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Presence of a dysfunctional form of CD59 on a CD59$^+$ subclone of the U937 cell line

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SUMMARY

U937 cells are known to be relatively sensitive to C-mediated killing and have been reported to show variable expression of CD59. We have obtained stable CD59$^+$ and CD59$^-$ sublines of the U937 cell line. Expression of other C-regulatory proteins, decay-accelerating factor (DAF), MCP and CR1, was similar on both cell lines. Although the sublines were morphologically similar and expressed similar amounts of most surface antigens, qualitative difference in expression of CD13 and CD64 and a quantitative difference in CD15 expression was observed. Sensitivity to C-mediated killing of the cell lines was measured using classical pathway activation. Both cell lines appeared to be equally sensitive to C-mediated killing. Monoclonal antibodies against CD59, which neutralize CD59 and enhance killing of most cell lines (including K562, HL60 and Molt4), did not enhance the killing of the CD59$^-$ cells but, surprisingly, also did not enhance killing of the CD59$^+$ U937 subline. CD59 was expressed on the U937 subline at similar levels to that on HL60 and K562 cells, was glycosyl-phosphatidylinositol (GPI) anchored and could be immunoprecipitated from cell extracts. However, unlike these other cell lines, U937 cell extracts were negative in a Western blot using a variety of anti-CD59 antibodies even when ultrasensitive detection methods were used. These results indicate that the CD59$^+$ U937 cell expresses a form of CD59 which is dysfunctional and structurally abnormal.

INTRODUCTION

We are currently investigating the routes by which nucleated cells signal the removal of complement (C) membrane attack complexes (MAC) during the process of recovery.[1] In particular, we are examining the potential role of the glycosyl-phosphatidyl-inositol (GPI)-anchored inhibitor of the MAC, CD59, in these events. CD59 binds to C8 in the forming MAC and limits incorporation of C9, thereby preventing the formation of a lytic lesion (reviewed by Holguin and Parker[2]). We have recently shown that cross-linking of CD59 on nucleated cells signals release of calcium stores through activation of tyrosine kinases and have suggested that cross-linking of CD59 in the MAC or clusters of MAC might similarly signal calcium store release, an essential component of the recovery process.[3]

To elucidate further the importance of CD59 in recovery we require cell lines which differ only in the expression of CD59. One possible source was suggested by reports that U937, a stable promonocytic cell line, showed variable expression of CD59.[4] This cell line has previously been shown to be relatively susceptible to killing by complement and to respond to non-lethal attack by removal of MAC from the membrane,[5] making it a good candidate for our planned studies.

Here we describe the derivation and characterization of stable CD59$^+$ and CD59$^-$ sublines from the U937 line described as showing variable expression of CD59 by Davies *et al.*[4] The susceptibility of each of these sublines to complement killing and the capacity of endogenous and incorporated CD59 to protect the cells has been examined. We conclude that the CD59$^+$ subline expresses a dysfunctional form of CD59 which does not inhibit C killing and interferes with the inhibitory action of active, incorporated CD59. These findings make the sublines unsuitable for examination of the role of CD59 in recovery processes but may provide additional information on the structural requirements for complement inhibition by CD59.

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Abbreviations: C, complement; GVB, gelatin veronal buffer; MAC, membrane attack complex of complement; PI, propidium iodide.

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MATERIALS AND METHODS

Cells

The original U937 cell line used in this study was a kind gift of the laboratory of Professor P. J. Lachmann (Cambridge, U.K.). A second U937 line, K562, and HL60 cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, Salisbury, U.K.). Molt4 cell line was a kind gift from the laboratory of Professor L. K. Borysiewicz (Cardiff, U.K.). All cells were grown at 37° and 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 2 mM sodium pyruvate, 100 U/ml

penicillin, 100 U/ml streptomycin, 2·5 μg/ml amphotericin and kept at a density of 10^6–10^7 cells/ml. Cells were washed three times in phosphate-buffered saline (PBS) before use. CD59$^-$ and CD59$^+$ sublines were obtained by a single limiting dilution step. Thirty subclones were prepared and analysed by FACScan analysis with anti-CD59 monoclonal antibody (mAb) BRIC229 and GAM/IgG–FITC (fluorescein isothiocyanate) as described below. Subclones did not change their phenotype even after many months in culture.

