ELSEVIER

Biochimica et Biophysica Acta 1279 (1996) 137–143

BBA Biochimica et Biophysica Acta

Target-sensitive immunoerythrocytes: interaction of biotinylated red blood cells with immobilized avidin induces their lysis by complement

Vladimir R. Muzykantov [a,c,*], Alla B. Zaltsman [a,b], Mikhail D. Smirnov [a], Gennady P. Samokhin [a], B. Paul Morgan [b]

[a] *Institute of Experimental Cardiology, Russian National Cardiology Research Center, Moscow, Russia*
[b] *University of Wales College of Medicine, Cardiff, UK*
[c] *Institute for Environmental Medicine, University of Pennsylvania Medical Center, 36th Street and Hamilton Walk, Philadelphia, PA 19104-6068, USA*

Received 20 July 1995; revised 2 October 1995; accepted 1 November 1995

Abstract

Red blood cells (RBC) coated with antibody (immunoerythrocytes) may be useful for drug targeting. Previously we have developed a methodology for avidin (streptavidin)-mediated attachment of biotinylated antibodies (b-Ab) to biotinylated RBC (B-RBC). We have observed that binding of avidin to B-RBC in suspension leads to their complement-mediated lysis by autologous serum. In the present work we have studied the interaction of B-RBC, which are not complement susceptible, with immobilized avidin and their consequent susceptibility to lysis by complement. B-RBC adhered tightly to avidin-coated surfaces and were rendered susceptible to lysis by autologous serum. A long biotin ester provided more effective binding of the B-RBC to immobilized avidin and greater lysis by complement, than a short biotin ester. Based on these results, we have hypothesized that targeting of serum-stable drug-loaded B-RBC attained by step-wise administration of b-Ab and streptavidin may provide target-sensitive lysis of B-RBC. To confirm this hypothesis, we have studied b-Ab and streptavidin mediated targeting of B-RBC to immobilized antigen. Step-wise addition of biotinylated antibody, avidin or streptavidin and b-RBC caused specific binding of B-RBC to immobilized antigen and their subsequent lysis by autologous serum. Therefore, our results obtained in an in vitro model demonstrate that B-RBC might be used for targeting and local release of drug.

Keywords: Drug targeting; Complement; Membrane lysis

1. Introduction

The aim of targeting is to achieve an accumulation of a drug at a specific site after systemic administration [1,2]. Several methods for drug targeting have been proposed, including packaging in liposomes and red blood cells (RBC) bearing antibody to target antigens [1–3]. Targeting of immunoliposomes loaded with various drugs has been studied in various animal models, including targeting to tumors [4–6], endothelial cells [7] and macrophages [8,9]. Recent studies demonstrate that modification of liposomes with polyethylene glycol [5,10,11] and gangliosides [12], as well as utilization of specific lipid composition [13] provide prolongation of the lifespan of liposomes in the bloodstream. Meanwhile, immunogenicity and the uptake of liposomes by reticuloendothelial tissue are of concern in terms of the safety of liposomes utilization [14].

RBC represent a second potential carrier for drug delivery. The most important difference between liposomes and RBC is their size: RBC are particles of 5–10 μm in various animal species, whereas liposomes vary in the range 100–1000 nm. Therefore, liposomes are capable of filtration into tissues, and as such are preferable for the targeting of drugs to tissues (e.g., for tumor targeting [15]). As RBC can not filtrate in the tissues, they can interact with intravascular targets only. Association with RBC should reduce the filtration of a drug in the tissues and, therefore, reduce the side effects of a drug.

Autologous RBC are a non-immunogenic, physiologic and available carrier for drug targeting [3,16]. RBC possess a large inner volume and thus offer the advantage of prolonged circulation of a large dose of encapsulated drug in the organism [17]. RBC may be used for drug delivery to various intravascular targets including endothelial cells,

Abbreviations: RBC, red blood cell; B-RBC, biotinylated red blood cell; Av, avidin.

* Corresponding author at address c. Fax: +1 (215) 8980868.

SSDI 0005-2736(95)00260-X

macrophages [18], blood cells [19] and immunocompetent cells [20]. Components of the extracellular matrix exposed to the bloodstream in sites of vessel injury (collagen, e.g.) may also represent a target for immunoerythrocytes [21]. For example, immunoerythrocytes could be used for the delivery of inhibitor(s) of arachidonic acid metabolism to the sites of the vascular injury, in order to prevent the aggregation of platelets and reduce inflammation [22]. Delivery of anticoagulant agents (hirudin, e.g., [23]) to the injured blood vessel(s) could also be considered as an area of application of RBC-mediated targeting.

During the last decade we have investigated a targeting strategy using avidin-mediated attachment of biotinylated antibody to the surface of biotinylated RBC (B-RBC). RBC biotinylation is a relatively simple procedure providing sites for high-affinity attachment of avidin or streptavidin [24–26]. There are two strategies for immunotargeting of B-RBC loaded with a drug. First, biotinylated antibody can be attached to B-RBC via streptavidin (one-step targeting). Such immunoerythrocytes possess high affinity to the targets [21,25,27]. Second, biotinylated antibody and streptavidin may be injected at the first stage followed by injection of drug-loaded B-RBC (two- or three-step targeting, developed recently in nuclear medicine [28–31]).

