Isolation and partial characterization of antibody- and globin-enriched complexes from membranes of dense human erythrocytes

Rama KANNAN, Jie YUAN and Philip S. LOW*

Department of Chemistry, Purdue University, W. Lafayette, IN 47907, U.S.A.

In previous studies we have described a process whereby an erythrocyte in biochemical distress can initiate its own removal by macrophages of the reticuloendothelial system. This process involves the clustering of the integral membrane protein band 3 by denatured haemoglobin and the subsequent recognition of the exofacial poles of clustered band 3 and associated proteins by autologous antibodies. To determine whether this clearance pathway might mediate normal cell turnover, the fraction of normal erythrocytes containing the 0.5 % densest cells, which are known to be destined for immediate removal, was isolated and characterized biochemically. This densest fraction was found to contain 6 times more membrane-bound globin (haemichromes) and 10 times more surface-bound autologous IgG than the other fractions containing cells of lower density. To determine whether the autologous IgG was physically associated with the haemichrome-stabilized membrane protein clusters, a procedure was developed for isolation and characterization of the microscopic aggregates. The isolated aggregates were found to contain a disulphide-cross-linked mixture of several membrane proteins, predominantly haemichromes, spectrin and band 3. Although the aggregates constituted only 0.09 % of the total membrane protein, they still contained ~ 55 % of the total cell-surface IgG. Since in control studies anti-(blood group A) antibodies, which are distributed randomly over the surface of type A cells, could not be recovered in the aggregate, we conclude that the autologous cell-surface IgGs were physically associated with the membrane protein clusters when they were co-isolated with them in our procedure. Thus the 640-fold enrichment of autologous IgG in the aggregates compared with regions of the membrane devoid of tightly clustered protein suggests that sites of integral protein clustering either are non-specifically sticky to IgG or are viewed as foreign or 'non-self' by the immune system and aggressively opsonized with IgG.

INTRODUCTION

We have presented a hypothesis which proposes that the lifespan of the circulating erythrocyte is determined, at least in part, by the stability of its encapsulated haemoglobin [1–5]. As pointed out by Morrison *et al.* [6], shortly before an erythrocyte is removed from the circulation, large amounts of globin precipitate on the membrane. We have found that this denatured haemoglobin (haemichrome) exhibits a high affinity for the cytoplasmic domain of band 3, associating with it in a manner that rapidly results in formation of copolymer [2,3,7]. This macroscopic band-3–haemichrome copolymer has been shown to manifest itself *in situ* as a globin-induced aggregate of band 3, where band 3 is clustered in the membrane directly over sites of haemichrome binding [1,8]. In sickle cells, where quantitative measurements have been made, the microscopic aggregates of band 3 and associated proteins bind autologous IgG, concentrating ~ 75 % of the total cell-surface IgG at regions comprising only ~ 1 % of the cell surface area [7]. Because artificial clustering of band 3 *in situ* can also strongly stimulate autologous IgG binding [1,9], we have postulated that localized sites of integral protein redistribution are somehow viewed as 'non-self', or antigenic, by the immune system. Once opsonized by autologous antibodies, these sites presumably trigger complement deposition [1] and macrophage recognition [10–12], and thereby promote elimination of the aged erythrocyte from circulation. Because the low quantities of denatured haemoglobin necessary to initiate this process are generated in many bio-

chemical defects [2,3,13–16], the above mechanism assures that an erythrocyte is removed as soon as it begins to falter biochemically.

While many aspects of the above hypothesis have been confirmed in sickle cells [7,8] and phenylhydrazine-treated cells [1], in which haemoglobin denaturation and cell removal occur rapidly [17,18], little evidence for the proposed mechanism currently exists for normal cells, in which globin binding to the membrane is only observed directly before cell removal [6]. For this reason, we decided to isolate from normal blood a population of erythrocytes enriched in cells destined for immediate elimination. The most dense 1 % of erythrocytes exhibit this unusual property, disappearing from the circulation immediately after they are re-infused into the donor [19,20]. Thus, while the densest erythrocyte population may not necessarily constitute the oldest cells *in vivo*, they do at least represent a population this is efficiently recognized by phagocytosing cells. We report here that this dense population is also characterized by the presence of membrane-bound haemichrome aggregates and that these aggregates, which comprise only 0.1 % of the total membrane protein, nevertheless contain the majority of cell-surface IgG.

