Effect of splenectomy on opsonization and deformability of erythrocytes in hereditary spherocytosis with band 3 and spectrin/ankyrin deficiency

Author(s):
Reliene, Ramune

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Effect of splenectomy on opsonization and deformability of erythrocytes in hereditary spherocytosis with band 3 and spectrin/ankyrin deficiency

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SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH

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presented by
Ramune Reliene
M.Sc. University of Vilnius
born June 9, 1970
Lithuanian citizen

Accepted on the recommendation of
Prof. Dr. Kaspar Winterhalter, examiner
Dr. Hans U. Lutz, co-examiner
Prof. Dr. Walter H. Reinhart, co-examiner
Prof. Dr. Ulrike Kutay, co-examiner

2001
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SUMMARY

The mechanisms that lead to premature clearance of erythrocytes in hereditary spherocytosis are poorly understood. We investigated whether naturally occurring antibodies (NAbs) are involved in opsonization of hereditary spherocytes and determined the amount of RBC-bound IgG in two types of HS, namely in band 3 and in spectrin/ankyrin deficiency before and after splenectomy.

The titers of anti-band 3 NAbs were upregulated by 2-3 fold in band 3 deficient patients before and normalized after splenectomy. Anti-band 3 levels were extremely low before and normal after splenectomy in the more severe anemia associated with spectrin/ankyrin deficiency.

The amount of firmly RBC-bound IgG was 2-3 times lower in unsplenectomized than in splenectomized HS patients, though these RBCs were cleared far more rapidly than those from splenectomized patients. The low degree of opsonization was not due to their low absolute cell age, it was an artifact introduced by washing, but induced by splenic conditioning. This was demonstrated by determining total RBC-bound IgG by a novel technique, which prevented mechanical damage and used whole blood freed from white cells. RBCs from blood were separated from unbound plasma components by passage through a phthalate oil mixture and were cross-linked to the remaining cell-associated molecules in the underlying dextran phase. Thereby we could demonstrate that total RBC-bound IgG was 1.6 times higher in unsplenectomized than splenectomized patients. Hence, RBCs from unsplenectomized patients were extremely fragile due to what is called "splenic conditioning" and lost most of their membrane associated, and heavily opsonized vesicles during washing. The ratio of total over firmly RBC-bound IgG was similar for RBCs of splenectomized HS patients and normal controls. These findings suggest that
conclusive studies on clearance and particularly opsonization can only be performed on HS RBC from splenectomized patients.

In splenectomized HS patients with a band 3 deficiency, where RBCs do not suffer from "splenic conditioning", RBC deformability inversely correlated with the number of firmly RBC-bound IgG reaching up to 140 molecules per cell. In spectrin/ankyrin deficient HS, however, deformability dropped slowly with increasing cell age and firmly RBC-bound IgG remained at control levels (≤ 40 IgG per cell for the given cell age). The low extent of opsonization in spectrin/ankyrin deficient HS RBC was paralleled by a continuous loss of band 3 containing membrane, presumably by releasing vesicles containing band 3. This process evidently rescued these RBCs from a rapid clearance. Thereby spectrin/ankyrin deficient RBC survived considerably longer than band 3 deficient RBC as judged from their band 4.1a/4.1b ratio.

This study shows for the first time a correlation between impairment in RBC deformability and the degree of opsonization for band 3 deficient HS. It further illustrates why spectrin/ankyrin deficient HS RBC profit more from splenectomy than band 3 deficient RBC, since they yet can get rid of their loosely anchored and opsonized band 3 in the form of vesicles and thereby reach a higher absolute cell age.
ZUSAMMENFASSUNG


Die Titer an Anti-Bande 3 NAbs waren vor Splenektomie um einen Faktor von 2-3 höher als bei Kontrollen und normalisierten sich nach Splenektomie. Die Anti-Bande 3 Antikörper Konzentration war extrem tief vor und normal nach Splenektomie bei den schwereren Anämien mit einem Spektrin/Ankyrin Mangel.

Die Menge an fest zell-gebundenem IgG war vor Splenektomie 2-3 mal tiefer als nach Splenektomie, obwohl diese Erythrozyten rascher entfernt wurden als in splenektomierten Patienten. Das geringe Ausmass an Opsonisierung war nicht Folge eines jungen Zellalters, es war ein Artefakt, welcher durch das Waschen der Erythrozyten ausgelöst, aber durch das Vorhandensein einer Milz induziert worden war (Konditionierung durch die Milz). Dies konnte durch Bestimmung der total zell-gebundenen IgG Moleküle mittels einer neuartigen Technik bewiesen werden. Diese Technik vermied eine mechanische Schädigung der Zellen und ging von filtriertem Gesamtblut aus, das frei von weissen Blutkörperchen war. Erythrozyten aus dem filtrierten Blut wurden von Plasmakomponenten abgetrennt, indem sie durch eine Phtalat-Oel Mischung hindurch zentrifugiert und in der darunterliegenden Dextranphase mit den verbleibenden, zell-assoziierten Molekülen chemisch vernetzt wurden. So konnten wir zeigen, dass die totale Menge der an Erythrozyten gebundenen IgG Moleküle vor Splenektomie 1.6 mal grösser war als nachher. Folglich waren Erythrozyten von nicht-splenektomierten HS Patienten durch die "Milz-

In splenektomierten HS Patienten mit einem Bande 3 Mangel, in denen die Erythrozyten nicht unter der Milz-Konditionierung leiden, verhielt sich die Deformabilität der Erythrozyten umgekehrt proportional zum Gehalt an fest zellgebundenem IgG, mit Werten von bis zu 140 IgG Molekülen pro Zelle. In HS mit einem Spektrin/Ankyrin Mangel jedoch nahm die Deformierbarkeit langsam mit dem Zellalter ab und die Menge an fest zellgebundemem IgG stieg nicht über jene von Kontrollzellen an. Das geringe Ausmass an Opsonisierung der HS Erythrozyten mit einem Spektrin/Ankyrin Mangel war begleitet von einem kontinuierlichen Verlust an Bande 3 in der Erythrozytenmembran, vermutlich durch die Abschnürung von Bande 3-haltigen Vesikeln mit ihren Opsoninen. Dieser Prozess verschonte diese Erythrozyten vor einer raschen Elimination. Dadurch überlebten Erythrozyten mit einem Spektrin/Ankyrin Mangel deutlich länger, was aus deren Verhältnis an Bande 4.1a/4.1b abgeleitet werden konnte.

1. INTRODUCTION

1.1 RBC membrane architecture and its defects

1.1.1 RBC membrane proteins and their interactions

The red cell membrane comprises a lipid bilayer, integral membrane proteins and an intracellular membrane skeleton (Fig 1). The membrane skeleton consists of \( \alpha \) and \( \beta \) spectrin that interact side-to-side to form flexible rod-like heterodimers which self associate head-to-head to form tetramers (1-3). Spectrin is the major building element and most abundant protein of the red cell skeleton present in approximately \( 2 \times 10^5 \) copies per cell. Electron microscopy visualization of spectrin reveals a long worm-like twisted structure measuring about 100 nm in length (4, 5). Up to six spectrin tetramers interact at their tail ends with short actin filaments and these with tropomyosin, adducin, band 4.9 to form junctional complexes (6). Protein 4.1 binds to \( \beta \) spectrin at the actin-binding domain and increases the affinity of the spectrin-actin interaction (7). The membrane skeleton is attached to the overlying plasma membrane at two sites. Skeletal protein 4.1, which interacts with \( \beta \) spectrin, also binds to the integral membrane protein glycophorin C (8). The second, more important membrane-skeleton linkage is provided by the cytoskeletal protein ankyrin containing two independent high affinity sites for the cytoplasmic domain of band 3 and for \( \beta \) spectrin (9, 10). The N-terminally myristoylated and palmitoylated peripheral protein 4.2 (11-13) binds to the cytoplasmic domain of band 3 (14, 15) as well as to ankyrin (10, 14, 16, 17) and may strengthen the band 3-ankyrin interaction.

The most abundant RBC membrane protein band 3, the anion exchanger, is present in \( 10^6 \) copies per cell and accounts for 30% of the membrane proteins (18). It is a 95 kD integral protein divided into two domains (3). The 43 kD cytoplasmic domain
binds ankyrin, band 4.1 and 4.2, various glycolytic enzymes, and hemoglobin (19). The 52 kD membrane domain, composed of 12-14 transmembrane helices, forms the physiologically important channel for chloride-bicarbonate exchange (20). Band 3 is glycosylated at a single external site (Asn-642) (21, 22) in approximately equal amounts by either a short complex oligosaccharide (23) or an extended polylactosaminyl oligosaccharide (24), which accounts for its diffuse migration on SDS-PAGE. Another two third of plasma membrane proteins consists of single-chain proteins glycophorins (A, B, C, D and E), which are highly glycosylated and carry most of the sialic acid residues (up to 30 groups per glycophorin A molecule) on the RBC surface.

Fig 1. Schematic model of the red cell membrane. The vertical and horizontal interactions of its components are indicated. Taken from (3).
1.1.2 Defects in the vertical membrane interactions result in hereditary spherocytosis

Disruption of the interactions between components of the red cell membrane skeleton and the integral membrane proteins may cause loss of structural and functional integrity of the membrane. Conceptually, there are two types of interactions: vertical interactions between the skeleton and the plasma membrane, and horizontal interactions among components that form the membrane skeleton meshwork (25-27) (Fig 1). The critical horizontal interactions occur between α and β spectrin, β spectrin and protein 4.1, protein 4.1 and actin. The horizontal protein contacts are important in the maintenance of the structural integrity of the cell, accounting for the high tensile strength of the erythrocyte (28). Defects in the proteins involved in the horizontal interactions lead to hereditary elliptocytosis or hereditary pyropoikilocytosis (26). The important links in the vertical interactions involve band 3, ankyrin, spectrin and protein 4.2. The vertical protein-protein, protein-lipid bilayer connections are critical in the stabilization of the lipid bilayer (25). The tight association of the lipid bilayer to the underlying skeleton is maintained via the binding of ankyrin to band 3 tetramers. Band 3 attachment to the skeleton may further be strengthened by band 3-band 4.2, band 4.1-glycophorin C, and spectrin-band 4.1 interactions (for review, see (2)). The vertical interactions are also supported by a small portion of spectrin (~10% of total spectrin) which inserts into the membrane due to its palmitoylation (29). Deficiencies or dysfunction in the proteins involved in the vertical membrane interactions result in hereditary spherocytosis (25).
1.1.3 Hereditary spherocytosis is a hemolytic anemia caused by inherited or de novo mutations

Hereditary spherocytosis (HS) is the most common cause of non-immune hemolytic anemia in people of Northern European ancestry, with a prevalence of approximately 1 in 2000 (3). About 75% of the cases are autosomal dominant, and about 25% occurrence of the disease is sporadic in nature (30, 31). About half of the sporadic cases are probably caused by a recessive form (compound heterozygotes and/or homozygotes) of HS and the rest by spontaneous new mutations (31, 32). Clinically, HS is characterized by the presence of sphrerocytes in peripheral smears with varying degrees of hemolysis and splenomegaly. In HS patients anemia (exaggerated RBC loss) is compensated by enhanced reticulocytosis. In severely affected patients splenectomy is indicated, since it ameliorates the degree of hemolysis, although the intrinsic abnormality of the red cell remains. HS sphrerocytes have increased fragility and decreased deformability, presumably due to the loss of surface area in the form of skeleton-free lipid vesicles (28, 33, 34). The osmotic fragility is improved (although not normalized) after splenectomy, consistent with the observation that HS RBC membrane lipid content increased after splenectomy (35). HS erythrocytes are dehydrated which is associated by an increased Na⁺ and decreased K⁺ concentration (36-38). K⁺ and water content are particularly diminished in HS red cells obtained from the splenic pulp (39). HS RBCs obtained from the splenic pulp during splenectomy are considerably more fragile, more spherical, and have lower net sodium and potassium contents than the cells obtained from the circulation (40). The nature of this conditioning effect of the spleen is not clearly defined. It is possible that a release of oxygen radicals from macrophages inflicts damage to intracellular proteins (41, 42) and enhances K⁺ loss and dehydration (43). Splenic conditioning is a consequence of multiple episodes of splenic
passages: the estimated residence time of HS RBC in the cords is only 10 to 100 min, and only 1 to 10 percent of blood entering the spleen is detained by congestive cords, while more than 90 percent is rapidly shunted into the venous circulation of the spleen (40, 44). As suggested by electron microscopy studies of the spleen in HS (45), macrophage phagocytosis is the final step of RBCs destruction. The signal that triggers HS RBC phagocytosis has not yet been elucidated.

The majority of HS cases are caused by dominantly inherited family-specific point mutations. These involve five proteins that link the membrane skeleton to the overlying lipid bilayer: α and β spectrin, ankyrin, band 3 and protein 4.2. As a rule, the defective genes are either not expressed or proteins encoded by these genes are not incorporated into the membrane. This results into a partial deficiency of a given protein estimated to 10-30 % in mild and to higher percentage in moderate to severe cases. This variability may be explained by different amounts of compensation by normal allele in single heterozygotes or by other mutations underlying the disease in compound heterozygotes (3). Several surveys using SDS-PAGE analysis have shown that 30-45% of HS have combined ankyrin and spectrin deficiency, about 30 % have an isolated spectrin deficiency, and about 20% have a band 3 deficiency (46-49). Isolated protein 4.2 deficiency, which accounts for about 5% of HS cases, was found in a small number of American and European patients, but is more common in Japan (50, 51).

Various types of mutations such as nonsense, missense or short in frame-deletions have been identified in patients with HS. Nonsense or frameshift mutations that result in either unstable ankyrin mRNA transcripts or truncated peptides are predominant in HS (52-55). The clinical expression of these mutations ranges from mild to severe transfusion-dependent anemia. Several ankyrin defects have been found in patients with recessive HS. Unlike the nonsense or frameshift mutations found in dominant HS, the recessive ankyrin mutations are usually missense or promoter mutations (52). In some HS families with the recessive inheritance pattern a second mutation in the other ankyrin allele was identified (52). In general, patients with
dominant defects are less affected than those with recessive mutations, however there is considerable overlap (3).

Most of the spectrin defects in HS lie in the β spectrin gene, since α spectrin is normally produced in excess in red cells (56) and β spectrin production is the rate-limiting step in biosynthesis of the membrane skeleton (3). More than 10 β spectrin alleles resulting in a null mutation (frameshift or nonsense) were associated with dominant HS. They either cause formation of an unstable transcript or a truncated polypeptide that is inefficiently incorporated into the red cell membrane (57-61). Several missense mutations have been identified in β spectrin. A point mutation near the N-terminus of β spectrin close to the putative protein 4.1 binding site (spectrin Kissimmee), causes defective binding to band 4.1, makes spectrin unstable and oxidant sensitive, and leads to spectrin deficiency (62). Clinically, HS varies form mild to severe in patients with β-spectrin defects (57-60, 62, 63). Spherocytosis is prominent and is associated with a subpopulation (8-15%) of dense spiculated RBCs and acantocytes that are not seen in other forms of HS (3).

Band 3 mutations are inherited as a dominant trait with relatively mild anemia and spherocytosis (3). Many unsplenectomized patients have a small population (0.2 – 2.3%) of “pincerred” erythrocytes (47, 64), which are not seen in other forms of HS (3). Substitution of highly conserved arginine residues (48, 65) or other conserved amino acids (66-68) positioned at the internal boundaries of transmembrane segments of the protein band 3 probably interferes with the cotranslational insertion of band 3 into the membrane of the endoplasmic reticulum or the stability of the inserted protein. A 22-residue insertion in the transmembrane domain of band 3 Milano prevents incorporation of the peptide into the membrane, since the mutant form of the protein was not found in the membrane of RBC of the affected patients (69). Band 3 protein with a missense mutation in the cytoplasmic domain of the protein, although incorporated in the lipid bilayer (47, 70, 71), is incapable to bind other membrane skeleton proteins. Partial deficiency of protein 4.2 is often seen in patients with
deficient ankyrin or band 3, secondary to the underlying defect (49, 72). Some patients have an isolated deficiency of protein band 4.2 with recessively inherited but clinically mild form of HS (50, 73-75).

1.2 RBC aging and turnover

1.2.1 RBCs lifespan is programmed

Normal human erythrocytes circulate for 120 days (76) and then are removed from the blood circulation by non-apoptotic mechanisms. Despite the lack of the nucleus and, therefore, a direct genetic trigger, the life span of mammalian red blood cells (RBCs) is limited. What processes contribute to define the red cell lifespan is a question that has been addressed by many studies, but has remained elusive. Once entered into the circulation, erythrocytes undergo constant shearing in the microvasculature and exposure to oxidative stress during blood passage through the reticuloendothelial system. In addition, due to the loss of the nucleus and other organelles RBCs can not replace their old or damaged proteins (77-81). Therefore, RBCs undergo a series of surface and intracellular alterations that accumulate with the circulation time (Table 1). To mention a few of them, such as cell shrinkage, decreased cellular deformability, protein modifications, and topological membrane alterations leading to increased levels of bound opsonins. All processes may well contribute to defining the red cell life span, but some of the many changes in the aging cell must serve as a signal of the end of its life leading to its elimination from the blood circulation. The final stage in destruction of a senescent or oxidatively damaged RBC is its phagocytosis by the mononuclear phagocytic cells in the spleen, liver, bone marrow, where these cells reside. In the normal animal and in man the spleen appears to be the major site of sequestration and catabolism of red (76, 82). It has been assessed that up to 60% of human RBCs are
eliminated in the spleen, whereas about 20 % of RBC sequestration takes place in the liver (83). Splenectomy extends red cell lifespan in the rat and mice (84, 85), rabbit (86, 87) and man (88, 89) in the order of 10-20%, (90). In HS RBCs with shortened survival, splenectomy prolonged the lifespan by up to 80 % of that of normal RBCs (91).

