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Attachment of antibody to biotinylated red blood cells: immuno-red blood cells display high affinity to immobilized antigen and normal biodistribution in rats

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Streptavidin-mediated attachment of biotinylated antibodies (b-Ab) to biotinylated red blood cells (b-RBC) is useful for preparation of immuno-red blood cells, a prospective vehicle for drug targeting. However, streptavidin (SA) induces lysis of extensively biotinylated RBC by complement due to cross-linking and inactivation of RBC complement regulators. To reduce cross-linking of RBC membrane proteins, we utilized mild biotinylation of RBC with 20 μM biotin ester (b_{20}-RBC). SA effectively binds to rat b_{20}-RBC (10^5 SA molecules/cell) and provides for following attachment of 5×10^4 molecules of b-IgG/SA per b_{20}-RBC. By *in vitro* assay, b-Ab/SA/b_{20}-RBC were stable in fresh rat serum. Serum-stable immuno-red blood cells (b-Ab/SA/b_{20}-RBC) specifically bound to antigen-coated surfaces, but not to BSA-coated surfaces. Biodistribution of ^{51}Cr-labelled b-Ab/SA/b_{20}-RBC in rats was similar to that of control RBC, with no indication of lysis *in vivo*. These results suggest b-Ab/SA/b_{20}-RBC may be explored as a vehicle for drug targeting.

Introduction

Red blood cells (RBC[2]) are a non-immunogenic, available and physiological vehicle useful for the prolongation of the lifetime of drug circulation in the bloodstream [1,2], drug targeting [3] and selective clearance of circulating pathogens from the bloodstream [4]. Antibody may be attached to RBC, to provide targeting or selective binding of pathogens. Such immuno-RBC ('immunoerythrocytes') must be stable *in vivo* and circulate in the bloodstream for a prolonged time. Since streptavidin (SA) possesses four biotin-binding sites [5], we utilized SA-mediated attachment of biotinylated antibodies (b-Ab) to biotinylated RBC (b-RBC) [6]. However, binding of SA to RBC modified with 1 mM hydroxysuccinimide biotin ester leads to b-RBC lysis by autologous complement [7]. The lysis is the result of cross-linking of membrane regulators of complement by SA [8]. We hypothesized that reduction of the surface density of biotin residues on b-RBC membrane may serve as an approach to minimize cross-linking of the b-RBC membrane by SA and obtain serum-stable immuno-RBC. In the present work we studied rat RBC biotinylated over a wide range of biotin ester concentrations. We have found that rat RBC biotinylated at 20 μM biotin (b_{20}-RBC) remain stable in serum *in vitro*, despite SA binding. We further characterize b-Ab/SA/b_{20}-RBC in terms of (i) stability in autologous serum, (ii) binding to immobilized antigen *in vitro*, and (iii) biodistribution in rats *in vivo*.

Materials and methods

SA, 6-biotinylaminohexanoic acid *N*-hydroxysuccinimide ester (long-arm biotin ester, BxNHS) and polyclonal goat antibody against mouse IgG were purchased from Calbiochem (San Diego, CA, U.S.A.). Iodogen was obtained from Pierce (Rockford, IL, U.S.A.). Both ^{51}CrCl and $Na^{125}I$ were from Amersham (Arlington Heights, IL, U.S.A.). Normal mouse IgG, BSA and dimethylformamide (DMF) were from Sigma (St. Louis, MO, U.S.A.). Proteins were radiolabelled with ^{125}I by the Iodogen method according to the manufacturer's recommendations. Goat antibody against mouse IgG, as well as non-immune mouse IgG, were biotinylated with BxNHS by a standard method [5].

