Stimulation of Eryptosis by Anti-A IgG Antibodies

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Key Words

Cell volume • Phosphatidylserine • Apoptosis • Gardos channel • Calcium

Abstract

Anti-A IgG antibodies have previously been shown to stimulate Ca^{2+} entry into red blood cells. Increased cytosolic free Ca^{2+} concentration is known to trigger eryptosis, i.e. suicidal erythrocyte death, characterized by exposure of phosphatidylserine at the erythrocyte surface. As macrophages are equipped with phosphatidylserine receptors, they bind, engulf and degrade phosphatidylserine exposing cells. The present experiments have been performed to explore whether anti-A IgGs trigger phosphatidylserine exposure of erythrocytes. Phosphatidylserine exposure was estimated from annexin-V binding as determined in FACS analysis. Exposure to anti-A IgGs (0.5 $\mu\text{g}/\text{ml}$) indeed significantly increased annexin-V binding in erythrocytes with blood group A, but not in erythrocytes with blood group O. According to Fluo3 fluorescence, anti-A IgGs increased cytosolic Ca^{2+} concentration. Whole cell patch clamp recordings revealed

the activation of a Ca^{2+} -permeable cation channel following treatment with anti-A-IgGs. Annexin-V binding following anti-A IgG exposure was blunted by Ca^{2+} removal while anti-A IgG-stimulated cation channel activity was not dependent on extracellular Ca^{2+} . Osmotic shock (exposure of erythrocytes to 850 mOsm) increased annexin binding, an effect further enhanced by exposure to anti-A IgGs. In conclusion, anti-A IgGs activate erythrocyte cation channels leading to Ca^{2+} entry and subsequent erythrocyte cell membrane scrambling. The effect most likely contributes to the elimination of erythrocytes following an immune reaction against the A antigen.

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Introduction

An increase of erythrocyte cytosolic Ca^{2+} activity leads to cell membrane scrambling [1, 2], thus triggering exposure of phosphatidylserine at the cell surface [3-7]. Erythrocyte cytosolic Ca^{2+} activity may be increased by

activation of a Ca^{2+} -permeable cation conductance which is activated by osmotic shock, oxidative stress and energy depletion [8-11]. Cell membrane scrambling could further be elicited by ceramide, a proapoptotic lipid metabolite and signaling molecule which is formed following activation of a sphingomyelinase [12].

As macrophages are equipped with receptors specific for phosphatidylserine [13-15], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [16, 17] and are thus expected to be eliminated from circulating blood. Accordingly, the decreased life span of erythrocytes with sickle cell disease, thalassemia and glucose-phosphate dehydrogenase deficiency has been shown to be paralleled by accelerated phosphatidylserine exposure [18]. Moreover, phosphate depletion [19], iron deficiency [20], Hemolytic Uremic Syndrome [21], sepsis [22], malaria [4], Wilson disease [23], accumulation of methylglyoxal [24] or amyloid [25], treatment with paclitaxel [26], chlorpromazine [27], cyclosporine [28] or curcumin [29], as well as intoxication with lead [20] or mercury [30] lead to enhanced phosphatidylserine exposure and thus accelerated clearance of peripheral red blood cells.

Besides increase of cell membrane scrambling, Ca^{2+} activates the Ca^{2+} -sensitive K^+ channels [31, 32], which trigger cell shrinkage and contribute to the stimulation of phosphatidylserine scrambling [33, 34]. Cellular loss of K^+ has been demonstrated to parallel and to support apoptosis of a variety of nucleated cells [35-41].

Phosphatidylserine exposure and decrease of cell volume are hallmarks of apoptosis [6]. To reflect the similarities with and differences from apoptosis of nucleated cells, the term eryptosis has been coined [42]. Eryptosis displays similarities to but may be distinct from erythrocyte senescence or neocytolysis [42-46].

