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2,5-HEXANEDIONE MODIFIES SKELETAL PROTEINS OF THE RED BLOOD CELLS AND INCREASES THE BINDING OF HEMOGLOBIN TO THE MEMBRANE

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Abstract—The effects of 2,5-hexanedione (2,5 HD) on skeletal proteins of red blood cells (RBCs) were investigated both *in vitro* (human RBCs) and *in vivo* in male Sprague–Dawley rats which had been treated with the drug for several days. We found that 2,5 HD induced the following major changes in the electrophoretic pattern of the skeletal proteins: (i) the appearance of high-molecular weight bands, (ii) a dose-dependent decrease in spectrin Bands 1 and 2, and (iii) a dose-dependent increase in the amount of hemoglobin (Hb) associated with the membrane. Membranoskeletons, prepared from resealed ghosts which had been previously treated with 2,5 HD, were able to bind an increased amount of Hb from untreated RBCs, thus suggesting a drug-induced modification of the membrane. Extraction of spectrin and actin from ghosts did not remove the membrane-bound Hb and, furthermore, Hb bound to 2,5 HD-treated membranes mainly bearing Band 3 and free of peripheral proteins. These data suggested a 2,5 HD-induced modification of an intrinsic membrane protein, probably Band 3. This hypothesis was consistent with the observation that 2,5 HD also induced a modification of Band 3 aminogroups, as evidenced by a dose-dependent decrease in the binding of eosin probes. Furthermore, RBCs treated *in vitro* with 2,5 HD bound an increased amount of autologous immunoglobulins (IgG). As reported by Kay [10–13] and Low *et al.* [14] the binding of autologous IgG is a phenomenon associated with the aging process of RBCs and may involve a modification of Band 3. Our data show that RBCs treated with 2,5 HD acquired various characteristics of senescent cells such as spectrin cross-linking, Hb-membrane binding and increased IgG binding, and suggest that 2,5 HD treatment might affect RBC survival.

n-Hexane and methyl-butylketone (solvents largely used in industrial settings) are known to cause polyneuropathy in persons and in experimental animals repeatedly exposed through the respiratory route. The two solvents share a series of metabolites, one of which, 2,5-hexanedione (2,5 HD),‡ is considered the primary neurotoxic agent [1]. Evidence is accumulating in favour of the hypothesis that its mechanism of action involves a chemical modification of neurofilaments and/or of other axonal cytoskeletal proteins [2] through the formation of pyrrole groups on lysine side-chains [3, 4]. Cytoskeletal proteins are responsible for the stabilization of cell shape and for the regulation of cell motility and of the mobility of transmembrane proteins. Among the various types of cytoskeleton of mammalian cells, that of red blood cells (RBCs), known as membranoskeleton, has received particular attention in this last decade, and much is known about its constituent proteins and construction (for a review see Marchesi, Ref. 5). Furthermore, reactive amino groups of Band 3, the major skeleton-associated transmembrane protein of RBCs [6], can be selectively modified by some fluorescent probes [7–9] and tested as possible target sites for 2,5 HD.

The aim of this work was to study, *in vitro* and *in vivo*, the effects of 2,5 HD treatment on the skeletal proteins of RBCs. We found that drug treatment produced an increase in both the crosslinking of spectrin and the amount of hemoglobin (Hb) associated with the membranes. In addition, Band 3 was modified by 2,5 HD treatment as shown by the reduced binding of specific eosin probes and by the increased ability of *in vitro* treated cells to bind autologous antibodies. All the observed changes are characteristics of senescent RBCs [10–17] and, therefore, might be indicative of drug-induced cell aging. These findings should also be considered in relation to the general symptoms which accompany polyneuropathy in persons exposed to toxic concentrations of *n*-hexane or of methyl-butylketone.

MATERIALS AND METHODS

Chemicals. 2,5-hexanedione or acetonylacetone was purchased from Fluka (99% purity, Buchs, Switzerland) and eosin-isothiocyanate (EITC) from Sigma Chemicals Co. (St Louis, MO). Eosin–maleimide (EMI) was obtained from Molecular Probes, Inc. (Junction City, OR). [^{35}S]Na_2SO_4, [^{35}S]Protein A and [^{125}I] Protein G were purchased from Amersham International (Amersham, U.K.).

Animals. Male adult Sprague–Dawley rats (Charles River, Italy) weighing initially around 260 g were individually housed in plastic cages in the following conditions: temperature 21 ± 1°, relative

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‡ Abbreviations: 2,5 HD, 2,5-hexanedione; RBC, red blood cell; IOVs, inside-out vesicles; Hb, hemoglobin; PMSF, phenylmethyl-sulfonylfluoride; DFP, diisopropyl-fluorophosphate; EMI, eosin–maleimide; EITC, eosin-isothiocyanate; SDS, sodium dodecylsulfate.

