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Band 3/Complement-mediated Recognition and Removal of Normally Senescent and Pathological Human Erythrocytes

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Key Words

Erythrocyte • Band 3 • RBC • Erythrocyte senescence • G6PD-deficiency • Falciparum malaria • Malaria anemia • Hemolytic anemia

Abstract

Band 3 modifications that normally occur during physiological red blood cell (RBC) senescence in humans, and occasionally in pathological conditions are described in the context of their role in enhancing RBC recognition and phagocytic removal. Band 3 modifications are mostly due to oxidative insults that gradually accumulate during the RBC lifespan or impact massively in a shorter time period in pathological conditions. The oxidative insults that impact on the RBC, the protective mechanisms that counteract those damages and the phenotypic modifications that accumulate during the RBC lifespan are described. It is shown how specific oxidative as well as non-oxidative band 3 modifications enhance RBC membrane affinity for normally circulating anti-band 3 antibodies, and how membrane-bound anti-band 3 antibodies bring about a limited complement activation and membrane deposition of complement C3 fragments. The partially covalent complexes between anti-band 3 antibodies and complement C3 fragments are very powerful opsonins readily recognized by the CR1 complement receptor on the phagocyte. Band 3 modifications typically encountered in old RBCs have crystallized to a number of band 3-centered models of RBC senescence. One of those band 3-centered models, the so-called "band 3/complement RBC removal model" first put up by Lutz et al. is discussed in more detail. Finally, it is shown how the genetic deficiency of glucose-6-phosphate dehydrogenase (G6PD) plus fava bean consumption, and a widespread RBC parasitic disease, *P. falciparum* malaria, may lead to massive and rapid destruction of RBCs by a mechanism comparable to a dramatic, time-compressed enhancement of normal RBC senescence.

Introduction

Until the end of its life span of 120 ± 4 days with 120 miles of travel and $1.7 \cdot 10^5$ circulatory cycles the human RBC successfully copes with a number of dangers, such as passage across narrow capillaries and splenic slits, periodic high turbulence and high shear stress, and extremely hypertonic conditions. The external RBC

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surface is non-immunogenic and non-adhesive, to avoid adhesion to endothelia and phagocytosis by spleen, liver and bone marrow macrophages, ready to phagocytose any cell showing even subtle membrane alterations. Internally, the continuous formation of oxidant species during the oxygenation-deoxygenation cycles of hemoglobin (Hb) are opposed by powerful detoxication and protection mechanisms. Mechanical stresses are met by a resilient, mechanically rugged yet flexible and highly deformable membrane envelope and underlying cytoskeleton, while a complex system of pumps and transporters counterbalances osmotic changes.

The adult RBC lacks protein synthesis and is unable to restore inactivated enzymes or damaged cytoskeletal and membrane proteins. To cope with these inabilities, the RBC is equipped with high activity of protective enzymes fully adequate to sustain even excessive oxidative stress for limited time periods. Table 1 shows data on metabolic fluxes, enzyme activities and metabolite levels and briefly summarizes RBC metabolism. It is of note that at the end of RBC life span, its enzyme activities, ATP and other crucial metabolites are still present in sufficient amounts and do not justify RBC death. What determines the RBC demise is not its fragmentation but its rapid transformation into a non-self cell, recognized as such by the phagocytic system and removed. This transformation is likely due to rather subtle modifications of the RBC membrane and expression of neo-antigenic sites that lead to opsonization or direct recognition by phagocytes. A number of inherited or acquired RBC defects accelerate or magnify the normal process of senescence and enhance the precocious removal of chronologically young, yet immunologically and biologically old cells. The term "hemolytic anemia" usually adopted to indicate enhanced RBC removal is misleading, because in most cases RBCs do not lyse but are removed by phagocytosis.

In the following, we will first briefly summarize the kind of (mostly) oxidative insults that impact on the RBC membrane and will describe the protective mechanisms that counteract those damages. We will then describe the phenotypic modifications that accumulate during the RBC lifespan eventually transforming the viable RBC into a non-self cell bound to phagocytic removal. We will show how one or more of the modifications typically encountered in old RBC have crystallized to "models" of RBC senescence. We will then concentrate on the description of one of those model, the so-called "band 3/ complement RBC removal model". Finally, we will show how the genetic deficiency of glucose-6-phosphate dehydrogenase (G6PD) plus fava bean consumption, and a widespread RBC parasite disease, falciparum malaria, may lead to massive and rapid destruction of RBC by a mechanism comparable to a dramatic enhancement of the normal RBC senescence (Table 1).

Cumulative Damages to the RBC During its Lifespan

During the binding of oxygen to form oxy-Hb, one electron is transferred from iron to the bound oxygen forming a ferric-superoxide anion complex. The shared electron is normally returned to the iron when oxygen is released during deoxygenation. However, a fraction of the electrons remains and transforms oxygen into a superoxide anion radical (superoxide or O_2^-). In this process, iron is left in the ferric state and Hb is transformed into met-Hb. The autoxidation of Hb occurs spontaneously and transforms 0.5–3% of Hb into met-Hb per day. In addition to this physiological process, met-Hb may be generated by endogenous oxidants, such as hydrogen peroxide (H_2O_2), nitric oxide (NO) and hydroxyl radicals ($OH^\bullet$), formed by granulocytes/monocytes or endothelial cells (for review, see [17, 57]). Exogenous oxidants, such as drugs or food components may also produce met-Hb directly or via some of their metabolites. Both superoxide and met-Hb are potentially dangerous to the RBC membrane. Met-Hb is unable to bind oxygen and is the first step in the formation of harmful hemichromes. Superoxide is easily transformed into the potent oxidant H_2O_2 by superoxide dismutase, abundantly present in the RBC. Under normal conditions, the RBC is able to reduce met-Hb back to ferrous-deoxyHb by the NADH-dependent met-Hb reductase. H_2O_2 is detoxified by catalase and by GSH peroxidase (GPOx). Catalase is now believed to be very important in cellular antioxidant defence (24). H_2O_2 is broken down to water and oxygen with the stoichiometry:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

in a rapid two-step reaction. Interestingly, catalase is protected against inactivation by NADPH. In case of insufficient NADPH generation, catalase is inactivated and thus the anti-oxidant defence of the RBC impaired [24]. The GPOx-dependent peroxide detoxication route is also dependent of efficient supply of NADPH, the reducing cofactor in the GSH-regenerating reaction catalyzed by glutathione reductase:

$$\text{Oxidized glutathione} + 2\ NADPH + H^+ \rightarrow$$
$$2\ GSH + 2\ NADP^+$$

Table 1. Selected metabolic fluxes, enzyme activities and metabolite levels of glycolysis, hexose monophosphate shunt (HMS) and anti-oxidant defense system in human RBC.

