Differential sensitivity to photohemolysis of erythrocytes enriched with some liposome-carried substances[1]

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Summary. The sensitivity of human erythrocytes to photohemolysis sensitized by addition of protoporphyrin IX can be selectively affected by their enrichment with substances carried by cationic liposomes. In particular the enrichment wich superoxide dismutase is accompanied by a copper-related greater sensitivity toward photohemolysis, as observed in the Down's syndrome (mongolism). Instead it is possible to protect the erythrocytes against the phototoxic effect of protoporphyrin by enrichment with small amounts of β-carotene.

Photohemolysis induced by photosensitizers has been studied 'in vitro' in view of its relevance to pathogenetic mechanisms[2] or to clinical applications[3]. Various aspects of the problem have been studied: a) identification of early damage to the erythrocyte: b) identification of primary toxic species produced by light in the presence of sensitizers; c) development of more specific sensitizers; d) agents affecting the rate of hemolysis. The latter point concerns the identification of intracellular or extracellular factors which directly or indirectly interact with the active oxygen species elicited by photosensitization. However, while it is possible to vary the composition of the medium outside the erythrocyte over a wide range, the study of the role of intraerythrocytic factors is hampered by the relative constancy of their concentration.

In the present paper we report on the enrichment of the erythrocyte, by means of liposomes, with molecules which may selectively alter the sensitivity of these cells to photohemolysis.

Materials and methods. Human red blood cells were obtained from healthy volunteer donors. Crystalline catalase (bovine liver), protoporphyrin IX (sodium salt), L-α-phosphatidyl choline-dipalmitoyl and stearylamine were from Sigma (St. Louis, Mo., USA). Human and bovine RBC Cu, Zn superoxide dismutases and human liver Mn superoxide dismutase were generous gifts of Prof. G. Rotilio, University of Rome. These enzymes were obtained in a homogeneous form according to established purification methods[4-6]. Copper-free erythrocytic superoxide dismutase was prepared according to Rigo et al.[7].

Carbonic anhydrase was purified in the native (Zn-containing) form from bovine erythrocytes[8], and the Cu-substituted form prepared according to Lindskog and Malmström[9]. Both samples were a gift of Dr L. Morpurgo, CNR Center for Molecular Biology, Rome.

Liposomes were prepared as described by Michelson et al.[10] with minor modifications. Satisfactory preparations were obtained with cationic liposomes containing superoxide dismutase and carbonic anhydrase. With catalase, however, highly aggregated material was obtained, which was not suitable for further use.

The liposomes obtained were incubated with erythrocytes for 180 min at 20 °C under continuous stirring. Afterwards the red blood cells were centrifuged off at $80 \times g$, washed 3 times with isotonic saline and resuspended to a final concentration of 10^6/ml. To the suspension of erythrocytes, protoporphyrin IX in dimethylformamide was added (final concentration 0.25 μM). A series of 10 ml samples were irradiated for 15 min with 2 150 W Hanovia Flood lamps at a distance of 15 cm. Control samples were stored in the dark or illuminated without protoporphyrin IX. After irradiation all samples were kept in the absence of light, and then aliquots were centrifuged at various time intervals. The extent of hemolysis was measured by the OD of the supernatants at 410 nm and expressed as percentage of that obtained by osmotic shock.

The activity of superoxide dismutase was determined in packed erythrocytes and in the supernatant according to Concetti et al.[11].

Results and discussion. The photosensitized damage to cells has been related to the formation of oxygen reactive species, in particular singlet oxygen[12,13] which may attack both membranes and intracellular structures[14-18]. In the present paper we have investigated the possibility of increasing the intraerythrocytic concentration of molecules known to interact with singlet oxygen or species derived from it. In particular, we tried the enrichment of red blood cells with the enzyme superoxide dismutase and with β-carotene.

Following the method of Michelson[10] we were able to increase to variable extents the activity of Cu, Zn superoxide dismutase bound to the erythrocytes.

