Cold Agglutinin-Mediated Autoimmune Hemolytic Anemia

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KEYWORDS

• Autoimmune hemolytic anemia • B lymphocytes • Cold agglutinin
• Cold agglutinin disease • Cold agglutinin syndrome • Complement
• Lymphoproliferative disorders • Therapy

KEY POINTS

- Primary chronic cold agglutinin disease (CAD) is a clonal lymphoproliferative disorder and a distinct clinicopathologic entity.
- Secondary cold agglutinin syndrome (CAS) occasionally complicates specific infections or aggressive lymphomas.
- In both CAD and CAS, hemolysis is entirely complement dependent.
- Hemolysis is predominantly extravascular, mediated by the classical complement pathway.
- Targeting the pathogenic B-lymphocyte clone has resulted in successful therapy for CAD. Complement modulation is promising in specific situations, but has to be further developed and documented before clinical use.

INTRODUCTION

Cold antibody types account for approximately 25% of autoimmune hemolytic anemias (AIHA) and are classified as shown in **Box 1**.[1–3] Most cold-reactive autoantibodies are cold agglutinins (CA). CA are antibodies that bind to erythrocyte surface antigens at low temperatures, causing agglutination and complement-mediated hemolysis. We review the etiology, pathogenesis, clinical features, and therapy of CA-mediated AIHA, highlighting the role of complement involvement. Paroxysmal cold hemoglobinuria is not addressed, because it is described elsewhere in this issue and the involved autoantibodies are not agglutinins.

The authors have nothing to disclose.
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Hematol Oncol Clin N Am 29 (2015) 455–471
http://dx.doi.org/10.1016/j.hoc.2015.01.002 **hemonc.theclinics.com**
0889-8588/15/$ – see front matter

Box 1
Autoimmune hemolytic anemia

- Warm antibody type
  - Primary
  - Secondary
- Cold antibody type
  - Primary chronic cold agglutinin disease
  - Secondary cold agglutinin syndrome
    - Associated with malignant disease
    - Acute, infection associated
  - Paroxysmal cold hemoglobinuria
- Mixed cold and warm antibody type

Data from Refs.[1–3]

COLD AGGLUTININS

Cold hemagglutination was first described in 1903.[4] CA are determined semiquantitatively by their titer, based on their ability to agglutinate erythrocytes at 4°C.[5] A proportion of the adult population has demonstrable CA in serum without any evidence of hemolysis or disease; a frequency of positive screening tests at 0.3% has been reported in a cohort of patients with nonrelated disorders.[6,7] These normally occurring CA are polyclonal and are found in low titers, usually below 64 and rarely exceeding 256.[6,8] In 172 consecutive individuals with monoclonal immunoglobulin (Ig)M in serum, on the other hand, significant CA activity was found in 8.5% with titers between 512 and 65,500, and all individuals with detectable CA had hemolysis.[9] Thus, monoclonal CA are generally far more pathogenic than polyclonal CA.

The thermal amplitude is defined as the highest temperature at which the CA reacts with the antigen. In general, the pathogenicity of CA depends more on the thermal amplitude than on the titer.[10–12] The normally occurring CA have low thermal amplitudes. If the thermal amplitude exceeds 28°C or 30°C, erythrocytes agglutinate in the circulation in acral parts of the body, even at mild ambient temperatures and, often, complement fixation and complement-mediated hemolysis ensues. CA should not be confused with cryoglobulins. In rare cases, however, the cryoprotein can have both CA and cryoglobulin properties.[13,14]

Most CAs are directed against the Ii blood group system.[5,15] The I and i antigens are carbohydrate macromolecules and the density of these antigens on the erythrocyte surface are inversely proportional. Neonatal red blood cells almost exclusively express the i antigen, whereas the I antigen predominates in individuals of 18 months of age and older.[16] Therefore, CA with anti-I specificity are more pathogenic in children as well as adults than those specific for the i antigen. Occasionally, CA show specificity against the erythrocyte surface protein antigen designated Pr and such CA can be highly pathogenic.[17,18] Other specificities have been reported, but are probably very rare.[8] More than 90% of pathogenic CA are of the IgM class and these IgM macromolecules can be pentameric or hexameric.[19–21] Hexameric IgM is more pathogenic than pentameric IgM.[20]