MATERIALS AND METHODS

Materials

Fresh human blood was obtained from the Central Indiana Regional Blood Bank in citrate/dextrose solution and was used within 10 days of withdrawal. Octaethylene glycol mono-n-

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; GAR-IgG, goat anti-rabbit immunoglobulin G; GAH-IgG, goat anti-human immunoglobulin G; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4); IOVs, inside-out vesicles; TBS, Tris-buffered saline (20 mM-Tris/500 mM-NaCl, pH 7.4); HRP, horseradish peroxidase; C₁₂E₈, octaethylene glycol mono-n-dodecyl ether.

* To whom correspondence should be addressed.

dodecyl ether ($C_{12}E_8$) was obtained from Nikko Chemicals Co., and phenylmethanesulphonyl fluoride (PMSF) was purchased from Research Organics. Iodo-Beads were obtained from Pierce Chemical Co., and $Na^{125}I/NaOH$ was obtained from Amersham. Nitrocellulose of pore size $0.2 \mu m$ was purchased from Schleicher and Schuell. PD-10 desalting columns and goat anti-rabbit IgG (GAR-IgG) were obtained from Bio-Rad, and goat anti-human IgG (GAH-IgG) was purchased from Miles Biochemicals. The latter antibody reacts with the heavy chain of human IgG, but not with IgM or IgA. Crude stractan was purchased from Sigma, and purified further as described [21]. Anti-band 2.1, anti-band 4.1 and anti-(glycophorin C) antibodies were kindly provided by Bernard Thevenin and Chris Lombardo of our laboratory. All other chemicals were reagent grade or better and were purchased from major suppliers.

Isolation of protein aggregates from density-fractionated erythrocytes

Normal human blood was washed in phosphate-buffered saline (PBS) to remove the plasma and buffy coat and then fractionated on iso-osmotic stractan gradients with densities ranging from 1.07 to 1.11 g/litre, according to the method of Corash *et al.* [21]. After pouring the above gradient into centrifuge tubes, 2.5 ml of erythrocytes at 60 % haematocrit in PBS was layered on top and the tubes were spun at 62 500 *g* in an SW 28.1 rotor for 45 min. The densest fraction of erythrocytes, which constituted less than 1 % of the total cells, was washed thoroughly in PBS to remove any adhering stractan. Fractions of other densities, when desired, were treated similarly.

Protein aggregates from the densest erythrocytes and from unfractionated (control) erythrocytes were isolated as described earlier [7]. Briefly, immediately after removal from the stractan gradients, erythrocytes were lysed by washing 3 times in 40 vol. of cold 5 mM-sodium phosphate, pH 8, in the presence of PMSF (20 $\mu g/ml$). The derived membranes were immediately depleted of spectrin and actin by incubating at 37 °C for 30 min with 40 vol. of 0.5 mM-EDTA, and the resulting inside-out vesicles (IOVs) were collected by centrifugation at 35 000 *g*. The protein content of the various IOV fractions was then determined by the method of Lowry *et al.* [22].

Dense and control IOVs of equal protein concentrations (~ 6.2 mg/ml) were then incubated on ice in 5 vol. of 5 mM-sodium phosphate, pH 8.0, containing 1 % $C_{12}E_8$ (18.5 mM) with occasional mixing on ice for 10 min. The insoluble aggregate was collected by ultracentrifugation at 70 000 *g* for 1 h in an SW 28.1 rotor and washed in 3×10 ml of 5 mM-sodium phosphate, pH 8.0, to remove any loosely bound detergent.

