Low Membrane Protein Sulfhydrils but not G6PD Deficiency Predict Ribavirin-Induced Hemolysis in Hepatitis C

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Hemolysis is a frequent adverse effect of ribavirin (RBV). It has been suggested that oxidative stress plays a role, but mechanisms and predictive risk factors for severe forms remain unknown. Markers of redox status were determined in erythrocytes of 34 patients with hepatitis C—four of them with glucose-6-phosphate-dehydrogenase (G6PD) deficiency—before and during treatment with RBV and interferon (IFN) and were compared with 10 healthy control subjects. In addition, erythrocytes were incubated with RBV, and the effects of dipyridamole (DPD), diethylmaleate (DEM), and glutathione ester (GSHE) were studied *in vitro*. Of the 30 patients without G6PD deficiency who were treated with RBV and IFN-α, five developed major hemolysis (Δ hemoglobin > 6 g/dL) and 25 developed minor hemolysis (Δ hemoglobin < 2.5 g/dL). Patients with major hemolysis had lower median pretreatment values of membrane protein sulfhydrils than patients with minor hemolysis (28.4 vs. 36.7 nmol/mg, $P < .001$). Erythrocytes of G6PD-deficient patients were not more susceptible to RBV-induced hemolysis. In *in vitro* incubations of erythrocytes, DEM enhanced the RBV-induced decrease of glutathione, protein sulfhydrils, and osmotic resistance. Supplementation of GSHE and DPD prevented the RBV-induced decrease in osmotic resistance, adenosyl triphosphate (ATP), and 2,3-diphosphoglycerate (DPG), the loss of glutathione and protein sulfhydrils, and the formation of thiobarbituric acid reactive substances (TBARs). In conclusion, the data indicate that low membrane protein sulfhydrils prior to therapy but not G6PD deficiency are predictive of RBV-induced major hemolysis. *In vitro*, GSHE and DPD reduce the RBV-associated oxidative stress in erythrocytes and prevent the increase in osmotic fragility, suggesting that these compounds might decrease the risk of hemolysis in patients. (HEPATOLOGY 2004;39:1248–1255.)

Ribavirin (RBV) is a synthetic nucleoside analogue used in the treatment of chronic hepatitis C in combination with interferon (IFN)-α. The addition of RBV has increased the probability of sustained response to approximately 40% if combined with conventional IFN[1,2] and to over 50% if combined with pegylated IFN.[3] RBV actively enters cells by way of the es-nucleoside membrane transporter[4] and subsequently undergoes phosphorylation at the expense of adenosyl triphosphate (ATP).[5,6] In contrast to nucleated cells such as hepatocytes, phosphorylation is irreversible in the anucleate erythrocytes, and phosphorylated RBV accumulates because it cannot be exported by the nucleoside carrier. Therefore, the concentration of RBV in erythrocytes is 50- to 70-fold higher than the therapeutic plasma concentration of 9–15 μM.[5,7,8] The high concentration of RBV in erythrocytes is probably responsible for the hemolysis occurring during RBV therapy. A decrease in hemoglobin (HB) is frequently observed in patients who receive RBV,

Abbreviations: RBV, ribavirin; IFN, interferon; ATP, adenosyl triphosphate; HB, hemoglobin; G6PD, glucose-6-phosphate-dehydrogenase; GSH, total glutathione; DPD, dipyridamole; GSSG, glutathione disulfide; PSHs, protein sulfhydrils; TBARs, thiobarbituric acid reactive substances; DPG, 2,3-diphosphoglycerate; DEM, diethylmaleate; GSHE, glutathione ester.

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and in one clinical trial this decrease required dose reduction or even treatment discontinuation in 7% of patients.[1] Some risk factors for the development of RBV-induced hemolysis have been identified, such as low pretreatment platelet count and haptoglobin phenotype or, after liver transplantation, decreased creatinine clearance.[9,10] Yet severe RBV-induced hemolysis remains unpredictable in clinical practice, and the underlying mechanisms are poorly understood.

