Naturally Occurring Human Anti-Band 3 Autoantibodies Accelerate Clearance of Erythrocytes in Guinea Pigs

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A variety of naturally occurring autoantibodies (NOAs) have been found in sera of animals and humans. Although their specific homeostatic role in the clearance of altered or senescent cells has been proposed and in vitro studies support such functions, in vivo evidence has been lacking. We studied the effect of affinity-purified human anti-band 3 NOA on the survival of untreated and diamide-treated erythrocytes in normal and complement C3-deficient guinea pigs. In vitro exposure to diamide, an oxidative agent, severely reduced the erythrocyte deformability and increased the amount of high-molecular-weight forms of band 3 protein and band 3-hemoglobin adducts in erythrocyte membranes, thereby markedly shortening the survival of these cells in vivo. Human anti-band 3 NOA bound in a dose-dependent manner to erythrocytes, and binding increased with exposure to diamide. In normal guinea pigs anti-band 3 NOA significantly accelerated the clearance of erythrocytes that were mildly damaged by iodine surface labeling and of those that were further oxidized by diamide. However, the anti-band 3 effect was transient and small. In contrast, anti-band 3 NOA did not significantly alter erythrocyte survival in functionally C3-deficient guinea pigs, thereby supporting the C3b requirement for anti-band 3 NOA activity. On the other hand, a pretreatment of animals with purified human band 3 protein slowed down the clearance of erythrocytes incubated with IgG depleted of anti-band 3 NOA. These results provide the first in vivo evidence of a role for anti-band 3 NOA in the clearance of erythrocytes.

Erythrocytes from humans and most other mammals are not randomly removed from the circulation but have a fixed life span that varies among species.1,2 While approaching the end of their life span, they undergo a multitude of changes, many of which can be regarded as signs of an aging process of cells that have lost their regenerating potential. Erythrocytes approaching their finite life span represent senescent or aged cells, as they show several distinct hallmarks of oxidative damage (lipid peroxidation, hemichrome binding to band 3 protein, and protein oligomerization),3,4 they have lost enzyme activities5 and membrane in the form of vesicles6 and have extruded ions and cell water7 thereby becoming more dense, less deformable,8,9 and evidently more susceptible to clearance.10

Clearance of senescent erythrocytes occurs by phagocytosis1; thus, the endogenous trigger for removal has to be signaled to the erythrocyte surface. Experimental evidence from Kay11,12 suggested a selective recognition of senescent and of in vitro aged erythrocytes by autologous or homologous serum IgG, implying that age-related changes on the erythrocyte surface allow binding of naturally occurring autoantibodies (NOAs), which facilitate the specific clearance of senescent cells by the macrophage system.

NOAs directed against many conserved self-antigens have been found in sera from healthy humans and animals and have been shown to be exact translation copies of germ line Ig genes.13 The serum concentrations of NOAs are normally kept low, their affinities are 10- to 1,000-fold less than those of induced antibodies, and many are bi- or polyspecific.14 It has been hypothesized that NOAs are involved in the removal of senescent or altered self-antigens by providing specificity for these tissue homeostatic clearance mechanisms. Of the presently characterized NOAs against specific erythrocyte membrane constituents, anti-band 3 autoantibodies (anti-band 3 antibodies)14 appear most likely to be involved in opsonization, phagocytosis, and in vivo clearance of erythrocytes by extravascular hemolysis.15,16

