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Avidin acylation prevents the complement-dependent lysis of avidin-carrying erythrocytes

Vladimir R. MUZYKANTOV, Mikhail D. SMIRNOV and Gennady P. SAMOKHIN
Institute of Experimental Cardiology, U.S.S.R. National Cardiology Research Center of the Academy of Medical Sciences, 3-rd Cherepkovskaya Street 15A, Moscow 121552, U.S.S.R.

Non-covalent binding of avidin to biotinylated erythrocytes results in complement-dependent haemolysis. Biotinylated erythrocytes, as well as native cells, are not lysed by complement. Complement activation requires a tight contact between avidin and the erythrocyte membrane, since avidin does not in itself activate complement and does not inhibit lysis of sensitized sheep erythrocytes. The efficiency of haemolysis depends on avidin's surface density. When the avidin concentration in the reaction mixture is less than 15 μg/ml, erythrocyte lysis is not induced. However, the attachment of biotinylated antibodies to avidin-carrying erythrocytes decreases dramatically. Acylation of avidin with succinic anhydride strongly decreases its ability to induce complement-dependent haemolysis. However, the ability of avidin to cross-link the biotin-containing structures decreases after acylation. A 50% modification of avidin by succinic anhydride (pI about 7.0) allows preparation of 'immunoerythrocytes', which retain their affinity to antigen and stability in the presence of complement.

INTRODUCTION

The use of microcontainers (erythrocytes, liposomes) bearing specific antibodies has been regarded as a prospective approach to targeted drug delivery [1]. We have proposed the use of erythrocytes that carry antibodies attached via the avidin–biotin pair ('immunoerythrocytes') [2]. Such a container has the advantages of a firm and specific binding to the target, and the high efficiency of antibody immobilization considerably decreases the amount required for the procedure [3]. Furthermore, the avidin–biotin pair provides co-immobilization of both antibodies and active enzymes on erythrocyte surface, which allows a direct delivery of enzymic activity into the site of immunoerythrocyte–target interaction [4,5]. Thus the use of the biotin–avidin pair enabled us to resolve the problems associated with the specificity of targeting. At the same time it was clear, *a priori*, that modification of the erythrocyte membrane (a covalent modification with biotin ester followed by non-covalent binding of avidin) may considerably change the stability of the immunoerythrocyte obtained. In fact, avidin-carrying erythrocytes were lysed in homologous serum, in contrast with biotin-carrying and native erythrocytes [6]. If serum was preincubated at 56 °C or if EDTA was added, the lytic activity was lost, which indicates its complement-dependent nature. Obviously, uncontrolled immunoerythrocyte lysis and concomitant activation of complement is undesirable. In the present work we tried to develop a non-lysing avidin attachment to biotinylated erythrocytes and showed that avidin acylation prevents lysis by complement.

MATERIALS AND METHODS

Avidin was purified from hen's-egg white [7] and its activity was determined by spectrophotometric titration with biotin [8]. BSA, biotin *N*-hydroxysuccinimide ester (BOSu), trinitrobenzene sulphonic acid (TNBS) and buffer components were from Sigma. Human type I collagen and the corresponding rabbit antiserum were generously given by Dr. S. P. Domogatsky. Blood from healthy volunteers was collected in acid/citrate/dextrose anticoagulant. Fresh human and guinea-pig sera were used as sources of complement.

Avidin acylation

Succinic anhydride (0–80 mM) in 50 μl of dimethylformamide was added to 1 ml of avidin (1 mg/ml in 0.1 M-sodium tetraborate, pH 9.2) and incubated at 0 °C for 1 h. The number of substituted amino groups was determined by titration with TNBS [9]. The reaction mixture was exhaustively dialysed against phosphate-buffered saline (PBS; 138 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4).

