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Targeting of mouse erythrocytes by band 3 crosslinkers

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Abstract

Chemical conditions of crosslinking mouse erythrocytes with BS^3 and DTSSP have been studied. These two crosslinking reagents seem to react with band 3 protein in mouse erythrocytes membrane. Extent of crosslinking is dependent on the concentration of the reagent used. Similar cell volumes were observed in crosslinked erythrocytes with respect to control erythrocytes. In vivo behaviour of these modified erythrocytes revealed prominent targeting of crosslinked erythrocytes to liver. This effect is clearly evident when concentrations of 5 mM BS^3 or DTSSP were used and can be dependent of reagent concentration. Consequently, from our results BS^3 and DTSSP can be considered as very useful tools to control and modulate targeting of crosslinked erythrocytes.

Keywords: Crosslinking; Erythrocyte; Band 3

1. Introduction

The development of new drug delivery systems has taken advantage of the physiological nature of the erythrocyte to use these cells as carriers [1–5]. Chemical treatments of erythrocytes with crosslinkers may be a method to modify erythrocytes which could be used separately or in combination with standard hypotonic encapsulation methods to prepare carrier erythrocytes [6–12]. Extensive crosslinkers which react with several proteins in erythrocyte membrane have been used [7,11,13]. Band 3 protein is a prominent protein in erythrocytes membrane which is involved in several erythrocyte functions [14–16]. Conditions have been used where a high extent of crosslinking of human band 3 protein can be obtained [17–19]. Chemical modification of this protein can control the release of compounds introduced into erythrocytes [20,21]. Chemical crosslinking of mouse erythrocyte band 3 could be of relevant interest to characterize this animal as an alternative model for the development of physiological delivery systems in therapy. We have focused this study on the action of two crosslinkers which interact with band 3 protein in the erythrocyte membrane: bis(sulfosuccinimidyl)suberate (BS^3) and 3,3′-dithiobis(sulfosuccinimidyl)-propionate (DTSSP) [10,14–16]. These bifunctional reagents are relatively safe in comparison to other crosslinking reagents which can produce some toxic effects. In our knowledge, no author has extensively described the effects of the only action of these crosslinkers on mouse erythrocytes. Furthermore, the necessity of the combined use of aggregating reagents ($ZnCl_2$) and crosslinkers (BS^3) to make these systems useful for clinical applications has been described [22,23]. Interestingly, the use of chemical reagents which react mainly with mouse band 3 could be applied to control targeting of modified erythrocytes on the basis of the extent of crosslinking of this protein.

Phagocytic removal of mouse erythrocytes has also not been so far described previously as a consequence of the exclusive action of band 3 crosslinkers. Extensive erythrocyte membrane proteins modification produces targeting of the modified erythrocytes [4,13]. A selective band 3 crosslinking can be used to control the removal of mouse modified erythrocytes. In humans, intraperitoneal injection is applied for treatments in several diseases [24–27]. Intraperitoneal injection is a convenient way of administration in animal model studies [28,29]. Thus, intraperitoneal in-

Abbreviations: BS^3, bis(sulfosuccinimidyl)suberate; DTSSP, dithiobis(sulfosuccinimidylpropionate); GSH, reduced glutathione; MCV, mean corpuscular volume; PBS, saline phosphate buffer; SDS, sodium dodecyl sulfate

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jection of mouse band 3 crosslinked erythrocytes can be eventually applied to study targeting of substances.

In this paper, we have analyzed the action of these crosslinkers on band 3 in mouse erythrocytes. We have also studied the differential uptake of mouse crosslinked erythrocytes by different organs in order to claim for a possible feasibility of these crosslinked erythrocytes as carriers of substances with action on some organs.

2. Materials and methods

Bis(sulfosuccinimidyl)suberate (BS^3) and 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) were supplied by Pierce Rockford, IL, USA.

2.1. Isolation of erythrocytes

Erythrocytes suspensions were prepared from CD1 mouse whole blood. Plasma, white blood cells and platelets were separated by centrifugation ($500 \times g$ for 10 min at 4°C). Erythrocyte pellets were washed three times in saline phosphate buffer (PBS) solution containing 145 mM NaCl, 5 mM glucose, 5 mM K_2HPO_4/NaH_2PO_4 pH 7.4, and centrifuged as above.

