Iron release, membrane protein oxidation and erythrocyte ageing

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Abstract The aerobic incubation of erythrocytes in phosphate buffer for 24–60 h (a model of rapid in vitro ageing) induced progressive iron release and methemoglobin formation. Membrane proteins showed electrophoretic alterations and increase in carbonyl groups (as documented by IR spectroscopy). None of these phenomena were seen when the erythrocytes were incubated under anaerobic conditions. The membranes from aerobically incubated cells bound a much higher amount of autologous IgG than those from anaerobically incubated ones, suggesting that the aerobic incubation gives rise to the senescent antigen. The addition of ferrozine during the aerobic incubation prevented both the IgG binding and the protein alterations seen in the IR spectra, suggesting an intracellular chelation of the released iron by ferrozine.

Key words: Erythrocyte; Iron release; Senescent cell antigen; Protein oxidation; Iron chelation

1. Introduction

The homeostasis of the circulatory system largely depends on the removal of aged or damaged cells from the bloodstream. The removal of aged cells is accomplished by mononuclear phagocytes which can distinguish mature from senescent cells. It is generally accepted [1–4] that the recognition of senescent cells depends on the appearance of a neo-antigen on the surface of senescent cells (senescent cells antigen) which acts as a specific signal for the termination of aged cells. The antigen in fact is recognized by the Fab region of autologous specific IgG [5,6] which attaches to it and initiates the removal of cells by macrophages. Senescent cell antigen is immunologically related to band 3 [3,7] and is located in an extracellular portion of it [3]. The molecular changes underlying the formation of senescent cell antigen and marking erythrocytes as aged are under investigation.

It has been suggested that an oxidative mechanism underlies erythrocyte ageing [8,9] and it has been shown [10] that erythrocytes from vitamin E deficient rats behave like old erythrocytes from normal rats with regard to susceptibility to phagocytosis, IgG binding, anion transport ability, etc. Furthermore, increased breakdown products of band 3 were observed in membranes of erythrocytes from vit. E deficient animals [10]. Recently it has been shown [8] that incubation of erythrocytes with oxidizing systems, such as ADP/Fe^{3+} or xanthine/xanthine oxidase/Fe^{3+}, promotes the binding of autologous IgG and that the antibody involved in the binding is anti-band 3. On the base of additional experimental results it has been suggested [11] that the essential alteration leading to the formation of band 3 antigenic sites (band 3 senescent antigen) is an oxidation of protein -SH groups and that the antigen is formed following moderate oxidation.

In previous studies [12–14] we have shown that iron is released in a 'free' (desferrioxamine, DFO, chelatable) form when mouse erythrocytes are incubated with oxidizing agents such as phenylhydrazine, acrolein, divicine and isouramil, and that such a release promotes lipid peroxidation and hemolysis when the cell is depleted of GSH. With human and calf erythrocytes phenylhydrazine induces iron release, but not lipid peroxidation nor hemolysis [15]. Nethertheless in human erythrocytes phenylhydrazine induces the clustering of band 3 and promotes the binding of autologous IgG [16].

In view of the possibility that the release of reactive iron is a relevant factor in the generation of senescent antigen from band 3, we first investigated whether a release of iron occurs in human erythrocytes aged in their own plasma for several weeks. Subsequently we studied, in a model of rapid in vitro ageing of erythrocytes, the relationships among iron release, oxidation of membrane proteins, breakdown of band 3 and binding of autologous IgG. The results indicate that release of iron, alterations of membrane proteins and binding are dependent on aerobic conditions and that the release of iron may be related to the antigen formation and the subsequent binding.

2. Materials and methods

2.1. Materials

Desferroxamine (Desferal, DFO) was kindly supplied by Ciba-Geigy (Basel, Switzerland). Rabbit anti-bovine IgG antibodies conjugated with alkaline-phosphatase were from Sigma Immuno Chemicals. ^{125}I Protein A was from NEN (Radiochemicals). The specific activity was 9.01 μCi/μg. 3-(2-pyridyl)-5,6 bis(4 phenyl sulfonic acid) 1,2,4 triazine (ferrozine) was from Sigma. Electrophoresis standards and reagents were from Bio-Rad Laboratories (Richmond, CA). The solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade.