Recent data obtained in erythrocytes of patients treated with RBV who experienced no or little hemolysis suggest that erythrocytes might be damaged by RBV-associated oxidative stress and that this may result in an increased consumption of glutathione with a compensatory activation of the hexose monophosphate shunt.[11] If this hypothesis is correct, glucose-6-phosphate-dehydrogenase (G6PD)-deficient erythrocytes—which have an impaired hexose monophosphate shunt with a reduced capacity to compensate for an increase in total glutathione (GSH) oxidation—should be particularly susceptible to RBV-induced hemolysis. Yet a considerable number of patients with (often undiagnosed) G6PD deficiency must have been treated with RBV—particularly in the Mediterranean, which has a high prevalence of both hepatitis C and G6PD deficiency—but no published reports of RBV-induced severe hemolysis associated with G6PD deficiency are available.

It is not known whether certain characteristics of erythrocytes (*e.g.*, G6PD deficiency) predispose them to RBV-associated hemolysis and whether or not such predisposing features, if identified, could be modified to prevent hemolysis. In this study, we prospectively determined the metabolic and redox status of erythrocytes in consecutive patients with hepatitis C with and without major RBV-associated hemolysis and in patients with hepatitis C and G6PD deficiency. In addition, the effects of depletion and supplementation of glutathione and of dipyridamole (DPD), an inhibitor of the nucleoside transporter,[12] on RBV-induced changes in erythrocytes were studied *in vitro*.

Experimental Procedures

Subjects and Study Design

The demographic data of the studied subjects are summarized in Table 1. No differences regarding transaminases or Knodell index of liver histology were observed between those who later developed major hemolysis and those who developed minor hemolysis. The study protocol conformed to the declaration of Helsinki, and all subjects were carefully informed about the study protocol and possible risks before they gave their written informed consent to participate.

Table 1. Demographic Data: Characteristics of Patients With Chronic Hepatitis C and Healthy Control Subjects With and Without G6PD Deficiency

	Age (Range)	Sex (Female/Male)
Patients without G6PD deficiency		
Patients with minor hemolysis (n = 25)	25–47	12/13
Matched patients with minor hemolysis (n = 5)	31–45	3/2
Patients with major hemolysis (n = 5)	26–49	3/2
Healthy controls (n = 10)	28–54	8/2
Patients with G6PD deficiency		
Patients with hepatitis C (n = 4)	38–55	2/2
Patients without hepatitis C (n = 3)	28–37	2/1

In Vivo *and* In Vitro *Studies in Patients Without G6PD Deficiency.* Thirty consecutive patients with chronic noncirrhotic hepatitis C (genotype 1b) eligible for treatment with pegylated IFN-α2b and RBV were followed in the Department of Internal Medicine of the Bari University Hospital. Patients with chronic or hemolytic anemia, G6PD deficiency, or HB abnormalities were excluded based on pretreatment HB levels, G6PD enzyme activity, and HB electrophoresis. Red blood cell indices (mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration) and the redox status of erythrocytes were determined in each patient prior to therapy as outlined below.

All 30 patients underwent treatment with RBV at 12–15 mg/kg/d (Rebetol, Schering-Plough, Milano, Italy) and IFN at 1.5 μg/kg/wk (Pegintron, Schering-Plough) for 12 months, and were prospectively followed during this time with monthly clinical visits and routine blood tests (transaminases, blood cell count, HB and red blood cell indices). Five patients showed a decrease of HB of more than 6 g/dL between the third and fifth month of treatment, which required a dose reduction of RBV. The redox status of erythrocytes of these five patients was reassessed during treatment with RBV and compared with five age- and sex-matched patients who had shown a minor decrease of HB ($<$2 g/dL) after at least 120 days of treatment. The redox status was also determined in 10 healthy control subjects.

To assess the redox status of erythrocytes prior and during treatment, a venous blood sample was drawn at 8.00 A.M. into a heparinized vial from a cubital vein after fasting overnight and was immediately centrifuged at 4,000*g* for 5 minutes to separate erythrocytes from plasma and cells. In the packed erythrocytes, GSH, glutathione disulfide (GSSG), "ghost" membrane protein sulfhydrils (PSHs), thiobarbituric acid reactive substances (TBARs), ATP, 2,3-diphosphoglycerate (DPG), and osmotic resistance were determined.