2.2. Cell counting and cell size determination

These two parameters were determined in a Hematology Analyzer (Serono-Baker diagnostics System 2000 Plus). For cellular yields analyses, cell numbers were counted before and after crosslinking. The number of whole cells remaining in the sample after crosslinking is referred to this value as a percentage.

2.3. Chemical crosslinking of erythrocytes

Treatments with BS^3 and DTSSP were accomplished as described [17,18]. Crosslinking of mouse erythrocytes with bifunctional reagents was carried out by adding erythrocytes ($1.7 \cdot 10^9$ cell/ml in phosphate buffer containing 5 mM glucose, 106 mM Na_2HPO_4/NaH_2PO_4 pH 7.4) to BS^3 or DTSSP in 53 mM phosphate buffer in a 1:1 ratio, followed by an incubation for 30 min at room temperature with gentle agitation. Three different final concentrations of bifunctional reagent were used (5 mM, 0.5 mM or 0.25 mM). The reaction was stopped by adding approx. 20 vol. of 0.15 M NaCl, 25 mM Tris pH 7.4 buffer.

2.4. Isolation of membranes

Erythrocyte membrane isolation was performed as described previously [17,18]. Suspensions of untreated, BS^3 or DTSSP modified erythrocytes were used. Erythrocytes (1 vol.) was lysed in 80 vol. of an hypotonic solution containing Na_2SO_4EDTA 0.1 mM, *N*-ethylmaleimide 20 mM pH 7.4 (adjusted with a solution of Tris), for 1 h at 4°C. After centrifugation ($20000 \times g$ for 20 min at 4°C), the ghosts were washed twice in NaCl 15 mM, Tris 1.7 mM, Na_2SO_4EDTA 0.1 mM pH 7.4. The final pellet was suspended in PBS and kept frozen at −80°C until use.

2.5. Electrophoretic analyses and densitometric scanning

Analytical SDS-polyacrylamide gel electrophoresis was performed as described [30]. β-Mercaptoethanol was avoided in the case of DTSSP-crosslinked mouse erythrocyte membranes in order to prevent disruption of crosslinker structure by reducing reagents. For densitometric scanning of the gels, a LKB Ultroscan XL Densitometer, with Gelscan XL programme, was used. Erythrocyte membranes from mouse untreated and crosslinked erythrocytes were run on SDS-polyacrylamide gel electrophoresis. To analyze the crosslinking of band 3 protein, it was measured the proportion of band 3 monomer present in the densitometric scans of membranes from untreated erythrocytes with respect to the total membrane proteins present in the gels by integration of the area under the peaks. This value corresponds to the percentage of band 3 protein monomer in relation to the whole protein present in the untreated sample (a). Similarly, it was calculated the proportion of band 3 monomer present in the densitometric scans of membranes from crosslinked erythrocytes with respect to the total membrane proteins present in the gels by integration of the area under the peaks. This value correspond to the percentage of band 3 protein monomer in relation to the whole protein present in the crosslinked sample (b). This second value (b) was always lower than the first one (a). From these two values, it can be calculated the reduction of the proportion of band 3 protein monomer in the crosslinked samples. $(b/a) \times 100$ gives the percentage of band 3 protein monomer which remains uncrosslinked after the crosslinking treatment. $100 - [(b/a) \times 100]$ could be used as a value for estimation of the extent of crosslinking.

2.6. Cromium labeling of erythrocytes

Erythrocytes were labeled with radioactive chromium (^{51}Cr) by mixing 100 μl $Na_2^{51}CrO_4$ in isotonic saline (1.0 mCi/ml, Dupont NEN, Brussels, Belgium) with one milliliter of erythrocytes (10^{10} cells/ml). The cells were radiolabeled by incubation for 30 min at 4°C with gently agitation. The labeled cells were washed three times in PBS, to remove free ^{51}Cr.

2.7. In vivo uptake of crosslinked radiolabeled erythrocytes

For in vivo experiments, ^{51}Cr-erythrocytes were crosslinked as described above using two final crosslinker concentrations (5 mM and 0.25 mM). ATP 0.25 mM and

reduced glutathione (GSH) 0.5 mM were added to the solutions used in cell isolation, labeling and washing, in order to prevent cell oxidation processes. The labeled erythrocytes were washed 2 times in PBS and resuspended to around 10^{10} cells/ml for experimental use.