Table 1 reports the relevant data together with the sensitivity of the treated erythrocytes to photohemolysis expressed as half-time of lysis. It is apparent from these data that the red blood cells become more fragile as a function of the increased SOD activity. The time course of hemolysis for SOD-enriched erythrocytes vs controls is shown in figure 1. Identical results were obtained using either human or bovine Cu, Zn SOD. The reason for this effect was not

Table 1. Half-time of red blood cell hemolysis as a function of their SOD activity

Sample	SOD activity (%)[a]	t/2 of lysis (min)
RBC	100	130±8
RBC+L[b]	100	125±5
RBC+L[b] SOD (bovine)	128	53
RBC+L[b] SOD (bovine)	135	35
RBC+L[b] SOD (bovine)	146	24
RBC+L[b] SOD (bovine)	153	17
RBC+L[b] SOD (bovine)	177	13
RBC+L[b] SOD (human)	130	52
	135	41
RBC+L[b] SOD (Cu-free)	106	96
	111	72
RBC+SOD[c]	100	127

[a]Erythrocyte-bound activity. [b]Erythrocytes treated with empty or loaded liposomes. [c]Externally added SOD.

Table 2. Photohemolysis of RBC from patients with Down's syndrome

Subject[a]	SOD activity (%)[b]	t/2 of hemolysis (min)
Patient No. 1	122	43
Patient No. 2	112	52
Patient No. 3	131	37
Parent No. 1	100	128
Parent No. 2	100	134
Parent No. 3	100	132

[a]Blood samples from patients and one of their parents were drawn and stored under the same conditions. The time elapsed before the hemolysis experiment was variable, but constant for each pair. [b]Percent activity with respect to matched controls.

immediately apparent, as superoxide dismutase was expected to increase the resistence of RBC to photohemolysis[19]. Thus control experiments were done to understand the phenomenon. Erythrocytes enriched with Mn-containing superoxide dismutase showed neither a significant increase in SOD activity nor an increased sensitivity to photohemolysis. It should however be pointed out that the activity of Mn SOD is about $^1/_{10}$ that of Cu, Zn SOD[19]. Externally added Cu, Zn SOD was also uneffective[10].

In another set of control experiments we enriched red blood cells with carbonic anhydrase, a protein, like SOD, present in erythrocytes and with a similar molecular weight. This protein did not show any effect on the rate of photohemolysis either. On the other hand the enrichment of red cells with Cu-substituted carbonic anhydrase, a derivative devoid of superoxide dismutase activity, produced a marked increase in the photohemolysis rate (fig. 1). Thus it appears that the enrichment with a copper protein rather than the SOD activity 'per se' is responsible for the increased sensitivity of erythrocytes to photohemolysis. It should however be pointed out that erythrocytes obtained from children affected by Down's syndrome (mongolism) which are naturally richer in Cu, Zn SOD[20,21] than normal controls (in our case their parents) also show a greater sensitivity to photohemolysis (table 2).

It appears therefore that an increased amount of copper-containing protein present within the erythrocyte causes a greater sensitivity to hemolysis. While an unspecific role of copper, like the so-called metal-catalyzed Haber-Weiss reaction[22] or copper-linked lipid peroxidation[23] seems to be involved in this increased sensitivity, a role of SOD itself cannot be ruled out. In fact it has been reported that the set of enzymes protecting against oxidative damage, namely SOD, catalase and glutathione peroxidase, afford maximum protection when the ratio of their activities is adequate, while an unbalance of this ratio could be harmful to the cell[24].

A well known quencher of singlet oxygen is β-carotene. It is possible to convey this substance into the erythrocyte using loaded liposomes. In this way we obtained a protection of red cells against the photosensitizing effect of protoporphyrin IX which was much larger than that observed by simple addition of carotene to the medium (fig. 2).