The terms *cold agglutinin disease* (CAD) and *cold agglutinin syndrome* (CAS) have been used in the literature in a rather random way. We should distinguish, however,

between these concepts. CAD is a well-defined clinicopathologic entity, as shown herein and should be called a disease, not syndrome.[3,22] The term CAS is appropriate for the secondary CA-mediated syndrome occasionally complicating specific infections or malignancies.

CHRONIC COLD AGGLUTININ DISEASE

Epidemiology

Primary CAD has been reported to account for about 15% of AIHA.[1,2] In Scandinavia, the prevalence has been estimated to 16 per million inhabitants and the incidence rate to 1 per million per year, which is probably a slight underestimation.[21] There seems to be a slight female preponderance with a male-to-female ratio of approximately 0.6:1. In the same population-based, descriptive study, the median age of the patients was 76 years (range, 51–96) and the median age at onset of clinical symptoms 67 years (30–92).[21]

Clonality and Histopathology

The first monoclonal protein ever described was a CA from a patient with CAD[23]; early studies showed that, in most patients, the CA was monoclonal IgM with kappa light chain restriction.[24,25] In a more recent study of 86 patients, the CA was found to be monoclonal IgM-kappa in more than 90% of patients.[21] Monoclonal IgG, IgA, or lambda light chain restriction were rare findings.[21] In 6% of the patients, monoclonal Ig could not be detected despite otherwise typical primary CAD. This is probably a matter of sensitivity. Furthermore, 90% of patients in whom flow cytometry of bone marrow aspirate had been performed, had a clonal expansion of kappa-positive B cells.[21] The CA in CAD are almost always specific for the I antigen and show restriction to the *IGHV4-34* gene segment.[5,22,26] These findings have led to the conclusion that patients with CAD must have a clonal B-cell lymphoproliferative disorder, which has not been elucidated fully until recent years.

Two large, retrospective studies of consecutive patients found signs of a bone marrow clonal lymphoproliferation in most patients.[21,27] Undoubtedly, this majority represents the same group of patients that has traditionally been diagnosed with “primary” or “idiopathic” CAD. Within each series, however, the individual hematologic and histologic diagnoses showed a striking heterogeneity.[21,27] In 1 series, lymphoplasmacytic lymphoma was the most frequent finding, whereas marginal zone lymphoma, unclassified clonal lymphoproliferation, and reactive lymphocytosis were also reported frequently.[21]

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 reexamined systematically by a group of lymphoma pathologists using a standardized panel of morphologic, immunohistochemical, flow cytometric, and molecular methods.[22] 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 lymphoplasmacytic lymphoma, marginal zone lymphoma, and other previously recognized lymphoma entities (**Fig. 1**). The MYD88 L265P somatic mutation, typical for lymphoplasmacytic lymphoma, could not be detected in any of 15 samples from patients with CAD tested for this mutation, even though a sensitive, polymerase chain reaction-based method was used.[22,28,29]

Complement-Mediated Hemolysis

Cooling of blood during passage through acral parts of the circulation allows CA to bind to erythrocytes and cause agglutination (**Fig. 2**). Being a strong complement