Despite the anti-band 3 antibodies' low concentration of 50 to 100 ng/mL serum in humans and their weak affinity to band 3 protein, with an estimated association constant of 5 × 1010 to 7 × 1010 L/mol,17 these antibodies apparently reach functionality by at least two additive mechanisms. Oligomerization of band 3 protein (without chemical modification) within the erythrocyte membrane allows for firm, bivalent binding of anti-band 3 IgG18 to protein rather than to carbohydrate of band 3.19 Because senescent or oxidatively stressed erythrocytes contain band 3 oligomers,20,21 these antibodies preferentially bind in small amounts to them.22,23 Furthermore, after forming clusters on membranes, anti-band 3 antibodies not only initiate complement deposition, but preferentially form C3b-IgG complexes,24 as they have a second binding affinity for C3 that is independent of the band 3 binding site.25 In analogy to antigen-bound C3b-IgG complexes from induced antibodies,26,27 C3b–anti-band 3 antibody complexes on erythrocytes represent strong opsonins and nucleate alternative C3 convertases, thereby further augmenting C3b deposition and leading to enhanced phagocytosis. However, the tissue homeostatic role of anti-band 3 antibodies in the selective opsonization and clearance of senescent and altered erythrocytes has not yet been documented in vivo.

In many inherited and acquired erythrocyte disorders,
damage to erythrocyte membranes accelerates clearance of altered erythrocytes by phagocytes. Damage by several oxidants causes peroxidation of lipids and/or crosslinking of proteins. For instance, in vitro exposure of erythrocytes to diamide selectively oxidized protein SH-groups and drastically shortened the survival of canine erythrocytes in vivo. Exposure of human erythrocytes to diamide also caused disulfide-linked band 3 proteins; the preferential phagocytosis of these oxidatively stressed erythrocytes by adherent monocytes was dependent on the presence of serum and active complement and accelerated by the addition of anti-band 3 antibodies. Because the oxidative damage by diamide is similar to changes observed in senescent erythrocytes, the mechanisms of in vivo clearance of senescent and oxidatively stressed erythrocytes may be similar.

Here we provide the first evidence that affinity-purified human anti-band 3 antibodies accelerate the clearance of labeled and oxidatively stressed erythrocytes in vivo. To demonstrate the effect of anti-band 3 antibodies, guinea pigs appeared to be most suitable for the following reasons: (1) Because only small quantities of purified anti-band 3 antibodies were available, small-sized animals, from which repeated blood samples could be conveniently obtained, were chosen. (2) Guinea pigs have previously been used to study antibody-mediated clearance of erythrocytes. Complement component C3-deficient guinea pigs have recently been identified, allowing the study of the role of complement on the effect of anti-band 3 antibodies in vivo.

**MATERIALS AND METHODS**

**Guinea Pigs**

Healthy 3- to 4-month-old Dunkin-Harley guinea pigs (Wiga, Zurich, Switzerland) and complement C3-deficient guinea pigs from the Robert Koch Institute in Berlin, Germany, were housed and used according to the guidelines of Zürich’s Animal Care and Use Committee. For all blood collections and administrations, guinea pigs were anesthetized either by short ether inhalation or subcutaneous injection of 0.6 mg/kg ketamine and 0.1 mg/kg xylazine, and was calculated from bound label and the specific radioactivity of the cells with PBS containing glucose, and O.

**Diamide Treatment of Erythrocytes and Deformability**

Based on our in vitro human erythrocyte studies and in vivo canine erythrocyte studies by Johnson et al., diamide was used to oxidatively stress erythrocytes. Affluents of a freshly prepared 20 mmol/L diamide (Sigma) solution in 150 mmol/L NaCl were added to labeled or unlabeled 5% erythrocyte suspensions in HEPES buffer to reach a final concentration of 0 to 400 μmol/L. After a 1-hour incubation at 37°C, erythrocytes were washed three times in cold PBS-glucose and once in PBS-HSA before resuspending them at a hematocrit of 35% in PBS-HSA. Erythrocytes were counted with a Sysmex T-800 (TOA Medical Electronics, Japan). Deformability of labeled and unlabeled erythrocytes treated with various concentrations of diamide was assessed by an ektacytometer (Technicon, Hamburg, Germany) by using the osmotic mode as we previously described for human erythrocytes.