Metabolic fluxes of fresh RBCs in plasma, 46% hematocrit (μmoles per hour per ml RBCs at 37°C)	Maximum activity of energy metabolism enzymes in fresh RBC (μmoles per hour per ml RBCs at 37°C)	Maximum activity of anti-oxidant defense enzymes in fresh RBCs (μmoles per hour per ml RBCs at 37°C)	Steady-state levels of metabolits in fresh RBCs (μmoles per ml RBCs)
Glucose consumption: 1-2	Hexokinase: 37		
Lactate production: 2-4			Lactate: 932
			Pyruvate: 53
HMS flux: 0.08-1.8	G6PD: 173		Glucose-6-phosphate: 28
	Glucosephosphate isomerase: 1265		NADPH: 32
			NADP: 1.4
	Phosphoglycerate kinase: 6656		ATP: 1350
	Pyruvate kinase: 312		ADP: 216
	Na^+, K^+-ATPase: 2.5		AMP: 21
	Mg^{2+}, Ca^{2+}-ATPase: 10-20		
DPG shunt: 0.4-1.6	DPG-mutase: 99		DPG: 4171
		Catalase: $3.2 \cdot 10^6$	
		Glutathione peroxidase: 640	GSH: 2200-2500
		Glutathione reductase: 140-216	GSSG: 3.6
		Superoxide dismutase: 46900	
		NADH-met-Hb reductase: 395	

Glucose is the main energy source of the RBC. Glucose can be transformed to lactate via glycolysis, or to ribulose-5-phosphate via the oxidative section of HMS (glucose-6-P-dehydrogenase, G6PD, and 6-phosphogluconate dehydrogenase, 6-PGD). Ribulose-5-phosphate can re-enter glycolysis via the nonoxidative section of HMS. Glutathione cycling from oxidized (GSSG) to reduced form (GSH) is dependent upon NADPH generation by via G6PD and 6-PGD. NADH is utilized to reduce met-Hb to Hb. Net output of glycolysis is 2 moles of ATP per mole of glucose metabolized. The main glycolytic pathway has two branching points: in the first one the product of hexokinase, glucose-6-phosphate can be diverted to the HMS by G6PD. Under normal steady-state conditions, 92% of glucose is metabolized along glycolysis and 8% along HMS. Under oxidant conditions up to 90% of glucose can be metabolized along HMS. In the second branching point, 1,3-diphosphoglycerate can be diverted by DPG-mutase to produce DPG. Five metabolic intermediates are particularly important: ATP, the primary energy intermediate is essential to ensure the active cation transport across the membrane, to preserve RBC shape and flexibility, to affect the position of the oxygen dissociation curve; DPG, in association with pH and HCO_3^-/CO_2 modulates position and shape of the oxygen dissociation curve; NADH is the substrate for met-Hb reductase to keep Hb in a functionally competent state; NADPH is the substrate for glutathione reductase to regenerate GSH after oxidant insults and protect catalase from inactivation; GSH is the main protector of thiol groups, scavenger of oxides, peroxides, oxidant radicals, and detoxicant of foreign compounds. Data were recalculated and adapted from references [4, 9, 10, 28].

Therefore, reduced co-enzymes play essential roles in anti-oxidant RBC defence: NADH, produced by the glycolytic pathway is the main reductant of met-Hb to Hb, while NADPH, produced by the first two reactions of HMS, is co-substrate of glutathione reductase and the protector of catalase efficiency [17, 57, 60, 77].

The Damaging Role of Hemichromes, Free Heme and Free Iron

In normal, unstressed RBCs spontaneous production of met-Hb on autoxidation of Hb and conversion of met-Hb back to its normal functional state are in balance. Any pathological situation, which increases the turnover of this cycle whether increased oxidative stress or impaired antioxidant defences will enhance production of met-Hb and generation of active oxygen species. Enhanced formation of met-Hb leads to formation of hemichromes. Hemichromes are ferric Hb derivatives with characteristic absorption and electron spin resonance spectra in which the sixth heme ligand is either the distal histidine, in which case the process is reversible ("reversible hemichromes") or another aminoacid residue from the globin ("irreversible hemichromes") [17, 77]. Hemichrome formation depends on the amount of met-Hb formed and is accelerated by oxidants such as superoxide or H_2O_2 that enhance the formation of met-Hb. The damaging activity of hemichromes derives from

their potential role as generators of powerful oxidant hydroxyl radical via a so-called iron-catalyzed Haber-Weiss reaction:

Reaction 1: $O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

Reduction of Fe^{3+} by O_2^- to form Fe^{2+} and provide the "Fenton reagent"

($H_2O_2 + Fe^{2+}$)

Reaction 2: $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^\bullet + Fe^{3+}$

Iron-catalyzed Haber-Weiss reaction with production of hydroxyl radical ($OH^\bullet$)

Reaction 3: $O_2^- + H_2O_2 \rightarrow OH^- + OH^\bullet$

Non-catalyzed Haber-Weiss reaction less likely to occur in the cell.

In the Haber-Weiss reaction 1, O_2^- produced continuously by univalent reduction of oxygen would reduce ferri-hemichrome to ferro-hemichrome. In reaction 2, the "Fenton reagent" ferro-hemichrome would catalyze decomposition of H_2O_2 to hydroxyl radical and regenerate ferri-hemichrome. In this sequence, the "Fenton reagent" ferro-hemichrome generates hydroxyl radical upon reaction with H_2O_2 (for review, see [17, 29, 57, 60]). Hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion-controlled rates. For example, hydroxyl radical may abstract hydrogen from unsaturated fatty acids and start lipoperoxidation. Iron is bound tightly to Hb but may be released from heme under specific circumstances (e.g. oxidant insult), yielding free, reactive iron. A second mechanism by which hemichromes exert a damaging effect is due to their liberation of free oxidized heme (hemin). Oxidized Hb or hemichromes easily lose hydrophobic heme that readily associates with membrane lipids. RBCs of all pathologic conditions characterized by increased susceptibility to oxidation such as sickle cell anemia, thalassemia and G6PD-deficiency contain increased amounts of membrane-bound hemin [18, 19, 29]. Reports indicate that micromolar hemin can induce potassium leak, decrease osmotic fragility, cause RBC swelling, interact with spectrin, actin and protein 4.1, mediate dissociation of membrane skeletal proteins, and destabilize the RBC membrane. Hemin is an extremely potent catalyst of peroxide-induced lipid peroxidation in RBC membrane, and oxidizes thiol groups of membrane proteins dose-dependently [18, 19].