To investigate further the mechanisms of RBV-induced toxicity in erythrocytes, and to explore potentially protective interventions against RBV toxicity, we performed *in vitro* incubations of erythrocytes. A blood sample was obtained from three of the five patients who had experienced major hemolysis after termination of RBV treatment and complete recovery of HB. Erythrocytes from these patients and three healthy volunteers were incubated for 18 hours in round-bottom glass vials placed in a heated (37°C) shaking water bath. One milliliter of packed erythrocytes was incubated with 2 mL of plasma and 7 mL of the following buffer (pH 7.4): 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, supplemented with 10 mM glucose, containing (1) RBV (1 mM, final concentration); (2) RBV (1 mM) and diethylmaleate (DEM, 0.2 mM); (3) RBV (1 mM) and glutathione ester (GSHE, 3 mM); and (4) RBV (1 mM) and DPD (1 mM). The erythrocytes were then analyzed directly after incubation as described below, and the mean values of triple incubations performed for each subject were used for further analysis.

Studies in Patients With G6PD Deficiency. Four patients with G6PD deficiency and hepatitis C with an indication for antiviral treatment and three healthy controls with G6PD deficiency but without hepatitis C were studied. G6PD deficiency was confirmed by determining the enzyme's activity using a routine method in the hospital's central laboratory. The activity of G6PD ranged from 0.28–1.58 IU/g HB (normal: 3.85–5.45 IU/g HB). In three of these patients and their controls, a blood sample was obtained after an overnight fast for determination of the same erythrocyte parameters as in patients without G6PD deficiency. The patients with hepatitis C then underwent treatment with RBV and pegylated IFN-α2b and were closely followed with clinical visits and laboratory determinations during the whole treatment period, with parameters of redox status redetermined 6 months after the start of antiviral treatment.

Analytical Methods

Determination of GSH and GSSG. One hundred microliters of packed erythrocytes were lysed by addition of 900 μL of ice-cold bidistilled water. Proteins and HB were precipitated with 4% (wt/vol) sulfosalicylic acid and centrifuged at 12,000*g* for 3 minutes. The supernatant was analyzed for total GSH by using the glutathione-disulfide reductase 5,5-dithiobis-nitrobenzoic acid recycling procedure.[13] For the determination of GSSG, lysed erythrocytes were treated with 25% (wt/vol) sulfosalicylic acid and centrifuged as described above. The supernatant was incubated for 1 hour with 2-vinylpyridine and triethanolamine before measuring the absorbance at 412 nm by using the GSSG reductase recycling procedure.[14]

Preparation of Erythrocyte Ghosts and Determination of PSHs. To determine PSHs, 1 mL of packed erythrocytes was lysed in 40 volumes of 5 mM sodium-phosphate buffer (pH 8.0) under vigorous agitation and centrifuged at 50,000*g* for 30 minutes at 4°C. The pellet (membrane) was washed twice with 40 mL of the same buffer and centrifuged again.[15] The final pellet was then resuspended in 500 μL of 0.1 M phosphate buffer containing 5 mM ethylenediaminetetraacetic acid. The membrane proteins in the suspension were then precipitated with 4% sulfosalicylic acid and centrifuged at 10,000*g* for 5 minutes. The resulting pellet was washed twice with 2% sulfosalicylic acid and finally resuspended in 800 μL of 6 M guanidine. Absorbance was measured at 412 and 530 nm before and 30 minutes after incubation with 50 μL of 10 mM 5,5-dithiobis-nitrobenzoic acid.[16]

TBARs. TBARs are a marker of lipid peroxidation. For their assessment, 100 μL of packed erythrocytes were hemolysed by shaking with 900 μL of ice-cold bidistilled water. Proteins were precipitated with 50% trichloroacetic acid, and the resulting supernatant was incubated 1:1 with 0.67% thiobarbituric acid in water at 100°C for 45 minutes. After cooling, the chromophore was extracted with 2 mL of N-butanol and the absorption was measured at 532 nm.[17]

Measurement of DPG and ATP. DPG—which affects erythrocyte capacity to release oxygen in tissue and generally increases when HB decreases—and ATP in erythrocytes were measured spectrophotometrically.[18,19]

Determination of Osmotic Resistance. One hundred milliliters of packed erythrocytes were carefully added to 13 tubes containing 1 mL each of 0.85%–0% saline in distilled water. After gentle mixing, the tubes were kept at room temperature for 20 minutes and then centrifuged at 3,000*g* for 5 minutes. The absorbance of the supernatant was measured at 540 nm. Total hemolysis was assumed to occur in tubes containing only distilled water, and hemolysis was then calculated using the formula $a = (b*100)/c$, where b is the absorbance of each sample and c the absorbance of totally hemolyzed samples.

