Role of Complement in Autoimmune Hemolytic Anemia

Sigbjørn Berentsen

Department of Research and Development, Haugesund Hospital, Helse Fonna HF, Haugesund, Norway

Our knowledge about the essential role of complement in subgroups of AIHA is also expanding [6–8], and possible therapeutic options for complement-modifying therapy are being investigated [9–11]. Moreover, although paroxysmal nocturnal hemoglobinuria (PNH) is not an autoimmune disorder, the entirely complement-dependent pathogenesis and the success of therapeutic complement inhibition in this disease makes it possible to learn lessons from PNH that might prove useful in treating AIHA [12, 13].

This review will address the pathogenetic mechanisms of AIHA, focusing on the role of complement in RBC destruction and possible implications for the potential therapeutic use of complement modulators. Diagnostic procedures and established, non-complement-targeted therapies will only be briefly mentioned.

The Complement System

The classical complement pathway is initiated by binding of complement protein 1q (C1q), part of the C1 complex, to an antigen-antibody (AgAb) complex at a pathogen or host cell surface, allowing activation of C1r. Activated C1r cleaves C1s, generating an active serine protease that in turn cleaves C4 and C2.

Introduction

Autoimmune hemolytic anemia (AIHA) is a collective term for several diseases characterized by autoantibody-initiated destruction of erythrocytes [1–5]. AIHA can be classified as shown in table 1. The insight into the etiology, pathogenesis, and therapy of these disorders is rapidly growing [3–7].

Keywords
Complement · Autoimmune hemolytic anemia · Cold agglutinin disease · Therapy · Complement inhibitors

Summary
The classification of autoimmune hemolytic anemias and the complement system are reviewed. In autoimmune hemolytic anemia of the warm antibody type, complement-mediated cell lysis is clinically relevant in a proportion of the patients but is hardly essential for hemolysis in most patients. Cold antibody-mediated autoimmune hemolytic anemias (primary cold agglutinin disease, secondary cold agglutinin syndrome and paroxysmal cold hemoglobinuria) are entirely complement-mediated disorders. In cold agglutinin disease, efficient therapies have been developed in order to target the pathogenetic B-cell clone, but complement modulation remains promising in some clinical situations. No established therapy exists for secondary cold agglutinin syndrome and paroxysmal cold hemoglobinuria, and the possibility of therapeutic complement inhibition is interesting. Currently, complement modulation is not clinically documented in any autoimmune hemolytic anemia. The most relevant candidate drugs and possible target levels of action are discussed.

© 2015 S. Karger GmbH, Freiburg
actions result in the formation of C3 convertase, which cleaves C3 into C3a, an anaphylotoxin, and C3b, which binds covalently to the pathogen or cell surface and acts as an opsonin as well as a further proteolytic enzyme [14–17].

Two other initial complement pathways are also known; the lectin pathway initiated by mannose-binding lectin and the alternative pathway triggered by the binding of spontaneous activated C3 in plasma to a pathogen surface. Like the classical pathway, these two reaction chains lead to the production of C3 convertase and, in turn, deposition of C3b on the cell surface. Thus, the formation of C3b is the point of convergence between the three initial complement pathways; and the classical pathway-initiating event of binding C1q represents a link between the adaptive immune system and the complement system [14, 16, 17]. The lectin and alternative pathways, believed to be less important for the pathogenesis in AIHA, will not be further addressed here.

Pathogens, or host cells in the case of autoimmunity, opsonized by C3b can bind to complement receptors on phagocytes. Such binding results in removal of C3b-opsonized cells by the reticuloendothelial system, in particular in the spleen and/or liver. Concomitantly or alternatively, surface-bound C3b can be further degraded [3, 14, 16]. In the AIHA setting, such phagocytosis is known as extravascular hemolysis.

In addition to allowing phagocytic removal, surface-bound C3b can bind to complement receptors on phagocytes. Such binding results in removal of C3b-opsonized cells by the reticuloendothelial system, in particular in the spleen and/or liver. Concomitantly or alternatively, surface-bound C3b can be further degraded [3, 14, 16]. In the AIHA setting, such phagocytosis is known as extravascular hemolysis.

