Biochimica et Biophysica Acta, 691 (1982) 300–308
Elsevier Biomedical Press

BBA 71291

ALTERATIONS OF RED CELL MEMBRANE PROTEINS AND HEMOGLOBIN UNDER NATURAL AND EXPERIMENTAL OXIDANT STRESS

N. ALLOISIO [a,*], D. MICHELON [b], E. BANNIER [b], A. REVOL [b], Y. BEUZARD [c] and J. DELAUNAY [a]

[a] *Laboratoire de Chimie Biologique, Faculté de Médecine Grange Blanche, 69373 Lyon Cedex 8,* [b] *Laboratoire de Biochimie, Centre Hospitalier Lyon Sud, 69310 Pierre Benite and* [c] *INSERM U 91, Hôpital Henri Mondor, 94010 Créteil (France)*

(Received September 14th, 1981)
(Revised manuscript received March 12th, 1982)

Key words: Erythrocyte membrane protein; Hemoglobin; Hemolytic anemia; Oxidant stress

We compared on red cell membrane proteins and hemoglobin (Hb) the effects of (i) natural oxidant stress that has been suggested to occur in a variety of oxidative hemolytic anemias, and (ii) experimental stress induced by hydrogen peroxide. SDS-polyacrylamide gel electrophoresis was used for protein analysis. Under natural conditions (thalassemias, hemoglobinopathies with Hb unstability), a high molecular weight polymer (HMWP) and variable amounts of globin mono- and dimers became apparent. Furthermore, a major 12 kDa polypeptide, its dimer, and conspicuous spectrin degradation products in the band 2.2–2.6 region occurred in a patient carrying the highly unstable Hb Hammersmith. Under experimental conditions, incubation of erythrocyte ghosts with H_2O_2 in the presence of minimal concentration (25 μM) of Hb generated a HMWP at the expense of membrane proteins, mainly spectrin. Incubation of a diluted (200 μM) membrane-free hemolysate with H_2O_2 induced a HMWP, an array of globin oligomers and a 12 kDa polypeptide similar to that mentionned above. Therefore, the damage to the red cell membrane present in various oxidative hemolytic anemias, including polypeptide polymerisation and breakdown, can be produced by experimental oxidant stress. These observations support the view that the alterations described in the patients result directly from oxidative reactions. However, we did not observe in the patient the sharp breakdown of polyunsaturated fatty acids that was triggered in vitro by H_2O_2 in the presence of Hb acting as a catalyst. In most cases, oligo- and polymers were resistant to β-mercaptoethanol, and the chemical nature of the underlying cross-links is discussed. To our knowledge, the 12 kDa polypeptide, that we consider as arising from globin proteolysis, has never been reported under pathological conditions.

Introduction

Biological membranes are threatened by oxidant reactions. Due to the high intracellular oxygen concentration [1], the red cell membrane is particularly exposed. Proteins may be oxidized through the formation of disulfide bonds. Polyunsaturated long chain fatty acids may undergo complex radical reactions requiring catalytic activation of oxygen. Among other degradation products, they yield malonyldialdehyde [2], a bifunctional reagent. It has been suggested that malonyldialdehyde, in turn, could generate β-mercaptoethanol-resistant cross-links [3–5]. Still another type of covalent bond, generated in the presence of trans-

* To whom correspondence and reprint requests should be addressed.

Abbreviations: DEGS, diethylene glycol succinate; EDTA, ethylene-diaminetetraacetic acid; Hb, hemoglobin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamide; TMCS, trimethylchlorosilane.

0005-2736/82/0000–0000/$02.75

glutaminase [6,7], may appear. Nevertheless, the relationship, if any, of this type of linkage to oxidant reaction is not direct.

Although the red cell is protected against oxidant stress by powerful reducing systems, these may be overwhelmed in such conditions as oxidative hemolytic anemias [8], that include thalassemias and hemoglobinopathies with hemoglobin unstability [1]. Oxidant stress that results in vivo from these conditions will be referred to as natural. Membrane lipid and protein alterations have been reported in β-thalassemia major [4,9,10]. The purpose of this work was to further investigate the damage undergone by red cell membrane proteins and by hemoglobin (Hb) in a variety of oxidative hemolytic anemias. We also tried to elucidate the mechanism of their occurrence, using one type of experimental oxidant stress, e.g. incubation of erythrocyte ghosts, membrane-free hemolysates or intact red cells with H_2O_2. A general protein alteration pattern could be worked out that is valid under both in vivo and in vitro conditions.