Table 1. Postulated cell age-dependent RBC modifications

<table>
<thead>
<tr>
<th>Physico-chemical modifications on aging RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Increase of density by loss of K⁺ and water (77, 92-94)</td>
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</tr>
<tr>
<td>- glycation of N-terminal amino groups of proteins and the ε- amino group of lysyl residues (105, 106),</td>
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1.2.2 Selective elimination of senescent RBC is due to a gradual accumulation of surface alterations

1.2.2.1 Exposure of Phosphatidylserine in the outer leaflet of human RBCs

Phosphatidylserine (PS) is almost exclusively located in the inner membrane leaflet of mature RBC and of most other cells (120, 121). This asymmetry is maintained by the ATP-dependent aminophospholipid translocase, or flipase, (122, 123) and can be disrupted by action of the scramblase (124), provided that the flipase activity is inhibited. Exposure of PS on the cell surface as a result of "scrambling" appears to be an important signal for ingestion of apoptotic nucleated cells by macrophage (125). Erythrocytes with an abnormal, symmetric distribution of their membrane phospholipids were phagocytosed in vitro four times more readily than normal asymmetric, intact erythrocytes (126). RBCs whose surface has been altered by incorporation of lipid vesicles containing PS or its analog, the 1-acyl-2[(N-4-nitrobenzo-2-oxa-1,3 diazole) aminocaproyl] phosphatidylserine (NBD-PS), showed increased binding to macrophages (127, 128) and rapid clearance in syngeneic mouse with approximately 30% of the injected cells localizing in the spleen and about the same percentage in the liver within 60 min (129). Such rapid clearance occurred when RBCs have incorporated about 80 ng of NBD-PS /10^7 cells. Some years later the same group has demonstrated that the PS content in the outer monolayer of the densest (presumably oldest) murine RBCs was about twice as high (104 versus 55 ng/10^7 RBC) as that of the youngest cells (115). The halflife of light cells was about twice that of dense cells, when studied in a wild type as well as in antibody deficient SCID mice. It has been postulated that the presence of PS in the outer leaflet of RBC might serve as a signal for triggering their recognition by macrophages. When annexin V, a calcium
dependent protein of the annexin family (130, 131) was found to bind to activated platelets (132) and this binding was attributed to the exposure of PS resulting from the activation of platelets (132), annexin V became a useful reagent to monitor PS exposure in numerous normal and pathological cells, like apoptotic cells, cancer cells, sickle and thalassemic erythrocytes. Initial studies utilized 125I-labelled annexin V, but later the assay was adapted successfully to flowcytofluorimetric studies using FITC-labeled annexin V to follow the changes at the cellular level. Using the annexin V-FITC label and flow cytometry, it has been demonstrated that red cells from patients with thalassemia (125, 133, 134) and sickle cell anemia (114, 135, 136) have elevated levels of PS levels on the outer leaflets of their membrane (1.5 - 6.8% of cells exposed PS), perhaps accounting for their shortened circulating lifetime. Interestingly, red cells of patients with hereditary spherocytosis, hereditary elliptocytosis, and G6PD deficiency have as little PS exposed as normal RBCs (0.0 - 0.5 % of cells), so that some other signal must be responsible for their reduced red cell life span (125, 137). Flow cytometric analysis of biotinylated rabbit RBCs drawn at 10-days interval up to 70 days after biotinylation indicated that older, biotin-labeled cells exposed more PS and its level increased in an exponential manner (125). At day 70 retrieved cells exposed maximal amounts of PS which was detected on 15 % of the cells, whereas until 50 days post biotinylation PS was detected only on 3% of RBCs. Exposure of PS paralleled the rate at which biotinylated red cells were removed from circulation. Thus, PS exposure correlates with phagocytic removal of thalasemic, sickle and senescent RBCs in vivo. It can not, however, be excluded that actual recognition was antibody mediated, since those RBCs also showed a high degree of opsonization by IgG (116-118, 138-141).
1.2.2.2 Membrane "pattern" formation

Oxidation of plasma membrane and intracellular components is a common feature of cell damage and senescence (109, 110). Aged erythrocytes demonstrate peroxidation of their cell membrane lipids (142) and cross-linking of hemoglobin (143, 144). One group suggested that oxidative damage of RBC results in the formation of lipid-protein conjugates closely related to that found in oxidized LDL, making the oxidized RBC a ligand for the macrophage scavenger receptors for oxidized LDL (145). In accordance, oxidized LDL but not acetylated or native LDL inhibited the binding and uptake of oxidized RBC, when assayed in vitro with mouse peritoneal macrophages in the absence of opsonizing antibodies. In contrast, it was suggested by another group that the receptor for oxidized LDL is identical to the macrophage Fc receptor for IgG (146). In a more recent study a murine model was used in which 51Cr-labeled red blood cells oxidized with Cu2+ plus ascorbic acid were injected intravenously, and the cellular uptake sites and the potential involvement of scavenger receptor were analyzed (147). After 10 minutes of injection more than 50% of damaged, but no native cells were removed. The main site of uptake of damaged RBC were the liver Kupffer cells, which contained 24% of the injected dose. The uptake was inhibited up to 50-80% by typical ligands for scavenger receptor, such as polyinosinic acid, liposomes containing PS, oxidized LDL. More important, mice lacking scavenger receptors of class A type I and II showed no significant decrease in the ability to take up damaged cells from the circulation. The authors suggested that the scavenger receptors, different from class A type I and II, may serve as pattern-recognizing receptors and play an important role in vivo in the removal of damaged cells from the blood circulation. Whether this receptor also takes part in the physiological clearance of senescent or pathologic RBCs in
humans is not so obvious, since oxidation of RBCs induced in the course of aging or
due to pathology may not compare to that artificially provoked with Cu\(^{2+}\) ascorbic
acid, which leads to extensive membrane cross-linking (145).

1.2.2.3 Glycosylation of RBC proteins

Glucose can react nonenzymatically with amino groups of a wide range of proteins
including intracellular proteins as hemoglobin (148, 149), extracellular proteins such as
collagen (150), cell membrane proteins on red cells (151) and endothelial cells (152).
Initially a reversible adduct, the almidine is formed (153). With time these adducts
undergo Amadori rearrangements to form irreversible ketoamine, advanced
glycosylation endproducts (AGE), which accumulate as a function of time and glucose
concentration (154-156). A high affinity macrophage receptor has been identified in the
murine macrophages that recognized proteins modified by this process of long-term
AGE formation (157, 158). This receptor did not bind to LDL, acetyl-LDL,
mannose/fucose, and formaldehyde-treated proteins (159). It has been reported that
AGE accumulation accelerated by exposure to high glucose levels led also to increased
monocyte AGE receptor binding and ingestion of glucose-modified human erythrocytes
in vitro (106). About 20% of human monocytes ingested glucose-treated RBCs versus
4% that phagocytosed BSA-treated RBCs and 60% IgG-opsonized RBCs (106). In
these experiments, RBCs were incubated with about 20-fold of the physiologic glucose
concentration (100 mM), and phagocytosis assays were performed in a serum–free
media. Diabetic mouse RBCs were phagocytosed by nearly three times the extent of
normal mouse RBCs, when exposed to syngeneic mouse macrophages (106).
Phagocytosis was inhibited to 70% by addition of excess AGE coupled to BSA (106).
Unfortunately, phagocytosis of glycosylated erythrocytes was not studied by adding
various serum constituents as potential competitors. This mechanism may in part
account for the increased removal of red cells observed in diabetic mice, where prolonged hyperglycemia is associated with AGE formation and shortened red cell survival (160). Nonenzymatic glucosylation also results in increased cross-linking of proteins via the formation of disulfide bonds (106, 161). Such a glucose-dependent cross-linking may add to the clustering of RBC membrane proteins, and thus the appearance of a signal of senescence, which is recognized by IgG autoantibodies that mediate subsequent recognition and uptake by macrophages (162-164).

1.2.2.4 Loss of CD47

Very recently it has been hypothesized that CD47 (integrin-associated protein) functions as a marker of self on murine red blood cells and prevents RBC from elimination (165). CD47 is an immunoglobulin family member with a five fold membrane-spanning domain and a short cytoplasmic tail (166). It is physically and functionally associated with β3 integrins and is expressed on virtually all cells, including erythrocytes, which lack integrins (167, 168). Mouse red cells that were completely deficient in CD47 were rapidly cleared from the bloodstream by splenic red pulp macrophages, when transfused into wild type mice (165). Clearance of CD47−/− RBCs was rapid also in antibody-deficient Rag−/− and C3-deficient mice. CD47 on normal red blood cells is thought to prevent this elimination by binding to the inhibitory receptor signal regulatory protein alpha (SIRPα) on macrophages. Unexpectedly, gene targeted knockout CD47−/− mice, though having a defect in host defense due to delayed granulocyte migration to sites of infection, are viable, have normal blood counts, and RBC parameters (169). When CD47−/− RBCs were transfused into wild type mice, they were rapidly eliminated (165). Antibodies to SIRPα that block CD47–SIRPα interactions increased phagocytosis of wild type RBC to the same extend as that seen with CD47−/− targets. This indicated that inhibition of phagocytosis...
by CD47 on the target RBC required SIRPα and SIRPα-CD47 interaction and suggested that CD47−/− RBCs were cleared because of the absence of CD47 on their surface, rather than because of a possible secondary effect of CD47 deficiency on the RBCs. Since CD47 binds to SIRPα, regulation of CD47 expression might serve as a mechanism to control elimination of damaged or senescent murine RBC. Heterozygote cells, however, expressed about 60% of wild type CD47 (169) and yet showed normal survival in wild-type mice (165). Thus, these results argue against a gradual removal of CD47 as the mechanism regulating RBC life span. It is unclear what level of CD47 expression is sufficient to allow phagocytosis. There is no hint why CD47−/− RBCs survive normally in CD47-deficient mice with virtually identical level of SIRPα on both CD47−/− and wild type macrophages. Whether CD47-SIRPα may operate in humans and represent a potential pathway for the control of hemolytic anemia, remains to be elucidated.

1.2.2.5 Topological membrane protein alteration followed by binding of antibodies and complement

Topological membrane alteration, such as protein and lipid aggregation, occurs in certain hemolytic anemias, in the course of RBC aging and can be artificially induced (170). The fraction of high molecular weight protein aggregates has been studied on RBC from patients with hemoglobinopathies (138, 140, 171, 172) density separated normal (173, 174) and artificially oxidized RBC (164, 175). The high molecular weight material, comprising 1.3% in sickle erythrocyte, or 1.6% in ß-thalassemic cells of the total membrane proteins contained primarily globin, band 3 protein, glycophorin, some minor cytoskeletal proteins, and 3/4 of the cell-bound IgG. The composition of these aggregates was similar in senescent normal RBC, but comprised only 0.09% of the total
membrane protein and was 640 fold enriched in surface IgG (174). High molecular weight material from senescent erythrocytes, which was purified by gelfiltration in SDS, also contained covalently linked (C3b)n-IgG complexes (176). IgG functions as an adaptor, which specifically binds to target cells and promotes contact to phagocytic cells via Fcγ receptors. In contrast to most other phagocytic mediators, they can further enhance their effect by stimulating complement deposition to target cells (177-181). Their ability to activate complement is crucial, since physiologic concentrations of monomeric IgG completely inhibited macrophage and neutrophil phagocytosis, when mediated by antibody alone (182-184). On the other hand, physiologic concentration of IgG did not significantly inhibit internalization when target cells were opsonized with antibody and complement together (183). Thus, physiological phagocytosis requires participation of two independent receptors for IgG (Fcγ) and C3b (CR1)(185-187) or iC3b (CR3) (188). Erythrocytes oxidatively damaged by diamide and opsonized with serum were phagocytosed in vitro (162, 163) as well as in vivo (190). Phagocytosis of diamide-treated RBC required opsonization by C3b, since complement inactivation abolished phagocytosis (163). When affinity purified anti-band 3 antibodies from pooled human IgG were added to serum, they further stimulated opsonization by C3b favoring C3b deposition by activating complement amplification (163). Other oxidative agents were used to further explore the role of membrane protein oligomerization on binding of IgG and complement components. A treatment of RBC with 1mM ZnCl2, followed by a chemical cross-linking of the topologically altered organization, generated band 3 oligomers and enhanced binding of autologous IgG and C3d from serum (175). Cleavage of the cross-linked complexes with mercaptoethanol reversed the effect of clustering, lowered binding of IgG and C3d by 80%, and abolished phagocytosis (164).

One hypothesis to explain the age-dependent clearance of red blood cells proposes that denatured/oxidized hemoglobin (hemichromes) arising late during the RBC life-span induces clustering of integral membrane protein, band 3 (191). This, in
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turn, stimulates anti-band 3 IgG binding and complement deposition to the red cell surface. The hypothesis on stabilization of band 3 clusters by hemichromes was recently tested in a canine model, where denatured hemoglobin bound to RBC membrane was measured on in vivo -aged biotinylated RBC (192). Senescent dog RBCs contained measurably elevated amounts of membrane-bound (denatured) globin and 7-fold higher numbers of RBC-bound IgG. Denaturation of hemoglobin may, indeed, account for band 3 oligomer induction, enhanced opsonization, and accelerated RBC clearance in some anemia. The level of RBC-bound IgG from patients with unstable hemoglobin, such as sickle cell anemia or hemoglobin Köln disease was increased several-fold as compared to that in healthy controls (138). By using immunofluorescence microscopy evidence was presented that membrane attached hemichromes were associated with band 3 and clusters of surface bound IgG.

1.2.3 RBC clearance is mediated by phagocytosis

1.2.3.1 Fcγ receptor-mediated phagocytosis

Fc receptors (FcR) comprise two general classes - those involved in effector functions (particle internalization and cell activation) and those that transport immunoglobulins across epithelial barriers (193). There are two major classes of Fcγ receptors (FcγR): receptors that activate effector functions and receptors that inhibit these functions (194-196). FcγRs that mediate effector functions in human macrophages fall within the activation class and include FcγRI, FcγRIIA, FcγRIII (195). The human FcγRIIA is a single chain protein with an extracellular Fc binding domain, a transmembrane domain, and a cytoplasmic tail containing two YXXL ITAM motifs (immunoglobulin gene family tyrosine activation motif). Murine as well as human macrophages express FcγRIIB, an inhibitory receptor that does not contain ITAM motifs and does not
participate in phagocytosis (197). FcγRI and FcγRIIIA have extracellular Fc binding domains, but lack ITAMs on their cytoplasmic tails (197, 198). Alternatively, these receptors are associated to homodimers of γ subunits that contain ITAMs. For proper signaling, FcγRI and FcγRIIIA must interact with the dimers of γ subunits or ζ subunits of FcγRIIA, small transmembrane proteins that contain the ITAMs needed for signal transduction (199, 200). Ligand (IgG Fc portion) binding results in receptor cross-linking, which causes tyrosine phosphorylation of the ITAMs. The tyrosine kinase syk is then recruited to the phosphorylated ITAM domain, and upon its activation, it is thought to mediate particle internalization by activating PI3-kinase and phospholipase C.

The human FcγRs are constitutively expressed on monocytes and macrophages with about 2-6 x 10^4 copies/cell. FcγRI binding affinity is high (10^8-10^9 M^-1), this is attributed to the third extracellular domain, which is lacking in all other human FcγR. FcγRII exhibits the broadest cellular distribution among all FcγR. It is expressed on monocytes, macrophages, neutrophils, eosinophils, basophils, B cells, platelets, follicular dendritic cells, Langerhans cells, blood vessel endothelial cells. It binds IgG with low affinity (<10^7 M^-1) only in complexes not in monomeric form (201).

1.2.3.2 Complement receptor-mediated phagocytosis

Several complement receptors, CR1, CR3 and CR4, are expressed on macrophages and participate in phagocytosis of complement-opsonized particles (202, 203). CR1 is a single chain transmembrane protein with an extracellular portion composed of 30 short consensus repeats (204). CR1 has a large extracellular lectin-like complement-binding domain and a short 43 amino acid cytosolic domain. CR1 binds C3b, C4b and C3bi, and participates mainly in particle binding (205). CR3 and CR4 are integrin family members made up of heterodimers of different α chains and a shared β chain. These
two receptors bind specifically to C3bi and are responsible for particle internalization. While FcRs are constitutively active for phagocytosis, the CRs of resident peritoneal macrophages bind, but do not internalize particles in the absence of additional stimuli (206, 207). Particle ingestion by CRs can be induced by protein kinase C activators, as well as by TNF-α, GM-CSF, or attachment to laminin- or fibronectin-coated substrates (206, 207). Although all types of phagocytosis require actin polymerization at the site of ingestion (208), results of electron microscopy studies demonstrate that IgG- and complement-opsonized particles are internalized differently by macrophages (209, 210). During FcγR-mediated phagocytosis, veils of membrane rise above the cell surface and tightly surround the particle before drawing it into the body of the macrophage. Complement-opsonized particles appear to sink into the cell with elaboration of small, if any, pseudopodia (209, 210).

### 1.2.3.3 Scavenger receptor-mediated phagocytosis

In order to phagocytose an effete cell, receptors on the macrophage must see a ligand found on senescent or apoptotic cells that is not present on healthy cells. Ligands fitting these criteria that have been implicated in the recognition of apoptotic cells include PS in the outer leaflet of the plasma membrane, changes in the pattern of glycosylation of cell surface proteins, and surface charge (211, 212). Receptors demonstrated to participate in phagocytosis of apoptotic cells by macrophages include class A and B scavenger receptors (213, 214), and CD14 (215).

Scavenger receptors (SRs) are a family of structurally diverse receptors having broad ligand specificity that includes LDL, PS, and polyanionic compounds (216). Scavenger receptors SR-AI and SR-AII are alternatively spliced products of the same gene. These receptors are homotrimeric glycoproteins with a short (about 50 amino acid) amino terminal cytoplasmic domain and a carboxy terminal extracellular ligand-
binding domain. SR-A bind acetylated, oxidated low-density lipoproteins, and polyanionic compounds such as polyinosinic acid (217). Direct evidence for involvement of SR-A in macrophage phagocytosis of apoptotic cells comes from studies on the phagocytosis of apoptotic thymocytes by thymus-derived macrophages in vitro (218). Internalization of the thymocytes could be substantially inhibited with monoclonal antibody to SR-A or polyanionic ligands, and phagocytosis was inhibited by 50% in thymic macrophages derived from SR-A null mice.