B-RBC are stable in serum and do not undergo intravascular lysis in either animals [32–34] or humans [35]. However, addition of avidin or streptavidin to a suspension of B-RBC renders the cells susceptible to lysis by autologous complement via the alternative pathway [36]. If interaction of avidin (streptavidin) with B-RBC could be restricted to the target, then consequent lysis by complement could be utilized to cause local release of drug. One possible strategy would be to deliver complement-stable, drug-loaded B-RBC to a target by sequential administration of biotinylated target-specific antibody, avidin (streptavidin) and B-RBC. Systemic injection of biotinylated antibodies followed by streptavidin has been shown to provide targeting of streptavidin in animals [28,29] and humans [30,31]. Importantly, excess streptavidin and biotinylated antibody are rapidly eliminated from the circulation due to polyvalent interaction of avidin with biotinylated antibodies and fast liver uptake of the complexes [37]. B-RBC injected into the circulation would then bind to the target due to interaction with avidin already bound to the target. Subsequently, complement-mediated lysis of the target-bound B-RBCs would release the drug, thus providing high local concentration of a drug in the target zone.

A requirement for this strategy is that B-RBC attached to avidin already bound to the target antigen are rendered susceptible to lysis by complement. However, interaction of B-RBC with immobilized avidin has not been previously investigated and the resultant susceptibility to complement lysis may differ from that in suspension due to steric considerations. In the present work, we have investigated this possibility using RBC biotinylated with biotin *N*-hydroxysuccinimide ester ('short biotin ester, SB-RBC') and with 6-biotinylamidocaproic acid *N*-hydroxysuccinimide ester ('long biotin ester, LB-RBC'). We have examined in vitro the targeting of B-RBC to immobilized avidin and also to immobilized antigen via biotinylated antibody and avidin (streptavidin). The results demonstrate that B-RBC bound to avidin immobilized either directly or via biotinylated antibody are specifically rendered susceptible to lysis by complement.

2. Materials and methods

2.1. Materials

Avidin was purified from hen egg white as described in [38], streptavidin was from Calbiochem (San Diego, CA). RBC were obtained from healthy volunteers. Fresh autologous serum or pooled human serum of the same blood group (prepared from the blood of healthy volunteers) was used as a source of complement. Bovine serum albumin (BSA), biotin *N*-hydroxysuccinimide ester (short biotin ester, SB), 6-biotinylamidocaproic acid *N*-hydroxysuccinimide ester (long biotin ester, LB) and human fibrinogen were from Sigma (St. Louis, MO). Avidin was labeled with [^{125}I]-Bolton-Hunter reagent from Amersham (UK), according to the manufacturers instructions. Isotonic veronal-buffered saline (3 mM diethylbarbituric acid, 1 mM sodium salt of diethylbarbituric acid, 145 mM NaCl, 1.8 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.4; VBS) containing 1 mg/ml BSA (VBS-BSA) was used as diluent in all studies.

2.2. Biotinylation of RBC

Biotinylation of RBC was performed as described previously [25]. Briefly, sodium tetraborate (0.1 ml 0.1 M, pH 9.0) and biotin ester (3 μl of 0.1 M solution in dimethyl formamide, DMF) were added to 1 ml of 10% suspension of RBC in saline. After 30 min incubation at room temperature, B-RBC were washed by centrifugation in VBS. To estimate number of biotin residues on the B-RBC membrane we used the method of Suzuki and Dale [32,33]. Briefly, we have incubated B-RBC with radiolabeled avidin for 1 h at room temperature, eliminated non-bound avidin via standard washing by centrifugation and determined B-RBC-bound radioactivity in a gamma-counter. By this assay, the standard procedure of biotinylation provides about 10^6 avidin-accessible biotin residues per RBC.

2.3. Binding of B-RBC to immobilized avidin

For avidin immobilization, avidin (5 μg in 0.25 ml VBS) was incubated overnight at 4°C in multiwell culture plates ('Nunc', USA). Control wells were incubated with VBS-BSA instead of avidin. The plates were washed 5

times with distilled water to remove unbound avidin, incubated for 1 h with VBS-BSA to block non-specific binding sites and washed 5 times with distilled water. To determine the surface density of immobilized avidin, radiolabeled avidin was immobilized in the wells as described. After washing out of non-bound avidin, immobilized radiolabeled avidin was eluted from wells by boiled SDS solution and bound radioactivity was measured in a RackGamma counter (LKB, Sweden).