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Kingston, NY, U.S.A.) weighing 200–300 g, were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). To obtain serum, non-hepari-

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[2] Abbreviations used: SA, streptavidin; BxNHS, 6-biotinylaminohexanoic acid *N*-hydroxysuccinimide ester (long-arm biotin ester); DMF, dimethylformamide; RBC, red blood cell(s); b-RBC, biotinylated RBC; b-Ab, biotinylated antibody, b_{20}-RBC, RBC biotinylated with 20 μM BxNHS; GVB, gelatin/Veronal buffer.

nized blood was collected from the peritoneal cavity after dissection of the descending aorta. After a 2 h incubation of 4 °C, serum was separated by centrifugation. To obtain RBC, blood from the peritoneal cavity was collected in heparin-treated tubes. Fresh rat RBC in heparinized blood were washed four times with PBS before biotinylation. BxNHS in DMF was added to 10% suspensions of rat RBC at alkaline pH (pH 9.0), and after 30 min incubation at room temperature the RBC were washed four times by centrifugation with BSA/PBS (PBS containing 2 mg/ml BSA). Then SA was added to a 10% suspension of b-RBC and, after 30 min incubation at room temperature, non-bound SA was removed by centrifugation. In a separate experiment, radiolabelled SA was added to b-RBC to estimate SA binding.

Lysis of rat RBC, b-RBC, SA/b-RBC and b-Ab-coated SA/b-RBC by fresh rat serum was studied in 96-well microtest plates by estimation of the light transmission of the RBC suspension as described previously [7]. Standard gelatin/Veronal buffer containing Ca^{2+} and Mg^{2+} (GVB, pH 7.4) was used as a diluent for serum and RBC. Diluted serum was incubated with a 1% suspension of RBC preparations for 1 h at 37 °C, and the lysis was estimated by reading the A_{630} in an ELISA reader. To calculate degree of lysis, water-containing wells were used as a standard for 100% lysis, while serum-free GVB-containing wells were used as a standard for zero lysis.

Immuno-RBC were prepared as described previously [6]. Biotinylated goat antibody against mouse IgG (b-anti-IgG) was attached to SA/b-RBC by incubation of indicated amounts of b-anti-IgG with a 10% suspension of SA/b-RBC for 1 h at room temperature. Non-bound b-anti-IgG was eliminated by centrifugation with BSA/PBS. To estimate binding of biotinylated immunoglobulins to SA/b-RBC, radiolabelled biotinylated mouse IgG (b-IgG) was used as a tracer in a separate experiment.

To study binding of immuno-RBC to the target, mouse IgG was immobilized in the plastic wells of 24-well culture plates as described previously [6]. Indicated amounts of mouse IgG, used as an antigen, were added to wells in 0.5 ml of PBS. After overnight incubation at 4 °C, non-bound antigen was eliminated by washing and sites of non-specific binding in wells were blocked by a 1 h incubation with BSA/PBS. Indicated amounts of immuno-RBC (b-anti-IgG/SA/b-RBC) were added to wells in 0.5 ml of BSA/PBS. After incubation for 2 h at room temperature, non-bound RBC were eliminated by washing with BSA/PBS. To estimate RBC targeting, bound RBC were lysed by addition of 1 ml of water to each well. The amount of bound RBC in wells was determined spectrophotometrically according to haemoglobin absorbance in lysates at 405 nm.

To trace immuno-RBC after *in vivo* administration, ^{51}Cr isotope was added to SA/b_{20}-RBC suspension simultaneously with b-Ab and incubated for 1 h at room temperature. Excess isotope was eliminated by centrifugation. Effectiveness of RBC radiolabelling was 35–50%. Animal experiments were designed as described previously [9]. Briefly, ^{51}Cr-labelled b-Ab/SA/b_{20}-RBC were injected through the tail vein in anaesthetized rats. At indicated time after injection, anesthetized rats were killed by exsanguination. Blood and internal organs were collected, the latter were also rinsed with saline until free of blood. Radioactivity of ^{51}Cr in blood, serum and internal organs was measured in a γ-radiation counter.

