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Binding of human and rat CD59 to the terminal complement complexes

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SUMMARY

CD59-antigen (protectin) is a widely distributed glycolipid-anchored inhibitor of complement lysis. CD59 interacts with complement components C8 and C9 during assembly of the membrane attack complex (MAC). To evaluate species specificity of these interactions we have in the present study examined cross-species binding of isolated human and rat CD59 to the terminal complement components C8 and C9. By using primarily soluble CD59 isolated from urine ($CD59_U$) potentially non-specific binding interactions of the phospholipid portion of the membrane forms of CD59 could be avoided. Sucrose density gradient ultracentrifugation analysis showed that human $CD59_U$ bound to both human and rat C8 in the SC5b–8 complexes. Similar binding occurred when rat $CD59_U$ was used. The degree of binding did not significantly differ between the heterologous and homologous CD59–C8 combinations. C9 from both species inhibited the binding of CD59 to soluble SC5b–8. In ligand blotting analysis human and rat $CD59_U$ bound to human and rat C8$\alpha\gamma$-subunit and C9. Binding of human and rat $CD59_U$ was stronger to human than rat C9. In plate binding assays the erythrocyte form of CD59 ($CD59_E$) bound to both human and rat C8. Binding of $CD59_E$ to heterologous C9 was considerably weaker than to homologous C9. Our results imply that the reciprocal binding sites between C8 and CD59 and to a lesser degree between CD59 and C9 are conserved between human and rat. Interactions of CD59 with the terminal C components are thus species selective but not 'homologously restricted'.

INTRODUCTION

The complement system in human plasma is a first line defence system against invading micro-organisms and a central mediator of inflammation. To avoid destruction by autologous complement normal human erythrocytes and nucleated cells are protected against lysis by specific membrane proteins. CD59-antigen (protectin) is the major inhibitor of the membrane attack complex of complement (MAC). CD59 is anchored to cell membrane phospholipids via a glycophosphoinositol (GPI)[3] moiety.[1–3] Haemopoietic and endothelial cells in most tissues express CD59 on their membranes.[3–8] CD59 inhibits complement lysis by binding to the C5b–8 and C5b–9 complexes and preventing formation of the polymeric C9 complex during the final steps of MAC assembly on cell membranes.[9,10]

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Abbreviations: C8DS, C9DS, C8 or C9 depleted serum; C89DS, serum depleted of C8 and C9; $CD59_E$, CD59 isolated from erythrocyte membranes; $CD59_U$, CD59 isolated from urine; GPI, glycophosphoinositol; MAC, membrane attack complex of complement; SC5b–9, soluble terminal complement complex; TCC, terminal complement complex.

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Most studies so far have suggested that the activity of CD59 is species restricted, as indicated by the ability of human CD59 to preferentially inhibit complement lysis when C8 and C9 are of human or primate origin.[2,10–12] The studies of Rollins *et al.*[13] showed that inhibition of C5b–9 induced haemolysis was maximal when C8 and C9 were derived from human or baboon serum. By contrast, human CD59 showed a reduced activity when C8 and C9 were derived from dog or sheep serum and no activity when C8 and C9 were from rabbit or guinea-pig serum. Rollins *et al.*[13] also demonstrated equal binding of ^{125}I-labelled erythrocyte CD59 ($CD59_E$) to C5b–9 complexes containing C8 and C9 from baboon or human serum. No binding was seen when guinea-pig or rabbit serum was used as a source of C8 and C9. A view in contrast to the 'homologous restriction' concept was provided by a recent study which suggested that CD59 analogues from rat, sheep and pig protected guinea-pig erythrocytes against lysis by complement from homologous and heterologous sources.[14] However, when erythrocytes from species other than guinea-pig were used the lysis inhibitory effect of incorporated CD59 was lost. This was suggested to be due to the presence of endogenous inhibitors that overcame the effect of the incorporated CD59.[14]

In our earlier study we showed that soluble human CD59 binds with high affinity to the terminal complement complex (TCC) intermediate SC5b–8.[15] Binding occurs predominantly during formation of the complex. The primary interaction site of CD59 in the soluble TCC appears to be on C8 for which

CD59 and C9 compete.[15] These experiments, however, did not exclude an interaction of CD59 with C9 in membrane-associated complexes, which has been demonstrated in other studies.[9,13] The binding sites of CD59 on human C8 and on C9 have been localized to the C8α-chain and C9b fragment.[16] Recent studies have mapped the CD59 binding site to residues 320–415 in C8α[17] and to residues 320–411 in C9b.[18] It has clearly been shown that human CD59 does not interact with rabbit C8[17,19] or C9.[16,20] On the other hand, Tomlinson *et al.*[21] recently showed that horse C9, which has a weak haemolytic activity against most mammalian erythrocytes, binds human CD59.

