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Regulation of the Complement-Mediated Elimination of Red Blood Cells Modified with Biotin and Streptavidin

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Red blood cells (RBC) modified with biotin and streptavidin (SA) present an interesting potential drug delivery system. Biotinylation and SA attachment, however, alter the biocompatibility of RBC. We have reported that polyvalent SA attachment induces lysis of biotinylated RBC (b-RBC) by homologous complement via the alternative pathway. Lysis occurs due to inactivation of the membrane regulators of complement, DAF and CD59, cross-linked by SA. However, monovalent SA attachment does not induce lysis. On the basis of these findings we hypothesized that reduction of the biotin surface density on b-RBC would allow for monovalent SA attachment to b-RBC and that such SA/b-RBC should then be stable in the circulation.

In the present work we injected into rats several different radiolabeled RBC probes: rat RBC biotinylated to varying degrees (b_n-RBC, where b_n represents the input micromolar concentration of biotinylating agent), as well as SA/b_n-RBC. Extensively biotinylated rat RBC (b_{700}-RBC, stable in serum *in vitro*) were rapidly cleared from the bloodstream. We further found that extensively biotinylated human b_{1000}-RBC bound C3b from serum *in vitro* without detectable lysis, and that rat b_{700}-RBC bound to isolated macrophages in a complement-dependent fashion. Therefore, nonlytic C3b fixation and uptake of C3b-carrying b_{700}-RBC by macrophages appears to be the mechanism leading to clearance of b_{700}-RBC *in vivo*. Moderately biotinylated RBC (b_{70}-RBC and b_{240}-RBC) were stable in serum *in vitro*. SA attachment to b_{240}-RBC led to their rapid lysis in serum *in vitro*, lysis in the bloodstream, and clearance by the liver and spleen. SA attachment to b_{70}-RBC led to fast elimination of SA/b_{70}-RBC from the bloodstream, while *in vitro* SA/b_{70}-RBC were stable in serum. Modestly biotinylated RBC (b_{23}-RBC) demonstrated only marginally decreased 60-min survival in the bloodstream regardless of SA attachment. Our *in vitro* studies indicate that b_{23}-RBC bound approximately 10^5 SA molecules per cell, and the resulting SA/b_{23}-RBC bound 5×10^4 molecules of biotinylated IgG (b-IgG) per cell. About 60% of the injected dose of b-IgG/SA/b_{23}-RBC labeled with ^{51}Cr was detected in the rat blood cells 1 day after iv injection. To assess whether b-IgG/SA/b_{23}-RBC circulate in the bloodstream as a stable complex, we have injected ^{125}I-labeled b-IgG/SA/^{51}Cr-labeled b_{23}-RBC in rats. Up to 60 min after injection, both radiolabels display similar level in bloodstream. Up to 3 h after injection, about 70% of ^{125}I was detected in the blood cells. In contrast, 100% of ^{125}I was detected in plasma after injection of nonconjugated ^{125}I-labeled b-IgG. Thus, major portion of SA/b_{23}-RBC-attached b-IgG circulates as a complex with RBC. About 30% of RBC-bound b-IgG undergoes detachment from the carrier b-RBC, probably in the pulmonary capillaries, because lung level of ^{125}I was twice as high as that of ^{51}Cr. Therefore, the surface density of biotin on the b-RBC membrane appears to play a key role in regulating complement-mediated clearance of b_n-RBC and SA/b_n-RBC from the bloodstream. Modest biotinylation generates b-IgG/SA/b_{23}-RBC circulating for several hours as stable immunoerythrocytes without detectable lysis or marked elimination, and it may be possible to use these RBC in a drug delivery system.

Biotinylation of red blood cells (RBC)[2] has been studied during the past two decades as a tool for the *in*

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[2] Abbreviations used: RBC, red blood cells; b-RBC, biotinylated RBC; SA, streptavidin; SA/b-RBC, b-RBC carrying streptavidin; b-IgG, biotinylated IgG; DAF, decay-accelerating factor; C3b, active subcomponent of the third component of complement; MAC, membrane attack complex of complement; BxNHS, long-arm succinimide biotin ester; BSA, bovine serum albumin.

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vitro investigation of the RBC membrane (1–5). More recently biotinylated RBC (b-RBC) have also been utilized in a series of *in vivo* experiments which traced the RBC in the bloodstream in animals (6, 7) and in humans (8). Streptavidin (SA)-mediated attachment of biotinylated molecules (antibodies, antigens, enzymes, etc.) to b-RBC has been suggested as a strategy to achieve RBC-mediated drug targeting (9), selective delivery of antigens to immunocompetent cells (targeted immunization (10)) and specific elimination of circulating pathogens from blood (11).

Biotinylation of RBC followed by attachment of avidin or SA may, however, alter the biocompatibility of RBC prepared at high levels of biotinylation. Several authors have demonstrated that biotinylation does not substantially alter the life span of RBC in the bloodstream (6, 8, 12). However, SA attachment (generating SA/b-RBC) leads to lysis of these cells by autologous serum *in vitro* (13) and to their rapid clearance from the circulation *in vivo* (12). Therefore, SA reduces the biocompatibility of b-RBC, which would obviously eliminate the possible use of SA-coated b-RBC (SA/b-RBC) *in vivo*.

In order to solve this problem we previously examined the mechanism by which complement induces lysis of SA/b-RBC (13–17). Our studies indicated that attachment of SA to b-RBC transforms these cells into activators of the alternative pathway of complement and renders b-RBC susceptible to lysis by autologous complement (13). It is likely that SA-induced cross-linking and subsequent inactivation of biotinylated regulators of complement, decay-accelerating factor (DAF) and CD59 in the membrane of b-RBC (14), is responsible for this effect. Evidence which supports this mechanism includes our observations that monovalent attachment of SA to b-RBC, as well as SA attachment to RBC via biotinyl-lipid, tannic acid, and biotinylated antibody against RBC antigens, does not induce lysis by complement *in vitro* (15–17). Thus, monovalent attachment of SA may provide complement-resistant SA/b-RBC suitable for *in vivo* exploration.

Based on these *in vitro* observations, we have now extended our studies to an *in vivo* model. We have biotinylated rat RBC at various levels of biotinylation (generating rat b_n-RBC, where *n* represents the micromolar input of biotinylating reagent) and studied the properties of these RBC as well as SA/b_n-RBC, in the circulation of rats. Unexpectedly, we observed that RBC biotinylated at relatively high levels (b_{700}-RBC, which are stable in serum *in vitro*), undergo rapid elimination from the bloodstream, as do complement-sensitive SA/b_n-RBC. To clarify the mechanism of elimination of b_{700}-RBC, we have studied the interaction of complement component C3b with b_n-RBC *in vitro* and the effect of complement on the binding of b_n-RBC to isolated macrophages. Finally, we have evaluated *in vivo* fate of the complex b-IgG/SA/b_{23}-RBC, where b-IgG and b_{23}-RBC were labeled with ^{125}I and ^{51}Cr, respectively.