**Anti-Band 3 Antibodies and Their Binding to Cells and Blotted Protein**

Naturally occurring anti-band 3 antibodies were affinity-purified from pooled human IgG (Sandoglobulin; provided by the Central Laboratory of the Swiss Transfusion Services) on columns with immobilized band 3 protein as previously described, except that pooled IgG was first depleted of antiidiotype and antispectrin antibodies as outlined elsewhere. The unbound fraction representing human IgG depleted of anti-band 3 antibodies was used as an IgG control.

For binding studies with erythrocytes from guinea pigs, human anti-band 3 antibodies were iodinated using chloroaurate T as an oxidant to a specific activity ranging from 2.5 × 10^6 to 6 × 10^6 cpm/μg IgG. Equal numbers (2 × 10^6) of packed, unlabeled guinea pig erythrocytes, previously treated with 0, 100, 200, and 400 μmol/L diamide and washed once in binding buffer (10 mmol/L phosphate, 150 mmol/L NaCl, 1 g/L D-glucose, 0.1% gelatin, 2 mmol/L MgCl2, pH 7.4), were mixed with 60 μL of binding buffer containing labeled anti-band 3 antibodies at 500 × 10^6 to 750 × 10^6 cpm. Unlabeled anti-band 3 antibodies to achieve a given total concentration, 5% HSA, and 12.5 mg/mL human IgG. After incubation for 30 minutes at 37°C, the binding reaction was stopped by adding 120 μL of cold PBS-glucose containing 10 mmol/L EDTA, layering 150 μL of this suspension over 200 μL of a phthalate oil mixture (70% (vol/vol) d buzyl- and 30% bis[3,5,5-trimethyl-phthalate], and centrifuging for 4 minutes at 10,000 rpm in a Beckman Microfuge II (Beckman Instruments, Geneva, Switzerland). The tubes were frozen in methanol/dry ice, and the tube tips containing the erythrocyte pellets were cut off and counted for radioactivity. Binding of anti-band 3 antibodies to guinea pig erythrocytes is given in nanograms per 10^6 cells and was calculated from bound label and the specific radioactivity of anti-band 3 antibodies.
For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, membranes from either labeled or unlabeled guinea pig erythrocytes that had been treated with 0 to 400 μmol/L diamide were prepared and stored as previously described for human erythrocyte membranes.17 SDS-PAGE was performed with 7-μg aliquots of membrane protein that were unreduced or reduced with dithiothreitol and alkylated by N-ethylmaleimide as previously described.19 Gels were blotted onto nitrocellulose and then incubated with 100 ng/mL 125I-labeled anti-band 3 antibodies (0.5 × 10⁶ to 0.7 × 10⁶ cpm/mL) as outlined elsewhere.17 Bound radioactivity was shown by Phosphorlmager screens from Molecular Dynamics (Sunnyvale, CA).

**Determination of In Vivo Clearance**

Mixtures of 131I and 125I surface-labeled erythrocytes were administered to determine the effect of anti-band 3 antibodies on the survival of untreated and diamide-treated cells in the same animal. On the day of allogeneic transfusions a mixture containing equal numbers of untreated and diamide-treated and differently labeled erythrocytes was prepared. Aliquots containing 3.5 × 10⁸ to 5 × 10⁸ cells of each type were incubated in vitro with either 20 μg/mL human anti-band 3 antibodies or control IgG at a hematocrit of 20% to 25% in PBS-HSA at 37°C for 30 minutes immediately before injecting a total of 5 × 10¹² erythrocytes containing 8 × 10⁸ to 12 × 10⁸ cpm 125I and 2 × 10⁹ to 4 × 10⁹ cpm 131I. Normal and C3-deficient guinea pigs were studied simultaneously. Where indicated, 4 hours before injection of labeled cells, animals received 200 μg of purified human band 3 protein,24 which was mixed with twice the amount of HSA and dialyzed extensively against PBS-glucose to deplete it of nonionic detergent before injection. Retroorbitally collected and EDTA-anticoagulated blood was kept at 4°C until analyzed. Cell numbers were determined and radioactivity was measured on whole blood by incubation with 400 pmol/L diamide and then incubated with 100 ng/mL 125I-iodinated anti-band 3 antibodies and 100% value for oxidatively stressed, labeled erythrocytes was calculated as the higher counts per minute value of the measurements at 1 and 3 minutes as the 100% value at time 0. The corresponding value at time 0 for untreated control cells. The Student's t test was used to analyze the data, and a P value of less than .05 was considered statistically significant.