Mice were injected with 100 μl of control or modified ^{51}Cr-erythrocytes intraperitoneally (i.p.). At time zero (within 10 min after injection) and at different times after injection (4, 24 h when a concentration of crosslinker of 5 mM was used and 4, 24, 48, 72, 96 h for 0.25 mM crosslinker concentration) from 3 to 10 mice of each group were bled and liver, spleen, lungs and kidneys taken. The radioactivity present in the organs and 10 μl of blood was determined using a LKB-Wallac 1282 compugamma CS counter.

3. Results

3.1. Electrophoretic analysis of the crosslinking reaction

We have first studied the extent of crosslinking of band 3 when mouse erythrocytes were incubated with the bifunctional reagent (either BS^3 or DTSSP). Since crosslinking of mouse erythrocytes at a cell/crosslinker concentration ratio equal to that described for human erythrocytes [17,18] renders a lower crosslinking in mouse erythrocytes (not shown), we used here a reduced cell/crosslinker concentration in order to increase the extent of crosslinking of band 3 protein. Electrophoretic gels are shown in Fig. 1. In this figure, a reduction in the level of band 3 protein monomer is apparent in crosslinked mouse erythrocyte membranes. Band 3 protein monomer is increasingly more prominent when crosslinker concentration is progressively decreased. Formation of band 3 oligomers might be possible since high-molecular-weight aggregates seem to precipitate and to be unable to enter stacking gel (see also Fig. 1). Densitometric analyses of SDS-gels of erythrocyte membranes allowed an estimation of the extent of crosslinking of both reagents on band 3 protein in mouse erythrocytes (Table 1). Since no band 3 dimers or tetramers bands were observed and the relative proportion of the different erythrocyte membrane proteins others than band 3 are kept constant, it is arguable to estimate the extent of crosslinking of band 3 as the reduction on band 3 monomer in comparison with the total protein present in the gel as analyzed by densitometric scanning (see methods). The estimation of the crosslinking in mouse erythrocytes represented around 29% at a concentration of 5 mM. For lower crosslinker concentrations (0.5 mM and 0.25 mM), the extent of crosslinking was around 10% or less (see Fig. 1 and Table 1).

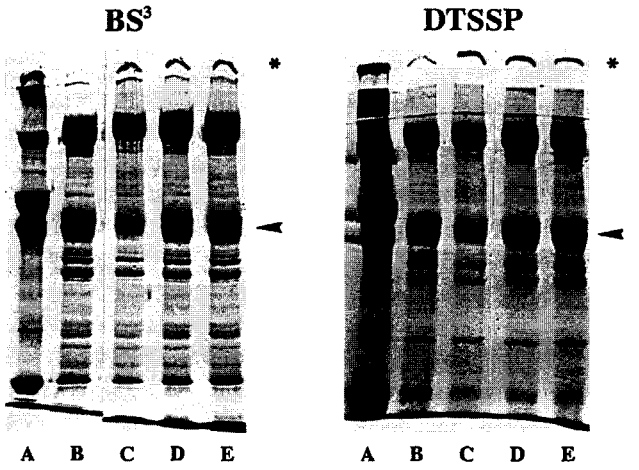

Fig. 1. Electrophoretic patterns of control and BS^3 and DTSSP crosslinked mouse erythrocytes membranes. Membranes from control and crosslinked erythrocytes were isolated and run on SDS-gels. 25 μg of sample were applied per well. Capital letters correspond to molecular mass standards (A), control (B), 5 mM crosslinked (C), 0.5 mM crosslinked (D) and 0.25 mM crosslinked (E). Molecular mass standards used were: myosin (205 kDa), β-galactosidase (116 kDa), Phosphorylase *b* (97 kDa) and ovalbumin (45 kDa). Arrowhead shows the position of band 3. Asterisk represent the position of the material of the sample which are unable to enter the polyacrylamide gels.