MATERIALS AND METHODS

Streptavidin (SA) and 6-biotinylaminocaproic acid *N*-hydroxysuccinimide ester (long arm biotin ester, BxNHS) were purchased from Calbiochem (San Diego, CA). Iodogen was obtained from Pierce (Rockford, IL). Both [^{51}Cr]Cl and Na[^{125}I] isotopes were from Amersham (Arlington Heights, IL). Normal mouse IgG, bovine serum albumin (BSA) and dimethyl formamide were from Sigma (St. Louis, MO). Human RBC and serum were obtained from the blood of healthy volunteers as described (15). Proteins were radiolabeled with ^{125}I by the Iodogen method according to the manufacturer's recommendations. Mouse IgG was biotinylated with BxNHS as described (18), and the molar ratio of BxNHS to IgG in the reaction mixture for biotinylation was 10.

Sprague–Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–300 g, were anesthetized with ip injection of sodium pentobarbital (50 mg/kg). To obtain serum, nonheparinized blood was collected from the peritoneal cavity after dissection of the descending aorta. After 2 h incubation at 4°C, serum was separated by centrifugation. To obtain RBC, blood from the peritoneal cavity was collected in heparin.

Fresh RBC in heparinized blood were washed four times with PBS before biotinylation. RBC were biotinylated with BxNHS as described previously (13). Varying amounts of BxNHS in DMF were added to 10% suspensions of RBC in PBS, and after 30-min incubation at room temperature the resultant b-RBC were washed four times by centrifugation with BSA–PBS (PBS containing 2 mg/ml BSA). Then 20 μl of SA stock solution (1 mg/ml in PBS) was added to 100 μl of 10% suspension of b-RBC. This provides addition of 1 μg of SA per 5×10^6 b-RBC or about 2×10^6 SA molecules per b-RBC. After 30-min incubation at room temperature unbound SA was removed by centrifugation. In a separate experiment, radiolabeled SA was added to b-RBC to assess streptavidin-accessible biotin residues on b_n-RBC, as described (13). According to this assay, streptavidin binding to b_0-RBC, $b_{2.4}$-RBC, b_7-RBC, b_{23}-RBC, b_{70}-RBC, b_{240}-RBC, and b_{700}-RBC was equal to 290, 14,000, 35,000, 90,000, 260,000, 290,000, and 510,000 molecules per RBC, respectively.

Lysis of RBC, b-RBC, SA/b-RBC, and b-IgG/SA/b-RBC by fresh homologous serum was studied in 96-well microtest plates, as described previously (19). 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 1% suspensions of RBC preparations for 1 h at 37°C and then the

degree of lysis was determined by measuring the OD of the suspensions at 630 nm in an ELISA reader. Standards for 100% lysis and background lysis were based on wells treated with distilled water and serum-free GVB, respectively.

Analysis of C3b binding to human RBC was performed using a radiolabeled monoclonal antibody against human C3b, mAb 7C12, as described previously (20). Briefly, 20-μl aliquots of 50% suspensions of RBC, b_n-RBC, or SA/b_n-RBC were incubated for 10 min at 37°C with 20 μl of normal human serum (final serum dilution 1/4). After this incubation, the RBC were washed in PBS–BSA, reconstituted at 10% and incubated for 1 h with the radiolabeled mAb 7C12. The amount of mAb bound to the RBC (proportional to the number of C3b bound per RBC) was determined by centrifugation of the RBC samples through oil followed by counting of the pellet.

Rat alveolar macrophages were obtained by bronchoalveolar lavage as described (21). Rat peritoneal macrophages were obtained by lavage of peritoneal cavity of rats which were treated with a 5-ml ip injection of sodium thioglycate (4 mg/ml in phosphate buffer, pH 7.4) 4 days earlier. Macrophages were washed by centrifugation (1300 rpm, 5 min) and resuspended in RPMI at 3.5×10^6 cells/ml. Two milliliters of this suspension was added to each well of a 6-well tissue culture multiwell dish and the cells were incubated overnight at 37°C in 5% CO_2. The next day nonadherent cells were removed by washing with Dulbecco's PBS, and the adherence assay was performed as follows: RBC or b_n-RBC were added to the wells along with 2 ml of culture medium. The samples were incubated for 2 h at 37°C in 5% CO_2, and then nonbound RBC were removed by washing with Dulbecco's PBS. One milliliter of lytic solution (1% Triton, 1 mM EDTA in water) was added to each well and after a 10-min incubation at room temperature the number of RBC bound to the macrophages was determined spectrophotometrically by measuring released hemoglobin (OD at 405 nm) in the lysates.

Immunoerythrocytes were prepared as described previously (22). In brief, after treatment of b-RBC with SA (generating SA-b-RBC), a 10% dispersion of the cells was incubated with biotinylated mouse IgG (b-IgG) for 1 h at room temperature to produce b-IgG/SA/b-RBC. Nonbound b-IgG was removed by washing with BSA–PBS. The amount of IgG bound to the RBC was quantitated by using radiolabeled and biotinylated mouse IgG (b-IgG) as a tracer in a separate experiment. For *in vivo* studies, the RBC were labeled with [^{51}Cr]Cl and animal experiments were designed as described (12). Briefly, ^{51}Cr-labeled b-IgG/SA/b_{23}-RBC were injected into the tail vein in anesthetized rats. At the indicated times after injection, the anesthetized rats were sacrificed by exsanguination. Blood and internal organs were collected, and the organs were also rinsed with saline to eliminate blood. The level of ^{51}Cr counts in the blood, serum, and internal organs was measured in a gamma counter. In a separate experiment we have injected in rats ^{51}Cr-labeled SA/b_{23}-RBC possessing ^{125}I-labeled b-IgG, in order to estimate whether b-IgG circulates as a complex with SA/b_{23}-RBC *in vivo*.

RESULTS

Effects of biotinylation and streptavidin attachment on RBC lysis by serum in vitro. Previously we have demonstrated that noncovalent attachment of SA or avidin to b-RBC leads to complement-mediated lysis of the cells *in vitro* (13). However, if the multivalent binding of avidin to the cells is reduced (attained by partial occupation of potential biotin-binding sites on avidin with free biotin, or by chemical modification of avidin), this leads to a significant decrease in the observed lysis (16, 23). We have also shown that the ability of SA to engage in polyvalent binding to b-RBC depends upon the surface density of biotin residues coupled to the RBC (16). The surface density of biotin on the b-RBC membrane may be regulated by variation of the extent of biotinylation of the RBC. Based on these considerations, we have hypothesized that the susceptibility of SA/b-RBC for lysis by complement may be regulated by varying the concentration of biotin ester in the reaction mixture used for RBC biotinylation. In the first series of experiments we have tested this hypothesis *in vitro*. We biotinylated human and rat RBC (to generate b_n-RBC) at various input concentrations of BxNHS. We define *n* as the micromolar concentration of BxNHS which is incubated with a 10% RBC dispersion.