Human blood was obtained from the blood bank of local hospital. Bovine blood (with which most of the experiments were performed) was obtained from local slaughter-house.

2.2. Erythrocyte incubations

Erythrocytes were prepared from heparinized human or bovine blood by centrifugation and removal of plasma and buffy coat. The cells were washed three times with buffered physiological solution and resuspended in 0.123 M NaCl, 27 mM sodium phosphate/potassium phosphate buffer (pH 7.4), as a 50% (v/v) suspension. Iron contamination was removed from the buffer as previously described [13]. The hemoglobin concentration, measured as in ref. [12] and expressed per heme, was 8.36 ± 0.16 μmol/ml.

In the experiments of in vitro ageing the suspension was incubated at 37°C in a Dubnoff shaker under aerobic or anaerobic conditions in the presence of antibiotics (20 units penicillin and 20 μg streptomycin/ml). Anaerobiosis was obtained by bubbling the erythrocyte suspension with a nitrogen stream for the entire incubation period. Samples were

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withdrawn at the indicated times for the determination of 'free' iron (DFO-chelatable iron), methemoglobin [17], GSH [18], malonaldehyde [19] and hemolysis [13]. Additional samples were withdrawn for ghost preparation and for the measure of IgG binding by radioimmunoassay (RIA) with ^{125}I Protein A. 'Free' iron was determined as DFO-iron complex (ferrioxamine) as previously reported [12,13].

In the experiments in which ferrozine was used to chelate intracellularly the released iron, 100 μM ferrozine was added to the aerobically incubated sample. In preliminary experiments it was ascertained that ferrozine enters the erythrocytes. To this end 1.0 ml of packed cells, previously incubated with ferrozine and washed once with the buffer, was lysed by addition of 3 ml of water. The hemolysate was centrifuged (10,000 × g, 20 min) and the supernatant was ultrafiltered (Centriflo CF 25, Amicon ultrafiltration cones). $FeSO_4$ (100 μM) was added to the ultrafiltrate and the ferrozine–iron complex was measured spectrophotometrically at 562 nm [20]. The intracellular concentration of ferrozine, measured with ferrozine–$FeSO_4$ standards, was 84 nmol/ml of packed cells indicating approximately an equilibrium between the outside and the inside of the cells. In these experiments carried out in the presence of ferrozine, 'free' iron was determined as a DFO complex as usually, for uniformity with the sample incubated without ferrozine. The procedure [12,13] was the same, except that the amount of DFO added to the erythrocyte lysate was higher (100 instead of 50 μM) than that currently used. In preliminary experiments it was ascertained that, under this condition, DFO was able to abstract all the iron from the ferrozine–iron complex.

2.3. Ghost protein preparation for electrophoresis and IR spectroscopy

Erythrocyte ghosts were prepared according to Doodge [21], resuspended in 50 mM NaCl, 10 mM sodium phosphate buffer, pH 7.35, and the protein concentration was determined according to Lowry et al. [22]. An aliquot was used for the electrophoretic analysis on 12% acrylamide gel (SDS-PAGE) according to Laemmli et al. [23]. Another aliquot was used for protein preparation for infra-red (IR) spectroscopy. To this end, ghosts were delipidated by extraction with a chloroform/methanol mixture (2:1, v/v) for 2 h, sedimented by centrifugation and washed three times with chloroform/methanol (2:1, v/v), chloroform/ethanol/ethyl ether (2:2:1, v/v/v) and ethyl ether, respectively. The delipidated membrane proteins were diluted with KBr and the Fourier Transform (FT) IR spectra were recorded on a Perkin-Elmer M 1800 spectrometer between 4,000 and 750 cm^{-1}. A mercurium cadmium telluride detector was used and the apparatus was purged with nitrogen. Typically 100 scans at a resolution of 2 cm^{-1} were averaged and the spectra stored at 1 cm^{-1} data interval. The frequency scale was internally calibrated with a reference helium-neon laser to an accuracy of 0.01 cm^{-1}. An apparatus for Diffuse Reflectance (DR) equipped with a microsample holder was used to record the spectra of the KBr diluted sample powders.