A class B scavenger receptor, CD36, which acts in conjunction with the vitronectin receptor, has also been implicated in phagocytosis of apoptotic cells. CD36 is a 88-kDa membrane glycoprotein expressed on a variety of cells including platelets, monocytes, endothelial cells, and erythroblasts (219). CD36 has been identified as one of the receptors for collagen type I, thrombospondin, oxidized LDL and phosphotidylserine (220). Since CD36 has a very short (4 amino acids) carboxy terminal cytoplasmic domain, it seems likely that it interacts with other transmembrane molecules to stimulate phagocytosis. Trombospondin has been suggested to bridge an apoptotic cell, CD36, and the vitronectin receptor, resulting in a phagocytic active ternary complex (213, 214), although the mechanism by which it interacts with phagocytic cells is obscure. This is at odds with the finding that macrophages from CD36 deficient patients show no defect in the phagocytosis of apoptotic neutrophils (212).

In addition to the above receptors, CD14, a molecule also known to transduce LPS signals, has been implicated in recognition and internalization of apoptotic cells (215).
1.3 Concept of autoreactivity

1.3.1 NAbs are compatible with normal life

Autoantibodies of the IgM, IgG and IgA classes, reactive with a variety of serum proteins, cell surface structures and intracellular components, are found in small quantities in all normal humans (221-226). Evidence has since accumulated to disfavor the clonal selection theory stating that autoreactive lymphoid cells termed “forbidden clones” are eliminated in embryonic life. First of all, mankind suffers from dozens of autoimmune diseases. Moreover, since autoantibodies are formed against the very same, disease-specific antigen in all patients with a given disease, it implies that autoimmunity is not random (227). To name a few other examples: lymphocytes from normal mice and humans can be stimulated in vitro to produce autoantibodies (228-230), autoantibodies can be induced in vivo in normal mice (230-233), and hybridomas can be formed between normal mice or human lymphocytes and non-secreting myeloma cell lines which then produce NAbs (224, 234-237). Antibodies of similar reactivities were not only found in conventionally bred animals, but also in germ-free mice (238). In addition, autoreactive antibodies were measured in mice early in neonatal life (239). This implied that the existence of NAbs is independent on external antigens. It was found that NAbs are encoded by non-mutated or minimally mutated germline Ig genes (240-242) and, once established early in ontogeny, remain conserved throughout life (226). They are also remarkably conserved among individuals (243) and across species (244). NAbs bind with low affinities (225, 245, 246) to phylogenetically conserved antigens like myosin, actin, spectrin (222, 223, 247), IgG F(ab′)2 fragments (248, 249), protein band 3 (163, 250, 251), and others. NAbs have low affinities (Ka=10^4-10^6 M^{-1}) but, in contrast, they have high avidities (252-254). Many NAbs,
primarily those of the IgM class are polyreactive (240, 255-259). Polyreactivity of NAbs is in part due to their specificity for conserved epitopes. Polyreactivity is primarily mediated by a high number of positively charged amino acids and those carrying side chain hydroxyl groups in the hypervariable region. This makes the active site of these antibodies hydrophilic and thus more flexible, capable of interacting with the antigen via ionic and hydrogen bonds (240, 260). NAbs are produced by conventional B lymphocytes, although in some instances, it has been reported that CD5+B cells were preferentially involved in their synthesis (255, 261, 262).

1.3.2 The origin of NAbs

In light of the impressive data demonstrating that humoral autoimmunity is common in humans and animals with a normal immune system, new theories were proposed to explain this “paradoxical” phenomenon. One of the theories proposed by Cunningham postulates that clonal deletion operates in early life, however, later in life all autoreactive B cells not eliminated during early ontogeny, are prevented from expanding by a “compensatory suppressor” (263). Therefore, NAbs are produced in minute concentrations (10-1000 ng/ml) in contrast to the large quantities of self antigens. Spleen cells from normal mice produced upon stimulation by polyclonal B cell activators (PBA) autoantibodies that are normally low or absent, such as anti-DNA antibodies (231, 264), rheumatoid factor (265, 266) and anti-thymocyte antibodies (233). This suggested that under normal conditions production of these autoantibodies is suppressed. The magnitude of the polyclonal in vivo response was ten times lower than the in vitro response and this could not be overcome by multiple PBA injection (230). This observation suggested that a natural suppressive mechanism is absent in vitro but exists in vivo. Another hypothesis on NAb origin suggests that the B cell population, which gives rise to NAbs carries a polyspecific receptor (224). Binding of a foreign antigen to such an Ig determinant would induce the B cell to undergo a series of
divisions and mutations and eventually lead to the production of a high affinity antibody against that antigen. In the absence of activation with foreign antigen, such B cells produce low affinity antibodies. The majority of low affinity NAbs are polyspecific, capable of binding a number of structurally related but not identical ligands (224, 228, 236, 267). Thus, polyspecific NAbs may constitute the antibodies secreted by these B cells prior to encounter a foreign antigen (224).

Later studies suggest (268-271) and others support (243, 272) the concept that naturally activated B cells with reactivity for self-Ag are selected early in development and persist throughout life. Thus, the repertoire of NAbs in the serum of healthy vertebrates may represent the end result of successive selective processes. The existence of a large number of autoreactive B cells, which express and secrete a variety of NAbs, led to the conclusion that they probably have important physiological functions. Indeed, several lines of evidence unequivocally demonstrated that NAbs play important biological roles.

1.3.3 The role of NAbs

Circulating antibodies against toxins and bacteria in the sera of naïve animals were described early in history of immunology (273). NAbs are viewed as part of the innate immune system which serves as a first line defense and may facilitate elimination of foreign agents on initial contact (274-278). Recent studies showed that antibody-deficient mice (μMT and Rag-1−/−) (279, 280) or mice that lacked only IgM but did produce all other Ig classes (IgM−/−) (281) infected with various viruses (vesicular stomatitis virus, lymphocytic choriomeningitis virus, vaccinia virus) or with Listeria monocytogenes showed already between 2 and 8 hours after infection elevated viral or bacterial titers in peripheral organs, which were 10 to 100 times greater than in antibody-competent mice (282). It has to be noted that in mice, NAbs belong exclusively to IgM class, whereas in humans both IgM and IgG NAbs are prevalent.
The authors suggested that natural antibodies play a crucial and immediate role in first line defense by preventing pathogen dissemination to vital organs and by improving immunogenicity through enhanced antigen trapping in secondary lymphoid organs. In addition to that, preexisting NAbs bound on foreign epitopes may initiate complement deposition and generate C3b-C3b-NAb complexes. The inactivated form of these complexes, C3dg-C3dg-NAb get associated with the non-self antigen, may bind to B cells via CR2 and stimulate B cell proliferation and secretion of antibodies against that antigen. Therefore, NAbs are capable of linking the innate to the acquired immunity.

An interesting hypothesis was proposed by Cohen that NAbs displaying low affinity for highly conserved self epitopes may prevent autoreactive clones from reacting vigorously with self antigens by binding to these antigens and masking their antigenic determinants. It is well known that self-mimicking epitopes on a pathogen can trigger a strong autoimmune response. NAbs were suggested to act as a filter that enables only non-self epitopes to induce an immune response while self resembling antigens are masked by these NAbs and thus fail to elicit an immune response. Lutz extended this hypothesis and suggested a mechanism by which NAbs against a conserved epitope on non-self antigen can direct the immune response to non-self epitopes. Thus NAbs may participate in achieving of peripheral tolerance.

NAbs comprise a part of an idiotypic-anti-idiotypic network and are involved in immune regulation. Normal human Igs of IgM and IgG class contain anti-idiotypic Igs which bind to F(ab')2 fragments of NAbs and/or to the Fab variable region of corresponding idiotypes. Pooled human IgG contains anti-idiotypes against a variety of autoantibodies from patients with autoimmune diseases. NAbs capable to interact with molecules present on the surface of B and T cells inhibit B cell growth or to suppress activation of T cells. Similarly, NAbs to various cytokines possess immunoregulatory function.
NAbs are also relevant in the disposal of cellular and plasma waste products, and thereby they are involved in tissue homeostasis. NAbs with specificities to a variety of intracellular components (actin, tubulin, spectrin, DNA) and plasma proteins (albumin, complement proteins) mediate an immune clearance of catabolic products, when they are released from cells during lysis or apoptosis or when plasma proteins are structurally altered and form aggregates in the course of oxidative damage (222, 223, 300, 301).

1.4 Anti-RBC NAb

1.4.1 Anti-band 3 NAb

RBC-bound IgG eluted from normal senescent (302), sickle cells (172) and erythrocyte with unstable hemoglobin (138) bound primarily to band 3 proteins on blots, suggesting that the majority of RBC-bound IgGs represented anti-band 3 NAbs. There is ample evidence for the existence of anti-band 3 NAb in normal human serum and pooled human IgG preparation (226, 251, 303, 304). Although anti-band 3 NAb exist in low concentration in plasma (<100 ng/ml) and have a weak affinity (Ka = 5-7 x 10⁶ M⁻¹) (305), they possess a biological efficiency. Once bound bivalently, low affinity NAb become powerful, since their dissociation is lowered by up to a 1000-fold (306, 307). They have an affinity for C3 at a site independent of the antigen-binding domain with an estimated association constant of 2-3 x 10⁵ M⁻¹ (305). Though weak, their binding to C3 was about 100 times higher than that of whole IgG (308). Due to their affinity for C3, anti-band 3 NAb become preferred targets of nascent C3b. This results in C3b-C3b-anti-band 3 complex formation (305). C3b-C3b-anti-band 3 complexes represent the most powerful mediators for phagocytosis, because they are capable of recruiting both FcγR and the complement receptors CR1 and CR3 on phagocytic cells.
Phagocytosis of C3b-C3b-IgG containing target was not inhibited by fluid phase IgG (183). In addition to that, these complexes can nucleate alternative complement pathway C3 convertases 30 times better than free C3b (309) and 7-11 times better than immobilized C3b (285), thus, further enhance RBC opsonization by C3b. Evidence from our and several other laboratories showed that naturally occurring anti-band 3 antibodies (303, 310) are involved in the clearance of senescent (173, 311, 312), oxidatively stressed human red blood cells (163, 313), and in clearance of malaria-infected RBC (314) and those with a hemoglobin defect (138, 315). Studies to elucidate the molecular target within band 3 molecule that is responsible for anti-band 3 NAb binding revealed rather controversial data. Initially, carbohydrate on glycoprotein band 3 was proposed as a potential target for anti-band 3 NAb (312, 316, 317). Affinity purified anti-band 3 NAb from pooled IgG had a very pronounced preference and bound exclusively to the 55 kDa chymotryptic fragment of band 3 and neither to carbohydrate (318) nor to carbohydrate-containing C-terminal 38 kDa fragment of band 3 (319). These findings did, however, not exclude the possibility that a minute fraction of anti-band 3 NAb obtained from IgG of particular donors might also interact with the 38 kDa fragments of band 3 (316). IgG eluted from senescent RBC appeared to bind to the loop between helices 5/6 and 7/8 in the C-terminal region of band 3, when probed with synthetic peptides of band 3 protein (320) (Fig. 2). In contrast to this, affinity purified anti-band 3 NAb did not bind to the 38 kDa chymotryptic fragments containing the loops between helices 7/8 in native arrangement (190). Since anti-band 3 NAb bound equally well to blotted band 3 protein from young and old RBCs (319), selective binding of NAb to intact RBC does not require exposure of neoantigens, resulting from proteolytic cleavage of band 3 as has been suggested by Kay (321), but rather oligomerized band 3. Low affinity NAbs become powerful, when they are bound bivalently, because bivalent binding enhances their binding by up to a 1000 fold by lowering dissociation (306, 307). Monovalent binding of NAbs is transient as these antibodies dissociate rapidly. Antigen oligomers are required for bivalent binding of the
low affinity, but highly specific anti-band 3 NAb. Studies in which band 3 was oligomerized either by oxidative damage by diamide (163, 313, 323) or non-oxidative damage by treatment with Zn\(^{2+}\), followed by cross-linking with BS3 (164, 189) demonstrated enhanced anti-band 3 binding and RBC clearance in vitro (10-20 folds) (163, 164) as well as in vivo (323). Saturating Zn\(^{2+}\) concentrations used in this system could, however, not only induce oligomer formation but perturb the system otherwise, because Zn\(^{2+}\) inhibits anion transport (324) and is not easily displaced by washing (325). Binding of affinity purified anti-band 3 NAbs was also studied on RBC in which band 3 was oligomerized with BS3 without addition of Zn ions (190) in a system approaching physiological conditions, namely in the presence of 25 mg/ml HSA and 5 mg/ml unlabelled human IgG. The binding was 10-fold higher to cells cross-linked at 37°C as compared to 0°C, while binding of IgG depleted of anti-band 3 remained low in both situations (326). This implies that oligomerization was diffusion controlled and anti-band 3 binding was mediated by band 3 oligomerization only and not by the BS3 modification as such. The number of bound anti-band 3 NAb exceeded that determined on oxidatively stressed erythrocytes (163) which amounted to less than 20 IgG per cell, but yet was almost two orders of magnitude lower compared to the total amount of IgG that bound from plasma in vitro to RBC with oligomerized band 3 (164). This suggested the involvement of yet other types of naturally occurring antibodies.
Fig 2. Topology of human band 3 protein.

Both the amino and carboxyl termini are located in the cytosol. N-linked oligosaccharide is attached to asparagine 642 facing the outside of the cell. T, trypsin cleavage site; C, chymotrypsin cleavage site; Pa, papain cleavage site; Pe, pepsin cleavage site. Taken from (327)
1.4.2 Anti-spectrin NAb

Besides anti-band 3 NAb human plasma contains IgG against other erythrocyte constituents, like spectrin (223), actin and band 4.2 (303). Though these NAbs are directed to intracellular components, IgG eluates obtained from thalassemic patients contained anti-spectrin NAbs (328). Some of these antibodies may have reacted with antigens liberated upon mechanical hemolysis and formed immune complexes that then bound to CR1 on RBC (247). On the other hand, some NAbs may cross-react with particular cell surface constituents due to their inherent polyreactivity. Many polyreactive NAbs belong to a group of strongly positively charged antibodies (240, 329). They tend to bind by charge-charge interactions to negatively charged cell surface components. In the presence of whole plasma or sera this type of binding is transient due to continuous exchange with other positively charged IgG. Affinity purified anti-spectrin NAb subfractionated into highly charged and less charged NAb were assayed for their binding to RBC and inside-out vesicles under physiological concentrations of HSA (50 mg/ml) and human IgG (15 mg/ml) (190). The binding was higher in case of highly charged NAb; it increased with decreasing ionic strength and was considerably lower on intact erythrocytes than inside-out vesicles. At physiological ionic strength anti-spectrin NAb binding amounted to about 1.3 molecules per RBC. Binding of affinity purified anti-spectrin NAb was also studied to RBC on which band 3 oligomers were generated by exoplasmic cross-linking with BS3. This pretreatment increased binding not only of anti-band 3, but also of anti-spectin NAb by 7-10 folds at 0° C in the presence of nearly physiological IgG and HSA concentrations. Binding of anti-spectrin NAb was not to spectrin as judged from surface labeling of RBCs that were treated with BS3. Binding of anti-spectrin was not competed by high concentrations of anti-band 3 NAbs and anti-spectrin NAbs even stimulated binding of anti-band 3
F(ab')$_2$ by 30%. This suggested that anti-spectrin NAb bound to band 3 or a protein associated with it by virtue of their inherent polyreactivity (326).

The role of anti-spectrin NAb in RBC clearance was supported by an animal model (330). In this study a population of aged RBCs obtained by hypertransfusion was injected into rats bearing a high level of anti-spectrin antibodies, following an immunization with spectrin. The clearance of the $^{51}$Cr-labeled aged and control RBC was measured in both spectrin-immunized and control rats. Aged RBCs were eliminated in spectrin-immunized rats at a significantly higher rate than in control rats with only naturally occurring anti-spectrin antibodies.
1.5 Aims and strategy of this study

The mechanism(s) by which senescent erythrocytes or erythrocytes with a membrane defect are cleared from the circulation are not conclusive. Whenever a new type of recognition principle between target cell and phagocyte has been discovered, clearance of young versus old, of intact versus damaged or of aberrant versus normal red blood cells was studied. The results remained contradictory for example in the case of PS exposure and recognition by one type of the scavenger receptors as outlined in the introduction. Likewise, in the case of pattern recognition, where mice lacking two scavenger receptors were not impaired in clearance of damaged RBC and clearance could not be inhibited to more than 50 % by adding high concentrations of a potent ligand. In the case of the negative regulator CD47, the studies were done on an animal model, which can not necessarily mimic the human system and RBC survived in a CD47⁻/⁻ animal. The only recognition principle which so far has repeatedly generated concordant, but not yet conclusive results in the hands of several investigators, is recognition by naturally occurring antibodies from total IgG or purified NAbs leading via complement deposition to binding of RBC to FcγR and complement receptors. Most of the work on the role of NAbs in RBC clearance was done on artificially damaged or oxidized cells, little on senescent, but almost no effort was taken to clarify their involvement in the premature clearance of RBCs from patients with hereditary spherocytosis. This is particularly striking, since a study on HS RBCs offers a multitude of controllable variables that may grossly alter opsonization, like the presence or absence of the spleen and the availability of several different and rather clearly defined membrane defects that lead to spherocytes and premature clearance by different routes. In contrast to this, a study on in vivo opsonization of aging RBCs offers a very limited number of controllable parameters in a human being. Hence, it was the aim of this study to address the question whether IgG NAbs are involved in in


*vivo* opsonization of RBC in hereditary spherocytosis. Though opsonization as such is not sufficient evidence for RBC clearance to occur by this mechanism, it is the first step and its elucidation alone possess multiple questions that no other group has tackled.

