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Chemical camouflage of antigenic determinants: Stealth erythrocytes

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ABSTRACT **In a number of clinical circumstances it would be desirable to artificially conceal cellular antigenic determinants to permit survival of heterologous donor cells. A case in point is the problem encountered in transfusions of patients with rare blood types or chronically transfused patients who become allosensitized to minor blood group determinants. We have tested the possibility that chemical modification of the red blood cell (RBC) membrane might serve to occlude antigenic determinants, thereby minimizing transfusion reactions. To this end, we have covalently bound methoxy(polyethylene glycol) (mPEG) to the surface of mammalian RBC via cyanuric chloride coupling. Human RBC treated with this technique lose ABO blood group reactivity as assessed by solution–phase antisera agglutination. In accord with this, we also find a profound decrease in anti-blood group antibody binding. Furthermore, whereas human monocytes avidly phagocytose untreated sheep RBC, mPEG-derivatized sheep RBC are ineffectively phagocytosed. Surprisingly, human and mouse RBC appear unaffected by this covalent modification of the cell membrane. Thus, mPEG-treated RBC are morphologically normal, have normal osmotic fragility, and mPEG-derivatized murine RBC have normal *in vivo* survival, even following repeated infusions. Finally, in preliminary experiments, mPEG-modified sheep RBC intraperitoneally transfused into mice show significantly improved (up to 360-fold) survival when compared with untreated sheep RBC. We speculate that similar chemical camouflage of intact cells may have significant clinical applications in both transfusion (e.g., allosensitization and autoimmune hemolytic disease) and transplantation (e.g., endothelial cells and pancreatic β cells) medicine.**

The transfusion of red blood cells (RBC) is the most common, and best tolerated, form of tissue transplantation. Indeed, in 1993, over 14 million units of blood were donated for transfusion in the United States alone (1). In most transfusions, simple blood typing (ABO/Rh-D) is sufficient to identify appropriate donors. Occasionally, however, appropriate donors for patients with rare blood types cannot quickly be found; a situation that may become life-threatening. More often, problems are encountered in individuals who receive chronic transfusions, such as patients with sickle cell anemia and thalassemia. In such patients, alloimmunization against minor RBC antigens can make it nearly impossible to identify appropriate blood donors (2–4). Previous studies in which purified proteins were covalently modified with poly(ethylene glycol) (PEG) provided a possible solution to this problem. PEG-modified proteins have been shown to have increased *in vivo* survival and to be nonimmunogenic, even with repeated infusions (5, 6). We therefore explored the hypothesis that the covalent binding of PEG to intact RBC might mask RBC surface antigens and thereby permit the survival of heterologous or even xenogeneic RBC.

To experimentally test this hypothesis, human, mouse, and sheep RBC were derivatized with methoxypoly(ethylene glycol) (mPEG) and the *in vitro* and *in vivo* effects of derivatization on cell structure, function, antigenicity, and survival were investigated. The results indicate that Type A or B human RBC covalently modified with mPEG resist agglutination by appropriate antisera, show decreased anti-A or anti-B antibody binding, and are structurally normal. Furthermore, mPEG-derivatized sheep RBC are resistant to phagocytosis by human peripheral blood monocytes. Finally, mPEG-derivatized mouse RBC have normal *in vivo* survival and mPEG-modified sheep erythrocytes exhibit significantly prolonged survival when transfused into mice. It is our belief that this procedure for antigen camouflage may have significant potential in transfusion and transplantation medicine.

MATERIALS AND METHODS

Following informed consent, venous blood was drawn in heparin from healthy laboratory volunteers. Serum was collected in serum tubes and, in some cases, complement was heat inactivated (56°C for 30 min). To prevent any storage artifacts, all samples were processed immediately. Care was taken to assure adequate representation of males and females and no individuals were excluded on the basis of race or gender. Statistical significance was determined by Student's *t* test or ANOVA (7). All biochemicals, unless otherwise noted, were obtained from Sigma.

mPEG Derivatization. The general protocol for mPEG derivatization of intact RBC was based on that previously developed for covalent modification of proteins with cyanuric chloride-coupled PEG (8, 9). mPEG (M_r 5 kDa) conjugated to cyanuric chloride was added to washed RBC suspended to a hematocrit of ≈12% in isotonic alkaline phosphate buffered saline (PBS; 50 mM K_2HPO_4/105 mM NaCl, pH ≈9.2) and the RBC were incubated for 30 min at 4°C. In some experiments, unactivated (i.e., no cyanuric chloride) mPEG was used to assess the effects of noncovalently bound polymer. Following derivatization, the RBC were washed three times prior to use. mPEG concentrations used ranged from 0 to 8 mg per ml of RBC suspension.