Figure 1 shows the effects of biotinylation and attachment of SA on the *in vitro* stability of b_n-RBC in autologous serum. For both rat and human RBC, covalent coupling of biotin did not lead to lysis by serum within the entire range of BxNHS inputs used for biotinylation. In the present study we added about 2×10^6 SA molecules per b-RBC. This addition of SA is enough to saturate streptavidin-binding capacity of RBC biotinylated at BxNHS concentrations 100 μM and below (not shown). Attachment of SA to RBC biotinylated at high BxNHS inputs (>200 μM BxNHS for rat RBC and >1000 μM BxNHS for human RBC) led to their lysis by autologous serum. Attachment of SA to human or rat b-RBC prepared at BxNHS input concentrations lower than 100 μM did not induce their lysis by serum. We have used radiolabeled SA and estimate that rat b_{20}-RBC specifically bind 10^5 SA molecules per RBC (not shown). Previously we have also reported that attachment of 10^5 SA molecules to human b_{3000}-RBC and b_{1000}-RBC leads to their complete lysis by serum *in vitro* (13). Thus, data presented in Fig. 1 confirm our hypothesis that reduction of the surface den-

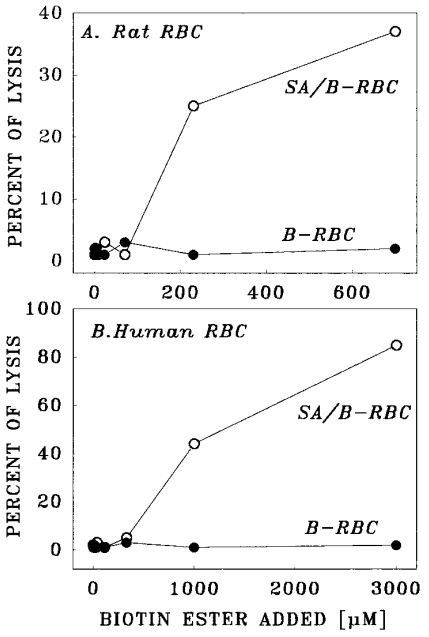

FIG. 1. Effect of modification of rat (A) and human (B) RBC with biotin hydroxysuccinimide ester (BxNHS) and streptavidin on RBC stability in homologous serum *in vitro*. Freshly obtained washed RBC (10% suspension in saline) were incubated for 30 min at RT with BxNHS at the indicated final concentrations. After elimination of nonreacted BxNHS, streptavidin (SA) was added to b_n-RBC (1.5×10^6 SA molecules/RBC). B_nRBC (closed circles) and SA/b_n-RBC (open circles) were incubated for 1 h at 37°C with normal fresh homologous serum and then lysis was determined by measuring the OD of the sample at 630 nm. Representative results from series of three experiments for each cell type.

sity of biotin residues on b-RBC generates SA/b-RBC which are complement-resistant. Using radiolabeled biotinylated IgG (b-IgG) we have found that both human SA/b_{100}-RBC (15) and rat (not shown) SA/b_{20}-RBC bind 5×10^4 molecules of b-IgG per RBC. Furthermore, the b-IgG/SA/b_{20}-RBC are also complement-resistant *in vitro*. Both control rat RBC and b-IgG/SA/b_{20}-rat RBC display no more than 1% lysis after incubation with autologous serum (1 h, 37°C, final serum dilution 1/5). Previously we have reported that human RBC prepared at even higher levels of BxNHS (b-IgG/SA/b_{300}-RBC) are similarly complement-resistant (15). Therefore, taken together, the results presented in this section and our previous data indicate that monovalent attachment of SA to b-RBC possessing low surface density of biotin residues on the membrane (facilitated by use of lower inputs of BxNHS) generates immunoerythrocytes resistant to complement *in vitro*.

In vivo injection of biotinylated RBC and the mechanisms of elimination of b_{700}-RBC from the bloodstream. Because the results of our *in vitro* experiments suggest that RBC biotinylated at BxNHS inputs lower than 100 μM may be stable in the bloodstream regardless of the level of SA attachment, we extended our work to an *in vivo* physiological model. We radiolabeled several different RBC preparations with ^{51}Cr and studied their fate in the bloodstream and their biodistribution in rats after iv injection. We first examined ^{51}Cr-labeled b_n-RBC. Figure 2 illustrates the kinetics of elimination from the bloodstream and lysis *in vivo* of radiolabeled b_n-RBC after injection in rats. Unexpectedly, rat RBC biotinylated at 700 μM BxNHS (b_{700}-RBC, stable in serum *in vitro*) were eliminated from the bloodstream immediately after injection. Approximately 20% of the recovered radioactivity in blood was detected in the plasma, indicating partial lysis of b_{700}-RBC *in vivo*. Figure 3, which is based on the same series of experiments, shows the biodistribution of b_n-RBC in rats. Hepatic uptake of b_{700}-RBC-associated radioactivity (a total of ca 80% of the injected radioactivity, rat liver weight is 10 g) was substantially higher than that of RBC, b_{20}-RBC, and b_{70}-RBC. Splenic uptake of b_{700}-RBC also was markedly higher than that of RBC, b_{20}-RBC, and b_{70}-RBC. Therefore, a high level of biotinylation of RBC

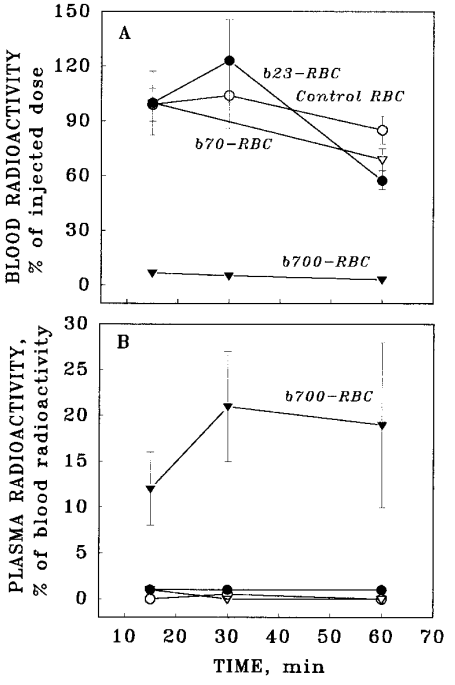

FIG. 2. Kinetics of blood clearance and lysis in the bloodstream of biotinylated RBC. Rat RBC, biotinylated at the indicated final micromolar concentration of BxNHS (b_nRBC) and radiolabeled with ^{51}Cr, were injected in rats via the tail vein. At the indicated time, blood was collected in heparin from the tail vein. Radioactivity in blood cells and in plasma (separated by centrifugation) was determined. (A) Total radioactivity in the blood, calculated assuming the blood weight as 7% of the total rat weight. (B) Radioactivity in plasma, presented as a percentage of that in blood. BxNHS concentrations for RBC biotinylation: 0 μM (open circles), 23 μM (closed circles), 70 μM (open triangles), and 700 μM (closed triangles). The data are presented as means ± SD, $n = 3$.