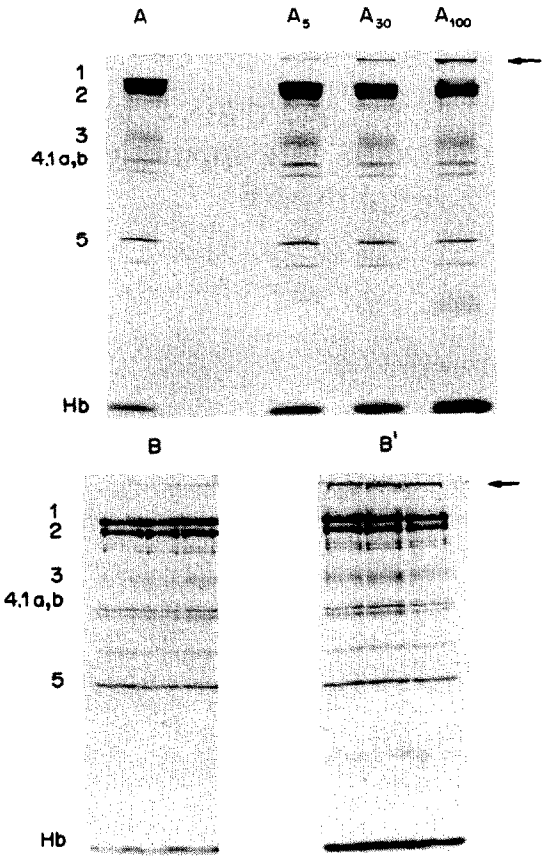

Fig. 1. Electrophoretic patterns of membranoskeletons prepared from human RBCs treated *in vitro* (A) and from RBCs of rats treated *in vivo* with 2,5 HD (B). Human RBCs were treated for 18 hr at 37° with: 0 mM (A); 5 mM (A_5); 30 mM (A_{30}); and 100 mM (A_{100}) 2,5 HD. RBC membranoskeletons from pair-fed control rats (B) and from rats treated with 2,5 HD 400 mg/kg per day for 12 days (B'). The arrows denote the position of the high molecular weight bands. Nomenclature of bands according to the electrophoretic mobility. Hb = hemoglobin. Proteins were visualized by Coomassie Blue staining. Preparation of membranoskeletons were performed as described in Materials and Methods.

humidity 95% and 12 hr light–dark cycle. They were randomly assigned to two groups; each animal in group one was matched to a pair-fed control in group two. The animals in group one were injected intraperitoneally daily with 400 mg/kg of redistilled 2,5 HD for 5 days/week for up to 3 weeks. Food and water were available *ad lib.* for treated rats and food intake was measured daily. Control rats in group two received the same amount of food as consumed by their partners the day before, while water was available *ad lib.* At the indicated days of treatment (with exclusion of the intervals), the rats were anaesthetized with ethyl ether and 1–1.5 ml of blood were drawn by cardiac puncture using EDTA as anticoagulant. Body weight was measured weekly.

Treatment of human RBCs with 2,5 HD. Freshly collected human RBCs were centrifuged at 2500 rpm for 5 min and washed three times with 5 mM sodium phosphate, 0.15 M NaCl, pH 8 buffer (PBS). Red blood cells were resuspended (1:25 v/v) in PBS containing the indicated amount of 2,5 HD, 15 mM glucose, and 100 U/ml streptomycin/penicillin to avoid bacterial growth. After 18 hr at 37°, RBCs were washed four times with PBS to remove unreacted 2,5 HD.

Preparation of ghosts, inside-out vesicles (IOVs) and membranoskeletons. Red blood cells were obtained by centrifugation of blood at 2500 rpm for 5 min and washed three times with PBS. Unsealed ghosts were prepared from RBCs by lysis and repeated washings at 0° with 10 vol. 5 mM sodium phosphate, pH 8 buffer (lysis buffer). Ghosts were resealed by incubation for 15 min at 37° with 10 vol. 130 mM KCl, 10 mM Tris, 20 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride (PMSF), 1 mM diisopropyl fluorophosphate (DFP), 2 mM ATP and 2 mM $MgCl_2$ pH 8 buffer. Spectrophotometric determination of bound Hb was performed after ghost solubilization with 0.1% sodium dodecyl sulfate (SDS). Inside-out vesicles were prepared from ghosts by extraction of spectrin Band 1 and 2 and actin at 37° for 30 min with 10 vol. 0.1 mM EDTA and 0.1 mM DFP pH 9.4.

Membranoskeletons were prepared from ghosts by extraction at 0° with 6 vol. 1% (v/v) Triton X-100 in 5 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 buffer. After centrifugation and removal of the supernatant, the Triton X-100 insoluble residues were washed at 0° with the lysis buffer containing 1 mM EDTA and 0.2 mM PMSF.

Rebinding of Hb to RBC membranes. Membranes (IOVs or membranoskeletons) were incubated with 10 vol. (10 times the original volume of ghosts) of RBC lysate for 1 hr at 37° and then washed three times with lysis buffer. Cell lysate was prepared from untreated RBCs by lysis at 0° with 10 vol. lysis buffer.

Gel electrophoresis. Membrane proteins were separated by SDS–polyacrylamide gel electrophoresis. About 0.1 mg of membrane proteins were boiled for 3 min with gel sample buffer (1:1 v/v), and the proteins were separated on 8–18% gradient slab gels using the discontinuous buffer system of Laemmli [18]. Gels were stained with Coomassie Blue R-250. Stained bands were quantified by densitometric analyses (2400 GELSCAN XL Laser Densitometer LKB, Broma, Sweden).

