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Original Paper

Sublytic Terminal Complement Components Induce Eryptosis in Autoimmune Haemolytic Anaemia Related to IgM Autoantibodies

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Key Words
Phosphatidylserine • C5b6 • C5b-7 • C5b-8 • Annexin • Haemolysis • IgM autoantibodies

Abstract
Background/Aims: Eryptosis, the suicidal death of red blood cells (RBCs), is characterized by phosphatidylserine (PS) exposure at the cell surface. It can be catalysed by a variety of abnormal conditions and diseases. Until now, the many questions surrounding the physiology and pathophysiology of eryptosis have not been sufficiently answered. Recently, we demonstrated IgM and IgA autoantibodies (aab) to induce PS exposure on circulating RBCs of patients with autoimmune haemolytic anaemia (AIHA). However, it remained unclear how these aab lead to eryptosis. ***Methods:*** Serum and plasma samples from patients with clinically relevant AIHA of cold type were used to induce eryptosis in O RBCs. Serum containing fresh complement from healthy donors, antibodies to complement component, and complement factor depleted sera were added to examine the influence of the complement on PS-exposure. RBC bound annexin V PE were analysed by flow cytometry. ***Results:*** Eryptosis related to IgM aab was found to be dependent on complement activation and could be effectively inhibited by EDTA, serum heat inactivation and anti-C5. PS exposure increased with sequential activation of the sublytic terminal complement components C5b6, C5b-7 and was most significant at the C5b-8 stage. A decrease was observed following the formation of the lytic membrane attack complex C5b-9, either because of lysis of eryptotic RBCs or because of inhibition of eryptosis by C9. ***Conclusion:*** Our findings reflect new aspects on RBC destruction in AIHA as well the impact of the terminal complement complexes on the RBC membrane. The striking differences to nucleated cell apoptosis may even have physiological meaning of RBC acting as a buffer of the complement system.

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Introduction

It has been generally accepted for long time that red blood cells (RBCs) in patients with autoimmune haemolytic anaemia (AIHA) are destroyed by phagocytosis due to their coating with IgG autoantibodies (aab) and / or C3b complement component or by cell lysis due to IgM-mediated activation of the terminal complement components C5b-9 (membrane attack complexes), which form pores on cell membranes [1-3]. A single pore in the RBC membrane has been demonstrated to lyse the affected cell [4]. IgA aab alone may also cause *in vivo* haemolysis [5, 6]. It remains speculative how IgA aab lead to RBC destruction in patients with AIHA [3].

We recently demonstrated for the first time that eryptosis, the suicidal death of RBCs resembling the apoptosis of nucleated cells [7, 8], is also involved in AIHA related to IgA and IgM but not IgG aab [9]. Eryptosis has also been described to occur in oldest erythrocytes triggered by oxidative stress [10], and in tumor suppressor protein p53 deficiency [11]. Also it has been related to patients with a variety of diseases, including metabolic syndrome, diabetes, malignancy, hepatic failure, heart failure, renal failure, sepsis, malaria, mycoplasma infection, iron deficiency, sickle cell anaemia, spherocytosis, thalassaemia, glucose-6-phosphate dehydrogenase deficiency, and Wilson's disease [7, 12, 13]. Upon Ca^{2+} influx, RBCs become eryptotic. This process is characterized by cell shrinkage and cell membrane scrambling, leading to the breakdown of the cell membrane's phospholipid asymmetry and exposition of phospholipid phosphatidylserine (PS) from the inner to the exterior leaflet [14-18]. These cells are usually recognized by macrophages and rapidly removed from the circulation [12].

The mechanisms by which IgM and IgA aab cause eryptosis remain unclear. The present study investigated the potential role of complement activation in these processes. The results provide insight into various aspects related to apoptosis, complement activation, and the interaction of lytic and sublytic terminal complement components with the RBC membrane.

Materials and Methods

Samples and reagents

Serum and EDTA samples were from two patients with clinically relevant AIHA of cold type [9] and from one patient who had severe AIHA due to IgA aab [19]. Fresh RBCs and serum from healthy blood donors were used as controls and source of complement, respectively. Anti-C8b and anti-C9 polyclonal antibodies were from Thermo Fisher Scientific, Rockford, Il, USA, and anti-C5 (Eculizumab, Soliris®) from Alexion Europe (Rueil-Malmaison, France). C5-, C6-, C7- and C8-deficient serum and complement C5 were obtained from Sigma-Aldrich (Saint Louis, MO, USA) and C9-depleted serum from EMD Millipore (Merck KgaA, Darmstadt, Germany).

