Naturally Occurring Anti-Band 3 Antibodies in Clearance of Senescent and Oxidatively Stressed Human Red Blood Cells

Hans U. Lutz

Institute of Biochemistry, ETH Zurich, Switzerland

Keywords

Naturally occurring antibody ·
Anion transport protein · Band 3 · Opsonization ·
Complement C3b deposition

Summary

Naturally occurring anti-band 3 antibodies (anti-band 3 NABs) are directed against the 55-kDa chymotryptic fragment of the anion transport protein (band 3) of red blood cells (RBCs). They bind to senescent and oxidatively stressed RBCs and induce their selective clearance. These IgG NABs exist at low concentrations, and have a weak affinity that prevents them from actively recruiting second binding sites. Cellular senescence or oxidative damage induces a cascade of biochemical events that results in the detachment of band 3 from the cytoskeleton and in clustering of band 3 protein by bound hemichromes and Syk kinase. Clustered band 3 proteins allow bivalent binding of anti-band 3 NABs. Bivalently bound anti-band 3 NABs have the unique capacity to stimulate C3b deposition by preferentially generating C3b₂-IgG complexes, which act as potent C3 convertase precursors of the alternative complement pathway. Antibody binding not only to clustered, but also to oligomerized band 3 protein further increases if the human plasma also contains induced anti-lactoferrin antibodies. These bind to the polylactosaminyl oligosaccharide, a carbohydrate that exists in lactoferrin and in the 38-kDa fragment of band 3 protein. Anti-lactoferrin antibodies are found primarily in plasma of patients with autoimmune diseases and who have anti-neutrophil cytoplasmic antibodies (ANCA).

Schlüsselwörter

Natürlicherweise vorkommende Antikörper ·
Anionentransportprotein · Bande 3 · Opsonisierung ·
Komplement-C3b-Deposition

Zusammenfassung

Natürlicherweise vorkommende Anti-Bande-3-Antikörper (Anti-Bande-3-NABs) sind gegen das chymotryptische 55-kDa-Fragment des Anionenaustauschproteins der roten Blutkörperchen (RBCs) (Bande 3) gerichtet, binden an alte und oxidativ geschädigte RBCs und induzieren deren selektive Phagozytose. Diese NABs sind IgG-Immunglobuline und haben eine schwache Affinität, welche sie daran hindert, aktiv Zweitbindungsstellen zu rekrutieren. Zelluläre Alterung oder oxidative Schädigungen induzieren eine Kaskade von biochemischen Vorgängen, die in der Ablösung der Bande-3-Proteine vom Zytoskelett resultieren und die Verklumpung von Bande 3 in der Membran durch die Bindung von Hemichromen und Syk-Kinase begünstigen. Verklumpte Bande-3-Proteine erlauben eine bivalente Bindung der Anti-Bande-3-NABs. Bivalent gebundene Anti-Bande-3-NABs haben die Fähigkeit, die C3b-Deposition zu stimulieren indem sie präferentiell C3b₂-IgG bilden, die als potente Vorläufer von alternativen C3-Konvertasen wirken. Die Antikörper-Bindung nicht nur an verklumpte, sondern bereits an oligomerisierte Bande-3-Proteine steigt weiter an, wenn humanes Plasma auch induzierte Anti-Laktoterrin-Antikörper enthält, die an das Polylactosylamin-Kohlehydrat binden, das in Laktoterrin vorkommt aber auch im 38-kDa-Fragment des Bande-3-Proteins. Anti-Laktoterrin-Antikörper treten bevorzugt im Plasma von Patienten mit Autoimmunkrankheiten auf, insbesondere solchen, die sich durch anti-neutrophile zytoplasmatische Antikörper (ANCA) auszeichnen.

Naturally Occurring Anti-Band 3 Antibodies Initiate Red Blood Cell Removal by Bivalent Binding to Oligomerized Band 3 Protein

It is now 30 years since I first described the findings from our ongoing research activities in a review article [1] (with an English summary), and stated that the signal that marks the clearance of senescent human red blood cells is probably not desialylation of glycophorins. Instead, this signal is generated by a cell-age-dependent oligomerization of an integral membrane protein with an apparent molecular mass of 100 kDa, a process that facilitates bivalent binding of a low-affinity naturally occurring IgG antibody (NAb) directed to this protein. Each of these claims was supported by experimental evidence. For example, the removal of senescent red blood cells (RBCs) is most likely not triggered by a loss of 10–15% of sialic acid from ageing RBCs (desialylation) because both dense and light RBC fractions have roughly the same electrophoretic mobility [2], and RBCs lose less than 3% of sialic acids along with glycophorins from the membrane while they age [3]. Based on convincing data from Kay [4], showing that autologous IgG plays a role in the selective clearance of senescent RBCs, we searched for naturally occurring IgG antibodies (NABs) directed against RBC proteins. Autologous IgG binds to several membrane proteins but most strongly to both spectrin polypeptides and band 3 protein on immunoblots from young and old RBCs [5]. IgG binding to band 3 on blots of young and old RBCs is of similar intensity. This finding makes it unlikely that a cell-age-specific antigen is exposed by a proteolytic modification, as suggested by Kay [6]. Instead, topological changes like oligomerization/aggregation of band 3 protein may provide the prerequisite for bivalent binding of anti-band 3 NABs. This possibility was investigated by comparing the binding of autologous IgG to spectrin-free vesicles with that to intact RBCs. Spectrin-free vesicles bud off spontaneously from ATP-depleted RBCs. They lack the cytoskeleton, but retain the full complement of integral membrane components [7]. Spectrin-free vesicles bind 14 times more autologous IgG than ATP-containing RBCs and 4 times more than the ATP-depleted RBCs from which they budded off [8]. Thus, oligomerization/aggregation of band 3 protein is enhanced in vesicles lacking cytoskeletal anchorage. Although experimentally convincing, these data could not be put into perspective with what was known about the topology of band 3 protein in RBC membranes. Rotational mobility measurements [9] and cross-linking with an impermeable, bifunctional cross-linker [10] suggested that band 3 protein exists as dimers. Since the cross-linking studies were performed for 30 min at room temperature, it was possible that cross-links occurred between mobile molecules. We therefore reinvestigated membrane glycoprotein topology in intact human RBCs on treatment with a highly reactive, impermeable cross-linker, disuccinimidyl 3,3'-dithiobispropionate (DTSP) at 0–4 °C for 8 min [11]. Using DTSP as a crosslinker, we studied membrane pro-