Preparation of EMI- and EITC-ghosts. Red blood cells labelling with eosin probes was performed according to Chiba *et al.* [8]. For EMI labelling, intact human or rat RBCs were washed with 20 mM histidine–HCl, 0.15 M NaCl pH 7.4 buffer and resuspended to 2.5% hematocrit in the same buffer containing 15 mM glucose, 0.01% NaN_3 and 1×10^{-5} M EMI. Eosin-isothiocyanate labelling was performed with 20 mM sodium carbonate, 0.15 M NaCl, 15 mM glucose, 0.01% NaN_3 and 1×10^{-5} M EITC pH 9 buffer. Both EMI and EITC labelling were allowed to proceed for 3 hr at 37° in the dark. Unreacted label was removed by three washes in 40–50 vol. PBS. Labelled RBCs were lysed at 0° in 10 vol. 5 mM phosphate 0.5 mM β-mercaptoethanol buffer pH 8. After ghost solubilization with 0.1% SDS, the amount of bound eosin was determined spectro-

Table 1. Densitometric analyses of membranoskeletons from human RBCs treated *in vitro* with 2,5 HD

2,5 HD (mM)	Areas (A.U. × mm)			Spectrin/Actin	Hb/Actin	Spectrin decrease (%)	Hb increase (%)
	Spectrin*	Actin	Hb				
0	1.84 ± 0.17	0.19 ± 0.001	0.80 ± 0.06	9.71 ± 0.90	4.20 ± 0.30	—	—
5	2.10 ± 0.30	0.19 ± 0.03	0.92 ± 0.06	10.68 ± 0.15	4.65 ± 0.35	—	11
10	2.07 ± 0.20	0.22 ± 0.01	1.03 ± 0.08	9.43 ± 0.50	4.70 ± 0.10	3	12
30	1.76 ± 0.20	0.21 ± 0.01	1.14 ± 0.10	8.40 ± 1.40	5.40 ± 0.20	14	29
60	1.06 ± 0.13	0.16 ± 0.02	1.28 ± 0.01	6.39 ± 0.01	7.90 ± 0.90	34	88
100	0.79 ± 0.08	0.22 ± 0.01	1.93 ± 0.17	3.59 ± 0.50	8.75 ± 0.55	63	108

Values are the means ± SD of three different experiments.
* Sum of band 1 and 2 areas.

photometrically (Perkin–Elmer MPF-44B Fluorescence Spectrophotometer, Norwalk, CT).

Sulfate-efflux. Intact RBCs were washed twice with 130 mM KCl, 10 mM Tris, 20 mM NaCl pH 8 buffer (iso–KCl) and resuspended in the same buffer to 10% hematocrit. After equilibration at 37° for 2 hr in the presence of NaN_3 (0.01%), [^{35}S] Na_2SO_4 was added (1 μCi/ml) to the cell suspension, which was then incubated for another 2 hr. In order to remove extracellular [^{35}S]Na_2SO_4 and to stop the transport, the cells were washed three times with ice-cold iso–KCl. Sulfate efflux was started by resuspending the cells at 5% hematocrit in the iso–KCl buffer at 37°. At the indicated time, the radioactivity of the deproteinized (2% trichloroacetic acid) cell supernatant was determined by liquid scintillation counting.

Circular dichroism. Circular dichroism measurements were made with a Jasco J-41 A spectropolarimeter (Tokyo, Japan) on EITC labelled ghosts; spectra were measured in thermostated quartz cells of 10 mm pathlength. Following the method of Sato *et al.* [7] the concentration of ghost proteins was adjusted to 0.15 mg/ml and that of EITC to 10^{-5} M.

Quantification of cell-bound antibodies. The amount of autoantibodies bound to intact RBCs was determined essentially as described by Low *et al.* [14]. Fresh human RBCs and serum from the same donor were kindly provided by the Department of Hematology, University of Rome "La Sapienza". Red blood cells were washed three times with 5 mM sodium phosphate, 0.15 M NaCl, 10 mM glucose and 2 mM adenosine pH 7.4 buffer (buffer A). These cells were suspended at 5% hematocrit in the serum and kept for 3 hr at room temperature; they were then washed five times with 40 vol. buffer A; the last wash contained 1 mg/ml bovine serum albumin (buffer B). Immunoglobulin-coated RBCs were incubated for 1 hr at room temperature at 4% hematocrit in buffer B which contained 0.4 μg/ml [^{35}S]protein A in the case of human RBCs and 0.4 μg/ml [^{125}I]protein G in the case of rat RBCs. Cells were washed six times with 40 vol. buffer B and then lysed at 0° in 10 vol. 5 mM phosphate buffer pH 8. After solubilization of ghosts with Soluene-350 (Packard, Frankfurt, F.R.G.), the amount of [^{35}S]protein A was determined by liquid scintillation counting (TRI-CARB 460C and 460CD liquid scintillation system, Packard Instrument Co. Inc. Dawners Grove, IL). The amount of [^{125}I]protein G bound to rat RBCs was determined on packed RBCs using a Gamma Counter (1282 Compugamma Universal, LKB, Broma, Sweden).

Statistical methods. Comparisons between groups (determination of the significance of the differences between means) were performed using a non-parametric method: Mann–Whitney's U-test. This is one of the most powerful non-parametric tests, and it is a useful alternative to the parametric *t*-test when the N-values are low [19].