2.4. IgG binding to ghosts as measured by ELISA test

Ghosts were diluted at the concentration of 150 μg protein/ml. IgG were prepared from the serum of the erythrocyte donor animal by precipitation in 40% ammonium sulphate according to Good et al. [24]. After dialysis, IgG in 50 mM NaCl/10 mM sodium phosphate, pH 7.35, were partially purified on DEAE column, equilibrated with the same buffer, as reported by Levy et al. [25]. The recovered IgG were measured spectrophotometrically at 280 nm using $E^{1\%} = 14.0$ and adjusted at the concentration of 10 μg/ml.

An aliquot (0.5 ml) of autologous IgG solution was added to an aliquot (0.5 ml) of the ghosts suspension and the mixture was incubated at 4°C overnight. The ghosts were washed four times with 20 ml of incubation buffer and sedimented each time at 10,000 × g for 20 min in Corex tubes.

Subsequently, the ghosts were resuspended in 1 ml of phosphate saline buffer, pH 7.35, containing rabbit antibovine IgG antibodies conjugated with alkaline phosphatase and incubated for 2 h at 4°C (total enzymic activity 0.16 units). At the end of incubation the ghosts were washed four times with the incubation buffer, resuspended in 1.0 ml of 100 mM NaOH glycine buffer pH 10.0, containing 1 mM $ZnSO_4$ and 1 mM $MgCl_2$ for 15 min at 37°C after the addition of 200 μl of 10 mM *p*-nitrophenyl-phosphate. The incubation was stopped by addition of 0.5 ml of 2 M NaOH. After sedimentation of ghosts at 10,000 × g for 5 min the *p*-nitrophenol formed was measured spectrophotometrically at 405 nm.

2.5. IgG binding to erythrocytes as measured by radioimmunoassay (RIA) with ^{125}I-protein A

In the experiments in which ferrozine was used to chelate intracellularly the released iron, the IgG binding was measured by RIA using ^{125}I protein A. This was done because the E.L.I.S.A. test, previously used with ghosts, showed substantial IgG binding even at 0 time and the difference with respect to the aerobically incubated sample (maximun two-fold) did not seem appropriate to appreciate possible protection by iron chelating agents. The measure of IgG binding with ^{125}I-protein A showed, on the contrary, very low levels at 0 time and large difference with respect to the aerobically incubated sample.

An aliquot of incubated erythrocytes were washed with the incubation buffer and resuspended in phosphate-buffered saline containing 5 mM $MgCl_2$ (PBS-Mg) and 1% bovine serum albumin (BSA) to make a 20% cell suspension. To 0.5 ml of this suspension, 0.5 ml of a solution of autologous IgG (2 mg/ml) were added in a plastic tube precoated with 0.75% gelatin solution. The mixture was incubated at 4°C overnight. The cells were washed four times with PBS Mg at 4°C by centrifugation. To 100 μl of packed cells 55 ng of ^{125}I-protein A (specific activity 9.01 μCi/mg) were added in Dulbecco-PBS containing 0.1% BSA (final volume 500 μl). After 2 h of incubation at room temperature, the cell suspension was washed four times with DPBS to remove free ^{125}I-protein A, and the radioactivity associated with 50 μl of packed cells, resuspended in 500 μl of DPBS, was measured by an automated g-counter. Results are presented as the number of protein A molecules bound per cell.

