Enhanced complement susceptibility of avidin–biotin-treated human erythrocytes is a consequence of neutralization of the complement regulators CD59 and decay accelerating factor

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Biotinylation of erythrocytes (E) followed by avidin cross-linking at specific sites has been suggested as a novel means of drug delivery. Upon avidin cross-linking, biotinylated E become complement-activating and highly susceptible to complement lysis, thus bringing about release of entrapped drug. We set out to examine the mechanisms of this biotin–avidin-induced lytic susceptibility, focusing on the effects of biotinylation and avidin cross-linking on the major E complement regulatory molecules, decay accelerating factor (DAF) and CD59. We demonstrate here that biotinylation of E, which does not render them complement activating, partially inhibits DAF but has little effect on CD59. Subsequent cross-linking with avidin causes complete inhibition of DAF and near complete loss of CD59 activity. Following cross-linking, DAF and CD59 become associated in high molecular mass avidin-containing complexes on the membrane. Incorporation of physiological amounts of CD59 into the membranes of biotinylated and avidin cross-linked E is sufficient to render these cells resistant to complement lysis whereas incorporation of DAF has relatively little effect. An understanding of the molecular mechanisms underlying complement susceptibility of biotin–avidin treated E should allow a rational design of strategies for drug delivery using E or other large, potentially complement-activating carriers.

INTRODUCTION

During the last few years the avidin–biotin complex has found wide application in different fields of biology and medicine [1]. The utility of avidin and biotin is based on the high affinity, high specificity and multivalency of the avidin–biotin interaction. Cells and other particles can be modified using biotin derivatives of different molecules, followed by avidin binding.

Our aim has been to utilize avidin–biotin interactions to deliver erythrocytes (E), carrying therapeutic agents, to specific sites *in vivo* [2]. A surprising finding from these early studies was that while biotinylated erythrocytes (BE) were not complement activating, subsequent binding of avidin to BE makes the cells efficient activators of the alternative pathway and additionally renders the cells highly susceptible to complement lysis [2,3]. These effects were shown to be independent of the charge on the avidin and of the sugar moiety, but were dependent on the mode and site of avidin–biotin binding to the membrane [3]. Avidin cross-linking on E biotinylated on NH_2-groups rendered cells complement sensitive, whereas attachment of avidin through a phospholipid derivative of biotin or through the cross-linking reagent tannin in the E membrane did not increase complement susceptibility [4,5]. The degree of complement susceptibility was also dependent on the length of the biotin residue attached through NH_2-groups on the E; the *N*-caprolamine-succinimide ester of biotin caused more efficient lysis than the *N*-hydroxy-succinimide ester, a consequence of the aminocaproate group which provides an additional 6 Å spacer arm, placing the biotin moiety further from the membrane [3]. Multivalency of avidin attachment to biotin on the E membrane also caused increased complement susceptibility [3]. Recently, we have begun to examine whether this phenomenon can be utilized to cause release of entrapped agent at targeted sites [6].

The precise mechanism by which avidin cross-linking of biotin on the E membrane causes activation of the alternative pathway and increased lytic susceptibility have yet to be ascertained. One possible explanation of the enhanced lytic susceptibility is that the cross-linking event inactivates membrane complement regulatory proteins [3,6]. Complement activation is controlled on the surface of E and other cells by several membrane proteins acting at two distinct stages in the activation cascade [7,8]. The complement 3 (C3) and C5 cleaving enzymes of the activation pathways are regulated by three molecules, decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and complement receptor 1 (CR1, CD35). The membrane attack pathway is regulated by two molecules, CD59 and homologous restriction factor (HRF). E do not express MCP and express CR1 in very low amounts (100–1000 copies per cell; [9,10]), probably insufficient to influence lysis [11,12]. HRF is poorly characterized and is probably of little relevance to E protection [13]. The major complement protective proteins on human E are therefore DAF (E expression 3.3×10^3 copies per cell; [14]) and CD59 $\{(1–4) \times 10^4$ copies per E; [15,16]$\}$. This contention is supported by the observation that E from patients with paroxysmal nocturnal haemoglobinuria (PNH), which are deficient in the glycosylphosphatidylinositol (GPI)-linked inhibitors DAF and CD59 but express CR1 in normal amounts, exhibit greatly enhanced lytic susceptibility [17].

