Volume 318, number 2, 108–112 FEBS 12116 March 1993

Avidin attachment to biotinylated amino groups of the erythrocyte membrane eliminates homologous restriction of both classical and alternative pathways of the complement

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Received 20 November 1992; revised version received 4 January 1993

Lysis of avidin-coated biotinylated sheep red blood cells (RBC) via the classical pathway of homologous (sheep) and heterologous (guinea pig) complement has been studied. The minimal surface density of avidin inducing antibody-dependent lysis via the classical pathway is smaller than that inducing antibody-independent lysis via the alternative pathway. Heterologous lysis via the classical pathway does not depend on the mode of avidin attachment: both biotinylation of membrane amino groups and insertion of biotinyl–lipid into the membrane provide the same lysis of avidin-coated RBCs by guinea pig serum in the presence of anti-avidin antibody. Avidin-free sheep RBC sensitized with hemolytic anti-RBC antibody were lysed by guinea pig, but not by sheep serum, confirming high efficiency of homologous restriction of the complement. However, avidin-coated RBCs were lysed by homologous serum in the presence of anti-avidin antibody at low surface density of avidin attached. The elimination of the homologous restriction depends on the mode of avidin attachment: biotinylation of membrane amino groups provides antibody-mediated lysis via the classical pathway of homologous complement, while insertion of biotinyl–lipid does not provide lysis.

Avidin; Complement; Biotinylated erythrocyte; Homologous restriction; Biotin ester; Biotinyl–lipid

1. INTRODUCTION

As described previously, avidin attachment to biotinylated RBCs induces their lysis by homologous complement activated via the alternative pathway (alternative pathway of the complement, APC) [1]. Only avidin attachment via amino groups of the RBC membrane biotinylated with biotin *N*-hydroxysuccinimide ester induces lysis [1–3], while avidin attachment via biotinyl–lipid [4] or via tannic acid [5] does not induce lysis. Avidin possesses four biotin-binding sites [6]; multivalent binding of avidin to biotinylated RBC membranes seems to be essential for lysis [7].

The cell membrane contains proteins inactivating the membrane-bound complement: a complement receptor type 1 (CR1), a decay accelerating factor (DAF), a factor of homologous restriction (HRF) and a membrane inhibitor of reactive lysis (MIRL) [8–13]. Some of these proteins are clusterized in the cell membrane; such a clusterization is important for the inactivation of the membrane-bound complement [9]. Avidin might rearrange biotinylated membrane proteins (cross-linking, destruction of clusters, etc.), leading to their inactivation and to the activation of the complement. There are two pathways of complement activation: the classical pathway of the complement (CPC, mediated by C1q binding with the antibody) and the alternative pathway (APC, mediated by direct binding of C3) [10]. Membrane inhibitors regulate both pathways of the complement: CR1 and DAF inactivate CPC-convertase (membrane-bound C4) as well as APC-convertase (membrane-bound C3), while HRF and MIRL inactivate the terminal components of the complement regardless of the pathway of complement activation [8,9,11–13]. Inactivation of the homologous complement is more effective compared with the heterologous one [13].

Since avidin attachment induces homologous lysis by complement via the APC (i.e. eliminates homologous restriction of the complement) [1], we have suggested that avidin attachment may also induce lysis of biotinylated RBCs by homologous complement via the CPC. Here we studied lysis of avidin-coated sheep RBCs by homologous and heterologous complement at reduced surface density of membrane-bound avidin, which does not induce lysis by complement via the APC. We found that in the presence of anti-avidin antibodies, these RBCs lysed by heterologous and homologous complement activated via the CPC.

2. MATERIALS AND METHODS

Avidin was purified from hen egg white [14]. Sheep RBCs were prepared by defibrinating the blood with glass beads. Lyophilized guinea pig serum or fresh autologous sheep serum was used as a source of complement. Rabbit antiserum to avidin was obtained and characterized as described earlier [7].