Antibodies

Polyclonal antisera against the CD59$^-$ U937 cell line, erythrocyte CD59 and MIP/HRF were raised in rabbits using standard procedures. Antisera were heat inactivated (30 min, 56°) before use. Monoclonal antibodies were kindly donated by V. Horejsi (Prague, Czech Republic): (MEM-43 and MEM-43·5, anti-CD59; MEM-118, anti-CD55); J. W. van de Winkel (Utrecht, The Netherlands) (2.2, anti-CD64/FcγRI; VI.3, anti-CD32/FcγRII); J. Atkinson (St Louis, MO) (GB24, anti-CD46/MCP). Other mAb were prepared in our laboratory (A35, anti-CD59; G3 and B5, anti-MIP) or were obtained from commercial sources: International Blood Group Reference Laboratory (IBGRL, Bristol, U.K.; BRIC229, anti-CD59; BRIC216 and BRIC110, anti-CD55; BRIC5, anti-CD58), Dako (High Wycombe, U.K.); antibodies recognizing CD2, CD3, CD4, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD25, CD32, CD35, CD45, CD61, anti-macrophage, anti-HLA-DR, GAM/IgG–FITC and GAR/IgG–FITC). GAM/IgG–HRP (horseradish peroxidase) and GAR/IgG–HRP were obtained from BioRad (Hemel Hempstead, U.K.). Bovine serum albumin (BSA) and human IgG were obtained from Sigma (Poole, U.K.). CD59 was purified from human erythrocytes using an immunoaffinity column (MEM-43) as described previously.[4]

Buffers

GVB: 2·8 mm barbituric acid, 145·5 mM NaCl, 0·8 mM $MgCl_2$, 0·3 mm $CaCl_2$, 0·9 mM Na–barbital, pH 7·2 (Oxoid Ltd, Basingstoke, U.K.) supplemented with 0·1% gelatin. FACS buffer: 1% BSA, 0·2% Na-azide in PBS. PBS: 8·1 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 137 mM NaCl, 2·7 mM KCl, pH 7·4 (Oxoid Ltd).

FACScan

Cells were harvested, washed three times with PBS and resuspended at 10^6 cells/ml in FACS buffer. All steps were carried out on ice. To prevent non-specific binding of antibodies to FcγR, cells were incubated with heat-aggregated IgG (final conc. 20 μg/ml) for 15 min on ice. Human IgG was aggregated at 63° for 15 min at a concentration of 20 mg/ml. Cells were incubated with the specific poly- or monoclonal antibody (1–10 μg/ml) for 30 min, washed three times with FACS buffer and incubated for 30 min with GAR/IgG–FITC or GAM/IgG–FITC. Cells were washed three times in FACS buffer and fixed with 1% paraformaldehyde in PBS. Fluorescence was measured using a Becton Dickinson FACScan (San Jose, CA).

Incorporation of CD59

CD59, purified from human erythrocytes was incorporated in the U937 sublines. Cells were harvested, washed three times in PBS and 1 μg/ml CD59 was added to 10^7 cells/ml in PBS/0·05% CHAPS. After 30 min at 37° cells were washed three times with PBS and resuspended in GVB.

Complement assays

Cells were harvested, washed three times in PBS and resuspended in GVB at 10^7 cells/ml. Heat-inactivated rabbit anti-U937 serum (raised against the CD59$^-$ cell line to avoid generation of anti-CD59 activity) was added (1/15) and after 30 min incubation on ice, cells were washed three times with GVB and resuspended to the original volume. To 100 μl of the cells 100 μl human serum (diluted in GVB) was added. After incubation for 1 hr at 37°, 200 μl of propidium iodide (PI; 0·5 μg/ml in FACS buffer) was added and killing was immediately estimated using a FACScan. Per tube 5000 events were counted. C8- and C9-depleted serum were prepared by passage of human serum over a monoclonal anti-C8 or anti-C9 column as previously described.[6]