**Plasma Anti-Band 3 Antibody Concentration**

Our recently developed radioimmunooassay for human anti-band 3 was modified to detect guinea pig anti-band 3 antibodies in plasma or serum by capturing anti-band 3 antibodies on covalently immobilized band 3 protein and detecting bound antibodies with 125I-labeled goat anti-guinea pig IgG (The Binding Site, Birmingham, UK). Binding was inhibitable to 50% to 60% by 200 μg/mL of fluid-phase human band 3 protein. Intracardial administration of 200 μg human band 3 protein to normal guinea pigs lowered the inhibitable portion of the endogenous anti-band 3 antibody concentration by about 50% at 4 and 24 hours (averaged data from two animals not being transfused).

**RESULTS**

**In Vitro Erythrocyte Studies**

Effect of diamide and labeling on erythrocyte deformability. The deformability of erythrocytes from guinea pigs as assessed by the osmocan mode with an ektacytometer (Fig 1A) was similar to that of human erythrocytes. Exposure to increasing concentrations of the oxidative agent diamide gradually reduced the deformability to approximately 60% of control values, as indicated by the dose-dependent lowering of the osmocans (Fig 1A). Surface iodination with either isotope did not affect the deformability of otherwise untreated cells (Fig 1B). As previously shown for other species,4243 the deformability of erythrocytes from guinea pigs was reduced by increasing concentrations of diamide. The diamide-induced reduction in deformability of either type of labeled erythrocytes (Fig 1B) was similar to that recorded for unlabeled cells (Fig 1A). However, the right arms of the osmocans of labeled and diamide-treated erythrocytes, were also markedly shifted to the left, indicating that these cells had lost a considerable amount of water and thereby became denser. This effect was likely caused by the isotopes or radicals within the solution, because identical incubating conditions without either isotope solution did not produce a left shift in the osmocans. Surface iodination of erythrocytes...
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Fig 2. Immunoblot with 125I-iodinated, human anti-band 3 antibodies of membrane polypeptides from guinea pig erythrocytes that were exposed to the given concentrations of diamide. Unlabeled erythrocytes from guinea pigs were subjected to the radiiodination procedure in the absence of radioisotopes, and then they were treated with diamide as indicated. Membranes were prepared as outlined, and equal amounts of membrane protein were run on SDS-PAGE in the presence (+) or the absence (−) of reducing agent. Membranes from untreated human erythrocytes containing roughly the same amount of protein were also electrophoresed and blotted (human). The polypeptides were transferred to nitrocellulose and immunoblotted with 125I-labeled, human anti-band 3 antibodies. The dried blot was exposed to a Phosphorimager screen, and the digitized picture is shown. In addition to band 3 and band 4.2 protein, an adduct of band 3 protein with hemoglobin is marked (band 3-hb). The asterisks mark the high-molecular-weight protein complexes to which anti-band 3 antibodies bound.

by either isotope generated similar labeling patterns of membrane proteins (in particular, band 3; data not shown).