Due to the amplifying nature of the cascades and positive feedback loops, activation of the complement system can lead to an accelerating, uncontrolled, and even fatal process of inflammatory reaction and cell lysis. Several physiologic complement inhibitors and negative feedback loops, however, prevent this from occurring under normal circumstances as well as in some pathologic conditions. Of relevance for complement-mediated hemolytic anemias, important cell-bound regulators are CD55, which has an inhibitory function at the C4-C2 level, and CD59, which prevents final assembly of the MAC at the C8-C9 stage [13, 14].
non-Hodgkin’s lymphomas (NHLs) is less common [1, 2, 19]. Furthermore, systemic lupus erythematosus, rheumatoid arthritis, Sjögren’s syndrome, primary biliary cirrhosis, hypothyroidism, inflammatory bowel disease, immune thrombocytopenia as well as primary hypogammaglobulinemia and other immunologic diseases can be associated with w-AIHA [1, 2, 20, 22]. Some patients have several associated diseases at the same time.

Autoantibody or complement fragment deposition on the RBC can usually be detected using polyspecific and monospecific direct antiglobulin test (DAT). The autoantibodies in w-AIHA are of the immunoglobulin G (IgG) class in most cases [4]. In up to 50% of w-AIHA, DAT is positive for complement fragments, most often C3d and usually in combination with IgG. IgA autoantibodies occur in 15–20% of the patients, either in combination with IgG or, less frequently, alone [23]. Cases with IgA as the sole autoantibody class may be misdiagnosed because reagents used in the polyspecific DAT do usually not contain anti-IgA. Warm autoantibodies of the IgM class have been assumed to be rare. Their frequency remains somewhat controversial, however, because they may have low affinity to the antigen and may have detached from the RBC surface before they can be detected by DAT [24, 25]. In 3–10% of patients with w-AIHA, DAT is found to be negative [4, 26]. The problem of ‘DAT-negative AIHA’ has been extensively discussed elsewhere in the literature [4, 26, 27, 28].

Erythrocyte Destruction and Role of Complement in w-AIHA

Erythrocytes coated with warm-reactive autoantibodies are sequestered and phagocytosed by macrophages, primarily in the spleen [29–31]. The macrophage surface expresses receptors for the Fc region of the immunoglobulin molecules, which enables trapping and ingestion of the opsonized RBCs [32, 33]. Often, however, phagocytosis is incomplete and results in formation of spherocytes [7, 32]. This has been explained in part by the removal of more membrane than volume. Furthermore, ectoenzymes on the macrophage surface cause microperforations of the cell membrane, increasing its permeability and thereby promoting the transition from a biconcave to a spherical shape of the cell [7, 29, 31]. Spherocytes are prone to further destruction during subsequent passages through the spleen [4, 7, 30].

On erythrocytes heavily coated with immunoglobulin, the amount of AgAb complex may be sufficient for binding C1q and activation of the classical complement pathway (fig. 1) [15, 34, 35]. Unlike IgG, IgM is a potent complement activator but, as mentioned above, usually not found on the RBC surface by DAT in w-AIHA [24]. Regarding the IgG subclasses, IgG3 activates complement more efficiently than does IgG1, while IgG2 is a weak activator and there is no good evidence for complement activation by IgG4 [36]. IgA does probably not activate complement. Despite this, however, IgA deposition on RBCs can lead to fulminant hemolysis [23, 37]. A probable explanation is involvement of IgM even in some cases where only IgG or IgA is detected, since IgM will often detach from the RBC before it can be detected by DAT [25]. Upon complement activation in w-AIHA, phagocytosis of C3b-opsonized erythrocytes by reticulo-endothelial cells in the liver is responsible for most of the hemolysis, while full-blown intravascular hemolysis mediated by the terminal complement pathway is usually not prominent [4, 7, 35]. The explanation is probably the modest activation of the complement pathway, combined with the protective effect of the physiological cell surface complement inhibitors CD55 and CD59 which, unlike in PNH, are intact in AIHA.