Results and discussion

In the first series of experiments we have studied the biotinylation of rat RBC in terms of consequent binding of SA to b-RBC, as well as binding of b-IgG to SA/b-RBC and stability of immuno-RBC in serum *in vitro*. We have biotinylated rat RBC in the range of BxNHS concentrations in the reaction mixture equal to 2–700 μM, designated as b_2-b_{700}RBC. Figure 1(A) shows that an increase in the biotin ester concentration upon biotinylation of rat RBC provides more binding sites for SA on the RBC membrane. Maximal SA attachment (5×10^5 molecules/b-RBC) was attained to b_{700}-RBC. B_{20}-RBC (i.e. RBC biotinylated at 20 μM BxNHS) bind about 10^5 SA molecules per b-RBC. Radiolabelled b-IgG effectively attached to b-RBC coated with SA (SA/b-RBC; Figure 1B). Previously we have described that a high degree of biotinylation of human RBC leads to reduced b-IgG-binding capacity of SA/b-RBC, although in the cited work we did not estimate SA attachment to b-RBC [10]. Confirming this observation, SA-mediated b-IgG attachment to b_{700}-RBC was markedly reduced as compared with RBC biotinylated at 20–230 μM BxNHS (Figure 1B), whereas SA surface density was maximal in SA/b_{700}-RBC (Figure 1A). This result indicates that the surface density of biotin residues coupled to the membrane of b_{700}-RBC is so high that b_{700}-RBC-associated biotin occupies most of the biotin-binding sites of SA, thus preventing subsequent attachment of b-IgG. Our recent study shows that multivalent binding of SA to b-RBC inactivates the biotinylated membrane complement regulators DAF and CD59 [8]. Figure 1(C) demonstrates that incubation with homologous serum leads to lysis of SA-coated rat RBC biotinylated at 230 and 700 μM BxNHS, while SA-free b_{200}-RBC and b_{700}-RBC were stable in the serum. Attachment of SA to rat RBC biotinylated at BxNHS concentrations less than 100 μM did not induce their lysis by homologous serum *in vitro*. Attachment of b-IgG did not affect the stability of SA/b_{20}-RBC and SA/b_{70}-RBC in serum (less than 1% lysis after 1 h incubation at 37 °C with serum at final dilution of 1:5).

Figure 2 shows attachment of radiolabelled b-IgG to serum-stable SA/b_{70}-RBC and SA/b_{20}-RBC. Both preparations demonstrated saturable attachment of b-IgG. Saturation level of b-IgG attachment was markedly higher for SA/b_{70}-RBC, presumably due to the higher surface density of membrane-bound SA (Figure 1A). About 40% of added b-IgG bound to both SA/b_{70}-RBC and SA/b_{20}-RBC. Maximal b-IgG attachment was 5×10^4 IgG molecules per SA/b_{20}-RBC and 9×10^4 IgG molecules per SA/b_{70}-RBC.

In order to test the ability of serum-stable immuno-RBC to recognize the target, we utilized a model developed in our previous study, i.e. binding of immuno-RBC to antigen immobilized on the bottom of plastic culture plates [6]. Figure 3 shows that b-Ab/SA/b_{20}-RBC bind to the antigen-coated, but not to a BSA-coated plastic surface. In contrast, b-IgG/SA/b_{20}-RBC do not bind to any surface. At the antigen surface density attained at overnight incubation of 3 μg of antigen in the well, about 5×10^6 immuno-RBC bind to an antigen-coated well with a bottom area of 2 cm^2. This indicates that more than 85% of the well bottom surface is occupied with bound b-Ab/SA/b_{20}-RBC. Reduction of the surface density of immobilized antigen leads to marked reduction of b-Ab/SA/b_{20}-RBC

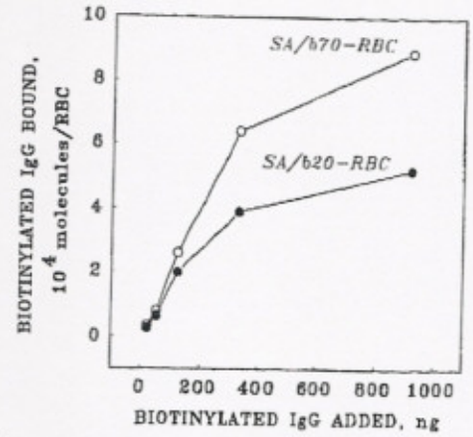

Figure 2

Streptavidin-mediated attachment of radiolabelled b-IgG biotinylated rat RBC. Indicated amounts of ^{125}I-labelled b-IgG were incubated for 1 h with SA-coated rat RBC biotinylated at 70 μM BxNHS (SA/b_{70}-RBC, ○) or with SA-coated rat RBC biotinylated at 20 μM BxNHS (SA/b_{20}-RBC, ●). After elimination of non-bound ^{125}I-labelled b-IgG, radioactivity associated with RBC has been determined.