The purpose of the present investigation was therefore to examine whether the enhanced complement susceptibility of E following biotin–avidin treatment was caused by the effects of

Abbreviations used: E, erythrocyte; BE, biotinylated E; AvBE, avidin treated BE; C, complement; DAF, decay accelerating factor; APB, alternative pathway buffer; MAC, membrane attack complex of complement; mAb, monoclonal antibody; VBS, veronal-buffered saline; MCP, membrane cofactor protein; CR1, complement receptor 1; HRF, homologous restriction factor; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal haemoglobinuria; FACS, fluorescence-activated cell sorting; NHS, normal human serum; FITC, fluorescein isothiocyanate; HRPO, horseradish peroxidase.

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biotin–avidin on the function of DAF and CD59. We demonstrate here that E treatment with the *N*-hydroxysuccinimide ester of biotin followed by avidin, cross-links both DAF and CD59 and destroys their complement-inhibiting activity. That this event is primarily responsible for the increased lytic susceptibility of avidin-treated BE is confirmed by the observation that incorporation of purified inhibitors into the treated cells decreases complement susceptibility.

MATERIALS AND METHODS

Chemicals and reagents

(6-Biotinylamido)-*N*-hydroxysuccinimide ester and hen egg avidin were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). PBS (10 mM sodium phosphate/140 mM NaCl, pH 7.4) was supplied in tablet form by Oxoid Ltd. (London, U.K.). Alternative pathway buffer was Veronal-buffered saline (VBS) (5 mM sodium barbitone/140 mM NaCl), pH 7.4, containing 7 mM $MgSO_4$ and 10 mM EGTA. Fluorescence-activated cell sorting (FACS) buffer was prepared from PBS containing 1% BSA and 0.1% NaN_3. Reagents for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.).

Blood, sera, antibodies and other proteins

Blood from healthy volunteers of blood group A or AB was collected in Alsever's solution (114 mM citrate/27 mM glucose/72 mM NaCl, pH 6.1) and stored at 4 °C. Normal human serum (NHS) was obtained from volunteers and stored in aliquots at −70 °C until use. Human serum depleted of C8 (HS-C8) and C9 (HS-C9) were produced by passage of human serum over immunoaffinity columns of anti-(human)-C8 or anti-(human)-C9 antibodies respectively as previously described [18,19]. Human CD59 was purified from E membranes by affinity chromatography as previously described [20]. DAF was affinity purified from the same membranes using the monoclonal antibody (mAb) BRIC110 (IgG1; IBGRL, Elstree, Herts., U.K.) immobilized on CNBr-activated Sepharose 4b (Pharmacia, Milton Keynes, Bucks., U.K.). Cobra venom factor was purified from venom of the Thai cobra, *N. naja kaouthia* (V9125; Sigma, St. Louis, MO, U.S.A.) as described [21]. Goat (anti-mouse)-IgG-horseradish peroxidase (HRPO) and streptavidin-HRPO were obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.) and rabbit (anti-mouse)-IgG-fluorescein isothiocyanate (FITC) from Dako (High Wycombe, Bucks., U.K.). IgM anti-human blood group A was from Sera-Lab (Crawley Down, Sussex, U.K.). BRIC229 (anti-CD59; mouse mAb IgG2b) and BRIC216 (anti-DAF; mouse mAb IgG1) were from IBGRL.

Biotinylation and avidin treatment of E

Biotinylation of E with (6-biotinylamido)-*N*-hydroxysuccinimide ester to form BE and subsequent avidin binding to form avidin-coated BE (AvBE) were performed as described previously [2]. In brief, 0.1 ml of 0.1 M sodium tetraborate (pH 9.0) and 3 μl of the biotin ester (0.1 M in dimethylformamide) were mixed with 1 ml of a 10% suspension of E in PBS. After 20 min of incubation at room temperature, BE were washed four times with PBS. Avidin (5 μl of 1 mg/ml in PBS) was added to 100 μl of a 10% suspension of BE and incubated for 20 min at 4 °C. AvBE were washed three times in PBS and resuspended in VBS.

Haemolytic assay

Haemolytic assays were carried out in 96-well microtitre plates. E, BE or AvBE from a donor of blood group A or AB were antibody-sensitized by incubation with IgM anti-A (1:1000 in VBS) for 10 min at 37 °C. This antibody is stated by the manufacturers to be non-complement-fixing but in our hands efficiently activates complement on E of group A and AB. Aliquots (50 μl) of sensitized cells (2% suspension in VBS) were incubated with dilutions of NHS (100 μl) as a source of complement for 60 min at 37 °C. When avidin-induced lysis was measured, unsensitized AvBE were incubated with dilutions of NHS as above. Plates were spun to remove intact cells and the absorbance in the supernatant was measured at 414 nm. E incubated without NHS were used as the standard for zero lysis and water-lysed E were used as the standard for 100% lysis.