Materials and Methods

Materials

The origin of some particular compounds was as follows: acrylamide and bisacrylamide, Serva; TEMED, Baker; β-mercaptoethanol, malonyldialdehyde and thiobarbituric acid, Merck. Most of other organic and mineral compounds were obtained from Merck.

Preparation of hemolysates and erythrocyte ghosts

Unless otherwise stated, all operations were carried out in ice. Blood was collected in citric acid-citrate-dextrose medium. In order to prepare hemolysates, red cells were washed three times in a buffer solution containing 5 mM Tris (pH 7.5), 145 mM NaCl and 4 mM NaN_3. White cells were removed by thorough aspiration of the buffy coat after the first centrifugation. Following further centrifugations, the top of the packed red cell fraction was carefully inspected and any remaining white spot was aspirated again. Hemolysis was generated by addition to packed erythrocytes of 10 vol. of distilled water containing 4 mM NaN_3. Stroma were separated by centrifugation at 15000 rpm, for 20 min.

Erythrocyte ghosts were prepared according to Dodge et al. [11], with some modifications [12]. After the last wash in hypotonic phosphate buffer, ghosts were washed with an isotonic buffer solution containing 5 mM Tris (pH 7.5), 150 mM NaCl. The ghosts were always prepared fresh prior to oxidant treatment. They were stored at −70°C for some control purposes (Control 1).

Abnormal erythrocyte ghosts were obtained from (i) four patients with β-thalassemia intermedia, (ii) four patients double heterozygous for the β^0-thalassemia and the HbE traits (genotype: β^0/β^E), (iii) one patient heterozygous for Hb Hammersmith (β42 Phe → Ser) and three patients heterozygous for Hb Köln (β99 Val → Met). Hb Hammersmith and Hb Köln are unstable variants of hemoglobin. Patients with the β^0/β^E association and the patient with Hb Hammersmith displayed a picture of severe hemolytic anemia that required frequent blood transfusions. In other patients, the condition was less severe and blood transfusions were not usually necessary. All patients had free diet and received no vitamin E supplementation. Serum iron ranged from normal values to 52 μM. Heinz bodies were present in the form of small inclusions in patients with β-thalassemia intermedia or with the β^0/β^E association, and also in the splenectomized patient with Hb Köln. In the Hb Hammersmith patient, Heinz bodies were represented on the contrary by enormous particles occupying a large fraction of the cell volume.

Peroxidation incubation

Erythrocyte ghosts were incubated at 37°C for 2 h with H_2O_2 (concentration: 0.5 or 5 mM) in a medium containing 5 mM Tris (pH 7.4), 145 mM NaCl and 2 mM NaN_3 (as a catalase inhibitor), at a final concentration of 1 mg membrane protein per ml. Hemolysate (25 μM Hb) was added in most experiments. Following immediately the incubation period, the reaction mixture was submitted to centrifugation (15000 rpm, 10 min, 4°C). Malonyldialdehyde was determined in the supernatant (see below). Pelleted ghosts were washed twice with 5 mM phosphate buffer (pH 8.0) in order to remove trace amounts of H_2O_2 that possibly remained. They were stored at −70°C in the presence of glycerol (10%, w/v) before further

analysis. Handling and storage times were strictly the same for all experiments.

In some experiments, intact red cells (hematocrit: 4%) were incubated with 0.5 mM or 5 mM H_2O_2 in a buffer solution containing 5 mM sodium phosphate (pH 7.4), 8 mM KCl, 140 mM NaCl and 2 mM NaN_3. Although substantial hemolysis occurred at 5 mM H_2O_2, malonyldialdehyde was determined in the post-incubation supernatant and ghosts were purified according to the standard procedure. Control 4 will refer to intact red cells incubated alone. The use of intact red cells more closely reproduced in vivo conditions. On the other hand, heavy contamination of the ghosts by the bulk of Hb could mask some features pertaining to membrane proteins.