IgM and IgG antibodies reactive against the erythrocyte A or B antigen are present in the immunoglobulin fraction of normal human serum. The reactivity of autoantibodies detecting self A and B antigens is probably restricted by complementary interactions between variable regions of antibodies [47]. Exposure of erythrocytes to anti-A IgG antibodies in auto-immune reactions [48], or after ABO incompatible transplantation [48-58] may lead to haemolytic anemia and hemolysis, respectively. Anti-A IgG antibodies have previously been shown to enhance cytosolic Ca^{2+} activity [59]. Nothing is known about the mechanism and consequences of immune globulin-induced Ca^{2+} entry. Thus, the present experiments have been performed to explore, whether exposure to anti-A IgG could trigger eryptosis, and to unravel the role of

Ca^{2+} in this process. To this end, we treated purified blood group A erythrocytes with anti-A IgG antibodies and measured eryptosis, Ca^{2+} entry and channel activity by flow cytometry (annexin binding and Fluo3-dependent fluorescence) and patch clamp analysis.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers and erythrocyte concentrates were obtained as described recently using standard leukocyte depletion filters [60]. The study was approved by the local ethics committee of the University of Tübingen (project#: 184/2003V). Experiments were performed at 37°C in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 . For the nominally calcium-free solution, CaCl_2 was replaced by 1 mM ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Osmolarity was increased to 850 mOsm by adding sucrose to the NaCl Ringer solution. Anti-A IgGs were added to the NaCl Ringer at final concentrations of 0.5 $\mu\text{g}/\text{ml}$ from an anti-A IgG stock solution (0.5 mg/ml; BD Pharmingen, Heidelberg, Germany).

FACS analysis

FACS analysis was performed as described [61]. After incubation in the presence or absence of anti-A IgG antibodies, cells were washed in annexin-binding buffer containing (in mM): 125 NaCl, 10 HEPES/NaOH (pH 7.4), 5 CaCl_2 . Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics; Mannheim, Germany) and annexin-binding buffer (dilution of 1:50). After 10 min of incubation, samples were finally diluted 1:5 in annexin-binding buffer, cells were thoroughly suspended by the use of a vortex mixer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Annexin fluorescence intensity was measured in fluorescence channel 1 (FL-1) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For FL-1 analysis, single cells were gated using FSC versus SSC two-dimensional diagrams to exclude erythrocyte conglomerates.

Fluorescence microscopy

The effect of anti-A IgG antibodies on annexin binding was further studied using fluorescence microscopy [20]. To this end erythrocytes were seeded on poly-L-lysine-coated glass cover slides (0.01% poly-L-lysine solution from Sigma) and treated for 24 h in Ringer in the presence or absence of anti-A IgG. After 24 h at 37°C, the cells were washed twice with annexin-binding buffer, stained with annexin-V-Fluos in annexin-binding buffer (1:50 dilution), and washed again. Finally, the cells were analyzed under a fluorescence microscope (Nikon, Düsseldorf, Germany and AHF Analysentechnik,

Fig. 1. Stimulation of phosphatidylserine exposure at the erythrocyte surface by anti-A IgG antibodies. A. Histograms of annexin-V binding in a representative experiment of erythrocytes with blood group A (upper panels) or blood group 0 (lower panels) incubated for 24 h in Ringer solution (left panels), or in Ringer solution containing anti-A IgGs (0.5 μ g/ml) (right panels). The numbers depict the percentages of annexin-V-positive cells. B. Arithmetic means $\pm$ SEM (n=8) of annexin binding of blood group A (left) or 0 (right) erythrocytes after a 24 h treatment with Ringer solution in the absence (open bars) or in the presence (closed bars) of anti-A IgGs. * indicates significant difference ($p < 0.05$) from the respective control (in the absence of anti-A IgGs); ANOVA.