3. Results

As shown in Table 1, a progressive release of iron occurs in human erythrocytes stored at 4°C for 15 or 35 days in their own plasma. Such a release is accompanied by a moderate increase

Table 1
Release of iron (DFO-chelatable iron), methemoglobin (Met-Hb) formation and GSH decrease in human erythrocytes stored in their own plasma at 4°C or incubated in phosphate buffer at 37°C

	Ageing time	'Free' iron (nmol/ml)	MetHb (nmol/ml)	GSH (nmol/ml)
Storing in plasma at 4°C	0 days	3.4 ± 0.4	115 ± 7	963 ± 68
	15 days	6.6 ± 1.6	141 ± 24	856 ± 52
	35 days	15.4 ± 3.2	179 ± 40	647 ± 101
Incubation in saline phosphate at 37°C	0 h	1.7 ± 0.03	162 ± 31	862 ± 42
	40 h	18.0 ± 2.3	324 ± 17	550 ± 85
	60 h	35.2 ± 4.1	2380 ± 106	338 ± 85

Results are the means ± S.E.M. of three to four experiments.

Table 2
Release of iron (DFO-chelatable iron), methemoglobin (Met-Hb) formation and GSH decrease, in calf erythrocytes incubated in phosphate buffer under aerobic or anaerobic conditions

	Incubation time (h)	'Free' iron (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)
	0	1.7 ± 0.3	169 ± 6	1255 ± 21
Aerobic incubation	24	5.2 ± 0.9	460 ± 30	337 ± 24
	48	11.6 ± 1.7	1680 ± 60	156 ± 24
	60	36.0 ± 2.0	3866 ± 121	171 ± 17
Anaerobic incubation	24	2.2 ± 0.3	130 ± 10	1018 ± 64
	48	3.1 ± 0.9	247 ± 37	563 ± 114
	60	5.0 ± 0.9	324 ± 45	418 ± 27

Results are the means ± S.E.M. of three to five experiments.

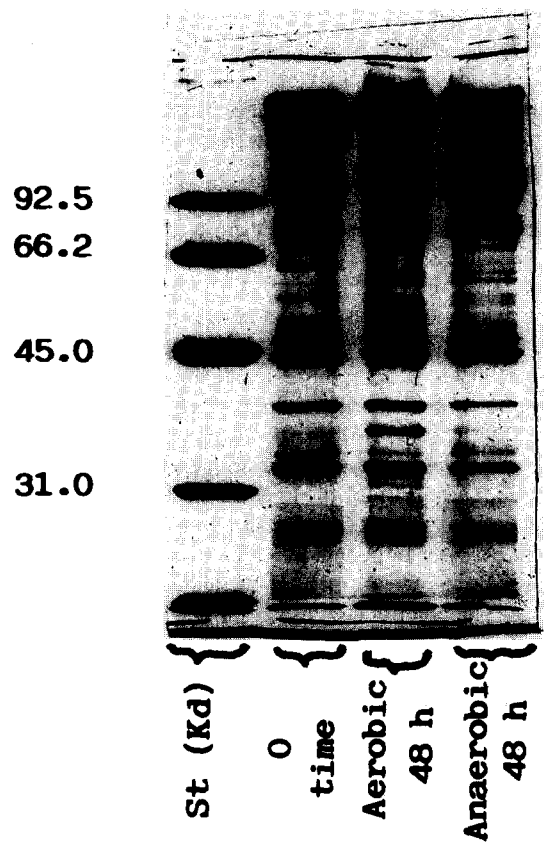

Fig. 1. Polyacrylamide gel electrophoresis of ghosts of erythrocytes incubated in phosphate buffer for 48 h under aerobic or anaerobic conditions.

in methemoglobin and decrease in cellular GSH. Therefore we set up a model of rapid in vitro ageing of erythrocytes to investigate whether a similar iron release could be detected during ageing and whether such a release could be related to the alterations of membrane proteins leading to the formation of senescent antigen. Since it has been reported [26–28] that the aerobic incubation in buffer markedly accelerates the ageing of erythrocytes as measured by vesciculation, we incubated calf erythrocytes in saline phosphate buffer for 24, 48 and 60 h under aerobic conditions and compared the results with similar incubates carried out under anaerobic conditions. As shown in Table 2, the aerobic incubation induced a marked and progressive release of iron, which was accompanied by an extensive methemoglobin formation and severe GSH depletion. A similar iron release was also seen in human erythrocytes incubated aerobically for 48 and 60 h (Table 1) and was equally accompanied by methemoglobin formation and GSH depletion (although less severe). On the other hand, no substantial iron release, nor extensive methemoglobin formation were seen when the incubation of calf erythrocytes was carried out under anaerobic conditions (Table 2). Also, the loss of GSH was much less severe than in aerobiosis. No substantial hemolysis was seen in both aerobic (2–4%) or anaerobic (4–7%) conditions, the low degree of lysis being probably due to shaking of the vessels and N_2 bubbling. Lipid peroxidation was never detected (not shown).