Effects of mPEG Derivatization on Antigenic Recognition. The effects of mPEG derivatization on the antigenicity of control and modified RBC were assessed by the reactivity of both major (ABO) and minor (Rh and MNS) antigens. ABO reactivity was determined by gross agglutination following addition of commercial blood typing antisera (Carolina Biological Supply). In addition, a novel microagglutination

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Abbreviations: RBC, red blood cells; mPEG, methoxy(polyethylene glycol); PBMC, peripheral blood mononuclear cells.

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method was developed using a platelet aggregometer (Chrono-Log, Havertown, PA) to follow the time-dependent agglutination of stirred RBC suspensions. Briefly, 450 μl of an RBC suspension (6% hematocrit in isotonic saline) was placed in an aggregometer cuvette at 37°C, with stirring, and 20 μl of anti-A and/or anti-B typing serum (or autologous/heterologous serum) was added. RBC agglutination was then followed over time. Reactivity of selected minor RBC antigens was tested in the Albany Medical Center Clinical Hematology Laboratory according to established American Association of Blood Banks methods and rated on the 4^{+s} system (10).

Direct RBC antibody binding was quantified by a sandwich ELISA (11). In brief, 96-well microtiter plates were pretreated with anti-λ and anti-κ human IgG/IgM antibodies. Washed mPEG-modified and control RBC were preincubated (60 min at 37°C) with anti-A and/or anti-B typing sera, washed (three times in isotonic saline), and added to the wells at a final concentration of 1×10^4 per well. After 60-min incubation (23°C), unbound RBCs were removed by washing the wells (three times). To each well, an alkaline phosphatase-conjugated goat anti-human Ig diluted in azide containing PBS (137 mM NaCl/0.3 mM KCl/0.15 mM KH_2PO_4/6.4 mM Na_2HPO_4/15.5 mM NaN_3/2 g% BSA, pH 7.4) was added and allowed to incubate for 60 min at 23°C. Excess (unbound) antibody was removed by washing the wells (three times) with PBS. To each well, 100 μl of ELISA buffer (1 M Na_2CO_3/20 mM $MgCl_2$/1 mg/ml phosphatase substrate, pH 9.8) was added to each well and allowed to develop for 30–60 min at room temperature. Bound enzyme activity was quantified spectrophotometrically at 405 nm on a standard microtiter plate reader (Molecular Devices).

The ingestion of control and derivatized RBC by human peripheral blood mononuclear cells (PBMC) was also examined. PBMC were prepared by the method of Pommier *et al.* (12). Briefly, whole blood was collected in acid–citrate-dextrose anticoagulant and gently layered over Histopaque (1:3 ratio of whole blood to Histopaque) and centrifuged to separate PBMC, which were subsequently washed three times in Hanks′ balanced salt solution. Washed, packed human and sheep RBC (treated as indicated) and PBMC (>95% viable as assessed by Trypan blue) (13) were prepared and mixed to achieve a ratio of 10 RBC per PBMC. PBMC concentration was held constant at 2×10^6/ml. The cell mixture was centrifuged ($120 \times g$ for 2 min) to pellet the cell mixture and to initiate cell:cell contact and phagocytosis. Following 30 min incubation at 37°C, 1 vol of water (4°C) was added to lyse nonphagocytosed RBC and, after 30 sec, 1 vol of 2× PBS was added to restore isotonicity. The total number of monocytes and the number of monocytes that had phagocytosed RBC were counted microscopically. The phagocytic index was calculated as the number of monocytes ingesting RBC per total monocyte number.

Effects of mPEG Derivatization on RBC Structure and Function. RBC morphology was examined by both light and scanning electron microscopy (SEM). For the SEM studies, mPEG-modified and control RBC (exposed to pH 9.2 in the absence of reactive mPEG) were prepared as described above, and immediately washed three times in isotonic saline. The cells were then fixed with 1% formaldehyde/1% paraformaldehyde and prepared using standard SEM procedures (14). Spontaneous RBC lysis was quantitated by measuring the amount of extracellular hemoglobin against the total hemoglobin concentration using the ferricyanide–cyanide (Drabkin's) method at 540 nm (15). The osmotic fragility of RBC in response to decreasing osmolarity was determined as described (16).