RESULTS

Effects of 2,5 HD on RBC skeletal proteins and binding of Hb to 2,5 HD-treated membranoskeletons

Treatment of human RBCs *in vitro* with 100 mM 2,5 HD for 18 hr at 37° produced ghosts which, even after 4–5 washings, maintained about 2.5 times more Hb than control RBCs incubated for the same time without the drug. The Hb content of SDS-solubilized 2,5 HD-treated ghosts was about 6.5 ± 0.3% of total ghost proteins. This result may have been due either to increased Hb-membrane binding or to the resealing of ghosts and trapping of cytoplasmic proteins. To distinguish between these two possibilities, ghosts were extracted with Triton X-100, a detergent known to produce membranoskeletons devoid of cytoplasmic proteins. Figure 1 shows that treatment with 2,5 HD induced the following major changes in the electrophoretic pattern of human RBC membranoskeletons: (i) the appearance of high-molecular weight bands not reducible with β-mercaptoethanol, (ii) a decrease in the amount of spectrin Band 1 and 2, and (iii) an increase in the amount of Hb associated with the membranoskeleton. The appearance of high molecular weight complexes was concurrent with a decrease in spectrin Band 1 and 2, thus suggesting the presence of this protein in the crosslinked material. The amount of actin did not change after drug treatment and it was chosen as an "internal standard" for quantitative densitometric analyses. Densitometric analyses of gels showed that 2,5 HD induced a dose-dependent decrease in spectrin Band 1 and 2 and a dose-dependent increase in the Hb bound to the membranoskeletons. At the highest dose that was tested, spectrin decreased by about 63% and Hb increased by about 108% (Table 1).

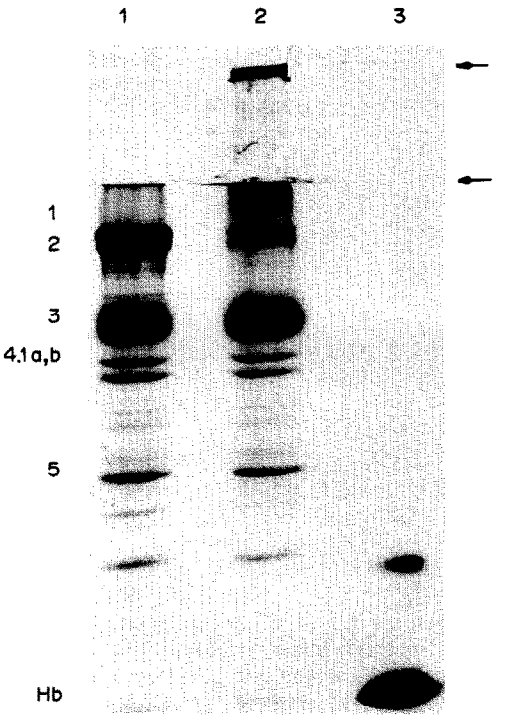

Fig. 2. Electrophoretic patterns of human RBC ghosts treated *in vitro* with 2,5 HD. Lane 1 control ghosts; lane 2 ghosts treated with 100 mM 2,5 HD for 18 hr at 37° in the presence of 1 mM EDTA, 1 mM PMSF, 1 mM DFP and 0.01% NaN_3; lane 3 Hb prepared by lysis of untreated RBCs. The arrows denote the position of the high molecular weight bands. Nomenclature of bands according to the electrophoretic mobility. Hb = hemoglobin. Proteins were visualized by Coomassie Blue staining.

Interestingly, RBCs of rats treated *in vivo* with 2,5 HD showed changes in the electrophoretic pattern of skeletal proteins comparable to those of human RBCs (Fig. 1). A statistically significant increase in the amount of Hb associated with the skeletal proteins was observed after 5 days of treatment, and, after 12 days, the amount of Hb associated with the skeletal proteins doubled (Table 2). The amount of spectrin began to decrease after 5 days of treatment, and a statistically significant decrease was observed after 12 days of treatment (Table 2). In this context it should be observed that the concentrations of 2,5 HD which proved effective on human RBCs *in vitro* are not too far from those which other authors have found in the blood of rats which had been treated daily with 400 mg/kg of 2,5 HD [20].

To investigate the role of cytoplasmic proteins in the process of spectrin crosslinking, we tested the effects of 2,5 HD on Hb-free resealed ghosts. As reported in Fig. 2, incubation of resealed ghosts with 100 mM 2,5 HD produced protein crosslinking and a decrease of spectrin Band 1 and 2 similar to those observed in intact RBCs, thus excluding a major role of cytoplasmic proteins in spectrin crosslinking.

The increased binding of Hb to RBC skeletal proteins is a phenomenon which has been observed in several other pathophysiological conditions [21–

Table 2. Densitometric analyses of membranoskeletons prepared from RBCs of rats treated *in vivo* with 2,5 HD as compared with pair-fed controls

Days*	Areas (A.U. × mm) (× ± SD) Spectrin†		Actin		Hb		Spectrin/Actin		Hb/Actin		Spectrin decrease (%)	Hb increase (%)
	C	T	C	T	C	T	C	T	C	T		
3	1.50 ± 0.30 (4)	1.54 ± 0.38 (4)	0.12 ± 0.02 (4)	0.13 ± 0.03 (4)	0.14 ± 0.03 (4)	0.10 ± 0.02 (4)	12.20 ± 1.5 n s	12.10 ± 1.77	1.18 ± 0.33 n s	0.81 ± 0.11	—	—
5	2.59 ± 0.35 (4)	2.50 ± 0.42 (4)	0.29 ± 0.01 (4)	0.27 ± 0.05 (4)	0.40 ± 0.06 (4)	0.60 ± 0.09 (4)	8.96 ± 1.3 n s	9.41 ± 1.75	1.37 ± 0.19 P < 0.05	2.20 ± 0.09	—	61
12	0.71 ± 0.16 (5)	0.54 ± 0.06 (4)	0.09 ± 0.01 (5)	0.11 ± 0.02 (4)	0.22 ± 0.05 (5)	0.57 ± 0.11 (4)	7.90 ± 2.3 P < 0.05	4.72 ± 0.94	2.40 ± 0.52 P < 0.01	5.30 ± 0.73	41	120