tein topology on cells on which glycoproteins were chemically or enzymatically oxidized and the generated aldehydes modified by either a ^{14}C -labeled arylamine or arylalkyldiamine. Incorporation of arylalkyldiamines into the glycoproteins/glycolipids provided each cell with up to 7.2×10^6 additional amino groups, helping to overcome a potential scarcity of reactive amines when studying the cross-linkability of glycoproteins and glycolipids. Following galactose oxidase treatment and the introduction of arylalkyldiamine, DTSP cross-linked 1.5% of total labeled material on young RBCs and 1.9% on old RBCs (different at a confidence level of 0.06), but 20% on spectrin-free vesicles [11]. Thus, band 3 protein cross-linking is slightly but significantly higher on old compared to young RBCs. However, glycoproteins on RBCs do not undergo substantial cross-linking regardless of whether they are supplemented with amino groups or not. These results imply that cross-linking does not occur between band 3 molecules belonging to the same dimer. Cross-linking between monomers and dimers, between dimers, or between dimers and tetramers occurred upon the loss of cytoskeletal anchorage during cell ageing or in vesicles budding off from ATP-depleted RBCs.

We also tried to immunoprecipitate the component of RBCs to which autologous IgG binds. The first approach published in 1981 [12] resulted in some artifacts, and taught us to introduce a number of precautions. Percoll gradients for cell separation were used following the removal of white cells [13]. Washed young and old RBCs were treated with the protease inhibitor diisopropylfluorophosphate (DFP) before lysis, and the purified membranes were alkylated with N-ethylmaleimide prior to solubilization with Triton X-100 [14]. Triton extracts of ^{125}I -iodinated RBCs were then immunoprecipitated using anti-IgG antibody. When applying all these precautions, after solubilization and subjecting to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitates from old RBCs revealed label at around 100 kDa, 200 kDa and at the top of the gel, whereas very little label at 100 kDa was seen with that from young RBCs [15]. Precipitated label comigrating with band 3 protein showed the same ^{125}I -iodinated peptides as band 3 protein from surface- ^{125}I -iodinated cells. The amount of band 3 protein that was immunoprecipitated from extracts of young RBCs increased on preincubation with 0.2 mg/ml autologous IgG. This series of findings therefore strongly suggested that senescent RBCs are recognized by low-affinity anti-band 3 NABs that bind to oligomerized/aggregated band 3 proteins that have lost their anchorage to the cytoskeleton.

Years later, once anti-band 3 NABs had been isolated and characterized [16], we studied their role in phagocytosis of oxidatively stressed human RBCs. Paolo Arese and his collaborators had developed an elegant assay using diamide as the oxidizing agent and measuring phagocytosis of opsonized [^{14}C]cyanate-labeled RBCs by adherent peripheral monocytes [17]. Opsonization of diamide-treated RBCs with up to 20 $\mu\text{g/ml}$ anti-band 3 NABs in 80% serum, and resuspension in

80% serum during phagocytosis stimulated phagocytosis 4- to 5-fold [18]. Anti-spectrin NABs had no effect. Increasing concentrations of diamide generated S-S-linked polypeptides that were highly enriched in S-S-bonded band 3 protein, reactive with anti-band 3 NABs. Effective phagocytosis required active complement, and the amount of bound C3b exceeded that of bound anti-band 3 NABs by 2 orders of magnitude, implying participation of the alternative complement pathway. The requirement of complement was later verified in experiments *in vivo* in guinea pigs. 20 µg/ml human anti-band 3 NAB accelerated *in vivo* clearance of diamide-treated guinea pig RBCs in normal, but not in C3-deficient animals [19].

These data provided direct proof of the initially hypothesized mechanism. This suggested mechanism has survived the test of time. It has triggered many follow up studies that have yielded *in vivo* evidence for the mechanism and clarified it in more detail (outlined below). However, opposing views on the fine specificity of anti-band 3 NABs have hampered a swift advance for many years. In fact, the task of writing this review has revived my interest in clarifying this issue from a distance and with the help of dozens of papers that have appeared since then on seemingly remote aspects.

Role of Naturally Occurring IgG Anti-Band 3 Antibodies and Disease-Induced IgG Antibodies to Lactoferrin

We have purified IgG anti-band 3 NABs 8 times from IgG of healthy blood donors (blood group O Rh⁺) on autologous band 3 protein and often from several lots of pooled human IgG for intravenous use (Sandoglobulin) on immobilized band 3 protein from healthy blood donors (O Rh⁺) [16]. The specificity of purified anti-band 3 NABs was always the same whether studied by labeled second antibody or by directly ¹²⁵I-labeled anti-band 3 NABs. Anti-band 3 NABs bound strongly to band 3 protein and weakly to bands 4.2, 5, and 6. Binding to these cytoplasmic proteins was fully suppressed on blots by adding equal concentrations of unlabeled IgG absorbed on band 3 protein or by adding whole, unlabeled IgG up to physiological concentrations. Using a chymotrypsin treatment of intact RBCs it was possible to differentiate between antigenic and non-antigenic peptides of band 3 protein. Anti-band 3 NABs bind exclusively to the 55-kDa transmembrane fragment (earlier named 62- or 65-kDa fragment), but not to the 38-kDa carbohydrate-containing fragment of band 3 [16]. Eight years after appearance of our paper, the group of Beppu [20] reported that anti-band 3 NABs, as purified by this group, bound to sialylated N-acetyl-lactosaminyl carbohydrate groups, localized in the 38-kDa fragment of band 3 protein. Since we did not observe any anti-band 3 binding to the 38-kDa fragment of band 3 protein in our first study, we reinvestigated binding of anti-band 3 NABs to band 3 protein [21]. Anti-band 3 NABs purified from IgG of pooled plasma from healthy humans (Sandoglobulin) again bound to the 55-kDa,