As is evident from above the degree of species selectivity of CD59 activity is still controversial, and the question needs to be examined at the level of specific protein–protein interactions. Comparison between CD59 molecules from different species will help in the identification of functionally important residues. The cDNA and amino acid sequences of rat CD59 have recently been determined.[22] Despite the low overall amino acid sequence similarity (44%) between rat and human CD59 several highly conserved stretches were apparent, particularly in the amino-terminal portion of the molecule. Since rat CD59 has been suggested to inhibit lysis by human complement we wanted to examine whether it directly interacts with the human terminal complement complexes and vice versa. The results show that the cross-species activity of CD59 does not apply only to species close to each other but is more random.

MATERIALS AND METHODS

Purification of human and rat CD59

Human erythrocyte CD59 ($CD59_E$) was purified from detergent-solubilized human red blood cell ghosts by YTH53.1-affinity chromatography as described.[9] Human urinary CD59 ($CD59_U$) was purified similarly from freshly voided urine obtained from healthy laboratory personnel.[15] Rat urinary CD59 was purified from mixed urine specimens collected from rats in the animal house of our department. The rat urine specimen containing a mixture of protease inhibitors and sodium azide (0·02%) was run through a Büchner funnel following filtration in a 0·22 μm pore size Millipore filter system (Bedford, MA). After adjusting the pH to 7·4 the rat urine was run through a 6D1-Sepharose 4B affinity column. 6D1 is a mouse anti-rat CD59 monoclonal antibody (mAb) of the immunoglobulin G1 (IgG1) isotype.[22] After a thorough washing of the column the bound antigen was eluted with 0·1 M glycine buffer, pH 2·5, in 2 ml fractions into tubes containing 100 μl 1·0 m Tris buffer, pH 9·0. The samples were pooled, concentrated and desalted with a Sephadex G25 column (Pharmacia LKB, Uppsala, Sweden). In sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis[23] under reducing conditions the purified rat $CD59_U$ gave a single band with an apparent MW of 21 000. $CD59_U$, $CD59_E$ and rat $CD59_U$ were radiolabelled to specific activities of 10^6–10^7 c.p.m./μg with Na[^{125}I] using the Iodogen method (Pierce Chemical Co., Rockford, IL). Purity of the radiolabelled proteins was analysed by SDS–PAGE and autoradiography.

Complement proteins and complement-deficient sera

Normal human sera (NHS) were obtained from healthy laboratory personnel by venepuncture. Sera were depleted of C8 and C9 (C8DS, C9DS) by running them through anti-C8 and anti-C9 affinity columns as described.[24,25] C89 depleted serum (C89DS) was prepared by depleting C8DS of C9 by immunoprecipitation at 4° with an IgG fraction of a polyclonal goat anti-human C9 antibody (Quidel Inc.) and ultracentrifugation at 200 000 *g*. Human C8 and C9 were purchased from Quidel Corp. (La Jolla, CA). Haemolytically active rat C8 and C9 were purified from rat serum according to the method of Jones *et al.*[26] C8αγ and C8β-deficient sera were a kind gift from Professor Francesco Tedesco (Institute of Pathology, University of Trieste, Italy).[27–29]

Binding of rat and human $CD59_U$ to $SC5b–8_{hu}$, $SC5b–8_{rat}$, $SC5b–8_{rat}\ 9_{rat}$, $SC5b–8_{rat}\ 9_{hu}$ and $SC5b–8_{hu}\ 9_{rat}$ complexes

Binding of human and rat $CD59_U$ to $SC5b–8_{hu}$ or $SC5b–8_{rat}$ complexes was analysed as follows: 60 μl of C89DS serum was first reconstituted with $C8_{hu}$ or $C8_{rat}$ (80 μg/ml) in 20 μl of phosphate-buffered saline (PBS) or with PBS alone as a control. After adding human or rat ^{125}I–$CD59_U$ ($0{\cdot}65 \times 10^{-8}$ M, 60 000 c.p.m.) the mixtures were activated with inulin for 30 min at 37° in a final volume of 120 μl.

Binding of human and rat $CD59_U$ to $SC5b–8_{rat}\ 9_{rat}$, $SC5b–8_{rat}\ 9_{hu}$ and $SC5b–8_{hu}\ 9_{rat}$ complexes was analysed as follows. Sixty microlitres of human serum depleted of both C8 and C9 (huC89DS) was reconstituted with $C8_{rat}$ or $C8_{hu}$ (80 μg/ml) and $C9_{rat}$ or $C9_{hu}$ (80 μg/ml). The mixtures were activated with inulin for 30 min at 37° in a volume of 100 μl and incubated thereafter with 20 μl of ^{125}I-$CD59_U$ or rat ^{125}I-$CD59_U$ ($0{\cdot}65 \times 10^{-8}$ M, 60 000 c.p.m.) for 30 min at 37°. As a control, C9-depleted human serum was incubated with ^{125}I-labelled human or rat $CD59_U$ and activated with inulin. All experimental mixtures were applied on 10–50% sucrose density gradients prepared into PBS. The gradient tubes were ultracentifuged for 17 hr at 200 000*g* using a SW50·1 rotor (Beckman Instruments, Palo Alto, CA) at 4°. Fractions of 200 μl were collected and counted for radioactivity.