Figure 2 summarizes the pathways of erythrocyte destruction in w-AIHA. In conclusion, complement activation does occur to some extent, at least in a proportion of the patients, but is hardly essential for hemolysis in w-AIHA. DAT positivity for C3 fragments is a marker of complement involvement.

Cold Agglutinin Disease

Etiology and Pathogenesis

Primary cold agglutinin disease (CAD) should be distinguished from secondary cold agglutinin syndrome (CAS) [5]. As will be further explained, CAD is a well-defined clinicopathological entity and should, therefore, be called a disease, and not a syndrome [5, 38]. Secondary CAS is a syndrome complicating a variety of infectious and neoplastic disorders, and not a well-defined disease. In a Norwegian population-based study, the prevalence of CAD was 16 per million, and the incidence was about 1 per million per year, making CAD account for approximately 15% of AIHA [1, 2, 39].

Cold agglutinins (CA) are autoantibodies that agglutinate RBCs with a temperature optimum of 3–4 °C but may also act in a warmer environment, depending of the thermal amplitude of the CA [5, 40]. If the thermal amplitude exceeds 28–30 °C, the CA will be pathogenic. Low-affinity CA also occur in many healthy individuals; these non-pathogenic CA are polyclonal, have low thermal amplitude and are present in low titers, not higher than 256 and...
usually lower than 64. More than 90% of pathogenic CA are of the IgM class, and these IgM macromolecules can be pentameric or hexameric [39, 41, 42].

In general, monoclonal CA are more pathogenic than polyclonal CA, and hexameric IgM is more pathogenic than pentameric IgM [5, 42, 43]. It has been known for decades that in patients with CAD, IgM antibodies with CA activity are monoclonal and, in more than 90% of the patients, show kappa light chain restriction [44]. Accordingly, CAD patients must have a clonal B-cell lymphoproliferative disorder which has not been fully elucidated until the last years. Two large, retrospective studies of consecutive patients with primary CAD found signs of a bone marrow clonal lymphoproliferation in most patients, but in both series the individual hematological and histological diagnoses showed a striking heterogeneity [39, 45]. In one of the series, lymphoplasmacytic lymphoma (LPL) was the most frequent finding, while marginal zone lymphoma (MZL), unclassified clonal lymphoproliferation, and reactive lymphocytosis were also frequently reported [39]. The explanation for this perceived heterogeneity was probably revealed by a recent study in which bone marrow biopsy samples and aspirates from 54 patients with CAD were systematically re-examined by a group of lymphoma pathologists, using a standardized panel of morphological, immunohistochemical, flow-cytometric and molecular methods [38]. The bone marrow findings in these patients were consistent with a surprisingly homogeneous disorder termed ‘primary CA-associated lymphoproliferative disease’ by the authors and distinct from LPL, MZL, and other previously recognized lymphoma entities. The MYD88 L265P somatic mutation, typical for LPL, could not be detected in the samples from patients with CAD [38, 46].

Role of Complement in CAD

CA are usually directed against the li blood group system, most CA in CAD being specific for the I carbohydrate antigen [47–49]. Cooling of blood during passage through acral parts of the circula-

Fig. 3. Complement-mediated hemolysis in cold agglutinin disease (CAD) and cold agglutinin syndrome (CAS). Explanation: See text. CA = Cold agglutinin; C = complement. Originally published in BioMed Res Int 2015 [28]. Copyright: S. Berentsen and T. Sundic. Re-used with permission.
montiae pneumonia. They do usually not give rise to significant hemolysis. In a few patients, however, production of high-titer, high-thermal amplitude CA results in hemolytic anemia which is transient but can be severe [5, 58, 59]. \(\text{CAS}\) complicating \textit{Mycoplasma pneumoniae} infection has been reported to account for approximately 8% of \text{AIHA} [2]. Still more uncommon but less severe, polyclonal anti-i specific CA of the IgM or IgG class can result in \(\text{CAS}\) in Epstein-Barr virus infection [5, 60]. Transient \(\text{CAS}\) has also been described following cytomegalovirus infection, varicella, rubella, adenovirus infection, influenza A, \textit{Legionella pneumophila} pneumonia, listeriosis and pneumonia caused by \textit{Chlamydia} species [5]. In \(\text{CAS}\) secondary to infection or aggressive lymphoma, the erythrocyte breakdown is complement-dependent, mediated by exactly the same mechanisms as in primary \(\text{CAD}\) (fig. 3) [5, 7].