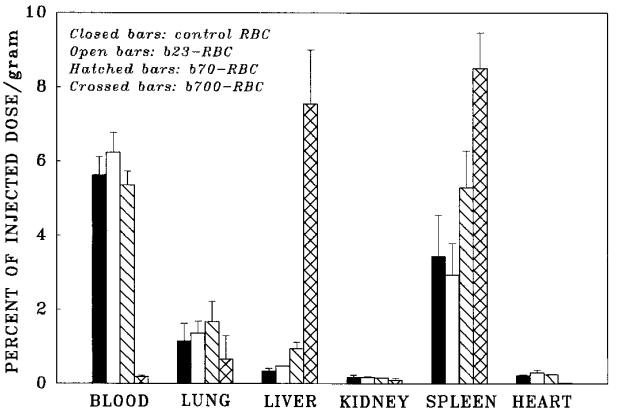

FIG. 3. Biodistribution of radiolabel after injection of ^{51}Cr-labeled b_n-RBC in rats. One hour after injection of b_n-RBC (see legend to Fig. 2), rats were sacrificed and radioactivity in the tissues was determined. Results are presented as a percentage of injected radioactivity per gram of tissue, means ± SD, $n = 3$.

induces their uptake by liver and spleen. Lower levels of RBC biotinylation (b_{70}-RBC and b_{20}-RBC) did not induce significant alterations in their life span and biodistribution, as compared with control RBC (Fig. 2).

The major cell types which eliminate senescent or chemically modified RBC in the body are hepatic and splenic macrophages (24). To clarify the mechanism of elimination and lysis of b_{700}-RBC *in vivo,* we have studied the interaction of these RBC with macrophages *in vitro.* Figure 4 shows that after treatment with serum b_{700}-RBC bound to alveolar macrophages and to peritoneal macrophages. Furthermore, inactivation of serum complement by heating or chelation of divalent cations abrogated binding (not shown). These results strongly suggest that uptake of rat b_{700}-RBC by homologous macrophages is mediated by complement. Thus, although complement does not induce lysis of extensively biotinylated RBC in serum *in vitro,* it apparently does mediate their binding to tissue macrophages.

Macrophages in the liver have a number of receptors for C3 activation and breakdown products, and it is known that these receptors play a key role in the clearance of complement-opsonized particles from the circulation (25). In view of the central role of C3b in both the classic and alternative pathway (26) and the effects of biotinylation on the activity of decay accelerating factor (see later discussion), it is likely that the enhanced uptake of rat b_{700}-RBC by macrophages is mediated by binding of C3b to b_{700}-RBC. We have used a monoclonal antibody specific for human C3b, mAb 7C12, to study the interaction of complement component C3b with human b-RBC which were exposed to serum. Figure 5A demonstrates that biotinylation of human RBC at BxNHS concentrations 1000 μM and higher followed by opsonization in serum leads to binding of C3b to the RBC. It should be noted that both human b_{3000}-RBC and SA/b_{3000}-RBC bound C3b to a similar extent (Fig. 5B). However, although human b_{3000}-RBC were stable in serum, SA/b_{3000}-RBC were lysed by complement *in vitro* (Fig. 5C). Therefore, substantial biotinylation of RBC does not lead to direct lysis of b-RBC by complement, but instead leads to nonlytic fixation of C3b. Based on our *in vitro* studies with macrophages (Fig. 4), it is likely that rat b_{700}-RBC bind via C3b-mediated fashion to macrophages in liver and spleen, which leads to their elimination from the bloodstream.

Circulation of serum-labile and serum-stable SA/b_n-RBC in the bloodstream. Figure 6 shows the results of experiments in which different preparations of rat SA/b_n-RBC were infused into rats. Both rat SA/b_{240}-RBC and SA/b_{70}-RBC were rapidly cleared from the circulation. Of the radioactivity remaining in the circulation, about 75% was detected in plasma after injection of SA/b_{240}-RBC, implying that complement-mediated lysis plays a major role in the elimination of these RBCs from the bloodstream. We note that Fig. 1A demonstrates that rat SA/b_{240}-RBC are lysed by complement *in vitro.* On the other hand, although rat SA/b_{70}-RBC were also rapidly cleared from the bloodstream, *in vitro* these RBCs demonstrated resistance to lysis by complement (Fig. 1A). Lysis of rat SA/b_{70}-RBC *in vivo* did not exceed 40% based on the distribution of radioactivity between plasma and blood cells. Taken together, these findings suggest that both complement-mediated lysis and complement-mediated uptake by

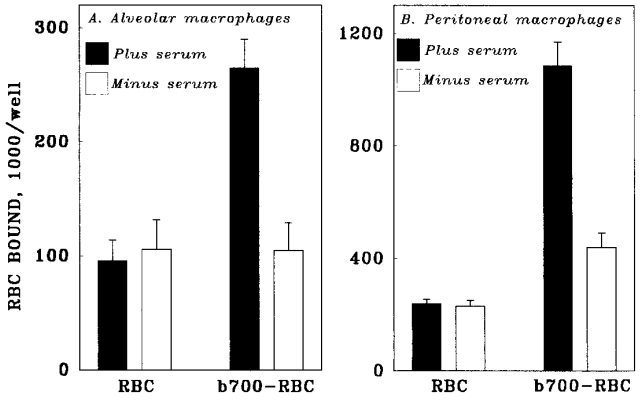

FIG. 4. *In vitro* binding of biotinylated rat RBC to isolated macrophages. (A) Alveolar macrophages from rat lungs; (B) peritoneal rat macrophages. Control RBC and RBC biotinylated with 700 μM BxNHS (b_{700}-RBC) were incubated for 2 h at 37°C with adherent macrophages in a 24-well culture plate (alveolar macrophages) or in a 6-well culture plate (peritoneal macrophages). Fresh rat serum (closed bars) or PBS–BSA (open bars) was added to the wells. After nonbound RBC were washed out, macrophage-bound RBC were lysed in the wells. The amount of bound RBC was determined based on the hemoglobin absorbance in the lysates. The data are presented as means ± SD, $n = 3$. Binding of b_{20}-RBC, b_{70}-RBC, and b_{240}-RBC to both types of macrophages was the same as that of control RBC.