Previous studies have shown that cyanuric chloride-activated mPEG reacts primarily with accessible ε amino acids (primarily lysine residues) on proteins. Thus, to determine possible sites of covalent mPEG derivatization, SDS/PAGE was done on membranes of control and treated cells. Briefly, membrane ghosts from the control and derivatized RBC were prepared by the method of Dodge *et al.* (17) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (100 μM). Protein concentration of the washed membranes was determined using Coomassie blue (Pierce) (18). Aliquots containing 30 μg of SDS-solubilized protein were loaded on 9% polyacrylamide gels as described (19, 20).

Effects of mPEG Derivatization on *in Vivo* Survival. The *in vivo* survival of mPEG-modified RBC was examined in BALB/c mice. RBC were obtained from multiple donor mice and pooled. Half the cells were derivatized as described above, and then both control and mPEG-modified RBC were labeled with PKH-26, a fluorescent lipid that inserts into the membrane. The labeled RBC were injected intraperitoneally (i.p.) into recipient mice. In similar studies, sheep RBC were mPEG derivatized, labeled, and injected i.p. into mice. Cell survival was followed up to 62 days (mouse → mouse) or 72 hr (sheep → mouse) by measuring PKH-26 fluorescence on a Becton Dickinson flow cytometer (21).

RESULTS

Covalent binding of mPEG to the membranes of intact Type A RBC prevents agglutination by anti-A antisera in a dose dependent manner (Fig. 1). This is apparent whether aggregation is measured by hemagglutination on a slide under static conditions (Fig. 1*A*) or using a platelet aggregometer (Fig. 1*B*). In additional studies (results not shown), mPEG-modified RBC were also resistant to aggregation in the presence of various "enhancing" agents, which ordinarily magnify hemagglutination (e.g., low ionic strength saline, high concentrations of albumin, or precedent neuraminidase treatment). Furthermore, testing of matched control and derivatized RBC (6 mg/ml RBC) by the Clinical Hematology Laboratory at the Albany Medical Center for selected minor RBC antigens also revealed significant decreases in the reactivity of minor blood group antigens on the modified RBC (Table 1). These antigens are typical of minor blood group determinants to which immunological responses occur in chronically transfused individuals (22).

As expected from the agglutination results, mPEG-modified human Type A RBC also bind significantly ($P < 0.01$) less anti-A antibody (Fig. 2*A*). Identical results (not shown) are obtained with Type B and Type AB RBC. Furthermore, mPEG-modified human RBC, following either opsonization with appropriate antisera or damage by heating (47°C × 30

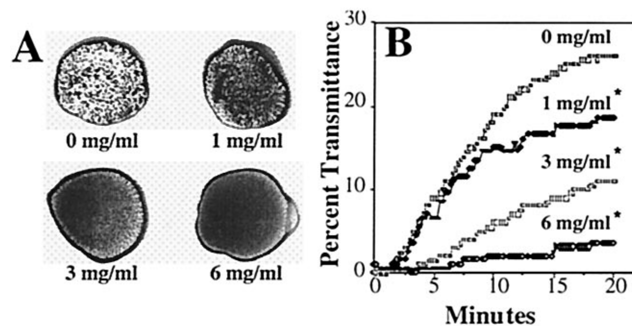

FIG. 1. Derivatization of RBC with mPEG prevents antibody-mediated aggregation. Type A$^-$ RBC were derivatized with 0, 3, and 6 mg/ml mPEG as described. (*A*) Gross RBC agglutination. Equal volumes of a 40% hematocrit of Type A$^-$ RBC were mixed with anti-A typing sera. As can be seen, increasing amounts of covalently bound mPEG inhibits RBC agglutination. (*B*) Microaggregation of control and mPEG-derivatized Type A RBC in response to anti-A serum as measured at 37°C in a platelet aggregometer. Shown is a representative experiment from over 20 independent assays. Significance ($P < 0.01$) is denoted by an asterisk.