but not the 38-kDa fragment of band 3 protein. Furthermore, the binding of purified anti-band 3 NABs to band 3 protein on blots from electrophoretically spread RBC membrane proteins was neither inhibited by pretreating RBCs with neuraminidase nor endo-β-galactosidase. Thus, the 2 anti-band 3 NAB preparations differed, most likely because the 2 preparations originated from different starting material. Our starting material was well defined, included RBCs from regular healthy blood donors with group ORh⁺ and pooled human IgG from healthy blood donors as worked up in Sandoglobulin. For anti-band 3 isolation, Beppu et al. [22] initially purified IgG from serum that was treated for 30 min at 56 °C to deplete of complement proteins and originated from donors with blood group AB (Rhesus antigens not indicated). In later papers, IgG purification apparently followed standard procedures, but the source of IgG remained poorly defined [20, 23]. Thus, it is possible that the starting material used by Beppu's group originated from apparently healthy people who had induced IgG antibodies that also bound to the carbohydrate of band 3 protein. This is very likely since: i) Ando et al. [23] could not only purify IgG anti-band 3, but also IgG anti-lactoferrin antibodies from the IgG preparation of the donors they used; and ii) both lactoferrin and the carbohydrate portion of the 38-kDa chymotryptic fragment of band 3 protein contain sialylated N-acetyl-lactosaminyl sugar chains [23, 24].

In support of this assertion, IgG anti-lactoferrin antibodies are normally not detected in healthy controls, but are found in a substantial, but varying, number of patients with anti-neutrophil cytoplasmic antibodies (ANCA) [25], ulcerative colitis [26], primary sclerosing cholangitis [27], systemic vasculitis [28], rheumatoid arthritis [25, 28, 29] or systemic lupus erythematosus (SLE) [30, 31]. Thus, in the experiments of Beppu and colleagues [23] immobilized band 3 protein captured not only anti-band 3 NABs from their IgG preparation, but also IgG anti-lactoferrin antibodies. In fact, 70% of this group's 'anti-band 3 NABs' bound to the carbohydrate portion of lactoferrin and 30% to a polypeptide of band 3 protein (presumably the 55-kDa fragment). Thus, it was wrong to name this eluate 'anti-band 3 NABs'.

However, the fact that the 'anti-band 3 NABs' of Beppu and colleagues contain IgG anti-lactoferrin antibodies gives these data an unexpected relevance. When this mixture of NABs and induced IgG antibodies is present with ageing RBCs, the 2 types of IgG together may accelerate clearance of senescent RBCs or may result in premature clearance of aging RBCs. The reason is that not only do the band 3 protein oligomers bind anti-band 3 NABs, but groups of band 3 protein carrying the anti-band 3 NABs can further be clustered by IgG anti-lactoferrin antibodies that bind to a different region of band 3 protein. Such complexation will most likely induce additional complement deposition and increased phagocytosis. Until now, this phenomenon has not been recognized or investigated, although there are a number of findings supporting this explanation. For example, disease activity was increased among SLE

patients with medium to high levels of IgG anti-lactoferrin antibodies [31] and 6 SLE patients who had IgG anti-lactoferrin but no other ANCA type antibody presented with hematological disorders (anemia) [32]. In 1 report, findings in pregnant women with iron-deficiency anemia treated for 30 days with bovine lactoferrin (30% iron saturated) were compared with those from anemic women treated with ferrous sulfate or nothing. The 60 patients treated with lactoferrin showed a significant increase in RBC numbers and hemoglobin concentrations, while those treated with ferrous iron alone had only slightly higher hemoglobin [33]. Thus, the curing effect of lactoferrin was probably not mediated by an improved bioavailability of iron, but by lactoferrin interfering with RBC clearance, as suggested by *in vivo* clearance experiments on rats in which lactoferrin inhibited phagocytosis of IgG-coated RBCs [34]. However, lactoferrin had no effect on the hematological parameters of healthy term infants younger than 6 months [35], presumably because their immune system is in early development and induced IgG anti-lactoferrin antibodies are definitely absent. The simplest explanation of all these findings is that the applied lactoferrin complexed the recipients' IgG anti-lactoferrin antibodies, whereby opsonization of aging RBCs by anti-lactoferrin antibodies was diminished, excessive clearance stopped, and so anemia reduced. It has not as yet been demonstrated whether the enhanced opsonization and RBC clearance was indeed due to N-glycan-specific anti-lactoferrin antibodies. For this, the IgG anti-lactoferrin antibodies in anemic patients, above all in patients with ANCA type of autoantibodies, need to be determined. Such assays would have to focus on the N-glycan specificity of anti-lactoferrin and need to be isotype specific because healthy people have a low titer of naturally occurring IgM anti-lactoferrin antibodies, which are directed to cryptic regions of lactoferrin that are exposed, e.g. on sperm heads during their interaction with oocytes [36]. If measurable titers of IgG anti-lactoferrin are found against N-glycans, clinicians should consider treating these patients with lactoferrin. Human or bovine lactoferrin can be applied *per os*, because a substantial portion of lactoferrin (64% of apo-lactoferrin; 79% of iron-saturated lactoferrin) escapes digestion and penetrates into other compartments [37].