Our study therefore concentrated on the NAbs that may be involved in clearance and on RBC-bound IgG. Among the first questions were the following: do the concentrations of the known anti-RBC NAbs differ in HS patients from those in normal donors? In trying to make use of the above mentioned variables all the parameters that we measured were determined as a function of them. Thus, NAbs and RBC-bound IgG were recorded for two types of HS, namely one with a band 3, the other with a combined spectrin/ankyrin deficiency which differ in their membrane defect and thus in their mode to reach the state of spherocytosis. In each of the groups we studied the effect of splenectomy. Furthermore, we aimed at relating the degree of opsonization to RBC function and membrane properties. Since the main membrane property of interest was the content of band 3 in relation to that of spectrin we determined this parameter in all RBC fractions on which we measured RBC-bound IgG. As a relevant RBC function we determined the cellular deformability, since the loss of deformability appears as the major trigger for induction of clearance. Wherever possible, we determined RBC-bound IgG not on the whole cells populations, since they differ greatly in their composition (mean cell age and reticulocyte number). Instead, we aimed at determining the parameters on cells of the same absolute cell age. As this is not feasible, we separated the cells according to their density, determined the given parameters on the obtained fractions and then determined the absolute cell age on the membranes of the fractionated RBCs. Following this strategy we could answer most of the questions raised as will be outlined.
2. MATERIALS AND METHODS

2.1 Patient material

Blood samples were obtained from patients with hereditary spherocytosis (HS) and hematologically normal controls. HS pairs (n=18) consisting of an unsplenectomized (A) and a splenectomized (B) family member were studied (e.g. A1B1,...,AnBn). Information on HS patients’ hematological parameters and familiar history were obtained from collaborating hematologists (Table 2 and 3).

Table 2. HS patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date of birth</th>
<th>Sex</th>
<th>Splenectomy</th>
<th>HS Pair member</th>
<th>Hematological parameters</th>
<th>Deficiency</th>
<th>Type of mutation</th>
<th>Studied</th>
</tr>
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<td></td>
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</tr>
<tr>
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<td>B3 Chur (66)</td>
<td>O+DF+NAb</td>
</tr>
<tr>
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<td>A2</td>
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<td>B3 Chur (66)</td>
<td>O+DF</td>
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<td>B3 Milano (69)</td>
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<td>BR</td>
<td>Age</td>
<td>Height</td>
<td>Rh</td>
<td>Antigen</td>
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<td>-----</td>
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<td>B17</td>
<td>15.9</td>
<td>1.4</td>
<td>B3</td>
<td></td>
</tr>
</tbody>
</table>
A1B1 to A19B19 are referred as HS pairs
Abbreviations: N.D. - not determined, B3 - band 3, Ank - ankyrin, Sp - spectrin, O - opsonization, DF - deformability, NAb - naturally occurring antibodies

Table 3. Mutations associated with HS

<table>
<thead>
<tr>
<th>Name of mutation</th>
<th>Mutation</th>
<th>Consequences</th>
<th>Membrane domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3-Chur</td>
<td>2426G→A</td>
<td>GGC→GAC, G771D</td>
<td>TM11</td>
<td>(66)</td>
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<tr>
<td>B3-Milano</td>
<td>1608ins69bp</td>
<td>Inframe insertion</td>
<td>TM4</td>
<td>(69)</td>
</tr>
<tr>
<td>B3 Napoli II</td>
<td>2462T→A</td>
<td>ATC→AAC, I783N</td>
<td>OL6</td>
<td>(68)</td>
</tr>
<tr>
<td>B3-Coimbra</td>
<td>1576G→A</td>
<td>GTG→ATG, V488N</td>
<td>OL2</td>
<td>(331)</td>
</tr>
<tr>
<td>Ank Stuttgart</td>
<td>2952del6bp</td>
<td>329-frameshift</td>
<td>-</td>
<td>(52)</td>
</tr>
</tbody>
</table>

Abbreviations: TM- transmembrane, OL- outer loop, ins-insertion, del-deletion
2.2 Red blood cell preparation

2.2.1 Blood collection and filtration

Blood collected from hereditary spherocytosis patients was filtered to remove white cells using blood filtration devices assembled by us. In brief, about 30 ml of blood was collected into a collection bag (Baxter, Switzerland) containing 4.2 ml of CPD-type (citrate-phosphate-dextrose) anti-coagulant. The bag with the anticoagulant was placed on a balance near the patient's bed to monitor the amount of blood collected. Once disconnected from the patient, the collection bag was closed and blood was mixed with anticoagulant. Blood filtration was performed within one hour by passive blood flow through a Sephacell R-200 filter (Asani Medical CO, LTD). The filter was connected to a sterile transfer bag in which filtered blood was collected. When all blood had left the collection bag, 14 ml of sterile isotonic sodium chloride was injected to the collection bag to rinse the filter free of residual cells. Blood filtration was performed at room temperature while filtered RBCs were kept on ice. After filtration the collection bag and the filter were disconnected from the transfer bag and most of the residual air was removed from the transfer bag before closure. The filtered blood samples were brought at 0-4 °C to Zurich within one day.

2.2.2 RBC isolation from whole blood units

Whole human control blood (0 Rh+) collected into CPD-adenine (Swiss Red Cross, Blood Bank, Zurich) was diluted at room temperature with one third the volume of PBS-G (10 mM NaKHPO₄, 150 mM NaCl, 1g/l D-glucose, pH 7.4) containing 30 µg/ml PMSF. Diluted blood was passed through a PBS-G equilibrated cellulose column
(α- and microcrystalline cellulose in a mass ratio 1:1) (332) to remove white blood cells. The filtrate was collected on ice into tubes containing 1/5 of the diluted blood volume of PBS-G containing 10 mM EDTA. RBC numbers, hct and MCV were measured using a Sysmex F-800 microcellcounter (TOA Medical Electronics Europe GmbH, Hamburg, Germany).

2.2.3 RBC density separation on self-forming Percoll gradients

Filtered blood was centrifuged at 478g for 10 min and the supernate was discarded. The pelleted cells were added to a Percoll buffer (854 g/l Percoll (Amersham Pharmacia Biotech AB, Sweden), 10 mM NaKHPO₄, 144 mM NaCl, 0.5 mM EDTA, 5 g/l D-glucose, 30 µg/ml PMSF, osmolality 320 mOsm/kg, pH 7.4) to a hematocrit of 15%. The suspension was mixed and centrifuged at 33000g for 30 minutes in SS 34 tubes in a Sorvall centrifuge (333). The banding pattern of RBC density gradients was photographed. Density separated RBC were fractionated in 4-6 portions from the top to the bottom of the tube (333). The fractionated RBCs were centrifuged at 478g for 10 min and washed with 5-10 volumes PBS-G by gentle RBC mixing and recentrifugation. The washing procedure was repeated three times.

2.2.4 Separation of unbound plasma proteins from RBC and subsequent RBC treatment with DTSSP

Where indicated, filtered RBCs were freed of unbound plasma material by a phthalate oil separation and were subsequently treated with a membrane impermeable cross-linker DTSSP (Pierce, Rockford, IL, USA) in a Dextran buffer. In brief, 5 mM DTSSP were dissolved in a Dextran solution (18 % Dextran T70 (Amersham Pharmacia Biotech AB, Upsala, Sweden), 10 mM NaKHPO₄, 150 mM NaCl, 1 g/l D-glucose, 300-
320 mOsm/kg, pH 7.45) just prior to use. Pre-cooled Dextran solution containing the
cross-linker (500 μl) were immediately placed in Eppendorf tubes and overlaid with
700 μl of a cold phthalate oil mixture (70 % dibutyl phthalate and 30 % di-isonyl
phthalate). Filtered RBCs (400 μl, 2 - 2.5 x 10⁹ RBC/ml) were applied to the phthalate-
dextran cushion and centrifuged for 15 seconds at 8000 RPM in a Beckman Mikrofuge
in order to pass the cells through the phthalate oil phase and to distribute them
homogeneously in the more viscous Dextran phase. The tubes were left for one hour on
ice to cross-link RBC associated proteins to the cell surface. The reaction was stopped
by pelleting the cells for 10 min at 8000 RPM, cutting the pellet, transferring the
material into a new tube containing a stopping buffer, Tris -G (170 mM Tris, 125 mM
HCl, 1 mg/ml D-glucose, pH 7.4), and mixing well. RBCs were washed twice, as
indicated, with PBS-G and used for the determination of total bound IgG and C3d.

2.3 Radioiodination of protein G and anti-C3d

Recombinant protein G (Sigma, St.Louis, MI, USA) and murine monoclonal antibody
to human C3d (Quidel, San Diego, CA, USA) were ¹²⁵I-iodinated (Amersham, Little
Chalfont, England) by using chloramine T as an oxidant (334). Before labeling proteins
were dialyzed against PBS. Chloramine T at a concentration of 1 mg/ml was dissolved
in PBS just before use. Four μl of 0.2M NaKHPO₄ (pH 7.4), 1mCi Na¹²⁵I and at the end
12.5 μl of chloramine T solution were added to 50-100 μl (30-80 μg) of protein, well
mixed and incubated at room temperature for 45 seconds. The reaction was stopped
with 12.5 μl of 1.4mg/ml Na₂S₂O₅, 1mM NaI and 1mM EDTA in PBS. The unreacted
free ¹²⁵I was removed by passage through a desalting column with Sephadex G-25
(10DG from Bio-Rad) equilibrated with PBS containing 1 mM NaI, 1 mM EDTA and
0.005 % gelatine in case of protein G, in case of anti-C3d antibodies a Sephadex G-75
column was used. Recovered protein amounted to about 50% of the original amount of
used for labeling protein; specific activities ranged from 15 to 40 x 10^6 cpm/µg. The labeled proteins were stored in aliquots at -20°C. Before use they were supplemented with ovalbumin (Sigma, St.Louis, MI, USA) to a final concentration of 10 mg/ml and dialyzed against PBS.

2.4 Radioimmunoassay for protein G and anti-C3d binding to RBC

Binding assays were performed by a phthalate oil separation method described previously (335). The assay was performed in PBS-G buffer. Washed RBCs were adjusted to a cell number of 2.0 to 3.0 x 10^9 RBC/ml. RBC suspensions of 25 µl were added to an equal volume of 125I-labeled protein (4 x 10^7 cpm/ml, 3-6 µg/ml) containing 10 mg/ml ovalbumin, mixed and incubated on ice for one hour. The reaction was stopped by adding 150 µl of PBS-G (four fold dilution) and centrifuging an aliquot of 150 µl through 200 µl of a pre-cooled phthalate oil mixture (70% dibutyl phthalate and 30% di-isonyl phthalate) in 400 µl polyethylene centrifuge tubes for 4 minutes at maximal speed in a Beckman Mikrofuge. The tubes were frozen on dry ice, the tips containing pelleted cells were cut and RBC-bound radioactivity was determined in the γ-counter (Kontron MR 480, Zurich, Switzerland).

2.5 Isolation of RBC membranes

RBC membranes were prepared according to Dodge (336). Washed packed RBC were lysed with 30 volumes of cold hemolysis buffer (5mM NaKHPO₄, 1mM EDTA, pH 7.4). The lysate was centrifuged at 23000g for 20 min and the supernate was removed. The pelleted membranes were resuspended and washed twice with 30 volumes of the same buffer, which in the first wash was supplemented by 0.8 mM DFP. The RBC
membranes were diluted with hemolysis buffer to the initial volume of packed cells and were solubilized and alkylated by adding SDS and NEM to a final concentration of 1% and 50 mM, respectively. Aliquots were stored at -70°C.

2.6 Stripping of peripheral proteins from RBC membranes

Peripheral membrane proteins were stripped from RBC membranes by low ionic strength buffer according to Bennett and Branton (16). RBC membranes were washed three times by resuspending them in 10 volumes of ice cold IOV buffer (0.3 mM NaKHPO₄, 0.2 mM EDTA, pH 8.0) and centrifuging at 40000 g for 20 min. The washed membranes were resuspended with five volumes of IOV buffer to achieve a protein concentration of about 1 mg/ml and proteins were extracted by gently shaking at 37°C for 30 min. The suspension was centrifuged at 100000 g for 30 min in a Kontron ultracentrifuge. The supernate containing 90% of the total membrane spectrin (29) was collected and supplemented by NaKHPO₄ (pH 7.4) to a final concentration of 5 mM and PMSF (50 μg/ml). The extract was stored overnight at +4°C.

2.7 Purification of RBC membrane proteins

2.7.1 Purification of band 3 protein by ion exchange and affinity chromatography

Band 3 protein was purified from Triton X-100 extracts of RBC membranes using anion exchange and affinity chromatography (337).

RBC membranes were prepared as described in the section 1.4. The membranes were suspended in six volumes of 5 mM NaKHPO₄ (pH 8), 150 mM NaCl and incubated for 20 min on ice to strip off band 6 protein. The membranes were centrifuged at 23400 g
for 20 min, the supernatant was removed, and the procedure described above was repeated. The pellet was resuspended in five volumes of a low salt detergent containing buffer (36 mM NaKHPO₄, 0.5% Triton X-100, pH 7.5) and kept overnight at +4 °C. This mixture was centrifuged at 23400g for 20 min, the supernate containing extracted proteins was passed through glass wool and applied to a DEAE-cellulose column (2.5 x 10 cm) equilibrated with the same buffer. Anion exchange chromatography was performed with a flow rate of 38 ml/h and monitored by the UV monitor (Single path monitor UV-1, Pharmacia, Sweden) with connected to it recorder (2-Channel recorder, Pharmacia, Sweden). The column was washed with two volumes of the low salt-Triton buffer and some protein eluted at this time. The remaining protein was eluted with a high salt Triton X-100 containing buffer (150 mM NaKHPO₄, 150 mM NaCl, 0.5% Triton X-100, pH 7.5). The peak fractions, which included band 3, band 4.2 and glycophorin were immediately loaded onto a p-CMB-agarose 4B column. Affinity chromatography was performed with a flow rate of 23 ml/h. The column was washed with five volumes of the high salt-Triton buffer and subsequently with the low salt-Triton buffer until no more material eluted. Pure band 3 was eluted from the column with 0.5 mM cysteine in the low salt-Triton buffer. Eluted material was collected into tubes containing β-mercaptoethanol to give a final concentration of 15 mM. The pooled protein was supplemented with 20 mM NEM, and was extensively dialyzed against 20 mM NaKHPO₄ (pH 7.4), 0.04% Triton X-100. Aliquots of band 3 were stored at -70°C at a concentration of 0.5 mg/ml.

2.7.2 Purification of spectrin by gelfiltration

Spectrin dimers (band 1+band 2) were isolated from the extracts of peripheral RBC membrane proteins. These proteins were obtained by stripping them from RBC membranes with a low ionic strength buffer as described in the section 2.6. The extract was concentrated about 20-fold (final protein concentration 1-2 mg/ml) by
ultrafiltration (YM 30 Filter, Amicon) and supplemented with 20xSepharose buffer to a final concentration of the Sepharose buffer (25mM Tris-HCl, 5 mM EDTA, 0.1 M NaCl, 0.01 mM DTT, 50 μg/ml PMSF, 0.02% Na azide, pH 7.6). The concentrated proteins (15 - 20 ml) were applied to a Sepharose CL-4B column (2.5 x 90 cm), equilibrated in Sepharose buffer (338). Gel filtration was performed with a flow rate of 23 ml/h. Fractions containing spectrin dimers (the 2nd peak on the flow chart) were pooled (29). The pooled protein was concentrated by ultrafiltration (YM 10 Filter, Amicon) to a concentration of 1-2 mg/ml, dialyzed against PBS and stored in aliquots at -70°C.

2.8 Covalent immobilization of RBC membrane proteins to microtiter plates

Proteins were immobilized on aldehyde-derivatized Chemobond plates (Dr. Ernst Fischer Laboratories, Dübendorf, Switzerland) using Schiff base chemistry (339). Proteins were diluted to 20 μg/ml into a coating buffer (10 mM NaKHPO₄, 0.5 M NaCl, 10 mM NaBH₃CN, pH 6.8) containing 0.1% Triton X-100 for band 3 and no Triton for spectrin. Protein solutions (150 μl/well) were loaded on the plates and left to react at room temperature overnight. The solution was decanted and unreacted aldehyde groups were reduced with 1 mg/ml NaBH₄ in PBS (pH 8.0) by incubating with 300 μl/well for 2 hours at +4°C. The plates were washed 15 times with TBS in a Skatron Microwash II (Skatron, Lier, Norway), filled with TBS containing 0.08% Na azide and 50μg/ml PMSF and used immediately or stored at +4°C for up to 6 month.
2.9 ELISA for determination of anti-band 3 and anti-spectrin NAb in sera

NAb concentrations in sera/plasma of HS patients and normal controls were determined by an ELISA method using spectrin or band 3 containing Chemobond plates. Sera treated with 5mM DFP were diluted in PBS buffer containing 0.08% Na azide and 50 mg/ml HSA to 1:250 and/or 1:500. Diluted sera (150 µl/well) were incubated at room temperature overnight with immobilized antigen. Wells were then washed twice with PBS containing 0.05% TWEEN 20 (Fluka, Switzerland), two times with TBS and incubated with 150 µl/well of a 1:5000 diluted stock solution of anti-human IgG-phosphatase conjugate (Sigma, St.Louis, MI, USA) in TBS containing 0.5% gelatin. After incubation for 3 hours at room temperature, the wells were washed twice with TBS - 0.5% gelatin, two times with TBS and once with bidistilled water. Substrate solution (20 mg/ml p-Nitrophenyl Phosphate (Sigma, St.Louis, MI, USA) in 100 mM Glycin, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4) was added (150 µl per well) and incubated for one hour at 37 °C. Absorbance was measured at 405 nm in a microplate reader Dynatech MR 5000.

NAb titers in sera/plasma of patients and healthy controls were analyzed on 2-3 independent occasions in triplicates including the same normal control sera (NC) in each assay. ELISA OD readings were normalized to those of NC and data points were expressed as the ratios of OD of HS plasma/sera to OD of normal control sera (OD₂₅/OD₂₃). To compare NAb levels in unspelenctomized versus splenectomized patient within HS pairs or in the groups of unspelenctomized versus splenectomized patients a paired two-sample Student`s t-test was used. P values smaller than 0.05 were considered to be significant.
2.10 RIA for IgG determination

IgG concentrations in human sera were determined by RIA based on competitive binding of test antibody to a limited quantity of antigen (340). Polystyrene tubes were coated with 1 ml of human IgG (35 µg/ml in PBS containing 0.1% gelatin) by incubating in 37 °C water bath for one hour. The tubes were washed twice with the same buffer. Different test sera dilutions (2 - 3) were prepared in PBS-0.1% gelatin to have an approximate concentration of 10 µg/ml of IgG. A series of standards (0.5 to 20 µg/ml) were prepared from human IgG (Sandoglobulin, a gift from the Swiss Red Cross, Zurich, Switzerland) of known protein concentration. One hundred µl of diluted sera and/or standards were loaded into IgG-coated tubes, 900 µl of ¹²⁵I-recombinant protein G (50000 CPM/ml) were added and the binding reaction was carried out for one hour in 37 °C water bath. The tubes were then washed twice with PBS - 0.1% gelatin and tube-bound radioactivity was measured by γ-counting. The IgG concentration in a sample was determined from the decrease of protein G bound to IgG coated tubes using a standard curve.