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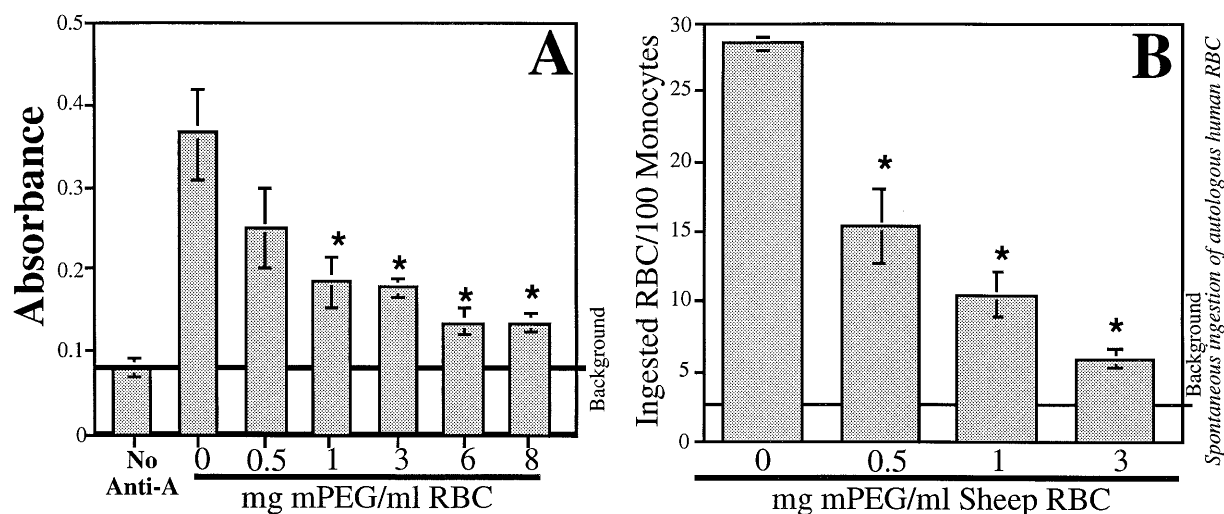

FIG. 2. mPEG derivatization decreases RBC blood group antibody binding and phagocytic destruction by heterologous phagocytes. (*A*) ELISA analysis of antibody binding to mPEG-treated human Type A^- RBC. The control and mPEG-derivatized RBC were mixed with anti-A serum and incubated for 30 min at 37°C. The samples were extensively washed and then incubated with a secondary antibody conjugated with alkaline phosphatase. The amount of bound antiblood Group A antibody was quantitated as described. (*B*) Phagocytic uptake of mPEG-derivatized sheep RBC by human peripheral blood monocytes. mPEG-modified sheep RBC were incubated with human peripheral blood monocytic cells for 30 min as described. Significance ($P < 0.01$) is denoted by an asterisk.

min), are significantly less susceptible to phagocytosis by autologous PBMC (not shown). Perhaps more surprisingly, mPEG-modified *sheep* RBC opsonized by prior incubation with human serum are highly resistant to phagocytosis by *human* peripheral blood monocytes (Fig. 2*B*); this decreased phagocytosis is closely correlated with the degree of derivatization. Importantly, addition of unactivated mPEG to intact RBC, followed by washing in saline (three times), has no inhibitory effect on antisera-induced hemagglutination, antibody binding, or phagocytosis (data not shown).

While mPEG derivatization significantly decreases RBC antigenicity, the covalent modification of RBC has no apparent morphological, structural, or functional effects on the cells. During the derivatization process itself, virtually no lysis (<1%) was observed in either the control or mPEG-modified RBC. Indeed, even mPEG concentrations of up to 50 mg/ml yielded no significant lysis during RBC derivatization. However, very high concentrations of mPEG (>30 mg/ml) resulted in significant lysis after 24 hr of incubation at 37°C (but not 4°C). In addition, as shown in Fig. 3, mPEG-derivatized RBC (6 mg/ml) are morphologically normal. Similarly, while mPEG modification of RBC does lead to a slight (though significant) increase in spontaneous hemolysis, this amounts to <5% after 48 hr at 37°C (Fig. 4*A*). Further supporting the normality/stability of the derivatized RBC, no differences in the osmotic fragility curves of the control and mPEG-modified RBC could be seen even after 48 hr of incubation at 37°C (Fig. 4*B*). Finally, the effects of mPEG modification on the hemoglobin oxygen affinity of treated cells were assessed. Oxygen equilibrium curves (obtained using a Hemox-Analyzer; TCS Medical Products, Southampton, PA) revealed that the oxygen affinity of mPEG-treated RBC was within the normal range (P_{50} = 25.6 mmHg; average of three determinations) and equivalent to both fresh control cells and untreated cells incubated at pH 9.2.