Band 3 Clustering: Oxidative Damage and Biochemical Help for Efficient Binding of Anti-Band 3 NABs

Despite the so-called 'uncertainty of whether anti-band 3 NABs bind only to the 55-kDa fragment, or to 55-KDa and the carbohydrate-containing 38-kDa fragment', many groups have since confirmed that band 3 protein oligomerization is a prerequisite for IgG NAB binding, and have eluted and characterized these NABs from *in vivo*-aged RBCs [22, 38–41]. These findings strongly suggest that anti-band 3 NABs are effective in clearance of RBCs *in vivo*. Several groups extended these findings by showing that oxidative damage to

RBCs results in hemichrome generation and its binding to the cytoplasmic portion of band 3 protein, which induces band 3 protein clustering in healthy subjects [42, 43], in anemia [42, 44], in malaria [45], and in dogs [40]. Schlüter and Drenckhahn [46] nicely demonstrated that aged RBCs from healthy donors and RBCs from splenectomized patients with unstable hemoglobins reveal hemichrome precipitates (Heinz bodies) that colocalize with clusters of band 3 protein and clusters of surface-bound Ig. Turrini et al. [47] used Zn^{2+} -ions to induce band 3 clusters, and studied binding of autologous IgG. Eluted material indeed contained anti-band 3 NABs and C3b. Together these data suggest that hemichrome-induced band 3 clustering facilitates efficient binding of anti-band 3 NABs.

However, for years it remained unclear how these band 3 oligomers and clusters are induced, because band 3 protein exists primarily as dimers and anchored tetramers in the membrane [48]. While the dimers are held together by helix/helix interactions within the cytoplasmic phase, the tetramers are formed from 2 dimers stabilized by interactions with cytoskeletal proteins [49]. The only region to which anti-band 3 NABs bind is located within the 55-kDa transmembrane portion of band 3. A bivalent binding of IgG anti-band 3 NABs to both band 3 proteins of a dimer does not occur (see first section) because it is sterically impossible. Thus, docking of 2 band 3 dimers provides the prerequisite for bivalent binding of anti-bands 3 NABs. Even if anti-band 3 NABs had a high affinity and could recruit binding partners upon monovalent binding, band 3 protein has first to become mobile within the plane of the membrane.

Upon oxidative damage by diamide and pervanadate as well as in ageing RBCs, band 3 tetramers are liberated from the cytoskeleton by a $p72^{syk}$ -dependent tyrosine phosphorylation of the N-terminal portion of band 3 [49–51]. This syk-dependent phosphorylation follows inhibition of phosphatases and syk kinase translocation to the membrane [50], phenomena which are differently induced by the various types of agents that can induce oxidative damage [52]. Tyrosine phosphorylation of band 3 protein lowers the affinity of ankyrin for band 3 [49, 53], and increases both its lateral mobility and the probability to be cross-linked by a non-penetrating bivalent cross-linker [53]. These biochemical changes primarily allow the formation of quasi linear oligomers from preexisting band 3 dimers and tetramers, where 1 anti-band 3 NAB may cross-link the 2 dimers within a tetramer, with each of the 2 remaining band 3 protein of a tetramer becoming connected to separate dimers or tetramers [54]. Clusters of band 3 protein are not readily generated by this process. The following conditions favor clustering: i) the presence of anti-lactoferrin antibodies, as is the case in patients with ANCA-type autoantibodies (as outlined); and ii) the absence of poly-N-acetyllactosaminyl groups on the 38-kDa portion of band 3 (as is the case in RBCs of CDA II (congenital dyserythropoietic anemia type II) patients [55]) favors band 3 clustering, opsonization and phagocytosis [56]. The poly-N-acetyllactosaminyl

groups sterically interfere with band 3 aggregate formation to the point that band 3 aggregates on ageing RBCs from healthy controls are generated primarily from band 3 proteins that lack poly-N-acetyllactosaminyl groups [50]. It appears that clustering is not only enhanced by bound hemichromes, but also by band 3-bound Syk kinase that catalyzes the phosphorylation of the N-terminal cytoplasmic tyrosine residues [50].

A Unique Mechanism by Which Anti-Band 3 NABs Stimulate Complement Amplification

Puzzled by the fact that old RBCs carry only 100–200 IgG molecules [4, 57] or only 60 to 120 molecules of protein A more than young cells [58], which are capable of inducing their selective clearance, we also looked for RBC-bound complement component C3 and noticed covalently linked high-molecular-weight complexes that were positive for anti-band 3 and C3 [59, 60]. This prompted us to investigate why activated C3 formed covalent complexes primarily with the rare anti-band 3 NABs. On the basis of what was known on complement amplification at that time, we discussed how such a complex may help to stimulate complement C3b deposition (for a review see [61]). Anti-band 3 NABs have an affinity for C3, which resides in the Fab portion independent of the binding site for band 3 because C3 cannot inhibit binding to band 3 protein [62]. This affinity for C3 is a prerequisite for these NABs to preferentially form C3b₂-IgG complexes [63]. IgG anti-spectrin NABs lack this affinity and do not form C3b₂-IgG complexes under the same conditions. In analogy to the first description of such complexes by Gadd and Reid [64], we continued to use the term ‘C3b-IgG complexes’, implying complexes containing either 1 or 2 C3b molecules [63], although our data showed that C3b₂-IgG complexes represented the dominant ones. Later, we generated C3b₂-IgG complexes from whole IgG and C3 by activation with trypsin. The purified complexes did not contain C3b-IgG complexes [65]. The dimeric C3b within such a C3b₂-IgG complex is not only far more stable than surface-bound C3b, it also has a unique effect on the assembly of an alternative C3 convertase. Unlike surface-bound C3b, the dimeric C3b in C3b₂-IgG complexes first binds properdin, which greatly stimulates factor B binding [65]. C3 activation by an alternative complement pathway C3 convertase assembled on C3b₂-IgG complexes is 1–2 orders of magnitude greater than on C3b. This enhancement effect is highest at low inputs of C3b₂-IgG complexes [65] (reviewed in [66]). Finally, we addressed the question whether C3b-IgG complexes represent a mixture of complexes with 1 or 2 C3b molecules. Detailed analyses by 2-dimensional SDS-PAGE, using reduced complexes in the first dimension and a hydroxylamine treatment between the dimensions to cleave ester bonds, were performed on complexes generated from either labeled C3, labeled IgG or labeled complexes [67]. The major component among these