Statistical Methods

We used the Mann-Whitney test for nonpaired comparisons between groups of subjects, the Wilcoxon signed-rank test for paired comparison before and during treatment, and the Kruskal-Wallis test (the nonparametric equivalent of ANOVA) for comparisons between incubation conditions. All analyses were performed in STATA, version 8.1 for MacOS X (STATA Corporation,

Table 2. Hematologic Values and Indices of Oxidative Stress in Erythrocytes From Healthy Controls and Patients With Hepatitis C

	Healthy Controls (n = 10)	Patients With Hepatitis C (n = 30)	*P* Value
HB (g/dL)	15.0 (14.6–15.4)	14.8 (14.2–15.9)	.17
Mean corpuscular volume (fl)	92 (87–95)	92 (84–97)	.95
Mean corpuscular hemoglobin (pg)	30.5 (28.6–32.1)	29.8 (26.9–32.3)	.42
Mean corpuscular hemoglobin concentration (g/dL)	35.2 (32.8–36.1)	35.6 (31.9–37.5)	.28
GSH (μmol/g HB)	6.0 (5.2–7.4)	6.1 (5.2–7.5)	.98
GSSG (nmol/g HB)	190 (140–235)	158 (117–216)	.01
PSH (nmol/mg protein)	41.2 (38.0–46.5)	36.1 (25.6–39.3)	<.01
TBARs (nmol/g HB)	23.0 (19.7–26.1)	25.9 (19.3–33.1)	.02
ATP (μmol/g HB)	3.2 (2.7–3.5)*	3.0 (1.3–4.1)	.81
DPG (μmol/g HB)	17.3 (14.6–18.6)*	18.0 (14.1–20)	.30
Hemolysis (% in 0.5% NaCl)	6.1 (5.1–6.8)	6.8 (5.1–9.4)	.02

NOTE: Ranges are given in parentheses.
*n = 5.

College Station, TX). No adjustments were made for multiple comparisons. The reported values are medians and ranges; all *P* values are two-sided.

Results

Factors Predisposing to RBV-Associated Hemolysis and Effects of RBV on Erythrocytes In Vivo

Prior to treatment, erythrocytes of the 30 patients with hepatitis C differed from healthy control subjects in terms of a lower concentration of membrane PSHs and GSSG and higher TBARs and osmotic hemolysis (Table 2).

During treatment with RBV and IFN, severe anemia with HB levels decreasing to values between 7.2 and 7.9 g/dL occurred in five of the 30 patients with hepatitis C. The five patients, who subsequently developed major hemolysis during treatment, had significantly lower concentrations of PSH before treatment than the 25 patients who experienced minor hemolysis during treatment (28.4 [range: 25.6–30.1] vs. 36.7 [range: 32.8–39.3] nmol/mg protein, respectively, $P < .01$) (Fig. 1). Other parameters before treatment did not significantly differ between erythrocytes of patients who later experienced hemolysis and those who did not (Table 3). The changes in the measured variables during treatment in the five patients with major hemolysis and their matched controls with minor hemolysis are presented in Table 3 and Fig. 2, respectively. DPG, TBARs, and osmotic hemolysis showed a more pronounced increase, and GSH showed a more pronounced decrease in patients with major hemolysis. The concentrations of PSHs and ATP showed similar moderate decreases in both groups, and no marked differences were observed for GSSG (data not shown). During treatment with RBV, red blood cell indices of patients with hepatitis C showed a decrease of the mean corpuscular hemoglobin concentration (35.5 [range: 32.8–36.4] vs. 31.6 [range: 29.1–35.9] g/dL, $P = .01$); a trend toward an increase of the mean corpuscular volume (91 [range: 86–97] vs. 95 [range: 89–104] fl, $P = .06$); and no change in the mean corpuscular hemoglobin (30.0 [range: 28.6–32.1] vs. 29.5 [range: 28.2–32.1] pg, $P = .80$). In addition, during treatment there were no significant differences of red blood cell indices between patients who had minor and major hemolysis.