Protein analysis

Proteins were assayed by the procedure of Lowry et al. [13]. SDS-polyacrylamide gel electrophoresis was carried out according to a technique derived from that of Fairbanks et al. [14]. Samples underwent only one freeze-thaw cycle in order to reduce or avoid artefactual proteolysis. They were pre-incubated (1 mg/ml) at 60°C for 15 min in the following medium: 1% SDS, 0.5 mM EDTA and 6, 100 or 500 mM β-mercaptoethanol. 15-μl aliquots were submitted to electrophoresis in a gel slab (5.6% acrylamide and 0.21% bisacrylamide monomers), in a medium (pH 7.4) containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA and 0.2% SDS. Staining with Coomassie blue and destaining were performed using standard procedures.

Lipid analysis

Malonyldialdehyde, reflecting oxidative breakdown of polyunsaturated long chain fatty acids, was determined with the thiobarbituric acid procedure [15,16]. Erythrocyte ghost lipids were extracted with chloroform-methanol [17]. Cholesterol was determined by gas-liquid chromatography (column: 2% OV 1; support: chromosorb WHP (Pierce)), after silylation of the lipid extract with bis(sialyl)acetamide/trimethylchlorosilane (BSA/TMCS) (4:1, v/v) mixture. Phospholipids were assayed according to Bartlett [18]. PC, PE and PS were separated by thin-layer chromatography on Merck silica gel slabs with chloroform/methanol/water (65:25:4, v/v). Bands were revealed with Bromophenol blue, scratched out and dessicated. After transesterification, the fatty acid methyl esters of each phospholipid class were separated by gas-liquid chromatography in a column whose support (Chromosorb W 80, 120 mesh) was coated with 10% DEGS (Carlo Erba Fractovap, Model 320). Each fatty acid was expressed as % of the total, derived from the integration of the peaks of the chromatogram.

Results

Lipid oxidation

In vivo, the spontaneous fatty acid distribution in PC and PE was found essentially unchanged in the patients examined (Fig. 1). In particular, no decrease of the 20:4 (*n*-6) and the 22:6 (*n*-3) fatty acids was recorded. Only was it, however, in PS from patients with β-thalassemia intermedia that a reduction of these fatty acids was visible (Fig. 1).

In vitro, lipid oxidation of erythrocyte ghosts was assayed chiefly on the basis of malonyldialde-

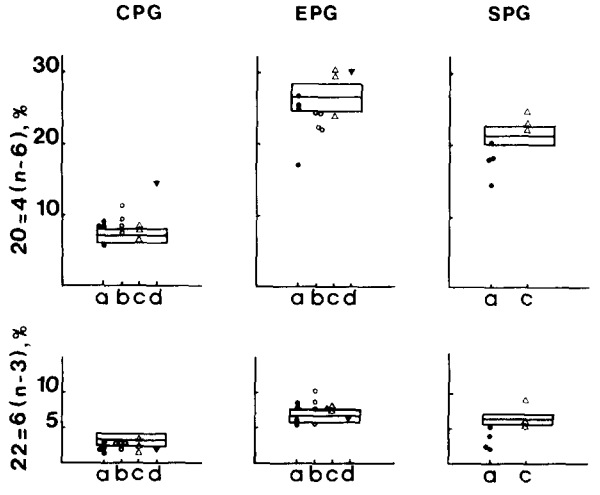

Fig. 1. Fatty acid distribution ((20:4 (*n*-6) and 22:6 (*n*-3)) in PC (CPG), PE (EPG) and PS (SPG) from patients with various oxidative hemolytic anemias. (a) β-Thalassemia intermedia, (b) β^{o}/β^{E} association, (c) Hb Köln, (d) Hb Hammersmith. Controls ($n = 11$ for PC and PE; $n = 8$ for PS) are indicated as the $m \pm \sigma$ interval with double rectangles. The percentage of the above polyunsaturated fatty acids failed to display any significant reduction, except for PS from patients with β-thalassemia intermedia. (Determination of fatty acids could not be made in PS in the β^{o}/β^{E} and the Hb Hammersmith patients.)

hyde formation. No oxidation occurred in the absence of Hb. Maximal oxidation (30 nmol malonyldialdehyde formed/mg membrane protein per 2 h) was obtained with 5 mM H_2O_2 in the presence of 25 μM Hb, e.g. 100-times less than the physiological concentration of Hb (Fig. 2). As is suggested by Fig. 3, the limited amount of malonyldialdehyde produced when the concentration of H_2O_2 or Hb increased results from the progressive exhaustion of the long chain polyunsaturated fatty acids serving as the major substrates of the reaction. When intact red cells were incubated with 5 mM H_2O_2 for 2 h, comparable malonyldialdehyde production occurred: 262 nmol/g Hb per 2 h (approx. 17.5 nmol/mg membrane protein per 2 h), values in agreement with those of Kahane et al. [4].