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Biotinylation of the RBCs and subsequent attachment of avidin were performed as described previously [15]. Briefly, 3 μl of 0.1 M biotin *N*-hydroxysuccinimide ester and 0.1 ml of 0.1 M sodium tetraborate were added to 10% suspension of RBCs washed with PBS (PBS, phosphate-buffered saline, pH 7.4). After a 20-min incubation at 20°C cells were washed with PBS (5 × 15 ml). Avidin was added to a 10% suspension of biotinylated RBCs and after 20–60 min of incubation at 4°C cells were washed with PBS (3 × 15 ml). Avidin was labeled with [^{125}I]-Bolton-Hunter reagent (Amersham) according to the manufacturer's recommendations.

A hemolytic assay was performed in microtitration plates as described previously, using standard veronal-buffered saline (VBS) [16]. The RBCs examined (50 μl of 2% suspension) were added to 50 μl of complement (2-fold serial dilution in VBS). Plates were incubated at 37°C for 60 min and the hemolysis degree was measured as absorbance at 630 nm in an MR-580 Micro Elisa Auto Reader (Dinatech, USA). Wells with complement-free VBS and RBCs were used as a reference point of 0% hemolysis, while lysis by distilled water served as a reference point of 100% hemolysis. For avidin-independent lysis via the CPC, sheep RBCs were routinely sensibilized with rabbit hemolytic antibody [10] and lysis by heterologous complement via the CPC was performed using lyophilized guinea-pig serum diluted in VBS as described earlier [16]. For antibody-mediated lysis of avidin-coated RBCs, these cells were added to the reaction mixture containing guinea pig serum (or sheep serum) and heat-inactivated rabbit anti-avidin antiserum (final antiserum dilution 1:80). Heat-inactivated pooled serum from normal non-immune rabbits added at the same dilution (1:80) was used as a control of antibody-mediated lysis.

Avidin attachment to RBCs via the biotinyl–lipid was performed as described earlier [4]. Biotin–phosphatidylethanolamine (biotinyl–lipid, biotinyl–PE) was prepared as described [17]. Biotinyl–PE was dispersed in PBS at 1–2 mg/ml and sonicated under a stream of argon gas at 4°C to clarity (5 × 30 s, 50 W, Labline Labsonic System with microtip). The lipid dispersion obtained was incubated in a 10% suspension of the washed RBCs for 1 h at 37°C with gentle rocking. Unbound lipid was removed by triple washing with PBS. All subsequent procedures (avidin binding to the RBC, hemolysis, etc.) were performed as described above for biotinylated RBC.

3. RESULTS AND DISCUSSION

To induce an antibody-mediated activation of the complement via the CPC we used a rabbit antiserum to avidin. However, since avidin attachment to biotinylated RBCs leads to their lysis via the APC even in the absence of anti-avidin antibody [1], at the first stage we should find conditions allowing lysis via the CPC without lysis via the APC. For this purpose we reduced the surface density of the avidin attached to biotinylated sheep RBCs. Attachment of less than $1.5–2.0 \cdot 10^5$ avidin molecules per RBC does not induce lysis by homologous serum in the absence of anti-avidin antibody (Fig. 1). Therefore, at low surface density of membrane-bound avidin we could avoid lysis via the APC. Very similar results were obtained using Mg-EgTA-VBS instead of calcium-containing complete VBS, supporting antibody-independent lysis by homologous complement activated via the alternative pathway (not shown).

In the presence of anti-avidin antibody, sheep RBCs carrying avidin attached at a low surface density were lysed, whereas in the absence of the antibody there was no lysis (Fig. 2, pooled serum from normal non-immune rabbits was used as a control and shown as closed squares in this figure). This indicates antibody-mediated activation of the complement via the classical, but not via the alternative pathway. As a source of the complement in this experiment lyophilized guinea pig serum was used, i.e. antibody-induced activation of the heterologous complement via the CPC was observed. Data shown in Figs. 1 and 2 suggest that the critical surface density of avidin for homologous complement activation via the APC is higher than that for heterologous complement activation via the CPC ($2 \cdot 10^5$ avidin molecules per cell vs. $2 \cdot 10^4$ molecules, respectively).