Binding of $CD59_U$ to terminal C complexes lacking C8αγ or C8β

C8αγ and C8β-deficient and normal human sera were depleted of C9 by immunoprecipitation at 4° with an IgG fraction of a polyclonal goat anti-human C9 antibody (Quidel Inc.) and ultracentrifugation at 200 000 *g*. Binding of human $CD59_U$ to TCC in C8αγ and C8β-deficient or C8αγ and C8β-deficient and C9-depleted sera was tested by incubating 60 μl of each serum with ^{125}I-$CD59_U$ ($0{\cdot}65 \times 10^{-8}$ M) and inulin for 30 min at 37° in a total volume of 120 μl. Binding of human $CD59_U$ to TCC in C8αγ and C8β-deficient and C9-depleted sera reconstituted with normal human C8 (80 μg/ml) was tested similarly. Normal human serum and C9-depleted NHS were used as controls. Binding was analysed by sucrose density ultracentrifugation as described above. The haemolytic activity of C8αγ and C8β-deficient sera with and without normal human C8 protein (Quidel Corp.) were tested on Quantiplate® Single Radial Diffusion Total Complement plates (Kallestad, Chaska, MN).

Ligand blotting assay for binding of human and rat CD59 to homologous and heterologous C8 and C9 immobilized on a nitrocellulose filter

Purified human and rat C8 and C9 were first denatured in non-reducing SDS buffer (containing 1% SDS) for 1 hr at 22°. Protein samples were run on a 10% SDS–PAGE gel whereafter the C8 and C9 protein bands were visualized with Coomassie

blue. Same amounts of proteins were transferred from other SDS–PAGE gels to nitrocellulose. After an overnight incubation in 3% bovine serum albumin (BSA)/PBS to prevent non-specific binding the filters were incubated for 2 hr at 37° with human or rat ^{125}I-CD59$_U$ or with human ^{125}I-CD59$_E$ diluted in 0·1% BSA, 0·1% Nonidet (NP40) in PBS. After washing to remove unbound ligand the membranes were analysed in a Bio-Imaging Analyser (Bas 1500 IP-reader, Fuji Photo Film Co., Japan) using Fujifilm MacBas 2·1 software. The images were printed on a 600 d.p.i. laser printer. As control proteins a mixture of high molecular weight standards was used. In some experiments the specificity of binding was examined by using a 100-fold excess of unlabelled CD59 during incubation with the labelled ligand.

Binding of human and rat CD59$_U$ to homologous and heterologous C8 and C9 immobilized on microtitre plates

A binding assay similar to that described by Ninomiya *et al.*[16,30] was used to study binding interactions of human CD59$_U$ and CD59$_E$ with isolated human and rat C8 or C9. Purified human and rat C8 and C9 were adsorbed to polystyrene microtitre plates (Nunc Inc.) by incubating (4°, overnight) 10 μg/ml of the proteins, in 0·1 M sodium bicarbonate, pH 8·2, on the plate wells. After incubation the wells were treated with 3% BSA in PBS and washed with 0·1% BSA, 0·1% NP40 in PBS. Next, 1·5–12 ng (12 500–100 000 c.p.m.) of ^{125}I-CD59$_U$ or ^{125}I-CD59$_E$ diluted in 0·1% BSA, 0·1% NP40 in PBS were added to the wells and incubated for 2 hr at 37°. After five washes with buffer the individual wells were cut out from the plate and counted for radioactivity. Specificity of the binding was tested using a 100-fold excess of unlabelled CD59. non-specific binding to BSA-coated wells was determined in each experiment and subtracted from the observed values.

RESULTS

Binding of rat and human CD59$_U$ to SC5b–8 complexes containing rat or human C8

In our recent study we demonstrated that human CD59 binds primarily to C8 in the soluble terminal complement complexes.[15] To examine binding of CD59 to heterologous C8 we performed fluid phase binding experiments using isolated, soluble human and rat CD59 and C89-depleted serum reconstituted with hu or rat C8. ^{125}I-labelled human and rat CD59$_U$ were observed to bind to both SC5b–8$_{hu}$ and SC5b–8$_{rat}$ complexes in reconstituted and inulin-activated sera (Fig. 1a and b). A similar result was obtained in two experiments performed in the same manner. Binding percentages ± SD were: 8·0 ± 1·0 (human C8 and human CD59$_U$), 7·8 ± 2·1 (rat C8 and human CD59$_U$), 8·3 ± 1·9 (human C8 and rat CD59$_U$), 6·8 ± 1·6 (rat C8 and rat CD59$_U$).