* Days of treatment.
† Sum of Band 1 and 2 areas.
Spectrin decrease and Hb increase were calculated from pair-fed controls.
C = pair-fed control group; T = treated group. Number of rats is shown in parentheses.
P values were calculated by Mann–Whitney's U-test; n s = not significant (see Materials and Methods).

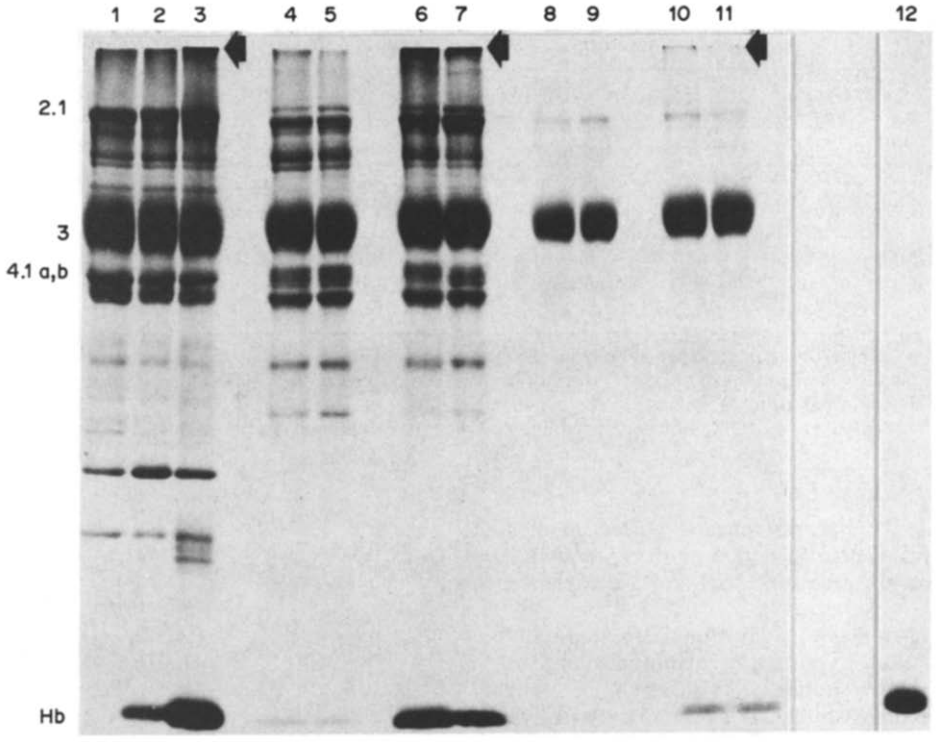

Fig. 3. Electrophoretic patterns of inside-out vesicles (IOVs) prepared from human and rat RBCs. Lane 1 untreated human IOVs; lane 2 IOVs from human RBCs incubated for 18 hr at 37° without 2,5 HD; lane 3 IOVs from human RBCs treated with 100 mM 2,5 HD for 18 hr at 37°; lanes 4 and 5 IOVs from RBCs of pair-fed control rats; lanes 6 and 7 IOVs from RBCs of rats treated with 2,5 HD 400 mg/kg/day for 12 days; lanes 8 and 9 IOVs from lanes 4 and 5 after extraction with 0.01 M NaOH; lanes 10 and 11 IOVs from lanes 6 and 7 after extraction with 0.01 M NaOH; lane 12 Hb prepared by lysis of untreated RBCs. The arrows denote the position of the high molecular weight bands. Nomenclature of bands according to the electrophoretic mobility. Hb = hemoglobin. Proteins were visualized by Coomassie Blue staining.

24] and shown to involve either spectrin-Hb cross-linking complexes [25] or the induction of new binding sites with the cytoplasmic domain of Band 3 [14, 17, 26].

In order to clarify which protein was involved in Hb-membrane binding, skeletal proteins were extracted from ghosts at low ionic strength. This treatment produces inside-out vesicles (IOVs) depleted of spectrin Band 1 and 2 and of actin. An increased amount of bound Hb was observed in the membranes of human RBCs treated *in vitro* with 100 mM 2,5 HD after extraction of peripheral proteins (Fig. 3). Densitometric analyses (Table 3) showed that the Hb/Band 3 ratio was about 5 times greater in 2,5 HD treated RBCs with than in control RBCs.