It should be noted that the antibody-induced lysis of avidin-coated RBCs by heterologous complement activated via the CPC does not depend on the mode of avidin attachment to the RBC. Fig. 2 compares two methods of avidin attachment: biotinylation of the membrane amino groups with *N*-hydroxysuccinimide

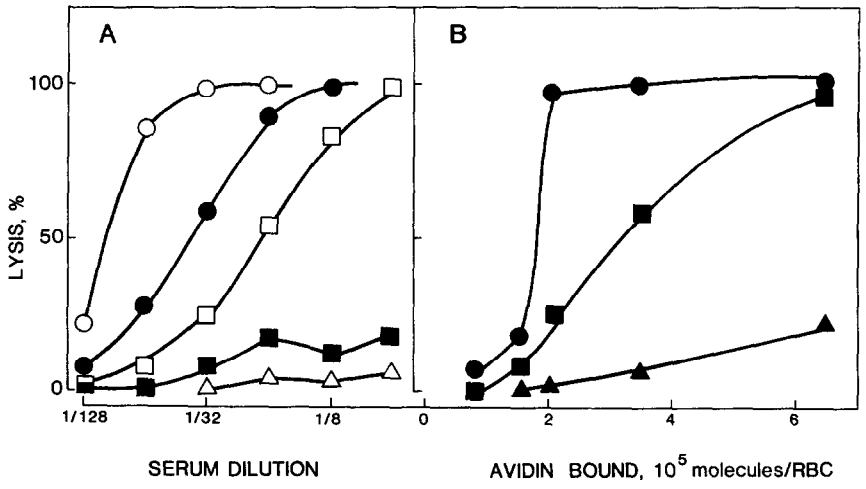

Fig. 1. Lysis of avidin-coated biotinylated sheep RBCs by autologous serum via the alternative pathway of complement (APC). A, dependence of lysis on serum dilution. Surface densities of avidin attached per RBC: $0.8 \cdot 10^5$ molecules (△); $1.6 \cdot 10^5$ molecules (■); $2.1 \cdot 10^5$ molecules (□); $3.5 \cdot 10^5$ molecules (●); and $6.5 \cdot 10^5$ molecules (○). B, lysis at serum dilutions: 1/4 (●); 1/32 (■) and 1/128 (▲).

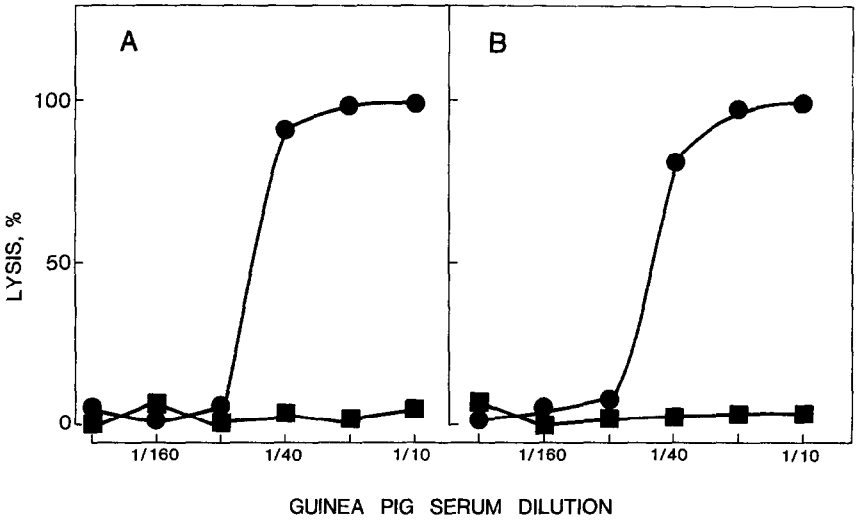

Fig. 2. Antibody-mediated heterologous lysis of avidin-coated sheep RBCs by guinea pig serum via the classical pathway of complement (CPC). Avidin ($2.2 \cdot 10^4$ molecules per cell) was attached via biotinylated amino groups (A) or via biotinyl–lipid inserted into the membrane (B). Incubation with guinea pig serum was performed in the presence (circles) or in the absence (squares) of heat-inactivated rabbit anti-avidin antiserum.