complexes was the α'_2 -heavy chain from C3b₂-IgG, which released 2 C3 α chains and 1 IgG heavy chain and a minor spot, previously thought to represent C3b-IgG, which instead contained α' - α' from dimeric C3b with an apparent molecular weight comparable to that of a C3b-IgG complex.

Other Mechanisms Suggested to Promote a Selective Clearance of Aged Erythrocytes

For many years it appeared as if exposure of phosphatidylserine (PS) on the outer leaflet of the membrane triggered a selective removal of senescent RBCs [68–70]. However, on determining PS exposure using the selective binding of annexin V [71] carrying a red fluorescent tag [72], rather than by measuring the exchange of labeled lipids [69], it became clear that PS exposure was by no means a phenomenon operating primarily on old RBCs. Experimental proof that PS is preferentially exposed on young rather than senescent RBCs has been provided by Khandelwal and Saxena [72] by quantifying binding of red-fluorescent annexin V to young, middle-aged and old RBCs that were biotinylated in vivo. Earlier measurements with green-fluorescent annexin V [70] gave higher readings for old RBCs because old RBCs have a significantly higher green autofluorescence than young ones [73]. PS exposure can be initiated by a variety of stimuli and requires activation of several enzymatic and transport processes that operate best in young RBCs. One of the first steps requires the entry of Ca²⁺ ions into the cell, which opens the Ca²⁺-dependent K⁺ channel and results in extrusion of both potassium and chloride ions followed by water [74]. The induced shrinkage of the cell stimulates scramblase activity, a specific phospholipid exchanger that exposes PS on the outer layer of the membrane. The entire process resembles that of apoptosis of nucleated cells. Lang and collaborators have therefore coined the term ‘eryptosis’ [74]. Ultimately, eryptotic RBCs are recognized by PS-specific receptors (TIM-4) on macrophages [75]. Eryptosis contributes to an accelerated clearance of RBCs in a number of diseases [76], but is clearly not the route by which senescent RBCs are recognized. Neocytolysis, an extreme form of PS exposure on young RBCs is induced by a 4-fold decrease in erythropoietin as experienced by acclimatized high-altitude climbers when descending to sea level [77].

Anti-gal directed against Gal α 1–3Gal β 1–4GlcNAc-R was suggested to represent an IgG NAB that selectively binds to a cryptic carbohydrate on senescent human RBCs (for a review see [78]). This IgG antibody exists at a concentration of 100 μ g/ml in human plasma. Anti-gal IgG may not represent NABs, but pathogen-induced IgG antibodies. Antibody eluates from senescent RBCs contained comparable numbers of IgG anti-band 3 and IgG anti-gal, although the anti-band 3 concentration in serum is more than 100 times smaller than that of anti-gal [79]. Considering the huge concentration difference, bound anti-gal may be an artifact due to insufficient washing.

Additional means to differentiate between self and non-self have been described by Oldenborg and collaborators [80], who discovered that the integrin-associated protein, also called CD47, functions as a marker of self on murine RBCs. These authors showed that normally opsonized RBCs were rapidly cleared by macrophages when the RBCs lacked CD47, but remained in circulation when they carried CD47. Thus, sufficient numbers of CD47 per RBC prevented RBC elimination. RBC-bound CD47 molecules exert this effect by interacting with the signal regulatory protein alpha (SIRP α) on macrophages. CD47 binding induces clustering of SIRP α , which triggers phosphorylation of its tail and this process recruits phosphatases (SHP-1) that prevent phagocytosis of the bound target. Indeed, a complete CD47 deficiency in mice with a spontaneously developing autoimmune hemolytic ane-

mia results in a lethal disease [81]. In contrast to this, CD47 does not operate as a marker of self in humans because, although humans lacking Rh or D antigens on their RBCs have 4 to 5 times fewer CD47, their RBCs are not phagocytosed more than RBCs with normal amounts of CD47 [82]. Likewise, CD47 was almost absent (1%) in a patient with spherocytosis due to the lack of band 4.2 [83]. Furthermore, low copy numbers of SIRP α on human phagocytes explain their inefficiency in clearing RBCs with reduced CD47 [84].

Disclosure Statement

The author declared no conflict of interest.