Avidin immobilization on erythrocyte surface

Sodium tetraborate (0.1 ml of a 0.1 M solution) and 3 μl of 0.1 M-BOSu in dimethylformamide were sequentially added to 1 ml of a 10% PBS-washed erythrocyte suspension. The reaction mixture was incubated at 20 °C for 20 min and the cells then washed with PBS (5 × 15 ml). Then avidin at concentration of 1 mg/ml (except in indicated cases) in 1 ml of PBS was added to 1 ml of 10% suspension of biotin-carrying erythrocytes with constant stirring. After a 20–60 min incubation at 4 °C with periodical shaking, erythrocytes were washed three times with 15 ml of PBS.

Antibody immobilization on erythrocyte surface

Attachment of antibody was performed via the biotin–avidin pair, as described previously [5]. Rabbit polyclonal antibody to human type I collagen was obtained from the corresponding antiserum by affinity chromatography. Before immobilization the antibody (0.5–1.0 mg/ml of PBS) was modified with 10 μl of 0.1 M-BOSu in dimethylformamide at 20 °C for 1 h [2]. Unbound biotin was removed by gel filtration and dialysis against PBS. The extent of the antibody amino group modification was determined by titration with TNBS [9].

To quantify protein binding to erythrocyte surface, the antibody was labelled with ^{125}I [10].

Abbreviations used: BOSu, biotin *N*-hydroxysuccinimide; TNBS, trinitrobenzenesulphonic acid; PBS, phosphate-buffered saline; VBS, Veronal-buffered saline (compositions of these buffers are given in the text).

Binding of immunoerythrocytes carrying anti-collagen antibody to collagen-coated surface

Binding was studied in collagen-coated multiwell plastic plates (Falcon) as described previously [4,5]. The plates were coated with human type I collagen or BSA by incubation with the protein solution (20 μg/ml) at 4 °C overnight. After removal of unbound protein by washing, the plates were incubated for 3 h with PBS containing 2 mg of BSA/ml (PBS/BSA). Then 400 μl of a 1% suspension of erythrocytes carrying either specific antibody to type I collagen or non-immune rabbit IgG was added to each well and incubated for 60 min with periodical stirring. Unbound erythrocytes were washed off. After washing, 250 μl of distilled water was added to each well, and the amount of bound erythrocytes was determined by reading the absorbance of haemoglobin at 405 nm as described in [5].

Lysis of avidin-carrying erythrocytes by complement

Haemolytic assays were carried out on microtitration plates as described previously [11]. Veronal-buffered saline (VBS) was used (4 mM-veronal buffer containing 3 mM-diethylbarbituric acid, 1 mM-diethylbarbituric acid sodium salt, 1.8 mM-$MgCl_2$, 0.25 mM-$CaCl_2$ and 145 mM-NaCl, pH 7.2). Serum was 2-fold serially diluted in 50 μl of VBS and then 50 μl of a 2% suspension of the erythrocytes being tested was added. Wells with serum-free VBS and erythrocytes were used as controls; 100% haemolysis was attained by the addition of distilled water instead of VBS. The plates were incubated at 37 °C for 60 min, and the degree of haemolysis was determined by measuring A_{630} with an MR-580 Micro Elisa Auto Reader (Dinatech).

Sheep erythrocytes were sensitized with rabbit haemolytic antibody by means of a routine procedure [11]. Haemolysis of sensitized sheep erythrocytes by guinea-pig serum was performed in microtitration plates as described above. To study the influence of free avidin itself on serum haemolytic activity, serum was preincubated with avidin (2 mg/ml) for 2 h before addition to sheep erythrocytes or avidin-carrying biotinylated human erythrocytes. On the other hand, stability of native human and sheep erythrocytes in fresh serum in the presence of free avidin (2 mg/ml) was also evaluated by using the method described.