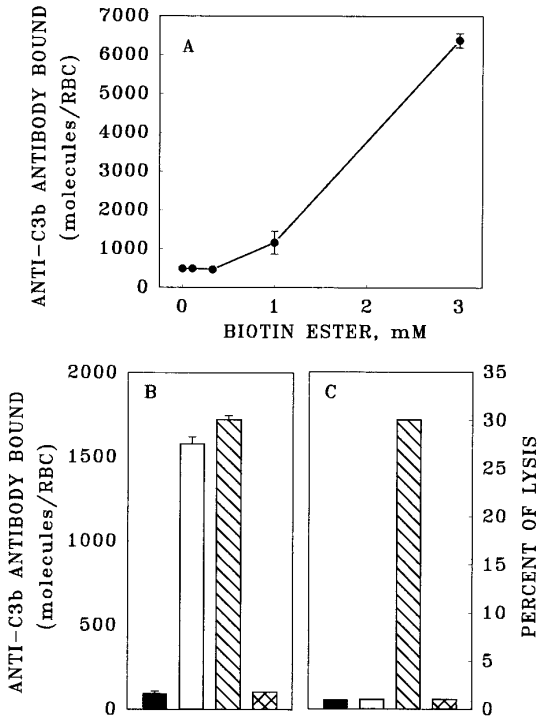

FIG. 5. *In vitro* binding of C3b to RBC modified with biotin and streptavidin. Human b_n-RBC and SA/b_n-RBC were incubated with normal human serum for 10 min at 37°C. Binding of C3b to RBC was determined using a radiolabeled monoclonal antibody specific for human C3b. (A) Binding of anti-C3b to b_n-RBC depends upon the extent of biotinylation. (B) Comparison of anti-C3b binding to control RBC (closed bar), b_{3000}-RBC (open bar), SA/b_{3000}-RBC (hatched bar), and SA/b_{100}-RBC (crossed bar). (C) Lysis of RBC modified with biotin and streptavidin in human serum. The key is the same as in B. The data are presented as means ± SD, $n = 3$.

macrophages contribute to the clearance of SA/b_{70}-RBC from the bloodstream. In contrast with SA/b_{240}-RBC and SA/b_{70}-RBC, serum-stable SA/b_{23}-RBC circulated in the bloodstream for at least 1 h without detectable lysis and with only mildly increased elimination. Figure 7 shows that hepatic uptake of radioactivity associated with SA/b_{240}-RBC and SA/b_{70}-RBC was dramatically higher than that associated with SA/b_{23}-RBC and RBC. Splenic uptake was enhanced for all preparations of streptavidin-coated RBC, including the SA/b_{23}-RBC. In view of the fact that the rat spleen weight is less than 1 g, no more than 10% of injected radioactivity was accumulated in the spleen 1 h after injection of SA/b_{23}-RBC.

Circulation of serum-stable immunoerythrocytes in rats. Because our major goal is the design of long-circulating immunoerythrocytes, i.e., RBC carrying biotinylated antibody, in the last part of this study we have examined the *in vivo* fate of serum-stable immunoerythrocytes, b-IgG/SA/b_{23}-RBC carrying 3×10^4 molecules of b-IgG per SA/b_{20}-RBC. Figure 8 shows that b-IgG/SA/b_{23}-RBC circulate in the rat's bloodstream for at least several hours without marked elimination. One day after injection, 60% of the injected radioactivity was still associated with circulating blood cells. Less than 1.5% of the blood radioactivity was demonstrable in the plasma, indicating that there was very little lysis of b-IgG/SA/b_{20}-RBC in the circulation. In contrast, complement-susceptible b-IgG/SA/b_{700}-RBC were lysed and eliminated from the bloodstream several minutes after injection. Figure 9 shows that the biodistribution of b-IgG/SA/b_{23}-RBC in rats is similar to that of control RBC. The only exception is splenic uptake of b-IgG/SA/b_{23}-RBC, which is higher than splenic uptake of control RBC.

To evaluate whether immunoerythrocytes circulate in the bloodstream as a complex possessing attached b-IgG, we have injected in rats b-IgG/SA/b_{23}-RBC containing ^{51}Cr-labeled b_{23}-RBC and ^{125}I-labeled b-IgG. Figure 10 shows that only 25–30% of iodine-125 could be found in the plasma several hours after injection of such a complex, whereas 100% of iodine-125 circulates

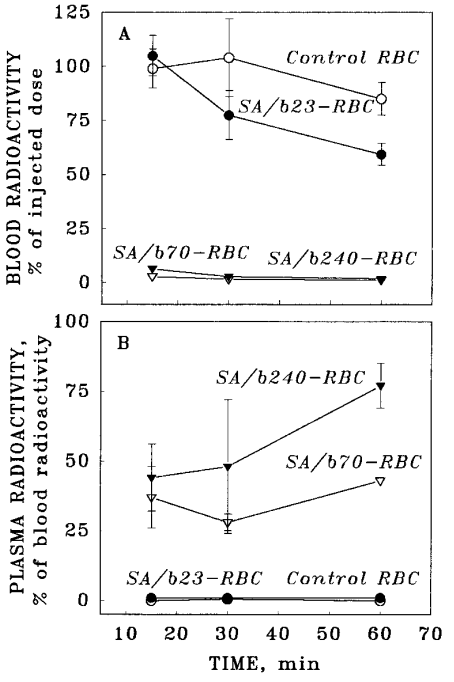

FIG. 6. Kinetics of blood clearance and lysis in the bloodstream of SA/b_n-RBC. Rat RBC were biotinylated at the indicated final micromolar concentration of BxNHS. After incubation with SA and radiolabeling with ^{51}Cr, SA/b_n-RBC were injected in rats via the tail vein. At the indicated times, blood was collected in heparin from the tail vein. Radioactivity in blood cells and in plasma (separated by centrifugation) was determined. (A) Total radioactivity in blood, calculated assuming blood weight as 7% of the total rat weight. (B) Radioactivity in plasma, presented as a percentage of that in blood. BxNHS concentrations for RBC biotinylation: 0 μM (open circles), 23 μM (closed circles), 70 μM (open triangles), and 240 μM (closed triangles). The data are presented as means ± SD, $n = 3$.

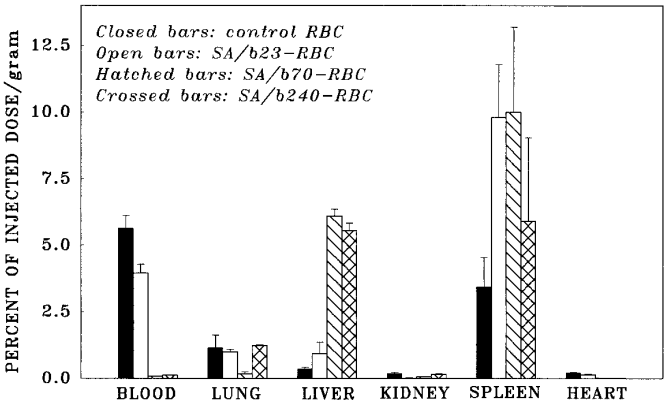

FIG. 7. Biodistribution of radiolabel after injection of ^{51}Cr-labeled SA/b_n-RBC in rats. One hour after injection of SA/b_n-RBC (see legend to Fig. 6), rats were sacrificed and radioactivity in the tissues was determined. Results are presented as a percentage of injected radioactivity per gram of tissue, means ± SD, $n = 3$.