The electrophoretic analysis (Fig. 1) of the membrane proteins of the same calf erythrocytes incubated aerobically for 40 and 60 h shows the appearance of new bands in the molecular range of 66–45 kDal and the increase of a band below 31 kDal. Such alterations were not seen when the erythrocytes were incubated under anaerobic conditions (Fig. 1). Many of these bands are considered, as previously stated, an index of erythrocyte ageing and are thought to originate from the oxidative degradation of protein band 3. Therefore the aerobic incubation of erythrocytes induces alterations of membrane proteins similar to those known to occur during erythrocyte ageing.

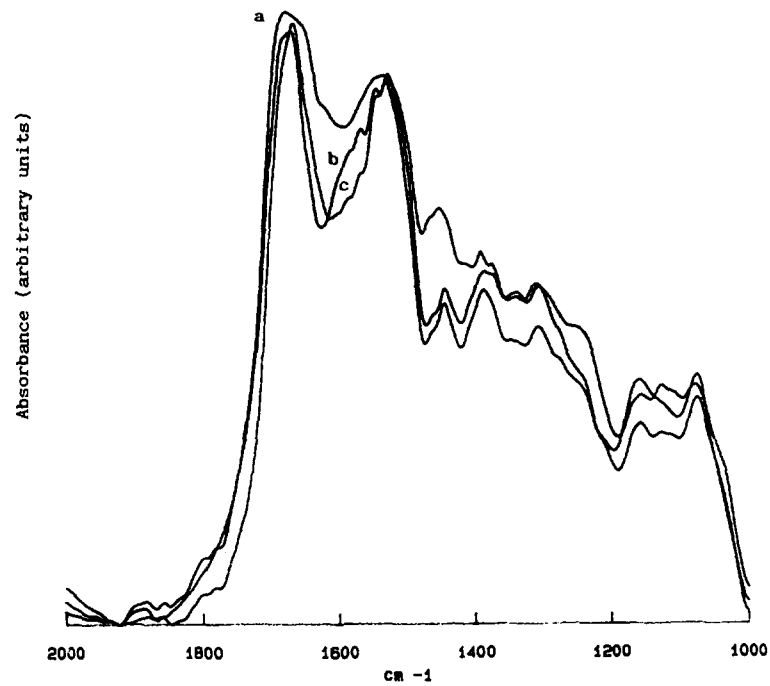

Fig. 2. IR spectra of ghosts proteins from erythrocytes incubated in phosphate buffer for 60 h under aerobic (a) or anaerobic conditions (b). The control (0 time) sample (c) is also reported. See section 2 for IR analysis conditions.