Evaluation of the agglutination activity of native and acylated avidin

Various dilutions of avidin in PBS/BSA (50 μl) were added to each well of a V-bottomed microtitration plate and 50 μl of a 1% suspension of biotin-carrying erythrocytes was added to each well, carefully mixed and left for 40–60 min at 20 °C. The agglutination titre was defined as the minimal avidin concentration in the well where erythrocytes still did not sediment to the centre of the bottom. The agglutination titre for native avidin was about 1 μg/ml.

RESULTS

A haemolysis curve showing the effect of avidin immobilization is shown in Fig. 1. It can be seen that the erythrocytes were lysed by fresh intact serum only after avidin attachment and that avidin-free biotinylated cells were not lysed as well as the native cells. The serum lytic activity was eliminated by 30 min incubation at 56 °C or by EDTA addition (results not shown). For fresh human serum, the concentration causing 50% haemolysis ('$CH_{50\%}$') was about 1/20, whereas for guinea-pig serum it was about 1/110. Serum preincubation with free avidin (up to 2 mg/ml) had no effect on consequent haemolysis of either antibody-sensitized sheep erythrocytes or avidin-carrying biotinylated human erythrocytes, i.e. there was no complement depletion in solution. On the other hand, such concentrations

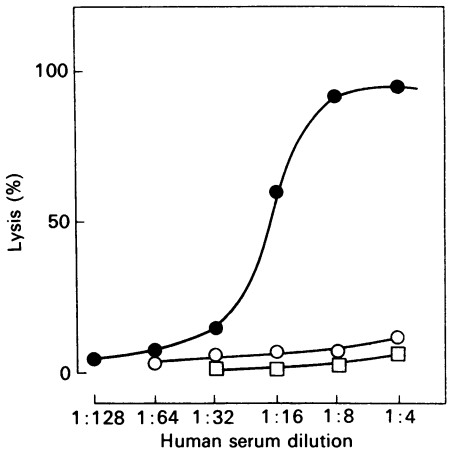

Fig. 1. Lysis of avidin-carrying erythrocytes in the presence of complement
●, Biotinylated avidin-carrying cells; ○, biotinylated avidin-free cells; □, native cells. The avidin concentration in solution upon attachment was 1 mg/ml. The result is a typical one from the three experiments.

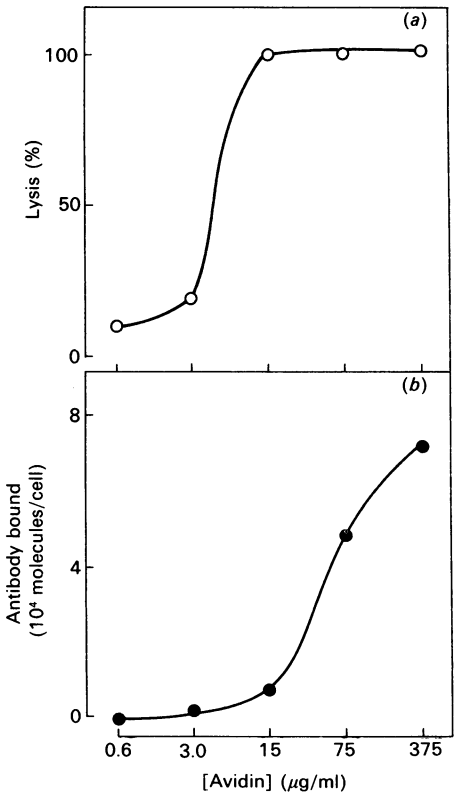

Fig. 2. Effects of avidin concentration in the reaction mixture during avidin attachment to biotinylated erythrocytes on haemolysis (*a*) and consequent attachment of biotinylated immunoglobulins (*b*).

Fivefold serial dilutions of avidin were used. Guinea-pig serum dilution in (*a*) was 1:40. Results are typical of those from three (*a*) and five (*b*) experiments.

(up to 2 mg/ml) of free avidin produce no haemolysis of both human and sheep intact cells in fresh serum. These findings indicate that haemolysis by complement is triggered only by avidin attachment to the biotinylated erythrocyte membrane, but not by the presence of avidin alone in serum.