in plasma after injection of nonconjugated ^{125}I-labeled b-IgG. This result indicates that a major portion of immunoerythrocytes circulates as a whole b-IgG/SA/b_{23}-RBC complexes carrying about 70% of attached immunoglobulins. Noteworthy, conjugation with SA/b_{23}-RBC prolongs circulation time of ^{125}I-labeled b-IgG. Three hours after injection, blood level of iodine-125 was 60 ± 7% of injected dose for ^{125}I-labeled b-IgG/SA/^{51}Cr-labeled b_{23}-RBC vs 25 ± 8% for nonconjugated ^{125}I-labeled b-IgG (mean ± SD, $n = 3$, $P < 0.05$). Therefore, conjugation with carrier RBC prolongs circulation time of b-IgG, probably due to reduction of its filtration in tissues. About 30% of b-IgG, however, detaches from SA/b_{23}-RBC during the first hours after injection; one day after injection, about 50% of RBC-conjugated b-IgG detaches from the carrier b-RBC (Fig. 10). Figure 11 shows distribution of both radiolabels in rats 20 min, 60 min, and 3 hours after iv injection. During first hour after injection, both labels display the same level in the blood. This supports the conclusion that the majority of immunoerythrocytes circulate as an intact complexes. Three hours after injection, however, blood level of iodine-125 was detectably lower than that of chromium-51, thus indicating a partial detachment of b-IgG from circulating immunoerythrocytes. Pulmonary level of iodine-125 was higher than that of chromium-51 and the difference between two labels increased with time. This result indicates that detachment of b-IgG may occur in the pulmonary vasculature, enriched by capillaries. In contrast, splenic level of iodine-125 was detectably lower than that of chromium-51. This result reflects a final splenic destination of injected RBC, regardless of presence of b-IgG on their membrane. Taken together, data presented in this section document that b-IgG/SA/b_{23}-RBC circulate

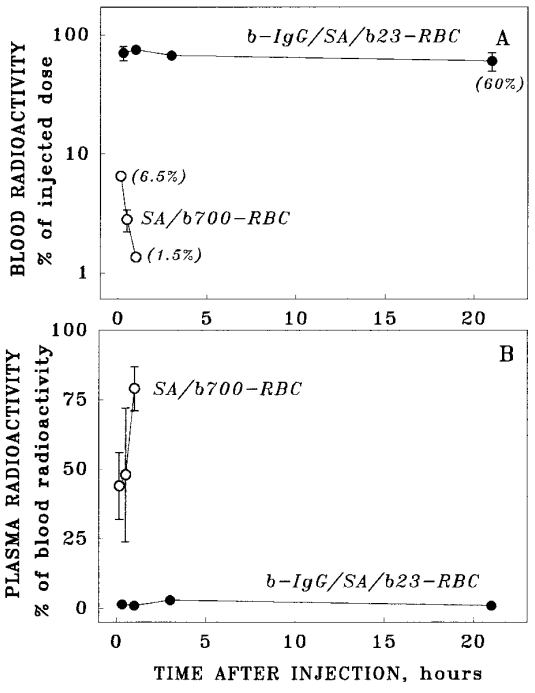

FIG. 8. Serum-stable immunoerythrocytes b-IgG/SA/b_{23}-RBC circulate in the bloodstream without marked elimination and detectable lysis. (A) ^{51}Cr-labeled b-IgG/SA/b_{23}-RBC, carrying 3×10^4 b-IgG per RBC were injected in rats via the tail vein. At the indicated times after injection, animals were sacrificed and ^{51}Cr in the tissues was determined in a gamma counter. Blood clearance and lysis of serum-labile SA/b_{700}-RBC are shown for comparison. Note the logarithmic scale in A; numbers in parentheses indicate actual values. Data are presented as means ± SD, $n = 3$.

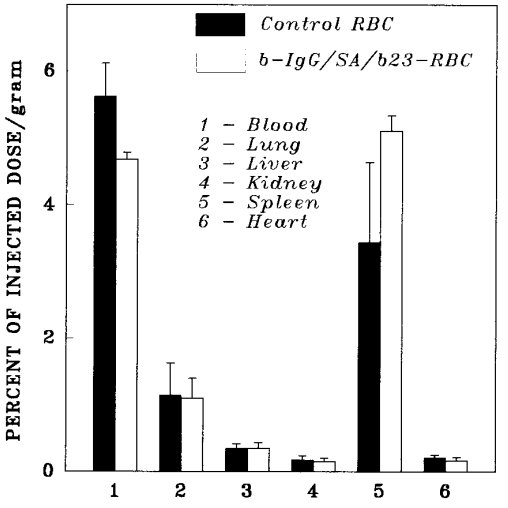

FIG. 9. Biodistribution in rats of serum-stable ^{51}Cr-labeled immunoerythrocytes b-IgG/SA/b_{23}-RBC and control RBC. Experimental design is the same as in Fig. 8. Closed bars: control RBC; open bars: b-IgG/SA/b_{23}-RBC. The data are presented as the percentage of injected dose per gram of tissue 1 h after iv injection of radiolabeled RBC and b-IgG/SA/b_{23}-RBC, means ± SD, $n = 3$.

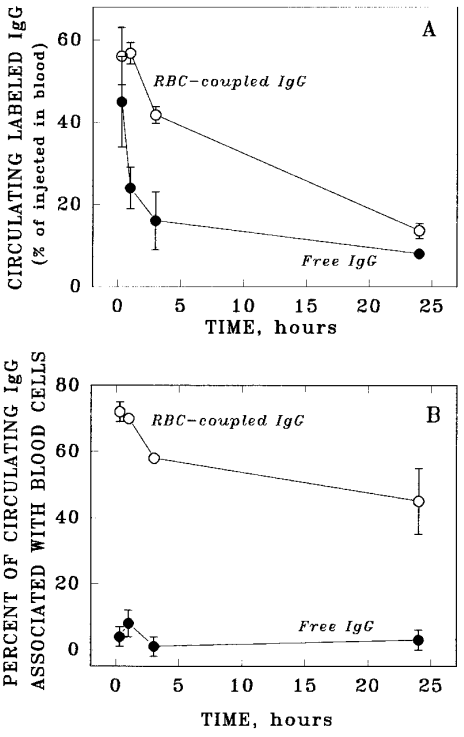

FIG. 10. Circulation of b-IgG and SA/b_{23}-RBC-bound b-IgG in rats after iv injection. ^{125}I-labeled b-IgG was conjugated with SA/b23-RBC as described under Materials and Methods. RBC-bound ^{125}I-labeled b-IgG (open circles) or nonconjugated ^{125}I-labeled b-IgG (closed circles) were injected in rats. At the indicated time, the level of iodine-125 was determined in the plasma and expressed as means ± SD, $n = 3$.

for at least several hours after iv injection as a stable immunoerythrocytes.

DISCUSSION

Biotinylation of RBC is a simple procedure which provides sites for high-affinity binding of SA on the b-RBC membrane. Although there are several functional groups on the RBC membrane which can be covalently linked to biotin (3–5, 27–29), most work in the field has focused on the use of succinimide esters of biotin (BNHS), which allow for covalent coupling of biotin to RBC amino groups (2, 6–8, 11–13). RBC modification with BNHS may be performed both *in vitro* (6) and *in vivo* (7, 30). RBC modified with BNHS were used to trace normal (6), senescent (31), and oxidized (32) RBC in the circulation.