References

1. Lutz HU: Elimination of old erythrocytes from the circulation: Exposure of a cell-age specific antigen on aging erythrocytes (in German). *Schweiz Med Wochenschr* 1981;111:1507–1517.
2. Luner SJ, Szklarek D, Knox RJ, Seaman GVF, Josefowicz JY, Ware BR: Red cell charge is not a function of cell age. *Nature* 1977;269:719–721.
3. Lutz HU, Fehr J: Total sialic acid content of glycoproteins during senescence of human red blood cells. *J Biol Chem* 1979;254:11177–11180.
4. Kay MMB: Mechanism of removal of senescent cells by human macrophages in situ. *Proc Natl Acad Sci U S A* 1975;72:3521–3525.
5. Lutz HU, Wipf G: Naturally occurring autoantibodies to skeletal proteins from human red blood cells. *J Immunol* 1982;128:1695–1699.
6. Kay MMB: Isolation of the phagocytosis-inducing IgG-binding antigen on senescent somatic cells. *Nature* 1981;289:491–494.
7. Lutz HU, Liu S-C, Palek J: Release of spectrin-free vesicles from human erythrocytes during ATP depletion. *J Cell Biol* 1977;73:548–560.
8. Mueller H, Lutz HU: Binding of autologous IgG to human red blood cells before and after ATP-depletion. Selective exposure of binding sites (autoantigens) on spectrin-free vesicles. *Biochim Biophys Acta* 1983;729:249–257.
9. Nigg E, Cherry RJ: Dimeric association of band 3 in the erythrocyte membrane demonstrated by protein diffusion measurements. *Nature* 1979;277:493–494.
10. Staros JV, Morgan DG, Appling DR: A membrane-impermeant, cleavable cross-linker. *J Biol Chem* 1981;256:5890–5893.
11. Schweizer E, Angst W, Lutz HU: Glycoprotein topology on intact human red blood cells reevaluated by cross-linking following amino-group supplementation. *Biochemistry* 1982;21:6807–6818.
12. Lutz HU: A cell-age specific antigen on senescent human red blood cells. *Acta Biol Med Ger* 1981;40:393–400.
13. Beutler E, West C, Blume K-G: The removal of leukocytes and platelets from whole blood. *J Lab Clin Med* 1976;88:328–333.
14. Pappert G, Schubert D: The state of association of band 3 protein of the human erythrocyte membrane in solutions of nonionic detergents. *Biochim Biophys Acta* 1983;730:32–40.
15. Lutz HU, Stringaro-Wipf G: Senescent red cell-bound IgG is attached to band 3 protein. *Biomed Biochim Acta* 1983;42:117–121.
16. Lutz HU, Flepp R, Stringaro-Wipf G: Naturally occurring autoantibodies to exoplasmic and cryptic regions of band 3 protein, the major integral membrane protein of human red blood cells. *J Immunol* 1984;133:2610–2618.
17. Bussolino F, Turrini F, Arese P: Measurement of phagocytosis utilizing ^{14}C -cyanate-labelled human red cells and monocytes. *Br J Haematol* 1987;66:271–274.
18. Lutz HU, Bussolino F, Flepp R, Fasler S, Stammler P, Kazatchkine MD, Arese P: Naturally occurring anti-band 3 antibodies and complement together mediate phagocytosis of oxidatively stressed human red blood cells. *Proc Natl Acad Sci U S A* 1987;84:7368–7372.
19. Giger U, Sticher B, Naef R, Burger R, Lutz HU: Naturally occurring human anti-band 3 autoantibodies accelerate clearance of erythrocytes in guinea pigs. *Blood* 1995;85:1920–1928.
20. Beppu M, Mizukami A, Ando K, Kikugawa K: Antigenic determinants of senescent antigen of human erythrocytes are located in sialylated carbohydrate chains of band-3 glycoprotein. *J Biol Chem* 1992;267:14691–14696.
21. Lutz HU, Gianora O, Nater M, Schweizer E, Stammler P: Naturally occurring anti-band 3 antibodies bind to protein rather than to carbohydrate on band 3. *J Biol Chem* 1993;268:23562–23566.
22. Beppu M, Mizukami A, Nagoya M, Kikugawa K: Binding of anti-band 3 autoantibody to oxidatively damaged erythrocytes. *J Biol Chem* 1990;265:3226–3233.
23. Ando K, Kikugawa K, Beppu M: Binding of anti-band 3 autoantibody to sialylated poly-N-acetyl-lactosaminyl sugar chains of band 3 glycoprotein on polyvinylidene difluoride membrane and sepharose gel: Further evidence for anti-band 3 autoantibody binding to the sugar chains of oxidized and senescent erythrocytes. *J Biochem* 1996;119:639–647.
24. Ando K, Kikugawa K, Beppu M: Involvement of sialylated poly-N-acetyl-lactosaminyl sugar chains of band 3 glycoprotein on senescent erythrocytes in anti-band 3 autoantibody binding. *J Biol Chem* 1994;269:19394–19398.
25. Chikazawa H, Nishiya K, Matsumori A, Hashimoto K: Immunoglobulin isotypes of anti-myeloperoxidase and anti-lactoferrin antibodies in patients with collagen diseases. *J Clin Immunol* 2000;20:279–286.
26. Roozendaal C, Pogany K, Horst G, Jagt TG, Kleibeuker JH, Nelis GF, Limburg PC, Kallenberg CGM: Does analysis of the antigenic specificities of anti-neutrophil cytoplasmic antibodies contribute to their clinical significance in the inflammatory bowel diseases? *Scand J Gastroenterol* 1999;34:1123–1131.
27. Peen E, Almer S, Bodemar G, Ryden BO, Sjolin C, Tejle K, Skogh T: Antilactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and crohns disease. *Gut* 1993;34:56–62.
28. Esnault VL, Short AK, Audrain MA, Jones SJ, Martin SJ, Skehel JM, Lockwood CM: Autoantibodies to lactoferrin and histone in systemic vasculitis identified by anti-myeloperoxidase solid phase assays. *Kidney Int* 1994;46:153–160.
29. Brimnes J, Halberg P, Jacobsen S, Wiik A, Heegaard NHH: Specificities of anti-neutrophil autoantibodies in patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 1997;110:250–256.
30. Näsberger L, Hultquist R, Sturfelt G: Occurrence of anti-lactoferrin antibodies in patients with systemic lupus erythematosus, hydralazine-induced lupus, and rheumatoid arthritis. *Scand J Rheumatol* 1994;23:206–210.
31. Caccavo D, Rigon A, Picardi A, Galluzzo S, Vadacca M, Ferri GM, Amoroso A, Afeltra A: Anti-lactoferrin antibodies in systemic lupus erythematosus: Isotypes and clinical correlates. *Clin Rheumatol* 2005;24:381–387.
32. Manolova I, Dancheva M, Halacheva K: Predominance of IgG1 and IgG3 subclasses of autoantibodies to neutrophil cytoplasmic antigens in patients with systemic lupus erythematosus. *Rheumatol Int* 2002;21:227–233.
33. Nappi C, Tommaselli GA, Morra I, Massaro M, Formisano C, Di Carlo C: Efficacy and tolerability of oral bovine lactoferrin compared to ferrous sulfate in pregnant women with iron deficiency anemia: a prospective controlled randomized study. *Acta Obstet Gynecol Scand* 2009;88:1031–1035.