During in vitro oxidation of erythrocyte ghosts, no change of fatty acid distribution occurred in PC, PE and PS when Hb was absent. In the presence of Hb, the 20:4 (*n*-6) and the 22:6 (*n*-3) fatty acid proportions decreased in those phospholipids where they exist in sufficient proportions, e.g. PE and PS (Fig. 2) [14]. In counterpart, the percentage of 16:0, 18:0 and 18:1 (*n*-9) fatty acids increased (not shown). This increase, for one part, is the arithmetical consequence of the decrease of the polyunsaturated fatty acids. For another part, it probably results from the presence of unidentified degradation products that appear in this region of the chromatogram and that may, in some cases, cochromatograph with the 16:0, 18:0 and 18:1 (*n*-9) fatty acids. The percentages

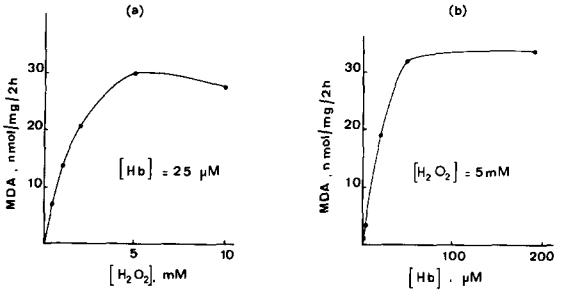

Fig. 2. Malonyldialdehyde (MDA) formation during incubation of erythrocyte ghosts with H_2O_2. Erythrocyte ghosts (1 mg protein/ml) were incubated at 37°C for 2 h in 5 mM Tris (pH 7.4), 145 mM NaCl, 2 mM NaN_3. (a) H_2O_2 concentration curve: Hb = 25 μM. No oxidation occurred in the absence of Hb. (b) Hemoglobin concentration curve: H_2O_2 = 5 mM.

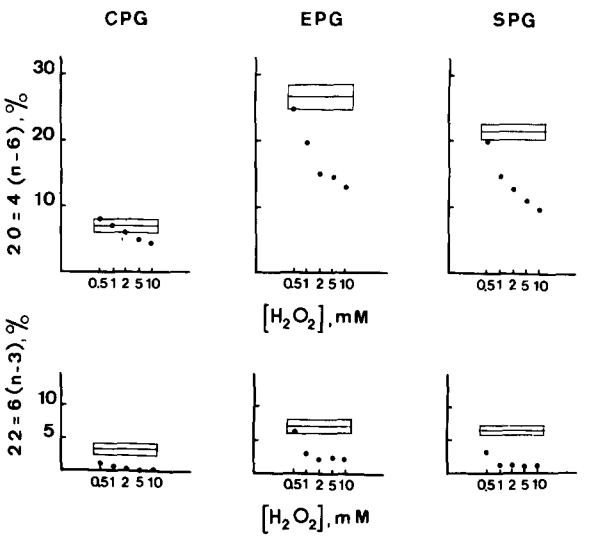

Fig. 3. Fatty acid breakdown (20:4 (*n*-6) and 22:6 (*n*-3)) during incubation of erythrocyte ghosts with H_2O_2. Erythrocyte ghosts were incubated as in Fig. 2, in the presence of 25 μM Hb. Controls as in Fig. 1. Fatty acids breakdown increased with increasing H_2O_2 concentrations. No breakdown occurred in the absence of Hb. CPG, PC; EPG, PE; SPG, PS.

of other fatty acids (18:2 (*n*-6), 20:3 (*n*-6), 22:4 (*n*-6), 22:5 (*n*-6 and *n*-3)) displayed either slight reduction or no detectable change (not shown).

In vivo protein alterations (Fig. 4)

In controls, the cholesterol/protein and the phospholipid/protein ratios were 0.216 ± 0.037 mg/mg ($n = 8$) and 0.468 ± 0.036 mg/mg ($n = 8$), respectively. In many patients, contamination by hemoglobin degradation products colored the ghosts pellets and reduced the above ratios. For example, the cholesterol/protein and the phospholipid/protein ratios were 0.131 and 0.259 mg/mg, respectively, in the patient with β-thalassemia intermedia presented in Fig. 4. These figures indicate that, even though the membrane cholesterol and phospholipids may also be increased [9], globin contamination was even higher on a weight ratio. Therefore, in contaminated ghosts, cholesterol and/or phospholipid determination represented a better (but still imperfect) criteria than the Lowry determination in order to estimate the amount of membrane protein to submit to electrophoresis.

