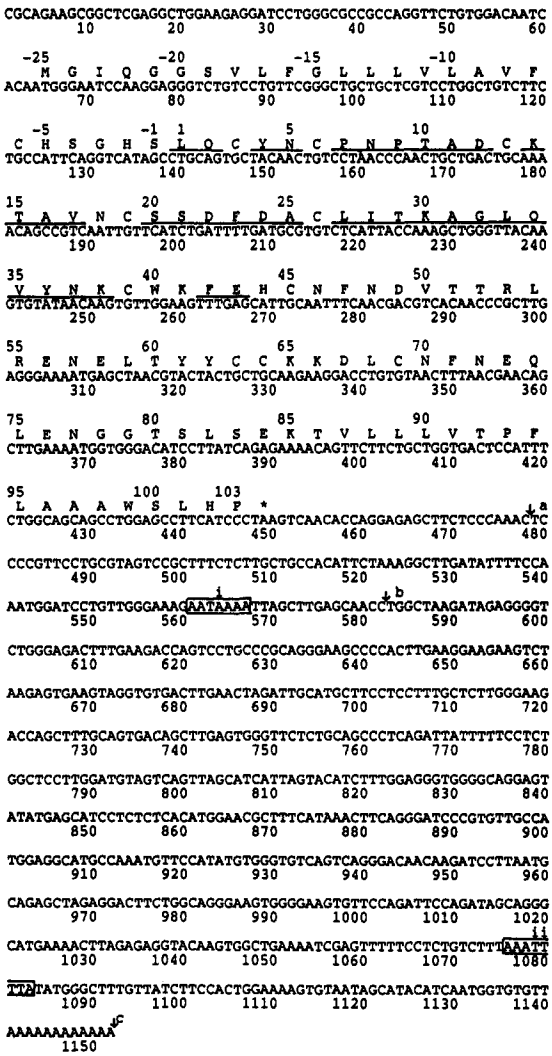

FIGURE 8. The nucleotide sequence shown is that of the longest clone, YTH 53.1/1, and encodes a precursor polypeptide of 128 amino acids. NH_2-terminal sequence analysis of CD59 antigen purified both from urine and erythrocyte membranes indicates the position of the NH_2 terminus of the processed protein (Leu-1). The probable initiation codon is met −25, with residues −25 to −1 representing a signal peptide. Residues underlined were determined by sequence analysis of the urine protein. Sequence analysis of the membrane protein (which was *s*-carboxymethylated before sequencing) confirmed residues 2–35, and also identified cysteine residues 3, 6, 13, 19, and 26. No signal was detected for either protein at position 18. *a*, 3′ end of clone YTH 53.1/6; *b*, 3′ end of clones YTH 53.1/4 and YTH 53.1/3 (with poly(A)$^+$ tail); *c*, 3′ end of clones YTH 53.1/1 and YTH 53.1/5; *i*, boxed nucleotides indicate polyadenylation signal utilized in clone YTH 53.1/3; *ii*, potential polyadenylation site utilized in clones YTH 53.1/1 and YTH 53.1/5.

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Position    1        10        20        30        40        50        60
LY-6E       LECYQCVGVPFETSCP-SITCPYPDGVCVTQEAAVIVGSQTRKVKNNLCLPI--CPFNIE
            * ** *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CD59        LQCYNC-PNPT-ADCKTAVNCSSDFDACLITKAGLQV--------YNKCWKFEHCNFN--

Position   61        70        80        90        100       110
LY-6E       SNEILGTKVNVKTSCCQEDLCN--AAVPNGGSTWTMAGVLLFSLSSVLLQTLL--
                      **  ****      ***       ***       *
CD59        DVTTRLRENELTYYCCKKDLCNFNEQLENGGTSLSEKTVLLLVTPFLAAAWSLHP
```

FIGURE 9. Alignment of CD59 antigen and murine LY-6E antigen amino-acid sequences. Numbering refers to the position in the aligned sequences, and does not therefore correspond with the residue number of either sequence. Residues conserved in both sequences are indicated by an asterisk.