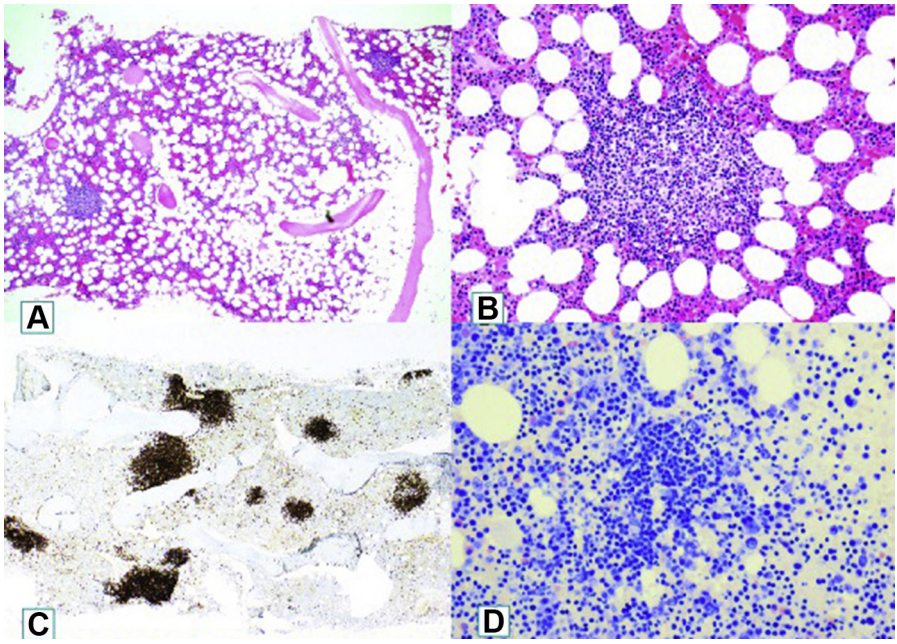

Fig. 1. Primary cold agglutinin-associated lymphoproliferative disease. Bone marrow trephine biopsy showing intraparenchymatous nodular lymphoid lesions (*A* and *B*; stain: hematoxylin and eosin; original magnification, ×40 and ×200, respectively). Immunoperoxidase staining for CD20 highlights clonal B-cell infiltration (*C*; original magnification, ×200). Mast cells are not usually discerned around the lymphoid lesions (*D*; stain: Giemsa; original magnification, ×200). (*From* Randen U, Troen G, Tierens A, et al. Primary cold agglutinin-associated lymphoproliferative disease: a B-cell lymphoma of the bone marrow distinct from lymphoplasmacytic lymphoma. Haematologica 2014;99(3):499, with permission.)

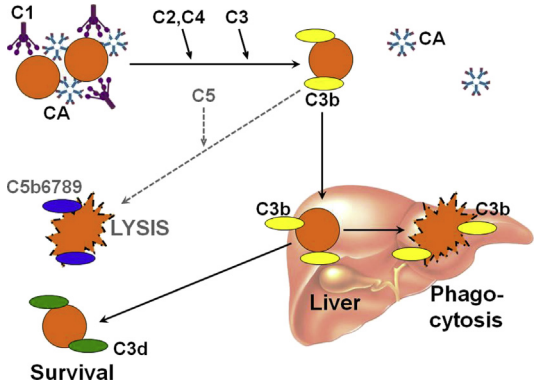

Fig. 2. Complement-mediated hemolysis in CA disease and CA syndrome. *Black arrows*, major pathway; *dotted arrows*, minor pathway; C, complement protein; CA, cold agglutinin.

activator, antigen-bound IgM CA on the cell surface binds complement protein 1 (C1) and thereby initiates the classical complement pathway.[30–32] C1 esterase activates C4 and C2, generating C3 convertase, which results in the cleavage of C3 to C3a and C3b. Upon returning to central parts of the body with a temperature of 37°C, IgM CA detaches from the cell surface, allowing agglutinated cells to separate, while C3b remains bound. A proportion of the C3b-coated erythrocytes is sequestered by macrophages of the reticuloendothelial system, mainly Kupffer cells in the liver. On the surface of the surviving red blood cells, C3b is cleaved, leaving high numbers of C3d molecules on the cell surface. These mechanisms explain why the monospecific direct antiglobulin test (DAT) is strongly positive for C3d in patients with CA-mediated hemolysis and, in the majority, negative for IgM and IgG.[21]