Analysis of effects of anti-inhibitor antibodies on lysis of E, BE and AvBE by complement

E, BE and AvBE (2% suspension in PBS), either unsensitized or sensitized with anti-A as described above were separately incubated with anti-CD59 (BRIC229; 10 μg/ml), anti-DAF (BRIC216; 10 μg/ml plus BRIC110; 10 μg/ml) or a combination of all antibodies (10 μg/ml of each antibody) in VBS for 30 min at 37 °C. After washing three times in PBS, cells were resuspended in VBS, incubated with dilutions of NHS for 60 min at 37 °C and lysis was measured as described above. The anti-DAF antibodies used recognize different parts of the DAF molecule [22] and together inhibit DAF function far better than either alone (A. B. Zaltzman, C. W. Van den Berg and B. P. Morgan, unpublished work).

Analysis of susceptibility to complement lysis induced by avidin binding after C5b-7 site formation

Sensitized E or BE (2% suspension in VBS) were incubated for 60 min at 37 °C with C8-depleted serum (1:5 v/v) in VBS. The intermediates so formed (EC5b-7, BEC5b-7) were washed three times with PBS. A portion of the BEC5b-7 was further treated with avidin to form Av(BEC5b-7). The intermediates were then incubated for 60 min at 37 °C with NHS (in PBS containing 5 mM EDTA) as a source of C8 and C9. Percentage lysis was determined as described above.

Effect of incorporation of CD59 and DAF on complement lysis of AvBE

BE (2% suspension in PBS) were incubated with purified human CD59 (5 μg/ml final concentration) and/or DAF (20 μg/ml final concentration) for 1 h at 37 °C. These conditions and concentrations were chosen in preliminary experiments to produce a doubling of the amount of the inhibitors on the cell membrane as assessed by flow cytometry. After washing three times in PBS, E were treated with avidin by the standard procedure to cross-link endogenous (biotinylated) but not incorporated (non-biotinylated) proteins, and susceptibility to complement was determined as described above.

SDS/PAGE and Immunoblotting

SDS/PAGE was carried out using standard methods [23] under non-reducing conditions. After treatment of E with biotin and avidin, cells were washed in PBS and lysed by adding 10 volumes of 10 mM Na-phosphate, pH 7.4 (lysis buffer). Ghosts were spun down in an Eppendorf centrifuge and washed 3× with the lysis buffer. The pelleted ghosts were mixed 1:1 with electrophoresis sample buffer. Samples (15 μl per lane) were loaded and run on 10 or 15% SDS/PAGE. Gels were electroblotted onto nitrocellulose using a Biorad electroblotter. Blots were blocked with 5% non-fat milk in PBS. CD59 and DAF were detected by

incubation for 2 h at room temperature with the specific mAbs (BRIC229 and BRIC216 respectively, both 1 μg/ml in PBS/5% milk). Blots were washed in PBS containing 0.1% Tween and incubated in GAM-IgG-HRPO (1:2000 in PBS/5% milk) for a further 2 hours at room temperature. Biotinylated proteins were detected by probing blots with streptavidin peroxidase in place of antibody (1 μg/ml; Sigma). All blots were developed using the enhanced chemiluminescence system (ECL; Amersham, Bucks., U.K.).

FACS analysis of CD59 and DAF expression

Expression of CD59 and DAF was measured using a Becton-Dickinson FACscan analyser. E, BE or AvBE (0.1% suspension in FACS buffer) were incubated with an equal volume of anti-DAF (BRIC216; 2 μg/ml) or anti-CD59 (BRIC229; 1 μg/ml) in FACS buffer for 30 min on ice. After washing three times in FACS buffer the cells were incubated with RAM-IgG-FITC (1:50 in FACS buffer) for 30 min on ice. The cells were then washed three times in the above buffer and fixed in 2% paraformaldehyde before FACS analysis.

RESULTS

Effects of anti-inhibitor antibodies on the lytic susceptibility of E, BE and AvBE

As in previous studies, biotin treatment alone did not induce spontaneous lysis of BE. However, antibody-sensitized BE were much more sensitive to lysis by NHS than antibody-sensitized E (Figure 1a). The antibodies BRIC229 and BRIC216/BRIC110, which are not themselves complement-fixing, neutralize CD59 and DAF respectively, and thereby render antibody-sensitized E and BE more susceptible to complement lysis (Figures 1b and 1c). Indeed, the combination of these antibodies rendered sensitized E almost as susceptible to lysis as AvBE (Figure 1b). The effect of blocking DAF on BE was small, suggesting that biotinylation effectively inhibited DAF (Figure 1c). In contrast to E and BE, AvBE were highly susceptible to complement lysis even in the absence of sensitizing antibody (Figure 1a). Anti-DAF antibodies caused no further enhancement of lytic susceptibility of AvBE, implying that this inhibitor had been completely neutralized by treatment with avidin–biotin (Figure 1d). However, anti-CD59 antibody did induce some increased complement susceptibility on AvBE, indicating that residual active CD59 was present on the cell (Figure 1d).