In vitro eryptosis experiments

RBCs from EDTA samples of healthy blood donors were washed two times in NaCl and once in HEPES-buffered Ringer's solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 $CaCl_2$ (p*H* 7.4). In general, ten µl of packed RBCs were used and 100 µl patient serum and where necessary 10 µl complement antibodies added as described. The mixture was incubated at 4 °C for 15 min, afterwards at 37 °C for 2 min, and then stored at 4 °C for 24–48 h. In the two-step experiments, 10 µl of RBCs were incubated with 50 µl heat-inactivated patient serum at 4 °C for 15 min, washed in 4 °C HEPES-buffered Ringer's solution, centrifuged, followed by the addition of 100 µl AB serum or complement factor-depleted sera. The mixture was incubated at 37 °C for 2 min and stored at 4 °C for 24–48 h. RBCs were handled with care and not pipetted vigorously to avoid physical damage. After incubation, RBCs were pre-warmed and washed three times at 37 °C to remove IgM aab. RBCs were diluted in 400 µl of HEPES-buffered Ringer's solution. Experiments were performed at least in triplicate.

Flow cytometry

Eryptosis was measured as previously described [9]. Annexin binding to extracellular exposed PS was used as a marker of eryptosis. A positive control was generated by incubating 5 µl of RBCs in 200 µl of a HEPES-buffered Ringer's solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 $CaCl_2$ (p*H* 7.4) with the Ca^{2+} ionophore ionomycin (1 µM) as described elsewhere [20].

Five µl of RBCs were diluted in 200 µl HEPES-buffered Ringer's solution. Therefrom five µl of RBCs were added to 5 µl of PE (phycoerythrin) annexin V in 45 µl of annexin V binding buffer (BD Biosciences, Heidelberg, Germany). After incubation at room temperature for 15 min, the mixture was diluted with 450 µl of annexin V binding buffer and analysed by flow cytometry (MACSQuant® Flow Cytometer, Miltenyi Biotech, Germany). At least 20 000 events were collected for each sample. Data were analysed using the FlowJo® software (FlowJo, LLC; USA) and the percentage of PE annexin V positive cells compared to the negative controls was calculated.

Statistics

Data are expressed as arithmetic means ± standard error of the mean (SEM) and statistical analysis was performed using IBM SSPS statistics software v24. Significance between two groups was determined using student's T test with probabilities of $P < 0.05$ considered statistically significant.

Results

IgM aab induce eryptosis in patients with autoimmune haemolytic anaemia

In our previous study, we could show that RBCs from patients with clinically relevant AIHA of cold type had significant increase of PS exposure [9]. Similarly, in the present study fresh serum samples from these patients induced significant PS exposure on O RBCs of healthy blood donors (Fig. 1A–E). The supernatant of the mixtures of RBCs with patient serum samples was haemolytic after incubation, indicating mild RBC lysis.

Effect of EDTA and serum heat-inactivation on eryptosis

In contrast, patient plasma as well as patient serum pre-treated with EDTA failed to provoke significant externalization of PS in RBCs (Fig. 1F–I, C). This can be explained by the fact that EDTA sequesters Ca^{2+} by forming a metal complex, thereby inhibiting Ca^{2+} influx into the cells as a trigger of eryptosis. However, the capability of patients' sera to induce eryptosis was also abolished after 2 days (Fig. 2A) but restored in the presence of fresh donor serum (Fig. 2B). Furthermore, heat-inactivation of fresh patient serum before its addition to RBCs did not lead to increased levels of exposed PS (Fig. 2C). The latter two findings indicate a further trigger of eryptosis in AIHA patients apart from Ca^{2+}.

IgM aab and fresh serum proteins are necessary for inducing eryptosis

To further subdivide the components necessary for inducing eryptosis in donor RBCs, heat-inactivated (i.a.) serum from a patient with IgM cold aab and fresh donor serum were added separately in a two-step experiment (Fig. 2D). As a result, RBCs were coated with IgM cold aab before adding fresh donor serum. The percentage of PS+ RBC was similar to that of the one-step addition of patient serum and fresh AB serum sera (Fig. 2E). The control, addition of i.a. patient serum and i.a. donor serum in two-steps showed no eryptosis (Fig. 2F). Taken together, the results indicate that fresh serum is necessary to induce significant eryptosis in patients with IgM aab (Fig. 2G).