Peroxidation of membrane lipids, most notably poly-unsaturated fatty acids arachidonic and linoleic acid, generates a wide array of molecules, such as lipid hydroperoxides; secondary lipid peroxidation products (for example malondialdehyde and 4-hydroxynonenal, HNE); volatile alkanes and conjugated diene hydroperoxides. Lipid derivatives of oxidant attack, most notably malondialdehyde, exert a number of detrimental effects on RBCs: they can damage membrane structure with formation of membrane pores, increase potassium leak and alter water permeability; polymerize membrane components and decrease cell deformability; cross-link membrane proteins; enhance IgG binding and complement activation; finally, they may enhance exposure of phosphatidylserine (PS) on the outer cell surface [18, 39, 40].

Lastly, oxidant activity against membrane components can be exerted by free non-heme iron. Free non-heme iron was found to accumulate in pathologic conditions such as sickle cell anaemia and thalassemia [16, 29, 55, 73]. Most likely, free iron enhances the oxidant activity of free heme and hemichromes by enhancing the formation of hydroxyl radicals. In conclusion, hemichromes, free heme and non-heme iron all contribute to generate oxygen-derived radicals of different potency that may hit and possibly damage any component of the RBC membrane or cytoplasm and produce a variety of functional impairments. The type of damage will depend on the intensity and specific potency of the oxidant bullets, on the sensitivity of the target, and on the functional role of the target [17, 29, 57, 60, 77].

The Human Senescent RBC

In the previous section an analytical overview was provided of oxidative changes occurring during the life span of the RBC. Basically due to the lack of protein synthesis and inability to regenerate effete protein molecules, most notably enzymes, a multitude of alterations accumulate as the end of the RBC life span approaches. In human and dog RBCs, alterations reach critical levels after approx 120 and 115 days after RBC entry into circulation, respectively. At the whole-cell level, terminal RBC have lost membrane in form of microvesicles, have extruded ions and cell water, and are thus smaller, more dense and less deformable. Two very important features of RBC death should be kept in mind [20, 37, 46, 47, 56]. First, terminal RBCs do not break down to fragments, but rapidly lose their identity, becoming non-self cells and ending up by phagocytosis. Removal of effete RBCs is basically an immunological process resulting from the generation of non-self identification sites on the RBC membrane and their recognition by

phagocytes. The first main problem of RBC senescence studies is thus to identify the non-self molecules on the RBCs and the recognition mechanism(s) on the phagocyte. The second important feature of terminal RBCs is the non-linear nature of alterations that lead to removal. It is evident that in the last few days of life some critical parameters change precipitously and non-linearly. Those parameter changes are the most likely responsible for the terminal phagocytic event. Due to the rapidity of those terminal events, and the small numbers of RBCs removed on a daily basis, it is expected that only a small fraction of circulating RBCs at any time (tentatively between 1 and 5% of the RBC mass) already carries the termination signals in analytically appreciable quantities and displays large differences in comparison to mature RBCs not yet ready for demise. Looking at those changes a few properties appear to show this kind of abrupt development in the most dense cells, considered to best reflect terminal RBCs. To summarize, studies indicate that the small most dense fraction of terminal RBCs had six times as many binding sites for IgG and complement as unseparated cells, sharply increased bound hemichromes, and a 25-fold higher phagocytic susceptibility [20, 45-47).

Band 3-Centered Models of RBC Removal

A very important feature of hemichrome/free heme/iron damage is its non-random occurrence in space. Specific location of attack and propagation are high-affinity binding sites for both heme and hemichromes localized on the long cytoplasmic domain of band 3. This domain is considered a major organizing centre of RBCs and an anchoring site for a number of membrane-associated proteins, such as protein 4.2, 4.1, glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, aldolase, Hb, hemichromes and protein tyrosine kinase (p72syk) [47, 79]. Detailed information on organization of the peripheral protein complex on the cytoplasmic domain of band 3 has been provided by the recently determined crystallographic structure [79]. The highly damaging properties of hemichromes result from their tight association with the cytoplasmic domain of band 3. This association propagates beyond the stoichiometric unit complex between the two moieties as it creates a polymer of macromolecular dimensions, promoting clustering of band 3 in the membrane [70, 71]. Several authors (for review, see [2-4, 33-36, 44-47]) have proposed mechanisms for recognition and phagocytic removal of senescent or damaged RBCs based on oxidative and non-oxidative clustering of band 3 as the starting event with subsequent opsonization and phagocytic removal by circulating or resident phagocytes. After the seminal papers by Kay and her group [33-36], the band-3-centered removal model has received attention by several groups, and will be presented in its most comprehensive formulation to include data and views from the original Kay's, Low's and Lutz's models.

Kay's model

Marguerite Kay was first to show that dense (presumably old) human RBC bound increased amounts of autologous IgGs [33, 34]. Those eluted auto-antibodies induced phagocytosis in an in vitro assay, re-bound only to band 3 and to a 62-kDa protein presumably derived from band 3 and including most of the 35-kDa carboxyl terminal fragment and the 17-kDa anion-transport region with the glycosylated side chain. The characteristics and origin of the 62-kDa fragment raised criticism [43, 44, 46] leading the seminal hypothesis of Kay to evolve into other models, also centered on the central role of band 3 but differing from Kay's model in several respects.

Low's model

According to Low and his group [32, 43-45, 70, 71], aggregation of band induces clustering of potential antibody-binding sites and promotes deposition of autologous IgGs. This mechanism does not imply covalent modifications of band 3 but simply assumes that antibodies with affinities too weak to bind to band 3 monovalently would react avidly with band 3 aggregates due to enhanced affinity of the bivalent interaction. The theoretical background was offered by the Brandts-Jacobson equation which predicts increase greater than three orders of magnitude in IgG affinity by band 3 aggregation. Typically, band 3 aggregation and ensuing deposition of autologous IgG was considered to be primarily elicited by hemichrome deposition. In studies performed in phenylhydrazine-treated RBCs and confirmed with hemoglobinopathic RBCs it was shown that clustered band 3 and surface-bound IgG had superimposable localization over Heinz bodies, extremely very large clumps of irreversible hemichromes. Protein aggregates were isolated by detergent disruption and further purification [32] from sickle and thalassemic RBCs, and from the 1% densest fraction of normal RBCs

(corresponding to senescent RBCs). Those aggregates contained approx. 1.3% of total membrane proteins but 67% of hemichromes (on total Hb basis) and large amounts of band 3 (13% of total membrane proteins), as well as abundant numbers of autologous IgG. As pointed out earlier [46] co-localization of Heinz bodies, band 3 clusters and anti-band 3 deposition in phenylhydrazine-treated RBCs might have been overshadowed by the artefactual liberation of spectrin from partially lysed RBCs and membrane deposition of spectrin-anti-spectrin immune complexes. According to Lutz *et al.*, formation of C3b immune complexes and their binding to complement receptor 1 (CR1) of RBC did also occur in the phenylhydrazine model [46, 48, 49].