Iodination of GAH-IgG

Affinity-purified GAH-IgG was iodinated using Iodo-Beads and $Na^{125}I/NaOH$ [23], with a specific radioactivity of 100 $\mu Ci/\mu l$, as follows. Iodo-Beads were washed thoroughly in PBS, pH 7.4, and dried on filter paper. To three Iodo-Beads in a Microfuge tube was added 0.75 ml of PBS, pH 7.4, and 300 μCi of $Na^{125}I$ solution. The mixture was allowed to sit at room temperature for 15 min, after which 100 μl (250 μg) of affinity-purified GAH-IgG was added. Following incubation with constant mixing at room temperature for 30 min, the free iodine was removed by passing the solution down a PD-10 desalting column. The resulting fractions were then assayed for protein content, and the radioactivity was measured using a Beckman V-4000 counter.

Quantitative analysis of autologous IgG on density-fractionated erythrocytes and their derived aggregates

Thoroughly washed density-fractionated erythrocytes were

incubated at 50 % haematocrit in PBS for 3 h with 25 μg of ^{125}I -labelled GAH-IgG/ml (1.46×10^5 c.p.m./mg), and the unbound antibody was removed by washing 3 times in PBS containing 1 % BSA. Spectrin-depleted IOVs were then prepared, assayed for protein content and counted in a Beckman V-4000 γ -radiation counter to quantify the tightly bound ^{125}I -GAH-IgG. Detergent-insoluble aggregates were then prepared as described above and the ^{125}I -GAH-IgG content in the derived aggregate was also determined by γ -radiation counting. The amount of ^{125}I -GAH-IgG bound per ghost was calculated from the c.p.m./mg of protein in the IOVs, assuming that 0.65 mg of vesicle protein is obtained from 1 mg of ghost protein, as determined experimentally.

Quantification of membrane-associated globin

The fractions of whole blood of various densities were collected as described above and then lysed and washed four times in 5 mM-sodium phosphate, 1 mM-EDTA and 20 μg of PMSF/ml, pH 8.0, to prepare membranes. SDS/PAGE was performed on these samples using 40 μg of protein per lane, as described by Laemmli [24]. The running gel contained 12 % polyacrylamide and the stacking gel 6 %. After electrophoresis, the gels were stained with Coomassie Blue, and the membrane-associated globin was determined by densitometry of the 16 kDa haemoglobin band using a Shimadzu CS-9000 dual-wavelength scanner.

Analytical procedures

The aggregates were solubilized in SDS electrophoresis buffer containing 5 % 2-mercaptoethanol, sonicated for 5 s to disrupt tightly associated proteins, heated for 10 min in a boiling water bath and analysed on 6–12 % gradient polyacrylamide gels in the presence of SDS according to the method of Laemmli [24]. For immunoblotting analyses, proteins were transferred to nitrocellulose using the buffer system of Towbin *et al.* [25] and blocked with 3 % gelatin in Tris-buffered saline (TBS), pH 7.4. The nitrocellulose strips were then rinsed with TBS, pH 7.4, containing 0.05 % Tween 20, and labelled with the desired antibody diluted in TBS. After further washing and labelling with the second antibody conjugated to horseradish peroxidase (HRP), the immunoblots were developed using 4-chloronaphthol as substrate [26].

RESULTS

Because of their very short life expectancy, the densest ~ 0.5 % of normal erythrocytes were isolated on stractan gradients and examined for features which might predispose them to rapid phagocytosis by macrophages. As shown in Table 1, this fraction of cells was characterized by a significantly greater number of bound cell-surface IgGs, exceeding that in the next most dense cell fraction by more than 10-fold. Importantly, when membranes were prepared from these same cells, separated electrophoretically on SDS/polyacrylamide slab gels and then scanned by densitometry to quantify the abundance of each polypeptide, membrane-bound haemoglobin was also seen to rise precipitously in the same fraction. Thus, although the gross polypeptide profiles of the membranes of the dense and the lighter control cells were similar except for the band 4.1a/4.1b ratio, the quantity of bound globin in the former fraction surpassed that of all other fractions by at least 6-fold (Table 1). This crude correlation between cell-associated autologous IgG and membrane-bound globin is consistent with the proposed causal relationship between the two parameters described previously [1,4,5].