Complement activation may proceed beyond the C3b formation step, resulting in C5 activation, formation of the membrane attack complex (MAC), and intravascular hemolysis. Owing to surface-bound regulatory proteins such as CD55 and CD59, however, the complement activation is usually not sufficient to produce clinically significant activation of the terminal complement pathway. The major mechanism of hemolysis in stable disease, therefore, is the extravascular destruction of C3b-coated erythrocytes.[30,32,33] Obviously, however, C5-mediated intravascular hemolysis does occur in severe acute exacerbations and in some profoundly hemolytic patients, as evidenced by the observation of hemoglobinuria in 15% of the patients,[27] the rather frequent finding of hemosiderinuria (M. J. Stone 2014, personal communication) and the beneficial effect of C5 inhibition in at least occasional patients.[34]

Clinical Features

By definition, all patients with CAD have hemolysis, but occasional patients are not anemic because the hemolysis is fully compensated. Most patients, however, have manifest hemolytic anemia. The anemia can be more severe than often stated in textbooks and review articles. Of 16 patients described in an early publication, 5 had hemoglobin (Hgb) levels below 7.0 g/dL and 1 had levels below 5.0 g/dL.[25] Hgb levels ranged from 4.5 g/dL to normal in a more recent, population-based, descriptive study of 86 Norwegian patients.[21] The median Hgb level was 8.9 g/dL and the lower tertile was 8.0 g/dL. Hemoglobinuria has been reported in at least 15% of the patients.[27] About 50% of the patients are considered transfusion dependent for shorter or longer periods during the course of the disease.[21,27] In many patients, therefore, CAD is not an indolent disease in terms of clinical symptoms and quality of life.

Approximately 90% of the patients experience cold-induced acrocyanosis and/or Raynaud phenomena.[21] The circulatory symptoms can range from slight to disabling. In cool climates, characteristic seasonal variations in the severity of hemolytic anemia have been well-documented.[35] In at least 70% of patients, exacerbation of hemolytic anemia is also triggered by febrile infections or major trauma.[21,36,37] The explanation for this paradoxical exacerbation is that, during steady-state CAD, most patients are complement depleted with low levels of C3 and, in particular, C4. During acute phase reactions, C3 and C4 are replete and complement-induced hemolysis increases.[5,37]

The median overall survival of patients with CAD has been estimated to be 12.5 years, similar to that of a general age- and sex-matched population.[21] Transformation of the lymphoproliferative bone marrow disorder to aggressive lymphoma is rare, probably with a cumulative rate of 3% to 4%. The clinical course is unpredictable; patients can experience either worsening or improvement with time, quite stable disease or, occasionally, a shift in the respective clinical manifestations.[21,36]

Diagnosis

The diagnosis of CAD should be based on history and clinical findings, assessment of hemolysis, the DAT pattern, and the CA titer. An additional electrophoretic, histopathologic, and flow cytometric workup should always be done, but demonstration of clonality may sometimes be difficult and is not absolutely required for diagnosis in the routine clinical setting. **Table 1** shows the diagnostic criteria.[3,38] Correct handling of samples as indicated in **Table 1** is essential for reliable assessment.[3,38]

Nonpharmacologic Management

Given that drug therapy has been largely ineffective until the last 10 to 15 years, counseling has been considered the mainstay of management.[25,39] Owing to the high thermal amplitude of the CA, however, the physiologic cooling of the blood in the peripheral vessels is usually sufficient to cause hemolysis and circulatory symptoms even at mild ambient temperatures.[10,21]

Most authors agree that patients should avoid cold exposure, particularly of the head, face, and extremities.[25,39–41] Those living in cool climates often, even before the diagnosis has been established, tell the physician that they use warm clothing and, in many cases, stay indoors during winter. Many patients experience improvement of Hgb levels and circulatory symptoms when temporarily relocating to a warmer climate during the cold season, but severely symptomatic CAD does exist even in the subtropics. Any liquids infused should be prewarmed, and surgery under hypothermic conditions should be avoided or specific precautions undertaken.