To study the binding of b-RBC to avidin-coated surfaces, 0.3 ml of VBS-BSA and 25 μl of 10% B-RBC or native RBC suspension were added to the wells and incubated for 1 h at room temperature. Unbound cells were washed away with saline. Then 0.3 ml of distilled water was added to each well. 10 min later, hemoglobin absorbance at 405 nm was measured in lysates to determine the amount of RBC bound to avidin-coated and to albumin-coated control surfaces. Calibration curves of absorbance at 405 nm in lysates of known amounts of RBC were made in each experiment to calculate the RBC binding.

2.4. Lysis of B-RBC bound to immobilized avidin

To study lysis by homologous serum of B-RBC bound to immobilized avidin, two methods were employed. The first was used to compare lysis of B-RBC incubated in avidin-coated and albumin-coated wells, i.e., avidin-bound B-RBC and unbound B-RBC. In this case, unbound B-RBC were not washed off after the binding step and serum at the chosen dilution was added directly to VBS-BSA in the well. After a 1 h incubation with serum at 37°C, aliquots of supernatants were withdrawn from the wells, avoiding disturbance of sedimented cells, and hemoglobin absorbance at 405 nm was measured. To correct for hemoglobin in serum, the absorbance at 405 nm in corresponding cell-free serum dilutions was subtracted from that obtained in test wells. Wells with serum-free VBS-BSA were used as zero lysis standards; 100% lysis was attained by the addition of distilled water to the same number of B-RBC. The degrees of lysis by water of B-RBC bound with immobilized avidin and avidin-free B-RBC were the same.

To evaluate lysis of B-RBC bound to the avidin-coated surfaces, unbound B-RBC were washed off after incubation of B-RBC with the immobilized avidin. VBS-BSA containing human serum was added and wells were incubated under standard conditions (1 h, 37°C). Lysis was estimated by hemoglobin absorbance at 405 nm in supernatants, as described above. Binding of B-RBC to the immobilized avidin was determined by water lysis in parallel wells as described above.

2.5. Binding of B-RBC to immobilized antigen through biotinylated antibody

Human IgG was immobilized in the wells by overnight incubation at 4°C (5 μg in 250 μl of VBS per well). The plates were washed with water, blocked with VBS-BSA and washed again with water. Biotinylated sheep anti-human IgG (prepared by standard method [39]) was incubated in the wells (4 μg in 250 μl of VBS-BSA/well, 1 h at room temperature), washed and avidin or streptavidin (5 μg in 250 μl of VBS-BSA/well) was added. After a 1 h incubation wells were washed 5 times with water, B-RBC were added and incubated for 1 h at 37°C. Unbound B-RBC were removed by washing. Binding of B-RBC to wells and their subsequent lysis by autologous complement

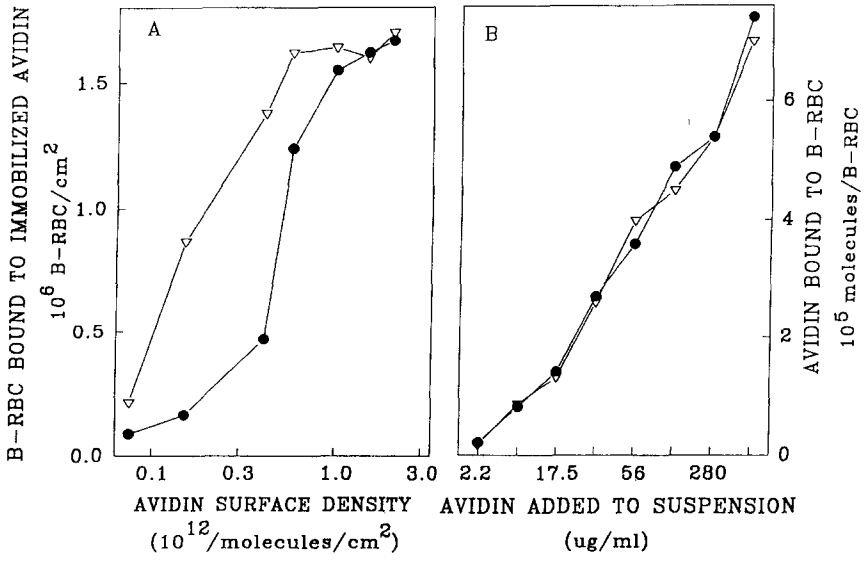

Fig. 1. Interaction of biotinylated human red blood cells with avidin. RBC modified with short biotin ester (SB-RBCs, circles) or with long biotin ester (LB-RBCs, triangles) were incubated with immobilized avidin (A) or with avidin in suspension (B). Avidin surface density and RBC binding were measured as detailed in Section 2. Typical results from a series of four experiments.

was measured as described. In a separate experiment, control RBC were incubated in the presence of complement in the antigen-coated wells pre-treated with biotinylated sheep anti-human IgG and avidin. According to hemoglobin absorbance in supernatants, lysis of control RBC in these wells did not exceed that in BSA-coated wells.