Human anti-band 3 antibody binding to band 3 protein and to erythrocytes. Binding of labeled human anti-band 3 antibodies to membrane proteins from erythrocytes of guinea pigs and humans is shown on immunoblots in Fig 2. Naturally occurring anti-band 3 antibodies, affinity-purified from pooled human IgG, bound to blotted band 3 protein and band 4.2 (copurified antibodies) of membranes from erythrocytes. The degree of binding of human anti-band 3 antibodies to band 3 protein from guinea pigs was markedly less than to human band 3 protein, but still highly specific as it bound in the presence of 10 mg/mL unlabeled human IgG. On blots from membranes of guinea pig erythrocytes exposed to diamide, a band above band 3 became evident that might represent a band 3-hemoglobin adduct (band 3-hb, Fig 2). Furthermore, three other high-molecular-weight proteins bound labeled anti-band 3 antibodies when membrane proteins were blotted from erythrocytes treated with increasing concentrations of diamide. Band 3-hb and other high-molecular-weight complexes could be completely reduced, whereby the anti-band 3 binding disappeared from high-molecular-weight complexes and was again confined to band 3 protein (Fig 2). This supports the hypothesis that band 3 protein was bound through disulfide bridges in the high-molecular-weight complexes. In fact, the high-molecular-weight complexes (Fig 2, marked with asterisks) had apparent molecular masses reminiscent of multiples of band 3 protein, thereby likely representing band 3 oligomers. Two-dimensional gels with radiolabeled membranes run first unreduced and then reduced confirmed these results (data not shown). The binding of human labeled anti-band 3 antibodies to intact erythrocytes, measured by incubating erythrocytes from guinea pigs at physiologic conditions in the presence of 50 mg/mL albumin and 10 mg/mL unlabeled human IgG, was dose-dependent (1 to 5 μg/mL) and enhanced by prior exposure of erythrocytes to diamide (Fig 3).

In Vivo Erythrocyte Studies

Clearance of iodinated erythrocytes and effect of diamide. The survival of a mixture of 125I or 131I surface-labeled erythrocytes, representing ~1/100 of the animal’s erythrocyte mass (5 × 10⁸ cells) of which one type of labeled cells was

![Image of membrane polypeptides from guinea pig erythrocytes exposed to diamide concentrations.](image_url)

Fig 3. Binding of 125I-iodinated human anti-band 3 antibodies to guinea pig erythrocytes. Binding of anti-band 3 antibodies was determined as outlined at the given concentrations to guinea pig erythrocytes treated with the indicated concentrations of diamide. The results were averaged from duplicates or triplicates of the given number of experiments (n). Standard deviations (SD) are given for 5 μg/mL anti-band 3 antibodies. (△) 1 μg/mL anti-band 3 (n = 3); (○) 2.5 μg/mL anti-band 3 (n = 2); (●) 5 μg/mL anti-band 3 (n = 6).
exposed to diamide, was observed by repeated small blood sample collections. The apparent mean half-life of surface-iodinated erythrocytes in guinea pigs was 8 days, independent of whether they were labeled with \(^{125}\text{I}\) or \(^{131}\text{I}\). Furthermore, the erythrocyte survival in normal guinea pigs receiving autologous cells or normal and complement C3-deficient guinea pigs transfused with allogeneic erythrocytes was not different (data not shown). An apparent half-life of 8 days was considerably shorter than the previously published half-life of 42 days obtained by a cohort labeling with \(^{57}\text{Fe}\), therefore, these cells must have been mildly damaged by the iodination process as shown under in vitro Erythrocyte Studies. Based on the negligible amount of plasma radioactivity (\(\leq\)1%), these cells were mostly cleared by extravascular hemolysis; however, partial or complete intravascular lysis of some erythrocytes cannot be ruled out. The half-lives of diamide-treated erythrocytes ranged from 10 minutes to 20 hours (mean, 5 hours), and the diamide-induced shortening of the mean half-life of erythrocytes correlated with a reduction in mean cell deformability.