Despite this restriction, the membrane protein

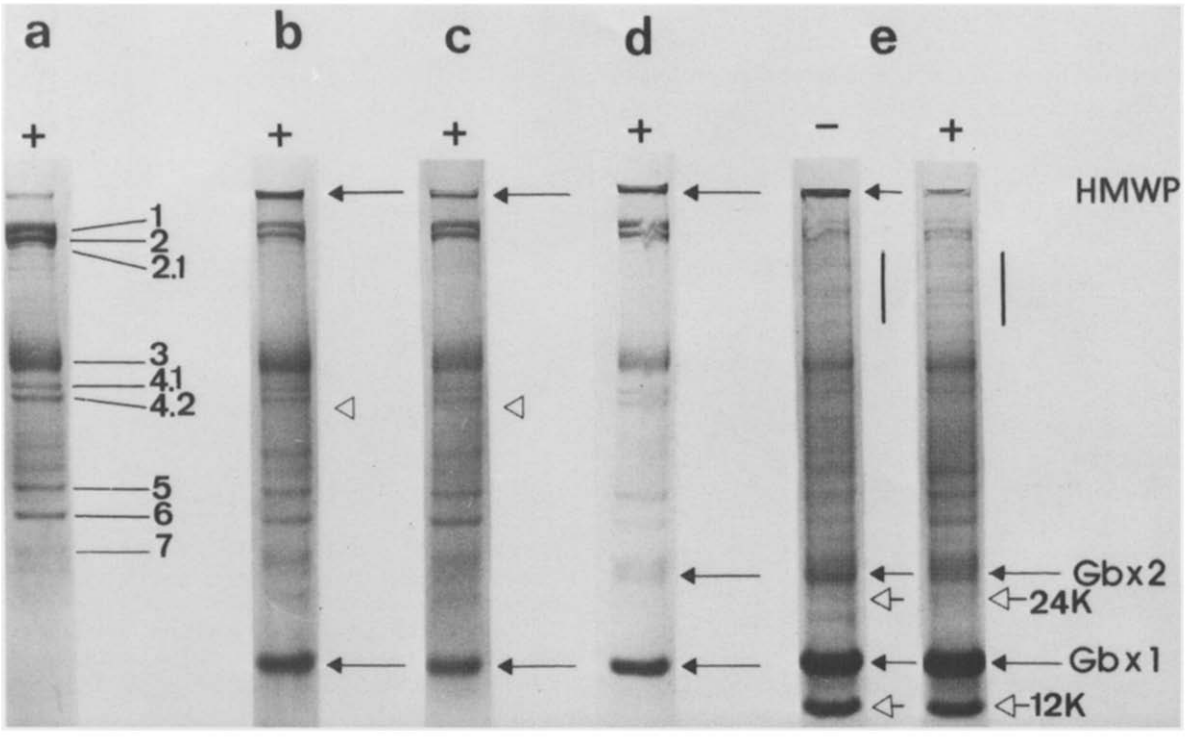

Fig. 4. Erythrocyte ghost proteins in a variety of oxidative hemolytic anemias. a, Control 1, freshly thawed ghosts; b, β-thalassemia intermedia; c, β^{o}-thalassemia/Hb E association; d, Hb Köln (splenectomized patient); e, Hb Hammersmith. Prior to electrophoresis, samples were treated with 6 mM (−) or 100 mM (+) β-mercaptoethanol (unless otherwise stated). Protein bands were numbered after Fairbanks et al. [14]. HMWP=high molecular weight polymer remaining on top of the gels.
← =Gb×1, Gb×2: globin mono- and dimers.
← =12 K and 24 K: major 12 kDa polypeptide and its dimer, observed in Hb Hammersmith patient.
| =spectrin degradation products in the band 2.2–2.6 region.
◁ =duplication of band 4.2.

profiles appeared obviously altered, sometimes dramatically. An HMWP developped on top of the gel. It was mostly resistant to 100 mM β-mercaptoethanol (in the Hb Hammersmith patient, however, the HMWP was reducible). Globin monomers were also visible in many patients, especially in the Hb Hammersmith patient. β-Mercaptoethanol-resistant dimers were more rarely present, but they were clearly visible again in the Hb Hammersmith patient.