Tübingen, Germany) with 440/480 nm excitation and 535/550-nm emission wavelength. Digital pictures were taken using a digital imaging system (Visitron Systems, Puchheim, Germany) equipped with the MetaVue software.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} measurements after treatment of erythrocyte concentrates with anti-A IgG antibodies were performed as described [60]. Briefly, erythrocytes were loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) by addition of 2 μ l of a Fluo-3/AM stock solution (2 mM in DMSO) to 1 ml erythrocyte suspension (0.16% hematocrit in NaCl Ringer solution). The cells were incubated at 37°C for 15 min. An additional 2 μ l aliquot of Fluo-3/AM was added, with incubation carried out for 25 min. Fluo-3-loaded erythrocytes were centrifuged at 1000g for 5 min at 22°C and washed two times with NaCl Ringer solution containing 0.5% bovine serum albumin (Sigma, Taufkirchen, Germany) and one time with albumin-free Ringer. For flow cytometry, Fluo-3-loaded erythrocytes were re-suspended in 1 ml Ringer (0.16% hematocrit) in the presence or absence of anti-A IgGs, the Ca^{2+} ionophore ionomycin (1 μ M; Sigma) or vehicle alone and incubated for different time periods at 37°C. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1.

Patch clamp recordings

Patch pipettes made of borosilicate glass (150 TF-10, Clark Medical Instruments) were pulled using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 8 to 12 MOhm were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA) as already described [8]. For current measurements (room temperature), erythrocytes were held at a holding potential (V_h) of -20 mV and 400 ms pulses from -100 to +100 mV were applied in increments of +20 mV. The original whole-cell current traces are depicted without filtering (acquisition frequency of 5 kHz). The currents were analysed by averaging the current values measured between 350 and 375 ms of each square pulse (I-V relationship). The applied voltages refer to the cytoplasmic face of

the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solution, and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch [62]. Data were corrected for liquid junction potentials. Standard NaCl Ringer or nominally Ca^{2+} -free NaCl Ringer solution (in which 1 mM $CaCl_2$ was replaced by 0.5 mM EGTA) was used in the bath in combination with Na-D-glucuronate pipette solution (in mM: 125 Na-glucuronate, 10 NaCl, 1 $MgCl_2$, 1 $Mg-ATP$, 1 EGTA, 10 HEPES/NaOH, pH 7.4). Cells were pre-incubated in NaCl-Ringer or in Ca^{2+} -free NaCl Ringer solution for 1 h at room temperature with anti-A IgGs (0 and 0.5 μ g/ml, respectively). Whole-cell recordings were obtained thereafter during constant superfusion with IgG-free NaCl-Ringer or in Ca^{2+} -free NaCl Ringer solution.

Determination of ceramide formation

For determination of ceramide after anti-A IgG treatment, a biochemical method using diacylglycerol (DAG)-kinase and [^{32}P]gamma-ATP was used [12, 60]. After stimulation, the cells were extracted in chloroform:methanol:1N HCl (100:100:1), the lower phase was collected and dried. The samples were re-extracted, dried again, resuspended in 20 μ l of detergent solution containing 7.5% (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC) and sonicated for 10 min. The kinase reaction was initiated by addition of 70 μ l reaction mix consisting of 10 μ l DAG-kinase (GE Healthcare Europe GmbH; München, Germany) in 5 mM potassium phosphate buffer (10% glycerol, 1 mM 2-mercaptoethanol, 0.005 M imidazole/HCl, 0.5 mM DETAPAC

Fig. 2. Fluorescence microphotographs of phosphatidylserine exposure at the erythrocyte surface following exposure to anti-A IgG antibodies. Fluorescence images (right panels) and transmission light images (left panels) of blood group A (BGA, lower panels) or blood group 0 (BG0, upper panels) erythrocytes treated in Ringer solution containing anti-A IgGs (0.5 μ g/ml). Annexin binding was visualized under a fluorescence microscope (magnification 100 fold, bar size = 10 μ m).

(pH 6.6)), 50 μ l assay buffer (0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM $MgCl_2$ and 2 mM DTT, 2.8 mM DTT, 5 μ M ATP and 10 μ Ci [32 P]gammaATP (in 10 μ l of water). The kinase reaction was performed for 30 min at room temperature. The reaction was terminated by addition of 1ml of chloroform:methanol: 1 N HCl (100:100:1). 170 μ l of a buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES (pH 7.2)) and 30 μ l of a 100 mM EDTA-solution were added. The samples were extracted, the lower phases collected, dried, dissolved in 20 μ l of chloroform:methanol (1:1) and separated on a Silica G60 TLC plate with chloroform: methanol:acetic acid (65:15:5). The plate was exposed and ceramide-phosphate identified by co-migration with an identical standard. [32 P]-ceramide-phosphate was scraped from the plate and quantified by liquid scintillation counting. The amount of ceramide was determined by comparison with a ceramide standard curve using C_{16} -ceramide (Axxora Deutschland GmbH; Grünberg, Germany).