biotin ester (part A) and insertion of biotinyl–lipid into the membrane (part B). Both methods of avidin attachment lead to lysis by heterologous complement in the presence of anti-avidin antibody (no lysis was observed in the absence of antibody). As it was described previously, avidin attachment via the biotinyl–lipid (up to $5 \cdot 10^5$ avidin molecules per cell) does not induce lysis via the alternative pathway [4]. Therefore, antibody- induced lysis of avidin-coated RBCs by heterologous complement via the CPC differs from homologous lysis via the APC; heterologous lysis via the CPC does not depend on the mode of avidin attachment to RBC, while homologous lysis via the APC depends on the mode of avidin attachment.

We compared antibody-induced lysis of sheep RBCs by heterologous and homologous complement activated via the classical pathway. Fig. 3 shows lysis of avidin-free sheep RBCs treated with rabbit hemolytic antibody against sheep RBC. Only heterologous serum induced lysis, supporting the high efficiency of the homologous restriction of complement. However, avidin-coated biotinylated RBCs were lysed by homologous complement in the presence of anti-avidin antibody, while in the absence of anti-avidin antibody no lysis was observed (Fig. 4, pooled serum from normal non-immune rabbits was used as a control and shown as closed squares). This result supports the suggestion that avidin attachment to the biotinylated RBC membrane eliminates homologous restriction of both classical (CPC) and alternative (APC) pathways.

As mentioned above, the mode of avidin attachment is essential for the activation of homologous complement via the APC [4], but has no influence on the activation of heterologous complement via the CPC (Fig. 2). In contrast to heterologous complement, lysis by homologous complement via the CPC depends on the mode of avidin attachment. Fig. 4 compares avidin attachment via biotinylated amino groups ($1.6 \cdot 10^5$ avidin molecules per cell) and via biotinyl–lipid inserted into the membrane ($3.8 \cdot 10^5$ avidin molecules per cell). In contrast with avidin attached via biotinylated amino groups, avidin attached via biotinyl–lipid does not induce lysis by homologous complement regardless of the presence of anti-avidin antibody.

Summarizing the results of this and previous work, we can make the following conclusion.

First, avidin attached to biotinylated RBCs at high

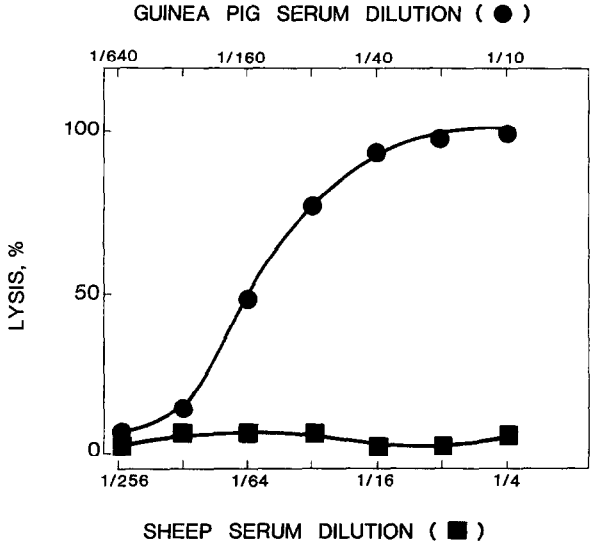

Fig. 3. Antibody-mediated lysis of sheep RBCs by homologous (sheep serum, ■) or heterologous (guinea pig serum, ●) complement activated via the classical pathway. Sheep RBCs were sensitized with rabbit hemolytic antibody by standard procedure.