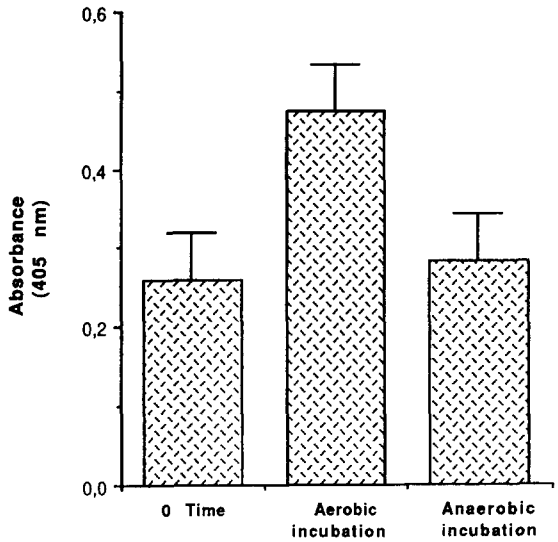

Fig. 3. Binding of autologous IgG to ghosts of erythrocytes incubated in phosphate buffer for 60 h under aerobic or anaerobic conditions. The erythrocyte ghosts (prepared at the end of the incubation) were incubated with autologous IgG as reported in section 2. The membranes were then washed and incubated with rabbit anti-bovine IgG antibodies, conjugated with alkaline phosphatase. After extensive washing, the substrate for alkaline phosphatase, *p*-nitrophenyl-phosphate, was added to the ghosts. The *p*-nitrophenol released from the substrate was measured spectrophotometrically.

Metal-catalysed oxidation of proteins has been described as a post-translational covalent modification which may be important in several physiopathological processes, including ageing [29–31]. Introduction of carbonyl groups into amino acid residues of proteins is a hall-mark for oxidative modification. It is known [32] that such carbonyl groups can be accurately detected by infrared (IR) spectrophotometric analysis of proteins. Therefore, we examined the proteins prepared from the ghosts of erythrocytes incubated under aerobic (aer) or anaerobic (anaer) conditions and of control, non incubated erythrocytes (0 time) by IR-spectroscopy. The spectra (Fig. 2) were recorded in the 2,000–1,200 cm^{-1} wavenumber range (Amide I, II e III regions). The spectrum of aer-sample (a) exhibits an increase of the C=O stretching absorption in the 1,685–1,650 cm^{-1} range with respect to that of 0 time sample (c), suggesting an increase of carbonyl groups. At the same time, a higher content of C=C bonds seems to occur in the aer sample with respect to the 0 time one, as evidenced by the intensity increase in the region centered at about 1,619 and 1,413 cm^{-1} absorptions (due to C=C stretching) in the spectrum. The possibility also exists that the increase of the intensity around 1,413 cm^{-1} absorption is due, at least in part, to the increase in the C-N bonds, which might results from oxidative processes leading to the formation of additional ring structures. All these alterations are consistent with protein oxidation according to the mechanism proposed by others [33].

In contrast to the aerobically incubated sample, the anaer sample (b) shows an IR spectrum very close to that of the control (0 time), indicating that anaerobiosis prevents, to a large extent, the chemical alterations mentioned above.

In order to investigate whether the protein alterations seen during the aerobic incubation give rise to the appearance of senescent cell antigen, the membranes of erythrocytes incubated under aerobic or anaerobic conditions were challenged with autologous IgG. As can be seen in Fig. 3, the membranes from aerobically incubated cells bind a substantially higher amount of autologous IgG than those from anaerobically incubated ones, indicating that the aerobic incubation gives rise to the senescent antigen.

Further studies were carried out in order to investigate whether the release of iron seen during the aerobic incubation could be related to the appearance of senescent antigen. Table 3 shows that the incubation of erythrocytes with phenylhydrazine, which induces a marked release of iron and methemoglobin formation, also promotes a considerable binding of autologous IgG to the erythrocyte membranes. This result, in addition to confirming the binding of IgG to phenylhydrazine-treated erythrocytes [16], further supports the idea that iron release is correlated to senescent antigen formation.

Additional support for the hypothesis that the release of iron is indeed causally related to the formation of senescent antigen was offered by experiments in which iron chelators were used in the attempt to chelate intracellularly the iron released during the aerobic incubation. The use of DFO was unsuccesful, since, contrary to what seen with mouse erythrocytes [12], the chelator does not enter at all calf or human erythrocytes. Therefore we used ferrozine, a Fe^{2+} chelator, which enters the erythrocytes reaching an equilibrium between the outside and the inside of the cells (see section 2). As shown in Table 4, the binding of autologous IgG to erythrocytes, that is the formation of senescent antigen during the aerobic incubation, was almost completely prevented in the presence of ferrozine, in spite of the fact that the amount of released iron and the level of methemoglobin formation were comparable to those seen in the absence of the chelator. It is therefore possible to postulate that intracellular ferrozine is capable to chelate immediately the relased iron and to prevent the membrane protein alterations possibly produced by its redox cycling and related to the antigen formation.In fact, as shown in Fig. 4, most of the alterations seen after the aerobic incubation (a) in the IR spectrum of ghost proteins were prevented in the presence of ferrozine (b). In particular, no increase in carbonyl groups was evident, nor an increase in C=C bonds, suggesting that no severe oxidative damage of proteins had occurred.