Table 1
Extent of crosslinking in band 3 protein in erythrocyte membranes obtained from control, BS^3- and DTSSP-crosslinked mouse erythrocytes

	Crosslinker concentration		
Crosslinker	5 mM	0.5 mM	0.25 mM
BS^3	29.3	11.5	7.8
DTSSP	28.1	9.0	5.8

Three crosslinker concentrations were used (5 mM, 0.5 mM and 0.25 mM). These values correspond to the mean of 2–5 independent experiments, each one in triplicate. To analyze the crosslinking of band 3 protein, it was measured by the proportion of band 3 monomer present in the densitometric scans of membranes from untreated erythrocytes with respect to the total membrane proteins present in the gels by integration of the area under the peaks. This value corresponds to the percentage of band 3 protein monomer in relation to the whole protein present in the untreated sample (*a*). Similarly, the proportion of band 3 monomer present in the densitometric scans of membranes from crosslinked erythrocytes was calculated with respect to the total membrane proteins present in the gels by integration of the area under the peaks. This value corresponds to the percentage of band 3 protein monomer in relation to the whole protein present in the crosslinked sample (*b*). This second value (*b*) was always lower than the first one (*a*). From these two values, the reduction of the proportion of band 3 protein monomer in the crosslinked samples can be calculated. $(b/a)\times 100$ gives the percentage of band 3 protein monomer which remains uncrosslinked after the crosslinking treatment. $100-[(b/a)\times 100]$ could be used as a value for estimation of the extent of crosslinking.

3.2. Cell yields and cell size determination after crosslinking treatments

In the use of crosslinked erythrocytes from species others than human as carriers the yield of unlysed cells resulting from the crosslinking procedure should be considered. Human erythrocytes remain basically unlysed (around 95–100%) when crosslinked at a reagent concen-

Table 2
Cell yields and cell volume analyses of control, BS^3 and DTSSP-crosslinked mouse erythrocytes

	Crosslinker		
	control	BS^3	DTSSP
Cell yield (%)	83.04 ± 0.77	55.91 ± 6.3	56.43 ± 9.77
MCV (fL)	47.18 ± 0.58	46.35 ± 0.8	44.69 ± 0.34

The number of unlysed cells and hematological parameters were determined in an Hematology Analizer. These values correspond to mean ± S.E.M. of 3 independent experiments, each one in duplicate.

tration of 5 mM (unpublished results). Lower cell yields were obtained for mouse erythrocytes (56% of unlysed cells in the case of BS^3- and DTSSP-treated erythrocytes). Reduction of the crosslinker concentration in the incubation mixtures gave rise to an increase in cell yield reaching values around 70% of unlysed cells for the lowest concentrations used (0.5 and 0.25 mM) (unpublished results). Anyhow, cell lysis can be partially due to the incubation procedure (Table 2), since removal of the crosslinker in the incubation medium produced 17% of cell lysis. This can be due to an influence of incubation temperature or other factors on cell stability which can produce changes in cell fragility.

Mean corpuscular volume (MCV) analyses of BS^3 and DTSSP erythrocytes showed similar values between control erythrocytes (which have been incubated in the same buffer for the same period of time than crosslinked erythrocytes although crosslinker has not been included in the incubation medium) and crosslinked erythrocytes (Table 2). These values are similar to those of native (obtained and isolated directly from the animal) erythrocytes (around 49.5 fL for mouse erythrocytes). For crosslinking at lower reagent concentrations (0.5 and 0.25 mM) values around 45–46 fL were obtained for both crosslinkers (unpublished results).

3.3. *In vivo uptake of crosslinked labeled erythrocytes*

Mice were injected with control and crosslinked ^{51}Cr-erythrocytes and measurements of radioactivity present in blood were carried out at times 0, 4 and 24 h. As shown in Fig. 2, a very low level of 5 mM-crosslinked ^{51}Cr-erythrocytes, in comparison to control (untreated) ^{51}Cr-erythrocytes was observed. BS^3 and DTSSP crosslinked erythrocytes did not circulate remaining an extremely low level of radioactivity in blood at 4 and 24 h (Fig. 2) at a crosslinker concentration of 5 mM. The radioactivity present in the collected organs (liver, spleen, kidneys and lungs) was also studied (Fig. 2). Measurements of radioactivity showed that BS^3 and DTSSP treated erythrocytes are similarly targeted to liver and spleen, mainly to liver, while no relevant differences in comparison to control ^{51}Cr-erythro-