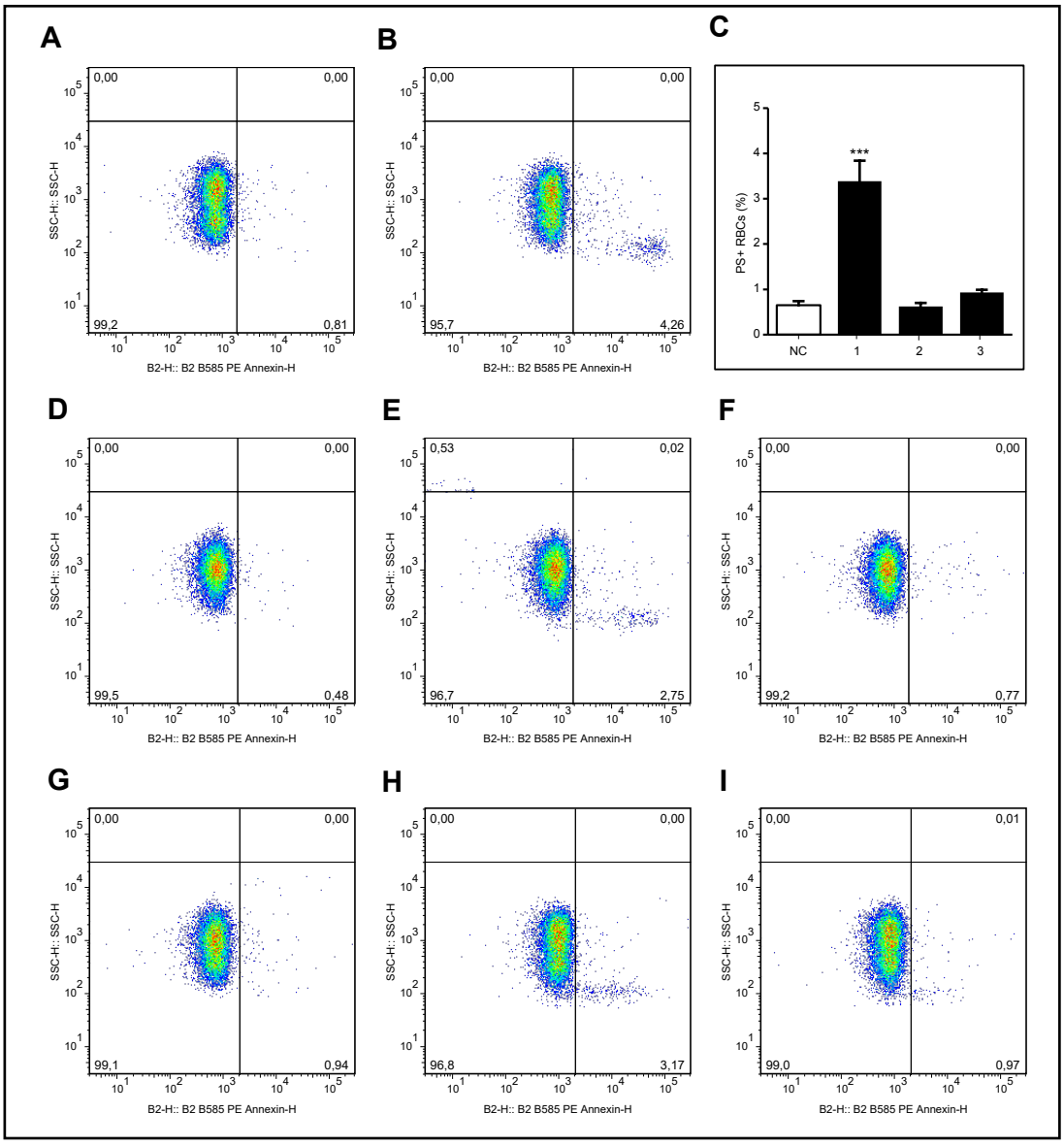

Fig. 1. Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs after incubation with serum/plasma from patients with cold AIHA. Compared to negative controls (A, D, G, and C bar "NC"), phosphatidylserin (PS) exposure was significantly ($p < 0.001$) increased in the presence of fresh serum (containing complement) from patients with cold AIHA (B, E, H, and C bar "1"). Incubation with patient EDTA plasma (F, and C bar "2") or addition of EDTA to patient serum (I, C bar "3") did not result in an increase of PS exposure. (***) $p < 0.001$.

IgA-induced eryptosis is independent of fresh serum

In a complementary experiment, serum and plasma from a patient with strongly agglutinating fatal IgA aab [19] were incubated with donor O RBCs. Although strong eryptosis was induced, the strength of PS exposure was not related to fresh serum but was equal after incubation with plasma or heat-inactivated serum (data not shown).

Inhibition of IgM-related eryptosis by complement C5 antibody and C5-depleted serum

Our results indicate that Ca^{2+}, IgM cold aab, and a number of heat-sensitive compounds in fresh serum are necessary to induce eryptosis in IgM AIHA patients. Since IgM cold aab were recently shown to activate complement even at core temperatures [21], we hypothesized