Table 1. Clinical laboratory detection of Rh, Kell, and MNS RBC antigen-mediated agglutination of control and mPEG-derivitized human RBC

	C	c	E	e	K	S	s
Control	0	4^+	0	3^+	0	3^+	3^+
mPEG-treated	0	1^{+w}	0	1^{+w}	0	1^+	1^{+w}

Agglutination response is measured macroscopically with a 4^{+s} rating being the strongest and a 1^{+w} being the weakest positive agglutination response. mPEG concentration was 6 mg/ml. C, c, E, e, Rh antigens; S, s, MNS antigens; K, Kell antigens.

To determine the site(s) of membrane modification, RBC membrane proteins from control and derivatized cells were analyzed by SDS/9% PAGE. As shown in Fig. 5, there are distinctive shifts in the apparent molecular weights of several protein bands in the area of Band 3 (the anion transport channel). The extent of these changes is mPEG dose dependent. For example, two bands (denoted by asterisks) occurring in the Band 3 area show approximate 2-fold differences in intensity when membranes from cells incubated with 3 vs. 6 mg/ml mPEG are compared by densitometry. In addition, a new band slightly above Band 4.1 is also observed in mPEG-derivatized samples. This band is probably not mPEG-modified Band 4.1 (an internal cytoskeletal protein), but more

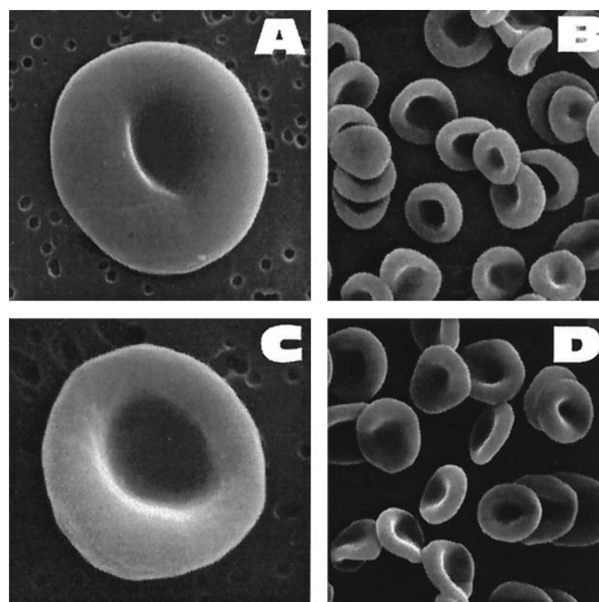

FIG. 3. Human mPEG-derivatized RBC are morphologically normal. Shown are control RBC (×5,500; *A*) and (×1,900; *B*) and mPEG-derivatized (6 mg/ml) RBC at (×5,500; *C*) and (×1,900; *D*). mPEG-modified RBC were prepared as described.

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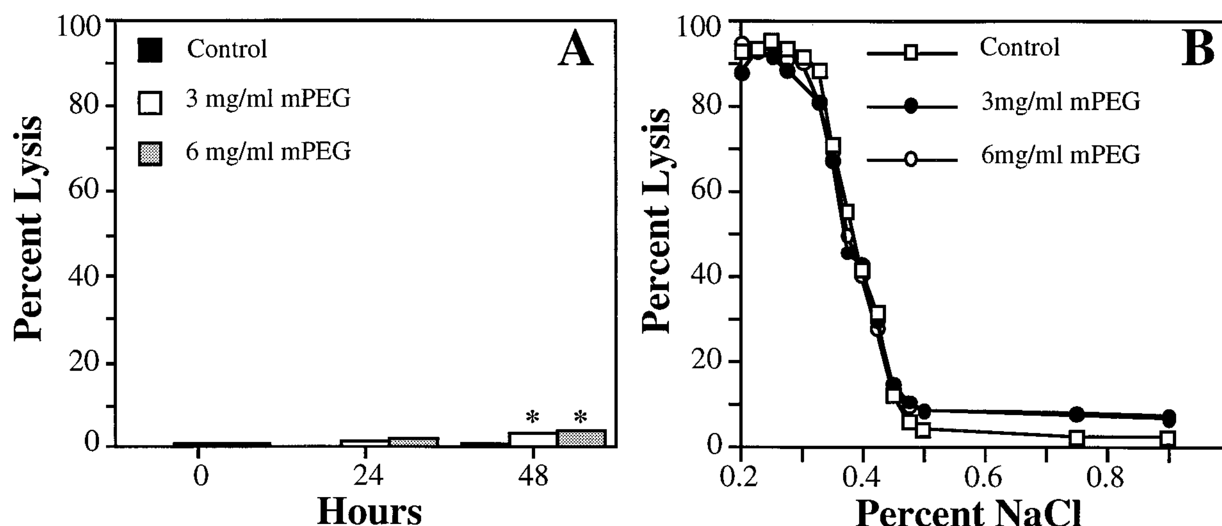