Statistics

Data are expressed as arithmetic means $\pm$ SEM. Statistical analysis was made by paired or unpaired *t*-test, or analysis of variance (ANOVA) as appropriate. $p < 0.05$ was considered as statistically significant.

Results

Incubation of erythrocyte concentrates in Ringer solution for 24 h resulted in low but appreciable phosphatidylserine exposure with subsequent annexin

binding in $1.3 \pm 0.3\%$ ($n = 10$) of the cells. Addition of anti-A IgG antibodies (anti-A IgG, 0.5 μ g/l) to the Ringer solution significantly increased the percentage of annexin binding erythrocytes of blood group A (Fig. 1A, upper right histogram, and Fig. 1B, left columns). In these experiments, the exposure to anti-A IgG for 24 h increased the percentage of annexin binding cells to $8.4 \pm 1.1\%$ ($n = 8$). In contrast, exposure of erythrocytes of blood group 0 to anti-A IgG was not followed by phosphatidylserine exposure (Fig. 1A, lower right histogram, and Fig. 1B, right columns). Thus, the proeryptotic effect of anti-A IgGs is blood group specific and depends on the presence of the A-antigen (Gal, Fuc, GalNAc) on the surface of the erythrocytes. In the experiments shown in Fig. 1A, only gated erythrocyte populations were analysed. The respective gates of the FSC versus SSC two-dimensional flow cytometry diagrams were set in a way to exclude all cellular aggregates (not shown). By this procedure, it was ruled out that the increase of annexin-V-dependent fluorescence of anti-A IgG-treated erythrocytes was simply a consequence of cell aggregation.

The anti-A-IgG-stimulated phosphatidylserine exposure of the erythrocytes was confirmed by fluorescence microscopy. Again, anti-A IgGs stimulated phosphatidylserine exposure of erythrocytes with blood group A (Fig. 2, lower right) but not with blood group 0 (Fig. 2, upper right). In this experimental setup, a weak aggregation of the anti-A IgG-treated blood group A erythrocytes was observed (Fig. 2, lower left), which was ab-

Fig. 3. Non-selective cation conductance activated by anti-A IgG antibodies. **A.** Whole cell macroscopic current traces recorded successively with Ca^{2+} -containing NaCl Ringer solution in the bath from blood group A (left) and blood group 0 (right) erythrocytes pre-incubated 1 h with 0.5 $\mu\text{g}/\text{ml}$ anti-A-IgG. The pipette contained a Na-gluconate solution. The dotted line indicates the zero current value. Membrane voltage was held at -20 mV. **B.** Mean current-voltage relationships ($\pm$ SEM) obtained from blood group A (left) and blood group 0 (right) erythrocytes under control conditions (closed triangles, $n = 6$ for blood group A and $n = 4$ for blood group 0) and after 1 h incubation with anti-A-IgGs (open squares, $n = 8$ for blood group A and $n = 5$ for blood group 0) measured between 350 and 375 ms after the onset pulse recorded as in (A) with Na-gluconate pipette solution. **C.** Mean whole-cell conductance ($\pm$ SEM) obtained from the blood group A (left, right) and blood group 0 (middle) erythrocytes under control conditions (closed bars, $n = 6$ for blood group A and $n = 4$ for blood group 0) and after 1 h incubation with anti-A-IgGs (open bars, $n = 5$ –8 for blood group A and $n = 5$ for blood group 0). Cells were either pre-incubated and measured in Ca^{2+} -containing NaCl bath solution (left and middle) or in nominally Ca^{2+} -free NaCl Ringer solution (right). The conductances were calculated from (B) by linear regression. ** indicates significant difference, $p < 0.01$; unpaired two-tailed t-test.

sent in erythrocytes with blood group 0 (Fig. 2, upper left).