The same increase in Hb-membrane binding was observed in the RBCs of rats treated *in vivo* for 12 days with 2,5 HD. As shown in Fig. 3 and Table 3, the Hb/Band 3 ratio in the RBCs of treated rats was about 4 times greater than in pair-fed control rats. In addition, the RBC membranes of rats treated *in vivo* with 2,5 HD were subjected to massive extraction of peripheral proteins with 0.01 M NaOH at 0°. This treatment is known to produce "stripped" vesicles depleted of all peripheral proteins (also protein 2.1 and protein 4.1 are extracted), with Band 3 accounting for 70–80% of total membrane proteins. No detectable amount of bound Hb was observed in the vesicles of control rats, whereas the Hb bound to vesicles obtained from *in vivo* 2,5 HD-treated rats accounted for about 6 ± 2% of total proteins (Fig. 3). These data suggested that the increase in the amount of Hb bound to 2,5 HD-treated membranes may be due to the modification of an intrinsic membrane protein, probably Band 3. It should be noted that in membranoskeletons about 70% of Band 3 had been extracted using Triton X-100 and it is likely, therefore, that the amount of membrane-bound Hb was underestimated in Fig. 1 and Table 1.

To further study the membrane protein(s) modified by 2,5 HD, rebinding experiments were performed with Hb prepared by lysis of untreated RBCs. We observed that IOVs prepared from untreated RBCs bound a considerably high amount of Hb. After rebinding, Hb accounted for 7% of total proteins in both 2,5 HD-treated and untreated IOVs (data not shown). This result may be due to the unmasking of new binding sites on Band 3 that in native RBCs had been occupied by other proteins and/or to the clustering of Band 3 in the IOVs. The IOVs Band 3 cytoplasmic domain, in fact, was the major binding site for several proteins including protein 2.1, protein 4.1, protein 4.2, Hb and glycolytic

Table 3. Densitometric analyses of inside-out vesicles (IOVs) prepared from 2,5 HD-treated human RBCs and from RBCs of rats treated *in vivo* with 2,5 HD

Sample	2,5 HD (mM)	Areas (A.U. × mm) Band 3	Hb	Hb/Band 3	Hb increase (%)
IOVs*	0	3.84 ± 0.37	1.02 ± 0.14	0.26 ± 0.06	—
IOVs*	100	3.40 ± 0.24	5.43 ± 0.47	1.61 ± 0.11	520
IOVs C (5)†		2.10 ± 0.24	0.24 ± 0.07	0.11 ± 0.03	—
IOVs T (5)†		1.62 ± 0.39	0.84 ± 0.15	0.53 ± 0.09 $P < 0.01$	380

* Values are the means ± SD of four different experiments.

† C = pair-fed control group; T = group treated for 12 days with 2,5 HD; number of rats is shown in parentheses; values are the means ± SD.

P value was calculated by Mann–Whitney's U-test (see Materials and Methods).

enzymes (Ref. 27 and references reported therein). It is conceivable that in native erythrocytes only a few proteins are accessible to Band 3 cytoplasmic domain.

Rebinding studies with Hb from untreated RBCs were also performed with membranoskeletons, although these membranes are not the ideal model for Band 3 binding studies (this protein is extensively extracted with Triton X-100). Membranoskeletons prepared from ghosts of untreated RBCs bound only a small amount of Hb (less than 1% of total proteins) but membranoskeletons from ghosts treated with 2,5 HD showed about two times more bound Hb (data not shown).

2,5 *HD treatment of RBCs inhibits the binding of eosin derivatives to Band 3*

A great deal of information about the properties of Band 3, the anion transporter of RBCs, has been obtained using selective inhibitors of the anion transport which covalently bind its functional amino groups [28]. Eosin derivatives are very interesting inhibitors of the anion transporter because their inhibitory activity depends on substituent groups and involves multiple binding sites [7–9]. Eosin-maleimide (EMI) and eosin–isothiocyanate (EITC) specifically label Band 3 [7]. As was shown by electrophoretic analysis of EMI- and EITC-ghosts only a single fluorescent band in the area of Band 3 was visible (data not shown). Figure 4(a) shows that 2,5 HD pretreatment produced a dose-dependent decrease in the binding of both eosin derivatives, with a 50–60% inhibition at 100 mM 2,5 HD. At the highest concentration tested, about half of the eosin binding sites on Band 3 were no longer available. Sato *et al.* [7] showed that EITC binding to ghosts induced a circular dichroism (CD) spectrum with a positive peak at 520 nm which was due to the chiral conformation of the EITC produced by the binding with Band 3. We observed that the pretreatment with 2,5 HD did not change either the chirality sign or the wavelength of CD maxima of the EITC bound to ghosts. Moreover, in accordance with the fluorescence measurements, a dose-dependent decrease of the intensity of the 520 nm CD peak due to the decrease of EITC bound to ghosts was observed (Fig. 4c).