Lutz's model

This model shares important elements with Low's model. In fact, Lutz et al. [48, 49] demonstrated increased binding of anti-band 3 antibodies to band 3 oligomers in senescent RBC, a correlation between cross-linkability of band 3 and anti-band 3 binding, and enhanced binding of anti-band 3 to aggregated band 3 on immunoblots. The fundamental difference with respect to the Low model is the crucial role firstly described by Lutz *et al.* [46, 48 ,49] of both anti-band 3 antibodies and complement deposition as mediators of opsonization and phagocytosis. Naturally occurring auto-antibodies (Nabs) are low-affinity and well below saturating concentration. Therefore they cannot operate as efficient opsonins. Their efficiency is increased by complement components that lower by about 100-fold the number of antibody molecules required for phagocytosis induction. As pointed out earlier [46, 48, 49-51] Nab and specifically antiband 3 Ab activate the classical complement pathway and stimulate complement amplification and overstoichiometric deposition of C3b component. Complexes betweenAbs and $C3b_2$ are formed that are far more resistant to inactivation by factors H and I than free C3b. In addition, $C3b_2$-Ab complexes are better activators of the amplifying C3 convertase than immobilized C3b. Therefore, complement amplification compensates low affinity of NAbs and generates very efficient opsonins. Older data from the blood transfusion field indicated that removal of transfused RBCs was remarkably delayed when complement was removed during blood storage [61] The essential importance of complement in mediating immuno-adherence and phagocytosis is a tenet in classical immunology. It has been shown in bacteria and other non-self cells, as well as with senescent autologous cells, that phagocytosis is greatly enhanced when target cells were opsonized with both antibodies and complement, whereby efficient deposition of both opsonins (IgG and C3, the most abundant complement protein) on target cells was dependent on activation of the alternative complement pathway. Autologous anti-band 3 antibodies appear to possess the unique ability to stimulate alternative pathway C3b deposition. It has been shown [48, 49] that naturally occurring anti-band 3 antibodies stimulated C3b deposition on oxidatively stressed RBCs in presence of 500- to 1000-fold excess of autologous or allogeneic IgG molecules. They have shown that naturally occurring anti-band 3 antibodies have high affinity to C3 and contain a binding site for C3 probably within the Fd region of IgG. The affinity for C3 is assumed to potentiate the effect of anti-band 3 by stimulating alternative pathway C3 deposition.

Oxidative damage to Hb and formation of hemichromes leads to the association between hemichromes and the cytoplasmic domain of band 3 [43, 79] and to band 3 oligomerization and subsequent clustering to large aggregates. These clusters show enhanced affinity for normally circulating anti-band 3 antibodies and these in turn activate the complement system. It has been shown that less than 1% oligomerized band 3 was sufficient to elicit deposition of autologous anti-band 3 IgG. These few molecules were able to induce generation of large amounts of complement fragments via activation of the alternative pathway. The ultimate signal for phagocytic removal are complement C3 molecules deposited on the RBC surface in their active form C3b (for review, see [45, 46]). This model was substantiated by converging evidence showing co-clustering of aggregated band 3 and hemichromes and induction of phagocytosis *in vivo* and *in vitro*, and was verified in different *in vivo* situations (favic crisis, thalassemic patients, canine senescence model) and *in vitro* models. Interestingly, in a canine RBC senescence model closely resembling human RBC senescence [56], senescent RBCs were found to contain abruptly elevated membrane-bound denatured globin and seven-fold higher amounts of membrane-associated autologous IgGs. In parallel, band 3 was significantly less restrained indicating substantial disruption of the membrane skeleton. Nabs with specificity to spectrin also exist in human plasma and were found to bind to crosslinked band 3 at quasi-physiological concentrations. Anti-spectrin Ab were also found to bind to RBC in patients with hemoglobinopathies and in phenylhydrazine-treated animals. This anomalous binding is probably due to Nab polyreactivity and may have a physiological significance in removal of damaged

or senescent RBC, securing removal even in case of lack of anti-band 3 Nabs and explaining why band 3 knockouts and band 3 deficient animals were still capable of clearing senescent RBCs [30].

Experimental Verification of the Band 3/ Complement RBC Removal Model

Oxidative Band 3 clusters are associated with NAbs/complement deposition and phagocytosis

The "classical paradigm" of band-3 dependent RBC removal has been verified by a number of different groups in senescent and oxidatively-damaged human [3, 6, 33-35, 38, 43-49, 59, 62, 66] and dog RBC [56], in mutant and pathological RBCs [1, 5, 15, 16, 22, 23, 32, 53, 68].

Nonoxidative Band 3 clusters are associated withNAbs/complement deposition and phagocytosis

While in senescent RBCs the sequence of events leading to opsonization and phagocytosis starts with progressive cumulation of oxidative damages to Hb ensuing in met-Hb and hemichrome formation and membrane deposition of the latter, and band 3 clustering, other methods of nonoxidative clustering of band 3 lead to the same final sequence. Nonoxidative clustering of band 3 has been studied by Turrini *et al*. [64], who used millimolar zinc or acridine orange, and micromolar melittin as non-oxidant band 3 clustering agents, and BS3/DSP as generators of irreversible/reversible crosslinked band 3. Covalent crosslinks by DSP could be reduced by appropriate reducing agents. Sequential application of clustering agents and crosslinkers induced formation of high-molecular weight band 3 aggregates, followed by binding of NAbs and complement binding and enhanced phagocytosis by human monocytes. Crosslinking alone without clustering agents was ineffective. Clustering and crosslinking followed by reduction of crosslinks was also followed by dissolution of clusters, abrogation of binding of NAbs and complement, and blockage of phagocytosis.