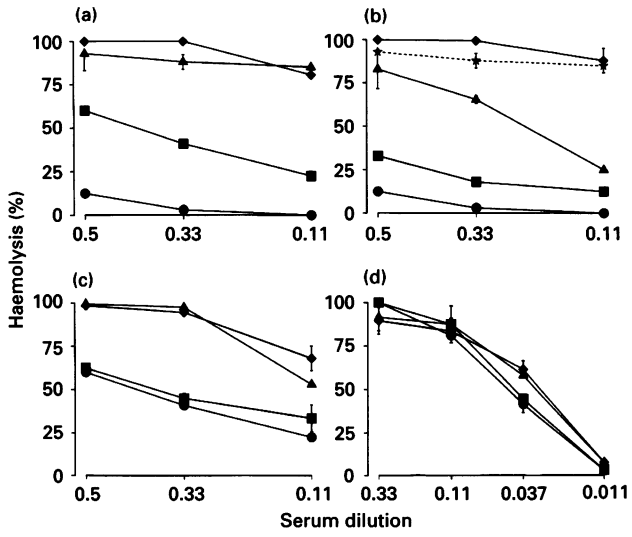

Figure 1 Complement lysis of E

(**a**) E, BE and AvBE were sensitized with anti-A antibody and incubated with various dilutions of serum at 37 °C. Lysis was measured as detailed in the Materials and methods section. Results are means of duplicates (±S.D.). ●, E; ■, BE; ▲, AvBE; ◆, AvBE unsensitized with anti-A. (**b**) Antibody-sensitized E were additionally incubated with blocking antibodies against CD59 (BRIC229, ▲) or DAF (BRIC216/BRIC110, ■) or a combination of these reagents (◆) (all at 10 μg/ml) or with buffer (control, ●), before incubation with serum and measurement of haemolysis. For comparison, lysis of antibody-sensitised AvBE is also shown (–★–). Results are means of duplicates (±S.D.). (**c**) Antibody-sensitized BE were incubated with buffer (control, ●), anti-DAF (■), anti-CD59 (▲) or a combination of these reagents (◆) and incubated with human serum. Results are means of duplicates (±S.D.). (**d**) Unsensitized AvBE were incubated with buffer (control, ●), anti-DAF (■), anti-CD59 (▲) or a combination of these reagents (◆) and then incubated with human serum. Note the lower serum dilutions in this frame. Results are means of duplicates (±S.D.).

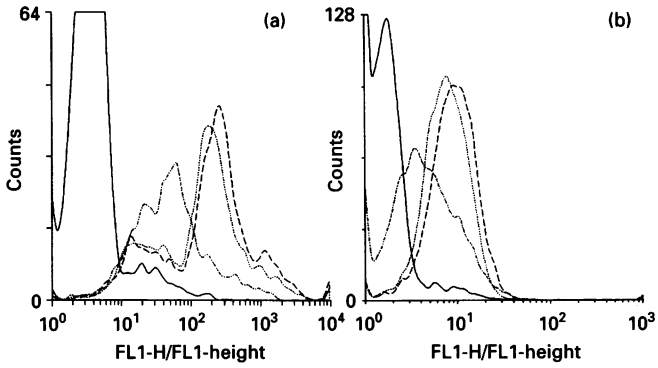

Figure 2 FACscan analysis

(**a**) Detection of CD59 using BRIC229 staining on E, BE and AvBE. (**b**) Detection of DAF using BRIC216 staining on E, BE and AvBE. E, broken lines; BE, dotted lines; AvBE, broken/dotted line. Units: *x*-axis, fluorescence (arbitrary units); *y*-axis, number of cells.

FACscan analysis of CD59 and DAF expression on E; effects of biotinylation and avidin treatment

The effects of biotinylation and of subsequent avidin cross-linking on the accessibility of E membrane CD59 and DAF to specific mAbs was studied by FACscan analysis. Biotinylation alone caused a decrease in antibody staining (median fluorescence of population) of about 20% for CD59 and nearly 30% for DAF. Biotinylation and avidin treatment further reduced binding of anti-CD59 (60% reduction in median fluorescence compared with control E) whereas binding of anti-DAF was not further diminished (32% reduction compared with controls) (Figure 2).