2.11 SDS-PAGE

SDS-PAGE was performed according to the Neville procedure (341) optimized by Fasler (342). The stacking gel contained 3.6% acrylamide - bis-acrylamid solution (the mass ratio of bis-acrylamide:acrylamide was 0.026:1), 0.1% SDS, 66 mM Tris-HCl, pH 6.8. The separating gel contained 6 - 8% acrylamide - bis-acrylamid solution, 0.1% SDS, 0.38 M Tris-HCl, pH 8.8. The electrode buffer contained 25 mM Tris, 0.19 M glycine and 0.1% SDS. Samples were heated for 3 min in a boiling water bath in a sample buffer (60 mM Tris-HCl, pH 6.8, 10 mM EDTA, 5% glycerin, 2% SDS, 0.002% bromphenol blue (Sigma), +/- 40 mM DTT) and then were alkylated with 50
mM NEM. Gels (0.5 mm thick minigels) were run for 45 - 90 min at 50 mA in a BioRad gel electrophoresis apparatus (BioRad, Hercules, CA), stained with 0.28 g/l Coomassie brilliant blue R-250 (Sigma) in 50% methanol, 7% acetic acid, and destained in 20% methanol, 10% acetic acid.

2.12 Protein quantification

2.12.1 Protein measurement using the Folin reagent

Protein concentrations were determined with the Folin reagent (Merk, Switzerland) according to Lowry (343) using BSA as a standard.

2.12.2 Densitometric quantification of proteins on SDS-PAGE

SDS polyacrylamide gels stained with Coomassie brilliant blue R-250 were scanned with a Personal densitometer (Molecular Dynamics, Sunnyvale, Ca, USA). The protein concentration was quantified with the program Image Quant (Molecular Dynamics) considering a signal that gave a corresponding protein of known concentration.

Band 4.1 appears in two bands (4.1a and 4.1b) on SDS-PAGE (344). The chemical conversion from the form 4.1b to 4.1a occurs in a time-dependent manner (345). Thus, the ratio of the amount of band 4.1a to band 4.1b is considered to be an excellent absolute red cell age parameter. Band 4.1a/band 4.1b ratios were quantified with the Image Quant program. The background signal was subtracted.
2.13 Measurement of erythrocyte deformability by ektacytometry

RBC deformability was measured using a laser diffraction technique employing an Ektacytometer (Technikon products, Bayer, Germany). In this instrument RBC are suspended in a viscous solution and subjected to a laminar fluid shear stress in a concentric cylinder (346). In response to the shear stress, an erythrocyte is deformed to an ellipsoid. Laser diffraction patterns of cells in their resting and deformed states are then measured. A quantitative assessment of the cell deformability is derived from photometric measurement of the light intensity at selected points on the diffraction patterns. The signal is zero for nondeforming cells that maintain a circular diffraction. Relative deformability is expressed as a ratio: the difference in light intensities along two orthogonal axes at points equidistant from the center of the diffraction image, divided by sum of the two intensities. The ratio is referred to as the Elongation Index (EI).

As a viscous solution to suspend RBC, Dextran T70 (Amersham Pharmacia Biotech AB, Upsala, Sweden) was used. Dextran T70 initially dissolved in bidistilled water at a concentration of 20% was diluted to 18% by adding NaKHPO₄ (pH 7.45) to have a final concentration of 10 mM. D-glucose was added to 1g/l of final concentration. The concentration of NaCl was adjusted to give the desired final osmolality. Red cells (3-4 x 10⁸ RBC/ml) were suspended in 4 ml of isotonic dextran solution (300-320 mOsm/kg) immediately before subjecting them to a constant shear stress (150 RPM, 159 dynes/cm²) over an osmolality gradient from 120 to 750 mOsm/kg. The gradient was created by adding continuously a Dextran solution of high osmolality (~750 mOsm/kg) to the Dextran solution of low osmolality (~120 mOsm/kg) and mixing them. Osmoscans of the red cells were recorded.
3. RESULTS

3.1 Opsonization of HS RBCs before and after splenectomy

3.1.1 A strategy to measure firmly RBC-bound opsonins before and after splenectomy

The degree of RBC opsonization before and after splenectomy was studied on RBCs from pairs of HS patients (HS pairs) which consisted of an unsplenectomized and a splenectomized patient from the same family, rather than by following the same patient through splenectomy. This approach enabled us to compare the level of RBC-bound opsonins before and after splenectomy at the same time, but in two different individuals suffering from the same mutation.

The number of RBC-bound opsonins was referred to an absolute cell age parameter. As the best RBC age parameter that depends solely on the RBC circulation time, the ratio of the protein content in band 4.1a and band 4.1b was used. The cytoskeletal RBC membrane protein 4.1 undergoes a chemical, time-dependent conversion from the form “b” to “a” form (344) due to deamidation at Asn 502 (345). Deamidation at this single amino acid residue alters the electrophoretic mobility of the protein so that form “a” can be resolved from form “b” on SDS-PAGE. The ratio of band 4.1a/band 4.1b was shown to serve as an excellent cell age parameter (333, 347-349). Referring the data to an absolute cell age parameter was necessary, because HS RBCs have a shortened survival, their populations and that of haematologically normal controls differ in their cell age distributions (Fig 3). The RBC age distributions also differ between unsplenectomized and splenectomized HS patients, since HS RBC survival is prolonged after splenectomy (91, 350). The density of normal erythrocytes
progressively increases with cell age (333). The RBC density also increases in ageing HS RBCs (351), but RBC density distributions in HS and normal donors are different (Fig 29). HS RBCs have higher densities than RBCs of a normal population. Furthermore, splenectomy alters HS RBC density so that RBCs from splenectomized patients distribute at lower densities towards normal ones (Fig 29). Thus, all three RBC populations have an entirely different density distribution and survival time. Consequently, RBCs having the same density do not have the same cell age (Fig 4). Therefore, RBCs were fractionated in 4-6 fractions according to their density, bound opsonins were determined, and these values were referred to the cell age parameter determined subsequently on membranes from each fraction. This approach enabled us to determine RBC-bound opsonins as a function of absolute cell age. Extrapolations between these values further allowed a direct comparison of the properties of RBC at a comparable cell age.
Fig 3. Theoretical distribution of HS and normal RBCs as a function of cell age. The RBC distribution is depicted: for unsplenectomized HS cases by a solid black line, for splenectomized HS by a solid grey line, and for a normal control by a dashed line. A RBC population of an unsplenectomized HS patient comprises a high number of reticulocytes (~15%) and RBCs with a very short life span. Reticulocytosis in splenectomized HS patients is reduced and the RBC lifespan is prolonged. Normal RBCs are cleared from the circulation after about 120 days.
Fig 4. Band 4.1a and 4.1b in membranes of RBCs having the same density obtained from HS patients and a normal control. Filtered RBCs were centrifuged on Percoll density gradients and RBCs having comparable density from each gradient were collected. RBC membranes were prepared and their proteins were run on SDS-PAGE. A region of the stained gel is shown: A, RBCs from an unsplenectomized HS patient (FR1) with a spectrin/ankyrin deficiency; B, RBCs from a splenectomized HS patient (FR2) from the same family; C, from a normal control. The absolute cell age parameter, the ratio of band 4.1a to band 4.1b, was determined by densitometric quantification of the content of protein in band 4.1a and 4.1b on stained SDS-PAGE. Averaged 4.1a/4.1b ratios are given from three independent determinations (0.46 ± 0.12 for A, 1.38 ± 0.18 for B, and 2.94 ± 0.48 for C).
3.1.2 Firmly bound IgG and C3d on HS RBCs before and after splenectomy

3.1.2.1 Firmly bound IgG on density fractionated RBCs in band 3 deficient HS patients

The extent of firmly bound IgG was 2 to 4 fold higher on RBCs from splenectomized than from unsplenectomized band 3 deficient HS patients from the same family (Fig 5). The difference was not due to chronological age, since this pattern was found irrespective of whether the unsplenectomized patients were younger or older than the splenectomized pair partners. The difference was neither associated with alterations in total serum IgG concentrations, since none of the splenectomized patients had a hypergammaglobulinemia, a possible side effect of splenectomy (352) and all patients studied had normal IgG concentrations in their plasma. The difference in RBC-bound IgG between unsplenectomized and splenectomized patients remained significant (P < 0.0001) when plotted against the absolute cell age parameter determined by the ratio of band 4.1a/band 4.1b (Fig 6). In addition, RBCs from splenectomized patients carried 2-3 fold more IgG than the cells from normal control (P < 0.0001). In unsplenectomized HS patients, the degree of RBC opsonization was similar or below that of RBCs from healthy individuals with a considerably higher cell age. In fact, HS RBCs from the most dense Percoll gradient fractions with 4.1a/4.1b values below 1 had not reached the cell age of normal younger erythrocytes (Fig 6). Nevertheless, the extent of RBC-bound IgG increased significantly within a very narrow range of absolute cell age in both unsplenectomized and splenectomized HS patients, but not in healthy controls (Fig 7). This showed that erythrocytes in HS were extensively opsonized by IgG in a
cell-age dependent manner during their short life span. In contrast, IgG binding to normal RBCs did not increase significantly for the fractions analyzed, since we did not enrich for the densest cells.

Fig 5. Firmly bound IgG on RBCs from pairs of band 3 deficient HS patients before and after splenectomy. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected for each type of sample. Cell-bound IgG was measured on density fractionated RBCs by $^{125}$I-labeled protein G and is expressed as the mean of bound protein G/RBC ± SD for 3-5 fractions. HS pairs consisted of unsplenectomized (filled bars) and splenectomized (hatched bars) HS patients from the same family (1, AZ1/AZ2; 2, AZ5/AZ4; 3, AZ8/AZ10; 4, WR3/WR1; 5, WR3/WR2).
Fig 6. Firmly bound IgG on RBCs from band 3 deficient HS patients and normal controls as a function of cell age. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected from each gradient. RBC-bound IgG was determined by $^{125}$I-labeled protein G on density fractionated RBCs, obtained from unsplenectomized (triangles), splenectomized HS patients (squares), and normal controls (open circles). The data are plotted against the absolute cell age parameter determined from SDS-PAGE of membrane proteins of the corresponding RBC fractions.
Fig 7. Cell-age dependent increase in IgG opsonization of HS (band 3 deficiency) and normal RBCs. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBC-bound IgG was determined by $^{125}$I-labeled protein G on density fractionated RBCs obtained from unsplenectomized (triangles) and splenectomized (squares) HS patients. Corresponding normal controls (open circles) were included. Data from two independent families are shown in different colours: HS pair AZ1/AZ2 is shown in light color, AZ8/AZ10 in dark colour. The data are plotted against the absolute cell age parameter.
3.1.2.2 Firmly bound IgG on density fractionated RBCs in spectrin/ankyrin deficient HS patients

The amount of firmly bound IgG was up to 2 folds higher on RBC from splenectomized than from unsplenectomized spectrin/ankyrin deficient HS patients from the same family (Fig 8). The extent of RBC-bound IgG was significantly higher on RBCs from splenectomized than from unsplenectomized HS patients (P < 0.001), when plotted as a function of the cell age parameter (Fig 9). IgG binding to RBCs slightly increased during cell aging in unsplenectomized spectrin/ankyrin deficient HS patients (Fig 10). In contrast, RBC-bound IgG did not significantly increase with cell age in splenectomized family members and was comparable to that in normal controls (Fig 10).
Fig 8. Firmly bound IgG on RBCs from pairs of spectrin/ankyrin deficient HS patients before and after splenectomy. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected for each type of sample. Cell-bound IgG was measured on density fractionated, washed RBCs by $^{125}$I-labeled protein G and is expressed as the mean of bound protein G/RBC ± SD for 3-5 fractions. HS pairs consisted of unsplenectomized (filled bars) and splenectomized (hatched bars) HS patients from the same family (1, AZ15/AZ16; 2, EG5/EG6; 3, AZ17/AZ18; 4, SE1/SE2).
Fig 9. Firmly bound IgG on RBCs from spectrin/ankyrin deficient HS patients and normal controls as a function of cell age. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected from each gradient. RBC-bound IgG was determined by $^{125}$I-labeled protein G on density fractionated, washed RBCs obtained from unsplenectomized (triangles) and splenectomized HS patients (squares), and normal controls (open circles). The data are plotted against the absolute cell age parameter.
Fig 10. Cell-age dependent increase in IgG opsonization of HS (spectrin/ankyrin deficiency) and normal RBCs. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBC-bound IgG was determined by $^{125}$I-labeled protein G on density fractionated RBCs obtained from unsplenectomized (triangles) and splenectomized (squares) HS patients. Corresponding normal controls (open circles) were included. Data from two independent families are shown in different colours: HS pair AZ17/AZ18 is shown in light colour, AZ15/AZ16, in dark colour. The data are plotted against the absolute cell age parameter.
3.1.2.3 Firmly bound C3d on density fractionated HS RBCs

The extent of firmly RBC-bound C3d did not differ significantly for RBCs from unspenectomized and splenectomized pair partners (Fig 11). Density fractionated RBCs carried similar amounts of C3d molecules in both unspenectomized and splenectomized HS patients, irrespective of cell age (Fig 12). The small difference in cell-bound C3d between these two RBC populations was not significant (P = 0.052). The amount of C3d on HS RBCs did not differ significantly from that on control RBCs neither in unspenectomized (P = 0.062) nor in splenectomized cases (P = 0.8). Thus, complement deposition to HS RBCs did not follow the binding pattern observed for IgG.
Fig 11. Firmly bound C3d on RBCs from pairs of HS patients before and after splenectomy. Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected for each type of sample. Cell-bound C3d was measured on washed density fractionated RBCs by $^{125}$I-labeled anti-C3d Ab and is expressed as the mean of bound anti-C3d Ab/RBC ± SD for 3-5 fractions. HS pairs consisted of unsplenectomized (filled bars) and splenectomized (hatched bars) HS patients from the same family (1, WR3/WR1; 2, WR3/WR2; 3, EG5/EG6, 4, AZ17/AZ18; 5, WR4/WR5; 6, SE1/SE2).
Fig 12. **Firmly bound C3d on RBCs from HS patients and normal controls as a function of cell age.** Blood was filtered and RBCs were fractionated on Percoll density gradients. RBCs from 3-5 density fractions were collected from each gradient. RBC-bound C3d was determined by $^{125}$I-labeled anti-C3d Ab on washed density fractionated RBCs obtained from unsplenectomized (triangles), splenectomized HS patients (squares), and normal controls (open circles). The data are plotted against the absolute cell age parameter.
3.1.3 Total IgG and C3d bound to HS RBCs before and after splenectomy

3.1.3.1 A method to preserve all HS RBC-bound opsonins

It was reported that HS RBCs release membrane vesicles in vivo (350, 353, 354) and under a variety of conditions in vitro (28, 33, 355-357). RBC vesiculation leads to decrease in surface area and concomitant loss of vesicle-bound opsonins. Moreover, RBC vesicles released from ATP-depleted RBCs were enriched in bound IgG by up to 14 fold as compared to the remaining RBCs (358). In addition, naturally occurring antibodies (NAb) may dissociate from RBCs during washing because of their low affinity (319) and/or band 3 oligomers that provide the high avidity binding sites for NAb may dissociate (326). To preserve weakly RBC-associated opsonins and to prevent fragmentation of HS RBCs that may occur during RBC handling before determining cell bound opsonins, we have developed a novel technique (Fig 13). Blood devoid of white cells was layered on a phthalate oil cushion and RBCs were centrifuged through the phthalate oil into a slightly denser cushion of dextran, containing the homobifunctional, non-penetrating cross-linker dithiobis(sulfo succinimidyl)propionate (DTSSP). During phthalate oil passage RBCs were freed from unbound plasma components (359). RBCs then dispersed in the dextran phase and reacted in the cold with the cross-linker. Cross-linked RBCs were then washed three times with PBS-G before cell bound IgG and C3d were measured.
Fig 13. An approach to preserve weakly RBC-bound opsonins. Whole blood filtered from white cells was loaded onto the cushion of two non-mixing phases: the lower phase contained PBS-buffered dextran T70 solution supplemented with the cross-linker DTSSP, the upper phase contained a phthalate oil mixture. During a short centrifugation (=20s) RBCs passed through the phthalate oil and dispersed in the more viscous dextran phase. The suspended RBSs reacted with DTSSP for 1 hour one ice, and then they were spun down by centrifugation for 10 min. The pelleted cells were removed, the reaction was stopped as described, and the recovered RBCs were washed 3 times with PBS-G buffer. ⇆ = centrifugation.
3.1.3.2 Total IgG bound on HS RBCs before and after splenectomy

In contrast to firmly bound IgG, the degree of total RBC-associated IgG was significantly higher in unsplenectomized patients than in splenectomized family members (Fig 14). Whole RBC populations from unsplenectomized HS patients carried significantly higher numbers of IgG than those from normal controls (P = 0.036), despite the average age of normal RBC population was significantly higher (compare the ratio band 4.1a/band 4.1b for HS and normal RBCs in Fig 15). The amount of IgG was 1.6-times higher in 3 of 4 unsplenectomized than in splenectomized cases (Fig 15). Thus, HS RBCs of unsplenectomized patients had a high number of IgG deposited on their surface than those of splenectomized patients.