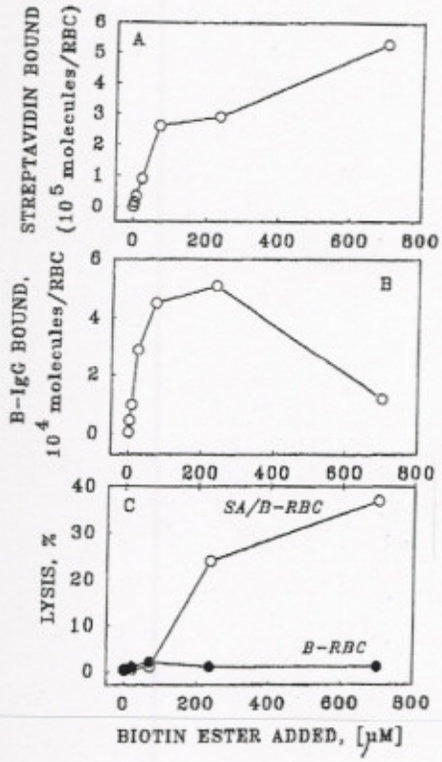

Figure 1

Biotinylation of rat RBC with long-arm biotin hydroxysuccinimide ester. Fresh rat RBC washed with PBS were biotinylated with BxNHS at the indicated final concentrations in the reaction mixture. After elimination of non-bound BxNHS, streptavidin (1.2×10^6 molecules/RBC) was added. (A) Binding of radiolabelled SA to b_n-RBC. (B) Binding of radiolabelled b-IgG to SA/b_n-RBC (1.25×10^5 molecules of ^{125}I-labelled b-IgG per SA/b_n-RBC were added for attachment). (C) Lysis of SA/b_n-RBC (○) and b_n-RBC (●) by serum *in vitro*.

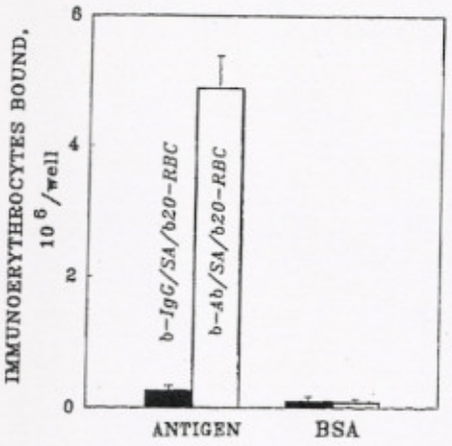

Figure 3

Binding of serum-stable immuno-RBC (b-Ab/SA/b_{20}-RBC) to immobilized antigen. Wells of 24-well culture plates were coated with mouse IgG by overnight incubation with 3 μg of IgG/well, as described in the Materials and methods section. Immuno-RBC carrying biotinylated polyclonal goat antibody against mouse IgG (open bars) and control immuno-RBC carrying biotinylated non-specific mouse IgG (closed bars) were incubated for 2 h in wells coated with antigen or with BSA. After washing of non-bound RBC, water was added to wells and binding of RBC in wells was determined by measurement of haemoglobin absorbance in lysates. The data are presented as means ± S.D. ($n = 3$).