4. Discussion

In agreement with a number of previous studies [8–10], the present results clearly show that the senescent antigen is formed

Table 3
Iron release (DFO chelatable iron), methemoglobin (Met-Hb) formation and binding of autologous IgG to ghosts in erythrocytes incubated with phenylhydrazine (Phz)

	'Free' iron (nmol/ml)	Met-Hb (nmol/ml)	IgG binding (absorbance at 405 nm)
0 time	3.0	154	0.144
Incubated − Phz	4.6	154	0.136
Incubated + Phz	46.4	4531	0.332

Erythrocytes were incubated aerobically, at 37°C, in phosphate buffer for 2 h, in the presence or in the absence of 10 mM phenylhydrazine. Iron release and methemoglobin formation were determined and the ghosts were prepared as reported in section 2. Autologous IgG binding to ghosts was determined as reported in Fig. 3.

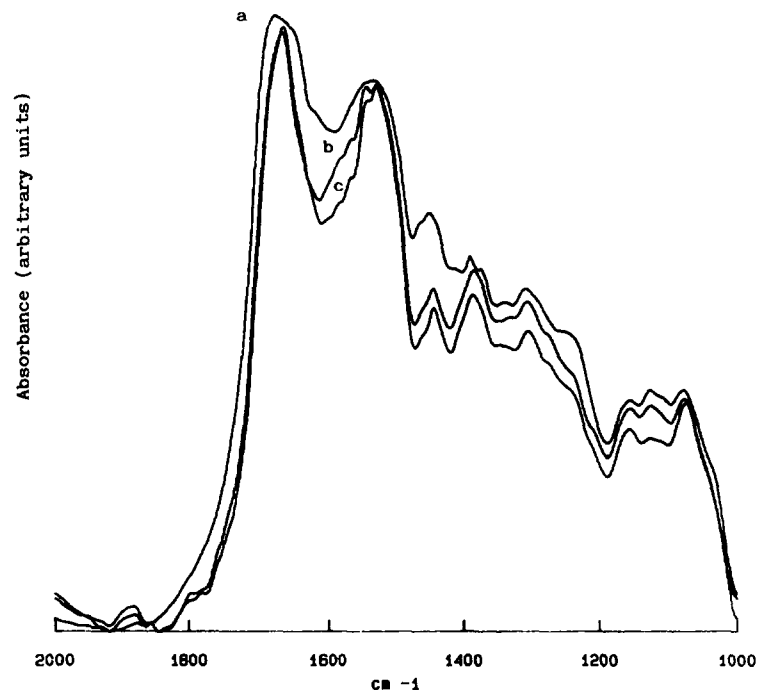

Fig. 4. IR spectra of ghost proteins from erythrocytes incubated in phosphate buffer for 60 h under aerobic conditions in the presence (b) or in absence (a) of ferrozine (1 mM). The control (0 time) sample (c) is also reported. See section 2 for IR analysis conditions.

in the erythrocyte membrane following an oxidative stress. In fact, in the model of erythrocyte ageing used in this study, the antigen is formed during the aerobic incubation, while its formation is prevented under anaerobic conditions. The formation of senescent antigen is accompanied by oxidative damage to membrane proteins leading to the formation of protein carbonyls as well as to the appearence of new electrophoretic bands, many of which likely originate from the fragmentation of protein band 3.