3. Results

3.1. Binding of biotinylated RBC to immobilized avidin

Table 1 shows that SB-RBC and LB-RBC bound specifically to avidin-coated plates, but not to the BSA-coated control plate. Native RBC did not bind to either plate. The standard procedure (overnight incubation with 5 μg avidin per well) provided immobilization of $2 \cdot 10^{12}$ molecules of avidin/cm^2 as calculated from binding of radiolabeled avidin. At this surface density of immobilized avidin, about $2 \cdot 10^6$ B-RBC bound per cm^2 for both LB and SB forms of biotin. Thus, at maximal binding the surface area available for each B-RBC is calculated to be 70 μm^2. The human RBC is a disc of 8 μm diameter and would be predicted to occupy an area of about 50 μm^2. Therefore, under these conditions, bound B-RBC occupy approx. 70% of the available surface.

Reduction of the surface density of immobilized avidin (by reducing the quantity of avidin incubated in the wells) caused a decrease in the binding of B-RBC. However, this decrease was not the same for SB-RBC and LB-RBC, the latter bound better at reduced density of immobilized avidin (Fig. 1A). In contrast, when free avidin was added to a suspension of B-RBC it bound to a similar degree to both SB-RBC and LB-RBC over the range of avidin concentrations examined (Fig. 1B).

3.2. Lysis by homologous serum of biotinylated red blood cells bound to immobilized avidin

While B-RBC were stable in serum in the absence of avidin, homologous serum rapidly lysed B-RBC bound to immobilized avidin (Fig. 2). No lysis was observed in the absence of serum. Serum induced negligible lysis of native (non-biotinylated) RBC incubated in the avidin-coated wells (not shown). Lysis of B-RBC incubated in BSA-coated wells was negligible as compared with lysis of the same cells in the avidin-coated wells.

Comparison of LB-RBC and SB-RBC revealed that the former were rendered more sensitive to complement lysis when bound at the same cell density to identical avidin-coated surfaces. While absolute values of lysis varied for different sera, maximal lysis of immobilized avidin-bound SB-RBC was never higher than 60%, while almost 100% lysis was achieved with immobilized avidin-bound LB-RBC (Fig. 2). Because LB-RBC were more susceptible to complement lysis than SB-RBC, subsequent studies utilized LB-RBC.

At the standard surface density of avidin used in this study $(2–2.5) \cdot 10^{12}$ molecules/cm^2) almost complete lysis of avidin-bound LB-RBC was achieved (85–95% of

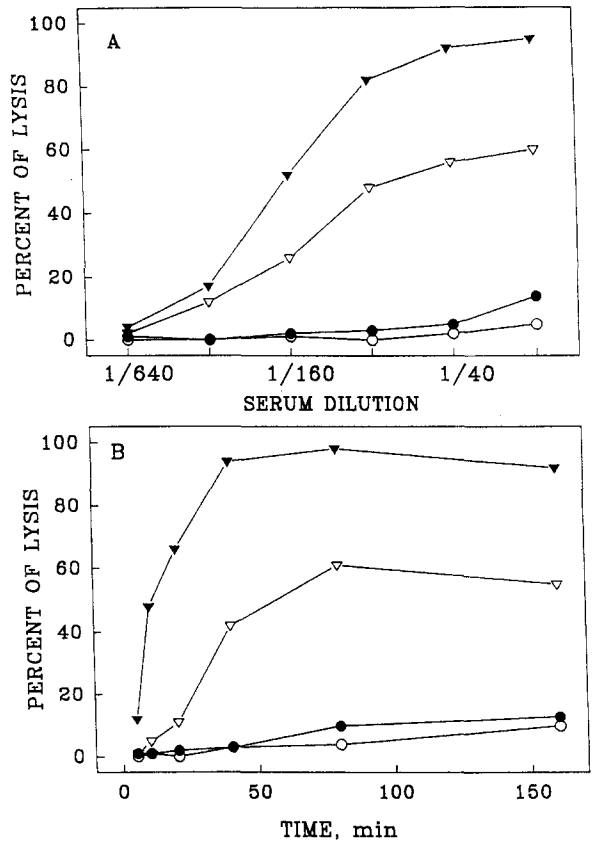

Fig. 2. Biotinylated RBCs are lysed by normal human serum after binding to immobilized avidin. (A) SB-RBC (open symbols) and LB-RBC (closed symbols) were incubated for 1 h in avidin-coated wells (triangles) or in BSA-coated wells (circles). Normal human serum was then added to the wells to attain the indicated final dilutions and incubated for a further 1 h at 37°C. Hemolysis was measured by hemoglobin release into supernatants as detailed in methods. Results are representative of those obtained in 3 separate experiments. (B) Time course at 37°C of serum lysis of B-RBC bound to immobilized avidin. Symbols are the same as in A. Hemolysis was measured by hemoglobin release into supernatants as detailed in methods. Results are representative of those obtained in 3 separate experiments.