FIG. 4. Erythrocyte stability is minimally affected by covalent attachment of mPEG. (*A*) RBC lysis of mPEG-modified RBC was slightly, though significantly, increased. However, even after 24 hr of incubation at 37°C (or storage at 4°C) lysis was less than 5%. (*B*) Osmotic fragility of the mPEG-treated cells is also unaffected. Shown are representative osmotic fragility profiles of the control and mPEG-modified (3 and 6 mg/ml) RBC. A 40% hematocrit of the control and mPEG-modified cells was incubated in Hanks′ balanced salt solution (pH 7.2; supplemented with 5 mM glucose and containing 100 units of penicillin and 100 μg of streptomycin per ml) for up to 48 hr at 37°C. Significance ($P < 0.05$) is denoted by an asterisk.

likely represents a modification of another, at least partially external, membrane protein.

In support of the lack of significant RBC damage occasioned by mPEG derivatization, no significant differences in the *in vivo* survival of mPEG-modified mouse RBC were observed even after repeated long-term administration. As shown in Fig. 6, the rates of removal of the fluorescently tagged cells in the primary transfusion were 2.33 and 2.31% per day, respectively, in the control and mPEG-modified (3 mg/ml) RBC. Similarly, a tertiary infusion of control and mPEG-modified (3 mg/ml) RBC administered 18 weeks following the primary infusion, demonstrated very similar rates of clearance (2.10 and 2.23% per day, respectively). Importantly, these data demonstrate that the covalent modification of the RBC surface with mPEG does not elicit an immune response. Similarly, repeated doses of mPEG-modified cells over the short term also does not alter *in vivo* survival.

However, as noted in Fig. 6, a somewhat greater variation in the $t_{1/2}$ of survival between the control [$t_{1/2} \approx 24$ (1°) and 25 (3°) days] and mPEG [$t_{1/2} \approx 22$ (1°) and 27 (3°) days]-treated RBC was observed. This may reflect some minor injury to the cells—perhaps due to differences in the preparation and

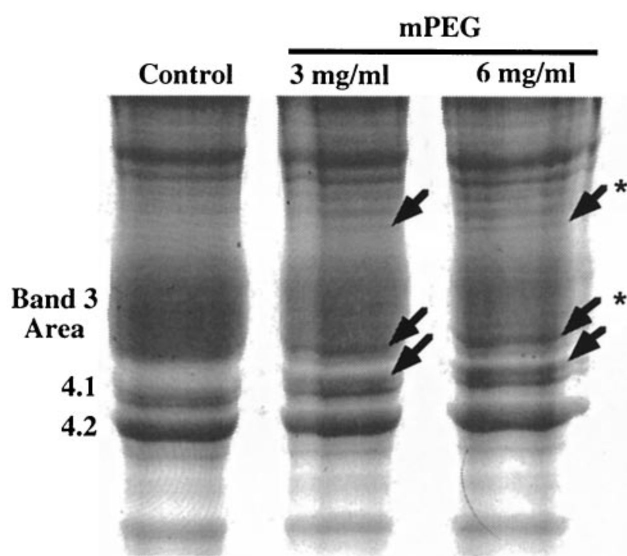

FIG. 5. SDS/PAGE analysis shows dose-dependent differences in the electrophoretic mobility of membranes from control and mPEG-derivatized human RBC. Note especially the distinct mobility shifts in proteins in the area of Bands 3 and 4.1 (arrows). Arrows denoted by an asterisk further demonstrate clear dose dependence as measured by gel densitometry. SDS/PAGE analysis was done as described.