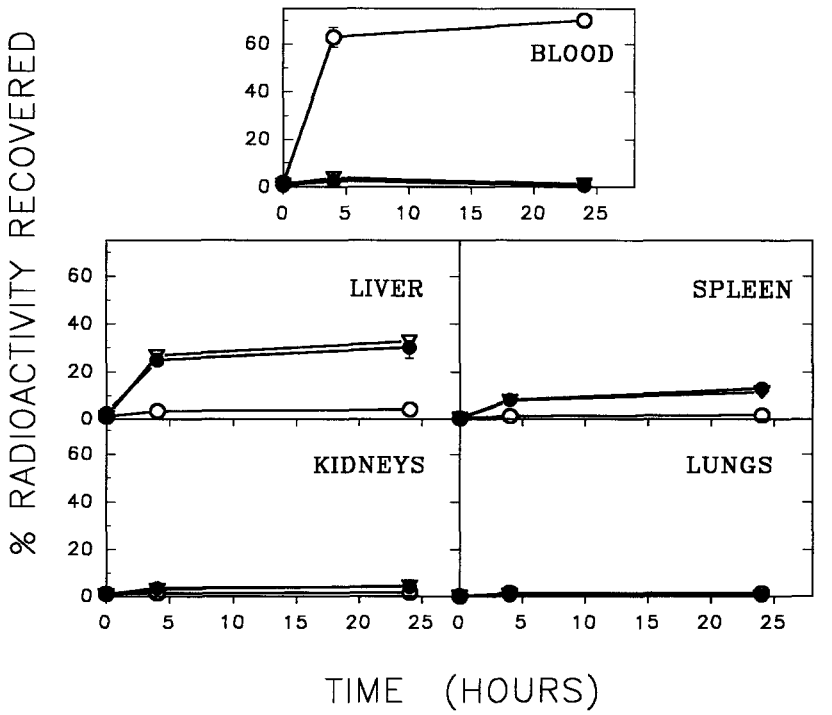

Fig. 2. Time-course of radioactivity localization of crosslinked erythrocytes in different organs (crosslinker concentration 5 mM). This figure shows a diagrammatic scheme showing the percentages of radioactivity present in different organs at different times after injection of labeled erythrocytes. The percentage of radioactivity from labeled erythrocytes collected in blood and in different organs is shown. The percentages represent absolute values in relation to the total injected radioactivity. The values correspond to the mean value ± S.E.M. From 3 to 10 animals were used. Symbols represent: control (○), BS^3- and DTSSP-crosslinked erythrocytes (● and ▽, respectively).

cytes were observed in kidneys and lungs. BS^3 and DTSSP crosslinked erythrocytes did not circulate remaining an extremely low level of radioactivity at 4 and 24 h (Fig. 2) at a crosslinker concentration of 5 mM.

When a lower concentration (0.25 mM) of both reagents was used, crosslinked erythrocytes can be seen in circulation (Fig. 3). This is particularly clear for DTSSP-erythrocytes, which still seemed to remain in circulation (30% of the total injected radioactivity) 4 days after injection. No circulation of BS^3-erythrocytes can be observed at that time. As observed for 5 mM-crosslinked erythrocytes (Fig. 2), liver and spleen are again the main organs where radioactivity is collected (Fig. 3). The only difference is that BS^3-erythrocytes seem to be targeted to liver and spleen in a higher proportion if compared to DTSSP-erythrocytes at all times studied. At low BS^3 concentration, a similar behaviour is clearly visible (Fig. 3) at long times (48 h) after injection. At this concentration, a relevant proportion of DTSSP crosslinked erythrocytes remained in circulation even at 96 h after injection.