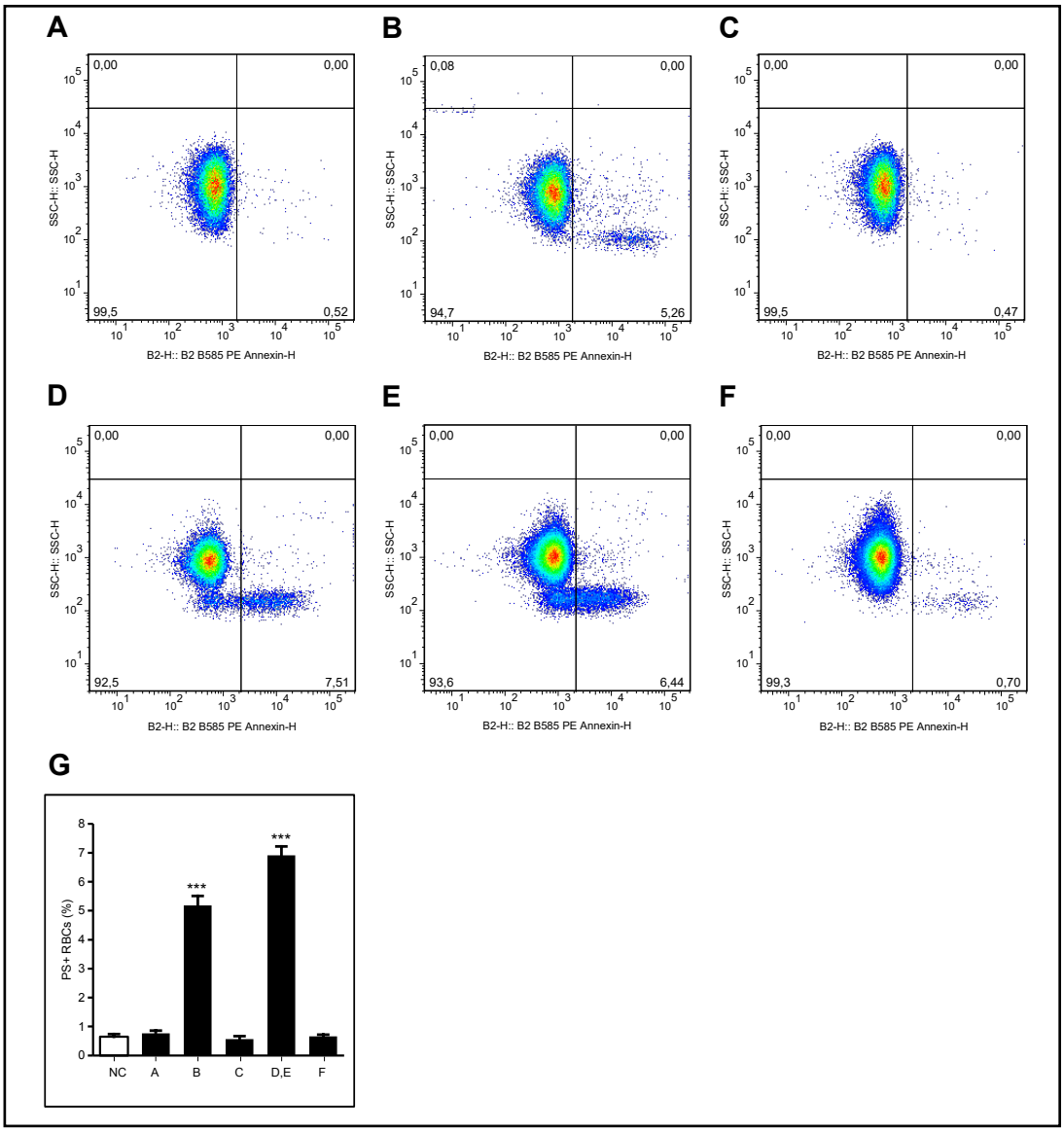

Fig. 2. Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and its dependence on serum complement activity. O RBCs incubated with serum (stored at 4° C for 48 h) from a patient with cold AIHA did not show increased PS exposure (A). Whereas supplementary addition of fresh AB serum as a source of complement induced significantly ($p<0.001$) PS exposure (B, G), no effect could be seen after addition of heat-inactivated (i.a.) AB serum (C, G). Similarly, i.a. serum from a patient with cold AIHA did induce significantly ($p<0.001$) PS exposure in O RBCs after subsequent addition of fresh AB serum (D, G) or simultaneous addition of fresh AB serum (E, G), but not after subsequent addition of i.a. AB serum (F). (***) $p<0.001$.

that the complement system is most likely involved in eryptosis. To test this hypothesis, fresh donor serum (Fig. 3A, negative control), a mixture of patient serum and fresh donor serum (Fig. 3B), or patient serum and fresh donor serum incubated prior to its addition to RBCs with anti-C5 antibody (Eculizumab, Soliris®) was added to RBCs (Fig. 3C). Whereas patient serum and fresh donor serum together induced strong eryptosis, PS externalization was almost completely prevented by pre-incubation with anti-C5 (Fig. 3G). Next, we used C5-depleted serum for independent evidence of the involvement of complement factor C5 in IgM aab-induced eryptosis. As expected, incubation with patient serum and C5-depleted