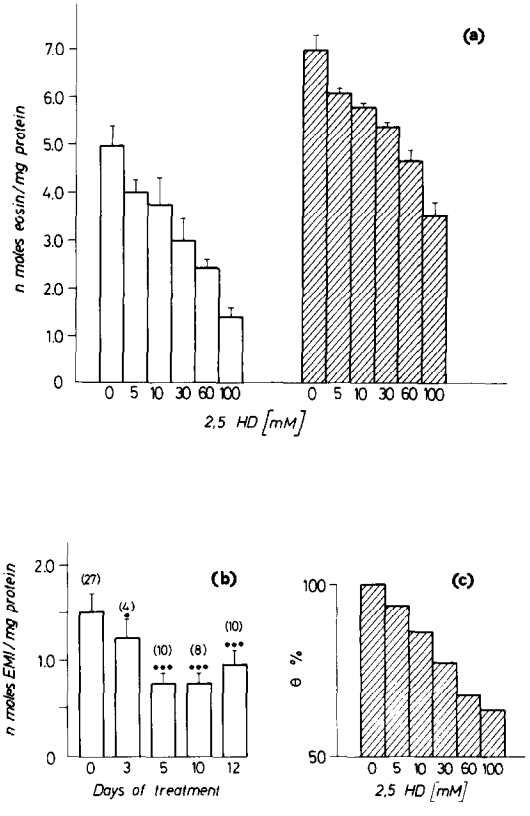

Fig. 4. Effect of 2,5 HD treatment, *in vitro* and *in vivo*, on the binding of eosin probes to Band 3. (a) Binding of EMI (white columns) and EITC (stippled columns) to human RBCs treated with the indicated concentrations of 2,5 HD. Values are the means ± SD of four experiments. (b) Binding of EMI to RBCs of rats treated *in vivo* with 400 mg/kg/day of 2,5 HD for the 3, 5, 10 and 12 days. The value relative to 0 days of treatment was obtained by collecting the values from pair-fed controls in each experiment. The values are means ± SD; the number of rats is reported in parentheses. Statistical significance: * $P < 0.05$, *** $P < 0.001$ (Mann–Whitney U-test [19]). (c) Decrease of 520 nm circular dichroism peak in EITC-labelled ghosts from human RBCs treated *in vitro* with the indicated concentrations of 2,5 HD. Ellipticity (θ) is expressed as percentages of control sample. All experiments are described in Materials and Methods.

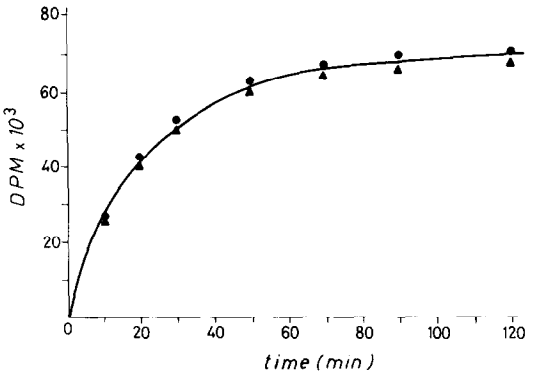

Fig. 5. Effects of 2,5 HD on the SO_4^{2-} efflux from rat RBCs. Sulfate efflux curve of RBCs from rats treated *in vivo* with 400 mg/kg/day of 2,5 HD for 10 days (▲) and from pair-fed control rats (●). Each point is the mean of duplicate determinations.

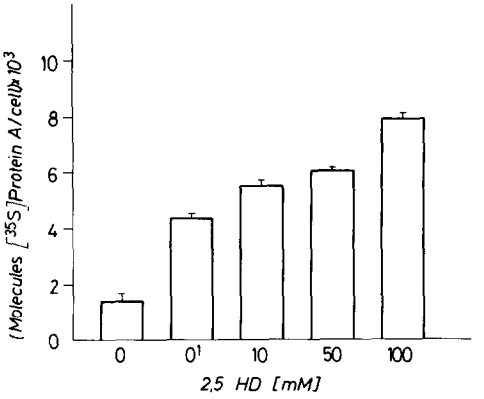

Fig. 6. Effects of 2,5 HD on the binding of autologous immunoglobulins (IgG) to human RBC membranes. The amount of IgG was measured by the binding of [^{35}S]protein A. The cells were immediately analyzed for IgG binding (0) or incubated for 18 hr at 37° without 2,5 HD (0¹) or treated with the indicated concentrations of 2,5 HD. Data represent the mean ± SD of triplicate determinations made in a single donor. Note that untreated RBCs (0) from different donors (N = 3) showed a great variability in the amount of IgG bound (i.e. 1.45×10^3, 3.7×10^3 and 4.7×10^4 molecules [^{35}S]protein A/cell). After 100 mM 2,5 HD treatment, however, bound IgG were 7.8×10^3, 1.9×10^4 and 2.5×10^5 molecules [^{35}S]protein A/cell respectively, with an increase in the IgG binding comparable to that shown in the figure.

The effects of 2,5 HD on eosin derivatives binding to RBCs were also studied on rats treated for 3, 5, 10 and 12 days. After 3 days of treatment a statistically significant decrease in the binding of EMI was observed (Fig. 4b). Maximum inhibition was observed after 5 days of treatment and did not change significantly thereafter.

To study the effects of 2,5 HD on the anion transport process, we compared the SO_4^{2-} efflux from the RBCs of rats treated for 5, 10 and 15 days with that from the RBCs of control rats. As shown in Fig. 5, the RBCs of rats treated with 2,5 HD for 10 days showed a sulphate efflux comparable to that of control cells. Similarly, *in vitro* 2,5 HD-modified human RBCs did not show changes in the SO_4^{2-} efflux (data not shown).

Effects of 2,5 HD treatment on the binding of autologous immunoglobulins to the RBC membrane

Recent data has clearly established that during the RBC aging process [10–14] and in chemically modified cells [29, 30] there is an increase in the binding of autologous immunoglobulins (IgG class). As shown in Fig. 6, *in vitro* treatment with increasing concentrations of 2,5 HD produced a dose-dependent increase in the binding of [^{35}S]protein A to human RBCs. At the highest dose tested, 2,5 HD treatment doubled the amount of membrane-bound autologous antibodies.