4. Discussion

The extent of the action of the two used crosslinkers could allow to control some properties of the carrier. Extensive crosslinking with chemical reagents such as glutaraldehyde and dimethylsuberimidate (DMS) which react with several proteins on erythrocytes have been previously done [7,11,13]. We have analyzed the action of two crosslinkers: BS^3 and DTSSP on mouse erythrocytes. Both reagents are considered as impermeant reagents which react with amino groups of proteins. In human erythrocytes, it has been postulated that these two crosslinkers react predominantly with band 3 although in the case of DTSSP other proteins can be also reactive [17,18]. This behaviour described by Staros et al. [17,18] can be understood on the basis of the much larger amount of band 3 protein as a integral protein in comparison to other proteins of erythrocyte membrane. Thus, the use of crosslinkers which react with a protein or a reduced group of proteins on erythrocyte membrane can be extremely interesting in order to be eventually applied for preparing erythrocytes as carrier delivery systems.

Identification of band 3 as preferential target of the crosslinkers used in this study was described in human erythrocytes [17,18]. One of the final aims of our work is to establish how some animal models like mice can be used to assess the behaviour of carrier systems. The final effects of the only action of these crosslinkers on mouse erythrocytes must be considered. The application of the combined use of aggregating reagents ($ZnCl_2$) and

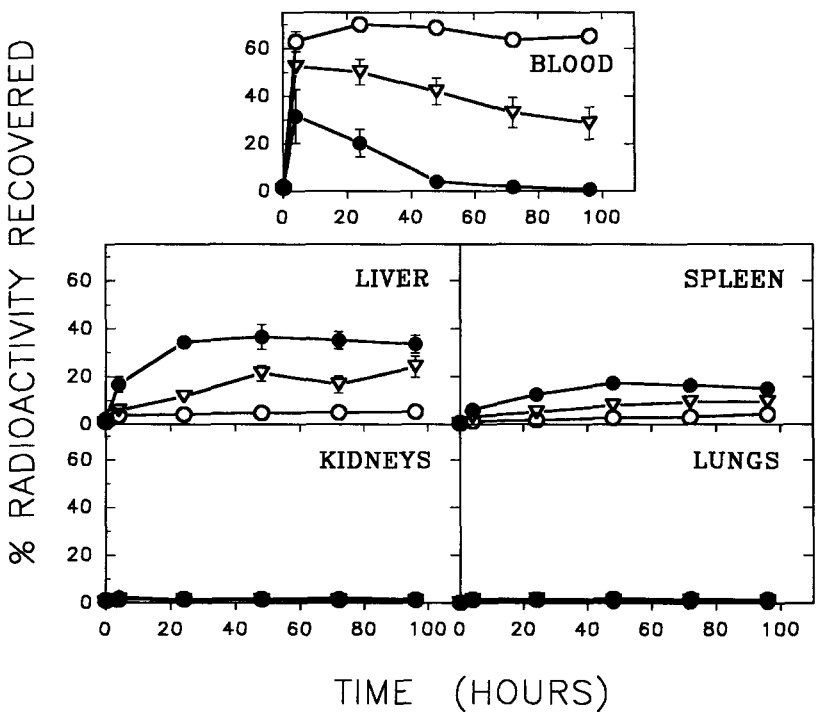

Fig. 3. Time-course of radioactivity localization of crosslinked erythrocytes in different organs (crosslinker concentration 0.25 mM). This figure shows a diagrammatic scheme showing the percentages of radioactivity present in blood and in different organs at different times after injection of labeled erythrocytes. The percentage of radioactivity from labeled erythrocytes collected in blood and in different organs is shown. The percentages represent absolute values in relation to the total injected radioactivity. The values correspond to the mean value ± S.E.M. From 3 to 10 animals were used. Symbols represent: control (○), BS^3- and DTSSP-crosslinked erythrocytes (● and ▽, respectively).

crosslinkers (BS^3) to make these systems useful for clinical applications has been described [10,22,23]. We demonstrated the accuracy of the unique crosslinking action of these reagents to target these modified erythrocytes.

The extent of crosslinking has been estimated as the ratio of band 3 protein to total protein in crosslinked erythrocytes compared to control untreated erythrocytes. This way of quantitation was chosen because in SDS-polyacrylamide gel electrophoresis is clearly visible the decrease of band 3 protein monomer subsequent to chemical crosslinking treatment. The disappearance of band 3 monomer band can only be the result of the oligomerization of this protein. Other possible causes can be disregarded since, i.e., proteolytic digestion of several membrane proteins or even of specifically band 3 protein would have resulted in the presence of several bands of lower molecular weight which are not apparent in electrophoresis gels. The electrophoresis conditions used (7.5% polyacrylamide) should be adequate to show at least some of these bands. Furthermore, in former studies on BS^3 and DTSSP action on human erythrocytes, Staros and coworkers [17,18] followed crosslinking by looking at the disappearance of band 3 monomer on the electrophoretic gels.