The haemolytic efficiency depends on the avidin density on the surface of biotin-carrying erythrocytes. If the avidin concen-

Table 1. Effect of the extent of avidin acylation by succinic anhydride on the pI of avidin

[Succinic anhydride] (mM)	Extent of acylation (%)	pI
0	0	10.0–11.0
0.3	25	8.0–9.0
1.0	50	6.0–8.0
2.75	75	5.0–6.0
4.0	90	3.0–5.0

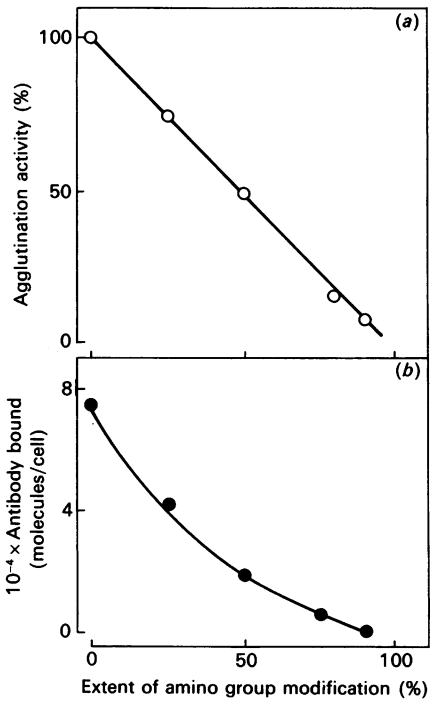

Fig. 3. Changes in agglutination activity of avidin (*a*) and binding of biotinylated immunoglobulins to avidin-carrying erythrocytes (*b*) upon avidin acylation

Agglutination activity is presented as a percentage of that for the native avidin, which was calculated as a ratio of agglutination titres for native and modified avidin. Results are typical of those from the four (*a*) and three (*b*) experiments.

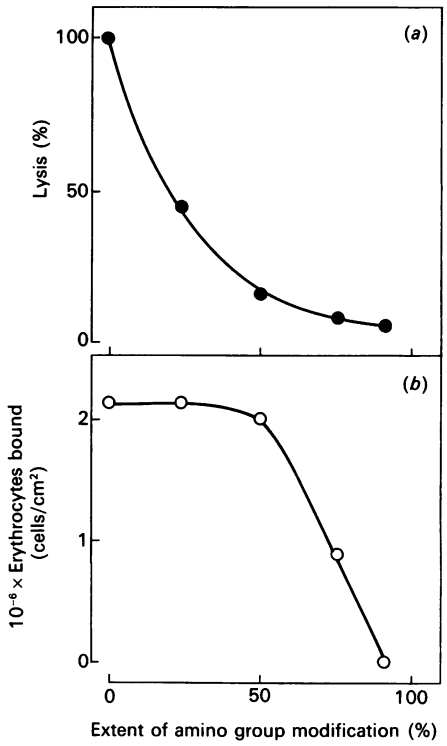

Fig. 4. Effect of the degree of avidin acylation on immunoerythrocyte stability in the presence of complement (*a*) and on immunoerythrocyte affinity (*b*)

The data for Figs. 3 and 4 were obtained with the same preparations of acylated avidin. The serum dilution in (*a*) was 1:40. Results are typical of those from three experiments.

tration used in the immobilization procedure is lower than 15 μg/ml, the degree of lysis decreases drastically (Fig. 2*a*). On the other hand, there is a marked decrease in the binding of biotinylated antibody to avidin-carrying erythrocytes at avidin concentrations higher than those giving decreased lysis (Fig. 2*b*). Therefore a decrease in avidin density on the erythrocyte surface cannot produce a complement-resistant immunoerythrocyte with the proper affinity.