Effect of avidin binding after C5b-7 formation on lysis by the membrane attack complex of complement (MAC)

In order to focus on the effects of avidin cross-linking on MAC-mediated lysis, C5b-7 sites were formed on E or BE using C8-depleted serum. Neither EC5b-7 nor BEC5b-7, made as described in the Materials and methods section, were lysed upon subsequent addition of EDTA serum as a source of C8 and C9. However, BEC5b-7, subjected to avidin cross-linking after formation of C5b-7 sites, were rendered susceptible to lysis upon subsequent addition of C8 and C9 (Figure 3). BE cross-linked with avidin before C5b-7 site formation were, as anticipated, even more susceptible to lysis upon subsequent addition of C8 and C9 (results not shown)

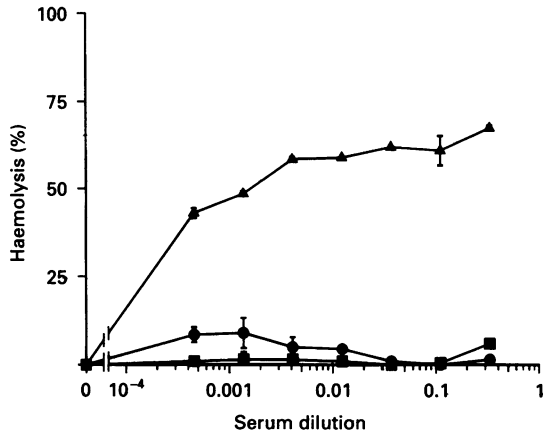

Figure 3 Effects of biotinylation and avidin cross-linking on MAC lysis

C5b-7 sites were formed on E and BE as detailed in the Materials and methods section. AvBE-bearing C5b-7 sites were produced by incubating BEC5b-7 with avidin. The intermediates were then incubated with various dilutions of EDTA serum as a source of C8 and C9 and haemolysis was measured. ●, EC5b-7; ■, BEC5b-7; ▲, AvBEC5b-7. Results are the mean of duplicates (±S.D.).

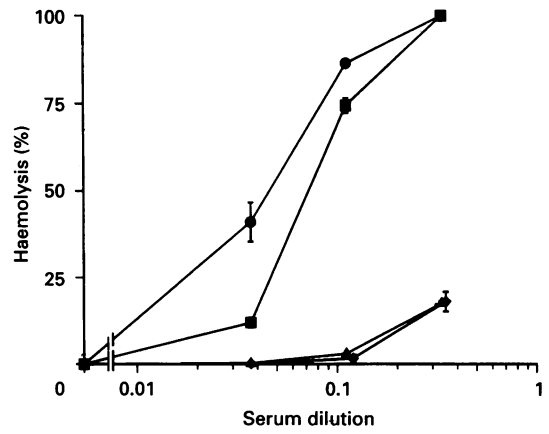

Figure 4 Effect of incorporation of CD59 and DAF upon lysis of AvBE

BE were incubated with buffer alone (●), with CD59 (5 μg/ml; ▲), DAF (20 μg/ml; ■) or both (◆) for 30 min at 37 °C. After washing, the cells were treated with avidin as described above, then incubated with various dilutions of NHS and lysis measured. Results are the mean of duplicates (±S.D.).

Inhibition of lysis by incorporation of CD59 and DAF in AvBE

BE were incubated with purified human CD59 and/or DAF at a concentration chosen to double membrane expression of the inhibitors. Endogenous, biotinylated membrane proteins were then cross-linked with avidin and the cells incubated with complement. Incorporation of either CD59 or DAF caused an inhibition of AvBE lysis by complement, CD59 being by far the more efficient, providing 100% protection under the conditions used (Figure 4).

Immunoblotting of CD59 and DAF on modified (BE, AvBE) and non-modified E

The effect of biotinylation and avidin binding on the electrophoretic mobility of CD59 and DAF was investigated by immunoblotting. Membrane ghosts from untreated E, BE and AvBE were prepared and subjected to SDS/PAGE and Western blotting. Probing of Western blots of E membranes with anti-CD59 or anti-DAF antibodies revealed bands of the appropriate size for the native proteins (Figures 5a and 5b; lane 1). Biotinylation (molecular mass of biotin ester used approx. 340 Da) did not cause a detectable change in the molecular masses of either CD59 or DAF (Figures 5a and 5b; lane 2) but for both DAF and CD59 the bands detected from BE were of diminished intensity when compared with the same amount of E membranes, despite identical protein loading. Probing of blots of AvBE membranes with anti-CD59 revealed a major high molecular mass band near the stacking/running gel interface with a weaker band in the correct region for native CD59 (Figure 5a, lane 3). Probing of blots of AvBE membranes with anti-DAF revealed only a high molecular mass band with nothing detectable at the correct region for native DAF (Figure 5b, lane 3). Probing of blots of BE membranes with streptavidin peroxidase revealed the full range of biotinylated proteins and highlighted bands of identical sizes with those detected by anti-CD59 and anti-DAF (indicated by arrows in Figures 5c and 5d), confirming that these proteins were efficiently biotinylated at the E surface.