SDS–PAGE and Western blotting of cell lysates

Cell lysates were prepared as described by Cinek and Horejsi.[7] Briefly cells were resuspended in ice-cold lysis buffer (20 mM Tris, 140 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (NP-40); pH 8·2) at 5×10^7/ml. After 30 min on ice, lysates were spun in an eppendorf centrifuge at 13,000 ***g*** for 3 min at 4°. The supernatant was mixed 1 : 1 with electrophoresis sample buffer, heated to 100° and run on a 15% gel under non-reducing conditions.[8] After blotting onto nitrocellulose and blocking with 5% milk/PBS, blots were subsequently incubated with anti-CD59 (BRIC229 1 μg/ml in 5% milk/PBS) washed with PBS/0·1% Tween-20, incubated with GAM/IgG–HRP (1/2000 in 5% milk/PBS), washed with PBS/0·1% Tween-20 and PBS. Blots were developed using the enhanced chemiluminescence technique (Amersham International, Amersham, U.K.). Hyperfilm (Amersham) was exposed to the blot for different time periods.

RESULTS

Derivation and immunochemical characterization of CD59$^+$ and CD59$^-$ U937 cell lines

U937 cells were prepared for FACScan with monoclonal and polyclonal anti-CD59 antibodies as described. The cells showed

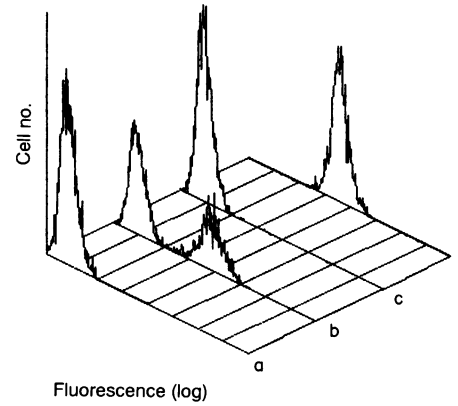

Figure 1. Cell-surface expression of CD59 on U937 cell lines as determined by FACScan. Cells were stained for CD59 with mAb BRIC229. (a) Background fluoresence (second Ab only); (b) mixed cell population of original U937 cell line; (c) CD59$^-$ cell line; (d) CD59$^+$ cells after limiting dilution.

Table 1. Expression of cell-surface antigens on U937 sublines as determined by FACScan. Cells were prepared for FACScan using various antibodies as described in the Materials and Methods. Those antibodies giving negative staining are marked (−). For all positive antibodies the median fluorescence is given. All analyses were performed at the same machine settings

CD antigen no.	CD59⁻ cell line	CD59⁺ cell line
control	–	–
2	–	–
3	–	–
4	18	13
11a	49	34
11b	–	–
11c	–	–
13	35	–
14	–	–
15	25	140
16	–	–
18	43	28
19	–	–
25	–	–
32	30	10
35	–	–
45	19	22
46	40	57
55	10	15
58	14	15
59	–	61
61	–	–
64	10	–
MIP	–	–
mph	–	–
HLA-DR	–	–

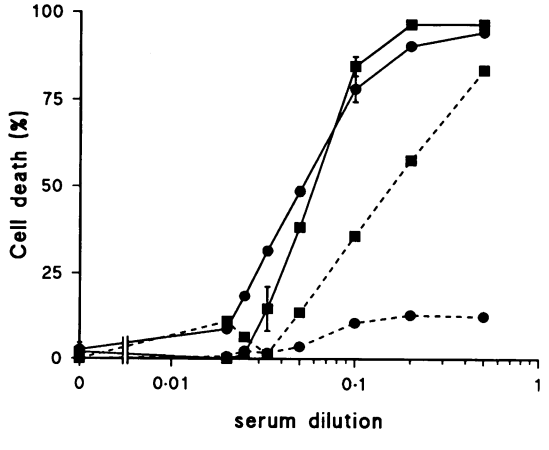

Figure 2. Killing of CD59⁺ (●) and CD59⁻ (■) U937 cell lines by human serum and C9-depleted serum. Cells were sensitized and incubated with various dilutions of human serum (——) or C9-depleted serum (- - - -). Killing was determined by PI uptake. Results are mean of duplicates ±SD and are corrected for background cell death.