Because SA possesses four high-affinity biotin-binding sites, various biotinylated molecules may be attached to b-RBC via SA. This strategy was suggested in the early eighties by two groups as an approach for the selective delivery of drugs (loaded into b-RBC) to the target cell, tissue, or organ (9, 33). Immunoerythrocytes, i.e., antibody-carrying RBC, have also been examined for the possibility of selective delivery of antigens to immunocompetent cells (10) and for the elimination of circulating molecules and particles from the bloodstream (11). Therefore, SA-mediated attachment of biotinylated antibodies is a key aspect in the design of immunoerythrocytes for drug targeting, targeted immunization, and selective blood clearance. Obviously, the potential binding capacity of immunoerythrocytes for the target(s) will be enhanced when large numbers of antibody molecules are bound to the RBC. However, modification of RBC with biotin and SA can indeed alter the biocompatibility of RBC, and this could fundamentally compromise their potential as a therapeutic agent.

Previous reports from our group and others indicated that biotinylation does not lead to enhanced elimination of RBC from the bloodstream or to their lysis *in*

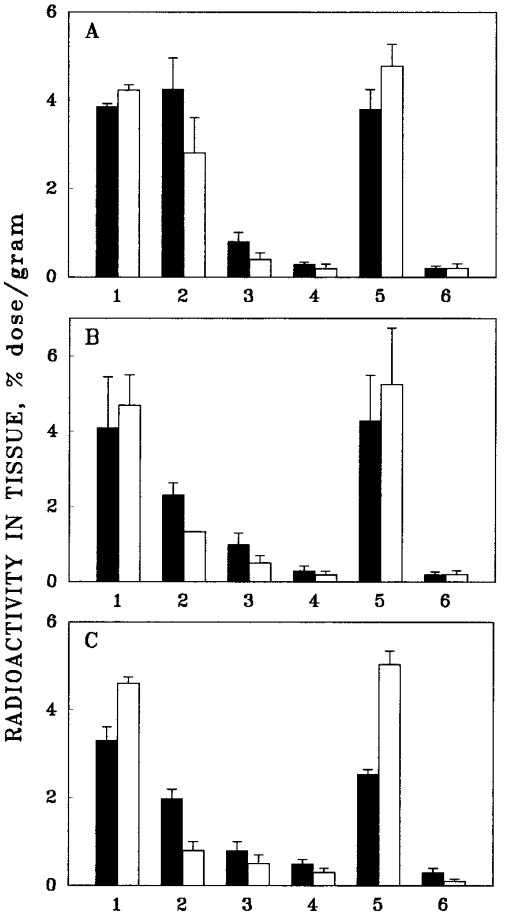

FIG. 11. Biodistribution of double-labeled b-IgG/SA/b_{23}-RBC in rats. Rats were injected with immunoerythrocytes containing ^{51}Cr-labeled b_{23}-RBC and ^{125}I-labeled b-IgG. Twenty minutes (A), 1 h (B), and 3 h (C) after injection, animals were sacrificed and both isotopes were counted in the blood (1), lung (2), liver (3), kidney (4), spleen (5), and heart (6). The data are presented as a percentage of injected ^{125}I (closed bars) or ^{51}Cr (open bars) found per gram of tissue, means ± SD, $n = 3$.

vivo or *in vitro* (6–8, 12, 34). Suzuki and Dale have reported that increased levels of biotinylation result in a moderately decreased *in vivo* life span for b-RBC (6). However, a systematic study of the effects of biotinylation on the biocompatibility of RBC has not been reported.

This study demonstrates that a high level of biotinylation of RBC does lead to their elimination from the bloodstream (Figs. 2 and 3), and several of our experiments suggest that complement activation plays a key role in this process. In fact, our recent work provided several lines of evidence which suggest that extensive biotinylation inhibits the activity of one of the regulators of complement in the RBC membrane, decay-accelerating factor (DAF) (14). DAF controls activation of complement in both the classical and alternative pathways at the level of C3b formation (35), and therefore we have hypothesized that a high level of biotinylation of RBC would render them susceptible to deposition of C3b on the RBC membrane. This would not lead to RBC lysis, however, because the activity of CD59 (which inhibits the lytic step due to the membrane attack complex) is less affected by biotinylation (14).

The results of the present study confirm this hypothesis. Extrapolation of our data obtained using human RBC (Fig. 5) indicates that rat b_{700}-RBC may fix C3b without detectable lysis *in vitro*. Deposition of C3b, however, reduces the biocompatibility of rat b_{700}-RBC even in the absence of their lysis. Figure 4 demonstrates that macrophages bind b_{700}-RBC in the presence of serum in a complement-dependent fashion. It is well known that C3b-opsonized particles (including RBC) are cleared by the liver in a process that presumably depends upon C3b receptors on liver macrophages (24, 25). Therefore, our data show that nonlytic, ("frustrated") activation of complement and C3b-mediated phagocytosis underlies the mechanism for elimination of b_{700}-RBC from the circulation (see scheme in Fig. 12).

Attachment of SA imposes further limitations on the biocompatibility of b-RBC. SA cross-links biotinylated components of the RBC membrane, including complement inhibitors DAF and CD59 (14). This leads to reduction of the RBC defense against complement at the level of both C3b formation/deposition (due to inactivation of DAF) and nonrestricted membrane attack complex (MAC) formation on the RBC membrane (due to inactivation of CD59) (14). Polyvalent attachment of SA to b-RBC leads to their lysis in serum *in vitro* by complement activated via the alternative pathway (13). Serum-labile rat SA/b_{700}-RBC and SA/b_{240}-RBC undergo extremely rapid elimination from the bloodstream (Figs. 6 and 8). Direct lysis by complement seems to be a major mechanism for the elimination of these RBC *in vivo* (see scheme on Fig. 10). Reduction of the BxNHS levels used for RBC biotinylation diminishes SA-induced lysis by complement *in vitro* (Fig. 1). This finding supports our hypothesis that complement-mediated lysis of SA/b_n-RBC may be regulated by the reduction of the surface density of biotin residues on the b-RBC membrane. Serum-stable SA/b_{70}-RBC, however, were rapidly eliminated from the bloodstream (Fig. 6). We did not address specifically the mechanism(s) of SA/b_{70}-RBC elimination in the present work. However, the high level of the hepatic and splenic uptake of SA/b_{70}-RBC (Fig. 7) suggests that uptake by liver macrophages with receptors for C3b (or perhaps by splenic receptors for C3bi or C3dg (36)) plays a major role in their elimination.