All changes observed in erythrocytes isolated from patients during RBV treatment had returned to baseline 6 months after cessation of therapy (data not shown). All patients had a biochemical response with normalization of transaminases. Two of the five patients with major hemolysis and 17 of the 25 patients with minor hemolysis had a virological response (*i.e.*, plasma samples were negative for hepatitis C virus RNA at the end of treatment).

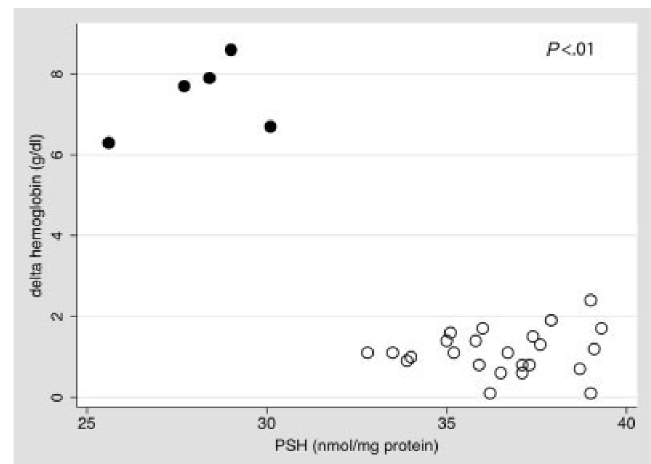

Fig. 1. Protein sulfhydrils before treatment and decrease in hemoglobin during treatment in five patients with hepatitis C who subsequently developed major hemolysis (**filled circles**) and 25 patients who developed minor hemolysis (**open circles**) during treatment. Abbreviation: PSH, protein sulfhydril.

Table 3. Hemolysis and Indices of Oxidative Stress in Erythrocytes Before and During Treatment

	Before Treatment With RBV			During Treatment With RBV		
	Hepatitis C Patients Who Later Had Minor Hemolysis During Treatment (n = 25)	Hepatitis C Patients Who Later Had Major Hemolysis During Treatment (n = 5)	*P* Value	Hepatitis C Patients With Minor Hemolysis Matched to Those With Major Hemolysis (n = 5)	Hepatitis C Patients With Major Hemolysis (n = 5)	*P* Value
HB (g/dL)	14.7 (14.2–15.9)	15.2 (14.2–15.8)	.67	14.4 (13.2–14.8)	7.5 (7.2–7.9)	–
GSH (μmol/g HB)	6.2 (5.3–7.5)	5.7 (5.2–6.1)	.07	6.7 (6.1–7.6)	3.8 (3.5–4.4)	<.01
GSSG (nmol/g HB)	158 (117–202)	143 (129–216)	.96	140 (120–150)	120 (110–140)	.09
PSH (nmol/mg protein)	36.7 (32.8–39.3)	28.4 (25.6–30.1)	<.001	33.1 (31.8–36.2)	26.9 (24.4–28.1)	<.01
TBARs (nmol/g HB)	26.7 (19.3–33.1)	24.6 (22.9–26.3)	.25	37.7 (33.3–40.1)	46.7 (42.2–48.9)	<.01
ATP (μmol/g HB)	2.9 (1.3–3.9)	3.7 (2.8–4.1)	.09	2.4 (2.3–3.3)	3.1 (2.6–3.5)	.05
DPG (μmol/g HB)	17.9 (15.6–20.0)	18.5 (14.1–19.3)	.70	15.9 (14.6–17.4)	21.6 (17.1–23.6)	.02
Hemolysis (% in 0.5% NaCl)	6.8 (5.1–9.4)	7.0 (5.1–8.9)	.87	7.5 (6.8–8.1)	12.1 (10.9–13.5)	<.01

NOTE: Parameters determined in whole blood (HB) and erythrocytes (all other parameters) of patients with chronic hepatitis C and healthy controls before and during treatment with IFN and RBV (*P* values are from the Mann-Whitney test). For logistic reasons determinations during RBV treatment were done in 5 out of the 25 patients with minor hemolysis that were matched for age and sex to those with major hemolysis. See Fig. 3 for within-group comparisons before and during treatment. Ranges are given in parentheses.