Effect of anti-band 3 antibodies in normal guinea pigs. Untreated and diamide-treated, labeled erythrocytes were incubated with either human anti-band 3 IgG or control IgG\(^{-}\) (depleted of anti-band 3 antibodies) before administration of the mixture. The maximal plasma concentration achieved by the injection of approximately 5 \(\mu\)g anti-band 3 antibody per guinea pig was estimated to be 100 ng/mL, representing about twice the normal anti-band 3 concentration in humans. Anti-band 3 antibodies enhanced the clearance of erythrocytes, when compared with IgG\(^{-}\), in normal guinea pigs, whether the cells were mildly oxidized by the labeling procedure or heavily oxidized by exposure of labeled cells to diamide. Data from a typical experiment is shown in Fig 4, and averages from 11 experiments are shown in Fig 5. The difference in erythrocyte clearance between animals receiving anti-band 3 antibodies or control IgG\(^{-}\) was evident in each of the 11 pairs of simultaneously studied guinea pigs, making the slightly accelerated clearance by anti-band 3 antibodies significant. However, this effect was relatively small and transient.

Effect of anti-band 3 antibodies in C3-deficient guinea pigs. Because anti-band 3–mediated phagocytosis of erythrocytes appears to be dependent on C3b\(^{18}\), we investigated the effect of anti-band 3 antibodies on the clearance of untreated and diamide-treated erythrocytes simultaneously in normal and complement C3-deficient guinea pigs.\(^{35,36}\) The survival of untreated and diamide-treated erythrocytes in C3-deficient and normal guinea pigs was similar (Figs 4 and 5). Interestingly, exogenous anti-band 3 antibodies had no effect on the survival of erythrocytes in C3-deficient animals (Figs 4 and 5). Thus, the addition of anti-band 3 antibodies did not significantly accelerate the clearance of untreated or diamide-treated, labeled erythrocytes in C3-deficient guinea pigs.

Effect of exogenous band 3 protein on erythrocyte clearance. To lower the endogenous concentration of anti-band 3 antibodies in normal guinea pigs, we injected 200 \(\mu\)g highly purified human band 3 protein 4 hours before infusing labeled erythrocytes. This pretreatment lowered the endogenous anti-band 3 antibody concentrations by about 50% at 4 to 24 hours, as assessed by a radioimmunoassay (see Materials and Methods). Administration of band 3 protein slightly delayed the clearance of erythrocytes when incubated with control IgG\(^{-}\) (Fig 6A, B). This effect of band 3 protein was not only seen with mildly oxidized labeled cells, but also with erythrocytes that were heavily oxidized by exposure to diamide. The delay of clearance introduced by injecting band 3 protein was abolished by the presence of exogenous anti-band 3 antibodies, irrespective of whether erythrocytes were exposed to diamide or not (Fig 6C, D). These results suggest that exogenous band 3 protein modulated the endogenous anti-band 3 antibody concentration.

**DISCUSSION**

Recent in vitro studies from our laboratory and others indicate that human naturally occurring anti-band 3 autoantibodies are involved in the recognition, opsonization, and phagocytosis of altered and senescent erythrocytes.\(^{15,16}\) The herein described experiments with guinea pigs indicate that human naturally occurring anti-band 3 antibodies can accelerate the extravascular clearance of labeled and oxidatively stressed erythrocytes in the presence of functional complement in vivo. In addition, a delayed clearance of labeled and oxidatively stressed erythrocytes was observed by prior administration of band 3 protein and further supports a tissue homeostatic role of anti-band 3 antibodies in the clearance of altered and senescent erythrocytes.
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Fig 5. The effect of anti-band 3 antibodies on the mean survival of untreated and diamide-treated erythrocytes in normal and C3-deficient guinea pigs as in Fig 6. The clearance of erythrocytes was significantly (P < .05) accelerated by preincubation with anti-band 3 antibodies in normal guinea pigs (n = 10), but not in C3-deficient animals (n = 5), when areas under the curve were compared in a paired t test. (C) 20 μg/mL IgG-; (D) 20 μg/mL anti-band 3.