a sample of the membrane-derived protein that had been reduced and alkylated. Sequence data for the two samples are shown in Fig. 8, in comparison with the sequence deduced from the cDNA isolated as described above. The urine- and membrane-derived CD59 antigens had identical NH_2-terminal sequences; these were in agreement with the residues predicted by the cDNA sequence for positions 1–43. This indicates that the probable initiation codon is at met −25, and that residues -25 to −1 represent a signal peptide. This sequence comparison also demonstrates that the predicted residue at position 18, asn-18, occurs within a glycosylation signal sequence (N-C-S). The lack of a detectable residue at this position in the protein sequence indicates that it is N-glycosylated in the mature protein. In contrast, while asn-8 also occurs within a potential glycosylation site (N-P-T), it is recovered in appropriate yield during sequence analysis of both urine- and membrane-derived proteins, indicating that it is not glycosylated to any significant degree in CD59 antigen.

Searches of sequence databases did not demonstrate a high degree of sequence homology of CD59 antigen with other proteins. However, the best match (26% overall identity) was to a murine lymphoid cell protein, the LY-6E antigen (36). The aligned sequences are shown in Fig. 9.

RNA and DNA Blot Analyses. RNA blot analysis on a range of human cell lines revealed four species of RNA at ~600 bp, 1.2 kb, 1.9 kb, and 2.2 kb present in varying amounts in different cells (data not shown). The lower bands at 600 bp and 1.2 kb probably correspond to the clones YTH 53.1/3 (547 bp) and YTH 53.1/1 and /5 (1,154 bp), respectively; the larger bands do not correspond to any of the cDNAs so far isolated. Southern blot analysis on human placental genomic DNA revealed a simple pattern consistent with a single gene copy (data not shown).

Release of CD59 Antigen from the Surface of COS Cells by PI-PLC. Incubation of COS cells transfected with the cDNA encoding CD59 antigen with PI-PLC from *B. thurin-*

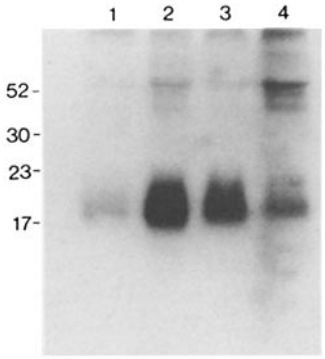

FIGURE 10. Immunoprecipitation of CD59 antigen from the supernatant or lysate of radioiodinated COS cells after incubation at 37°C for 30 min in the presence (lanes *3* and *4*) or absence (lanes *1* and *2*) of 10 U of PI-PLC (*B. thuringiensis*). (Lanes *1* and *3*) Samples of cell supernatant after incubation. (*Lanes 2* and *4*) Samples of cell lysate after incubation. The mobilities of molecular weight markers are as indicated.

giensis caused the release of almost all of the antigen from the cell surface into the supernatant (Fig. 10). Cells incubated in parallel but without PI-PLC retained their surface expression of CD59 antigen. These data show that CD59 antigen utilises a glycolipid anchor for attachment to the cell surface.

Discussion

We have reported herein the identification of a 20-kD membrane protein that is capable of inhibiting complement-mediated lysis. This protein was detected on a broad range of cell types using the mAb YTH 53.1, which was designated CD59 at the 4th Leucocyte Workshop (19). In this present study, YTH 53.1 was found to enhance the lysis of human erythrocytes by human complement in two separate hemolytic assay systems, thereby indicating a role for CD59 antigen in protecting against complement-mediated lysis. The fact that YTH 53.1 enhanced lysis in a reactive lysis system, which uses pre-activated $C\overline{56}$ complex, indicates that protection against lysis mediated by CD59 antigen takes effect in the later stages of complement activation, during assembly of the MAC. Moreover, comparative assays using different species of $C\overline{56}$ and of serum used as a source of C7-9 revealed that the inhibitory capacity of CD59 antigen is effective only against homologous complement.