Erythrocyte transfusions can be given safely, provided that appropriate precautions are undertaken.[40,42] In contrast with the compatibility problems encountered in warm

Table 1
Diagnostic criteria for primary chronic CA disease

Level	Criteria	Procedures and Comments
Required for diagnosis	Chronic hemolysis	
	Polyspecific DAT positive	
	Monospecific DAT strongly positive for C3d	DAT is usually negative for IgG, but occasionally weakly positive
	CA titer $\geq$64 at 4°C	Blood specimen must be kept at 37°C–38°C from sampling until serum is removed from the clot
	No overt malignant disease	Clinical assessment; radiology as required
Confirmatory but not required for diagnosis	Monoclonal IgMκ in serum (or, rarely, IgG, IgA, or λ phenotype)	Serum must be obtained as for CA titer Immunofixation should be done even if no band is visible on electrophoresis
	Cellular κ/λ ratio >3.5 (or, rarely, <0.9) in B-lymphocyte population	Flow cytometry in bone marrow aspirate
	CA-associated lymphoproliferative bone marrow disorder by histology	Trephine biopsy

Abbreviations: CA, cold agglutinin; DAT, direct antiglobulin test; Ig, immunoglobulin.

Data from Berentsen S, Tjonnfjord GE. Diagnosis and treatment of cold agglutinin mediated autoimmune hemolytic anemia. Blood Rev 2012;26(3):107–15; and Berentsen S, Beiske K, Tjonnfjord GE. Primary chronic cold agglutinin disease: an update on pathogenesis, clinical features and therapy. Hematology 2007;12(5):361–70.

antibody AIHA, it is usually easy to find compatible donor erythrocytes, and screening tests for irregular blood group antibodies are most often negative. Antibody screening and, if required, compatibility tests should be performed at 37°C. The patient and, in particular, the extremity chosen for infusion should be kept warm, and the use of an in-line blood warmer is recommended.[39,40,42] Failure to adhere to required precautions has resulted in dismal or, very rarely, even fatal outcomes.[42,43] Because complement proteins can exacerbate hemolysis, transfusion of blood products with a high plasma content should probably be avoided.[37]

Based on theoretical considerations and clinical experience, plasmapheresis is regarded an efficient "first-aid" in acute situations or before surgery requiring hypothermia, because almost all IgM is located intravascularly.[44] Such remissions, however, are very short lived and concomitant specific therapy should usually be initiated.[39,42] Complement inhibitor-based alternative approaches to this situation are discussed elsewhere in this article. Given that the extravascular hemolysis predominantly takes place in the liver, splenectomy should not be used for the treatment of CAD. Three splenectomized patients were registered in our population-based descriptive study; none of them responded.[21] Improvement after splenectomy has been reported occasionally among the rare patients with CAD mediated by an IgG CA instead of IgM.[45]

Unspecific Immunosuppression and Supportive Drug Therapy

In textbooks and review articles, it is often postulated that typical patients with CAD are just slightly anemic and do not require pharmacologic therapy. Based on the Hgb levels and clinical features described herein, this holds true for a minority only. In Norway as well as the United States, drug therapy had been attempted in 70% to 80% of unselected patients studied in 2 relatively large, retrospective series.[21,27]

In contrast with warm antibody AIHA, corticosteroids are of little or no value in CAD.[21,27,39,40,46] Monotherapy with alkylating agents has shown some beneficial effect on laboratory parameters and clinical improvement has been observed.[47,48] The clinical response rates, however, are probably in the same low order of magnitude as for corticosteroids.[21] In 2 small series of patients treated with interferon-α or low-dose cladribine, these drugs were not shown to be useful, although some conflicting data do exist for interferon-α.[49–51] Only a few patients treated with azathioprine have been reported; none of them responded.[21]

Exacerbations precipitated by febrile illnesses should warrant immediate treatment of any bacterial infection.[37,38] Supportive therapy with erythropoietin or its analogs seems to be used widely in North America, but less often in Scandinavia and Western Europe. No studies have been published to support or discourage its use. Although poorly documented, folic acid supplements have often been recommended.[39]