To learn whether the cell-surface IgG might be directly associated with globin-containing membrane protein aggregates,

Table 1. Comparison of IgG binding and membrane-associated haemoglobin content of density-separated erythrocytes

Density-fractionated erythrocytes were analysed for cell-bound IgG by measuring the content of ¹²⁵I-GAH-IgG as described in the Materials and methods section. The same erythrocyte fractions were also lysed in 5 mM-sodium phosphate/1 mM-EDTA, pH 8.0, and the membrane-associated haemoglobin content in 40 µg of membrane protein was determined by SDS/PAGE followed by densitometry. The various fractions are as follows: control (unfractionated) cells: fraction 1, $d < 1.08$, containing the lightest 44 % of cells; fraction 2, $1.080 < d < 1.0875$, containing the next 49 % of cells; fraction 3, $1.0875 < d < 1.095$, containing the next 6 % of cells; fraction 4, $1.095 < d < 1.103$, containing the next 0.5 % of cells; fraction 5, $1.103 < d$, containing the densest 0.5 % of cells.

Fraction no.	Proportion of total cells (%)	IgG (molecules/cell)	Globin content (µg/10 ⁹ cells)
Control	100	20	2.9
1	44	21	2.1
2	49	27	3.0
3	6	20	2.7
4	0.5	20	4.8
5	0.5	283	30.0

Fig. 1. Isolation of detergent-insoluble aggregates from erythrocytes (a) Determination of optimal detergent concentration for isolating detergent-insoluble aggregates from unfractionated erythrocytes. IOVs (10 mg) prepared from unfractionated erythrocytes were incubated with a 5-fold volume excess of 5 mM-sodium phosphate, pH 8.0, containing various concentrations of C₁₂E₈ on ice for 10 min with occasional mixing. The samples were then examined in an IBM u.v./visible spectrophotometer, model 9420, at 550 nm to determine the turbidity. The data from two separate samples are plotted, with the line intersecting the midpoint of the two values. (b) Determination of optimal centrifugation conditions for isolating detergent-insoluble aggregates from dense and control IOVs. Dense and control IOVs (2 ml; ~ 6.2 mg/ml) were treated with detergent as described in the Materials and methods section. The samples were then centrifuged at various g forces for 1 h and the aggregate concentrations were determined as described. The data from two independent preparations are plotted, with the line intersecting the midpoint of the two values.

Fig. 2. Gel electrophoresis of ghosts from normal and dense erythrocytes and of the detergent-insoluble macromolecular aggregates isolated from equal amounts of control and the 0.5% densest fraction of erythrocytes

The total protein aggregate isolated from 14 mg of protein of the 0.5% densest and also unfractionated (control) erythrocyte membrane IOVs was suspended in 2 vol. of SDS electrophoresis buffer containing 5% 2-mercaptoethanol, sonicated for 5 s to disrupt tightly associated proteins, heated for 10 min in a boiling water bath, and analysed on 6–12% gradient polyacrylamide gels according to the method of Laemmli [24]. Lane a, dense erythrocyte ghosts (60 µg); b, control erythrocyte ghosts (60 µg); c, dense erythrocyte IOVs (60 µg); d, control erythrocyte IOVs (60 µg); e, dense erythrocyte aggregates (30 µg); f, control erythrocyte aggregates (not detectable). Hb, haemoglobin.

as observed previously in sickle cells [7], a method for isolating the detergent-insoluble aggregates was developed. IOVs were first prepared from unfractionated (control) erythrocytes and then treated with increasing concentrations of a non-denaturing detergent (C₁₂E₈) to determine where maximal solubilization of normal vesicles might occur. As seen in Fig. 1(a), all C₁₂E₈ concentrations greater than 10 mM were effective in dissolving the vesicles, leading to a suspension which yielded no pelletable material even after centrifuging at speeds as great as 80 000 g (Fig. 1b). Therefore 18.5 mM-C₁₂E₈ (1%) was chosen for all subsequent studies, since it not only achieved the desired solubilization but also avoided the denaturing effects of high concentrations of C₁₂E₈ detergent.