We attempted to resolve the above problem by chemical modification of avidin. Avidin is a glycoprotein (molecular mass 66 kDa) consisting of four subunits, each with a single biotin-binding site. Avidin has a relatively high positive charge (pI about 11) [12]. The membrane sensitivity to complement lysis is known to be to a great extent dependent on its surface charge [13], and the activity of artificial or natural regulators of the complement system also depends on membrane charge [14]. Therefore we have suggested that a high positive charge of avidin may have an important role in the lysis of immunoerythrocytes and decided to change the avidin pI. For this purpose avidin was acylated with succinic anhydride. The degree of modification can be varied from 10 to 90% over a range of anhydride concentrations (0.1–4.0 mM). The pI value of the modified protein decreased in proportion to the degree of modification, as evidenced by isoelectrofocusing (Table 1).

Acylation of avidin decreases the complement-dependent lysis of avidin-carrying erythrocytes: at 75% modification of avidin amino groups the haemolytic value was 8% as against 100% for native avidin after 60 min incubation with 1:40 dilution of fresh guinea-pig serum at 37 °C. The native and biotinylated cells exhibit 0% lysis under these conditions. This result suggests that the positive charge of avidin may be involved in the triggering of immunoerythrocyte lysis by complement. A high reproducibility of the acylation reaction and its effects was obtained using several different preparations of avidin. In the present work guinea-pig serum was used as a source of complement (see Figs. 4 and 5 below), since serum of inbred animals provides much more reproducible results than serum from human volunteers; however, it was shown in a separate experiment that 75% avidin acylation drastically decreases lysis in homologous human serum (results not shown).

Modification by acylation was chosen on the basis of the finding that it was reported to have virtually no effect on the binding of biotin to avidin [15]. However, we have noticed that the cross-linking ability of avidin decreased, depending on the degree of its modification. Fig. 3 shows that there is a decrease both in the ability of avidin to agglutinate biotinylated erythrocytes (Fig. 3*a*) and in the ability of biotinylated antibody to bind to avidin-coated erythrocytes (Fig. 3*b*). Since these changes in the properties of acylated avidin might have changed the affinity of the immunoerythrocyte, we examined lysis and

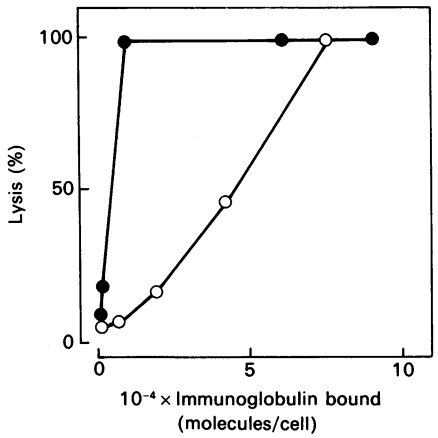

Fig. 5. Dependence of lysis values on the amount of biotinylated IgG attached to biotinylated erythrocytes via the native (●) and acylated (○) avidin

The data presented in Figs. 2, 3 and 4 were used. Serum dilution was 1:40.

binding to the antigen-coated surface of immunoerythrocytes prepared with avidin with various degrees of modification. It has been shown previously that the standard procedure employing the avidin–biotin pair provides immobilization of up to 200000 antibody molecules on the erythrocyte surface, and the affinity of immunoerythrocytes remained virtually unchanged when the antibody surface density was reduced to 20000 molecules/cell [5].

Fig. 3(*b*) shows that such an attachment of IgG can be achieved if the degree of avidin acylation does not exceed 50%. In accordance with this fact, immunoerythrocyte binding to the antigen-coated surface is virtually unchanged at these levels of avidin modification (Fig. 4*b*), whereas the extent of lysis decreases by an order of magnitude (Fig. 4*a*).