a bimodal distribution with CD59⁺ and CD59⁻ cells using each of the six available monoclonal and polyclonal anti-CD59 antibodies tested (Fig 1b). The CD59⁺: CD59⁻ ratio was 3:7 and was stable over many months in culture. Cloning by limiting dilution yielded stable CD59⁺ and CD59⁻ cell lines (Fig. 1c,d). These stable sublines were of similar gross morphology and differentiated into macrophage-like cells after stimulation with phorbol 12-myristate 13-acetate (PMA) (not shown). The sublines were examined for expression of other C-regulatory proteins and other cell-surface markers by FACScan (Table 1). Cells had similar levels of expression of DAF and MCP and were both negative for CR1 and MIP. The sublines differed qualitatively in CD13, CD64 expression (the CD59⁺ subline was negative for both markers), while a marked quantitative difference in expression of CD15 was observed (sixfold higher expression on the CD59⁺ subline). Treatment with phosphatidyl inositol-specific phospholipase C (PIPLC) reduced CD55 expression on both cell lines by more than 50% and reduced the expression of CD59 on the CD59⁺ subline by 80% (not shown).

Complement-mediated killing

The CD59⁻ and CD59⁺ cell lines were tested for their susceptibility to C-mediated killing. Rabbit polyclonal antibodies were raised against the CD59⁻ cell line in order to avoid the generation of anti-CD59 reactivity. Both cell lines bound the same amount of polyclonal antibody as assessed by FACScan. Sensitized cells were incubated with various dilutions of human serum and killing was estimated by measuring PI uptake (Fig. 2). The cell lines were found to be equally susceptible to killing by human C, although the background cell death of the CD59⁺ cell line was much higher (25% compared with 3% for the CD59⁻ line). Neither cell line was killed by C8-depleted serum but the CD59⁺ cells were sensitive to killing by C9-depleted human serum (Fig. 2).

Blocking CD59 with mAb

Functional activity of CD59 on the CD59⁺ cell line was assessed by incubation with the blocking mAb BRIC229.[8-10] Preincubation of the cell lines with mAb BRIC229 did not affect the killing of the CD59⁺ or CD59⁻ U937 cell lines by human complement (Fig. 3a,b). Under identical conditions the killing of all other CD59⁺ cell lines tested was enhanced (Fig. 3c–e). To demonstrate that all CD59 was efficiently blocked by the first incubation with mAb BRIC229, cells were either incubated with a second dose of BRIC229 or with buffer alone and subsequently stained with GAM/IgG–FITC and prepared for FACScan. As shown in Fig. 4 no increase of fluorescence was observed after the second incubation with anti-CD59 antibody, confirming that all endogenous CD59 was blocked during the first incubation.

Protection from killing by exogenous CD59

The CD59⁻ and CD59⁺ cell lines were incubated with 1 μg/ml CD59 purified from human erythrocytes. This amount of CD59 caused an almost ninefold increase in the amount of CD59 on the CD59⁺ cell surface and gave the CD59⁻ subline a level of