Spectrin degradation products were often observed in the band 2.2–2.6 region. Band 4.2 was duplicated in one patient with β-thalassemia intermedia and in two patients with the β^{o}/β^{E} association. The abnormality may correspond to increased band 4.3 described by Kahane et al. [4]. In the Hb Hammersmith patient, spectrin bands, and most of the other major bands, had nearly completely disappeared at the benefit of multiple minor bands spanning the gel. In addition, a very intense 12 kDa polypeptide occurred in this patient (its β-mercaptoethanol-resistant 24 kDa dimer was also discernable). We consider that these alterations are the consequence of enhanced proteolysis, that appears, therefore, together with polymerization reactions. In particular, we consider that the 12 kDa polypeptide arises from globin proteolysis (see below).

In vitro protein alterations (Fig. 5)

Incubation of ghosts with H_2O_2 in the presence of 25 μM Hb (Fig. 5b) induced the formation of

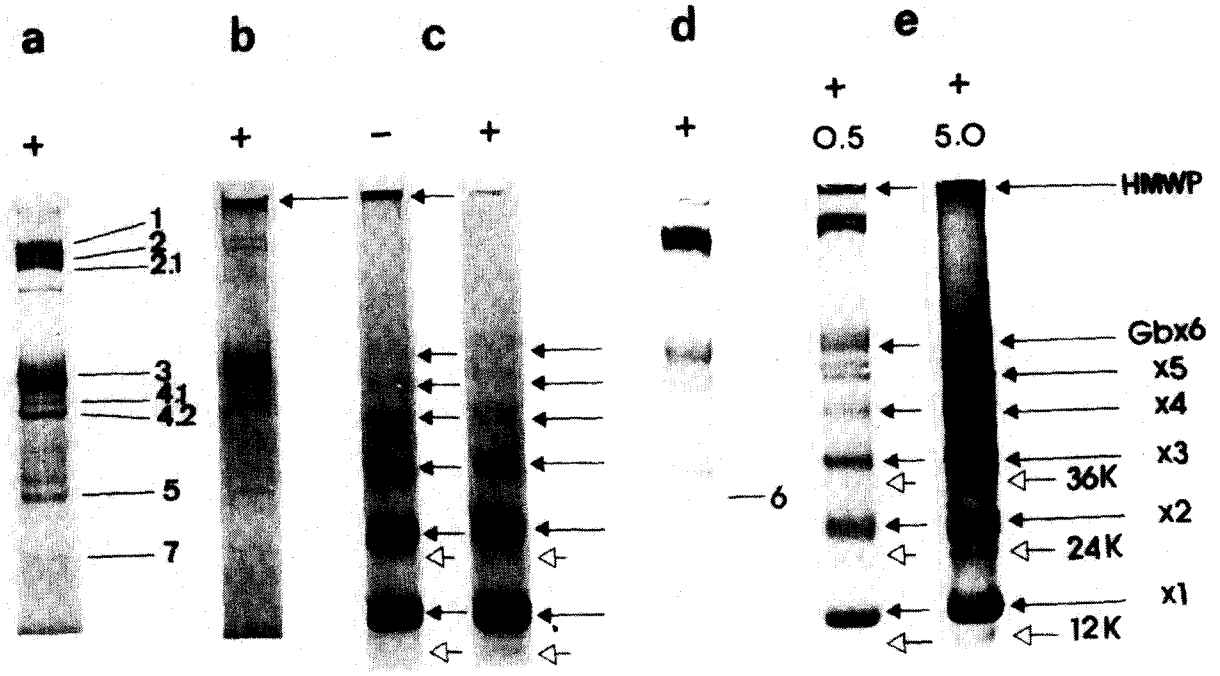

Fig. 5. Erythrocyte ghost proteins after in vitro H_2O_2 treatment. Conditions of experiment, control 1 and symbols are the same as in Fig. 4. a, Controls 2 and 3, ghosts incubated in the absence or in the presence of 25 μM Hb respectively (they yielded identical patterns). b, Ghosts+5 mM H_2O_2 +25 μM Hb (globin monomer appeared in the form of a very faint band). c, Membrane-free hemolysate (200 μM Hb)+5 mM H_2O_2. d, Control 4, intact red cells incubated without H_2O_2. e, Intact red cells incubated with 0.5 (left) or 5 mM (right) H_2O_2 (substantial hemolysis occurred at 5 mM); in these experiments, samples were treated with 500 mM β-mercaptoethanol (instead of 100 mM) prior to electrophoresis. Since ghosts were incubated in isotonic conditions in (a) and (b), they lack band 6 [14].