Table 1
Binding of biotinylated RBC to avidin-coated surface

Coating	RBC	SB-RBC	LB-RBC
Avidin	< 0.1	2.01 ± 0.2	1.9 ± 0.1
Albumin	< 0.1	< 0.1	< 0.1

Wells coated with 5 μg of avidin or albumin as control as described in Section 2 were incubated with suspensions containing $1.5 \cdot 10^7$ RBC, SB-RBC or LB-RBC for 1 h. After washing to remove non-bound RBCs, binding of RBCs was measured by detection of hemoglobin absorbance in wells after addition of distilled water for hypotonic lysis of bound cells. SB, short biotin ester; LB, long biotin ester. Binding is shown as RBC bound per cm^2 $\times 10^{-6}$, mean ± S.E., $n = 3$.

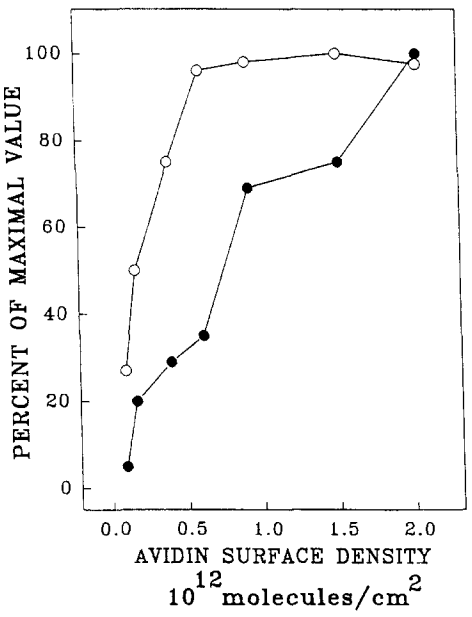

Fig. 3. Influence of the surface density of immobilized avidin on B-RBC binding and serum lysis. LB-RBC binding to avidin coated wells (open circles) and subsequent lysis by serum (closed circles) were measured as detailed in methods. The data are presented as percentage of maximal values of binding ($8 \cdot 10^5$ LB-RBC/cm^2) and lysis (78% lysis) attained at avidin surface density of $2.5 \cdot 10^{12}$ molecules of avidin/cm^2. Representative results from series of two separate experiments.

lysis at a serum dilution of 1:20). A reduction in surface density of immobilized avidin to $0.6 \cdot 10^{12}$ molecules/cm^2 caused a decrease in lysis to 40% of that attained at a surface density of $2 \cdot 10^{12}$ molecules/cm^2. In contrast with lysis, binding of LB-RBC to avidin remained unchanged over this range (Fig. 3). Further reduction of the avidin density caused a marked decrease in LB-RBC binding and almost complete elimination of lysis.

3.3. LB-RBC bound to immobilized antigen through biotinylated antibody and avidin are rendered susceptible to lysis by autologous serum

To test our strategy for local lysis of targeted LB-RBC, we studied binding to immobilized antigen through biotinylated antibody and avidin and subsequent susceptibility to lysis by autologous serum. Table 2 shows that step-wise incubation of antigen-coated wells with biotinylated antibody and avidin (or streptavidin) provides specific binding of LB-RBC. There was no binding of LB-RBC to antigen-coated wells if avidin or biotinylated antibody were omitted (not shown). Addition of autologous serum caused efficient lysis of target-bound LB-RBC in this model. Thus, biotinylated LB-RBC, which are stable in serum, can be targeted to an antigen-bearing surface and, after binding to the target, will be rendered susceptible to complement-mediated lysis by autologous serum.

4. Discussion

Avidin-biotin technology has achieved a high profile in biomedical sciences in the last 15 years. The high affinity polyvalent interaction between avidin (streptavidin) and biotin or biotinylated molecules has been exploited in many immunochemical techniques [39]. During the last five years we and others have attempted to explore this technology in vivo. Biotinylated antibodies and streptavidin are used for gamma-immunoimaging in animal models and in patients [28–31]. Streptavidin and avidin are used for immunotargeting of liposomes [40], for clearance of radiolabeled biotinylated antibodies from the circulation [37] and for targeted delivery of antigens to immunocompetent cells [41]. Avidin/biotin technology has been utilized to attach biotinylated antibodies to RBC for RBC-mediated elimination of pathogens from the bloodstream [42,43]. Avidin- and streptavidin-mediated conjugation of biotinylated DNA, peptides and enzymes is useful for targeting of these agents to the various organs and tissues including brain [44], lung [45] and tumors [46].