\textbf{Paroxysmal Cold Hemoglobinuria}

In paroxysmal cold hemoglobinuria (PCH), polyclonal cold-reactive IgG antibodies bind to the RBC surface protein antigen termed P but does not agglutinate the erythrocytes. The resulting hemolysis is entirely complement-dependent, and the temperature optimum for complement activation is at \(37^\circ \text{C}\) [61, 62]. Such biphasic antibodies are called Donath-Landsteiner hemolysins. In the Donath-Landsteiner’s test, one sample of patient blood is incubated at \(4^\circ \text{C}\) and then at \(37^\circ \text{C}\), while another sample is incubated at \(37^\circ \text{C}\) without having been pre-incubated in the cold [61, 62]. If biphasic autoantibodies are present, hemolysis will be observed only in the sample pre-incubated at \(4^\circ \text{C}\). The sensitivity is limited because the patient blood is often complement-depleted; and in more sensitive modifications of the test, complement is added and/or papain-pretreated RBCs are used [62].

50–100 years ago, PCH was associated with tertiary syphilis, but this form is hardly seen anymore. In the 21st century, PCH occurs almost exclusively in children and accounts for 1–5% of childhood AIHA, making it a rare disease [63]. It appears as an acute, postinfectious complication – in most cases following a virus infection [62]. Single cases have also been reported in \textit{Haemophilus influenzae} infection and visceral leishmaniasis [63, 64].

The P-anti-P complex is a very strong complement activator, resulting in full-blown activation of the classical and terminal pathways (fig. 4). The hemolysis, therefore, is intravascular and massive; the onset is usually sudden, and the clinical features include fever, pallor, jaundice, severe anemia, and macroscopic hemoglobinuria [62, 64]. Even though PCH is a transient complication with good prognosis, most patients will need transfusions, which can safely be given provided the same precautions are undertaken as in other cold-antibody AIHA [5].

\textbf{Established Therapies}

Established therapies for w-AIHA has been extensively reviewed elsewhere [3, 4]. The cornerstone of such therapy is unspecific immunosuppression and/or B-lymphocyte suppression [65] in addition to treatment of any underlying or associated disorder.

In primary CAD, rituximab monotherapy has yielded about 50% response rates and a median 1-year response duration according to two prospective trials [66, 67]. Combination therapy for CAD with rituximab and fludarabine in order to target the pathogenic B-cell clone even more efficiently resulted in a 75% response rate, 20% complete responses according to strict criteria and an impressive median response duration of more than 66 months, however with some toxicity [68]. Single case observations with bendamustine- or bortezomib-based therapies as alternative ways of targeting the lymphoproliferative bone marrow disease have reported favorable outcomes [69, 70].

For secondary \(\text{CAS}\) as well as PCH, no documented therapy exists apart from treating the underlying disease when relevant and feasible [5, 62].

\textbf{Therapeutic Complement Modulation}

\textit{Candidate Substances, Experimental Studies, and Case Observations}

The potential of complement modulation for the treatment of AIHA will depend on i) the type of AIHA and extent and level of complement involvement, ii) the availability, safety and efficacy of complement inhibition on immune hemolysis are, however, still being developed [10, 71]. Novel in vitro and in vivo models for testing the impact of specific complement inhibition on immune hemolysis are, however, still being developed [10, 71].

C1-esterase inhibitor (C1-INH) has been available for decades and is being successfully used for the treatment of hereditary angi-
oedema (HAE) [72]. Although not a complement-mediated disorder, HAE is caused by lack or deficiency of endogenous C1-INH, and replacement therapy has been well studied. In AIHA, on the other hand, endogenous C1-INH production is normal, indicating that physiological concentrations of the inhibitor will not block complement-mediated hemolysis.