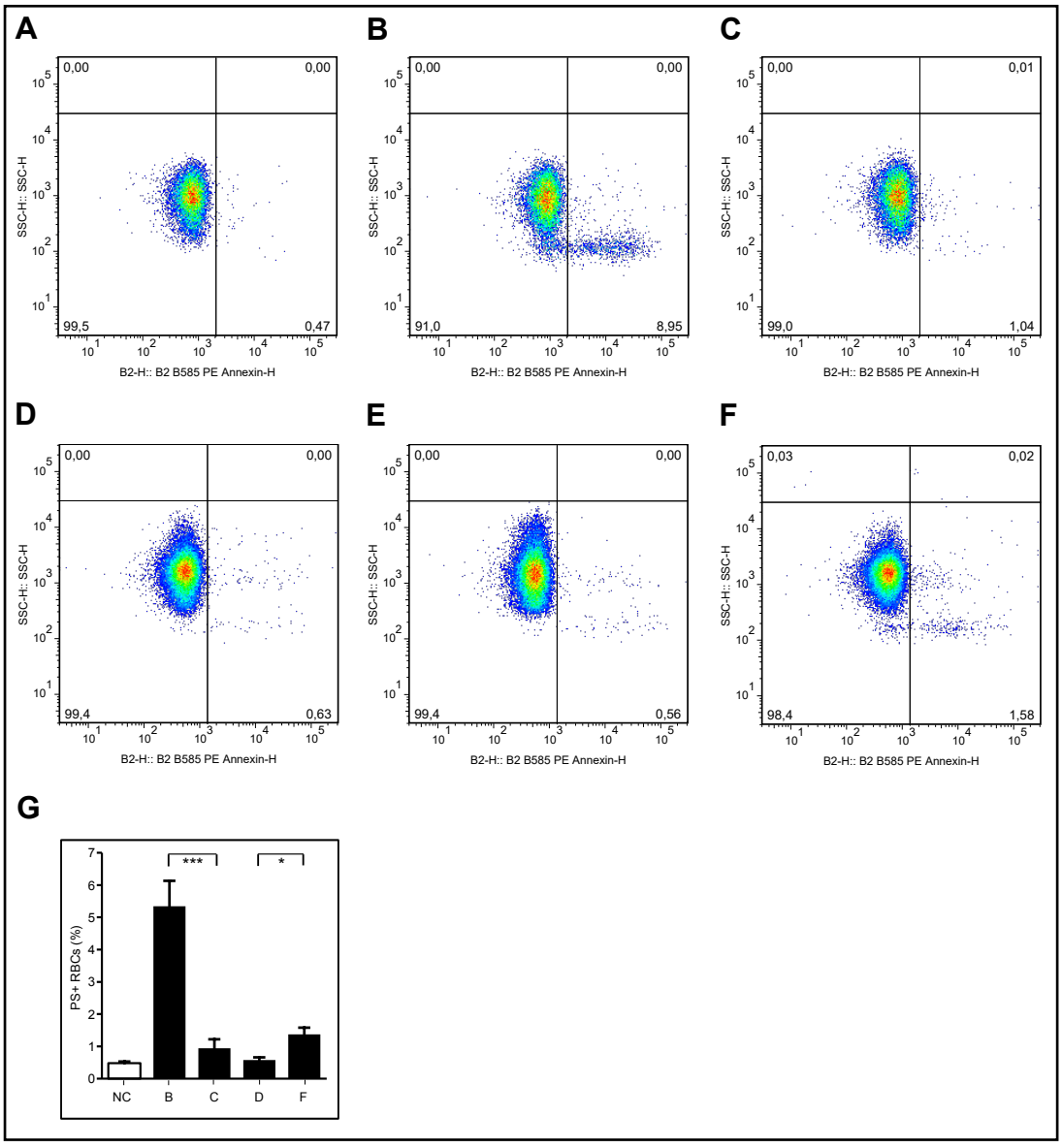

Fig. 3. Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and effect of C5 complement component. Compared to negative control (A), phosphatidylserin (PS) exposure was significantly ($p<0.001$) increased after incubation with fresh serum from patients with cold AIHA (B, G). Pre-treatment of patient serum with anti-C5 (Eculizumab, Soliris®) significantly ($p<0.001$) reduced PS exposure (C, G). Heat-inactivated patient serum and C5-depleted serum were incapable of inducing PS exposure (D) as compared to negative control (E, G) but supplementation with the missing C5 complement component resulted in a significant ($p<0.05$) increase of PS exposure (F, G). (***) $p<0.001$, (*) $p<0.05$ between the bracketed bars.

serum (Fig. 3D) did not result in PS externalization above the negative control level (Fig. 3E). However, the addition of C5 to this mixture restored significantly the eryptosis-inducing capacity (Fig. 3F, 3G), providing strong evidence of the specific role of the complement system in the eryptosis of RBCs from patients with IgM AIHA.