The antibody YTH 53.1 was used both to purify CD59 antigen by affinity chromatography and to isolate the corresponding cDNA from a human T cell library. Antigen extracted from human erythrocyte stroma or from human urine has an approximate molecular mass of 19–20 kD, while the isolated cDNA encodes a protein of ~11.5 kD molecular mass. This discrepancy between predicted size and apparent molecular mass as judged by SDS-PAGE may partly be due to N-glycosylation of the asparagine residue at position 18, as suggested by the lack of a signal at this residue during sequencing of the purified protein. Furthermore, the posttranslational addition of a glycolipid anchor may cause the molecular mass of the mature protein to be increased, though it is not possible to calculate exactly how much this modification might change the molecular mass without knowing how much of the protein COOH terminus is removed during the process.

RNA blot analyses on a range of human cell lines revealed the existence of longer mRNA transcripts that do not correspond to any of the isolated cDNAs, presumably because they are either untranslated or encode polypeptides that are not expressed on the cell surface and therefore would not be detected by the panning procedure. Polyacrylamide gel electrophoretic analysis of affinity-purified CD59 antigen from human urine also revealed a slightly larger component (45 kD) in some preparations, and on two occasions YTH 53.1 in solid phase extracted an 80-kD component from human urine. The reason why an 80-kD component was observed only in two instances during repeated preparations of CD59 from human urine, and on one of those occasions in the absence of the usual 20-kD protein, is not clear, and currently under investigation. Some preliminary results indicate that the polyclonal anti-CD59 antiserum recognizes an 80-kD component in urine and serum by immunoblotting (data not shown), suggesting that this component can be detected directly by antibody against CD59 antigen, rather than indirectly by means of an association with CD59 antigen. It is not known whether these larger bands represent larger versions of the CD59 antigen or are caused by the crossreaction of YTH 53.1 with related proteins in the urine.

Several other membrane proteins have been described that protect against the lytic action of the MAC. These include homologous restriction factor (HRF)/C8 bp (7, 13) and MIP (14). Since HRF/C8bp has an approximate molecular mass of 70 kD, CD59 antigen is clearly distinguishable from HRF/C8 bp by virtue of its size: both protein and cloning data indicate that CD59 antigen is a much smaller molecule, though its relationship to the larger bands occasionally associated with it remains obscure. As there are no sequence data available for HRF/C8bp, the distinction between HRF/C8bp and CD59 antigen cannot yet be confirmed by direct comparison of the primary amino acid sequences. With a molecular mass of 55/65 kD, MIP has a size approaching that of HRF/C8bp (Watts, M. J., and B. P. Morgan, personal communication). The relationship of MIP to HRF/C8bp is unclear at present, but Ouchterlony tests using polyclonal antisera to CD59 antigen and to MIP have established that the native proteins do not share epitopes. Recently, Sugita et al. (37) reported the isolation from human erythrocyte stroma of an 18-kD protein capable of inhibiting the reactive lysis of guinea pig cells by human complement. It seems likely that this protein, named P-18, is the same as CD59 antigen, though as yet no structural data for P-18 are available for comparison.

An 18–25-kD glycoprotein broadly expressed on human leucocytes and erythrocytes has recently been identified by Štefanová et al. (18) using their mAb MEM-43. This antibody was placed in a cluster (CD59) with YTH 53.1 at the 4th Leucocyte Workshop, and comparison of the NH_2-terminal amino acid sequence of the MEM-43 antigen (18) with the sequence for the 20-kD protein described here indicates that the two antibodies do in fact recognize the same molecule. Štefanová et al., (18) also noticed the sequence similarity, at the NH_2 terminus, between the glycoprotein isolated using MEM-43 and the LY-6 antigens. The overall degree of homology between the murine LY-6E and CD59 antigens is not high (26% identity). Two further LY-6 sequences have recently been published. One, Ly-6A, has only two differences to LY6-E (38, 39). The second, LY6-C, while only 62% homologous to LY6-E (40), has a similar (26%) degree of homology to CD59. In addition, both LY6 and CD59 antigens are of similar length (108 or 105 vs. 103 residues), both contain 10 cysteine residues, and interestingly, both are expressed as PI-linked cell membrane proteins. Taken together these data are strong evidence for a distant evolutionary relationship between the proteins. Both CD59 and LY-6 antigens are broadly expressed on a variety of cell types. However, while our data demonstrate that CD59 antigen plays a role in protection of the host cell against lysis by the MAC of homologous complement, LY-6 antigens have been implicated in augmentation of response to TCR stimulation. Although a human homologue of LY-6 has not yet been described, it therefore appears unlikely that the relationship of CD59 and LY-6 antigens extends beyond a structural similarity. A possible evolutionary relationship between a squid glycoprotein (SGP2) and LY-6 antigens has also been suggested (41). These and other homologies are discussed more fully elsewhere (Harrison, R. A., D. L. Simmons, A. Davies, G. Hale, P. J. Lachmann, and H. Waldmann, manuscript in preparation).