Decomplementation abrogates phagocytosis

The essential importance of complement deposition in eliciting phagocytosis has been shown by abrogating both complement activation and phagocytosis. Probably the most conclusive evidence was provided by Lutz *et al*. [48, 49] who induced full abrogation of convertase activity by diisopropylfluorophosphate (DIPFP) tretment and showed that opsonization of diamide-treated RBC with DIPFP serum abrogated phagocytosis by >85%. Similar results were obtained by opsonizing thalassemic or malaria-parasitized RBC with decomplemented serum [5, 65].

Blockage of complement receptor CR1 on phagocyte abrogates phagocytosis

The most important phagocyte receptor involved in RBC phagocytosis appear to be the C3b receptor (CR1). In fact, up to 90% phagocytic activity could be blocked by treating phagocytes with the J3.D3 anti-CR1 monoclonal antibody developed by Kazatchkine's group [21]. The blockage was effective with the senescence oxidative model, with diamide-treated, thalassemic, sickle-cell-trait and P. falciparum ring-form parasitized RBCs [2, 5, 26, 48, 49, 53].

Phagocytosis intensity is proportional to band 3 clustering, IgG and complement deposition

One paper shows a mutual quantitative correlation between the amount of membrane-deposited hemichrome, percentage of aggregated band 3, and phagocytosis intensity, expressed in terms of number of RBC ingested per monocyte [16]. Another paper also indicates mutual proportionality between the same parameters in three different cases of mutant RBC, affected by heterozygous sickle-cell anemia, beta-thalassemia and Hb-H disease [5]. To the best of our knowledge those are the only studies where phagocytosis was studied in a short-term period in order to have no heme oxygenase expression in the phagocyte and where phagocytic activity was determined in terms of average number of ingested RBC per phagocyte [58].

The band 3-clustering model is not necessarily exclusive of other explanations of senescent/damaged cell recognition. Thus opsonization of a cryptic galactosyl residue or recognition of deglycosylated, cleaved or oxidized cell surface protein by autologous IgG may also allow the more avid bivalent IgG attachment (for review, see [12, 20, 37]).

Magnification and Acceleration of Normal RBC Senescence in Pathological Situations

Oxidative damage outlined above represents normal events during RBC life. In the course of its life in the circulation, damage cannot be completely repaired due

to the progressive decrease in activity of defensive enzymes and accumulates until reaching a threshold value. As it has been shown in the dog RBC senescence model [56], a precipitous increase in protein aggregates occurs at the end of the RBC life-span accompanied by deposition of removal markers. Homeostatic mechanisms that cannot be discussed here keep a precise balance between RBC removal and erythropoiesis, such that a daily demise of 1% old RBCs is exactly matched by entry into circulation of a corresponding number of young RBCs. A number of genetically-determined or acquired conditions may enhance the formation of oxidant compounds or impair antioxidant defence. In both cases the consequence is accelerated senescence and removal, and increased hemolysis. If the number of removed cells or the kinetics of RBC removal exceeds the erythropoietic capacity, anaemia is the consequence. In quantitative terms, according to the intensity of oxidative stress and the degree of inactivation of the anti-oxidant defences, anemia can be imperceptible, acute and dramatically severe, or chronic.

Based on well-established evidence from various groups, it is reasonable to assume that the same events that induce removal of normal RBCs are present in a magnified and accelerated form in a series of genetic RBC defects affecting metabolism or Hb structure or synthesis, and in metabolic disorders not primarily involving RBCs but secondarily impacting on RBCs functionality and survival. An incomplete listing of genetic RBC defects affecting anti-oxidant defence would include genetically determined deficiency of HMS enzymes (G6PD, 6-phosphogluconate dehydrogenase)and anti-oxidant defence enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase, met-Hb reductase). Hb synthesis defects may consider the various thalassemias; Hb molecular defects would include sickle cell anemia, ovalocytosis, unstable Hbs, possibly Hb C and Hb E; metabolic disorders or generalized diseases impacting on RBCs include diabetes, alcoholism and cancer (for review, see [4, 11, 52, 73]).

In the following, we will concentrate on two model examples. We will consider deficiency of G6PD as an example of a genetic defect accompanied by insufficient anti-oxidant defence, and *falciparum* malaria, as an example of a general disease with distinct RBC damages. We will show that in both cases enhanced RBC destruction and anemia occurs basically by magnification and acceleration of the same mechanism responsible for the normal removal of senescent RBCs. We will show that the physiological mechanism designed to daily handle approx. 1% of the total RBC mass is extremely flexible and can be upgraded to govern the demise of up to 80% of total RBC mass in a few days as in the case of favism or acute malaria anemia.