DISCUSSION

The concept of using BE as carriers for therapeutic agents is attractive in that BE are easy to produce, can trap large amounts of the therapeutic agent and are well tolerated. In rats, BE have been shown to have a lifespan similar to that of unmodified E [24]. The therapeutic strategy would be, first to infuse a tissue- (or tumour-) specific antibody conjugated with biotin, follow this with avidin to localize avidin in the correct site and finally add BE which will localize in the same region as the antibody [6]. Release of therapeutic agent requires that the AvBE is lysed at that site. When biotin is attached through amino groups and at sufficient density to E membrane proteins, this will occur through the induction of complement-activating capacity and lytic susceptibility upon cross-linking with avidin [4–6,24]. In the present study we set out to investigate the roles of E membrane complement regulatory molecules in this avidin-induced complement lytic susceptibility.

The major membrane complement regulators on E are DAF, inhibiting at the C3/C5 convertase stage and CD59, which inhibits assembly of the lytic MAC [8]. Both of these proteins are abundantly expressed and anchored by GPI on the E surface and their importance in the regulation of E lysis is dramatically illustrated in the disease PNH, where both these proteins are deficient on the abnormal clone [17]. We therefore focused our studies on the effects of biotinylation and subsequent avidin cross-linking of these two molecules.

Although BE are not spontaneously lysed by complement, their susceptibility to lysis in the presence of a complement-activating antibody had not previously been examined. We found that BE were substantially more susceptible to antibody-mediated complement lysis than were untreated E, implying that biotinylation alone had a significant inhibitory effect on complement regulation on the E surface. The antibodies BRIC229 (IgG2b, anti-CD59) and the combination of BRIC110 and BRIC216 (both IgG1, anti-DAF) neutralize the complement-inhibiting activities of their respective ligands and render target cells more susceptible to complement lysis. None of these antibodies has any intrinsic complement activating capacity [25–27]. Antibody neutralization of CD59 on BE caused a further marked enhancement of lytic susceptibility, demonstrating that the activities of these inhibitors was not much affected by

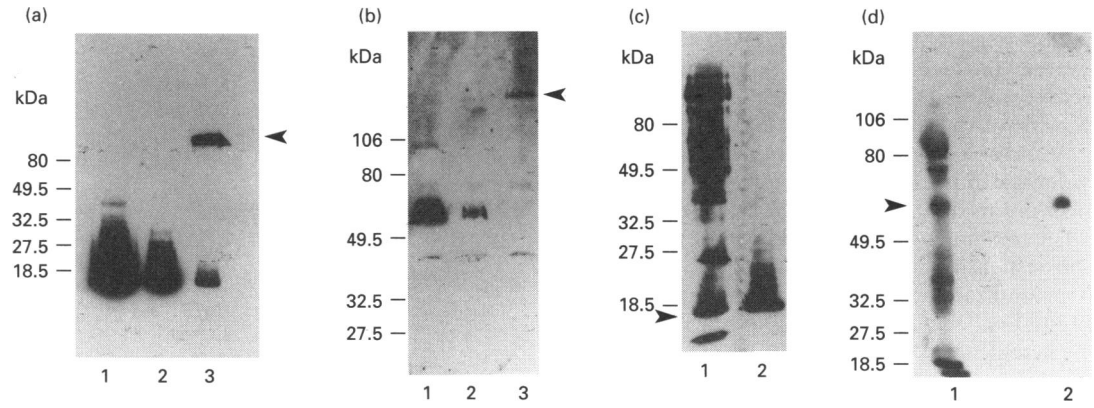

Figure 5 Western-blot analysis of E treated with biotin and avidin

(**a**) Effect of biotin and avidin on electrophoretic mobility of CD59. Washed membranes from treated and untreated were loaded onto 15% SDS/PAGE, run and blotted onto nitrocellulose. Blots were probed with BRIC229 anti-CD59. Lane 1, E; lane 2, BE; lane 3, AvBE. The high molecular mass CD59-containing complex is indicated by an arrow in lane 3. (**b**) Effect of biotin and avidin on electrophoretic mobility of DAF. Washed membranes were loaded onto 10% SDS/PAGE, run and blotted onto nitrocellulose. Blots were probed with BRIC110 anti-DAF. Lane 1, E; lane 2, BE; lane 3, AvBE. The high molecular mass DAF-containing complex is indicated by an arrow in lane 3. (**c**) BE membranes were run on 12.5% SDS/PAGE, blotted onto nitrocellulose and probed with streptavidin–HRP (lane 1) or anti-CD59 (lane 2). (**d**) BE membranes were run on 10% SDS/PAGE, blotted onto nitrocellulose and probed with streptavidin–HRP (lane 1) or anti-DAF (lane 2). Arrows in (**c**) and (**d**) indicate the position of biotinylated CD59 and DAF respectively.