Interestingly, an 80-kD component has also been associated with the MEM-43 antigen: immunoprecipitation from HPB-ALL cell lysates using MEM-43 detected an 80-kD band as well as the usual band within the 18–25-kDa region (18). However, this 80-kD band component was never observed in preparations of the MEM-43

antigen obtained by affinity chromatography, contrary to our experience during routine preparation of CD59 antigen from urine using the same technique.

The removal by PI-PLC of CD59 antigen expressed on the surface of COS cells suggests that it is linked to the cell surface by means of a glycolipid anchor. This was also demonstrated by Štefanová et al. (18) using MEM-43 to measure the release of CD59 antigen from the surface of PBMC by PI-PLC, and was confirmed by workshop studies (19). In view of this, it can be expected that CD59 antigen will be absent from the surface of affected erythrocytes in patients suffering from paroxysmal nocturnal hemoglobinuria (PNH), since such cells lack proteins that use the glycolipid anchor, for example, DAF and HRF/C8bp (42–44). An additional deficiency of CD59 antigen in PNH would be particularly significant, since CD59 antigen may act in synergy with HRF/C8bp to protect cells from complement-mediated lysis.

Complement-mediated cell lysis is used as a means of purging the bone marrow of unwanted cells in preparation for transplantation. The use of human complement for this purpose is not always practical because of the reduced efficiency with which complement lyses homologous cells; this can be a particular problem if the antibody that needs to be used to sensitize the target cell is not itself very lytic. However, the use of human complement is, in principle, preferable to that of rabbit complement because of the variable nonspecific toxicity that is associated with the latter (45), so a means of augmenting the lytic potential of human complement against autologous cells would be useful. In this respect, antibodies that can inhibit the effect of complement regulatory proteins may be of benefit therapeutically, to increase the susceptibility of target cells to lysis by autologous complement. YTH 53.1 may be one such antibody.

It is hoped that further investigations using purified CD59, as well as antibodies directed against it, will help to reveal the mechanism by which CD59 inhibits complement-mediated lysis.

Summary

A novel cell surface antigen has been identified on a wide range of lymphoid cells and erythrocytes. A mAb YTH 53.1 (CD59) against this antigen enhanced the lysis of human red cells and lymphocytes by homologous complement. Studies of reactive lysis using different species of $C\overline{56}$, and of whole serum used as a source of C7-9, indicated that the inhibitory activity of the CD59 antigen is directed towards the homologous membrane attack complex. CD59 antigen was purified from human urine and erythrocyte stroma by affinity chromatography using the mAb YTH 53.1 immobilized on Sepharose, and, following transient expression of a human T cell cDNA library in COS cells, the corresponding cDNA also identified using the antibody. It was found that the CD59 antigen is a small protein (~20 kD as judged by SDS-PAGE, 11.5 kD predicted from the isolated cDNA) sometimes associated with larger components (45 and 80 kD) in urine. The sequence of CD59 antigen is unlike that of other complement components or regulatory proteins, but shows 26% identity with that of the murine LY-6 antigen. CD59 antigen was released from the surface of transfected COS cells by phosphatidylinositol-specific phospholipase C, demonstrating that it is attached to the cell membrane by means of a glycolipid anchor; it is therefore likely to be absent from the surface of affected erythrocytes in the disease paroxysmal nocturnal hemoglobinuria.

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