Liposomes appear to be a useful tool for transporting antioxidant species inside the cells. This observation could be important in order to reduce the dose of β-carotene used in photosensitizing diseases[25]. However, the enrichment of erythrocytes with SOD showed that an effect anticipated on the basis of experiments conducted on soluble systems could not be easily reproduced in cells, but even an opposite effect can be obtained.

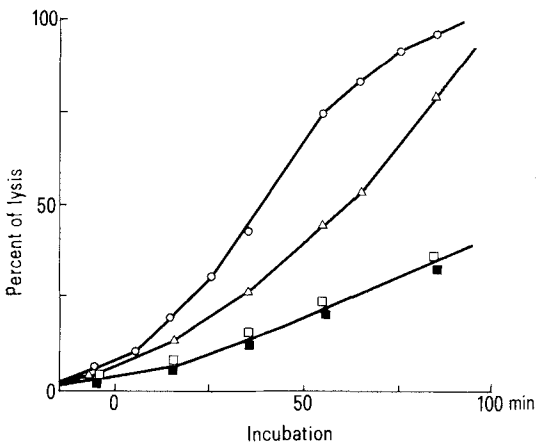

Figure 1. Time course of hemolysis for protein-enriched erythrocytes. Photohemolysis sensitized by 15 min irradiation in the presence of protoporphyrin IX (0.25 μM). Erythrocytes as such: (■); erythrocytes treated with empty liposomes: (□); erythrocytes enriched with SOD (bovine, activity 128% of the untreated erythrocytes: (○); and erythrocytes enriched with Cu-carbonic anhydrase: (△).

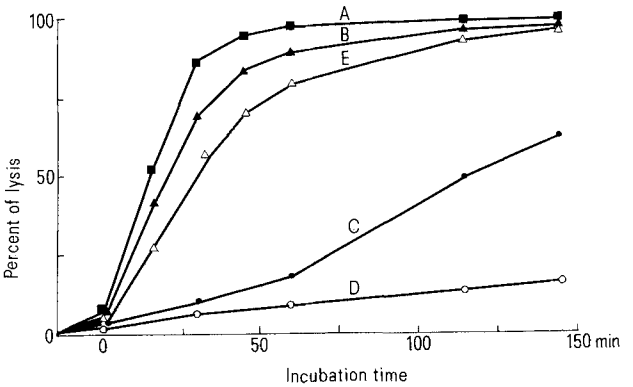

Figure 2. Time course of hemolysis for β-carotene treated erythrocytes. Photohemolysis obtained as in figure 1 but with a double concentration of protoporphyrin IX. Curve A: untreated erythrocytes; curves B-D: erythrocytes treated with liposomes containing β-carotene; 0.3 μM (curve B); 0.6 μM (curve C); 1.2 μM (curve D). Curve E represents the hemolysis of erythrocytes irradiated in the presence of 2 μM externally added β-carotene.

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Iodine-induced changes in thyroglobulin half-sized subunits[1]

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Summary. The two half-sized subunits of 19 S thyroglobulin have been separated and analyzed. They share the same peptide composition and carbohydrate content. The only difference was the iodine level, which was about three times higher in the faster electrophoretic subunit.

Thyroglobulin is the major iodoprotein synthesized in the thyroid gland as a framework for thyroid hormones. It is a large glycoprotein of 660,000 and has a sedimentation coefficient of 19S[2]. Van der Walt et al.[3] have shown that, upon reduction, this molecule is easily dissociated into 2 non-identical half-sized subunits having different electrophoretic mobilities in sodium dodecyl sulfate gel electrophoresis. The same authors inferred that the different electrophoretic behavior observed was due to some minor differences resulting from the amino acid analysis of the 2 separate polypeptides. In contrast with this finding we now show that these 2 half-sized molecules have similar peptide and carbohydrate compositions. Since the only remarkable difference found is in the iodination level, it is suggested that iodination is a specific post-translational event which is responsible for the observed changes in the electrodynamic properties of thyroglobulin apparent subunits.