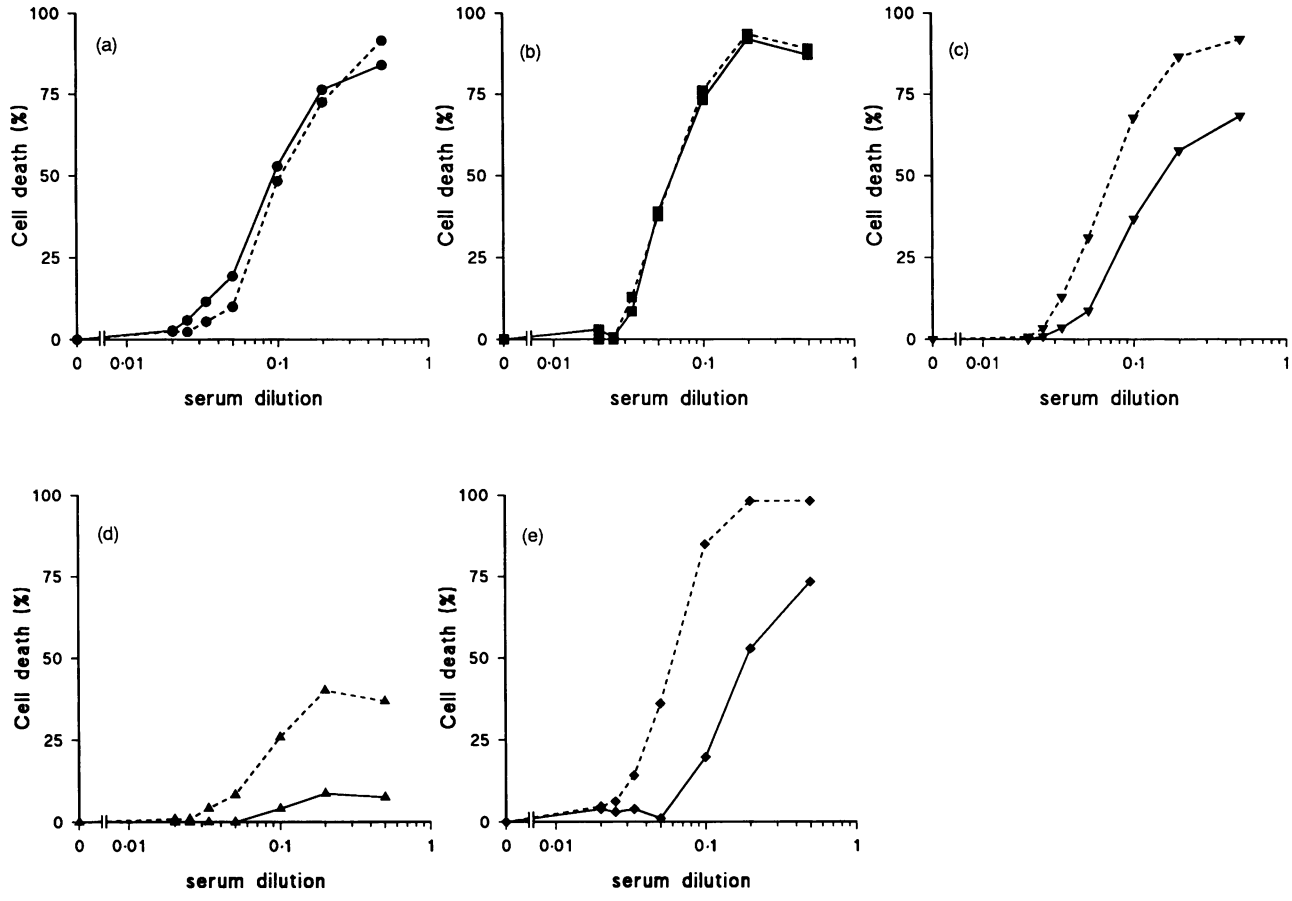

Figure 3. Effect of anti-CD59 mAb BRIC229 on killing of different cell lines by human serum. Cells were incubated with buffer (——) or anti-CD59 (- - - -) and incubated with various serum dilutions. Killing was determined by PI staining and is corrected for background cell death. (a) CD59$^-$ U937; (b) CD59$^+$ U937; (c) K562; (d) HL60; (e) Molt4.

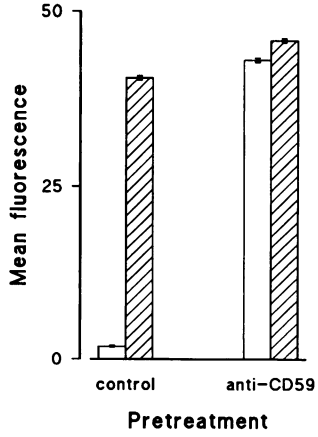

Figure 4. FACScan of CD59 availability on CD59$^+$ cell lines after blocking of CD59 with mAb BRIC229. Cells were incubated with anti-CD59 mAb BRIC229 (1 μg/ml); control cells were incubated with PBS. Subsequently cells were incubated with GAM/IgG–FITC (□) or incubated with mAb BRIC229 (1 μg/ml) followed by GAM/IgG–FITC (▨) and analysed on the FACScan. Results are means of duplicates ±SD.

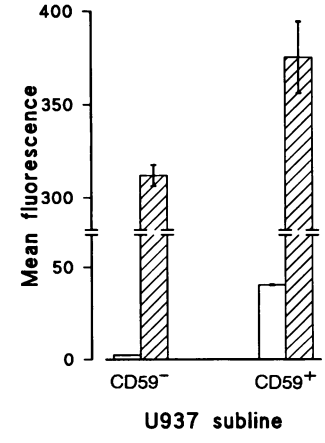

Figure 5. Incorporation of erythrocyte-derived CD59 in U937 sublines. Cells were incubated with purified CD59 (1 μg/ml; ▨) or PBS (control; □). Cells were stained with mAb BRIC229 and prepared for FACScan. Results are means of duplicates ±SD.