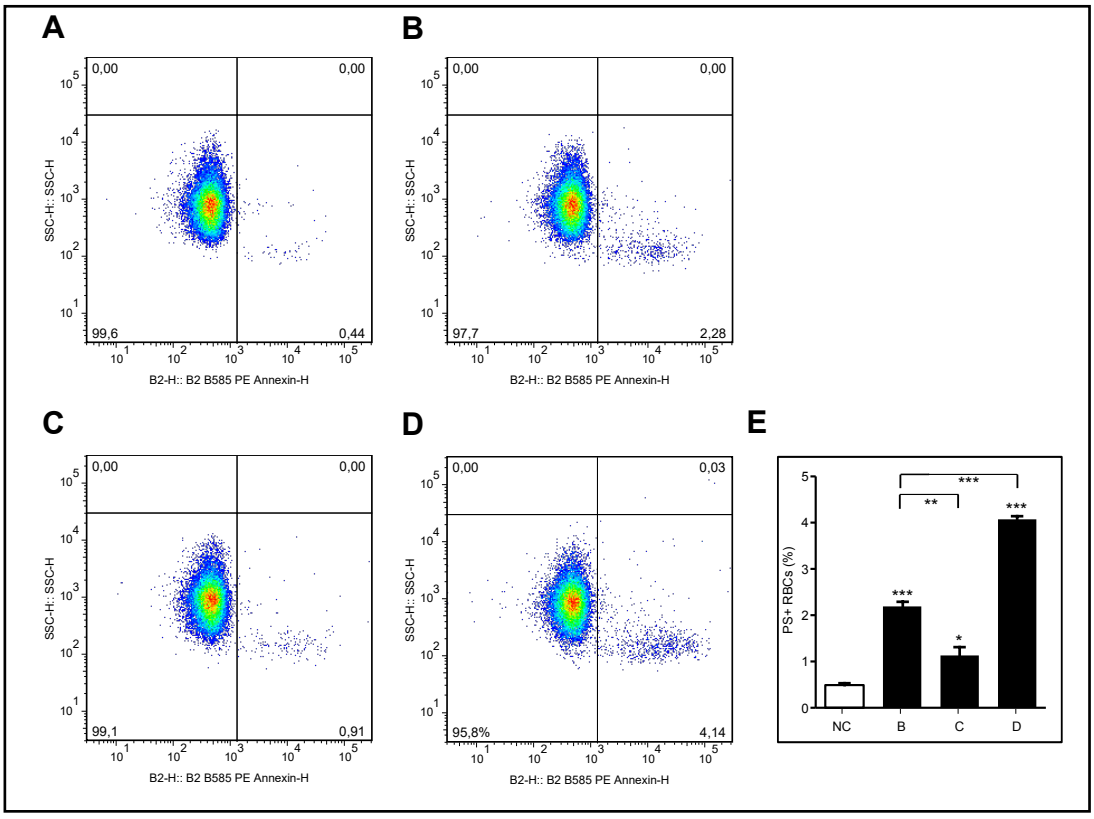

Fig. 4. Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and effect of anti-C8 and anti-C9. Compared to negative control (A), phosphatidylserin (PS) exposure was significantly ($p<0.001$) increased after incubation with fresh serum from patients with cold AIHA (B). Pre-treatment of patient serum with anti-C8 significantly ($p<0.01$) reduced PS exposure (C) but RBCs still had a significantly ($p<0.05$) higher level of eryptotic cells compared to negative control. Pre-treatment with anti-C9 further significantly ($p<0.001$) increased PS exposure (D) compared to untreated patient serum. (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ compared to negative control or between the bracketed bars, respectively.

Effect of anti-C8 and anti-C9 on eryptosis

To further elucidate the role of complement in eryptosis, particularly regarding the potential involvement of the MAC, anti-C8 and anti-C9 antibodies were used in an analogous experiment (Fig. 4A–E). The addition of anti-C8 inhibited significantly the formation of eryptotic cells; however, incompletely (Fig. 4C, 4E). In contrast, the addition of anti-C9 resulted in a significant increase of eryptotic cells (Fig. 4D, 4E). One potential explanation for this unexpected result lies in the presumption that C9 may not be required for inducing eryptosis. However, since C9 is responsible for forming the lytic pore in the final step of the MAC, part of the eryptotic cells may be haemolysed completely in presence of C9 and escape detection with Annexin-PE. Therefore, C9 inhibition by anti-C9 may prevent eryptotic cells from lysis *in vitro*. Altogether, our results indicate that C5 is necessary for inducing eryptosis in IgM AIHA and C8 may support the formation of eryptotic cells; however, C9 may not be involved.

Induction of eryptosis by the terminal complement complexes C5b/6, C5b-7, and C5b-8

To further confirm our hypothesis, heat-inactivated serum from patients with cold IgM AIHA were used with commercially available serum depleted of the single complement proteins C6, C7, C8, or C9. Compared to the negative control (i.a. serum from patient + i.a. AB serum; Fig. 5A), the use of C6-, C7-, C8-. or C9-depleted serum led to a steady rise in the