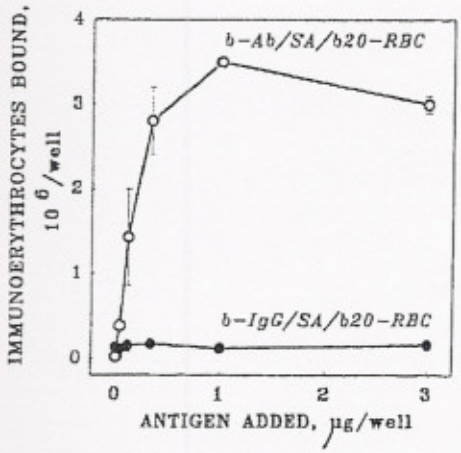

Figure 4

Influence of the surface density of immobilized antigen on binding of serum-stable immuno-RBC (b-Ab/SA/b_{20}-RBC). Wells of 24-well culture plates were coated with antigen by overnight incubation with indicated amounts of mouse IgG. Binding of b-Ab/SA/b_{20}-RBC (○) and b-IgG/SA/b_{20}-RBC (●) was determined as described in the legend to Figure 3. The data are presented as means ± S.D. ($n = 3$).

binding to the target surface (Figure 4). This result implies that: (i) b-Ab/SA/b_{20}-RBC targeting to immobilized antigen is specific, and (ii) multivalent interaction of RBC-bound antibody with immobilized antigen is important for targeting. It should be noted, however, that, even at minimal surface density of immobilized antigen, b-Ab/SA/b_{20}-RBC binding to antigen-coated wells was dramatically higher than that of b-IgG/SA/b_{20}-RBC [$(3.8 \pm 0.3) \times 10^5$ RBC/well versus $(3.4 \pm 1.5) \times 10^4$ RBC/well)] and than binding of b-Ab/SA/b_{20}-RBC to BSA-coated wells ($< 2 \times 10^4$ RBC/well).

To test biocompatibility of immuno-RBC in a physiological model, we have studied the biodistribution of serum-stable immuno-RBC after their intravenous injection into the bloodstream. For this purpose we have injected ^{51}Cr-labelled b-IgG/SA/b_{20}-RBC into rats. Previously we have used this model and demonstrated that injection of complement-sensitive avidin-carrying b_{1000}-RBC in rats leads to their fast lysis and elimination by the reticuloendothelial system after intravenous injection [9]. Table 1 shows the biodistribution of ^{51}Cr-labelled serum-stable b-IgG/SA/b_{20}-RBC and control RBC in rat tissues after intravenous injection. For both preparations, less than 1% of blood radioactivity was detected in plasma, thus indicating that there was no lysis of b-IgG/SA/b_{20}-RBC or control RBC *in vivo*. Enhanced uptake of radioactivity has been detected only in spleen, probably reflecting non-specific damage of RBC induced by multiple centrifugation. Thus serum-stable b-IgG/SA/b_{20}-RBC circulate in the bloodstream without marked elimination or lysis.

Table 1 Biodistribution of stable immuno-RBC and control rat RBC in rat tissues

The Table shows the biodistribution of ^{51}Cr isotope in rat tissues 1 h after intravenous injection of ^{51}Cr-labelled immuno-RBC or control rat RBC. The data are presented as percentages of injected radioactivity (ID) per g of tissue (means ± S.D., $n = 3$).

Tissue	b-IgG/SA/b_{20}-RBC (%ID/g)	RBC (%ID/g)
Blood	4.68 ± 0.11	5.63 ± 0.49
Lung	1.12 ± 0.49	1.15 ± 0.51
Liver	0.35 ± 0.09	0.35 ± 0.07
Kidney	0.15 ± 0.06	0.18 ± 0.06
Spleen	5.11 ± 0.23	3.44 ± 1.21
Heart	0.17 ± 0.05	0.22 ± 0.03

Modification of RBC with various cross-linking agents is a new approach for targeting of a drug and prolongation of its lifespan in the bloodstream [3,11]. SA–biotin cross-linker is useful for attachment of biotinylated proteins to RBC (e.g. antibodies for targeting) [6,12–14]. Results of the present study document that mild biotinylation of RBC provides immuno-RBC which satisfy requirements of applicability for drug targeting: b-Ab/SA/b_{20}-RBC bind to immobilized antigen and display normal biodistribution *in vivo*. Therefore these data open a new avenue for exploration of drug targeting by immuno-RBC.

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