an HMWP and a marked reduction of the membrane protein profile at the expense, mainly, of spectrin bands 1 and 2. The HMWP was essentially resistant to β-mercaptoethanol. Globin contamination was minimal, appearing in the form of a faint band (monomer). Incubation of diluted membrane-free hemolysate (200 μM Hb) with 5 mM H_2O_2 yielded a β-mercaptoethanol-sensitive HMWP necessarily formed from globin and an array of globin oligomers that were more and more sensitive to β-mercaptoethanol in proportion as they were larger (Fig. 5c). In addition, the same 12 kDa polypeptide and its β-mercaptoethanol-resistant dimers as those encountered in the Hb Hammersmith patient were visible (β-mercaptoethanol-resistant, 36 kDa trimers were also present). Incubation of intact red cells with 0.5 mM H_2O_2 roughly cumulated the above (Figs. 5b and 5c) effects on membrane proteins and hemoglobin (Fig. 5e). With 5 mM H_2O_2, membrane proteins became entirely cross-linked and the same profile as in Fig. 5c was observed, although the HMWP was resistant to β-mercaptoethanol, and the 12 kDa polypeptide and its oligomers appeared more pronounced (Fig. 5e).

Discussion

Unlike previous works on thalassemia major [9,20], we failed to detect a clear-cut change of the membrane fatty acids in the various cases of oxidative hemolytic anemias that we examined, except in PS from patients with β-thalassemia intermedia. This discrepancy cannot be accounted for by the fact that we did not analyse sphingomyelins. The latter are little represented in the cytoplasmic leaflet of the membrane [21], which is the most exposed to in vivo oxidant stress, and

contains low amounts of polyunsaturated fatty acids [22–24]. The above discrepancy, however, may be related to variations of the oxidant stress and of the vitamin E status of the different patients examined.

We confirmed [4] the existence of an HMWP in β-thalassemia intermedia, and extended this finding to other oxidative hemolytic anemias. The β-mercaptoethanol-irreducible nature of the polymer (except in the Hb Hammersmith patient) contrasts with the β-mercaptoethanol-sensitivity of the aggregate observed in glucose-6-phosphate dehydrogenase deficiency, another example of oxidative hemolytic anemia [25]. This suggests a markedly different contribution of disulfide bonding in the formation of polymers.

Nevertheless, the in vivo changes displayed substantial variability. Normal protein profiles were observed in patients with milder oxidative hemolytic anemias such as: β-thalassemic trait, Hb H disease, Hb E trait or disease, heterozygosity for Hb Hope (not shown). Even in a given category of patients, some variability was also possible due to factors other than the altered hemoglobin itself. For example, in one unsplenectomized patient with Hb Köln, a virtually normal profile was associated with white erythrocyte ghosts and the nearly complete absence of Heinz bodies, whereas his splenectomized mother (Fig. 4d) presented the HMWP, brown ghosts and numerous Heinz bodies: these facts emphasize the efficiency of the spleen in the removal of altered red cells.

Because we could create in vitro maximal oxidant conditions (as judged by malonyldialdehyde formation) in the presence of minimal hemoglobin concentration (1/100 of the physiological concentration), it was possible to better separate alterations pertaining to membrane proteins and others concerning hemoglobin. Nevertheless, we could miss some direct effects of (degraded) hemoglobin on membrane proteins when large amounts of Hb are present. The HMWP developped either from membrane proteins (Fig. 5b) or from hemoglobin (Fig. 5c). Apparently, globin HMWP were more sensitive than membrane protein HMWP to β-mercaptoethanol, indicating a larger contribution of disulfide bonding in the former. In the Hb Hammersmith patient, we suggest that membrane protein proteolytic degradation (see below) overtook polymerisation, thus preventing the formation of a β-mercaptoethanol-resistant HMWP. Neither in vivo nor in vitro did we observe the 180 kDa fragment (band 3 dimer) reported by Haest et al. [26], although under different oxidative experimental conditions.