As illustrated in Fig. 3A–C, the exposure of erythrocytes with blood group A to anti-A-IgG increased the activity of a Ca^{2+} -permeable cation conductance. After 1 h of incubation with anti-A-IgG antibodies, the overall conductance increased significantly about 2.6-fold as compared with control conditions (calculated between -100 and $+100$ mV, Fig. 3C left). In contrast, activation of cation channels was not observed in erythrocytes from blood group 0 (Fig. 3A–C, middle). To test the possibility that the anti-A-IgG-stimulated activation of the cation conductance is secondary to an increase in cytosolic free Ca^{2+} concentration, anti-A IgG incubation and whole cell recording was performed in Ca^{2+} -free Ringer solution. As depicted in Fig. 3C, right, anti-A IgGs stimulated the cation conductance also in the nominal absence of extracellular Ca^{2+} . This strongly suggests that activation of the cation conductance was not due to an increase in cytosolic free Ca^{2+} concentration.

To test whether the stimulation of non-selective cation channels by anti-A IgGs increased Ca^{2+} entry, intracellular free Ca^{2+} concentration was determined by Fluo3-dependent fluorescence. To this end, erythrocytes were loaded with the Ca^{2+} -sensitive fluorescence dye Fluo-3. Fluo-3-dependent fluorescence revealed an increase of the percentage of cells with enhanced cytosolic free Ca^{2+} concentration following exposure to anti-A IgGs (Fig. 4A, right histogram, and Fig. 4B, closed column). Thus, the stimulation of cation channel activity indeed led to an increase of cytosolic Ca^{2+} concentration, which could in turn account for the stimulation of phosphatidylserine exposure.

To determine the contribution of increased intracellular Ca^{2+} concentration to the anti-A IgG-induced eryptosis, additional experiments were performed in the nominal absence of Ca^{2+} . To this end, Ca^{2+} was removed from the incubation medium and replaced by EGTA. As shown in Fig. 5A–B, calcium removal significantly blunted the effect of anti-A IgGs on phosphatidylserine exposure.

Fig. 4. Increase of cytosolic Ca^{2+} activity in erythrocytes following exposure to anti-A IgG antibodies. A. Histograms of Fluo3-dependent fluorescence in a representative experiment of blood group A erythrocytes exposed for 6 h to Ringer in the absence (left histogram) or in the presence (right histogram) of 0.5 $\mu\text{g}/\text{ml}$ anti-A IgGs. The numbers depict the percentages of Fluo3-positive cells. B. Arithmetic means $\pm$ SEM ($n=7$) of Fluo3-positive cells in blood group A erythrocytes exposed for 6 h to Ringer in the absence (control, open column) or in the presence (antibody, closed column) of 0.5 $\mu\text{g}/\text{ml}$ anti-A IgGs. * indicates significant difference between presence and absence of anti-A IgGs (unpaired t-test $P<0.05$).

In this experimental series, the effect of anti-A IgG decreased from $24.8 \pm 1.6\%$ ($n=14$) to $13.7 \pm 1.2\%$ ($n=15$) (see Fig. 5B left, closed column and Fig. 5B right, closed column). Thus, anti-A IgG-induced increase of intracellular Ca^{2+} activity significantly contributes to the anti-A IgG-induced phosphatidylserine exposure.