Data from patients with G6PD deficiency are presented in Table 4. All four patients who had G6PD deficiency tolerated the treatment with RBV and IFN without a major drop of HB. Erythrocytes were investigated in three of the four patients, and no marked differences were found regarding HB, GSH, GSSG, TBARs, PSH, ATP, DPG, or osmotic resistance compared with G6PD-deficient subjects who did not have hepatitis C.

In Vitro *Incubations of Erythrocytes*

Results of the *in vitro* studies are presented in Fig. 3. After 18 hours of incubation without RBV, erythrocytes from patients who had recovered from RBV-induced major hemolysis had lower membrane PSH content and GSH and higher concentrations of GSSG and DPG than erythrocytes isolated from healthy subjects. Addition of RBV to the incubation medium was associated with a similar decrease in GSH, membrane PSHs, ATP, DPG,

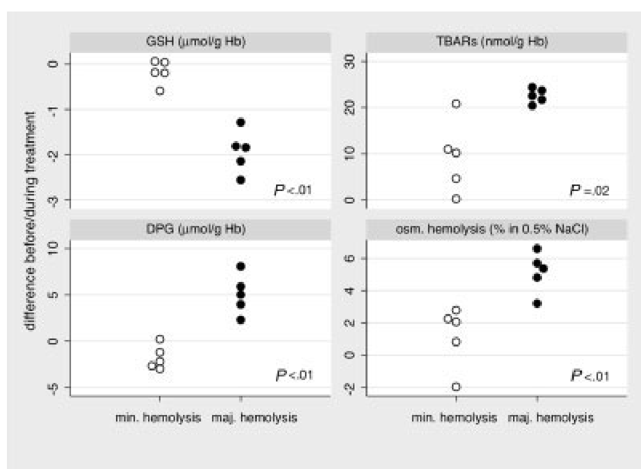

Fig. 2. Changes of markers of oxidative stress in erythrocytes in five patients showing major hemolysis (**filled circles**) and five matched control patients showing only minor hemolysis (**open circles**) during treatment with RBV and IFN. Symbols represent the difference between concentrations before and during treatment for individual subjects for each group. *P* values for differences between these groups are from the Mann-Whitney test. GSH, total glutathione; Hb, hemoglobin; TBARs, thiobarbituric acid reactive substances; DPG, 2,3-diphosphoglycerate; min., minor; maj., major.

Table 4. Hemolysis and Indices of Oxidative Stress in G6PD-Deficient Erythrocytes Before and During Treatment

	Subjects With G6PD Deficiency	Subjects With G6PD Deficiency and Hepatitis C	
		Before RBV Treatment	During RBV Treatment
HB (g/dL)	14.3	14.5	12.3
	14.8	15.1	11.7
	15.2	14.2	12.8
		15.2	12.7
GSH (μmol/g HB)	5.9	6.1	5.4
	6.2	6.0	5.7
	6.0	5.9	5.7
GSSG (nmol/g HB)	189	171	189
	172	150	201
	198	184	181
PSH (nmol/mg protein)	38.6	35.4	31.7
	41.4	32.3	25.2
	30.1	33.1	30.1
TBARs (nmol/g HB)	26.3	29.9	39.8
	29.1	26.6	45.4
	25.4	27.7	40.9
ATP (μmol/g HB)	2.7	2.9	2.3
	2.4	2.5	2.2
	2.6	2.9	2.4
DPG (μmol/g HB)	16.7	17.7	15.9
	15.6	18.4	16.4
	17.8	16.9	16.3
Hemolysis (% in 0.5% NaCl)	7.8	6.8	8.9
	6.4	7.4	10.4
	7.2	6.4	8.1

NOTE: For those with additional hepatitis C, values were determined before and during treatment with IFN and RBV.