Purified human anti-band 3 autoantibodies bind specifically to guinea pig band 3 protein on intact erythrocytes and on blots from electrophoretically separated erythrocyte membrane proteins. Its binding affinity was less than that previously described for human band 3 protein, presumably because of species differences in the structure of band 3 protein. The degree of binding of human anti-band 3 antibodies to erythrocytes was dependent on the antibody concentration. Therefore, anti-band 3 antibodies likely bind to preexisting antigenic sites on erythrocytes rather than requiring chemical modification for binding. The plasma concentration of anti-band 3 antibodies in healthy humans and guinea pigs is more than two orders of magnitude below saturation for these antibodies, with a known association constant of 5 × 10^6 to 7 × 10^6 L/mol human band 3 protein; therefore, the autoantibody concentration appears to be limiting and thereby influences the clearance of erythrocytes. The estimated number of bound antibodies was 22 to 35 molecules per cell at 5 μg/mL anti-band 3 antibody concentration, which seems, in the presence of complement, sufficient to enhance the clearance of erythrocytes for an antibody that preferentially forms C3b-IgG complexes. A similar number of complement fixing sites per cell opsonized with an induced antibody resulted in a half-life of less than 10 minutes in guinea pigs.

Similar to studies with human erythrocytes, exposure to diamide, an oxidative agent, increased the binding of human anti-band 3 antibodies to erythrocytes from guinea pigs. There was not only binding to band 3 protein, but also to high-molecular-weight band 3 protein complexes that may represent band 3 protein oligomers and band 3 protein-hemoglobin adducts. Oligomerization favors bivalent binding of antibodies and thereby reduces its dissociation by a factor of 100 to 1,000. This diamide-induced increased binding of anti-band 3 antibodies likely contributed to the accelerated clearance of oxidatively damaged erythrocytes, and because membrane changes induced by oxidative agents are similar to alterations observed in senescent erythrocytes, these mechanisms may play an important role in the clearance of aged and altered erythrocytes. The diamide treatment and oxidative damage by the iodination procedure may have had an additive effect on the clearance of erythrocytes, and other erythrocyte alterations affecting the clearance rate cannot be excluded. The diamide treatment and/or labeling procedure may have rendered erythrocytes more sensitive to complement by depolymerizing them of glycosylphatidylinositol-anchored proteins, in the same manner as the decay accelerating factor (protects from C3b deposition) or the homologous restriction factor (prevents autologous cell lysis).

Although erythrocyte clearance has not specifically been studied in guinea pigs, studies in rats, rabbits, and hypertransfused mice indicate that the mechanism involved in recognition and clearance of senescent erythrocytes is similar in rodents and other mammals, including humans. We chose surface iodination for erythrocyte labeling because the two available isotopes, I^+ and I^3+, allowed us to simultaneously...
observe the clearance of untreated and diamide-exposed erythrocytes in the same animal. In addition, elution of chromium label from erythrocytes was known to be considerable in guinea pigs, but negligible with surface iodination as shown in this study. Irrespective of the iodine isotope, the apparent half-life of labeled (autologous or allogeneic) erythrocytes in guinea pigs was approximately 8 days, comparable with that reported for chromium-labeled cells, but about five times shorter than the expected half-life based on cohort labeling. The rapid clearance of iodinated erythrocytes may be caused by radical formation in the isotope solution, which mildly altered the cells. The alterations by labeling alone did not change the in vitro deformability of erythrocytes and did not generate SS-linked band 3 oligomers, but clearly induced cell water loss when labeled cells were further exposed to diamide, thus making these cells denser and less deformable.