Treatment of IOVs from the densest fraction of erythrocytes with 18.5 mM-C₁₂E₈ yielded vastly different results from those obtained with control vesicles. Not only did the detergent solution remain slightly turbid, but upon centrifuging at speeds greater than 50 000 g, a pellet was invariably obtained which constituted ~ 0.1% of the total membrane protein (Fig. 1b). This high-density pellet was brown in colour, as noted previously for the haemichrome-enriched membrane aggregates from sickle cells [7], and relatively stable, as indicated by its ability to remain intact through three washings. The pellet was also fairly heterogeneous, as shown by the variety of bands in its SDS/polyacrylamide gel profile, and contained several of the major erythrocyte membrane polypeptides, together with a series of new bands not corresponding to any major proteins seen in whole ghost preparations (Fig. 2, compare lanes b and e). As anticipated, a densitometric scan of lane e showed the globin monomer to be the most abundant single species (19%), followed by spectrin (16%) and band 3 (15%). All other identifiable components contributed less than 20% to the integrated Coomassie Blue staining intensity (Table 2). Importantly, if the membrane-associated haemichromes (globin) were first removed

Table 2. Relative content of major erythrocyte membrane proteins in insoluble aggregates isolated from the 0.5% densest erythrocytes

Data were obtained by densitometric scanning of the gel in Fig. 2, lane e.

Protein	Relative content (% of total protein)
Spectrin ($\alpha + \beta$)	16
Ankyrin	3
Band 3	15
Band 4.1	1
Band 5	5
Band 6	5
Band 7	6
Globin	19
Unidentified polypeptides	30

Fig. 3. Immunoblotting analysis of the presence of erythrocyte membrane proteins in the insoluble aggregate isolated from IOVs derived from the densest 0.5% of cells and from control cells

Dense-cell IOVs (14 mg) were processed to yield ~ 30 µg of insoluble aggregate, which was directly loaded on to a polyacrylamide gel. Control cells usually yielded no detectable aggregate when processed similarly. After PAGE, the proteins were Western-blotted on to nitrocellulose and the nitrocellulose strips were incubated in the appropriate primary rabbit antibody for 3 h followed by GAR-HRP for 3 h. The primary antibodies used are specific for (a) band 3, (b) band 4.1, (c) ankyrin, and (d) glycophorin C (GC). The lanes contain: a, control ghosts (40 µg); b, dense cell aggregates (30 µg); c, control erythrocyte aggregates isolated from 14 mg of IOVs derived from unfractionated blood (results not detectable). In blot (d), 22 µg of aggregate was isolated from 12 mg of dense-cell IOVs, and this amount of aggregate was loaded on to the gel.

by incubating the IOVs in 100 mM-sodium phosphate, pH 8, for 20 min [7], no membrane protein aggregate could be harvested. This strong dependence on the presence of haemichromes for stability of the aggregate is consistent with the proposed role of haemichromes in generating the aggregate by clustering of band 3 and associated proteins [1–4,7].

Because several of the major membrane proteins were not highly resolved in the aggregate lane (Fig. 2, lane e), we decided to confirm their presence by staining immunoblots of the aggregates with antibodies to each protein. Figs. 3(a)–3(d) compare the staining patterns of monospecific antibodies to band 3, band 4.1, ankyrin and glycophorin C in blots of normal ghosts and of the dense cell aggregates. Not only are all four polypeptides represented in the aggregates, but their banding patterns also appear to be similar to the patterns seen in normal membranes. Thus, while the detergent-resistant aggregates may exhibit an altered protein composition and organization, the individual polypeptides seem to be neither heavily cross-linked nor unusually digested, at least if the samples are pretreated with 5% 2-mercaptoethanol. Importantly, under non-reducing conditions,

Table 3. Estimation of the percentage of autologous cell-surface IgG associated with dense cell aggregates

The aggregate-associated GAH-IgG represents the number of GAH-IgGs bound/cell. If more than one GAH-IgG can opsonize a surface-bound autologous IgG, as expected, then the total number of autologous IgGs will be much lower than this number. The enrichment factor represents the fraction of IgG in the aggregate ($f_{\text{IgG}}^{\text{A}}$) normalized to the fraction of protein in the aggregate ($f_{\text{Prot}}^{\text{A}}$) divided by the fraction of IgG in the remainder of the membrane ($f_{\text{IgG}}^{\text{M}}$) normalized to the fraction of protein in the remainder of the membrane ($f_{\text{Prot}}^{\text{M}}$) as described in the following equation:

$$\text{Enrichment factor} = \frac{f_{\text{IgG}}^{\text{A}} \cdot f_{\text{Prot}}^{\text{M}}}{f_{\text{IgG}}^{\text{M}} \cdot f_{\text{Prot}}^{\text{A}}}$$