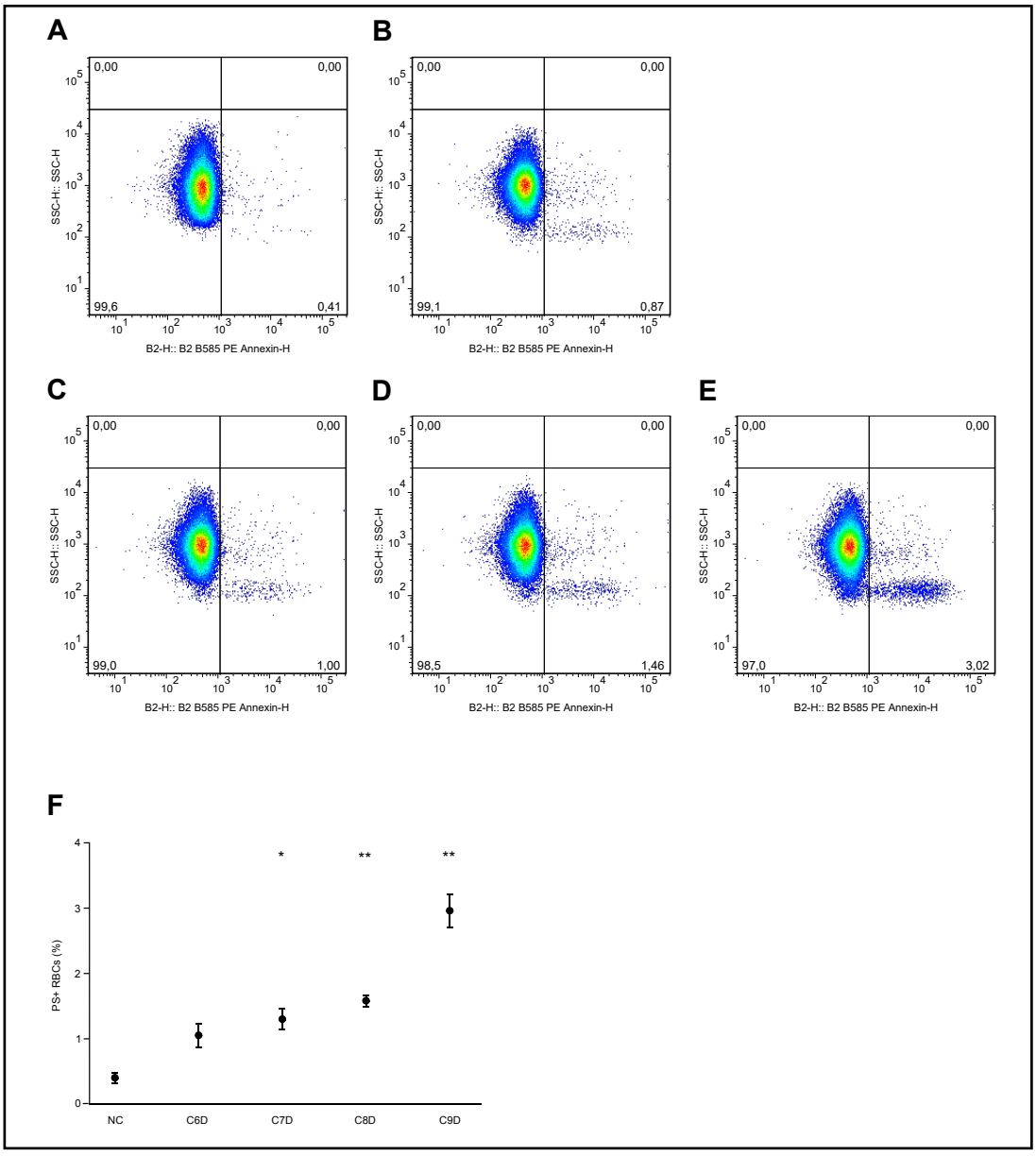

Fig. 5. Scatter plots showing annexin-PE binding to O RBCs and effect of the terminal complement components C5b/6, C5-7, and C5-8. Compared to negative control (A) PS exposure of RBCs incubated with i.a. patient serum gradually increased in the presence of C6-depleted serum (B), C7-depleted serum (C), C8-depleted serum (D), and C9-depleted serum (E). Graph showing increase of PS exposure in the presence of sera depleted of complement component C6, C7, C8 and C9, respectively (F). (*) $p<0.05$, (**) $p<0.01$.

number of PS+ cells (Fig. 5B–E) showing that the intermediate formation of distinct phases, collectively referred to as terminal complement complexes (TCCs) [22], increasingly induce eryptosis. Of notice is the strong increase in PS+ RBCs when C9-depleted serum was used and the TCC C5b-C8 complex is present (Fig. 5F).

Discussion

The obtained results are intriguing in many respects and may shed light on the diversity and complexity of RBC destruction in patients with AIHA and diseases associated with anaemia. In addition to the classical pathways Fc-, C3b-, and/or C5b-9-mediated RBC phagocytosis and/or lysis, respectively [2, 5], we recently demonstrated eryptosis to be involved in AIHA due to IgM and IgA aab [9]. However, how these aab cause PS exposure on RBCs and consequently eryptosis remained unknown. In the aforementioned study, three possible mechanisms by which these aab may lead to PS translocations were discussed. These include physical stress as a result of strong agglutination, the release of inflammatory signals, and Ca^{2+} influx. In the present study, we hypothesized whether complement activation may be involved in this process. Using EDTA, heat-inactivated serum, C5–C9 depleted sera, and antibodies to C5, C8, and C9, eryptosis due to IgM aab was found to depend on the activation of these components.