Material and methods. Purification of 19 S thyroglobulin. Hog thyroids were obtained at the local abattoir immediately after the death of the animals. Thyroids were minced with scissors and soaked for 15 min with 0.1 M sodium phosphate buffer, pH 7.2. After a fractionated (1.4–1.8 M) ammonium sulfate precipitation, the extracted proteins were filtered on a Sepharose 6B (1.3×80 cm) column equilibrated with the extraction buffer. The peak fractions were pooled and appropriately concentrated. The protein concentration was estimated spectrophotometrically using an E1% (1 cm) of 10 at 280 nm. Its homogeneity was tested by analytical ultracentrifugation in a Spinco model E ultracentrifuge.

Isolation of porcine thyroglobulin subunits. To separate the 2 major components of hog thyroglobulin observed under denaturating and reducing conditions (i.e. 0.1% sodium dodecyl sulfate, 0.01% 2-mercaptoethanol), preparative slab gel electrophoresis was employed. The slab gel (5% acrylamide) had the following dimensions: 0.3×14×12 cm. About 2 mg of sodium dodecyl sulfate-treated thyroglobulin were extensively reduced with 2-mercaptoethanol and layered on the top of the gel and electrophoresed for 4 h at 50 mA. At the end of this period the gel was removed and a side slice cut and briefly stained, to localize the 2 major peptides. On this basis the 2 portions of the gel, containing the faster and the slower moving bands, were cut into very small pieces, immersed in 2 ml of electrode buffer (without sodium dodecyl sulfate) and shaken vigorously for 12 h at room temperature. The extracted proteins were then concentrated by lyophilization.

Carbohydrate and iodine determinations. The carbohydrate content of both unfractionated hog 19 S and isolated polypeptides was estimated according to the method of Roe[4]. Organic iodine was determined by the procedure of Palumbo et al.[5].

Limited enzymatic proteolysis in sodium dodecyl sulfate by *Staphylococcus aureus* SV8 protease. Limited enzymatic digestion of the 2 peptides and the native protein was accomplished using the *Staphylococcus aureus* SV8 protease (EC 3.4.21., Miles Lab.). Each sample, consisting of a mixture of the enzyme and protein (0.1 mg/mg of protein), was incubated in 0.5% sodium dodecyl sulfate at 37 °C for 30 min according to the method of Cleveland et al.[6]. The digestion was stopped by adding an excess of (0.75 M) 2-mercaptoetheanol and boiling for about 1 min.

Analytical sodium dodecylsulfate polyacrylamide gel electrophoresis. Before electrophoresis samples, whose concentration in sodium dodecyl sulfate was adjusted to 1%, were incubated at room temperature for about 30 min, and then

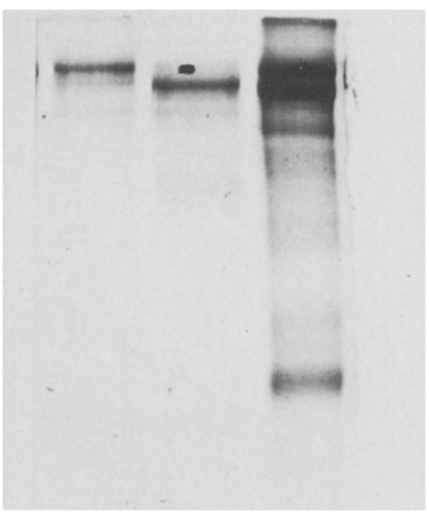

Figure 1. Purification of hog 19 S thyroglobulin reduced subunits by preparative gel electrophoresis. From the left: slots 1 and 2 show the isolated components (faster and slower migrating bands of the doublet of near 330,000). Slot 3 depicts the electrophoretic pattern of reduced hog 19 S thyroglobulin. Lighter materials, other than the 330,000 doublet may be noticed in this preparation. The gel contained 7.5% acrylamide.