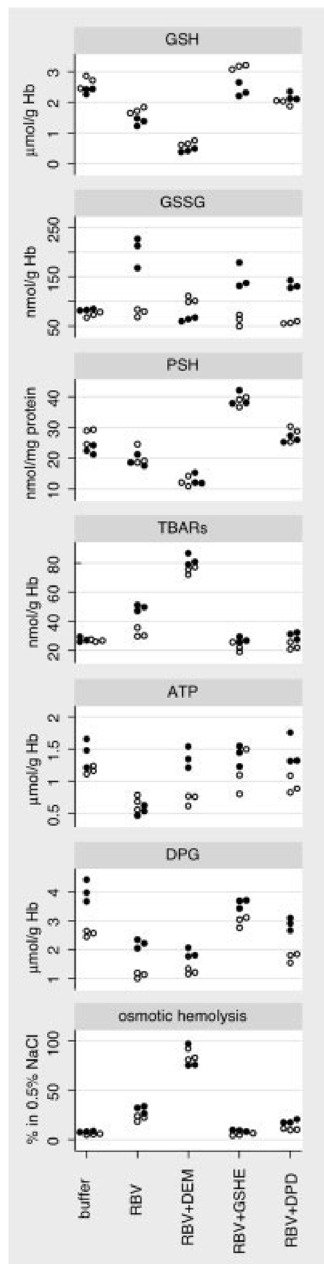

Fig. 3. Markers of oxidative stress in erythrocytes incubated *in vitro* from controls (**open circles**) and patients after recovery who had previously shown major hemolysis during treatment (**filled circles**). Incubations were performed with buffer, RBV, RBV and DEM, RBV and GSHE, and RBV and DPD. *P* values for overall differences between the five different incubation conditions (Kruskal-Wallis test) were reported separately for control erythrocytes (c) and erythrocytes of patients who had experienced major hemolysis under treatment with RBV (mh). GSH: c 0.01, mh 0.02. GSSG: c 0.02, mh 0.01. PSH: c 0.01, mh 0.01. TBARs: c 0.01, mh 0.02. ATP: c 0.02, mh 0.14. DPG: c 0.01, mh 0.01. Osmotic hemolysis: c 0.01, mh 0.01. Hb, hemoglobin; GSH, total glutathione; GSSG, glutathione disulfide; PSH, protein sulfhydril; TBARs, thiobarbituric acid reactive substances; ATP, adenosyl triphosphate; DPG, 2,3-diphosphoglycerate; RBV, ribavirin; DEM, diethylmaleate; GSHE, glutathione ester; DPD, dipyridamole.

and osmotic resistance in patient and control erythrocytes. Although the GSH loss was accompanied by a negligible increase of GSSG in control erythrocytes, a marked 2.6-fold increase of GSSG was seen in erythrocytes of patients who had experienced major hemolysis in which the oxidized/total glutathione ratio reached a median value of 0.15. Furthermore, the concentration of TBARs increased more in the erythrocytes of patients (1.8-fold vs. 1.2-fold in patients and healthy subjects, respectively). Addition of DEM together with RBV to the incubation medium further decreased the concentration of GSH, membrane PSHs, and the osmotic resistance in both groups; the concentration of GSSG, the oxidized/total glutathione ratio, and TBARs increased in both groups. In contrast, supplementation with GSHE increased the concentrations of GSH and the membrane PSH content and restored the osmotic resistance. GSHE prevented the increase in GSSG and TBARs and the decrease in ATP and DPG observed in the erythrocytes that were incubated with RBV alone. Incubation with DPD prevented a decrease in GSH, PSH, ATP, and DPG and an increase in GSSG and TBARs. A higher osmotic resistance was seen compared with the incubation with RBV alone.

Discussion

In the present investigation, five of 30 patients undergoing treatment with IFN and RBV for hepatitis C experienced major hemolysis. Interestingly, the decrease in HB did not show a continuous distribution, but there were two distinct groups: one with a major decrease in HB, and one with a minor decrease in HB. All patients who had a pretreatment PSH concentration below 32 nmol/mg protein developed severe RBV-associated hemolysis (see Fig. 1). PSH content was not only a predictor of major hemolysis but also one of the erythrocyte parameters that differentiated healthy controls from patients with hepatitis C.