CD59 seven times higher than the CD59$^+$ control cells (Fig. 5). Sensitized cells were attacked with complement and killing was estimated by PI uptake. The CD59$^-$ U937 cell line was much better protected by incorporated CD59 than the CD59$^+$ U937 cell line (Fig. 6).

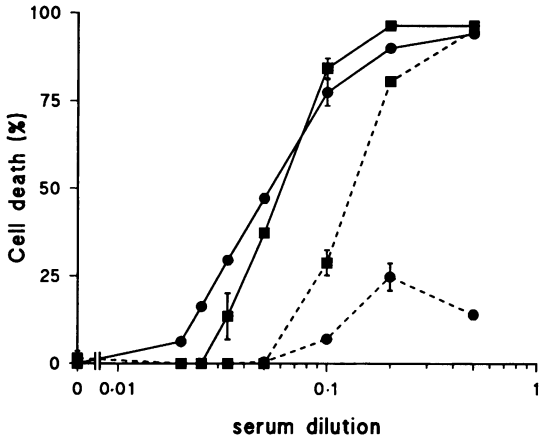

Figure 6. Protection from killing by human serum of CD59$^-$ (●) and CD59$^+$ (■) U937 cell lines by erythrocyte-derived CD59. Antibody-sensitized cells were incubated with 1 μg/ml purified erythrocyte CD59 (----) or PBS (——) and incubated with dilutions of human serum. Results are means of duplicates ± SD and are corrected for background cell death.

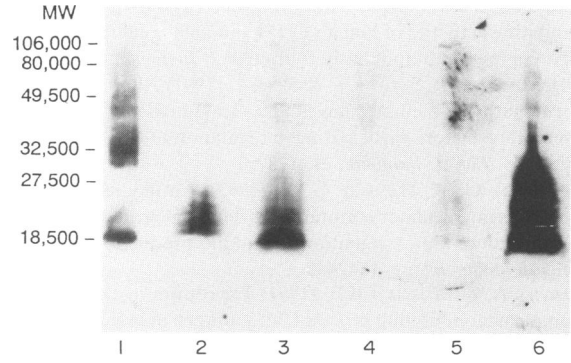

Figure 7. Western blot of CD59 purified from erythrocytes and CD59 from cell lysates, developed with the ECL method. Erythrocyte CD59 (10 ng/ml, lane 1) and cell lysates of Molt4 (lane 2), HL60 (lane 3), CD59$^-$ U937 (lane 4), CD59$^+$ U937 (lane 5) and K562 (lane 6) were run on a 15% SDS–PAGE under non-reducing conditions, blotted onto nitrocellulose and probed with anti-CD59 mAb BRIC229 and GAM/IgG–HRP. The blot was developed by the ECL method.

Western blotting

Cell lysates of the CD59$^+$ and CD59$^-$ U937 cell lines, Molt4, HL-60 and K562, and purified human erythrocyte CD59 were run on a 15% SDS–PAGE gel and blotted onto nitrocellulose. The blot was probed with anti-CD59 mAb BRIC229. Only cell lysates of HL60, K562, Molt4 and the HuE CD59 reacted with mAb BRIC229 after Western blotting (Fig. 7). The Molt4 lysate developed only very slowly. No CD59 reactivity was detected from the CD59$^+$ U937 subline, with either the mono- or polyclonal anti-CD59, even after over-exposure of the blots. The same results were obtained using a mAb which recognized a different epitope on CD59 (MEM-43·5) and with polyclonal anti-CD59 (not shown).