34. Malaise MG, Hoyoux C, Franchimont P, Foidart JB, Mahieu PR: Effects of mannose and mannose derivatives on the clearance of IgG antibody-coated erythrocytes in the rat. *Immunology* 1989; 68:126–132.
35. Hernell O, Lonnerdal B: Iron status of infants fed low-iron formula: No effect of added bovine lactoferrin or nucleotides. *Am J Clin Nutr* 2002;76:858–864.
36. Rodman TC, Winston R, Sullivan JJ, Yan XJ, Chiorazzi N: An innate natural antibody is reactive with a cryptic sequence of lactoferrin exposed on sperm head surface. *Proc Soc Exp Biol Med* 1997; 216:404–409.
37. Troost FJ, Steijns J, Saris WH, Brummer RJ: Gastric digestion of bovine lactoferrin in vivo in adults. *J Nutr* 2001;131:2101–2104.
38. Victoria EJ, Mahan LC, Masoureddis SP: The IgG binding function of the normal red cell plasma membrane: Identification of integral polypeptides that bind IgG. *Br J Haematol* 1982;50:101–110.
39. Turrini F, Arese P, Yuan J, Low PS: Clustering of integral membrane proteins of the human erythrocyte membrane stimulates autologous IgG binding, complement deposition, and phagocytosis. *J Biol Chem* 1991;266:23611–23617.
40. Rettig MP, Low PS, Gimm JA, Mohandas N, Wang JZ, Christian JA: Evaluation of biochemical changes during in vivo erythrocyte senescence in the dog. *Blood* 1999;93:376–384.
41. Pantaleo A, Giribaldi G, Mannu F, Arese P, Turrini F: Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions. *Autoimmun Rev* 2008;7:457–462.
42. Kannan R, Labotka R, Low PS: Isolation and characterization of the hemichrome-stabilized membrane protein aggregates from sickle erythrocytes. *J Biol Chem* 1988;263:13766–13773.
43. Waugh SM, Low PS: Hemichrome binding to band 3: Nucleation of Heinz bodies on the erythrocyte membrane. *Biochemistry* 1985;24:34–39.
44. Mannu F, Arese P, Cappellini MD, Fiorelli G, Cappadoro M, Giribaldi G, Turrini F: Role of hemichrome binding to erythrocyte membrane in the generation of band-3 alterations in beta-thalassemia intermedia erythrocytes. *Blood* 1995;86:2014–2020.
45. Turrini F, Giribaldi G, Carta F, Mannu F, Arese P: Mechanisms of band 3 oxidation and clustering in the phagocytosis of *Plasmodium falciparum*-infected erythrocytes. *Redox Rep* 2003;8:300–303.
46. Schlüter K, Drenckhahn D: Co-clustering of denatured hemoglobin with band 3: its role in binding of autoantibodies against band 3 to abnormal and aged erythrocytes. *Proc Natl Acad Sci U S A* 1986; 83:6137–6141.
47. Turrini F, Mannu F, Arese P, Yuan J, Low PS: Characterization of the autologous antibodies that opsonize erythrocytes with clustered integral membrane proteins. *Blood* 1993;81:3146–3152.
48. Casey JR, Reithmeier RAF: Analysis of the oligomeric state of band-3, the anion transport protein of the human erythrocyte membrane, by size exclusion high performance liquid chromatography – oligomeric stability and origin of heterogeneity. *J Biol Chem* 1991;266:15726–15737.
49. Willardson BM, Thevenin BJ, Harrison ML, Kuster WM, Benson MD, Low PS: Localization of the ankyrin-binding site on erythrocyte membrane protein, band-3. *J Biol Chem* 1989;264:15893–15899.
50. Pantaleo A, Ferru E, Giribaldi G, Mannu F, Carta F, Matte A, de Franceschi L, Turrini F: Oxidized and poorly glycosylated band 3 is selectively phosphorylated by Syk kinase to form large membrane clusters in normal and G6PD-deficient red blood cells. *Biochem J* 2009;418:359–367.
51. Harrison ML, Isaacson CC, Burg DL, Geahlen RL, Low PS: Phosphorylation of human erythrocyte band-3 by endogenous p72(syk). *J Biol Chem* 1994; 269:955–959.
52. Bordin L, Ion-Popa F, Brunati AM, Clari G, Low PS: Effector-induced Syk-mediated phosphorylation in human erythrocytes. *Biochim Biophys Acta* 2005;1745:20–28.
53. Ferru E, Giger K, Pantaleo A, Campanella E, Grey J, Ritchie K, Vono R, Turrini F, Low PS: Regulation of membrane-cytoskeletal interactions by tyrosine phosphorylation of erythrocyte band 3. *Blood* 2011;117:5998–6006.
54. Turrini F, Mannu F, Cappadoro M, Ulliers D, Giribaldi G, Arese P: Binding of naturally occurring antibodies to oxidatively and nonoxidatively modified erythrocyte band 3. *Biochim Biophys Acta* 1994;1190:297–303.
55. Fukuda MN, Masri KA, Dell A, Luzzatto L, Moremen KW: Incomplete synthesis of N-glycans in congenital dyserythropoietic anemia type-II caused by a defect in the gene encoding alpha-mannosidase-II. *Proc Natl Acad Sci U S A* 1990;87:7443–7447.
56. Defranceschi L, Turrini F, Delgiudice EM, Perrotta S, Olivieri O, Corrocher R, Mannu F, Iolascon A: Decreased band 3 anion transport activity and band 3 clusterization in congenital dyserythropoietic anemia type II. *Exp Hematol* 1998;26:869–873.