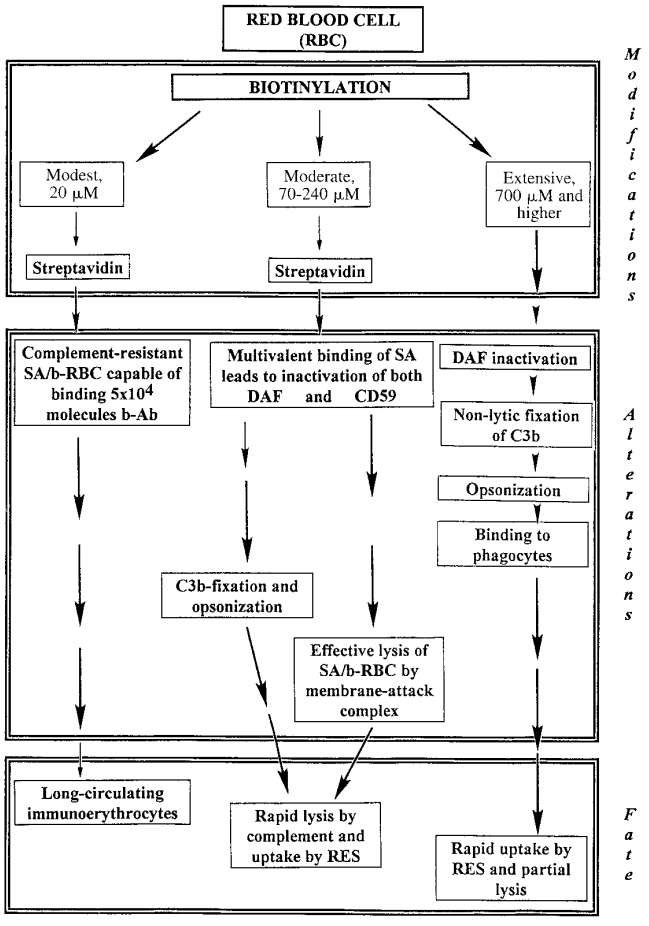

FIG. 12. Scheme of the mechanisms of complement-mediated elimination of red blood cells modified with biotin and streptavidin from the bloodstream. SA, streptavidin; SA/b-RBC, biotinylated RBC coated with streptavidin; DAF, decay-accelerating factor; RES, reticuloendothelial system. See explanation in the text.

The scheme in Fig. 12 illustrates our working model for mechanisms by which RBC modified with biotin and SA are cleared from the bloodstream. Extensive biotinylation of RBC (700 μM and more biotin ester in the reaction mixture) leads to DAF inactivation and fixation of C3b. Active CD59 in the b-RBC membrane

can prevent RBC lysis by complement *in vitro*. However, deposition of C3b opsonizes b_{700}-RBC and leads to the C3b-mediated uptake of b_{700}-RBC by tissue macrophages in the liver. Our preliminary observations indicate that isolated macrophages effectively phagocytize b_{700}-RBC in a complement-dependent fashion (Murciano, Herraez, and Muzykantov, unpublished data). Moderate biotinylation of RBC (70–200 μM biotin ester) does not markedly alter the biocompatibility of b-RBC. Both *in vitro* and *in vivo* these cells are resistant to lysis by complement and circulate in the bloodstream for a prolonged time. However, attachment of SA induces cross-linking of biotinylated DAF and CD59, thus inactivating both regulators of complement. This leads to both opsonization of SA/b-RBC by fixed C3b (due to DAF inactivation) and to lysis by MAC (due to CD59 inactivation). *In vitro* these SA/b_n-RBC demonstrate intermediate stability in serum: SA/b_{240}-RBC are susceptible to lysis, while SA/b_{70}-RBC are resistant to lysis. *In vivo,* however, both SA/b_{240}-RBC and SA/b_{70}-RBC undergo rapid elimination. Probably, both lysis by complement and macrophage uptake of C3b-opsonized SA/b-RBC contribute to this process. In addition to these complement-mediated mechanisms, some other effector systems recognizing cross-linking and/or clusterization of RBC membrane proteins (band 3, e.g., (37, 38)) might participate in the elimination of SA/b-RBC.

From the applications' point of view, the most important result of this study is the successful survival *in vivo* without detectable lysis manifested by the serum-stable SA/b_{23}-RBC (Fig. 6). This result demonstrates that monovalent binding of SA to b-RBC possessing biotin residues at low surface density does not inactivate membrane inhibitors of complement and that b_{23}-RBC carrying monovalently attached SA escape from clearance reactions *in vivo*. Moreover, attachment of 3×10^4 molecules of b-IgG to SA/b_{23}-RBC does not alter markedly their biodistribution, blood clearance, and rate of lysis *in vivo* (Figs. 8 and 9).

In the present study we also have addressed the issue about stability of b-IgG/SA/b_{23}-RBC complex in the bloodstream. This question is of essential importance because several groups have reported that b-RBC lose biotin residues in the circulation even without detectable lysis or elimination (8, 39). In comparison with short biotin ester, BNHS, spacered biotin ester BxNHS looks to be more susceptible for detachment from b-RBC and activity of serum biotinidase has been suspected to be responsible for this effect (39). Therefore, detachment of b-IgG from SA/b-RBC may occur in the bloodstream. To evaluate b-IgG detachment from circulating immunoerythrocytes, we have injected b-IgG/SA/b_{23}-RBC complex labeled with both chromium-51 (allowing tracing of RBC) and iodine-125 (allowing tracing of b-IgG). Our results (Figs. 10 and 11) document that a major portion of b-IgG circulates bound to SA/b_{23}-RBC for at least for several hours. Probably, molecules of streptavidin protects biotin residues of b-RBC from serum biotinidase. Partial detachment of b-IgG occurs, however. Recent study reported that GPI-anchored complement-controlling proteins (DAF and CD59) undergo transfer from RBC membrane to the pulmonary endothelium *in vivo* (40). Because we have documented that both of these proteins are biotinylated in b-RBC (14), it is reasonable to suggest that a portion of b-IgG anchored to these proteins also may transfer from RBC to the endothelium. After injection of ^{125}I-labeled b-IgG/SA/b_{23}-RBC in rats, we have observed a high iodine-125/^{51}chromium-51 ratio in the lungs, an organ containing about 30% of the total endothelial cells in the body. This result supports our hypothesis about transfer of RBC-coupled IgG to the vascular endothelium.

In previous publications we have reported that attachment of biotinylated antibody to RBC provides immunoerythrocytes with high binding capacity for the target antigens (15, 22). Our present results demonstrate that regulation of the surface density of biotin residues on the membrane of b-RBC provides immunoerythrocytes which display only marginally altered biocompatibility and lifetime in the circulation. Even these alterations (e.g., 40% elimination of b-IgG/SA/b_{23}-RBC 1 day after injection, 30% detachment of b-IgG from carrier SA/b_{23}-RBC) would restrict applications of b-IgG/SA/b_{23}-RBC and SA/b_{23}-RBC in terms of their injection for prolonged (several days) circulation in the bloodstream. However, data obtained with immunoliposomes demonstrate that several hours is enough for effective targeting to such targets as tumors (41). Because targets for immunoerythrocytes are restricted within the vascular system and, therefore, are more accessible for binding, b-IgG/SA/b_{23}-RBC would be applicable for intravascular drug targeting, antigen delivery, and clearance of circulating antigens.

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