The chemical nature of the protein cross-links is a critical question. Although sensitivity to β-mercaptoethanol (or other reducing agents [27]) indicates disulfide bonding, resistance to β-mercaptoethanol must be interpreted with caution. Activated oxygen may cause some disulfide groups to undergo further oxidation in the form of sulfoxide or sulfone groups [28]. When generated, malonylaldehyde can cross-link proteins [3–5] or lipids [29,30], presumably through Schiff base formation. Still another possibility is the formation of amide linkages between the γ-carboxyl group of glutamic acid residues and the ε-amino group of lysine residues in the presence of the enzyme transglutaminase [6,7,31,32]. Since transglutaminase is activated by calcium, one may rise the possibility that an increase of red cell calcium, as can be generated by red cell peroxidation [33], mediates the effects of oxidant stress on erythrocyte proteins in vivo. It should be pointed out, however, that elevated erythrocyte calcium (approx. 100 μM) in sickle cell disease [34] results in no apparent membrane protein polymerization [31,35]. In addition, Coetzer et al. [36] have pointed out that considerable increase of erythrocyte calcium (above 300 μM) was required to activate transglutaminase. In vitro, even though we did not use chelating agents, we can rule out the possibility that calcium, either due to the release of membrane-bound calcium or to contamination of commercial salts, would reach such high values.

To our knowledge, the 12 kDa polypeptide observed in the patient with Hb Hammersmith (Fig. 4e), or generated in vitro (Figs. 5c and 5e), has never been described before. Only did Pontremoli et al. [37] mention, but under very different conditions, the existence of globin proteolytic fragments with molecular weights of 13000 and 11000. The generation of the 12 kDa polypeptide from hemoglobin is the most likely possibility. The lack of the 4 kDa piece may be accounted for by the fact that it remains soluble in the cytosol. Further research will be needed to

locate accurately the peptide bond involved in the proteolytic cleavage. The lower intensity of the 12 kDa polypeptide in diluted membrane-free hemolysate (Fig. 5c) may depend on the lower hemoglobin concentration, but may also indicate that protease activity(ies) is (are) preferentially located in the membrane. This location would facilitate membrane protein degradation: spectrin breakdown, band 4.2 duplication, or such dramatic damages as those observed in the Hb Hammersmith patient. It is intriguing, however, that little or no membrane protein breakdown was observed in vitro. One may conceive that protease(s) is (are) specifically stimulated by oxidant stress. This property would represent an adaptive mechanism in a cell where a strong protection against oxidation and oxidative degradation products is needed. Although speculative, this hypothesis fits with the observation that red cell protease activities are inhibited by dithiothreitol [37]. Alternatively, the increase of Ca^{2+} influx, that can be generated by red cell peroxidation [33], would also stimulate some erythrocyte protease(s) [38,39] in vivo, at the same time as it would stimulate transglutaminase. For the reasons mentioned concerning the latter enzyme, however, Ca^{2+}-triggered proteolysis appears unlikely in vitro. Either in vivo or in vitro, it was not possible to ascertain whether globin mono- and polymers, as well as the 12 kDa fragment and its oligomers, were covalently linked to membrane proteins, or whether they merely cosedimented with erythrocyte ghosts in the form of inclusions of variable sizes.

Damages undergone by hemoglobin and membrane proteins turned out to exhibit striking similarities under both natural and experimental oxidant stress. This fact supports the view that pathological alterations directly result from oxidative reactions. The most salient features were protein polymerization and proteolysis. Since a particular class of erythrocyte membrane proteins, referred to as cytoskeletal proteins, controls red cell deformability [40], any change involving these proteins, especially cross-linking, is likely to reduce erythrocyte deformability [41] and, ultimately, reduce its life span. From a different viewpoint, limited globin proteolysis may provide some insight into endoerythrocytic protein digestion, an important physiological process involved, among other examples, in the clearing of ribosomes during the reticulocyte-erythrocyte transition.

Acknowledgements

We thank Drs. N. Philippe, P. Colonna and D. Bachir, for kindly providing us with abnormal blood samples. This work was supported by the Institut National de la Santé et de la Recherche Médicale (CRL 811 006) and by the UER de Biologie Humaine of the Université Claude Bernard, Lyon I.

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