We have explored the use of avidin (streptavidin) for targeted delivery of biotinylated RBC loaded with drug, using biotinylated antibody as the primary targeting agent [21,25,27]. While investigating the biocompatibility of avidin-coated B-RBC, we have observed that attachment of avidin or streptavidin to B-RBC in suspension caused

Table 2
Interaction of LB-RBC with avidin or streptavidin bound to immobilized antigen through biotinylated antibody cause their lysis by autologous serum

Coating		BSA	Avidin	IgG	IgG
Targeting		none	direct	B-anti-IgG + AV	B-anti-IgG + SA
LB-RBC bound		< 0.1	34.7 ± 0.7	32.4 ± 0.7	33.1 ± 1.2
	Serum dilution				
Lysis	1:5	9.5 ± 0.1	97.7 ± 0.0	81.2 ± 7.3	80.6 ± 7.8
	1:10	ND	100.0 ± 0.0	90.7 ± 5.3	45.7 ± 4.4
	No serum	< 5	< 5	< 5	< 5

Wells coated with human IgG were incubated with biotinylated sheep antibody to human IgG (B-anti-IgG) for 1 h, washed, incubated for 1 h with avidin (AV) or with streptavidin (SA) and washed again. Binding of LB-RBC is shown as a percent of added LB-RBC (10^6 LB-RBC were added per well), mean ± S.E., $n = 3$. Non-specific binding of LB-RBC in IgG-coated wells treated with B-&-IgG only or with avidin only was the same as in BSA-coated wells. Lysis by serum is shown as percent of lysis of bound LB-RBC at indicated serum dilution.

their lysis by autologous complement via the alternative pathway [36]. The combination of these properties of avidin interaction with B-RBC (affinity targeting and lysis by complement), provides a basis for a new stepwise targeting strategy to achieve local release of a drug from B-RBC. However, an obligatory requirement for this strategy is that B-RBC bound through immobilized avidin to the target cell are rendered susceptible to lysis.

The interaction of B-RBC with immobilized avidin and their consequent lysis differ in several respects from the previously described interaction of B-RBC with free avidin in suspension [36]. Notably, B-RBC formed using the long ester of biotin bound immobilized avidin more efficiently than did those formed using the short ester, and were more susceptible to serum lysis (Figs. 1 and 2). We suggest that these differences occur because the long biotin ester helps to overcome steric limitations imposed upon B-RBC binding to immobilized avidin. Presumably, binding of B-RBC to immobilize avidin (streptavidin) and consequent complement-induced lysis should depend on the degree of RBC biotinylation, i.e., on the surface density of biotin residues on RBC. In the present study we have used RBC biotinylated with 1 mM BxNHS, which provides coupling of about 10^6 streptavidin-accessible biotin residues per RBC. We did not address the interaction of B-RBC possessing lower surface densities of biotin residues with immobilized avidin. Our preliminary results, however, suggest that a decrease in the surface density of biotin residues on the B-RBC membrane leads to a reduction of B-RBC targeting to immobilized streptavidin and antigen (Muzykantov and Murciano, unpublished results).

We here demonstrate that B-RBC bind specifically to immobilized avidin and are consequently rendered susceptible to lysis by autologous serum. Both direct immobilization of avidin on the plastic surface and its targeting to immobilized antigen through biotinylated antibody gave similar results, confirming the validity of our strategy. Both avidin and streptavidin induced lysis, suggesting that streptavidin may be used for targeting and local lysis of B-RBC (Table 2). This is important because of the greater biocompatibility of streptavidin [47].

The concept of the target-sensitive vehicle for drug targeting was developed first for immunoliposomes by Huang and co-workers a decade ago. They have demonstrated that interaction of immunoliposomes with immobilized antigen induces alterations in the physical stability of the lipid vesicle and thus leads to target-induced lysis of liposomes [48]. We have extended this concept towards RBC. RBC are a non-immunogenic, biocompatible and available vehicle, possessing large inner volume which can be loaded with a drug [2]. The difference in the size of immunoliposomes (relatively small particles) and immunoerythrocytes (large particles) suggests that the former would be appropriate for targeting to antigens in tissues, whereas the latter appear to be appropriate for intravascular targeting of a drug. Target-induced lysis of immunoliposomes and immunoerythrocytes differ in their mechanisms, since the latter is mediated by a physiologic mediator, complement.

Our previous studies document that avidin attachment to RBC via biotinylated membrane proteins causes lysis, while avidin attachment via biotinylated lipids, biotinylated ricin or tannin does not, implicating a role for specific surface proteins in the process [49–51]. Analysis of b-RBC by SDS/PAGE, Western-blotting, and staining of nitrocellulose with streptavidin-peroxidase demonstrated that biotinylation leads to coupling of biotin to a variety of RBC proteins, including specific regulators of complement, decay accelerating factor and CD59 [52]. The functional assay performed in the cited work revealed that avidin cross-links RBC membrane regulators of complement, thus inactivating cellular defence against complement [52].

Our preliminary data suggest that interaction of B-RBC with immobilized streptavidin induces redistribution of biotinylated regulators of complement, DAF and CD59 in the RBC membrane. These regulators diffuse to the basal surface of the bound B-RBC, became tethered to avidin and thus effectively denude the apical surface of the B-RBC (A. Zaltsman, unpublished results). Therefore, the apical surface, depleted of membrane regulators of complement, may be spontaneously attacked by complement. As Fig. 3 shows, induction of hemolytic susceptibility required a much higher surface density of immobilized avidin than was needed for stable binding of B-RBC, presumably reflecting an increased capacity of immobilized avidin at higher densities to bind membrane proteins diffusing in from the apical surface of the bound B-RBC. Presumably, other biotinylated proteins in the B-RBC membrane also contribute to B-RBC anchoring to immobilized avidin. Moreover, these proteins also may diffuse from the apical surface of the target-bound B-RBC to their basal surface, and thus contribute to destabilization of the B-RBC membrane.