Eryptosis can be induced in donor O RBCs by incubation with fresh serum of patients with cold IgM aab. In contrast, an increase of PS exposure could not be induced in donor O RBCs by using patient plasma or heat-inactivated serum. Furthermore, there was no eryptosis following the addition of anti-C5 (Eculizumab, Soliris®) prior to incubation or when using C5-depleted serum as a source of complement. These data indicate that C5 may be mandatory for IgM-induced eryptosis. However, C5 activation does not suffice in significantly increasing PS exposure as demonstrated using C6-depleted serum. In contrast, the use of C7-depleted serum resulted in a further increase. This could be explained by the formation of labile C5b6 complexes. Though these complexes have not been reported to cause cellular changes [23, 24], they have the potential ability to interact with the hydrophobic domains of the lipid bilayer [22, 25, 26]. Whether this interaction could be enforced by the cell agglutination induced by the aab remains speculative. This assumption is supported by the observation that PS exposure can be stimulated by cell-cell adhesion of human RBCs [27-29], which is reflected in our experiment by the strong agglutination in the presence of the causative aab. In addition, C5b as a "collateral effect" of C5 activation has also been described to be involved in some apoptotic cell conditions [30]. Ultimately, it remains questionable whether all cellular responses induced by the sublytic MAC complexes including C5b6, C5b-7, and C5b-8 can be reproduced and/or measured *in vitro*.

PS exposure further increased as a result of the formation of C5b-7 and was most significant following C5b-8 complexes formation after the incubation of RBCs with IgM aab and C8- or C9-depleted serum, respectively. C5b-7 complexes adhere to the cell membrane [31, 32] and induce the generation of ceramide as well as Ca^{2+} influx, which are principal mediators of eryptosis [7, 8, 32-36]. Hence, PS exposure at this stage may be due to Ca^{2+} influx. This is not surprising since Ca^{2+} alone is sufficient to induce PS exposure in human RBCs [29, 37]. C5b-8 formation leads to a further increase of Ca^{2+} influx, membrane deformation, and may cause cell lysis [38-40]. Therefore, both sublytic C5b-7 and C5b-8 complexes are capable of inducing eryptosis. Future studies should focus on determining whether complement activation through the classical pathway, i.e., due to agglutinating IgM antibodies, might be more effective in causing eryptosis than the alternative pathway, i.e., in the absence of antibodies. The strong increase of PS exposure following inhibition at the last stage by anti-C9 antibody or the use of C9-depleted serum could be explained by the accumulation of C5b-8 sublytic complexes on the cell membrane. However, the decrease of PS on the cell membrane following C9 assembly and C5b-9 channel (MAC) formation could be explained by cell lysis or the formation of soluble sC5b-9 complexes, which are inhibitors of complement activation and sublytic C5b-8 complex formation [22].

In contrast to IgM aab, IgA aab do not appear to activate complement or require complement for inducing PS exposure and eryptosis, respectively. Their eryptotic effect remained unaltered through the use of EDTA plasma or heat-inactivated serum. This is supported by previous studies including ours [41] and from our group [19]. The question of whether IgA coated RBCs in patients with AIHA is eliminated by phagocytosis due

to PS exposure and, at least, in part due to autoagglutination remains unanswered. Haemagglutination due to IgM or IgA aab may result in RBC sequestration independent of Fc receptor and complement activation. This was previously demonstrated using a mouse model and polymeric forms of IgM and IgA aab [42]. In addition, Ca^{2+} influx, the principal stimulator of eryptosis, may occur as a result of mechanical RBC deformation [43]. Therefore, agglutinating IgA and IgM antibodies may simultaneously lead to RBC sequestration by eryptosis and or agglutination.

In conclusion, the present study provides evidence that IgM-induced eryptosis is complement-dependent and that the sublytic terminal complement complexes C5b/6, C5b-7, and especially C5b-8 are sufficient for eryptosis, potentially representing a third degradation pathway in patients with AIHA. Eryptotic cells are usually removed rapidly from the circulation by macrophages, suffering the same fate as RBCs that undergo extravascular hemolysis. However, if phagocytotic activity is diminished, eryptotic cells may undergo intravascular haemolysis. This may explain why a number of patients with AIHA cannot be treated successfully with drugs that intervene in the monocyte/macrophage system. Further studies are required to elucidate the significance of this third RBC degradation pathway and its use in drug therapy.

Unlike eryptosis, the apoptosis of nuclear cells does not seem to be complement-dependent and complement-induced cell death is rather necrotic [44]. Sublytic MAC doses failed to produce DNA-ladder formation as a typical indicator of apoptosis in nuclear cells [44] and may even protect cells from apoptosis [45]. Furthermore, apoptotic cells have been shown to activate complement [46] and on the other hand complement facilitates the clearance of apoptotic cells [47]. In that context, the exciting difference between apoptosis of nucleated cells and complement-induced eryptosis could even have a further significance; RBCs may act as a buffer of the complement system, preventing inflammation and necrosis in living tissue by self-sacrifice and fast macrophage clearance.

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All authors reviewed the manuscript and approved the final version.

Disclosure Statement

All authors declare that they have no competing interests.

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