Inhibition of rat and human CD59$_U$ binding to soluble SC5b–8 by C9

In our earlier study we observed that human C9 in soluble SC5b–9 inhibited binding of CD59$_U$ to the complexes.[15] Now we reconstituted human C89DS with rat C8 and rat C9 or with rat C8 and human C9 or with human C8 and rat C9 to obtain SC5b–8$_{rat}$ 9$_{rat}$, SC5b–8$_{rat}$ 9$_{hu}$ and SC5b–8$_{hu}$ 9$_{rat}$ complexes after activation with inulin. It was found that neither rat nor human ^{125}I-CD59$_U$ bound to any of these complexes.

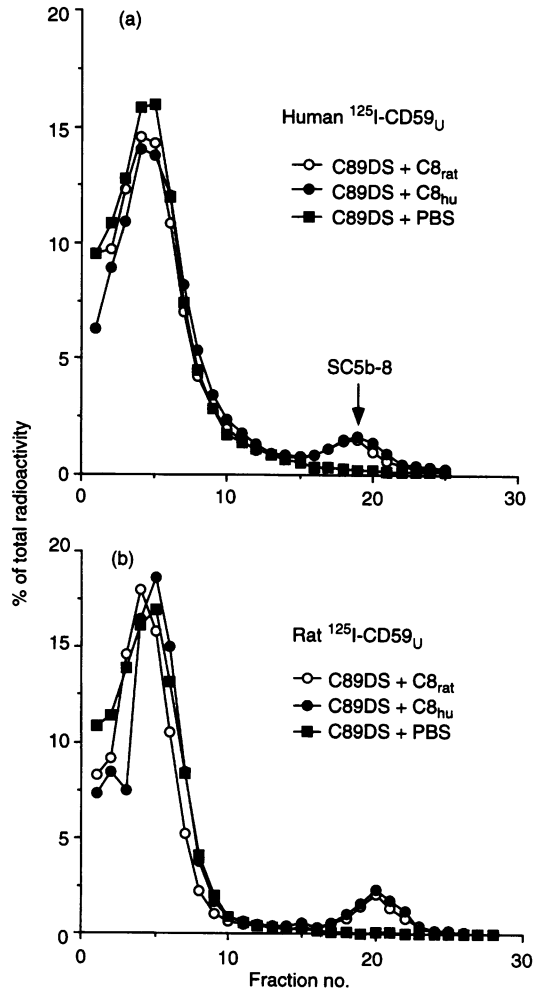

Figure 1. Binding of human (a) and rat CD59$_U$ (b) to SC5b–8$_{hu}$ and SC5b–8$_{rat}$ complexes. Human C89DS was incubated with C8$_{hu}$ (●), C8$_{rat}$ (○) or PBS (■) and activated (30 min, 37°) with inulin in the presence of human (a) or rat (b) ^{125}I-CD59$_U$. Mixtures were applied on 10–50% sucrose density gradients and centrifuged for 17 hr at 200 000 ***g***. Top of the gradient is to the left. The y-axis shows relative radioactivity in each of the fractions. Binding of ^{125}I-CD59$_U$ to SC5b–8 is detectable as a novel peak in fractions 17–21 (a) or 18–22 (b).

This suggests that human and rat C9 both inhibit binding of human and rat CD59 to SC5b–8. In simultaneous control experiments both human and rat CD59 bound to the SC5b–8$_{hu}$ complexes (Fig. 2a and b).

Lack of CD59$_U$ binding to TCC deficient in C8$\alpha\gamma$ or C8β

Patient sera deficient in C8$\alpha\gamma$ or C8β were next examined to see whether any interaction of ^{125}I-CD59$_U$ occurred with soluble TCC complexes generated in sera lacking C8$\alpha\gamma$ or C8β. Previous studies have suggested that binding of C8 to C5b-7 is mediated via the C8β-chain[31,32] and that CD59 recognizes a conformational epitope within human C8α.[17] No binding of ^{125}I-CD59$_U$ to the terminal complexes was observed when the deficient sera were activated with inulin (data not shown). When experiments were repeated after depleting C9 no binding occurred either (Fig. 3a). A third set of experiments