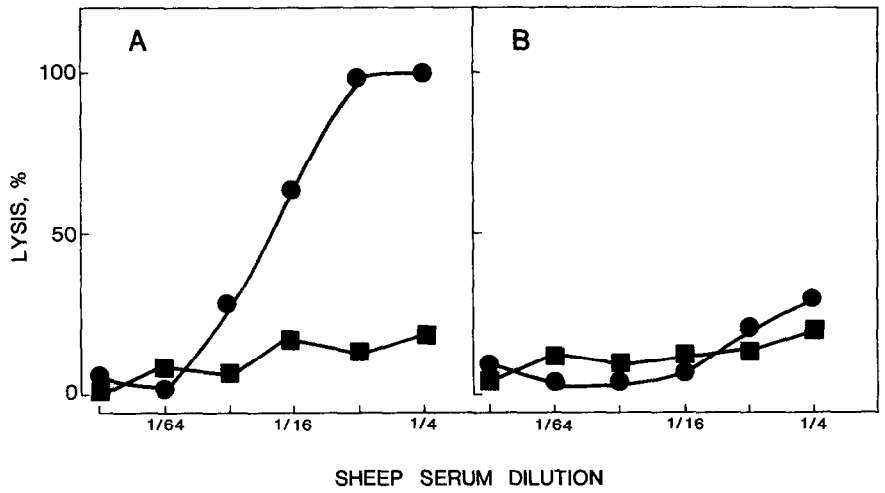

Fig. 4. Antibody-mediated lysis of avidin-coated sheep RBCs by homologous complement via the classical pathway. A, avidin attached to biotinylated amino groups of the membrane ($1.6 \cdot 10^5$ molecules per cell); B, avidin attached to biotinyl–lipid inserted into the membrane ($3.8 \cdot 10^5$ molecules per cell). Incubation in fresh sheep serum in the presence (●) or in the absence (■) of anti-avidin serum.

surface density eliminates homologous restriction of the complement and induces lysis via the APC [1]. This effect of avidin depends on the mode of its attachment. Only attachment to biotinylated amino groups is effetive, while attachment via biotinyl–lipid [4] or via tannic acid [5] is non-effective. Lysis by homologous complement via the APC does not occur at attachment of less than $2 \cdot 10^5$ avidin molecules per sheep RBC.

Second, avidin attached to RBCs might serve as a 'cellular antigen' and provide lysis of RBCs by heterologous complement activated by the anti-avidin antibody. This effect of avidin does not depend on the mode of its attachment: both biotin ester-anchored and biotinyl–lipid-anchored avidin induces lysis (Fig. 2), as well as tannin-anchored avidin [5]. Efficiency of antibody-mediated lysis of avidin-coated RBCs by heterologous complement via the CPC is very high and occurs at a low surface density of avidin attached ($2 \cdot 10^4$ molecules per sheep RBC).

Third, avidin attached to the biotinylated membrane at low surface density induces membrane rearrangement and elimination of the homologous restriction of the complement, while no lysis via the APC occurs. Elimination of homologous restriction at a low surface density of avidin may be revealed using anti-avidin antibody-mediated lysis by homologous complement activated via the CPC. Lysis by homologous complement via the CPC, as well as lysis via the APC, depends on the mode of avidin attachment. Attachment of avidin to biotinylated amino groups provides lysis, while attachment to biotinyl–lipids inserted into the membrane does not provide lysis. Efficiency of lysis of avidin-coated RBCs by homologous complement via the CPC is higher than via the APC.

Therefore, avidin induces considerable alterations in the RBC membrane leading to the elimination of the homologous restriction of the complement. This is of interest, since applications of the avidin–biotin complex for the investigation of circulating RBCs in vivo was recently suggested [18–20]. Bearing in mind the avidin-induced alterations in the RBC membrane, we should carefully study the biocompatibility of avidin-modified RBC and the safety of avidin applications in vivo [21]. In addition, the avidin-induced elimination of the homologous restriction seems to be an interesting model for the investigation of the mechanisms of cell lysis by complement, as well as mechanisms of cellular defence against homologous complement.

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