In conclusion, our present results clearly demonstrate that B-RBC bind to avidin targeted to biotinylated antibody bound to immobilized antigen. Bound cells activate complement and are readily lysed. This data should aid in the design of immunoerythrocytes for targeted delivery and local release of a drug. Further in vivo studies are needed to address the important issues of safety and effectiveness of this targeting strategy. Our preliminary data obtained in rats suggest that not only lysis by complement, but opsonization without lysis may contribute in liver- and spleen-mediated elimination of streptavidin-carrying B-RBC from circulation. Such uptake may lead to toxicity of drug loaded RBC towards liver and spleen. The results of our animal experiments, however, suggest that the major portion of streptavidin-free B-RBC injected intravenously circulates in the bloodstream for a prolonged time [34]. Therefore, a two-step strategy (injection of biotinylated antibody conjugated with streptavidin or con-

sequent injection of these proteins followed by injection of B-RBC) may be more safe than one-step injection of B-RBC carrying streptavidin and biotinylated antibody.

Acknowledgements

The authors thank Dr. Carmen W. van der Berg (UWCM, Cardiff) for experimental help. We also thank Drs. C. Esmon and D. Houston (Oklahoma Medical Research Foundation, Oklahoma City) and Drs. M. Beers and H. Ischiropoulos (Pennsylvania University, Philadelphia) for discussion and helpful comments. Dr. A. Zaltsman was supported in part by The Trust. Dr. V. Muzykantov was supported in part by Will Rogers Fellowship.