Eculizumab, a humanized monoclonal C5 antibody, blocks the terminal pathway and, thereby, prevents intravascular hemolysis by MAC. Therapy with eculizumab has been a great success in PNH, although complement-mediated hemolysis is not completely prevented [73]. The explanation for this is probably that patients with PNH lack physiological inhibitors both at a downstream level in the terminal pathway (CD59) and at an upstream level in the classical pathway (CD55). In consequence, a slight to moderate hemolysis mediated by phagocytosis of C3b-opsonized erythrocytes will still occur along the same pathway as described in CAD, independent of C5 activation or inhibition [12].

Some newer complement-modulating drugs have been studied with promising results in preclinical experiments but not yet in the in vivo setting. Compstatin Cp40 is a low-molecular-weight peptide complement inhibitor that blocks cleavage of C3 and has been found to efficiently prevent lysis of erythrocytes from PNH patients in vitro [74]. Peptide inhibitor of C1 (PIC1) is a recently described class of small molecules that targets C1q, blocking the activation of associated serine proteases (C1s-C1r-C1r-C1s) and subsequent classical pathway activation [75]. PIC1 has been studied in acute hemolytic transfusion reactions in animals but, so far, not in AIHA-related experiments.

TNT003, a mouse monoclonal anti-C1s antibody, has recently been shown to completely inhibit in vitro hemolysis induced by CA [10]. This antibody targets C1s serine protease activity. Using CA samples from 40 patients with CAD, the authors found that TNT003 prevented CA-induced deposition of C3 fragments on the RBC at the same concentration of antibody that stopped hemolysis. Furthermore, C1s inhibition by TNT003 resulted in prevention of in vitro erythropagocytosis by a phagocytic cell line. The classical-pathway-driven production of the anaphylotoxins C4a, C3a and C5a was also inhibited [10]. Favorable in vitro results have also been described with TNT003’s humanized counterpart: TNT009 [76].

Future Perspective

As shown above, complement activation plays a role in w-AIHA but is not essential for pathogenesis in most patients. Complement modulation may be expected, therefore, to be of limited therapeutic value in w-AIHA in general and of no value if DAT is negative for C3 fragments. Wouters and colleagues [77] described, however, a favorable effect of plasma-derived C1-INH in a patient with a C3d-positive, therapy-resistant severe w-AIHA secondary to an aggressive non-Hodgkin’s lymphoma. Although very high doses of C1-INH were required, hemolysis was efficiently controlled, and the efficacy of erythrocyte transfusion dramatically improved following treatment. No other clinical observations on the results of complement inhibition have been published in w-AIHA. In patients with a positive DAT for C3d and very severe hemolysis, further studies of complement inhibition even at a more downstream level would be of interest, mainly as an attempt to temporarily control hemolysis.

Given that hemolysis in CAD is entirely complement-dependent, studies of complement inhibition would be relevant in CAD. A case report by Röth and colleagues [52] described a favorable effect of therapy with eculizumab. This observation may seem somewhat surprising, since the predominant hemolytic pathway in CAD is not C5/MAC-mediated. A probable explanation is that activation of the terminal complement pathway does occur, after all, in acute exacerbations, in the chronic state of some severely affected patients, and, possibly, as a minor pathway even in less severely affected patients. Further studies will be of interest.

In theory, complement inhibition at the C1 level should be very promising in CAD because this will block the classical-pathway-dependent, C3b-mediated extravascular hemolysis without compromising the alternative and lectin complement pathways. The published in vitro studies of TNT003 and TNT009 are highly interesting therefore, and it is to be hoped that such a monoclonal anti-
body can be developed and further tested in the preclinical and clinical setting [10, 11, 75].

Given that immunotherapy directed at the pathogenic B-cell clone is efficient and requires administration only for a limited period of time, do we actually need complement-modulating therapies for CAD? First, in at least 25% of the patients, immunotherapy is unsuccessful because of treatment failure or toxicity [68]. Second, rapidly acting therapies should be developed for some specific clinical situations, for example acute severe exacerbations induced by infections, trauma or major surgery, and, possibly, before cardiac surgery in selected patients.