The interesting observation of the present work is that iron is released in a 'free' form during the aerobic incubation that results in senescent cell antigen formation, while no release occurs under anaerobic conditions in which no antigen is formed. A progressive and marked increase in 'free' iron content also occurs in erythrocytes aged for several weeks in their own plasma. In addition, the oxidizing agent phenylhydrazine induces both iron release and production of senescent antigen. It is therefore tempting to correlate the release of iron to the formation of senescent antigen, that is to the erythrocyte ageing. The present results clearly indicate that senescent antigen formation is prevented almost completely when the iron released during the aerobic incubation is chelated intracellularly by ferrozine. The presence of ferrozine during the aerobic incubation also prevents the alterations of membrane proteins, suggesting again that the release of iron is causally related to the oxidative damage of proteins and that such a damage finally results in the appearance of senescent antigen.

As is known, iron can be released in a 'free' form from its macromolecular complexes [34–38]. As mentioned above, in the erythrocyte an oxidative stress can lead to the release of iron in a redox active form as documented by its ability to induce lipid peroxidation in cell membranes [12–14].

As already stated, redox active iron, such as ADP/Fe^{3+} or xanthine/xanthine oxidase/Fe^{3+}, is able to induce the formation of senescent antigen by a moderate oxidation of membrane proteins [8]. In these experiments iron was acting from the outside of erythrocytes. It is therefore likely that iron released in a redox active form into the inside of the cell reaches the membrane proteins and causes the oxidation responsible for senescent antigen formation. Also it must be considered that GSH level of erythrocytes (and probably the antioxidant potential) is severely decreased following both the aerobic incubation and the storing in plasma.

The mechanism of metal-catalyzed oxidation of proteins has been extensively studied [29–31]. Briefly, a cation capable of

Table 4
Iron release (DFO-chelatable iron), methemoglobin (Met-Hb) formation and binding of autologous IgG in erythrocytes incubated aerobically in the presence of ferrozine

	'Free' iron (nmol/ml)	Met-Hb (nmol/ml)	Bound IgG No. of molecules per cell
0 time	1.6 ± 0.4	115 ± 30	3.3 ± 1.0
Aerobic incubation	26.1 ± 3.9	2363 ± 330	38.7 ± 9.7[a]
Anaerobic incubation	6.5 ± 0.6	344 ± 57	12.0 ± 3.4[b]
Aerobic incubation + ferrozine	23.0 ± 1.5	2361 ± 320	11.9 ± 5.5[c]

The erythrocytes were incubated aerobically or anaerobically for 60 h. Ferrozine was added at 1 mM concentration. Iron release and methemoglobin formation were determined as usually. Afterwards the erythrocytes were incubated with autologous IgG and then with ^{125}I-protein A. The radioactivity was measured by an automated γ-counter. See section 2 for additional explanations. Statistical significance for b – a, $P < 0.05$; c – a, $P < 0.05$.

redox-cycling (e.g., Fe^{2+}/Fe^{3+}) binds to a divalent cation binding site on the protein. Reaction with O_2 or H_2O_2 generates active oxygen species which oxidizes amino acid residues at or near that cation binding site. It is likely that the released iron is bound by the cation binding sites on proteins and that oxygen radicals are formed at those specific sites inducing damage primarily in the amino acid residues. The results of our IR spectroscopy studies are compatible with the scheme suggested by Dean et al. [33] for metal catalyzed oxidation of protein-bound 3,4-dihydroxyphenylalanine (DOPA), which is formed by hydroxyl radical-induced hydroxylation of tyrosine.

An additional comment is needed for the results obtained with ferrozine. As is known [20], ferrozine chelates Fe^{2+} and therefore the iron released during the aerobic incubation should be in the +2 form. However, as mentioned above, iron released from hemoglobin should be in the +3 form. It possible that the high concentration of reductants in the erythrocyte (ascorbic acid etc.) can reduce immediately iron thus rendering it accessible to ferrozine.

In conclusion the release of iron from hemoglobin may be a primary factor in the physiological ageing of erythrocytes. A mild but continous oxidation of hemoglobin may produce a continous iron release and consequent oxidative modifications of membrane proteins. The latter events may result in senescent antigen formation and IgG binding. The progressive iron release seen in erythrocytes stored in their own plasma supports such a view. Therefore iron release may be an important factor to be dealt with in blood storing.

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