In previous studies, it was shown that phosphatidylserine exposure following cellular stress is in part the result of platelet activating factor (PAF) release with subsequent activation of an erythrocytic

Fig. 5. Stimulation of phosphatidylserine exposure at the erythrocyte surface by anti-A IgG antibodies is blunted by removal of extracellular Ca^{2+} . A. Histograms of annexin-V binding in a representative experiment of erythrocytes in Ringer (upper histograms) or calcium-free solution (lower histograms) incubated for 24 h in absence (left histograms) or presence (right histograms) of anti-A IgGs (0.5 $\mu\text{g}/\text{ml}$). The numbers depict the percentages of annexin-V-positive cells. B. Arithmetic means $\pm$ SEM ($n=14-16$) of annexin-V binding in Ringer solution (Ringer, left) or calcium-free solution (0 Ca, right) for 24 h in the absence (control, open columns) or presence (antibody, closed columns) of anti-A IgGs. * indicates significant difference (ANOVA $P<0.05$) from control (Ringer in the absence of anti-A IgGs), # indicates significant difference (ANOVA $P<0.05$) from Ca^{2+} -containing Ringer solution in the presence of anti-A IgGs.

sphingomyelinase and accumulation of the pro-eryptotic lipid second messenger ceramide [12, 60]. We therefore determined the formation of ceramide in erythrocytes exposed to anti-A-IgG utilizing a biochemical method based on diacylglycerol kinase (for the ceramide standard curve see Fig. 6A). However, anti-A-IgGs did not enhance the formation of ceramide (Fig. 6B).

Further experiments were performed to explore whether anti-A IgG antibodies modified the stimulation of phosphatidylserine exposure following other cellular

Fig. 6. Lack of ceramide formation in erythrocytes following exposure to anti-A IgG antibodies. A. Calibration curve of C_{16} -ceramide using the DAG-kinase assay. The radioactivity scraped from the plate (cpm) is plotted as a function of the amount of C_{16} -ceramide (nmol). B. Arithmetic means $\pm$ SEM ($n = 5-6$) of ceramide formation in % of control in erythrocytes incubated for 24 h in control Ringer (open column) or in Ringer solution containing 0.5 μ g/ml anti-A IgGs (closed column). The ceramide level of Ringer-treated erythrocytes was 114 ± 18 pmol/ 10^6 cells ($n=6$).

Fig. 7. Stimulation of phosphatidylserine exposure at the erythrocyte surface by hyperosmotic shock in the presence or absence of anti-A IgGs. A. Histograms of annexin-V binding in a representative experiment of erythrocytes in isotonic Ringer (upper panels) or 850 mOsm (lower panels) incubated for 24h in absence (left panels), or presence (right panels) of anti-A IgGs (0.5 μ g/ml). The numbers depict the percentages of annexin-V-positive cells. B. Arithmetic means $\pm$ SEM ($n=4$) of annexin-V binding in isotonic (300 mOsm, left) or hypertonic (850 mOsm, right) solution for 24 h in the absence (control, open columns) or presence (antibody, closed columns) of anti-A IgGs. * indicates significant difference (ANOVA $P < 0.05$) from control (absence of anti-A IgGs), # indicates significant difference (ANOVA $P < 0.05$) from 850 mOsm in the absence of anti-A IgGs.

stressors, i.e. after hyperosmotic shock. As illustrated in Fig. 7A and B, an increase of extracellular osmolarity to 850 mOsm induced erythrocyte phosphatidylserine exposure, an effect that was significantly enhanced in the presence of anti-A IgG antibodies. Thus, erythrocytes which have been additionally challenged with anti-A antibodies are more sensitive towards cellular stress.

Discussion

The present experiments disclose a novel action of anti-A IgG antibodies on erythrocytes. The antibody leads to erythrocyte cell membrane scrambling with phosphatidylserine exposure at the cell surface. The effect of anti-A IgGs was paralleled by activation of cation

channels and Ca^{2+} entry as demonstrated by patch clamp analysis (see Fig. 3) and flow cytometry (Fig. 4). The increases of cation channel activity and of intracellular Ca^{2+} concentration clearly preceded the appearance of phosphatidylserine exposure, which was observed after 24 h. Apparently, a sustained rise in intracellular Ca^{2+} concentration is required for sizable phosphatidylserine exposure. Presumably, the breakdown of phosphatidylserine asymmetry following Ca^{2+} -stimulated lipid scrambling is counteracted by ATP-consuming aminophospholipid translocase activity. The increased Ca^{2+} -ATPase, Na^+/K^+ -ATPase and translocase activities are expected to eventually result in cellular ATP depletion with subsequent impairment of translocase activity.