In the uncommon cases of CAS secondary to specific infection, there is often no need for therapy for the CAS per se. However, this is not always the case. Particularly in CAS following Mycoplasma pneumoniae pneumonia, the patients can be profoundly anemic and transfusion-dependent for weeks until spontaneous resolution occurs [5]. Clinicians and patients would welcome a possibility for temporary control of this situation by complement inhibition along the same lines that may be developed in primary CAD. Systematic studies would be interesting but probably difficult to perform because of the rarity of the disorder.

In the rare cases of post-infectious PCH in children, measures for temporary control of the hemolysis will be valuable if such therapies can be developed. Since the terminal complement pathway is heavily involved, we do not necessarily need new substances; exploring the efficacy of eculizumab would be of great interest. Probably, however, prospective trials will never be performed because there are too few patients for such studies.

Present and future possibilities for therapeutic complement inhibition in AIHA are summarized in figure 5. It is important to ask whether such therapy will be dangerous. The complement system is, after all, an essential part of the innate immune system. Based on studies of eculizumab in PNH, we already have extensive information on the risk of severe infection following C5 inhibition. Provided the patients can be efficiently protected against meningococci, studies and clinical experience have shown that the risk of infection is negligible [73]. Complement inhibition at the C3 level may carry a much higher risk because efficient inhibition of C3 will completely block complement activation beyond this level, whether initiated by the classical, alternative or lectin pathway [11, 14, 74]. Interestingly, however, the still more proximal blockade at the C1 level achieved by TNT003 will selectively affect the classical pathway as required for control of hemolysis in CAD, while the lectin and alternative pathways will remain intact. Probably therefore, these pathways probably will still enable the system to generate the anaphylotoxins C5a and C5a in response to microbial stimuli, even though the production of these anaphylotoxins induced by the classical pathway will be blocked [10, 11, 14]. Although this selectivity may, theoretically, reduce the risk of infection, careful studies will be required to address this issue.

Disclosure Statement

S. Berentsen has received research support from Mundipharma and lecture honoraria and travel grants from Alexion.

References

1 Sokol RJ, Hewitt S, Stamps BK: Autoimmune haemolytic anaemia: a 18-year study of 865 cases referred to a regional transfusion centre. Br Med J (Clin Res Ed) 1981; 282:2023–2027.

2 Dacie J: The auto-immune haemolytic anaemias: introduction, in Dacie J (ed): The Haemolytic Anaemias, Vol. 3. London, Churchill Livingstone, 1992, pp 1–5.

3 Michel M: Classification and therapeutic approaches in autoimmune hemolytic anemia: an update. Expert Rev Hematol 2011;4:607–618.

4 Packman CH: Hemolytic anemia due to warm autoantibodies. Blood Rev 2008;22:17–31.

5 Berentsen S, Tjonnfjord GE: Diagnosis and treatment of cold agglutinin mediated autoimmune hemolytic anemia. Blood Rev 2012;26:107–115.

6 Garratty G: The James Blundell Award Lecture 2007: there are too few patients for such studies. Exploring the efficacy of eculizumab would be of great interest. Probably, however, prospective trials will never be performed because there are too few patients for such studies.

Role of Complement in Autoimmune Hemolytic Anemia

Transfus Med Hemother 2015;42:303–310
32 Gilsanz F, De La Serna J, Molto L, Alvarez-Mon M: Hemolytic anemia in chronic large granular lymphocytic leukemia of natural killer cells: cytotoxicity of natural killer cells against autologous red cells is associated with hemolysis. Transfusion 1996;36:463–466.

31 Kurlander RJ, Rosse WF: Monocyte-mediated destruction in the presence of serum of red cells coated with antibody. Blood 1979;54:1131–1139.

32 LoBuglio AF, Cotran RS, Jandl JH: Red cells coated with immunoglobulin G: binding and serum by mononuclear cells in man. Science 1967;158:1582–1585.

33 Abramson N, Lo Buglio AF, Jandl JH, Cotran RS: The interaction between human monocytes and red cells. Binding characteristics. J Exp Med 1970;132:1207–1215.

34 Meulebroek EM, Wouters D, Zeerleder S. Methods for quantitative detection of antibody-induced complement activation on red blood cells. J Vas Exp 2014; (83):e51161.