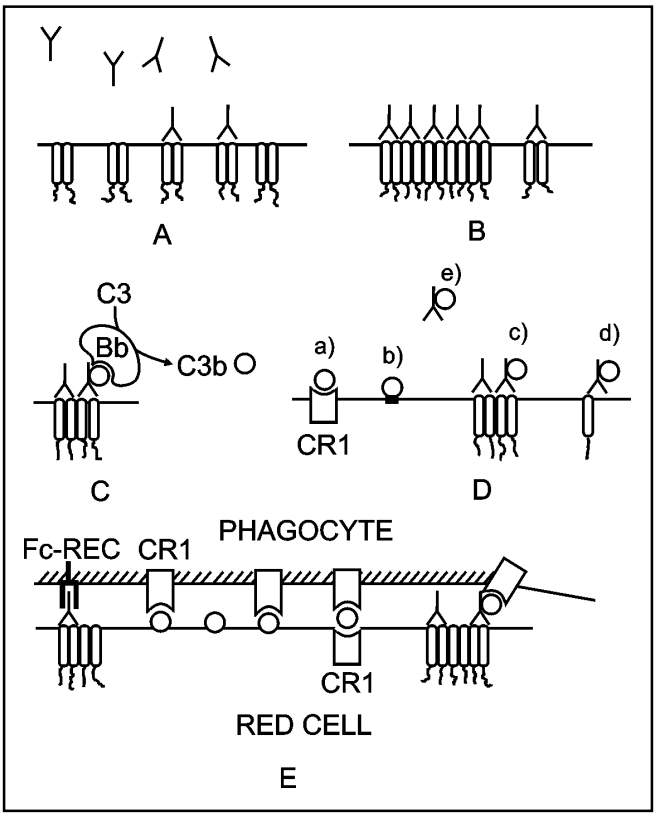

Fig. 1. The "band 3/complement RBC removal model". Sequence of events after oxidant damage of the RBC leading to opsonization with anti-band 3 IgG and complement, recognition and removal by the phagocyte. (A) Normal adult undamaged RBC. Band 3 is not aggregated and only a few molecules of anti-band 3 IgG are bound. (B, C) In senescent RBCs or after oxidant insult, aggregation ("clustering") of band 3 enhances binding of naturally-occurring anti-band 3 IgG and activates the alternative pathway of complement, leading to generation of C3b fragments. Clustered band 3 traps spontaneously generated complement fragments C3b. The binding prevents C3b from inactivation and allows formation of Bb convertase (Bb) that converts more C3 to C3b. (D) C3b can bind to complement receptor CR1 (*a*), bind covalently to RBC membrane (*b*), to band 3 clusters (*c*), to monovalently bound anti-band 3 IgG (*d*), or to other IgG (*e*). (E) Recognition of opsonized RBC occurs predominantly via CR1 or Fc receptor of the phagocyte. From (1), with permission. The model is based on ref. 1-3;46-50. See text for details.

G6PD-deficiency and favism

G6PD catalyzes the first and pace-making reaction of the HMS by which glucose-6-phosphate (G6P) is oxidized to 6-phosphogluconolactone with production of NADPH. In RBCs the production of NADPH is essential for the protection of the cell against oxidative stress. A major role of NADPH is to maintain GSH at a ratio grater than 500:1 over the oxidized form GSSG. GSH plays a vital role in antioxidant defence by reacting with H_2O_2 and organic peroxides, as well as maintaining thiol groups of Hb and other proteins and enzymes in the reduced state [1, 11, 52]. H_2O_2 can be also detoxified by catalase which is also stabilized by tightly bound NADPH (24). G6PD deficiency is the most common human enzyme defect, being present in probably more than 400 million people [1, 7, 8, 52]. G6PD is one of the most polymorphic proteins known. About 90 different variants have been detected in all coding regions and in the promoter. Widespread mutants with very low residual activity (1-10% residual activity, class II mutants) are: G6PD Mediterranean, frequent in populations facing the Mediterranean; G6PD A$^-$, frequent in Africans; G6PD Canton, frequent in Southern China and Taiwan; G6PD Markham, frequent in Papua New Guinea [7, 8, 11, 52].

The favic crisis is an acute hemolytic syndrome occurring in severely G6PD-deficient individuals after consumption of *Vicia faba major*. It can be considered as a paradigm for abruptely accelerated RBC senescence of human RBCs. The great majority of favism cases occur in hemizygous deficient males. The first symptoms of favism are hemoglobinuria, generalized weakness to severe lethargy, nausea and vomiting, headache, lumbar or abdominal pain and fever. After a delay of variable duration jaundice appears and may reach a very intense stage. It is accompanied by enlargement of spleen and liver. Hemoglobinuria may continue for several days [1, 52]. In the densest RBC fraction isolated during early favism, extremely low GSH levels, down to less than 10% of non-hemolytic G6PD-deficient controls have been noted. Anemia may be extremely severe and life-threatening. During severe attacks, RBC counts as low as $0.5 \cdot 10^6$ RBCs per µl have been measured. Fava beans are uniquely rich in two glucosidic compounds, vicine and convicine, which generate the redox aglycones divicine (2,6-diamino-4,5-dihydroxypyrimidine) and isouramil (6-amino-2,4,5-trihydroxypyrimidine) upon hydrolysis of the beta-glucosidic bond (Fig. 2). Fava beans also contain high amounts of ascorbate and varying amounts of L-DOPA glucoside (see [1], for review). Divicine and isouramil share common effects and similar mechanism of action with other oxidant drugs (1). In a cell-free system two mechanisms of autoxidation of divicine have been proposed (Fig. 2).

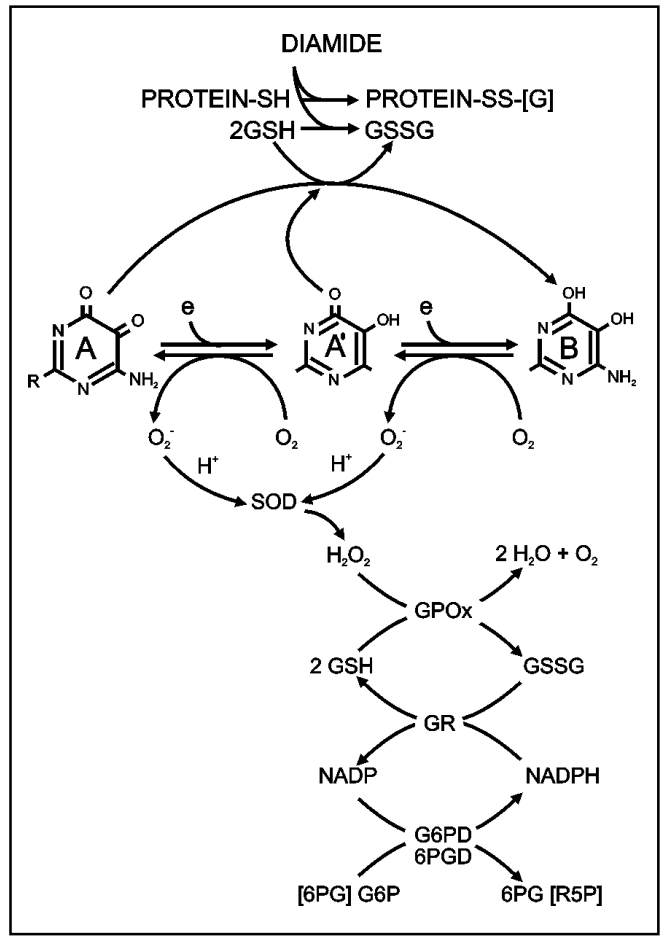

Fig. 2. Effect of oxidants and role of G6PD in antioxidant defence in human RBCs. Oxidant compounds such as diamide or quinones (*A*) oxidize GSH or protein –SH groups. Quinones in the figure are fava bean pyrimidine derivatives taken as an example. Quinone-generating drugs such as menadione, primaquine, doxorubicin, alloxan etc. behave similarly. In the process, quinone (*A*) is reduced to the corresponding hydroquinone (*B*) while 2 GSH or protein-SH are oxidized to GSSG or protein mixed disulfide (Protein-SS-[G]). The hydroquinone (*B*) is autooxidized in the presence of molecular oxygen back to the quinone (*A*) via a semiquinone radical intermediate (*A'*). During unielectronic autoxidation superoxide is generated. Superoxide dismutates spontaneously or by superoxide dismutase (SOD) catalysis to H_2O_2. H_2O_2 is reduced to water by glutathione peroxidase (GPOx) or by catalase (not shown), whereby 2 GSH are oxidized to GSSG. GSSG is reduced back to 2 GSH by NADPH-dependent glutathione reductase (GR). NADPH is produced by G6PD and 6-P-gluconate (6PGD) dehydrogenase. From Arese and De Flora (1), with permission.