3.1.3.3 Total C3d bound on HS RBCs before and after splenectomy

The degree of total RBC-bound C3d was similar in unsplenectomized and splenectomized patients from the same families (Fig 16). RBCs from HS patients carried similar amounts of C3d irrespective of splenectomy, while the amount of C3d on HS RBCs was higher than on normal RBCs (Fig 17). Thus, although HS RBCs were prone to complement deposition to a larger extent than normal RBCs, C3d binding to HS RBCs occurred in a similar manner in both unsplenectomized and splenectomized patients.
Fig 14. Total IgG bound on RBCs from pairs of HS patients before and after splenectomy. Filtered RBCs were separated from unbound plasma components and cross-linked with DTSSP. RBC-bound IgG was measured by $^{125}$I-labeled protein G. HS pairs consisted of unsplenectomized (filled bars) and splenectomized (hatched bars) HS patients from the same family (1, AZ17/AZ18; 2, AZ19/AZ20).
Fig 15. Total IgG bound on HS RBCs. Filtered RBCs were separated from unbound plasma components and cross-linked with DTSSP. RBC-bound IgG was measured on RBCs from unsplenectomized HS patients (triangles), splenectomized ones (squares) and normal controls (open circles) using $^{125}$I-labeled protein G. The data are plotted against the absolute cell age parameter. All HS cases have spectrin deficiency, except one with band 3, as indicated by grey triangle.
Fig 16. Total C3d bound on RBCs from pairs of HS patients before and after splenectomy. Filtered RBCs were separated from unbound plasma components and cross-linked with DTSSP. RBC-bound C3d was measured by $^{125}$I-labeled anti-C3d Ab on RBC from HS pairs consisting of an unsplenectomized HS patient (filled bars) and a splenectomized family member (hatched bars) (1, AZ17/AZ18; 2, AZ19/AZ20).
Fig 17. Total C3d bound on HS RBCs. Filtered RBCs were separated from unbound plasma components and cross-linked with DTSSP. RBC-bound C3d was measured on RBCs from unsplenectomized HS patients (triangles), splenectomized ones (rectangles) and normal controls (open circles) using 125I-labeled anti-C3d Ab. The data are plotted against the absolute cell age parameter. All HS cases have spectrin deficiency, except one with band 3, as indicated by grey triangle.

3.1.4 Total versus firmly bound IgG and C3d on HS RBCs before and after splenectomy

Washing of RBCs before the cross-linking step resulted in a dramatic loss of IgG from RBCs, while the amount of firmly cell-bound IgG was not affected by further washes (Fig 18 A). In contrast, cell-bound IgG remained high regardless of 1-4 washes, when RBCs were cross-linked first. The decrease of bound IgG by a pre-wash followed the same trend for splenectomized HS patients and controls and was about 14 times (Table 4). On the other hand, the pre-wash-induced loss of RBC-bound IgG amounted up to 28
fold for RBCs from unsplenectomized patients. Thus, RBCs from unsplenectomized HS patients tend to lose their IgG two times more easily during fractionation and washing than RBCs from splenectomized patients and controls.

Washing of RBCs also resulted in a loss of cell-bound C3d, as compared with the numbers of C3d molecules that were preserved on RBCs by cross-linking (Fig 18 B). The difference was not as high as that of RBC-bound IgG (Fig 18 A, 18 B). The ratio of total to firmly bound C3d was 1.6 times higher on HS RBCs when compared to normal RBCs (Table 4). This ratio remained the same in both unsplenectomized and splenectomized patients. Thus, “splenic conditioning” did not alter the binding pattern of C3d to HS RBCs.

Table 4. Total versus firmly bound IgG and C3d on HS RBCs. The total opsonins were measured on RBCs separated from unbound plasma components by centrifugation through phthalate oil, while firmly bound opsonins were determined on RBCs washed with PBS-G before centrifugation through phthalate.

<table>
<thead>
<tr>
<th>RBC-bound opsonins (total/firmly bound)</th>
<th></th>
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<tbody>
<tr>
<td>Red blood cells</td>
<td>HS</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>no</td>
</tr>
<tr>
<td>IgG</td>
<td>23±5</td>
</tr>
<tr>
<td>C3d</td>
<td>8±1.8</td>
</tr>
<tr>
<td>No of patients/controls studied</td>
<td>4</td>
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</table>
Fig 18. Washing effect on opsonin binding to RBCs. A. Washing effect on IgG binding to RBCs. B. Washing effect on C3d binding to RBCs. RBC-bound IgG was determined by $^{125}$I-labeled protein G, RBC-bound C3d was determined by $^{125}$I-labeled anti-C3d Ab. Bound opsonins were measured on normal RBCs under various cross-linking and washing conditions:

1. RBCs were cross-linked with DTSSP
2. RBCs were cross-linked with DTSSP and washed 1x with PBS-G
3. RBCs were cross-linked with DTSSP and washed 2x with PBS-G
4. RBCs were cross-linked with DTSSP and washed 3x with PBS-G
5. RBCs were washed 1x with PBS-G and then cross-linked with DTSSP
6. RBCs were washed 2x with PBS-G and then cross-linked with DTSSP
7. RBC were washed 3x with PBS-G and then cross-linked with DTSSP
3.2 Concentrations of naturally occurring IgG anti-RBC antibodies in HS patients before and after splenectomy

Naturally occurring antibodies (NAbs) to RBC were studied on pairs of HS patients comprising an unplenectomized and a splenectomized patient from the same family (HS pairs) or in the same patient before and after splenectomy (autologous cases). NAb titers were analyzed by ELISA on 2-3 independent occasions in triplicates including the same normal control sera (NC) in each assay (see methods). ELISA OD readings were normalized to those of the particular control and were given as a ratio $OD_{HS}/OD_{NC}$.

3.2.1 Anti-band 3 NAb in HS patients with a band 3 deficiency

Anti-band 3 NAb concentrations were 2-4 fold higher in unsplenectomized than in splenectomized HS patients with a band 3 deficiency (Fig 19). In 3 of 4 cases anti-band 3 titers were more than twise in unsplenectomized patients over normal controls suggesting that anti-band 3 NAbs were upregulated in these patients. In contrast to this, anti-band 3 concentrations were at the lower limit of the normal range in 3 of 4 cases after splenectomy. The difference in anti-band 3 NAb titers in unsplenectomized and splenectomized patients from the same families did not correlate with body age, since this pattern was found independent of the age of unsplenectomized patient. The low levels of anti-band 3 NAb after splenectomy were not due to low concentrations of total IgG. In fact, after splenectomy IgG concentrations remained unaltered in 3 of 4 cases and slightly increased rather than decreased in one case (Fig 21). The observed ELISA signals were inhibited by up to 90% ($70 \pm 19\%$, n=14) with 200 $\mu$g/ml of fluid phase band 3 protein. Thus, splenectomy was followed by a down regulation of band 3-specific NAbs.
3.2.2 Anti-spectrin NAb in HS patients with a band 3 deficiency

Anti-spectrin NAb concentrations were 1.5-3 fold higher in 2 of 4 unsplenectomized as compared to splenectomized HS patients (Fig 20). Anti-spectrin NAb titers were similar in 2 of 4 HS pairs. In contrast to anti-band 3 NAb, anti-spectrin levels were within the normal range in the majority (7 of 8) of HS patients. ELISA signals were inhibited by up to 67% (43 ± 24%, n=14) with 200 µg/ml of fluid phase spectrin protein. Further addition of spectrin did not enhance the degree of inhibition. Complement component C3, β2-glycoprotein I, myoglobin and casein did not inhibit anti-spectrin binding, when assayed in the presence of physiological concentration of human serum albumin (Fig 22).
Fig 19. Anti-band 3 NAb levels in band 3 deficient HS patients before and after splenectomy. NAb concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ8/AZ10; 2, LR1/LR2; 3, AZ6.1/AZ6.2; 4, AZ5/AZ4). The Y-axis represents the ratio of OD at 405 nm of HS to that of a control. Mean values of three determinations ± SD are shown. The range of NAb concentrations for normal controls (n=18) is indicated by horizontal lines above the X-axis. The OD values for normals were 0.122 ± 0.09 (n=18). Severity was determined based on hemoglobin concentration: 10-12 g/dl mild, 10-8 g/dl moderate, and <8 g/dl severe.
Fig 20. Anti-spectrin NAb levels in band 3 deficient HS patients before and after splenectomy. NAb concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ8/AZ10; 2, LR1/LR2; 3, AZ6.1/AZ6.2; 4, AZ5/AZ4). The Y-axis represents the ratio of OD at 405 nm of HS to that of a control. Mean values of 3 determinations ± SD are shown. The range of NAb concentrations for normal controls (n=18) is indicated by horizontal lines above the X-axis. The OD values for normals were 0.144 ± 0.05 (n=18).
Fig 21. IgG concentrations in band 3 deficient HS patients before and after splenectomy. IgG concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ8/AZ10; 2, LR1/LR2; 3, AZ6.1/AZ6.2; 4, AZ5/AZ4).
Fig 22. Anti-spectrin NAb binding to spectrin plates in the presence of various proteins. Binding of anti-spectrin in 0.2% of HS sera (AZ15) was studied in the presence of 200 mg/ml of antigen in PBS containing 50 mg/ml HSA.
3.2.3 Anti-band 3 NAb in HS patients with a spectrin/ankyrin deficiency

In contrast to band 3 deficiency, in unsplenectomized spectrin/ankyrin deficient cases, anti-band 3 NAb titers were extremely low and were below or at the lower limit for normal controls (Fig 23). These titers were similarly low in all splenectomized family members. In 4 of 5 patients of this group the low anti-band 3 titers were not associated with low IgG concentrations (Fig 25).

3.2.4 Anti-spectrin NAb in HS patients with a spectrin/ankyrin deficiency

Anti-spectrin titers in spectrin/ankyrin deficient patients did not reveal a clear tendency. Anti-spectrin titers were 2-3 fold higher than in normals in 2 of 5 unsplenectomized patients (Fig 24). Anti-spectrin NAb concentrations were within the normal range in 4 of 5 splenectomized cases.
Fig 23. Anti-band 3 NAb levels in HS patients with spectrin/ankyrin deficiency. NAb concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ15/AZ16; 2, EG2.1/EG2.4; 3, EG3/EG4; 4, EG5.1/EG6.1; 5, FR1/FR2). The Y-axis represents the ratio of OD at 405 nm of HS to that of a control. Mean values of three determinations ± SD are shown. The range of NAb concentrations for normal controls (n=18) is indicated by horizontal lines above the X-axis. The OD values for normals were 0.122 ± 0.09 (n=18). Severity was determined based on hemoglobin concentration: 10-12 g/dl mild, 10-8 g/dl moderate, and <8 g/dl severe.
Fig 24. Anti-spectrin NAb levels in HS patients with spectrin/ankyrin deficiency.

NAb concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ15/AZ16; 2, EG2.1/EG2.4; 3, EG3/EG4; 4, EG5.1/EG6.1; 5, FR1/FR2). The Y-axis represents the ratio of OD at 405 nm of HS to OD of a control. Mean values of three determinations ± SD are shown. The range of NAb concentrations for normal controls (n=18) is indicated by horizontal lines above the X-axis. The OD values for normals were 0.144 ± 0.05 (n=18).
Fig 25. IgG concentrations in HS patients with spectrin/ankyrin deficiency. IgG concentrations were determined in HS pairs consisting of an unsplenectomized (filled bar) and a splenectomized (hatched bar) patient from the same family or in the same patient (*) before (filled bar) and after (hatched bar) splenectomy (1, AZ15/AZ16; 2, EG2.1/EG2.4; 3, EG3/EG4; 4, EG5.1/EG6.1; 5, FR1/FR2).
3.3 RBC properties

3.3.1 Features of the osmotic deformability profiles of RBCs

HS RBC properties were studied by ektacytometry, since this proved to be a useful tool to characterize red cell deformability, osmotic fragility, and surface area-to-volume ratio (99, 360). RBC deformability was measured as a continuous function of suspension osmolality. The osmotic deformability profiles reveal several features (Fig 26):

1. The maximal RBC deformability, described by the elongation index (EI), is reached near 320-350 mosmol/kg, at a tonicity close to which RBCs are normally exposed (normal tonicity is ~290 mosmol/kg). The height of the EI maximum reflects the amount of membrane surface area available for cell deformation, although membrane deformability also depends on membrane viscoelastic properties and cell water content.

2. Osmoscans reveal two EI minima. The first EI minimum, O', is reached at about 160-170 mosmol/kg. This value coincides with that of the osmolality at which 50% of the cells are hemolyzed in an osmotic fragility assay (360).

3. The second EI minimum, O'', is reached in the hypertonic arm of the osmoscan. The osmolality value at which O'' is reached corresponds to the osmolality at which RBCs are no longer deformable and, thus, is inversely proportional to cell density. A progressive shift of the O'' values to lower osmolality is being observed as cell density increases.
Fig 26. Osmotic deformability profile of normal RBCs. Blood was filtered and ektacytometry was performed. The deformability, described by the elongation index (EI), was recorded as a continuous function of suspension osmolality. Features of the curve used to characterize the cells are: (1) the maximal EI which is reached near isotonic osmolality; (2) $O'$, osmolality at which EI reaches a minimum in the hypotonic arm of the curve. It corresponds to the osmolality at which 50% of the cells are lysed; (3) $O''$, osmolality at which EI reaches a minimum in the hypertonic arm of the curve representing a measure of cell density.
3.3.2 HS RBCs have impaired deformability, increased osmotic fragility, a reduced surface-to-volume-ratio, and higher density

The osmotic deformability profiles of the whole RBC populations from HS patients were compressed compared to that of normal RBCs (Fig 27). The lowered EI maximum of HS RBCs indicated that RBC deformability was significantly impaired in HS patients. The EI maximum was considerably lower in case of RBCs with a spectrin/ankyrin deficiency than a band 3 deficiency. The EI minimum $O'$, indicative of the osmolality at which 50% of RBCs are lysed, was shifted from $O'$ of normal RBCs to higher osmolalities by 27 mosmol/kg for RBCs in spectrin/ankyrin deficiency and by 20 mosmol/kg in band 3 deficiency. The shift implied that HS RBCs had impaired osmotic resistance, and this was more pronounced in spectrin/ankyrin than in band 3 deficient RBCs. The enhanced osmotic fragility of HS RBC with a spectrin/ankyrin deficiency is well known (361) and is in accordance with the severity of the disease. HS RBCs had a reduced $S/V$, since the ascending arms of the curves of HS RBCs were shifted to the right from that of normal RBCs. The reduced $S/V$ indicates a loss of RBC membrane and, in turn, transformation of the RBC shape from a disc to a sphere. In addition, HS RBCs also had higher densities, since their EI minima in the hypertonic region of the osmoscan, $O''$, were reached at lower values. RBC populations with a spectrin/ankyrin deficiency had a higher density than the cells with a band 3 deficiency. Thus, HS RBCs had impaired deformability, decreased osmotic resistance, a reduced surface-to-volume ratio, and higher density being more pronounced in spectrin/ankyrin than in band 3 deficiency.
Fig 27. Osmotic deformability profiles of whole RBC populations from patients with HS. Blood was filtered and ektacytometry was performed. The deformability, described by the elongation index (EI), was recorded as a continuous function of suspension osmolality. RBCs were obtained from an HS patient with a spectrin/ankyrin deficiency (AZ15), from the HS patient with a band 3 deficiency (LR1) and from a normal control (NC). O' value corresponds to the osmolality at which EI reaches a minimum in the hypotonic arm of the curve and at which 50% of cells are lysed, O'', the osmolality at which EI reaches a minimum in the hypertonic arm of the curve and represents a measure of cell density.
3.3.3 Splenectomy improved deformability and lowered density of the whole HS RBC population

Deformability of the whole RBC population was higher in splenectomized than in unsplenectomized HS patients from the same family (Fig 28). This difference was observed in both spectrin/ankyrin and band 3 deficiency. The EI maxima, however, increased more in spectrin/ankyrin than in band 3 deficiency, implying that the removal of the spleen had a more pronounced beneficial effect on HS RBC deformability in spectrin/ankyrin than in band 3 deficiency. Nevertheless, splenectomy did not restore the normal RBC deformability.

In addition to improve RBC deformability, splenectomy lowered HS RBC density. This was obvious from the osmotic deformability profiles, since the O'' values of RBC populations from splenectomized patients were shifted to higher osmolalities. The difference was twice as large in spectrin/ankyrin than in band 3 deficient RBC, when quantitatively assessed from the difference of the O'' values of RBC populations from splenectomized and unsplenectomized patients from the same families. Similar splenectomy-induced changes in RBC density were also observed by density centrifugation. RBCs from an unsplenectomized HS patient with a spectrin/ankyrin deficiency banded primarily at high densities (Fig 29A). The cells from the splenectomized family member distributed at lower densities. The splenectomy-mediated RBCs's shift from higher to the lower densities was also observed for HS RBCs with a band 3 deficiency, but the pattern differed from that of RBC with a spectrin/ankyrin deficiency (Fig 29B). Thus, splenectomy improved RBC density in HS.