Therapies Directed at the Pathogenic B-Cell Clone

The relative success in therapy for CAD during the last 10 to 12 years has been achieved by targeting the pathogenic B-cell clone.[52–54]

Rituximab monotherapy

Monotherapy with rituximab 375 mg/m^2 weekly for 4 weeks was studied in 2 prospective, uncontrolled trials of 37 and 20 treatment courses.[55,56] The response criteria used in the Norwegian study are shown in **Table 2**; similar strict definitions were used in the Danish study. The overall response rate was 54% and 45% in the 2 trials. With the exception of 1 complete response (CR) observed in the Norwegian trial, all remissions were partial responses (PR). Ten patients were treated for relapse after previously

Table 2
CAD: response criteria used in clinical trials

Response Level	Definition
Complete response (CR)	Absence of anemia No signs of hemolysis Disappearance of clinical symptoms of CAD No monoclonal serum protein No signs of clonal lymphoproliferation as assessed by bone marrow histology, immunohistochemistry and flow cytometry
Partial response (PR)	A stable increase in hemoglobin levels by ≥2.0 g/dL or to the normal range A reduction of serum IgM levels by ≥50% of the initial level or to the normal range Improvement of clinical symptoms Transfusion independence
No response (NR)	Any outcome not meeting the criteria for CR or PR

Abbreviations: CAD, cold agglutinin disease.

Data from Berentsen S, Ulvestad E, Gjertsen BT, et al. Rituximab for primary chronic cold agglutinin disease: a prospective study of 37 courses of therapy in 27 patients. Blood 2004;103(8):2925–8; and Berentsen S, Randen U, Vagan AM, et al. High response rate and durable remissions following fludarabine and rituximab combination therapy for chronic cold agglutinin disease. Blood 2010;116(17):3180–4.

having received rituximab therapy and 6 of them responded to a second course. In our study, the responders achieved a median increase in Hgb levels of 4.0 g/dL. We found a median time to response of 1.5 months (range, 0.5–4.0) and median response duration of 11 months (range, 2–42).[55]

In a population-based study of 86 unselected Norwegian patients with primary CAD, 40 patients were reported to have received rituximab monotherapy.[21] As far as permitted by available data, the same response criteria as previously published (see **Table 2**) were used for the retrospective analysis. Twenty-three patients (58%) were found to have responded; 2 (5%) achieving CR and 21 (53%) PR.[21] Responses had been observed after a second and even a third course of rituximab in patients who had relapsed after previous therapy. A descriptive, retrospective, single-center study from the United States noted an 83% overall response rate to single agent rituximab, although the response criteria were not specified.[27] These findings confirm the essential results of the prospective studies; rituximab monotherapy is an efficient treatment for primary CAD. CR is uncommon, however; the median response duration is relatively short and the number of nonresponders is considerable.

Adverse effects were few and tolerable in all 4 series.[21,27,55,56] Data from rituximab maintenance in patients with follicular lymphoma indicate that even prolonged or repeated administration of this monoclonal antibody is safe with regard to infections.[57] Very rare cases of progressive multifocal leukoencephalopathy and hepatitis B reactivation have been reported, however, in patients receiving rituximab for polyclonal autoimmune disorders.[58] Any causal associations are uncertain because of concomitant immunosuppressive therapies and immune dysregulation as part of the autoimmune disease itself.

Fludarabine and rituximab combination therapy

The safety and efficacy of combination therapy with fludarabine and rituximab was studied in a prospective, uncontrolled trial in 29 patients aged 39 to 87 years (median, 73) with primary CAD requiring treatment.[59] The participants received rituximab 375 mg/m^2 on days 1, 29, 57, and 85 and fludarabine orally 40 mg/m^2 on days 1

through 5, 29 through 34, 57 through 61, and 85 through 89. We used the same response criteria as published previously (see **Table 2**). Twenty-two patients (76%) responded, 6 (21%) achieving CR and 16 (55%) achieving PR. Among 10 patients nonresponsive to rituximab monotherapy, CR was observed in 1 patient and PR in 6. Median increase in Hgb level was 3.1 g/dL in the responders and 4.0 g/dL among those who achieved CR. Median time to response was 4.0 months. The lower quartile of response duration was not reached after 33 months, and estimated median response duration was more than 66 months (**Fig. 3**).