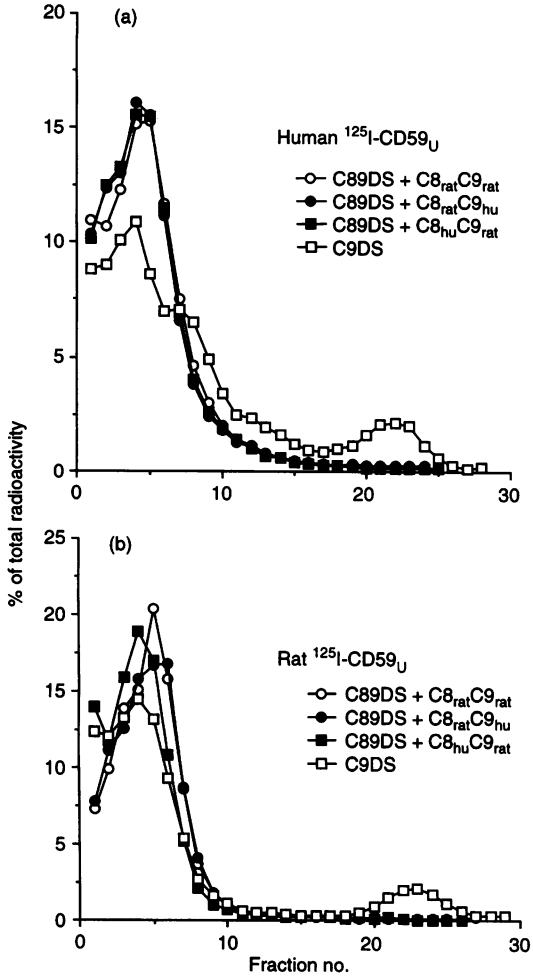

Figure 2. Analysis of binding of human (a) and rat $CD59_U$ (b) to SC5–8_{rat} $C9_{rat}$, SC5b–8_{rat} $C9_{hu}$ and SC5b–8_{hu} $C9_{rat}$ complexes. C89DS was first reconstituted with $C8_{rat}$ and $C9_{rat}$ (○), $C8_{rat}$ and $C9_{hu}$ (●) or $C8_{hu}$ and $C9_{rat}$ (■) and incubated with inulin for 30 min at 37° whereafter human (a) or rat (b) ^{125}I-$CD59_U$ was added following incubation for 30 min at 37°. As a control, human C9DS was activated with inulin in the presence of human (a) or rat (b) ^{125}I-$CD59_U$ (□).

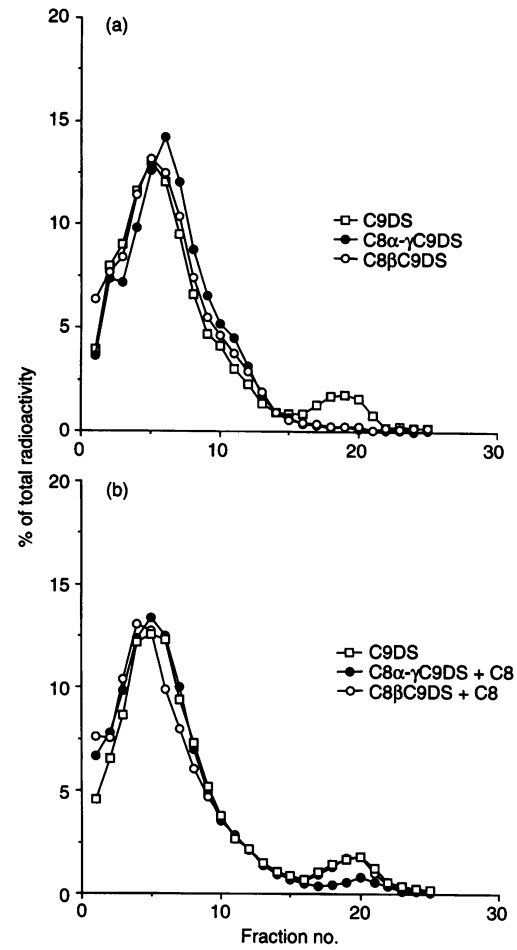

Figure 3. The effect of C8$\alpha\gamma$ and C8β deficiency on binding of $CD59_U$ to SC5b–8 complexes. (a) Inulin activated human C9-depleted C8$\alpha\gamma$- (●), C8β-deficient (○) sera and C9-depleted normal human serum (C9DS) were activated with inulin in the presence of ^{125}$CD59_U$ (□). (b) Human C9-depleted C8$\alpha\gamma$- (●) and C8β-deficient (○) sera or C9DS (□) were reconstituted with C8 (80 μg/ml) and activated with inulin. Binding of ^{125}I-$CD59_U$ to SC5b–8 was analysed as in Fig. 1.

was performed by reconstituting C9-depleted C8$\alpha\gamma$ or C8β-deficient sera with normal human C8. $CD59_U$ bound to both C8 reconstituted C8$\alpha\gamma$- and C8β-deficient sera, although binding was considerably stronger to the latter serum (Fig. 3b). A classical pathway haemolysis assay showed that both C8$\alpha\gamma$DS and C8βDS were haemolytically inactive prior to reconstitution with hu C8. However, after addition of an excess of human C8 protein to the deficient sera, the haemolytic complement activity was restored to levels equivalent with those in normal human sera.