When untreated or diamide-treated, labeled erythrocytes were incubated with anti-band 3 antibodies, their clearance was accelerated in normal guinea pigs compared with cells preincubated with anti-band 3-depleted IgG (IgG control). By comparing the effect of anti-band 3 antibodies with that of an identical amount of IgG depleted of anti-band 3 antibodies, the possibility of an accelerated clearance by guinea pig antibodies to human IgG molecules or an unspecific activation of the phagocyte system appears unlikely. In a recent study, up to 600 mg/kg/d of human IgG administered to guinea pigs neither mounted a severe immune complex disease nor a Forssmann-like shock syndrome, thereby ruling out preexisting strong anti-human IgG reactivity. Furthermore, because these experiments were of short duration (less than 5 days), induced antibodies against human IgGs could not have developed and interfered here. Although the effect of anti-band 3 antibodies was significant, the change in clearance of untreated and diamide-treated cells was small. This may in part be explained by (1) the lower binding of human anti-band 3 antibodies to band 3 protein of erythrocytes from guinea pigs compared with human beings, (2) the single administration of a near physiologic concentration of anti-band 3 antibody, and/or (3) the endogenous plasma anti-band 3 antibody content. In fact, prior administration of purified human band 3 protein in normal guinea pigs lowered the plasma concentration of anti-band 3 antibodies and thereby delayed the clearance of labeled erythrocytes. Although these data are the first evidence of an in vivo effect of anti-band 3 antibodies on the clearance by labeled, untreated and diamide-exposed oxidized erythrocytes in normal guinea pigs, future studies are needed to further elucidate the homeostatic role of anti-band 3 antibodies on the removal of senescent erythrocytes in health, as well as removal of altered cells in disease.

A major advantage of choosing guinea pigs was the availability of a C3-deficient strain, which allowed us to study the in vivo involvement of complement in the anti-band 3 antibody-mediated clearance of erythrocytes. Complement component 3-deficient guinea pigs have immunologically normal amounts of C3, but greater than 90% of it is functionally impaired by an inability of the nascent C3b to form covalent bonds. This dysfunction is apparently not caused by a mutation in the amino acid sequence of C3, but probably stems from a cotranslational or posttranslational defect in C3. These C3-deficient guinea pigs were stunted but not markedly susceptible to infection, and the mean survival of labeled and untreated or diamide-treated erythrocytes was similar in deficient and normal healthy guinea pigs.

Interestingly, in contrast to the accelerating effect of human anti-band 3 antibody on the clearance of erythrocytes in normal healthy guinea pigs, administration of these NOAs did not augment the erythrocyte clearance in C3-deficient guinea pigs in these short-term experiments. This finding suggests that the low affinity human anti-band 3 antibodies only gain in vivo functionality in the presence of active complement, as previously suggested by the enhanced in vitro phagocytosis of human erythrocytes exposed to complement and the ability of anti-band 3 antibodies to preferentially form C3b-IgG complexes, which are strong opsonins and activate alternative C3 convertase, thereby leading to further C3 deposition and enhanced phagocytosis. Thus, in C3-deficient guinea pigs, the concentration of active C3 was apparently too low to facilitate an anti-band 3 antibody-mediated clearance of erythrocytes.

The described in vivo activities of anti-band 3 NOAs suggest that the specificity of these antibodies, their preferred binding to band 3 protein clusters, and their unique ability to mobilize complement C3b deposition assure a highly controlled clearance of a specifically altered group of target erythrocytes. Although the selective clearance of altered and senescent erythrocytes may well represent their main function, these anti-band 3 antibodies may also regulate the clearance of other cells that express band 3 proteins. Other NOAs have also been implicated in the clearance of erythrocytes. In particular, naturally occurring antispectrin autoantibodies have been found to be associated with oxidatively stressed erythrocytes and erythrocytes from patients with hemoglobinopathies. However, human antispectrin antibodies appear to be functionally inefficient because they lack an affinity for C3 and, therefore, do not preferentially form C3b-IgG complexes in contrast with anti-band 3 antibodies. Furthermore, antispectrin antibodies belong to a group of cytophilic IgG molecules that associate unspecifically with erythrocytes, presumably via negatively charged sialic acid residues of glycoporphin rather than spectrin on the surface of erythrocyte membranes, thus making a key role of antispectrin antibodies in the clearance of erythrocytes unlikely.

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