Grade 3 and 4 hematologic toxicity occurred in 12 patients (41%); neutropenia accounted for all cases of grade 4 toxicity. Seventeen patients (59%) had infection grade 1 through 3, which was successfully treated in all except for 1 elderly, frail nonresponder who died of pneumonia 9 months after treatment. Cotrimoxazol or antiviral prophylaxis was not given routinely. Infection grade 4 or *Pneumocystis jirovecii* pneumonia did not occur, but 3 patients (10%) experienced herpes zoster reactivation. Transient exacerbation of hemolytic anemia was seen in 3 patients (10%). All 3 were found to have exacerbation of CAD precipitated by infection,[37] whereas fludarabine-induced warm antibody AIHA was not observed. Nearly one-half of patients had their doses of fludarabine reduced because of hematologic toxicity.[59]

Comparison of nonrandomized trials should be undertaken with care. Nonetheless, the baseline data reported in the fludarabine–rituximab trial matched well with those described in the trials of rituximab monotherapy.[55,56,59] The response criteria (see **Table 2**) were identical in the 2 Norwegian studies and very similar to those used in the Danish monotherapy trial. The 76% response rate and more than 66 months estimated response duration achieved by using fludarabine and rituximab in combination,

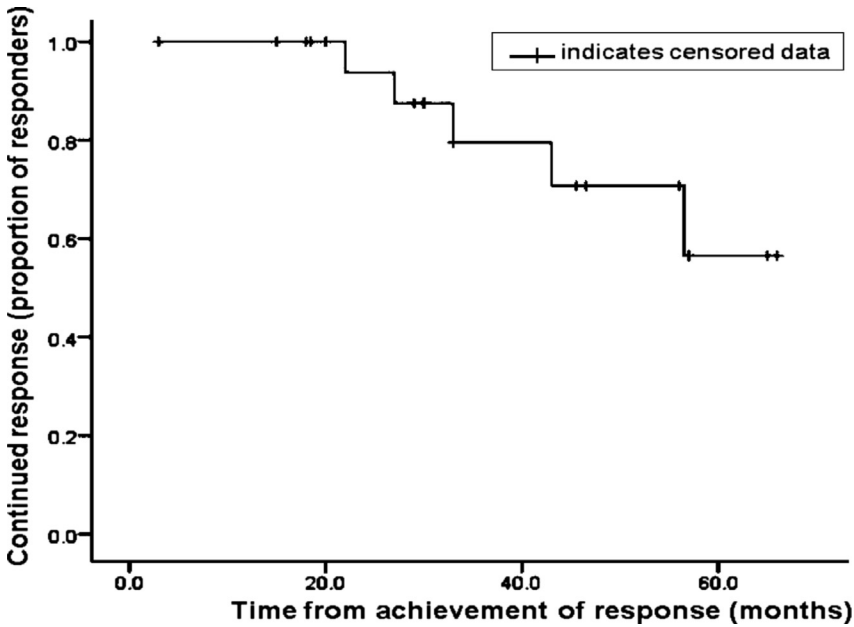

Fig. 3. Fludarabin and rituximab combination therapy for cold agglutinin disease: response duration. (*From* Berentsen S, Randen U, Vagan AM, et al. High response rate and durable remissions following fludarabine and rituximab combination therapy for chronic cold agglutinin disease. Blood 2010;116(17):3182, with permission.)

therefore, compare favorably with the 45% to 54% response rate and 11 months response duration observed after monotherapy with rituximab. Furthermore, CR was achieved in 21% of the patients after the combination therapy, whereas remissions are rarely complete after rituximab monotherapy. Ten patients acted as their own controls by receiving the combination after rituximab single agent therapy had failed.[59] With the achievement of 1 CR and 6 PR in this subgroup, the combination has been shown to be effective even in patients nonresponsive to monotherapy with rituximab.