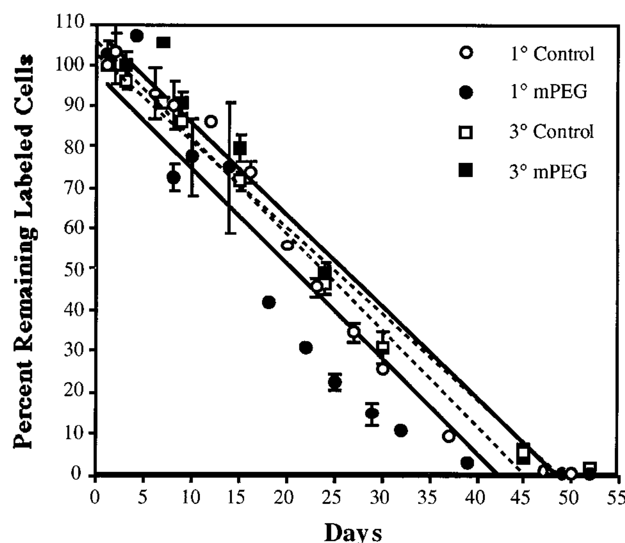

FIG. 6. *In vivo* survival of mPEG-derivatized mouse RBC is normal even after repeated infusions. Shown are the clearance rates of primary and tertiary i.p. infusions of control and mPEG-modified mouse RBC. Survival was followed using a fluorescent fatty acid label (PKH-26; Sigma) as described. Each infusion approximated 8–10% of the total mouse RBC mass (based on weight and calculated blood volume). Following each RBC administration, cell clearance was allowed to proceed until no detectable label remained (≈55 days) at which time the animals were retransfused. The total elapsed time between the primary and tertiary transfusions was ≈18 weeks. Each time point represents the mean ± SD of five BALB/c mice. The clearance rates of the control and mPEG-derivatized RBC were determined by linear regression analysis and are denoted by broken and solid lines, respectively.

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purification of the cyanuric chloride-activated mPEG. Such changes would be magnified in mouse RBC, which are much more fragile than human RBC. However, as demonstrated by the tertiary infusion of mPEG-modified cells shown in Fig. 6, this is not a consistent finding.

While the normal or near-normal survival of isogeneic (i.e., autologous) mPEG-derivatized mouse RBC indicates that derivatization *per se* causes no significant effects on RBC structure, function, or viability, it does not address what effect it may have on heterologous or even xenogenic transfusions. As an extreme test of this question, we elected to study the survival of mPEG-modified sheep RBC in mice. Control and mPEG-modified sheep RBC (mean cell volume $\approx 31\ \mu^3$; diameter $\approx 4.8\ \mu$) were injected i.p. into BALB/c mice (mean cell volume $\approx 49\ \mu^3$; diameter $\approx 6.6\ \mu$) (23). Surprisingly, as shown in Fig. 7, >4 times more mPEG-derivatized sheep RBC enter the murine circulation following i.p. injection and exhibit significantly longer *in vivo* survival. In contrast, far fewer control sheep RBC appear in the circulation and these are rapidly cleared. This is in accord with previous studies indicating that, following intravenous administration, sheep RBC have a half-life of <5 min in mice (Fig. 7 *Inset*) (24).

DISCUSSION

The effectiveness with which mammals immunologically respond to and destroy heterologous cells has been an important barrier in both blood banking and organ transplantation. Within the sphere of blood banking, effective typing for major blood groups does keep the incidence of severe transfusion reactions relatively low; the frequency of serious hemolytic transfusion reactions is estimated to be only ≈ 1 in 6,000, whereas fatal transfusion reactions occur in only ≈ 1 in 100,000 transfusions (25, 26). However, alloimmunization to minor RBC antigens is frequent in chronically transfused individuals, such as those with severe thalassemias and sickle cell anemia (27, 28). In such patients, 20–30% will ultimately show evidence of alloimmunization against minor blood group antigens making subsequent transfusions more problematic (2, 29). Finally, a large (but unknown) measure of morbidity and mortality may also be ascribed to the *lack* of transfusion. This is particularly true in patients with autoimmune hemolytic