Analysis of human and rat CD59 binding to C8 and C9 by ligand blotting

Because of the inability of CD59 to interact with C9 in the fluid phase TCC we examined binding of human and rat $CD59_U$ to C9 with a ligand blotting analysis modified from Ninomiya *et al.*[16] Human and rat $CD59_U$ as well as hu $CD59_E$ were isolated by affinity chromatography and radiolabelled with 125[I] (Fig. 4). Human and rat C8 and C9 were run on an SDS–PAGE gel and transferred to a nitrocellulose. When incubated on the nitrocellulose membrane human ^{125}I-$CD59_U$ was found to bind to both human and rat C8$\alpha\gamma$ but not to the C8β chain of either species. Binding to both human and rat C9 was also observed, but binding to human C9 was considerably stronger (Fig. 5a). Rat ^{125}I-$CD59_U$ bound to both human and rat C8$\alpha\gamma$ subunits. Like human $CD59_U$ rat ^{125}I-$CD59_U$ bound more strongly to human than rat C9 (Fig. 5c). The lipid-tailed human ^{125}I-$CD59_E$ bound to human and rat C8$\alpha\gamma$ and more strongly to human than rat C9 (Fig. 5d). Human and rat ^{125}I-$CD59_U$ and ^{125}I-$CD59_E$ bound also to *E. coli* β-galactosidase (MW 116200) and hen egg white ovalbumin (MW 42700) but not to other proteins in the high molecular weight standard lane.

Analysis of human and rat CD59 binding to C8 and C9 adsorbed on microtitre plates

Using a microtitre plate assay Ninomiya *et al.*[16] demonstrated that human $CD59_E$ bound to human C8 and C9 adsorbed on

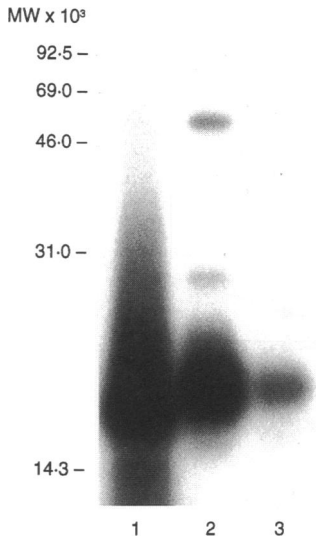

Figure 4. SDS–PAGE and autoradiography analysis of ^{125}I-labelled human $CD59_E$ ($\approx$100 000 c.p.m., lane 1), human $CD59_U$ (100 000 c.p.m., lane 2) and rat $CD59_U$ (30 000 c.p.m., lane 3).

plastic but not to rabbit C8 or C9. Our initial experiments performed with the non-lipid-tailed urinary form of CD59 failed to show any binding of ^{125}I-$CD59_U$ to either human or rat C8 or C9 (Fig. 6c and d). However, in subsequent experiments human ^{125}I-$CD59_E$ was observed to bind to both human and rat C8 and C9 (Fig. 6a and b). No significant difference in the binding of human $CD59_E$ to human versus rat C8 was observed in two different experiments (Fig. 6a). Binding to C9, however, seemed to favour the homologous protein since $CD59_E$ bound twice as strongly to human C9 as to rat C9 (Fig. 6b). Binding of ^{125}I-$CD59_E$ to human C8 and C9 was inhibited by a 100-fold excess of unlabelled $CD59_E$, implying specific association (Fig. 6a and b).

DISCUSSION

The contribution of CD59 to homologous restriction of complement lysis has recently been under reassessment. Previously, human CD59 had been reported to inhibit primarily human complement and complement from closely related species.[2,10–12,33] In contrast to this, recent experiments suggested that rat, sheep and pig CD59, when incorporated into guinea pig (GpE), protected the cells against complement from several species including human, rat, sheep, pig, rabbit, goat, bovine, mouse and guinea-pig.[14] It is apparent that evaluation of the degree of species selectivity of CD59 activity will require further studies and direct analysis of the binding interactions of CD59 with its target molecules. These studies on cross-species interactions are needed because they can give information about residues that are important (or unimportant) in the activity and species selectivity of CD59. Recent cloning of rat and many primate CD59 molecules allows comparison at the sequence level.[22] Using molecular modelling techniques this information can also be extrapolated to the three-dimensional structure of CD59[34,35] where it is possible to evaluate potentially active residues on the surface of the CD59 molecule. This information, in turn, could prove useful in the design of therapeutic inhibitors of complement lysis.

In the present study we show for the first time that human $CD59_U$ binds to both human and rat C8 in the SC5b–8 complexes. Similar binding occurred when rat $CD59_U$ was used. The degree of binding was not significantly different between heterologous and homologous CD59–C8 combi-