Lower erythrocyte concentrations of PSHs may reflect an increased oxidation of PSHs,[16] a deficiency in their synthesis, or a defect in the mechanisms regulating their reduced status. Previous investigations have demonstrated an increase in markers of oxidative stress associated with hepatitis C in liver, plasma, and lymphocytes.[20–22] Because there was no additional evidence for oxidative stress in erythrocytes (*e.g.*, an increase in TBARs or oxidized glutathione) prior to treatment, the lower concentrations of PSHs that are also present on the outer membrane of erythrocytes could be explained by an extra-erythrocytic oxidant stress. The low PSH concentrations in patients susceptible to RBV-associated hemolysis would then be due to a particularly high hepatitis C–related oxidative stress in plasma. Further studies will have

to show whether or not there is a correlation between markers of oxidative stress in plasma and low PSH content in erythrocytes.

In accordance with previous investigations,[11] the present *in vivo* and *in vitro* observations suggest that RBV causes an additional intracellular oxidative stress in erythrocytes. In the *in vitro* incubations, erythrocytes from patients who experienced major hemolysis as well as from patients who did not showed a decrease in GSH induced by RBV alone or in combination with DEM. This was associated with a decreased concentration of PSHs and an increase in TBARs and resulted in a decreased osmotic resistance. Supplementation of both GSHE and DPD in part prevented the decrease in GSH and PSHs and increased the resistance to osmotic hemolysis. GSHE protected PSHs from oxidation. Whether or not other sulfhydrils and precursors of GSH (*e.g.*, N-acetylcysteine or S-adenosylmethionine) would have similar effects remains to be established. The protective effect of DPD may be explained by a competitive inhibition of the membrane nucleoside transporter system[23] resulting in a reduced uptake of RBV into erythrocytes.[12] Although DPD may prevent RBV toxicity *in vivo*, its clinical use is likely to be limited because a reduced uptake of RBV into its target cells (hepatocytes and lymphocytes) might compromise the therapeutic effect of RBV.

Membrane PSHs play an important role in membrane fluidity and deformability[24] as well as in carrier and receptor systems, and compounds that are able to improve membrane fluidity have previously been shown to reduce RBV-induced hemolytic anemia.[25] Thus the lower PSH content found in patients who developed hemolysis might increase the fragility of erythrocytes and make them more susceptible to hemolysis during administration of RBV, which in addition to the extracellular oxidant stress will produce an intracellular oxidant stress. Indeed, the erythrocytes of patients who had experienced major hemolysis during treatment with RBV also showed more pronounced hemolysis *in vitro* when incubated with RBV. The addition of the sulfhydril-depleting agent DEM to RBV further decreased the concentration of PSHs and reduced osmotic resistance.

In the four patients with G6PD deficiency, no major hemolysis occurred during treatment with RBV and IFN. Furthermore, except for an increase in TBARs and a decrease in osmotic resistance similar to non–G6PD-deficient erythrocytes, no substantial changes occurred in the measured indices during treatment. Although the design of this investigation and the limited number of observations do not allow definite conclusions, our findings do not support the idea that the suggested up-regulation of the hexose monophosphate shunt[11] with a consequent increase in GSH production (which is dependent on G6PD and therefore is not possible in patients with a deficiency of this enzyme) is an important defense mechanism against RBV-induced oxidative stress.

In conclusion, the present study suggests that a low membrane PSH content of erythrocytes prior to therapy with RBV is a risk factor for major hemolysis during RBV therapy. Erythrocytes of patients with major hemolysis during RBV therapy show an increased osmotic fragility and seem to be more susceptible to oxidative stress as indicated by a larger increase in TBARs and a larger decrease in GSH compared with erythrocytes of patients with minor hemolysis. Erythrocytes of patients with G6PD deficiency are not at higher risk of RBV-induced hemolysis. Finally, GSHE and DPD modified the indices of oxidant stress associated with exposure to RBV and osmotic fragility *in vitro*, suggesting that these compounds might also decrease the risk of hemolysis in susceptible patients treated with RBV.

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