Donor no.	Total GAH-IgG/cell	Aggregate-associated GAH-IgG (no./cell)	IgG concentrated in the aggregate (%)	Membrane protein in the aggregate (%)	Enrichment factor
1	475	279	59	0.065	907
2	546	296	54	0.092	586
3	220	112	51	0.100	510
Mean	414	229	55	0.086	640

few polypeptides were seen to enter the gel (results not shown), suggesting that the aggregates are strongly stabilized by disulphide bonds. However, after reduction, solubilization in SDS and brief sonication, little evidence of residual aggregation can be detected, except for trace quantities of band 3 dimer seen above the monomer in the immunoblots (Fig. 3a).

As mentioned previously, a major goal of this study was to evaluate whether the haemichrome-containing aggregates present in the densest erythrocyte fraction were enriched in autologous IgG, like the corresponding aggregates in sickle cells [7]. To obtain this information, dense erythrocytes were incubated with ^{125}I -labelled GAH-IgG to allow quantification of cell-surface autologous IgG. Importantly, the specificity of this GAH-IgG antibody was confirmed by showing that it blotted a ~ 165 kDa IgG band and a 55 kDa heavy chain band in an immunoblot of the aggregates isolated from dense cells. After thoroughly washing the ^{125}I -GAH-IgG-labelled cells, IOVs and detergent-insoluble aggregates were prepared (see the Materials and methods section) and then counted for radioactivity. The number of autologous IgGs in the aggregate was calculated assuming that one goat anti-human IgG corresponded to one autologous IgG. The number of IgGs on the original cell surface was calculated from the c.p.m./mg of protein value in the IOVs, assuming that 1 mg of intact membrane protein corresponded to 0.65 mg of IOV, as determined experimentally. The reason for not measuring the c.p.m. on the intact cells directly was that some loosely associated IgGs (presumably non-specifically bound) were found to dissociate during ghost and IOV preparation. Because the nature of these easily elutable antibodies was unknown, we felt it would be wisest to assign them neither to the aggregate nor to the non-aggregated regions of the membrane.

Table 3 shows the total IgG/cell and the IgG/aggregate values, calculated as described above. The dense cells from three different donors contained on average 414 IgGs/cell. The detergent-insoluble haemichrome-enriched aggregates from the same cells contained ~ 229 IgG molecules, or ~ 55% of the total tightly bound IgG on the cell. Since the above aggregates comprised only 0.09% of the total membrane protein, it can be calculated that they are enriched more than 600-fold in auto-

logous IgG compared with the residual cell surface. It can, therefore, be concluded that these regions of abnormal integral protein distribution represent major sites of IgG binding on dense erythrocyte cell surfaces.

Finally, to ensure that unrelated antibody-antigen complexes that are not associated with haemichrome-stabilized membrane protein aggregates do not co-sediment with the aggregates, the same aggregate isolation procedure was conducted with control cells of blood type A opsonized with ~ 2000 anti-A antibodies per cell. Because the anti-(blood group A) antibodies would be expected to distribute randomly over the erythrocyte surface, few if any of these IgGs would be anticipated to be found in the detergent-insoluble pellet. Consistent with this view, the fraction of anti-A IgGs in the derived pellet was invariably less than 5% of the fraction in the parent IOVs. Thus random IgGs on a cell surface do not sediment at 70000 g unless they are directly associated with a macroscopic protein aggregate.