Formation of high molecular weight oligomers as a result of extensive crosslinking with these reagents can not be disregarded. Presumably, this can be the reason of the absence of a prominent band corresponding to band 3 dimer in electrophoretic gels. This can also justify the presence of protein precipitates which are unable to enter the stacking gel and which can be observed as irregular bands in upper region of the stacking gel (see Fig. 1).

It is not presently known the extent of crosslinking action exerted by BS^3 and DTSSP in erythrocytes from animal species. Thus, we have studied crosslinking on mouse erythrocytes. We evaluated the extent of crosslinking considering the progressive disappearance of band 3 monomer on electrophoresis gels as a consequence of the crosslinking reaction. Presence of higher molecular weight oligomers such as dimers has been observed by Staros et al. [17,18] when a low reagent/cell concentration ratio is used in human erythrocytes. In these conditions it is clearly visible the complete disappearance of band 3 monomer [17,18]. Crosslinking with these reagents is clearly lower in the case of mouse erythrocytes in comparison to human erythrocytes since the disappearance of band 3 monomer is not complete in mouse erythrocytes using the conditions described by Staros et al. [17,18] (not shown). We have used a high reagent/cell concentration ratio, to obtain around 28–29% of crosslinking of Band 3 protein in mouse erythrocytes. We have not observed in the electrophoresis gels concomitant increase of band 3 dimer or tetramer as a result of the crosslinking reaction. Band 3 protein structure has been described in mouse and other species [15,31–34]. Structural differences could account for the crosslinking shown by mouse erythrocytes lower than in the case of humans. Also, band 3 distribution along the membrane can be involved in differences in chemical crosslinking [35]. It would be plausible to consider that differences in band 3 structure and distribution among different species could determine the extent and other features of the crosslinking by these two reagents. As a matter of fact, we did not observe the presence of band 3 dimer in the case of mouse erythrocytes. Since these results are highly reproducible, we assumed that this species behaviour should be explained because of structural and distribution differences in band 3. Furthermore, the presence of band 3 tetramers is also difficult to observe because of their similar molecular weight to spectrin. The relative proportion of spectrin and the other membrane proteins others than band 3 is always kept constant among them after crosslinking and the unique change in the erythrocyte membrane protein pattern which can be observed is the decrease in the proportion of band 3 monomer with a concomitant presence of the precipitates which did not enter the gels in crosslinked erythrocytes. Thus, it would be rational to consider the formation of high-molecular-weight oligomers others than dimers or tetramers, at least in the case of mouse erythrocytes. Lower concentrations of crosslinker render correlated less crosslinking of band 3 as it could be expected. Thus, a correlation cause-effect is established. Furthermore, the conditions shown here can be applied to obtain erythrocytes with different degrees of crosslinking in band 3.

Chemical reaction with BS^3 and DTSSP produced cell lysis in mouse erythrocytes, which is correlated with morphological changes rendering echinocytic populations [36]. This lysis behaviour can reflect differences in the membrane properties of erythrocytes from different species. These membrane properties can be specially relevant in order to compare the introduction of several compounds and resealing of the hypotonically treated erythrocytes in different species [37]. However, crosslinking with both reagents did not alter cell volume. These data can indicate that some general features of erythrocytes are maintained after crosslinking what is in accordance with the preservation of other properties such as hypotonic resistance, etc. [36].

We have tested survival of modified erythrocytes in circulation after intraperitoneal injection into animals. It as been previously shown that either hypotonically or isotonically treated erythrocytes has a similar survival in circulation independently of the via of injection, intravenously o intraperitoneally [38]. Intraperitoneal injection with therapeutic usage is widely assessed in the literature. For instance, it is used in chemotherapy treatments of gastric carcinoma in humans, in radioimmunotherapy of ovarian cancer patients, in erythropoietin treatment of renal anemia subsequent to continuous ambulatory peritoneal dialysis (CAPS), in studies of cephalosporins effectiveness in the treatment of peritonitis associated with CAPS, in methotrexate treatments in lipid-based drug delivery systems, etc. [24–27]. Additionally i.p. injections are widely

described in the literature in animal models which are used to study therapy treatments with antitumor drugs in tumor bearing mice, in drug toxicity analyses, etc. [28,29].