DISCUSSION

In recent years the avidin–biotin pair has been attracting the attention of biochemists and immunologists [16]. Specific high-affinity interaction between these compounds has been employed in various immobilization systems (as 'cross-linking' and 'staining' agents etc.). Some researchers attempted to apply the avidin–biotin pair *in vivo* to the gamma-scintigraphic technique [17], acceleration of the clearance of labelled proteins in the circulation [18,19] and drug targeting [20]. However, the effects of biotin–avidin or of components carrying this pair on the effector systems of the organism are poorly understood. At the same time it was shown that avidin immobilization on the erythrocyte membrane markedly decreases cell stability in the presence of complement [6]. This can impose significant restrictions on drug targeting employing the avidin–biotin pair. Additionally, complement activation occurring at the container surface may produce undesirable effects resulting in chemo-attractant formation, changes in the balance of the plasma proteinase–antiproteinase activity and systemic activation or depletion of complement [21,22]. Therefore the investigation of the mechanisms of complement activation by avidin and the search for the means of control over this process are of paramount importance. We have examined the effect of chemical modification of avidin on complement activation by avidin-carrying erythrocytes. Since the affinity for the target is the most important parameter of immunoerythrocytes, and the avidin–biotin pair is employed to achieve such an affinity, we have also examined the effect of avidin modification on this parameter.

Our findings show that avidin acylation (a procedure that enabled us to decrease the positive charge of avidin) provides a decrease in the degree of the complement-dependent lysis of immunoerythrocytes, which is consistent with the suggestion that the avidin positive charge has a certain role in complement activation. At the same time, the cross-linking properties of avidin also change, and uncontrolled modification may lead to a considerable loss of immunoerythrocyte affinity, similar to that seen when the surface density of avidin on the erythrocyte membrane decreases. We have shown that a 50% modification of the avidin amino groups allows the preparation of stabilized immunoerythrocytes with retained affinity for the target.

The reason for lysis elimination by avidin acylation are still unclear. One of the possible explanations may be that avidin attachment to erythrocytes after acylation is decreased and, consequently, lysis is also diminished, similarly to the case of insufficient concentration of native avidin upon attachment (Fig. 2). However, a comparison of the data on attachment of the biotinylated IgG to biotinylated cells via native or acylated avidin with corresponding values for lysis does not support this suggestion. It can be postulated that at least equal amounts of cell-bound native and acylated avidin must exist for attachment of equal amounts of IgG. Moreover, bearing in mind the decreased cross-linking ability of acylated avidin (Fig. 3), we can suggest that equal attachment of biotinylated IgG might require a substantially greater amount of cell-bound acylated avidin in comparison with native avidin. At the same time the data of Figs. 2 and 4 show that, at a surface density providing the attachment of 7×10^3 molecules of biotinylated IgG, native avidin induces 100% haemolysis, whereas acylated avidin induces only 10% lysis. Fig. 5 shows that the dependence of the value for lysis on the amount of attached biotinylated IgG is drastically different for native and acylated avidin. In the range 0.1×10^3–5×10^4 molecules of biotinylated IgG attached per cell, the native avidin induces much greater lysis than acylated avidin at equal values of IgG attachment. Therefore changes in properties of cell-bound acylated avidin in comparison with native, but not simple changes in the amount of cell-bound avidin, seem to be responsible for elimination of the lysis.

The results obtained permits the suggestion that changes in avidin charge and/or in its cross-linking ability upon acylation may cause abolition of lysis. Therefore interaction of avidin with charged components of the erythrocyte membrane and/or cross-linking of biotinylated components of the erythrocyte membrane by avidin may induce erythrocyte susceptibility to lysis by complement. In the first case the avidin charge may play the main role, whereas in the second case avidin cross-linking ability may be paramount. We hope that an investigation of avidin influence on the features of cell membrane will provide an insight into the mechanisms of complement activation by the avidin-carrying cell. Additionally, the question of whether avidin can induce the complement-dependent lysis of nucleated cells is of particular interest.

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