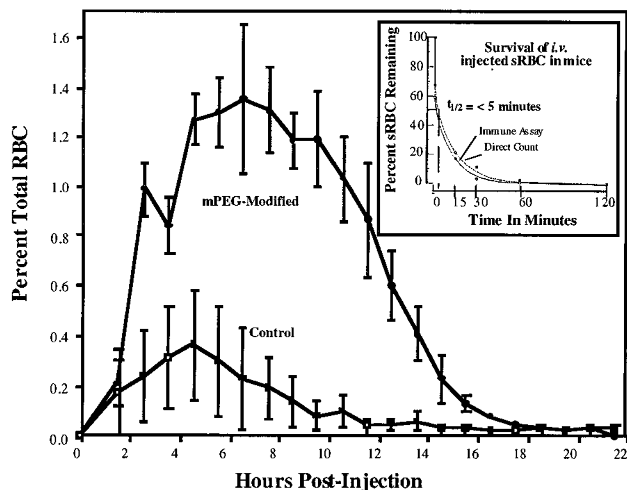

FIG. 7. *In vivo* survival of mPEG-modified sheep RBC is enhanced dramatically in mice. mPEG-derivatized sheep RBC were prepared as described and injected i.p. into naive BALB/c mice. Shown are the mean value ± SD (percent total RBC counted) of four mice per group. (*Inset*) Survival time ($t_{1/2} < 5$ min) of unmodified sheep RBC following direct intravenous infusion (modified from Sohnle and Sussdorf; ref. 24).

disease or those suffering severe trauma for whom blood typing and matching cannot be carried out quickly enough.

The above considerations have led to the our interest in hemoglobin-based blood substitutes. At present, preclinical and clinical tests of chemically and genetically manipulated hemoglobin solutions are being conducted. Despite substantial activity in this area, a safe and effective blood substitute, which can be used in compromised humans, has yet to be developed (30, 31). Indeed, because the RBC membrane serves to localize and protect the intracellular hemoglobin, it might be more desirable to employ antigenically silent RBC rather than engineered hemoglobin solutions. To this end, the selective exoglycosidase treatment of RBC to enzymatically remove A or B antigens is being tested (32). While significant success is being met with Type B erythrocytes, Type A RBC are more problematic. Furthermore, such treatments will have no effect on other blood group reactivities.

Consequently, we have attempted to produce antigenically silent RBC via the covalent attachment of nonimmunogenic materials such as mPEG. Previous studies with PEG, or PEG derivatives, coupled to purified proteins have demonstrated that the protein half-life within the circulation is greatly improved (5, 6, 8). Similarly, liposomes containing externally disposed PEG lipids have improved half-lives *in vivo* (33–35). It is also of interest that the covalent attachment of PEG usually does not impair the function of purified enzymes and appears to make foreign proteins nonimmunogenic even after repetitive intravenous administration (5, 6, 8, 36–39). The "camouflage" effected by PEG is imparted by the special physico-chemical nature of PEG (i.e., its size, large exclusion volume, and extensive hydration), which may prevent the interactions of large molecules such as antibodies with RBC and may as well hinder cell–cell interactions. Thus, we reasoned that mPEG derivatization might yield fully functional yet antigenically inert RBC.

Indeed, mPEG-modified human and mouse RBC are morphologically and structurally normal, exhibit decreased antigenicity and, in mice, demonstrate normal *in vivo* survival with no immunogenicity associated with the mPEG-modified RBC. Furthermore, in an extreme test of the potential for mPEG derivatization to retard recognition and destruction of xenogeneic cells, mPEG-derivatized sheep RBC have substantially improved survival in the circulation of mice. The eventual destruction of the derivatized sheep RBC in the mouse may not be immunologic in nature but may be mediated by structural changes induced by the foreign environment. For example, the lipid composition of mouse and sheep RBC is significantly different in that sheep RBC do not have phosphatidylcholine (40). This disparity in membrane composition may mediate the accelerated removal of the sheep RBC (41).

In summary, our findings suggest that mPEG-derivatized RBC may be an attractive alternative to hemoglobin-based blood substitutes in emergency situations and in the chronically transfused patient. This is largely because compartmentalized (i.e., intraerythrocytic) hemoglobin is preferable to free hemoglobin, which, even when modified to improve stability and increase molecular weight, can be toxic (42, 43). We find that the covalent modification of RBC surfaces with mPEG effectively obscures antigenic determinants while leaving the RBC structurally and functionally normal and nonimmunogenic. Furthermore, initial studies on xenogeneic transfusions have demonstrated increased survival of mPEG-modified RBC. The chemical camouflage of antigenic determinants on cell surfaces may permit not only heterologous RBC transfusions but may also have obvious utility in transplantation medicine.

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