35 Kurlander RJ, Rosse WF, Logue G. Quantitative influence of antibody and complement coating of red cells on monocyte-mediated cell lysis. J Clin Invest 1978;61:1309–1319.

36 Abramson N, Gelfand EW, Jandl JH, Rosen FS: The production of IgM hexamers by normal and autoimmune human subjects. J Immunol 1978;120:1207–1215.

37 Bazzoli B, Mengis C, Tschopp M, Wuelliem WA: Self-reacting auto-antibody autohemolytic anemia in a 48-year-old woman. Eur J Haematol 2003;70:60–63.

38 Randen U, Troen G, Tierens A, Steen C, Warsame A, Harboe M, Deverill J: Immunochemical properties of the patterns of hemolysis in vivo. J Clin Invest 1976;58:942–949.

39 Uvelstad E, Berentsen S, Molinos TE: Acute phase hemolysis in chronic cold agglutinin disease. Scand J Immunol 2001;54:239–242.

40 Røth A, Huttmann A, Rother RP, Duhuen H, Philipp T: Long-term efficiency of the complement inhibitor eculizumab in cold agglutinin disease. Blood 2009;113:3885–3886.

41 Uvelstad E: Paradoxic haemolysis in a patient with cold agglutinin disease. Eur J Haematol 1998;60:93–100.

42 Crisp D, Pruzanek WS: B-cell neoplasms with homogeneous cold-reacting antibodies (cold agglutinins). Am J Med 1982;72:915–922.

43 Naini F, Hammvirk OP, Gulmann C, Berthelsm A, Kelly J, Mc EP, et al: Diffuse large B-cell lymphoma with isolated bone marrow involvement presenting with secondary cold agglutinin disease. Int J Lab Haematol 2008;30:444–448.

44 Eskavan AE, Akmurad H, Ongoren S, Ozer O, Ferhanoglu B: Primary gastrointestinal diffuse large B-cell lymphoma with associated cold agglutinin disease. J Clin Immunol 1996;16:393–399.

45 Berentsen S, Ulvestad E, Langholm R, Beiske K, et al: Primary cold agglutinin-associated lymphoproliferative disease: a B-cell lymphoma of the bone marrow distinct from lymphoplasmacytic lymphoma. Haematologica 2014;99:497–504.

46 Berentsen S, Ulvestad E, Langholm R, Beiske K, Hjorth-Hansen H, Ghanima W, et al: Primary chronic cold agglutinin disease: a population based clinical study of 86 patients. Haematologica 2006;91:460–466.

47 Rosse WF, Adams JP: The variability of hemolysis in the cold agglutinin syndrome. Blood 1980;56:409–416.

48 Harboe M, Deveril J: Immunochemo-metric properties of cold haemagglutinins. Scand J Haematol 1964;61:223–237.

49 Hughey CT, Brewer JW, Colusol AD, Rosse WF, Coley RB: Production of IgM hexamers by normal and autoimmune red cells: implications for the physiologic role of hexameric IgM. J Immunol 1989;161:4091–4097.

50 Stone MJ, McAleery YG, Pestroneck A, Reynolds JL, New- man TT, Tong AW: Human monoclonal macroglobu- lins with antibody activity. Semin Oncol 2003;30:318–324.

51 Harboe M, van Faurth R, Schuette H, Lind K, Evans RS: Exclusive occurrence of K chains in isolated cold haemagglutinins. Scand J Haematol 1965;2:259–266.

52 Stone MJ, McElroy YG, Pestronk A, Reynolds JL, New- man TT, Tong AW: Human monoclonal macroglobu- lins with antibody activity. Semin Oncol 2003;30:318–324.

53 Ziman A, Hsu R, Goldfinger D: Transfusion medicine illustrated. Donath-Landsteiner antibody-associated hemolytic anemia after Haemophilus influenzae infection in a child. Transfusion 2004;44:1117–1128.

54 D’Angio M, Ceglie T, Giovannetti G, Neri A, Santolo I, Nunes V, et al: Visceral leishmaniasis presenting with paroxysmal cold hemagglutininaemia. Blood Transfus 2014;12(suppl 1):s141–s143.