DISCUSSION

In an effort to understand erythrocyte senescence, we have isolated a fraction of normal erythrocytes destined for immediate removal and have inquired as to the properties that might distinguish these cells from longer-lived cells in the same blood. As noted by other investigators, the densest fraction of erythrocytes was found to contain elevated levels of cell-surface autologous IgG [27] and membrane-bound haemichromes [28,29]. More importantly, however, these two compositional abnormalities were shown to be structurally related, in that they co-varied with erythrocyte density and could be co-isolated in the same immune complexes. This co-localization suggests either that the clustered membrane sites are non-specifically sticky to IgG or that the immune system views the haemichrome-enriched protein aggregates as non-self and responds by opsonizing the sites with IgG. Either way, because a clustered distribution of opsonizing antibodies on a cell surface is especially potent in triggering phagocytosis [30], the presence of haemichrome-induced protein aggregates should ensure rapid removal of the dying cell from the circulation. Taken together, the above observations describe a pathway by which a biochemical distress signal can be communicated from the cell interior to the reticuloendothelial system to promote removal of the faltering erythrocyte.

In view of the prominent role of haemoglobin denaturation in our senescence hypothesis, it is reasonable to compare the fraction containing the 0.5% densest erythrocytes with sickle cells, which also contain denatured haemoglobin and exhibit a shortened life expectancy [18,31]. As with dense erythrocytes, sickle cell haemoglobin precipitates on the cell membrane and forms disulphide-cross-linked aggregates with several membrane proteins, predominantly band 3 [7]. Although the aggregate in sickle cells is somewhat larger, sedimenting easily at 38000 g, it has a similar composition and is analogously dependent on association with haemichromes for stability. Thus if sickle cell haemichromes are first stripped from the membranes before aggregate isolation, no membrane proteins can be sedimented [7]. Furthermore, as with dense cells, the aggregates from sickle cells are highly enriched in antibodies, containing ~ 75% of the cell-surface IgG in only 1% of the membrane protein. Consistent with this premature deposition of clustered IgG over sites of haemichrome-induced protein aggregation, sickle cells also have a life expectancy of only 23 days [18], i.e. one-fifth of that of normal cells.

Although the role of antibody deposition in marking an aged/alterd erythrocyte for phagocytosis has been emphasized

here, one should not ignore other possible perturbations initiated by haemoglobin denaturation that might also promote macrophage recognition [2-5]. Thus haemichrome-containing cells are more rigid than normal [32,33], they leak cations more profusely [34-36], they generate reactive oxygen species more abundantly [37,38], their phospholipid asymmetry is often reduced [39], and their membrane skeletal structure is frequently abnormal [40]. To what extent these other lesions contribute to clearance of aged erythrocytes is still unclear. However, it would be very surprising if a process as essential to organism survival as aged/abnormal erythrocyte removal were not backed-up by one or more alternative pathways.

Probably the most reliable marker of a biologically old erythrocyte is a small increase in the band 4.1a/4.1b ratio [41,42]. In hypertransfused mice, which produce no new erythrocytes during hypertransfusion, the fraction of band 4.1a was found to increase from ~ 10% to 54% over the entire cell lifespan [41]. Densitometry scans of the membranes of our densest erythrocytes revealed a band 4.1a/total band 4.1 ratio that was ~ 25% higher than that in unfractionated blood. Although neither the source nor the consequence of this shift in the band 4.1 ratio is understood, the data at least suggest that dense cells are older than the average erythrocytes in circulation. Whether the densest 1% of cells are the absolute oldest cells chronologically is probably unimportant, since they are treated by the immune system as being biologically old and are rapidly removed [19,20].

Although we have identified a process by which membrane proteins can be clustered in senescent cells, the observation that a small fraction of integral membrane proteins might be abnormally distributed in such erythrocytes is also in accordance with other reports. Thus Brovelli *et al.* [43] have isolated a high-molecular-mass SDS-resistant disulphide-cross-linked aggregate of unidentified composition from aged erythrocytes *in vitro*. Gaczynska & Bartosz [44] further report that band 3 protein can be more readily cross-linked by cupric di-(1,10-phenanthroline) in old cells than in young cells. Also, Marikovsky & Danon [45] observe that colloidal iron stains young cell surfaces with an even distribution, but that electron micrographs of old erythrocytes show some regions of clustered cationic particles. We believe that all of these observations are causally related by the strong tendency of denatured haemoglobin to aggregate band 3 and associated proteins [2,3,8]. Perhaps the ability of phenylhydrazine, a potent haemoglobin denaturant, to promote rapid erythrocyte clearance [17] is also related to this pathway.

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