The present results are in line with a former study, which demonstrated that a monoclonal anti-A1 antibody accelerated the influx of radioactive Ca^{2+} into A1 erythrocytes [59]. However, the consequences of increased cytosolic Ca^{2+} concentration for erythrocyte physiology and regulation of the erythrocyte death program remained elusive. Here we provide a link between enhanced Ca^{2+} entrance (as evidenced by patch clamp and flow cytometry) and erythrocyte cell death (as evidenced from phosphatidylserine exposure).

Ca^{2+} entry and eryptosis are subject to regulation by several hormones and ligands. Erythrocyte Ca^{2+} uptake may be triggered by stimulation of specific epidermal growth factor (EGF) binding sites [63]. Furthermore, triggering of CD47 [64], glycophorin-C [65] and Fas [66] have been shown to induce erythrocyte phosphatidylserine exposure and concomitant loss of cell viability. On the other hand, Ca^{2+} entry and eryptosis are blunted by erythropoietin [67], which thus extends the life span of circulating erythrocytes.

Treatment with anti-A IgGs apparently did not lead to stimulation of ceramide formation, another signaling mechanism in the triggering of eryptosis [12, 60]. The stimulation of phosphatidylserine exposure may, however, not be entirely due to an increase in intracellular Ca^{2+} concentration. In the present study, anti-A IgG-stimulated phosphatidylserine exposure was blunted but not abrogated in nominally Ca^{2+} -free solutions (see Fig. 5). It should be kept in mind, though, that for determination of annexin binding Ca^{2+} had to be added shortly before FACS analysis. Nevertheless, further cellular mechanisms may contribute to the triggering of phosphatidylserine exposure. Sustained cation channel activity might lead to break down of the Na^+ and K^+ gradients across the erythrocyte membrane, to a loss of cellular K^+ , to enhanced Na^+/K^+ -ATPase activity and thus to ATP depletion. ATP depletion

in turn dis-inhibits glycolysis, which may result in a decreased hexose-monophosphate shunt-dependent glutathione (GSH) replenishment and subsequently to erythrocyte oxidation. Oxidation, ATP depletion and loss of intracellular K^+ may contribute to Ca^{2+} -independent eryptosis [11, 18].

Consequences of eryptosis include the clearance of affected erythrocytes from circulating blood, as macrophages equipped with receptors specific for phosphatidylserine [13–15] rapidly recognize, engulf and degrade erythrocytes exposing phosphatidylserine at their surface [16, 17]. Moreover, phosphatidylserine exposure may foster adhesion of erythrocytes to endothelial cells thus leading to impairment of microcirculation [17, 68].

On the other hand, eryptosis may serve an important physiological function, i.e. the removal of injured erythrocytes prior to hemolysis. Energy depletion, defective Na^+/K^+ -ATPase and enhanced leakiness of the cell membrane are followed by cellular gain of Na^+ and Cl^- and osmotically obliged water with subsequent cell swelling [69]. The swelling eventually results in hemolysis with release of cellular hemoglobin into the circulating blood. The hemoglobin could be filtered at the renal glomerula and subsequently occlude the renal tubules. Thus, the removal of injured erythrocytes by eryptosis is an important mechanism preventing, or at least decreasing, intravascular hemolysis. Accordingly, eryptosis may blunt the hemolysis but add to the anemia following a reaction against erythrocyte antigens.

In conclusion, exposure to anti-A IgGs activates cation channels leading to Ca^{2+} entry and subsequent phosphatidylserine exposure at the surface of the cell membrane. The affected erythrocytes are prone to be cleared from the blood stream, which presumably participates in the decrease of erythrocyte life span and development of anemia following exposure to anti-A IgGs.

Acknowledgements

The authors acknowledge the technical assistance of E. Faber and the meticulous preparation of the manuscript by Natalie Eckberger and Lejla Subasic. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/13-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research) 01 KS 9602 and the Biomed program of the EU (BMH4-CT96-0602). E.S. has been supported by a grant of the Alexander von Humboldt Foundation.

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