The osmotic deformability profiles revealed that splenectomy had an advantageous effect on deformability and density of total HS RBC population. The total RBCs comprise a series of subpopulations such as immature RBCs, reticulocytes, and
mature erythrocytes varying from young to senescent cells. HS RBCs from unsplenectomized patients comprised high numbers of reticulocytes with a higher number in spectrin/ankyrin (11 ± 3.4%, n=8) than in band 3 (5 ± 1.5%, n=8) deficiency. A high portion of reticulocytes (the lightest RBCs) apparently enhanced deformability and lowered density of the total RBC population. Hence, osmoscans from whole RBC populations should be compared at the same extent of reticulocytosis. Since reticulocytosis decreases following splenectomy, osmoscans from whole population can not be directly compared to assess the effect on mature RBCs. Furthermore, deformability and density of RBCs are heavily dependent on the age of the cell (deformability lowers and density increases with cell aging) (360), thus the cell age should be considered. To distinguish abnormalities in HS RBC deformability and density in the two types of deficiencies and to study the effect of splenectomy, ideally the cells of the same age had to be compared. Therefore, ektacytometry was also performed on age-matched RBC subpopulations from spectrin/ankyrin and band 3 deficient HS patients. The RBC osmotic deformability profiles were recorded for several RBC density fractions collected from Percoll gradients, and the absolute cell age parameter, the ratio of band 4.1a/4.1b, was determined for each available fraction.
Fig 28. Osmotic deformability profiles of whole RBC populations from HS patients before and after splenectomy. Blood was filtered and ektacytometry was performed. RBCs were obtained from an unsplenectomized spectrin/ankyrin deficient patient (AZ15) and from the splenectomized family member (AZ16), from an unsplenectomized band 3 deficient patient (LR1) and from the splenectomized family member (LR2), and from a normal control (NC).
Fig 29. HS RBC density distributions before and after splenectomy. A. RBC density distributions in a spectrin/ankyrin deficiency. B. RBC density distributions in a band 3 deficiency. Blood was filtered and RBCs were separated on Percoll density gradients. Labels on the gradient tubes stand for: AM, an unsplenectomized spectrin/ankyrin deficient patient (AZ15), BM the splenectomized family member (AZ16), CM a normal control, AL, an unsplenectomized band 3 deficient patient (LR1), BL the splenectomized family member (LR2), CL a normal control.
3.3.4 Splenectomy improved deformability of age-matched RBCs to a larger extent in a spectrin/ankyrin than in a band 3 deficiency

The deformability profiles of cell age-matched RBCs revealed that RBCs from patients with both types of deficiencies profited from splenectomy by having considerably higher deformability after splenectomy (Fig 30). The maximal RBC deformability (EI max) was 3 times higher after splenectomy in spectrin/ankyrin deficiency (n=2). In band 3 deficiency maximal RBC deformability of cell age matched RBCs was only 2 times higher in splenectomized cases (n=5). The increase in maximal deformability in spectrin/ankyrin deficiency was underestimated, since RBCs of unsplenectomized patients were younger (average 4.1a/4.1b = 0.55, n=2) than those of splenectomized patients (average 4.1a/4.1b = 0.9±0.012, n=4). It was not possible to compare RBCs of exactly the same cell age in this case, since in unsplenectomized HS patients with a spectrin/ankyrin deficiency RBCs with the band 4.1a/4.1b ratio higher than 0.55 were already eliminated in vivo. It was further not possible to collect enough cells from similarly young cells (4.1a/4.1b = 0.5) from control and splenectomized patients, since such cells comprised a very small portion of the whole cell populations. All other RBC fractions compared in Fig 30 had virtually the same absolute cell age, since the band 4.1a/4.1b ratios varied from 0.9 ± 0.14 to 1.08 ± 0.06. Hence, RBCs with both types of deficiencies in splenectomized patients had virtually the same density, surface/volume ratio and maximum deformability of comparable cell age.
Fig 30. Osmotic deformability profiles of cell age-matched RBCs from spectrin/ankyrin and band 3 deficient patients before and after splenectomy. Blood was filtered and RBCs were separated on Percoll density gradients. RBC density fractions were isolated and then ektacytometry was performed on 4-5 fractions from each run. RBC membranes were isolated from all fractions and the ratio of band 4.1a/4.1b (cell age parameter) was determined. Osmotic deformability profiles of RBCs with a comparable 4.1a/4.1b ratio in unsplenectomized and splenectomized patients in both types of deficiencies were selected and the following data computation was performed. The elongation index (EI) maximum values and the osmolality values at O' (EI minimum in the hypotonic arm of the curve), O'' (EI minimum in the hypertonic arm of the curve) and the minimal and maximal osmolality values where EI is maximal was determined. The averages of those data points were calculated for spectrin/ankyrin and band 3 deficient unsplenectomized and splenectomized patients. RBC
deformability, described by the EI, is plotted as a function of suspension osmolality. The average deformability profiles ± SD are given for all types of RBCs where more than two samples were studied. In the deformability profile of RBCs from unsplenectomized spectrin/ankyrin patients (n=2) minimal and maximal values are represented by error bars. The profile for unsplenectomized spectrin/ankyrin deficient patients (n=2) is depicted by a solid red curve with open symbols and for splenectomized ones (n=4) by a solid red curve with filled symbols, for unsplenectomized band 3 deficient patients (n=5) by a solid green curve with open symbols and for splenectomized ones (n=5) by a solid green curve with filled symbols, and for normal controls (n=3) by a dashed curve. The ratio of band 4.1a/4.1b is shown for every type of RBCs. The ratio of band 4.1a/4.1b was 0.55 in unsplenectomized spectrin/ankyrin deficient patients (n=2), 0.9±0.012 in splenectomized ones (n=4), 1.08±0.06 in unsplenectomized band 3 deficient patients (n=5), 1.05±0.17 in splenectomized ones (n=5), and 0.9±0.14 (n=3) in normal controls.
3.3.5 RBC deformability as a function of the absolute cell age in spectrin/ankyrin and band 3 deficiency

RBC deformability in unsplenectomized HS cases decreased steeply within a very narrow range of cell age as compared to normal controls, when the EI maxima were plotted as a function of the absolute cell age for band 3 deficient (Fig 31) as well as spectrin/ankyrin deficient RBCs (Fig 32). In contrast, deformability of normal RBCs decreased very little with the absolute cell age. Splenectomy resulted in a shift of those cells to higher cell age. In addition, splenectomy had a different effect on the deformability of RBC with the two kinds of deficiencies. In case of spectrin/ankyrin deficiency, splenectomy improved RBC survival such that RBC lost their deformability at a reduced rate as compared to RBC from unsplenectomized patients (Fig 32). RBCs with a spectrin/ankyrin deficiency lost less than half of their maximal deformability and reached a cell age corresponding to a band 4.1a/band 4.1b ratio of 1.5. The slope of the curve in spectrin/ankyrin deficiency flattened, but did not reach that of normal RBCs. In contrast to this, splenectomy did not improve the cell age dependent loss of deformability in band 3 deficiency. RBC from splenectomized patients lost their deformability with the same rate as those from unsplenectomized patients. Thus, splenectomy improved RBC deformability by two means:

1) By eliminating “splenic conditioning” in both types of deficiencies, resulting in RBC that in spite of having similar deformability had a considerably higher cell age (Fig 31 and 32).

2) By reducing the cell age dependent loss of deformability in case of spectrin/ankyrin deficient HS RBC (Fig 32).
Fig 31. RBC deformability as a function of cell age in unsplenectomized and splenectomized HS patients with a band 3 deficiency. Filtered RBCs were separated on Percoll density gradients and density fractions were collected. RBC deformability, described by the elongation index (EI), was determined by ektacytometry. The ratio of band 4.1a/band 4.1b was determined by densitometric quantification of the bands on SDS-PAGE of RBC membranes. The EI maximum values for the given RBC fraction are plotted against the cell age parameter (4.1a/4.1b). Open rectangles stand for RBCs from unsplenectomized patients, filled rectangles for splenectomized patients, open circles for normal controls. Data for five HS families is shown in different colors: (A) AZ1/AZ2 in red, AZ8/AZ10 in green, WR4/WR5 in brown, (B) LR1/LR2 in yellow, AZ3/AZ4 in blue.
Fig 32. RBC deformability as a function of cell age in HS patients with a spectrin/ankyrin deficiency before and after splenectomy. Filtered RBCs were separated on Percoll density gradients and density fractions were collected. RBC deformability, described by the elongation index (EI), was determined by ektacytometry. The ratio of band 4.1a/band 4.1b was determined by densitometric quantification of the bands on SDS-PAGE of RBC membranes. The EI maximum values for the given RBC fraction are plotted against the cell age parameter (4.1a/4.1b). Open triangles stand for RBCs from unsplenectomized patients, filled triangles for splenectomized patients, open circles for normal controls. The ratio of band 4.1a/band 4.1b was determined by densitometric quantification of the bands on SDS-PAGE of RBC membranes. Data for three HS families is shown in different colors: AZ15/AZ16 in blue, AZ17/AZ18 in green, FR1/FR2 in grey.
3.3.6 Band 3 content in density separated HS RBCs as an indication of membrane loss in spectrin/ankyrin and band 3 deficiency

Band 3 deficient (Fig 33 A) as well as normal RBCs (Fig 33 C) maintained band 3 protein during cell aging, as judged from the ratio of protein band 3 to spectrin determined by densitometric quantification of these proteins on SDS gels. In contrast, the band 3/spectrin ratio progressively decreased with cell age in spectrin/ankyrin deficiency (Fig 33 B). This implies that band 3 containing vesicles were released from spectrin/ankyrin deficient, but not from band 3 deficient RBCs.
Fig 33. The ratio of protein band 3/spectrin in the RBC membrane as a function of cell age. Blood was filtered and RBCs were separated on Percoll density gradients. RBC density fractions were isolated and RBC membranes were isolated from all fractions. The ratio of protein band 3/spectrin and the band 4.1a/4.1b was determined by densitometric quantification of the proteins on SDS-PAGE of RBC membranes. Open symbols stand for RBCs of unsplenectomized patients, filled symbols for splenectomized cases. A. **Band 3/spectrin ratio versus cell age in band 3 deficient RBCs.** Data for family AZ8/AZ10 is shown in green, LR1/LR2 in yellow color. B. **Band 3/spectrin ratio versus cell age in spectrin/ankyrin RBCs.** Data for family AZ15/AZ16 is depicted in blue, AZ17/AZ18 in green color. C. **Band 3/spectrin ratio versus cell age in normal RBCs.**
4. DISCUSSION

4.1 Splenectomy improves the hematological parameters in HS patients with band 3 and spectrin/ankyrin deficiency

Clinically, HS patients with both types of deficiencies profited equally from splenectomy. The Hb concentration in splenectomized patients was normal and reticulocytosis decreased to normal levels (Table 5). Spectrin/ankyrin deficient patients apparently profited more from splenectomy, since their reticulocyte counts decreased by 7-fold, while those in band 3 deficiency, only by 4-fold.

Table 5. Hematological parameters of HS patients before and after splenectomy

<table>
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<tr>
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<th>HS patients</th>
<th>Normal donor</th>
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<tr>
<td></td>
<td>Band 3</td>
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</tr>
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<td>yes</td>
</tr>
<tr>
<td>splenectomy</td>
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<td>n=8</td>
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<tr>
<td>Retic. No, %</td>
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<td>1.4±0.7</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>11.6±2.1</td>
<td>16±1.3</td>
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Abbreviations: Retic - reticulocyte, Hb - hemoglobin, F - female, M - male
4.2 Effect of splenectomy on functional properties of RBCs with spectrin/ankyrin and band 3 deficiency

The averaged deformability profiles of cell age-matched RBCs were similar for both types of deficiencies after splenectomy (Fig 30). Splenectomy improved deformability of RBC 2-fold in band 3 and 3-fold in spectrin/ankyrin deficiency (Fig 30). This effect could not be observed by analyzing total HS RBC populations, since the HS RBC populations before and after splenectomy have entirely different cell age distributions (Fig 3) and reticulocyte counts (Table 5). These findings suggest that splenic conditioning caused more damage to RBCs with a deficient skeleton than to those with an integral membrane protein deficiency. In accordance to this, Ingrosso et al reported that RBCs membrane proteins were highly methylated in unsplenectomized but not splenectomized cases with a spectrin/ankyrin deficiency and neither in unsplenectomized nor splenectomized band 3 deficient RBCs (362). Methylation is part of a cellular repair mechanism (363). Its activation in unsplenectomized patients suggests striking spleen-induced membrane skeleton disarray in spectrin/ankyrin deficiency. Thus, splenectomy was more beneficial in spectrin/ankyrin than band 3 deficiency.

Osmotic deformability profiles of RBCs with a comparable cell age also showed that pre-splenectomy RBCs had similarly high densities in band 3 and spectrin/ankyrin deficiency (Fig 30). Splenectomy lowered their densities to a similar degree in both types of deficiencies. This implies that both types of deficiencies suffered from splenic conditioning consisting of an excessive cation and water loss. Joiner et al. reported an increased red cell permeability to monovalent cations in three mouse mutants with a spectrin (sph/sph, sph^ha/sph^ha) or ankyrin (nb/nb) deficiency (364). These authors suggested that the cytoskeleton dysfunction resulting from these membrane protein
defects might be responsible for the abnormal cation permeability of the RBC membrane. We observed that the cell density was lowered to comparable values irrespective of whether the defect was in the skeleton or the plasma membrane. Thus, the pre-splenectomy loss of cations and water was not directly associated with a specific RBC membrane defect and appeared as the result of splenic conditioning. In agreement with our finding Defranceschi et al reported that the increased Na\(^+\) and low K\(^+\) content in HS RBCs can be attributed to an abnormal membrane permeability to cations, which is not related to a specific membrane protein defect (365). It has to be mentioned that pre-splenectomy spectrin/ankyrin deficient RBCs had a lower cell age parameter than band 3 deficient RBCs (Fig 30). It was not possible to collect sufficient numbers of RBCs with the same absolute cell age in spectrin/ankyrin deficiency from the small amount of blood available. This problem was not taken care of in comparing pre-splenectomy HS RBCs. As cell density correlates with cell age in a given cell population, it is tempting to speculate that ion and water loss would have been more pronounced in spectrin/ankyrin deficient RBCs having a band 4.1a/4.1b ratio of 0.9-1. Thus their deformability would have been lower and their density higher than those given for cells with an averaged cell age parameter of 0.5. This implies that the loss of cations and water though primarily due to splenic conditioning was further aggravated by a skeletal defect.

Plots depicting the maximal deformability as a function of absolute cell age for density-fractionated RBC (Fig 31, 32) yet reveal another beneficial effect of splenectomy. Although pre- and post-splenectomy RBCs having the same density had similar deformability, the absolute cell age of post-splenectomy RBCs was, however, higher by 0.4 units of the band 4.1a/4.1b ratio. The finding implies that the functional impairment is significantly delayed following splenectomy irrespective of the type of defect. Hence, splenectomy did not simply prolong the survival of RBC, but rather prevented an early elimination of a substantial fraction of young cells. This may not be the only possible explanation for the shift in cell age of the lightest fractions in both
types of deficiencies. It is, however, a likely explanation, since reticulocytes and young RBCs are known to have the highest KCl cotransport potential that is rapidly activated by swelling or low pH (366, 367), adverse conditions as they may exist in the splenic cords. Thus, the portion of young cells, which traversed the spleen, suffered considerably more from splenic conditioning than cells that entered the spleen at a higher cell age. Splenectomy also had the presumed effect and prolonged the survival of RBCs, but only in spectrin/ankyrin not in band 3 deficiency. Band 3 deficient RBCs lost their deformability with cell age as rapidly in splenectomized as in unsplenectomized patients. This implies that the rapid loss of deformability is a membrane inherent rather than a spleen induced phenomenon in this deficiency. In contrast to this, the deformability decreased at a considerably slower pace with cell age in splenectomized than unsplenectomized patients with a spectrin/ankyrin deficiency (Fig 32).

4.3 Spectrin/ankyrin and band 3 deficient RBCs acquire the spherocytic phenotype by different molecular mechanisms

The spherocytic phenotype in HS appears to develop due to reduced lipid content (35, 354) and a surface area loss in the form of microvesicles (2). Indeed, the band 3 protein to spectrin ratio markedly decreased with cell age in spectrin/ankyrin (Fig 33 B), but not in band 3 deficient RBCs (Fig 33 A). Since RBC vesicles contain integral membrane proteins, but are devoid of spectrin (368, 369), the decrease in the band 3/spectrin ratio in RBC membranes can be considered to reflect a surface area loss. Hence, spectrin/ankyrin deficient RBCs progressively released plasma membrane. In contrast to this, the band 3/spectrin ratio did not significantly alter in band 3 deficiency, when plotted as a function of the absolute cell age (Fig 33 A). Thus, band 3 deficient RBCs did not lose band 3 protein, while in circulation, and contain about half as many
spherocytes as spectrin deficient RBCs (5 versus 9%) (personal communication with G. Mariani). We postulate that band 3 deficient RBCs become spherocytes by having a reduced lipid content and by releasing of lipid vesicles lacking protein band 3. In spectrin/ankyrin deficiency, membrane surface loss was concomitant to the loss of band 3 protein.

Since the molecular basis of the disease is different in the two types of deficiencies, the surface area deficiency in HS may be a consequence of several distinct molecular processes. Several models have been proposed for the formation of surface area deficiency in HS associated with band 3 defects (2). One of them postulates that the lipid/protein ratio is considerably impaired, since incorporation of lipids into the plasma membrane requires the presence of band 3 proteins. Consequently, an underexpression or misincorporation of band 3 may lead to a reduced incorporation of lipids into the plasma membrane. This hypothesis is based on the observation that overexpression of fumarate reductase, a membrane protein in *E. coli*, is associated with a proportional increase in membrane lipids. Another hypothesis suggests the release of protein depleted vesicles, namely that a decrease in band 3 within the membrane, i.e. a reduced density of band 3 containing intramembrane particles (IMPs) may lead to an increase in IMP-free areas which lack skeletal support and may be prone to bud off. This is based on the current opinion that the tight association of the lipid bilayer to the underlying skeleton is primarily maintained via the binding of ankyrin to band 3 tetramers (2, 3). These interactions may involve a larger percentage of the band 3 molecules than in normal cells, since the number of skeletal proteins is virtually normal, whereas band 3 molecules is reduced by 15-30%. Our finding that band 3 deficient RBCs maintain the initial band 3 content in the membrane is compatible with both postulated mechanisms.

The membrane skeleton instability and the surface area deficiency are more prominent in spectrin/ankyrin deficiency than in band 3 HS (370). In normal RBCs, the skeleton forms a nearly monomolecular submembraneous layer, occupying more than
half of the inner surface of the membrane (28). Consequently, spectrin deficiency leads to a decreased density of this network. As a result, areas of the plasma membrane that are not directly supported by the skeleton are prone to be released from the cells. It has been reported that band 3 lateral mobility is regulated by the spectrin content of the red cell membrane (371), whereas ankyrin was found to reduce band 3 rotational mobility (372). Lateral band 3 mobility was greater in RBCs from patients with spectrin- and ankyrin-deficient RBCs than in control cells and the magnitude of the increase correlated with the degree of spectrin deficiency (373). Thus, the observed cell-age dependent loss of band 3 in spectrin/ankyrin deficient RBCs might be due to weakened skeletal constraints on band 3 protein, which allowed its loss along with the plasma membrane.