The amount of autologous IgG bound on the RBCs membranes of rats injected with 2,5 HD was also evaluated with [^{125}I]protein G. At variance with *in vitro* studies, we found that the amount of [^{125}I]protein G bound to RBCs of pair-fed control rats and that of rats treated for 3, 5, 11, 13 and 15 days was similar ($7.33 \pm 1.47 \times 10^4$ molecules of [^{125}I]protein G/cell).

DISCUSSION

Data reported in this paper show that 2,5 HD treatment of human RBCs produces several changes in the skeletal proteins. It is worth noting that similar changes also occurred in the RBCs of rats treated *in vivo* with 2,5 HD. Both spectrin and Band 3, the two major proteins of the RBCs' membrane, were affected by the drug and, furthermore, a dose-dependent increase in the amount of Hb bound to the membrane was observed.

The effect of the drug on spectrin was to promote the formation of crosslinked complexes as indicated by the dose-dependent reduction of spectrin Band 1 and 2 and the appearance of high molecular weight bands. Protein crosslinking was also observed on Hb-free resealed ghosts, a fact which rules out any major contribution of cytoplasmic proteins to the crosslinked material. The increased binding of Hb seems to be a 2,5 HD-induced modification of a membrane protein rather than a modification of Hb itself. In fact, rebinding studies with 2,5 HD-treated membranoskeletons showed that these membranes were able to bind a greater amount of Hb prepared by lysis of untreated RBCs.

It has been suggested that the binding of Hb to the RBCs' membranes involves the cytoplasmic domain of the transmembrane protein Band 3 [26] or crosslinked spectrin [25]. Hemoglobin binding to Band 3 is low under physiological conditions [31] and increases substantially in aged RBCs [14]. We found that membranes from RBCs treated both *in vitro* and *in vivo* with 2,5 HD, after selective extraction of peripheral proteins, still maintained an increased amount of Hb. Moreover, rat RBCs treated *in vivo* with 2,5 HD showed bound Hb also in vesicles stripped of all peripheral proteins and these vesicles showed mainly the Band 3 protein. These results suggest that Hb binding might be due to a drug-induced change in a membrane intrinsic protein,

perhaps precisely Band 3. Though direct proof that 2,5 HD can modify Band 3 in such a way as to increase its binding to Hb is still missing, we showed, using selective eosin tools, that the drug does, in fact, modify the amino groups of Band 3. It is interesting to note that decreased eosin binding to human and rat RBCs was the most sensitive change due to 2,5 HD treatment which we observed.

The inhibition of EITC binding to RBCs after *in vivo* and *in vitro* treatment with 2,5 HD suggests that the drug might react with those functional amino groups of Band 3 involved in the binding of the protein with eosin derivatives. Studies reported by Aubert *et al.* [32] demonstrated that EITC binding to Band 3 is based on a three-point attachment model. According to the model, the isothiocyano groups combine covalently with lysine amino groups in the hydrophobic region [33], whereas the carboxyl anion at the eosin phenyl ring and the oxyo anion at the xanthene skeleton bind non-covalently to histidine and arginine residues of Band 3 [8, 9]. Although —SH groups rather than —NH_2 groups are involved in the covalent coupling of EMI with Band 3, we found that this coupling also decreased after 2,5 HD treatment. As shown by competition experiments by Chiba *et al.* [9], these results can be reconciled by assuming that —SH groups and —NH_2 groups are located close to each other. The lysine residue of Band 3 which binds covalently with EITC does not play an essential role in the anion transport, since sulfate efflux can be observed in EITC-modified cells [9]. We found that the anion efflux of *in vivo* 2,5 HD-modified rat RBCs was similar to that of untreated cells. In conclusion, our data support the hypothesis of Graham *et al.* [3] that 2,5 HD can react with the lysine-charged amino groups of skeletal proteins. We suggest that the lysine residues involved in the binding of EITC with Band 3 are modified by the drug.

A second possible function, in addition to the anion transport activity, is probably associated with Band 3. This protein, in fact, appears to change during the RBC life span in such a way as to expose neoantigenic sites on the cell surface which are recognized by autologous IgG [10–14]. Not only senescent RBCs, but also chemically modified cells have been shown to bind increased amounts of IgG [30]. The appearance of a senescent cell antigen is one of the signals that may initiate RBCs removal by cells of the reticuloendothelial system [10]. However, other hypothesis on RBCs aging and removal have been advanced [22, 29, 30] and the mechanism at the molecular level remains to be clarified.

We found that human RBCs treated *in vitro* with 2,5 HD bind an increased amount of IgG in a dose-dependent way. Rats treated with the drug *in vivo*, however, did not show any increase in the binding of autologous IgG to the RBC membrane. It is conceivable that *in vivo* the reticuloendothelial system may have removed immunoglobulin-loaded RBCs from circulation. This is still only a hypothesis, however, and the possible role of autologous IgG in cell removal needs additional experimental investigation.

However, RBCs treated with 2,5 HD acquired other characteristics of senescent cells as showed by the presence of crosslinked spectrin and an increased amount of Hb bound to the membrane. Both these "signals" are considered a relatively "late" event in the process of RBC aging [15, 17, 22] and might be indicative of a drug-induced cell aging.

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