biotinylation. In contrast, neutralization of DAF caused little further increase in lytic susceptibility, suggesting that DAF was strongly inhibited by biotinylation alone (Figures 1a and 1c). DAF operates predominantly in the classical pathway [8], which explains the finding that biotinylation had its most marked effect on lysis of antibody-sensitized E (classical pathway; Figure 1a).

AvBE spontaneously activated complement and were easily lysed even in the absence of activating antibody. Addition of anti-DAF antibody to AvBE caused no further enhancement of lytic susceptibility, implying that this inhibitor was completely blocked by avidin–biotin treatment, whereas addition of anti-CD59 did further increase lysis, indicating that CD59 was incompletely blocked by avidin–biotin (Figures 1a and d). E express about 3×10^3 molecules of DAF and between 1×10^4 and 4×10^4 molecules of CD59 [14–16]. Given this almost 10-fold excess of CD59 and its smaller molecular size (approx. 20 kDa as opposed to 60 kDa for DAF) it is perhaps not surprising that neutralization by avidin–biotin was less complete. FACScan analysis revealed that biotinylation alone caused a decrease in antibody staining of about 20% for CD59 (BRIC229) and nearly 30% for DAF (BRIC216); subsequent cross-linking with avidin further reduced binding of anti-CD59 (60%) whereas binding of anti-CD55 was little changed (32%) (Figure 2). Hence, complete inhibition of DAF activity was achieved with only a 32% loss of the BRIC216 epitope whereas CD59 was incompletely inhibited despite a 60% loss of the BRIC229 epitope. These findings again reflect the difference in size between these two proteins.

In order to confirm that CD59 was significantly inhibited on AvBE we made C5b-7 sites on BE before cross-linking with avidin. BE bearing C5b-7 sites were not lysed upon subsequent addition of C8 and C9 whereas BEC5b-7 cross-linked with avidin were efficiently lysed by C8 and C9. We have previously demonstrated that AvBE are not more susceptible to 'non-specific' lysis mediated through osmotic shock, melittin or perforin [6]. These results thus demonstrate that avidin cross-linking does neutralize a substantial proportion of the CD59 and render E susceptible to MAC lysis (Figure 3). BE which were cross-linked with avidin before the formation of C5b-7 sites were extremely susceptible to MAC lysis, presumably as a consequence of increased complement activation and DAF neutralization, allowing the formation of more C5b-7 sites on the cell.

The fate of CD59 and DAF on the membranes of BE and AvBE was further explored by immunoblotting. Biotinylation (molecular mass of biotin moiety approx. 340 Da) caused no discernible alteration in the molecular mass of either DAF or CD59 (Figures 5a and 5b; lane 1), suggesting that only a few copies of biotin were bound per protein molecule. However, the signal obtained on blotting was diminished, suggesting that there was some hindrance of antibody binding to biotinylated protein, as was noted for FACS analysis. Avidin cross-linking caused a dramatic change in the pattern for DAF and CD59; all the DAF migrated as a high molecular mass complex (> 200000 Da), as did the bulk of the CD59 (Figures 5a and 5b; lane 3), indicating that these two proteins were entrapped in the same or similar complexes on the E surface. Probing of blots of BE membranes with streptavidin peroxidase revealed the full range of biotinylated proteins and highlighted bands of identical sizes with those detected by anti-CD59 and anti-DAF (indicated with an arrow in Figures 5c and 5d), demonstrating that these proteins were efficiently biotinylated at the E surface.

Although the above findings implicate neutralization of DAF and CD59 in the enhanced complement lytic susceptibility of AvBE they do not rule out the possibility that other surface changes contribute. In order to assess this possibility, purified CD59 and DAF were incorporated into BE at doses sufficient to double the surface expression of these inhibitors. Incorporation of either DAF or CD59 caused diminished complement lytic susceptibility when the cells were subsequently cross-linked with avidin, CD59 being the more efficient inhibitor (Figure 4). However, even combined incorporation of the two inhibitors at a dose sufficient to double inhibitor levels on the cells ([27] and C. W. Van den Berg and B. P. Morgan, unpublished work) did not decrease lytic susceptibility to the level of untreated BE, suggesting that additional factors were also involved. It is possible that HRF is one of these factors but HRF plays a minor role in the protection of E [13], hence other factors may also contribute.