57. Szymanski IO, Odgren PR, Fortier N, Snyder LM: Red blood cell associated IgG in normal and pathologic states. *Blood* 1980;55:48–54.
58. Muller H, Lutz HU: Binding of autologous IgG to human red blood cells before and after ATP-depletion. Selective exposure of binding sites (autoantigens) on spectrin-free vesicles. *Biochim Biophys Acta* 1983;729:249–257.
59. Lutz HU, Stammler P, Furter C, Fasler S: Alternative complement pathway activation by anti-band 3 antibodies (in German). *Schweiz Med Wochenschr* 1987;117:1821–1824.
60. Lutz HU, Stammler P, Fasler S: How naturally occurring anti-band 3-antibodies stimulate C3b deposition to senescent and oxidatively stressed red blood cells. *Biomed Biochim Acta* 1990;49:224–229.
61. Lutz HU: Erythrocyte clearance; in Harris JR (ed): *Blood Cell Biochemistry*, vol 1, Erythroid Cells. New York and London: Plenum Press, 1990, pp 81–120.
62. Lutz HU, Nater M, Stammler P: Naturally occurring anti-band 3 antibodies have a unique affinity for C3. *Immunology* 1993;80:191–196.
63. Lutz HU, Stammler P, Fasler S: Preferential formation of C3b-IgG complexes in vitro and in vivo from nascent C3b and naturally occurring anti-band 3 antibodies. *J Biol Chem* 1993;268:17418–17426.
64. Gadd KJ, Reid KBM: The binding of complement component C3 to antibody-antigen aggregates after activation of the alternative pathway in human serum. *Biochem J* 1981;195:471–480.
65. Jelezarova E, Vogt A, Lutz HU: Interaction of C3b₂-IgG complexes with complement proteins properdin, factor B and factor H: implications for amplification. *Biochem J* 2000;349:217–223.
66. Jelezarova E, Lutz HU: Assembly and regulation of the complement amplification loop in blood: The role of C3b-C3b-IgG complexes. *Mol Immunol* 1999;36:837–842.
67. Jelezarova E, Luginbuehl A, Lutz HU: C3b₂-IgG complexes retain dimeric C3 fragments at all levels of inactivation. *J Biol Chem* 2003;278:51806–51812.
68. Schroit AJ, Madsen JW, Tanaka Y: In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. *J Biol Chem* 1985;260:5131–5138.
69. Connor J, Pak CC, Schroit AJ: Exposure of phosphatidylserine in the outer leaflet of human red blood cells – relationship to cell density, cell age, and clearance by mononuclear cells. *J Biol Chem* 1994;269:2399–2404.
70. Boas FE, Forman L, Beutler E: Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci U S A* 1998;95:3077–3081.
71. vanEngeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM: Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998;31:1–9.
72. Khandelwal S, Saxena RK: A role of phosphatidylserine externalization in clearance of erythrocytes exposed to stress but not in eliminating aging populations of erythrocyte in mice. *Exp Gerontol* 2008; 43:764–770.
73. Khandelwal S, Saxena RK: Age-dependent increase in green autofluorescence of blood erythrocytes. *J Biosci* 2007;32:1139–1145.
74. Foller M, Huber SM, Lang F: Erythrocyte programmed cell death. *IUBMB Life* 2008;60:661–668.
75. Miyaniishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S: Identification of Tim4 as a phosphatidylserine receptor. *Nature* 2007;450:435–439.
76. Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378–388.
77. Risso A, Turello M, Biffoni F, Antonutto G: Red blood cell senescence and neocytolysis in humans after high altitude acclimatization. *Blood Cells Mol Dis* 2007;38:83–92.
78. Galili U: The natural anti-gal antibody: Evolution and autoimmunity in man; in Bona CA, Kaushik AK (eds): *Molecular Immunobiology of Self-Reactivity*. New York, Marcel Dekker, 1992, pp 355–373.
79. Sorette MP, Galili U, Clark MR: Comparison of serum anti-band-3 and anti-gal antibody binding to density-separated human red blood cells. *Blood* 1991;77:628–636.
80. Oldenburg PA, Gresham HD, Lindberg FP: CD47-signal regulatory protein alpha (SIRP alpha) regulates Fc gamma and complement receptor-mediated phagocytosis. *J Exp Med* 2001;193:855–861.
81. Oldenburg PA, Gresham HD, Chen Y, Izui S, Lindberg FP: Lethal autoimmune hemolytic anemia in CD47-deficient nonobese diabetic (NOD) mice. *Blood* 2002;99:3500–3504.
82. Arndt PA, Garratty G: Rh(null) red blood cells with reduced CD47 do not show increased interactions with peripheral blood monocytes. *Br J Haematol* 2004;125:412–414.
83. Bruce LJ, Ghosh S, King MJ, Layton DM, Mawby WJ, Stewart GW, Oldenburg PA, Delaunay J, Tanner MJA: Absence of CD47 in protein 4.2-deficient hereditary spherocytosis in man: An interaction between the Rh complex and the band 3 complex. *Blood* 2002;100:1878–1885.
84. Subramanian S, Parthasarathy R, Sen S, Boder ET, Discher DE: Species- and cell type-specific interactions between CD47 and human SIRPalpha. *Blood* 2006;107:2548–2556.