Addition of divicine to G6PD-deficient RBCs is followed by GSH oxidation, with a stoichiometry of approximately one mol oxidized GSH per mol divicine. In the G6PD-deficient RBCs no regeneration of GSH occurs even after several hours incubation in presence of glucose. In normal RBCs, divicine and isouramil rapidly oxidized GSH to the same extent as in G6PD-deficient RBCs, but initial levels of GSH were rapidly re-established. As a consequence of unbalanced production of oxidants and defence by GSH, incubation of G6PD-deficient RBCs with 1 mM divicine for 12–24 hours produced large polypeptide aggregates unable to migrate into the separating gel [1, 68]. Bidimensional electrophoresis after reduction identified those aggregates as consisting largely of band 3 protein and to a minor extent of spectrin. Heinz bodies were formed in a time-dependent and dose-dependent manner [68].

A crucial question in oxidant-induced hemolysis such as favism concerns the type of hemolysis. The share of intravascular hemolysis in the favic crisis has been determined by balancing RBC loss from circulation and appearance of free heme or Hb in urine and blood plasma [1]. The results obtained by follow-up of seven favic crises demonstrate a low share, not exceeding 20% as a mean value, of intravascular hemolysis. It has been estimated that the same share is present in the physiologic removal of senescent RBCs [25]. The membrane alterations observed *in vivo* during the favic crisis were superimposable to changes observed in senescent RBCs. In summary, RBC membranes isolated from favic patients contained elevated amounts of complexes between IgG and the complement fragment C3b/C3c; the complex could be cleaved with hydroxylamine, which hydrolyzes primarily ester bonds, and liberated IgG exclusively bound to separated band 3, indicating that autologous anti-band 3 IgG specifically reacted to the aggregated band 3-complement complexes. The relative amount of the complexes correlated with the severity of the crisis and decreased to normal values within 4 to 5 days after fava ingestion. Additional evidence obtained from early favic crises indicated extensive clustering of band 3 (up to 30% of band 3 was aggregated to higher than 4,000 kDa-molecular mass clusters) and deposition of hemichromes to the RBC membranes. In parallel, severely damaged RBCs isolated from early crises had extensive cross-bonding and very low reduced glutathione levels (down to 25% of normal levels), and were intensely phagocytosed by adherent human monocytes (up to 9-fold compared to normal RBCs) (1,68). In conclusion, the favic crisis and other types of oxidant hemolysis such as those caused by oxidant drugs or chemicals, may be considered as massive and rapid senescence of a large fraction of the RBC population. Those pathologically "senescent" RBCs are removed similarly as in normal RBC senescence, but the rate of the process was magnified up to 15-fold, and greatly accelerated. Indeed, losses of as many as 20% per day of the total RBC mass can frequently be observed in massive favic crises [1, 68].

Falciparum malaria

The growth of *P. falciparum* induces profound modifications in the host RBC membrane. Progressively from ring-stage on, parasitized RBCs are transformed into non-self cells, opsonized and phagocytosed by circulating and resident phagocytes. Opsonization occurs even when parasitized RBC are incubated in nonimmune autologous serum. Autologous IgG with anti-band 3 specificity, and complement have been identified as the opsonins responsible for phagocytic recognition at ring-stage, the early parasite forms. At this stage, phagocytosis is modest and almost totally mediated by complement deposition and recognition by the phagocyte complement receptor type 1 (CR1). In fact phagocytosis was inhibited when phagocytes were pre-treated with blocking monoclonals against the CR1 receptor. At later stages of parasite maturation (trophozoite and schizont stage), phagocytosis was strongly increased and the role of complement in phagocytic recognition was reduced. At schizont stage, approx. 18 percent of phagocytosis was not accounted for either by complement-, Fc- or phosphatidylserine-receptor occupancy [65, 69].

The biochemical changes occurring in the parasitized RBC membrane that induce binding of opsonins and erythrophagocytosis were studied by our group. It was shown that growth of *P. falciparum* induced hemichrome formation in the host RBC. Hemichromes were isolated from large membrane protein aggregates that also contained band 3, IgG and the complement C3c fragment, the stable derivative of C3b. This finding suggests that hemichromes bound to its specific, high-affinity binding site on the cytoplasmic domain of band 3 and induced band 3 aggregation, in agreement with literature data [43, 70, 71] and previous observations by our group [64, 66, 67]. During the parasite development, the amount of hemichromes bound to the membrane progressively increased and paralleled the amount of aggregated band 3, deposited IgG and C3c and phagocytosis, suggesting mutual causal relationships between those events. The involvement of oxidative events in the formation of the

antigenic membrane aggregates was substantiated by the inhibition of their formation and abrogation of opsonin binding and phagocytosis when the parasites were cultivated in presence of beta-mercaptoethanol, known to be a powerful permeant reductant and oxidant scavenger [27].

Immunoprecipitation studies further substantiated the connection between membrane-bound IgG and band 3. Immunoprecipitated band 3 was oxidatively cross-linked, as no electrophoretic bands were obtained in absence of reductive pre-treatment, and migrated as a sharp band which corresponded to a fast-migrating, underglycosylated band 3 component and was absent in the unreduced membranes from parasitized RBCs [27]. The latter results indicated that membrane-bound IgGs were linked to band 3 and showed that apparently underglycosylated band 3 was preferentially incorporated into the membrane aggregates. Proteomic analysis confirmed that aggregates contained band 3, complement C3 fragment, IgGs and also small amounts of ankyrin, flotilin and protein P55. Presence of underglycosylated band 3 was deduced by the high abundance of the 1564-Da tryptic fragment with the sequence LSVPDGFKVSNSSAR displaying free amino groups usually engaged in N-glycosidic bonds with the carbohydrate moiety (F. Carta and F. Turrini, unpublished results). To characterize the band 3 modifications which lead to its recognition by naturally occurring antibodies, the IgGs bound to the surface of parasitized RBCs were eluted and challenged with membrane proteins extracted from nonparasitized RBCs, parasitized RBCs and chemically treated RBCs (diamide and zinc-BS3 treatments) to obtain well-defined oxidative (diamide) and non-oxidative (zinc-BS3) band-3 aggregation. This approach further demonstrated that surface bound IgGs had affinity to oxidatively aggregated band 3.