References

[1] Gregoriadis, G. (1977) Nature 265, 407–411.
[2] Blakley, D. (1992) Acta Oncol. 31, 91–97.
[3] Poznansky, M. and Juliano, L. (1984) Pharmacol. Rev. 36, 277–344.
[4] Jones, M. and Hudson, M. (1993) Biochim. Biophys. Acta 1152, 231–242.
[5] Park, J., Hong, K., Carter, P., Asgari, H., Guo, L., Keller, G., Wirth, C., Kotts, C., Wood, W. and Papahadjopoulos, D. (1995) Proc. Natl. Acad. Sci. USA 92, 1327–1331.
[6] Nassander, U., Steerenberg, P., DeJong, W., Van Overveld, W., Te Boekhorst, C., Poels, L., Jap, P. and Storm, G. (1995) Biochim. Biophys. Acta 1235, 126–139.
[7] Maruyama, K., Kennel, S. and Huang, L. (1990) Proc. Natl. Acad. Sci. USA 87, 5744–5748.
[8] Hoedemakers, R., Vossebeld, P., Daeman, T. and Scherphof, G. (1993) J. Immunother. 13, 252–260.
[9] Roester, J., Hockertz, S., Vogt, B. and Lohmann-Matthes, M. (1991) J. Clin. Invest. 88, 1224–1229.
[10] Papahadjopoulos, D., Allen, T., Gabizon, A., Maylew, E., Matthay, K., Huang, S., Lee, K., Woodle, M., Lasic, D. and Redemann, C. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
[11] Blume, G., Cevc, G., Crommelin, M., Bakker-Wondenberg, G., Kluft, C. and Storm, G. (1993) Biochim. Biophys. Acta 1149, 180–184.
[12] Allen, T. and Hansen, C. (1991) Biochim. Biophys. Acta 1991, 1068, 133–141.
[13] Blume, G. and Cevc, G. (1993) Biochim. Biophys. Acta 1146, 157–168.
[14] Alving, C. (1992) Biochim. Biophys. Acta 1113, 307–322.
[15] Jain, R. (1987) Cancer Metast. Rev. 6, 559–593.
[16] Tonetti, M., Astroff, A., Satterfield, W., DeFlora, A., Benetti, U. and DeLoach, J. (1991) Am. J. Vet. Res. 52, 1630–1635.
[17] Sprandel, U. (1990) Res. Exp. Med. 190, 267–275.
[18] Magnani, M., Rossi, L., Brandi, G., Schiavano, G., Montroli, M. and Piedimonte, G. (1992) Proc. Natl. Acad. Sci. USA 89, 6477–6481.
[19] Chiarantini, L., Drolesky, R., Magnani, M. and DeLoach, J. (1992) Biotech. Appl. Biochem. 15, 171–184.
[20] Magnani, M., Chiarantini, L., Vittoria, E., Mancini, U., Rossi, L. and Fazi, A. (1992) Biotech. Appl. Biochem. 18, 188–194.
[21] Smirnov, V., Domogatsky, S., Dolgov, V., Hvatov, V., Klibanov, A., Koteliansky, V., Muzykantov, V., Repin, V., Samokhin, G., Shekhonin, B., Smirnov, M., Torhcilin, V., Sviridov, D. and Chazov, E. (1986) Proc. Natl. Acad. Sci. USA 83, 6603–6607.
[22] Samokhin, G., Smirnov, M., Muzykantov, V. and Domogatsky, S. (1986) Bull. Cardiol. Res. Ctr. USSR 1, 84–89.
[23] Fernandez-Ortiz, A., Meyer, B., Mailhac, A., Falk, E., Badimon, L., Fallon, J., Fuster, V., Cheseboro, J. and Badimon, J. (1994) Circulation 89, 1518–1522.
[24] Godfrey, W., Doe, B., Wallae, E., Bredt, B. and Wofsey, L. (1981) Exp. Cell. Res. 135, 137–145.
[25] Samokhin, G., Smirnov, M., Muzykantov, V., Domogatsky, S. and Smirnov, V. (1983) FEBS Lett. 154, 257–261.
[26] Magnani, M., Chiarantini, L. and Mancini, U. (1994) Biotech. Appl. Biochem. 29, 335–345.
[27] Muzykantov, V.R., Sakharov, D.V., Smirnov, M.D., Samokhin G.P. and Smirnov, V.N. (1986) Biochim. Biophys. Acta 884, 355–363.
[28] Hnatowich, D., Virzi, F. and Ruskowski, M. (1987) J. Nuclear Med. 28, 1294–1302.
[29] Pimm, M.V., Fells, H.F., Perkins, A.C. and Baldwin, R.W. (1988) Nuclear Med. Commun. 9, 931–941.
[30] Paganelli, G., Riva, P., Deleide, G., Cliva, A. and Siccardi, A. (1988) Int. J. Cancer Suppl. 2, 121–125.
[31] Paganelli, G., Belloni, C., Magnani, P., Siccardi, A. and Fazin, F. (1992) Eur. J. Nuclear Med. 19, 322–329.
[32] Suzuki, T. and Dale, G. (1987) Blood 70, 791–793.
[33] Suzuki, T. and Dale, G. (1988) Proc. Natl. Acad. Sci. USA 85, 1647–1651.
[34] Muzykantov, V.R., Seregina, N.S. and Smirnov, M.D. (1992) Int. J. Art. Organs 15, 622–627.
[35] Cavill, I., Trevetz, D., Fisher, J. and Hoy, T. (1988) Br. J. Haematol. 70, 491–493.
[36] Muzykantov, V.R., Smirnov, M.D. and Samokhin, G.P. (1991) Blood 78, 2611–2618.
[37] Sinitsyn, V., Mamontova, A., Chekneva, E., Shnyra, A. and Domogatsky, S. (1989) J. Nuclear Med. 30, 66–69.
[38] Green, M. (1970) Methods Enzymol. 18a, 414–418.
[39] Wilchek, M. and Bayer, M. (1990) Methods Enzymol. 184, 14–48.
[40] Loughrey, H., Ferraretto, A., Cannon, A., Acerbis, G., Sudati, F., Bottiroli, G., Masserini, M. and Soria, M. (1993) FEBS Lett. 332, 183–188.
[41] Skea, D. and Barber, B. (1993) J. Immunol. 159, 3557–3568.
[42] Taylor, R., Sutherland, W., Otto, A., Labuguen, R. and Wright, E. (1992) J. Immunol. 148, 2462–2468.
[43] Muzykantov, V. and Taylor, R. (1994) Anal. Biochem. 223, 142–148.
[44] Pardridge, W., Boado, R. and Kang, Y. (1995) Proc. Natl. Acad. Sci. USA 92, 5592–5596.
[45] Muzykantov, V. and Danilov, S. (1995) In Targeted delivery of imaging agents (Torchilin, V., ed.), pp. 465–485, CRC Press, Boca Raton.
[46] Schechter, B., Arnon, R., Wilchek, M., Schessinger, J., Hurwitz, E., Aboud-Pirak, E. and Sela, M. (1991) Int. J. Cancer 48, 167–172.
[47] Duhamel, R. and Whitehead, J. (1990) Methods Enzymol. 184, 201–207.
[48] Rodney, J., Ho, Y., Rouse, B. and Huang, L. (1987) J. Biol. Chem. 262, 13973–13978.
[49] Muzykantov, V.R., Smirnov, M.D. and Samokhin, G.P. (1991) Biochem. J. 273, 393–397.
[50] Muzykantov, V.R., Smirnov, M.D. and Klibanov, A.L. (1993) J. Immunol. Methods 158, 183–190.
[51] Muzykantov, V.R., Smirnov, M.D., Zaltzman, A.B. and Samokhin, G.P. (1993) Anal. Biochem. 208, 338–342.
[52] Zaltzman, A., Van der Berg, C.W., Muzykantov, V.R., Morgan, B.P. (1995) Biochem. J. 307, 651–656.