55 Birgens H, Frederiksen H, Hasselbalch HC, Rasmussen IH, Nielsen OJ, Kjeldsen L, et al: A phase III randomized trial comparing glucocorticoid monotherapy versus glucocorticoid and rituximab in patients with autoimmunne haemolytic anaemia. Br J Haematol 2013;163:393–399.

56 Berentsen S, Ulvestad E, Giertsen BT, Hjorth-Hansen H, Langholm R, Knutsen H, et al: Rituximab for primary chronic cold agglutinin disease: a prospective study of 37 courses of therapy in 27 patients. Blood 2014;130:2925–2928.

57 Schollkopf C, Kjeldsen L, Bjerum OW, Mourits- Andersen HT, Nielsen JL, Christensen BE, et al: Rituximab in chronic cold agglutinin disease: a prospective study of 28 patients. Leuk Lymphoma 2006;47:253–260.

58 Berentsen S, Randen U, Vagan AM, Hjorth-Hansen H, Värk A, Dalgaard J, et al: High response rate and durable remissions following fludarabine and rituximab combination therapy for chronic cold agglutinin disease. Blood 2010;116:3180–3184.

59 Gueli A, Gottiardi D, Hu H, Ricca I, De Crescenzo A, Tarella C: Efficacy of rituximab-bendamustine in cold agglutinin haemolytic anaemia refractory to previous chemo-immunotherapy: a case report. Blood Transfus 2013;11:311–314.

60 Carson KR, Beckwith LG, Mehta J: Successful treatment of IgM-mediated autoimmune hemolytic anemia with bortezomib. Blood 2010;115:913–914.

61 Shah TA, Mauriello CT, Hair PS, Sharp JA, Kumar PS, Lattanzio FA, et al: Complement inhibition significantly decreases red blood cell lysis in a rat model of acute intravascular hemolysis. Transfusion 2014;54:2892–2890.

60 Carson KR, Beckwith LG, Mehta J: Successful treat- ment of IgM-mediated autoimmune hemolytic anemia with bortezomib. Blood 2010;115:913–914.

61 Shah TA, Mauriello CT, Hair PS, Sharp JA, Kumar PS, Lattanzio FA, et al: Complement inhibition significantly decreases red blood cell lysis in a rat model of acute intravascular hemolysis. Transfusion 2014;54:2892–2890.

62 Bor B, Steffensen I, Machin T: Treatment with C1- esterase inhibitor concentrate in type I or II hereditary angioedema: a systematic literature review. Allergy Asthma Proc 2013;34:312–327.

63 Hilleman P, Young NS, Schubert J, Brooksy RA, Socie G, Mure C, et al: The complement inhibitor ecu- lizumab in paroxysmal nocturnal hemoglobinuria. N Engl J Med 2006;355:1233–1243.

64 Risitano AM, Ricklin D, Huang Y, Reis ES, Chen H, Ricci P, et al: Peptide inhibitors of C3 activation as a novel strategy of complement inhibition for the treat- ment of paroxysmal nocturnal hemoglobinuria. Blood 2014;123:2094–2011.

65 Sharp JA, Whitley PH, Cunin KM, Krishna NK: Peptide inhibitor of complement C1r/C1s, a new suppres- sor of classical pathway activation: mechanistic studies and clinical potential. Front Immunol 2014;5:406.

66 Panicker S, Shi J, Rose E, Hussain S, Tom S, Strober W: TNT009, a classical complement pathway specific in- hibitor, prevents complement dependent hemolysis induced by cold agglutinin disease patient autoantibodies. Presentation at the 56th Meeting of the Ameri- can Society of Hematology, New Orleans, LA, USA. December 8, 2013 (Paper 64043). https://ash.confex.com/ ash/2013/webprogram/Paper64043.html (last accessed July 28, 2015).

67 Wouters D, Stephan F, Stengers P, de Haas M, Brou- wer C, Hagenbeek A, et al: C1-esterase inhibitor hyper- centrase rescues erythrocytes from complement-mediated destruction in autoimmune hemolytic anemia. Blood 2013;121:1242–1244.