In the present study we do not directly address the mechanism by which avidin cross-linking causes BE to become activators of

the alternative pathway. One possibility is that neutralization of DAF, described above, is itself sufficient to render the surface activating. However, this is unlikely as biotinylation alone apppears to effectively inhibit DAF, and neither this nor neutralization of DAF with mAb causes E to activate complement spontaneously. A direct complement-activating effect of the bound avidin is eliminated by the observation that avidin linked to E through other means does not render the cells complement activating [4,5,24]. Cross-linking of other biotinylated proteins is thus likely to be responsible for the activation event which, combined with neutralization of the complement regulatory molecules CD59 and DAF, renders AvBE exquisitely susceptible to destruction by complement.

This work was funded by The Wellcome Trust.

REFERENCES

1 Wilchek, M. and Bayer, E. A. (1988) Anal. Biochem. **171**, 1–32
2 Muzykantov, V., Sakharov, D., Smirnov, M., Samokhin, G. and Smirnov, V. (1986) Biochim. Biophys. Acta **884**, 335–363
3 Muzykantov, V., Smirnov, M. and Samokhin, G. (1991) Blood **78**, 2611–2618
4 Muzykantov, V., Smirnov M. and Klibanov, A. (1993) J. Immunol. Methods **158**, 183–190
5 Muzykantov, V., Smirnov, M., Zaltzman, A. and Samokhin, G. (1993) Anal. Biochem. **208**, 338–342
6 Muzykantov, V., Zaltsman, A., Van den Berg, C. W., Smirnov, M. and Morgan, B. P. (1994) Immunology, in the press
7 Morgan, B. P. (1992) Curr. Top. Microbiol. Immunol. **178**, 115–140
8 Morgan, B. P. and Meri, S. (1994) Springer Semin. Immunopathol. **15**, 369–396
9 Pangburn, M. K. and Müller-Eberhard, H. J. (1984) Spring. Sem. Immunopathol. **7**, 163–192
10 Fearon, D. T. (1980) J. Exp. Med. **152**, 20–30
11 Rothman, I. K., Gelfand, J. A., Fauci, A. S. and Frank, M. M. (1975) J. Immunol. **115**, 1312–1315
12 Pangburn, M. K., Schreiber, R. D. and Müller-Eberhard, H. J. (1983) Proc. Natl. Acad. Sci., U.S.A. **80**, 5430–5434
13 Watts, M. J., Dankert, J. R. and Morgan, B. P. (1990) Biochem. J. **265**, 471–477
14 Kinoshita, T., Medof, M. E., Silber, R. and Nussenzweig, V. (1985) J. Exp. Med. **162**, 75–92
15 Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, M. G., Waldmann, H. and Lachmann, P. J. (1990) Immunology **71**, 1–14
16 Fletcher, A., Bryant, J. A., Gardner, B., Judson, P. A., Spring, F. A., Parson, S. F., Mallinson, G. and Anstee, D. J. (1992) Immunology **75**, 507–512
17 Rosse, W. F. (1993) Semin. Hematol. **30**, 219–231
18 Abraha, A., Morgan, B. P. and Luzio, J. P. (1988) Biochem. J. **251**, 285–292
19 Morgan, B. P., Daw, R. A., Siddle, K., Luzio, J. P. and Campbell, A. K. (1983) J. Immunol. Methods **64**, 269–281
20 Davies, A., Simmons, D. L., Hale, G., Harrison, R. A., Tighe, H., Lachmann, P. J. and Waldmann, H. (1989) J. Exp. Med. **170**, 673–674
21 Vogel, C. W. and Müller-Eberhard, H. J. (1984) J. Immunol. Methods **73**, 203–220
22 Coyne, K. E., Hall, S. E., Thompson, E. S., Arce, M. A., Kinoshita, T., Fujita, T., Anstee, D. E., Rosse, W. and Lublin, D. M. (1992) J. Immunol. **149**, 2906–2913
23 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
24 Muzykantov, V., Seregina, N. and Smirnov, M. (1992) Int. J. Art. Organs **15**, 622–627
25 Rooney, I. A. and Morgan, B. P. (1992) Immunology **76**, 541–547
26 Tandon, N., Morgan, B. P. and Weetman, A. P. (1992) Immunology **75**, 372–377
27 Van den Berg C. W. and Morgan, B. P. (1994) J. Immunol. **152**, 4095–4101

Received 30 September 1994/23 December 1994; accepted 9 January 1995