Localization of injected erythrocytes to removal organs has been described in the case of erythrocytes crosslinked with glutaraldehyde or other reagents which react with a variety of proteins in erythrocyte membranes [13]. In the case of erythrocytes crosslinked only with reagents which react mainly with Band 3, this study has not been previously done. Testing survival of treated erythrocytes (Figs. 2 and 3), a targeting mainly to liver has been seen. We here clearly showed that BS^3 and DTSSP crosslinked erythrocytes are targeted to liver and spleen.

Phagocitic removal of mouse BS^3 and DTSSP crosslinked erythrocytes has also not been so far described in the conditions we have used. We included glutathione, glucose and ATP to the medium to preserve cell stability and functionality. Previous results indicating phagocitic recognition by macrophages did not use the above mentioned conditions. Modified erythrocytes could be recognized because of the crosslinking of band 3 protein or as a result of energy depletion of erythrocytes during the whole crosslinking procedure. It is relevant to indicate that it can be assumed that such a behaviour should be due to the action of the crosslinker and not to some other causes which could modify erythrocyte membrane and intracellular physiology such as energy depletion or oxidation processes. We have used stabilizing conditions in the crosslinking procedure of mouse erythrocytes, including ATP and reduced glutathione. The above mentioned targeting depends on the crosslinking action of BS^3 and DTSSP [36]. Thus, targeting can be assumed as a result of chemical crosslinking and not as a consequence of cell death. These results can be correlated with those described by other authors using $ZnCl_2$ as an agent which induces clustering of band 3 [10,22,23]. In the present work, we demonstrated that phagocitic removal is exclusively consequence of crosslinking of band 3 protein by the unique action of these two reagents.

The concentration of crosslinker may be of particular relevance to modulate the circulation of modified erythrocytes at least in the case of DTSSP. Also, the present results are comparable to others obtained for more extensive crosslinkers such as glutaraldehyde [4]. This indicates that even an specific alteration on a membrane protein such as band 3 can produce targeting of erythrocytes. As a matter of fact, band 3 protein has been claimed to play a key role in erythrocytes aging. Some authors have proposed the generation of senescence antigen as a factor which produces removal of erythrocytes from circulation [39]. This phenomena could be a consequence of the alteration of band 3 by the action of oxidant reagents, etc. [40]. Clustering of band 3 protein in erythrocyte membrane may also be responsible of rapid removal of aged erythrocytes [41,42].

The use of lower concentrations of crosslinking reagents can give rise to a longer circulation of modified erythrocytes (Fig. 3). This can be a consequence of a reduced direct crosslinking on band 3. The modulation of the presence of crosslinked erythrocytes in circulation (Figs. 2 and 3) might be of particular relevance in the use of carrier erythrocytes obtained by crosslinking with BS^3 and DTSSP.

Several reagents such as glucose, ATP and reduced gluthatione were included in survival studies in order to prevent cell death during the preparation procedure as a consequence of energy depletion. Although, the presence of GSH in the washing buffers used for erythrocyte preparation could produce some reduction of DTSSP crosslinks as a consequence of the rupture of this molecule, this reducing agent was maintained for avoiding possible cell oxidation in the preparation procedure. GSH could disrupt DTSSP and subsequently reduce the crosslinking effect of this reagent on erythrocyte membrane. Anyhow, this effect is not visible when this crosslinker is used to high concentration (5 mM). DTSSP can be used to target erythrocytes to liver. At lower reagent concentrations of DTSSP, there is a partial targeting to liver. Also, at low DTSSP concentration, a modulation of erythrocytes targeting can be obtained as observed in Fig. 2. These crosslinking conditions may be highly useful to target erythrocytes mainly to liver and to regulate the extent of uptake of modified erythrocytes by liver.

Acknowledgements

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