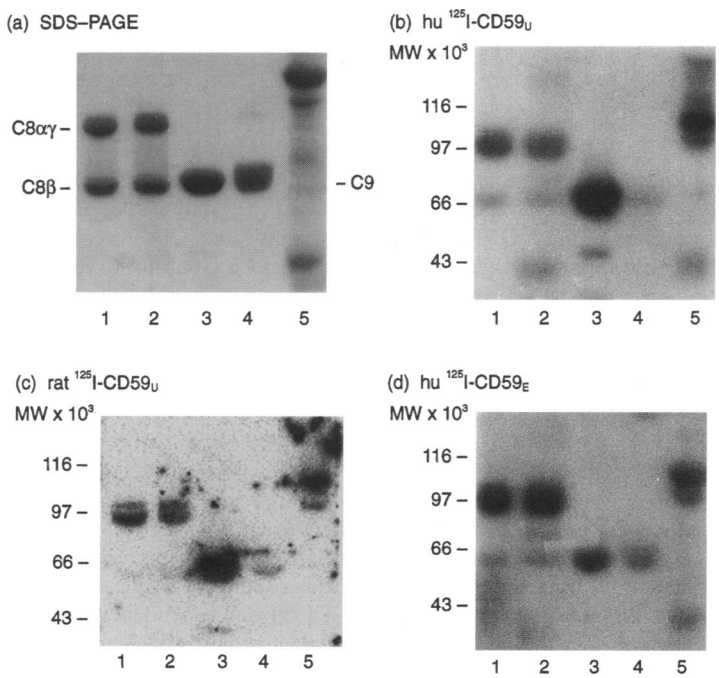

Figure 5. Analysis of CD59 binding to human versus rat C8 and C9. Multiple samples of human (lane 1) or rat C8 (lane 2) and human (lane 3) or rat C9 (lane 4) were run in SDS–PAGE gels (10%) and transferred to nitrocellulose filters. One gel was stained with Coomassie blue (a). The nitrocellulose filters were incubated with human ^{125}I-$CD59_U$ (b), rat ^{125}I-$CD59_U$ (c) or human ^{125}I-$CD59_E$ (d), washed extensively and subjected to autoradiography.

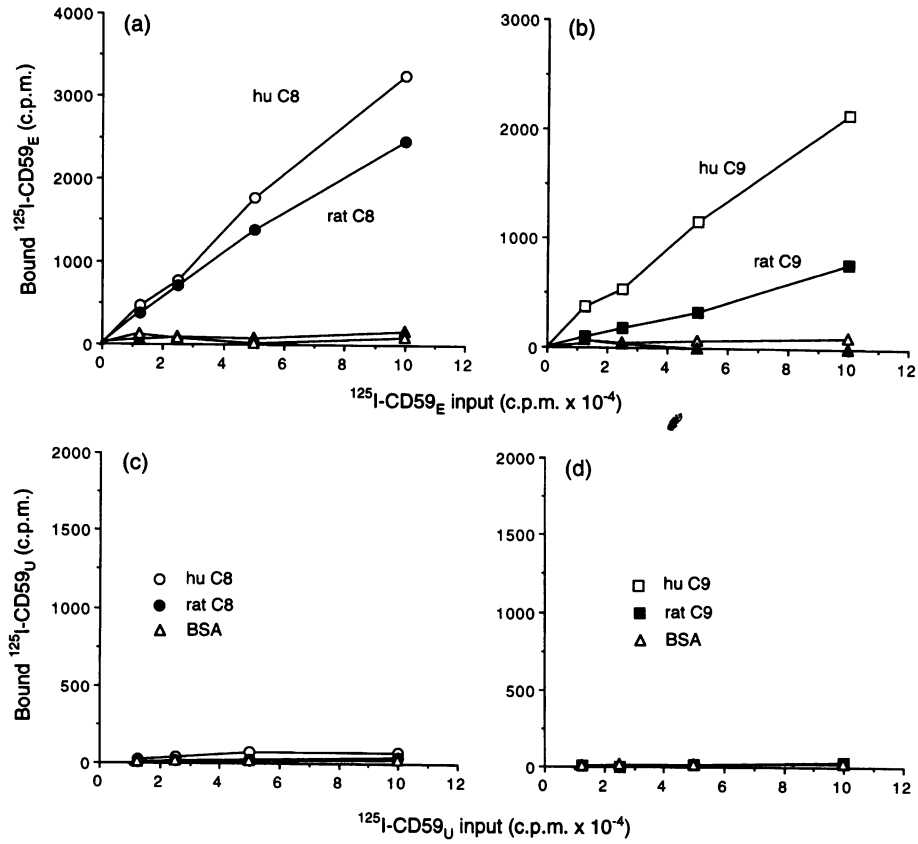

Figure 6. Binding of ^{125}I-CD59 to human and rat C8 and C9 adsorbed to plastic. Microtitre wells were coated with 10 μg/ml of C8, C9 and BSA (see Materials and Methods) and incubated with increasing amounts of radiolabelled human CD59$_E$ or CD59$_U$. (a) Binding of ^{125}I-CD59$_E$ to human (○) or rat C8 (●). As a specificity control the binding of labelled CD59$_E$ to human (△) or rat C8 (▲) was also examined in the presence of a 100-fold excess of unlabelled CD59$_E$. (b) Binding of ^{125}I-CD59$_E$ to human (□, △) or rat C9 (■, ▲) in the absence (□,■) or presence (△, ▲) of a 100-fold excess of unlabelled CD59$_E$. Data are corrected for non-specific binding to BSA. (c) Binding of ^{125}I-CD59$_U$ to human C8 (○), rat C8 (●) and BSA (△). (d) Binding of ^{125}I-CD59$_U$ to human C9 (□), rat C9 (■) and BSA (△).

nations. Cross-species binding of CD59 to C9 could also be demonstrated in ligand blotting and plate adsorption studies.