DISCUSSION

The original aim of this study was to investigate the role of CD59 in recovery from C attack. Cell lines that differed only in the expression of CD59 were required. Recovery from C attack in the U937 cell line has previously been studied by us and the line was shown to be susceptible to C killing.[5] However, reports regarding the presence of CD59 on U937 are confusing with various authors reporting that the cells have negative,[11] mixed[4] or positive[12] expression of CD59. We obtained a U937 cell line which showed bimodal expression of CD59. From this cell line stable CD59$^+$ and CD59$^-$ U937 cell lines were established by limiting dilution (Fig. 1). Most other cell-surface antigens, including the complement regulatory proteins DAF (CD55), MCP (CD46), CR1 (CD35) and MIP were expressed to the same extent. The cell lines only differed in expression of CD59, CD13 and CD64, while a quantitative difference was observed in expression of CD15 (Table 1) and both lines differentiated into macrophage-like end-cells upon stimulation with PMA, providing strong evidence that they were sublines of U937. PIPLC treatment of the cells removed CD55 from both lines and CD59 from the positive line indicating a simple GPI-anchoring for these proteins on both cell lines. Comparison of cell-surface expression of the proteins listed in Table 1 between our U937 sublines and a U937 cell line newly obtained from the ECACC, showed 100% concordance for this cell line and our CD59$^-$ subline (data not shown), suggesting that this is the common phenotype of U937 cells and that the CD59$^+$ subline has arisen during culture. Comparison with other cell lines showed that both U937 sublines differed markedly from HL60, Molt4 and K562 in their surface antigen expression (data not shown).

When the two U937 sublines were tested for their sensitivity to killing by human C, they were found to be equally sensitive to complete serum but the CD59$^+$ cell line was also efficiently killed by C9-depleted serum (Fig. 2). This observation confirms an earlier report which showed that U937 cells were highly susceptible to killing by serum in the absence of C9.[5] Blocking of CD59 on the CD59$^+$ U937 with mAb BRIC229 did not further enhance C susceptibility showing that CD59 on this cell line was not functionally active. CD59 on these cells was recognized by all monoclonal and polyclonal antibodies by FACScan and we have shown elsewhere that CD59 could be immunoprecipitated from the CD59$^+$ cell line.[3] However U937 CD59 could not be detected in a Western blot using several mAb recognizing different epitopes and polyclonal antibody and using the ultrasensitive enhanced chemiluminescence (ECL) technique even after over-exposure of the film (Fig. 7). In contrast, CD59 from HL60, K562 and, to a lesser extent, Molt4 cells reacted strongly on Western blots after electrophoresis under non-reducing conditions. All these observations indicate that the CD59 expressed on the U937 cell line is functionally inactive and the loss of immunogenic material after SDS denaturation suggests that this CD59 is structurally less stable than on other cell types. Deglycosylated CD59 is still recognized by the mAb[13] indicating that the differences in blotting of CD59 from the various cell lines is not due to differences in glycosylation. None of the available antibodies reacts with reduced CD59 on Western blotting indicating that the antibodies recognize epitopes which are lost on disulphide-bond cleavage. It is possible that abnormal disulphide bonding in the U937 CD59 renders these epitopes sensitive to denaturation by SDS even in the absence of reducing agent.

Addition of human CD59 to the U937 cell line in order to study the inhibitory activity of exogenous CD59 showed that the $CD59^+$ cell line incorporated more CD59 but was much less efficiently protected than the $CD59^-$ U937 cell line (Figs 5 and 6). The endogenous CD59, which was shown to be functionally inactive may still have the ability to bind into the MAC in a non-inhibitory fashion, thereby competing with the active exogenous CD59 and preventing efficient inhibition.

Our initial aim was to use the $CD59^-$ and $CD59^+$ sublines to examine the role of CD59 in recovery from C attack which we suggest is a second means by which CD59 limits C killing. Although it is likely that U937 CD59 is inactive, the possibility still exists that the CD59 on the U937 cell line is capable of inhibiting MAC formation but fails to interface with the recovery machinery,[1] perhaps because it is not associated with the effector molecules (e.g. tyrosine kinases) implicated in recovery.

In order to characterize fully the dysfunctional CD59 on U937 cells we are currently working to purify the molecule and clone the cDNA from the $CD59^+$ U937 subline. Incorporation of the purified inhibitor in guinea-pig erythrocytes will provide definitive evidence about the functional activity of this form of CD59. Aberrant forms of CD59 may exist on other cell types and should be considered in cells susceptible to killing by the MAC. The observation that the abnormal CD59 interferes with C inhibition by active incorporated CD59 is particularly noteworthy and may provide important clues to the mode of action of CD59.

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