4.4 HS RBC opsonization and its relationship to splenic conditioning

We observed that HS RBCs of unsplenectomized patients carried about 2-fold lower numbers of firmly bound IgG molecules than RBCs of splenectomized patients. This was the case for HS RBCs with a spectrin/ankyrin (Fig 9) and for those with a band 3 deficiency, which carried more opsonins (Fig 6). We speculate that pre-splenectomy HS RBCs might have low numbers of firmly bound IgG, since they have lost their membrane vesicles with membrane-bound opsonins during the experimental handling (washing and centrifugation steps). Centrifugation and washing might have accelerated vesiculation of HS RBCs with underlying spleen-induced instability, since exposure of RBC to shear stress is known to induce vesicle formation also on normal RBCs (374). Indeed, using a technique that prevents vesicle release and preserves RBC-associated molecules, we found that the ratio of total to firmly RBC-bound IgG was 1.6 times higher on RBCs from unsplenectomized than splenectomized cases.
(Table 4). The finding suggests that HS RBCs that were conditioned by the spleen are more fragile in vitro, and therefore vesiculate in the test tube unless hindered by cross-linking of RBCs membrane proteins. Thus, vesicle release from HS RBCs of unsplenectomized patients may have occurred during washing and/or centrifugation, while RBCs of splenectomized patients may have kept their membrane intact. Correspondingly, the ratio of total to firmly bound IgG was comparable for RBCs from splenectomized patients and normal controls.

These results shed considerable doubts on any determination of RBC-associated opsonins on washed RBCs from unsplenectomized patients (375). The ratios of total/firmly bound IgG strongly suggest that pre-splenectomy RBCs can not be washed without severely affecting their preformed and yet membrane bound vesicles including their opsonins.

The total IgG reached $4 - 8 \times 10^4$ IgG molecules per cell (Fig 15). This number might be a measurement of the potential NAb binding sites. It is not reflecting the number of unspecific sites for IgG, which was similar to that found for albumin, amounting to $4 \times 10^5$ molecules per cell (376). The total IgG binding was assessed on RBCs stripped from 1/2 diluted filtered blood containing a large excess of albumin and all other blood components in almost physiological concentration. Total IgG binding was dependent on the IgG concentration in filtered blood (not shown), and further studies will have to be performed to identify the specificity of RBC-bound IgG and whether their binding was exclusively or partially Fab mediated.

Vesicle release from HS RBCs was earlier reported to occur (34, 353, 377). The small amounts of blood available in these studies did not allow their detailed characterization. In case of spectrin/ankyrin deficiency we show evidence for the loss of band 3 protein suggesting that the released vesicles contained band 3, lacked spectrin and therefore may have had similar properties as those described for skeletal-free vesicles that were released from ATP depleted normal RBCs. For the latter type of vesicles it was shown that their band 3 proteins were oligomerized to 10-fold higher
degree than band 3 of RBCs membrane (378). Correspondingly, the vesicles bound 14 times higher amounts of autologous IgG than the remaining RBCs from which the vesicles were released (379). Vesiculation may operate by different modes before and after splenectomy. Spleen-induced metabolic deprivations consisting of hypoxia, decreased pH, reduced glucose concentration, and depleted ATP (82) may cause vesiculation, since ATP depletion (368) and decreased intracellular pH (pH 5 - 6) (380) artificially induce vesicle release. Thus, the splenic environment provides means to render HS RBCs extremely fragile.

RBC membrane vesiculation occurs not only in HS, but also in normal aged (96) or stored RBCs (97). It is possible that this phenomenon plays a physiological role. First, RBCs may escape IgG-mediated phagocytosis by shedding IgG-enriched vesicles. Second, membrane vesiculation also protects RBC from destruction by complement, since RBC can get rid off their MAC (C5b-9) in the form of microvesicles (381).

4.5 Anti-RBC NAb titers may be modulated by their consumption

In most HS patients, anemia is compensated by high reticulocytosis. Thus, reticulocyte counts parallel the extent of red cell clearance. Reticulocyte numbers may correlate with the degree of anti-RBC NAb consumption, if these NAbs were involved in RBC clearance. Elevated reticulocyte numbers (5.8 ± 2.6%, n = 8) in cases of unsplenectomized band 3 deficient patients were associated with high anti-band 3 NAb titers (Fig 34). The increased consumption appeared to be well compensated by an extra production of anti-band 3. Anti-band 3 pre-splenectomy levels were 2 to 3 times higher than the averaged level in normal donors (Fig 19, 34). In contrast to band 3 deficiency, reticulocyte numbers were significantly higher (11 ± 3.4%, n=8) in
unsplenectomized patients with a spectrin/ankyrin deficiency. High reticulocytosis correlated with low titers of anti-band 3 NAb (Fig 34). The dramatically increased demand of NAbs in spectrin/ankyrin deficiency most likely was not balanced by an extra NAb production and resulted in very low anti-band 3 NAb levels (Fig 23, 34). Splenectomy normalized reticulocyte counts and anti-band 3 NAb levels in all splenectomized HS patients, irrespective of the type of deficiency (Fig 34). Thus, NAb levels appear to be regulated predominantly by consumption. In addition, anti-RBC NAbs may be upregulated to a limited degree. A comparable phenomenon has been observed for anti-β2-glycoprotein I-antibodies in systematic lupus erythematosus (SLE) patients. These antibodies are strongly associated with thrombosis in SLE patients. Anti-β2-glycoprotein I levels were similarly high in SLE patients before and after thrombosis, but significantly dropped in the same patients during the thrombotic event (382).

Anti-spectrin NAbs did for most part not follow the same trend as anti-band 3 NAbs (Fig 19, 20, 23, 24). Anti-spectrin NAbs are directed to the skeletal protein spectrin that is not exposed under normal circumstances. Though anti-spectrin NAbs do bind to artificially oligomerized structures on the surface of RBC due to their polyreactivity (326), this phenomenon may not appreciably add to RBC opsonization in vivo. What portion of RBC-bound IgG they comprised was not studied.
Fig 34. Anti-band 3 NAb levels versus reticulocyte numbers in HS patients before and after splenectomy. Data for unsplenectomized patients are shown in triangles, that for splenectomized ones in rectangles. Black symbols stand for band 3 deficiency, grey symbols, for spectrin/ankyrin deficiency. A shaded area shows normal range for both NAb titers and reticulocyte number.
4.6 NAb titers and the spleen

Since the spleen is a secondary lymphoid organ responsible for a portion of antibody production in the body, its removal may also diminish NAb production in particular because the spleen homes primed T helper cells presumably required for the class switch (383). Likewise a series of immunologic abnormalities were associated with congenital or acquired absense of the spleen. The absence of the spleen induced decreased NK cytotoxicity and antibody-mediated cellular cytotoxicity (384). Splenectomized patients had decreased levels of serum IgM (385), and their B-cell function was impaired in vitro (386). It has been debated for a long time whether splenectomy also affects cell-mediated immunity. It has recently been demonstrated that post-traumatic splenectomy resulted in a significant and long-term reduction of the levels of CD4+ T cells, coexpressing the high molecular weight isoform of CD45, CD45RA, a subset of T cell critical to mount a primary immune response (387). A further functional abnormality was suggested based on the finding that antibody responses following primary immunization with a T-cell dependent antigen, hepatitis A vaccine, was significantly lower as compared to age- and sex-matched controls vaccinated in parallel (387). Whether splenectomy also diminished the concentration of pre-existing IgG NAbs is not known, but this possibility is worth considering. Anti-band 3 NAb concentrations significantly decreased after splenectomy in band 3 deficient cases (Fig 19). The polyreactive anti-RBC NAbs, anti-spectrin NAb, did not exhibit spleen-associated alteration in their titers (Fig 20, 24). This is possible, if, beside the spleen, other lymphoid organs were involved in the production of naturally occurring antibodies. The plasma cells of the lymphnodes or Peyer's patches (383) may take over the NAb generating function of the spleen. Moreover certain IgG may be generated by a T cell-independent process (388, 389). Irrespective of the mechanism of
IgG NAb production, their level in splenectomized patients and the high IgG binding to RBC from splenectomized patients exclude the possibility that prolonged survival of HS RBC in splenectomized patients were due to a lack of opsonins.

4.7 Postulated mechanisms for HS RBC clearance

The cell-age dependent increase in firmly RBC-bound IgG in splenectomized band 3 deficient patients correlated with the loss of deformability (Fig 35). This is the first direct correlation of a functional impairment with the degree of opsonization in HS RBCs. This increase in firmly bound IgG occurred without a loss of band 3 from the membrane and therefore followed membrane alterations that favor firm IgG binding, like lipid loss, increased band 3 concentrations, band 3 patch formation, shrinkage of the cell (Fig 36 A). Band 3 deficient RBCs had a 3-5 fold increase in IgG, when compared to normal RBC and disappeared from circulation as young cells (Fig 6, 35). These data suggest that accelerated clearance of HS RBC with a band 3 deficiency may be IgG NAb mediated. In contrast to band 3 deficiency, RBCs with a spectrin/ankyrin deficiency lost deformability at a lower rate and survived considerably longer (2-fold the 4.1a/4.1b) (Fig 35). Their longer survival might be due to their low numbers of firmly bound IgG, which did not increase significantly with cell age. Spectrin/ankyrin deficient RBCs progressively released band 3 containing vesicles, as judged from their decreasing band 3/spectrin content (Fig 35), presumably due to a weakened band 3 anchorage by a deficient skeleton (Fig 36 B). The loss of membrane vesicles enriched in band 3 oligomers and bound IgG might have accounted for the lower IgG numbers on the remaining RBC membrane and consequently resulted into a longer survival of these RBCs.

IgG NAbs may similarly be involved in opsonization of HS RBC in unsplenectomized cases, but can only be assessed on the basis of total RBC-bound IgG. Total RBC-bound IgG was 2-fold higher on HS than normal RBCs (P=0.036), despite
they were half as old (4.1a/4.1b) (Fig 15), and had yet 50-70 % of the deformability of control cells (Fig 27), as compared to those from splenectomized patients with about 70-80 % of the deformability of normal RBCs (Fig 28).

In summary, opsonization by IgG NAbs may be involved in the premature clearance of band 3 deficient RBCs. Based on earlier work we suggest that IgG NAb binding was promoted by generation of new types of interdimeric and intertetrameric band 3 oligomers, which were retained in band 3 deficient RBCs due to anchorage by the excess of skeletal proteins. Oligomerization of band 3 protein may have favored a strong, bivalent binding of IgG NAbs as has been exemplified for affinity purified anti-band 3 NAbs (303). Though we have no direct evidence for the involvement of anti-band 3 NAbs, their participation in the opsonization is likely, since spectrin/ankyrin deficient RBCs escaped from IgG opsonization by the release of band 3 containing vesicles. In keeping with this, the involvement of anti-band 3 NAbs was demonstrated in clearance of oxidatively stressed RBCs in vitro (163, 390) and in vivo (323). Likewise, the involvement autologous IgG as an opsonin in phagocytosis was further shown for malaria-infected RBCs (314) and those with a hemoglobin defect (315, 328, 391). These findings appear to contradict the currently discussed recognition of RBCs through exposed PS and recognition by scavenger receptors. Opsonin-independent RBC elimination may be mediated by the interaction of PS on RBC and scavenger receptors on phagocytic cell (129, 392). However, HS RBCs lack this clearance pathway, since they do not expose PS (125, 137, 393), in contrast to sickle (114, 136, 394, 395) beta-thalassemic (133, 395, 396), and aged normal RBCs (114, 115, 397).
Fig 35. Relationship between RBC opsonization, deformability and membrane surface loss in HS with a band 3 and spectrin/ankyrin deficiency. This figure is recombined from previously shown Figures 7, 10, 31, 32, 33. Only splenectomized cases are shown.
Fig 36. A model for band 3 protein dynamics and NAb binding in HS RBC membranes. A. Band 3 dynamics and NAb binding in band 3 deficient RBCs. Band 3 proteins exist as preformed dimers and tetramers (not depicted) in the membrane. In band 3 deficient RBCs, the fraction of immobilized band 3 is increased. Lateral mobility and reassociation of these elements results in new types of interdimeric band 3 oligomers, which favor bivalent NAb binding. B. Band 3 dynamics and NAb binding in spectrin/ankyrin deficient RBCs. In spectrin /ankyrin deficient RBCs the mobile fraction of band 3 dimer is enlarged, since the skeletal anchorage is partially deficient. This results in an extensive formation of interdimeric oligomers, which bind IgG NAbs and are released as skeleton-free vesicles, whereas the remaining cell is depleted of band 3 oligomers and bound opsonins.
4.8 HS RBC clearance after splenectomy

The numbers of firmly bound IgG on post-splenectomy band 3 deficient HS RBCs were significantly higher than those on control erythocytes (P<0.0001). Hence, RBCs continue to circulate in splenectomized patients despite carrying more opsonins than normal cells. This phenomenon may be related to the lack of the spleen, and therefore altered RBC clearance mechanisms and/or altered opsonic requirements. In humans, the spleen appears to be the major site of destruction for senescent, as well as damaged cells (82, 398). On the other hand, it has been reported that in patients with HS, where the spleen plays a major role in the destruction of red cells (45), only half the red cells were destructed in the spleen (83). After splenectomy, other reticuloendothelial organs, like the liver, lungs or bone marrow have to take over the splenic clearance function. A significant delay in antibody-mediated clearance was found for autologous erythrocytes sensitized with IgG in splenectomized patients (399). A fourfold increase in sensitizing antibody (reaching approximately 10⁴ molecules of IgG per RBC) was required to result in a rapid clearance that was due to increased hepatic sequestration. The prolonged contact between splenic macrophages and RBC allows phagocytosis of poorly opsonized particles. Presumably, for hepatic clearance to occur, a decreased contact time must be compensated by an increased opsonic potential. Thus, after splenectomy the increased IgG numbers are probably required for HS RBC clearance in non-splenic reticuloendothelial organs.
REFERENCES


## Buffers

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<th>Buffer</th>
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<tr>
<td><strong>Coating buffer</strong></td>
<td>10 mM Na$_2$HPO$_4$, 0.5 M NaCl, 10 mM NaBH$_3$CN, pH 6.8</td>
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<tr>
<td><strong>Dextran buffer</strong></td>
<td>18 % Dextran T70, 10 mM Na$_2$HPO$_4$, 150 mM NaCl, 1 g/l D-glucose, 300-320 mOsm/kg, pH 7.45</td>
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<td><strong>Hemolysis buffer</strong></td>
<td>5 mM Na$_2$HPO$_4$, 1 mM EDTA, pH 7.4</td>
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<td><strong>IOV buffer</strong></td>
<td>0.3 mM Na$_2$HPO$_4$, 0.2 mM EDTA, pH 8.0</td>
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<tr>
<td><strong>PBS</strong></td>
<td>10 mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.4</td>
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<tr>
<td><strong>PBS-G</strong></td>
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<tr>
<td><strong>Percoll buffer</strong></td>
<td>854 g/l Percoll, 10 mM Na$_2$HPO$_4$, 144 mM NaCl, 0.5 mM EDTA, 5 g/l D-glucose, 30 µg/ml PMSF, osmolality 320 mOsm/kg, pH 7.4</td>
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<tr>
<td><strong>Sepharose buffer</strong></td>
<td>25 mM Tris·HCl, 5 mM EDTA, 0.1 M NaCl, 0.01 mM DTT, 50 µg/ml PMSF, 0.02% Na azide, pH 7.6</td>
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<td><strong>Tris -G</strong></td>
<td>170 mM Tris, 125 mM HCl, 1 g/l D-glucose, pH 7.4</td>
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Acronyms

BS3    Bis(sulfosuccinimidyl)suberate
CPD    Citrate-phosphate-dextrose
DFP    Diisopropyl fluorophosphate
DTSSP  Dithiobis(sulfosuccinimidyl)propionate
DTT    Dithio-D, L-treitol
EDTA   Ethylene diamine-N,N,N',N''-tetraacetic acid
EI     Elongation index
HSA    Human serum albumin
IOV    Inside out vesicles
LDL    Low density lipoprotein
LPS    Lipopolysaccharide
NEM    N-ethyl maleimide
PMSF   Phenylmethyl sulfofluoride
PS     Phosphotidyl serine
RBC    Red blood cell
S/V    Surface to volume ratio
Sandoglobulin Whole human IgG for intravenous use
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sandoglobulin and HSA were gifts from the Central Laboratories of the Transfusion Service, Swiss Red Cross, Bern.

RBC membrane proteins (except spectrin) are named by positions of the protein band on SDS-PAGE, counted from the top.
### Immunological terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tr>
<td>B cell</td>
<td>Lymphocyte derived from bone marrow stem cells</td>
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<tr>
<td>C3</td>
<td>Complement component C3, cleaved to fragments a-g, part of convertases and opsonins</td>
</tr>
<tr>
<td>C3b</td>
<td>Highly reactive C3 breakdown product</td>
</tr>
<tr>
<td>C3d</td>
<td>C3 breakdown product</td>
</tr>
<tr>
<td>CR1, CR2, CR3</td>
<td>Complement receptors</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>FcγR</td>
<td>IgG Fc receptor</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>MAC</td>
<td>Membrane attack complex</td>
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<tr>
<td>NAb</td>
<td>Naturally occurring antibodies</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>Opsonization</td>
<td>Deposition of Ig and complement on an antigen thereby promoting tight contact with a phagocytic cell</td>
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<tr>
<td>T cell</td>
<td>Lymphocyte derived from thymic stem cells</td>
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<tr>
<td>T(_)(_) cell</td>
<td>Helper T cell</td>
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<tr>
<td>TNF-(\alpha)</td>
<td>Tumor necrosis factor (\alpha)</td>
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Curriculum vitae

Ramune Reliene

Data of Birth: June 9, 1970

Citizenship: Lithuanian

Address: Vogelsangstr.10-256
CH-8006 Zürich
Switzerland

Telephone: 01-255 99 07 (home) 01-632 30 07 (lab)

E-mail: Ramune.Reliene@bc.biol.ethz.ch

Education:


1996 Master of Science thesis "Construction of expression systems for foreign genes in the yeast Kluyveromyces lactis".

1994-1996 Master degree in Biochemistry, University of Vilnius, Lithuania.

1994 Bachelor thesis "Development of a detection method for E.coli".