C8 and C9 are amphipathic proteins that bind sequentially to the C5b67 complex. It has been shown that CD59 does not bind to soluble monomeric C8 or C9 apparently because the CD59 binding sites are not exposed on the native conformations of these molecules.[9,16] When C8 and C9 incorporate into the membrane attack complex conformational changes take place and CD59 is able to bind to exposed sites on C8 and C9.[9,13,16] Equivalent changes in C8 and C9 seem to occur during the immunoblotting procedure or when the proteins are adsorbed on plastic. While soluble CD59 binds readily to SC5b–8 no binding is seen to SC5b–9[15] suggesting either lack of a similar conformational change to that in the MAC or non-exposure of the CD59 binding site. Our ligand blotting studies and experiments with C8$\alpha\gamma$ and C8β-deficient sera are compatible with earlier studies showing that CD59 recognizes a conformational epitope within human C8α.[17] CD59$_U$ did not bind to TCC in C9-depleted C8$\alpha\gamma$ or C8β-deficient serum unless they were reconstituted with the normal C8 protein. CD59$_U$ bound more strongly to TCC in the C8 reconstituted C8β deficient than in the C8$\alpha\gamma$-deficient C9DS. This could be due to competition between C8β and C8 for binding to C7 in the forming TCC, which is consistent with the data that binding of C8 to C7 is mediated via the C8β subunit.[31,32]

In the study of Ninomiya *et al.*[16] ligand blotting was used to show that CD59$_E$ bound to human C8$\alpha\gamma$ and to isolated human C8α, but not to human C8β- or C8γ-chain. No binding occurred to rabbit C8 chains. CD59$_E$ bound also to human C9 but not to rabbit C9. Using a similar method we found that human CD59$_U$ and CD59$_E$ bound equally strongly to human and rat C8$\alpha\gamma$ but more strongly to human than rat C9. Like human CD59$_U$ and CD59$_E$, rat CD59$_U$ also bound to both human and rat C8$\alpha\gamma$. Somewhat surprisingly, rat CD59 bound more strongly to the heterologous human C9 than to rat C9. This may indicate that the CD59 binding epitopes are better exposed on human than rat C9. Alternatively, this result may indicate specificity problems in the somewhat artifical ligand blotting analysis. ^{125}I-labelled CD59 had a tendency for non-specific binding to *E. coli* β-galactosidase and hen egg white ovalbumin in the standard protein lane (see Fig. 5). Weak binding of CD59 to rat C9 may indicate that the interaction between CD59 and C8 is functionally more important than that between CD59 and C9.

In the plate binding experiments human CD59$_U$ failed to bind human or rat C8 or C9 adsorbed on the microtitre plate

wells. However, when ^{125}I-CD59$_E$ was used binding to both human and rat C8 was seen. Binding of ^{125}I-CD59$_E$ to C9 was also seen but was weaker to rat than to human C9. These binding interactions could be inhibited by a 100-fold excess of CD59$_E$ (Fig. 6). The data obtained are consistent with the ligand blotting results. It can be concluded that binding of CD59$_E$ does not differ between human and rat C8 but binding to C9 shows more variation. The reason why CD59$_U$ did not bind to C8 or C9 on the plate assay remains uncertain. It is possible that the folding conformations of C8 and C9 immobilized on plastic need contact with phospholipid to be able to bind to the protein part of CD59. Alternatively, hydrophobicity provided by the acyl groups of CD59$_E$ might be needed for a stable association with plate-adsorbed terminal complement components.

Human and rat CD59 show 45% sequence identity at the protein level[22] while human and baboon CD59 show 84% identity.[36] Ferriani *et al.*[37] observed that a N-terminal fragment of human CD59 comprising disulphide-linked residues 1–14, 15–30 and 39–41, bound to nascent C5b–8 whereas the major peptide derived from the carboxy-terminal region (44–53, 55–65/66, 66/67–77) did not. According to our studies[38] and those of others the active site of CD59 is conformation dependent because reduced and alkylated CD59 does not bind to SC5b–8 or inhibit complement lysis and peptides from trypsin cleavage of CD59 are not active either. Comparison between potentially surface-exposed amino acids in human and rat CD59 suggests that residues Leu 1, Ser-20, Ser-21 (replaced by Pro in rat), Asp-24, Asp-22 (Arg in rat), Phe-42, Lys-65 (Gln in rat) could be functionally important in CD59.[22]

Our study implies that the reciprocal binding sites between at least CD59 and C8 and possibly between CD59 and C9 are for the most part conserved between human and rat. Our observations support findings[14] that rat CD59 is an effective inhibitor of heterologous human complement but does not exclude incompatibilities between combinations of CD59 and complement from other species. The activity of CD59 is thus species selective but it is not restricted exclusively to the homologous and closely related species.

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