Several reports have described band 3 modifications in *P. falciparum* parasitized RBC. Band 3 had restricted mobility [63], was re-organized during parasite development [54] and was implicated in the expression of neo-antigens which bound naturally occurring auto-antibodies [74, 76]. The expression of neo-antigens increased with parasite maturation and the process was controlled by the RBC redox state [15, 65]. *P. falciparum*-induced band-3 modifications appear to be also involved in cytoadherence and sequestration [31, 74-76].

The sequence observed in parasitized RBCs, namely hemichrome formation, band 3 aggregation, opsonin binding and phagocytosis is quite similar to that observed in normally senescent RBCs [46, 47], oxidatively and non-oxidatively modified RBCs [6, 48, 49, 64] or pathologic RBCs, most notably sickle [64] and thalassemic RBCs [16, 53, 78], where membrane protein aggregates also contained large amounts of IgG and C3 complement fragments, and where deposition of IgG and complement C3c and phagocytic susceptibility were correlated to the amount of membrane-bound hemichromes [5, 16, 53].

Phagocytic recognition of ring-forms is low, but is strongly enhanced in parasitized mutant RBC, endogenously less defended against oxidative insult, such as G6PD-deficient RBCs [15], or in RBCs more prone to oxidant production, such as thalassemic or sickle RBC [5]. Enhanced phagocytosis of ring-forms developing in mutant RBC has been recently proposed as an alternative of the classical "malaria hypothesis" [5, 15, 69].

Concluding Remarks

Present review has focussed on band-3 related mechanism of RBC removal. We do not claim exclusivity for this mechanism, although a wide range of studies performed by independent groups over the last twenty years show agreement with the band 3 model. The band 3-related mechanism is itself not a monolytic paradigm: There is some controversy as to the band 3 epitope recognized by the NAbs, identified by Beppu *et al.* [6] within the oligosaccharidic portion of the protein and disputed by Lutz (for review, see [47]). Partly different complement fragments and receptors have been involved [62], and the possible coadjuvant role of naturally occurring anti-spectrin Ab has been mentioned in a recent study [30]. Slightly divergent views concerning the role of band 3 in malaria were expressed by Sherman's group [74]. On the whole, however, the band 3 model seems to be a widely accepted paradigm for RBC removal.

Recently, renewed emphasis has been put on exposure of PS (for review, see [39, 40]) as an alternative mechanism to explain RBC removal (Bratosin-Aminoff-Montreuil's and Lang's groups in France and Germany, respectively). This mechanism, called "eryptosis" by Lang's group and considered as a special form of apoptosis typical for the anucleated RBC, is characterized by phosphatidylserine exposure, cellular shrinkage, ceramide formation, opening of cation channels, increase of intracellular Ca^{2+} activity, and activation of intracellular proteases such as μ-calpain, in the absence of hemolysis but ensuing into phagocytic recognition of exposed phosphatidylserine by a scavenger receptor on the macrophage. This mechanism is possibly a parallel

pathway of removal alternative to the one detailed before. How and when both pathways operate, interact or possibly integrate might be the object of future endeavors. A detailed discussion of eryptosis is outside the main objective of this review, and the reader is referred to recent papers [12-14, 41, 42] specifically focussed on this interesting mechanism of RBC demise. Present authors, however, share with Lutz some recently expressed reservations [47] on the physiological significance of PS exposure in RBCs removal. Firstly, treatment of RBCs with oxidants and with phenylhydrazine, a strong oxidant and inducer of Heinz bodies and phagocytosis did not expose PS; secondly, patients with HbH, HbH/CS and beta-thalassemia intermedia did not manifest PS exposure but had aggregated band 3, deposition of complement and IgG and were intensely phagocytosed by human monocytes; thirdly, A. Skorokhod in our lab (unpublished data) has shown that ring-malaria parasitized RBC were distinctly phagocytosed but did not expose any PS; fourthly, opsonized normal RBCs treated with very low diamide concentrations (20-50 micromolar) were phagocytosed intensely but had no PS exposure. Finally, since PS exposure is a powerful thrombogenic stimulus, it appears unlikely that evolution would have selected such a potentially dangerous mechanism to ensure removal of RBCs, that constitute 99% of blood cells. Indeed, pathological RBCs characterized by massive PS exposure such as homozygous thalassemia and homozygous sickle cell anemia, suffer from chronic and severe thrombo-embolism.

Based on two distinct examples we have discussed how impaired RBC metabolism may negatively impact on RBC functionality. We have selected a genetic metabolic defect, G6PD-deficiency, and a primarily non-erythrocytic generalized metabolic disease with RBC consequences, because of their high prevalence and practical importance in medical practice. Besides their practical importance, both conditions are also classical subjects for genetic studies, protein modification studies, and analysis of cell-to-cell relationships. The parameter of functionality chosen here for discussion is certainly of paramount importance for the RBCs: the duration of its own life. We have shown that a healthy, non-immunogenic, asymmetric, non-glycated, flexible and resilient surface is essential for the RBC well-being and survival, and that, in turn, such an orderly façade is the manifestation of proper metabolism, sufficient anti-oxidant defence, and very low internal calcium. We have tried to show how the internal metabolic machinery and external appearance are connected. The two conditions discussed here indicate that the macrophage, the final executor of RBC demise, is prepared to accept an array of death signals: clustered band 3 and deposited anti-band 3 and complement fragments, predominant in oxidative damage, possibly exposed PS, on both. Other signals may also play supplementary roles, such as specific oligosaccharides recognized by macrophage mannose receptors.

Essentially the same mechanisms discussed here for G6PD-deficiency may apply to other common RBC mutations of genetic origin affecting several hundred million human beings characterized by disturbancies of Hb synthesis or structure. The thalassemias, sickle-cell anemia, Hb C, possibly Hb E and a host of less frequent unstable Hbs, are all characterized by increased production of oxidant radicals and unbalanced anti-oxidant mechanisms. All of them are in jeopardy of being phagocytically removed following similar paths as those discussed here for G6PD-deficiency. All of them are connected by the red thread of providing efficient protection against falciparum malaria [2, 5, 15, 69, 72].

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