Given the relative toxicity of the fludarabin–rituximab combination, the therapeutic potential should be weighed carefully against the short-term risks, particularly in very old and comorbid patients. We found, however, no association between adverse events and age itself.[59] The study was not designed to assess the risk of secondary hematologic malignancies. Although not specific to purine nucleoside analogs, late-occurring acute myelogenous leukemia and myelodysplastic syndromes have been observed after fludarabine-based therapy for Waldenström's macroglobulinemia.[60] This concern should not be prohibitive to the use of rituximab and fludarabine in combination, but rather lead to a balanced, individualized consideration of benefit versus long-term risk, particularly in the younger patients.

Other therapy targeting the clonal B cells

Favorable results of combination therapy with bendamustine and rituximab have been reported in 1 single patient, but no systematic study has been published to date.[61] Improvement after bortezomib-based therapy or a cyclophosphamide-containing combination has been described in single case reports.[14,62]

Future Perspectives: Complement-Directed Therapy?

Eculizumab, a monoclonal anti-C5 antibody, is a powerful therapeutic agent in paroxysmal nocturnal hemoglobinuria (PNH). In PNH, however, the predominant route of complement-mediated hemolysis is the intravascular cell destruction caused by activation of the terminal complement pathway, starting with C5 cleavage. Nevertheless, eculizumab therapy did produce major improvement of CAD according to 1 single case report.[9] Further studies may elucidate the representativeness of this observation.

The only classical pathway inhibitor currently available for clinical use is plasma-derived or recombinant C1-esterase inhibitor (C1-INH), which is approved for the treatment of hereditary angioedema (HAE). Although not a complement-mediated disorder, HAE is caused by lack or deficiency of endogenous C1-INH and replacement therapy is well-established.[63] In AIHA, on the other hand, endogenous C1-INH production is normal, indicating that physiologic concentrations of the inhibitor will not block complement-mediated hemolysis. Pharmacologic doses of C1-INH did, however, result in improvement in a well-described case of severe warm antibody AIHA.[64]

A recent in vitro study tested the effects of TNT003, a mouse monoclonal antibody targeting C1, on CA-induced complement activity against human erythrocytes.[30] This antibody has specificity for the complement serine protease C1s. Using CA samples from 40 patients with CAD, the authors found that TNT003 prevented CA-induced hemolysis. TNT003 also blocked deposition of C3 fragments on the erythrocytes at the same concentration of antibody that stopped hemolysis. Furthermore, the inhibition of C1s resulted in prevention of in vitro erythrophagocytosis by a phagocytic cell line. The monoclonal antibody also inhibited the CA-induced, classical pathway-driven generation of anaphylotoxins C4a, C3a, and C5a. Interestingly, CA from only 1 patient sample out of 40 was able to directly induce MAC-mediated hemolysis. These findings reveal an interesting potential for using a corresponding humanized antibody for the

treatment of CAD and, in addition, support the existing view that complement activity in CAD terminates before activation of the terminal pathway in most patients.[30,65,66]

Small molecule peptide inhibitors of the classical complement pathway are also of potential interest.[67] Peptide inhibitor of C1 (PIC1) is a recently described class of such molecules that targets C1q, blocking the activation of associated serine proteases (C1s–C1r–C1r–C1s) and subsequent downstream complement activation.[68] These molecules have also been shown to inhibit the lectin pathway.[68] PIC1 has been studied in acute hemolytic transfusion reaction in animal models but, so far, not in CA-related experiments. Another interesting low-molecular-weight inhibitor is compstatin Cp40, a peptide that blocks cleavage of C3. Although no specific data have been published for CA-induced hemolysis, compstatin has been found to efficiently prevent in vitro lysis of red blood cells from PNH patients.[69]

Given that the complement system is an essential part of the innate immune system, it is important to ask whether complement modulation will be too dangerous. Regarding eculizumab therapy in PNH, we know that the risk of severe infection after C5 inhibition is negligible, provided that the patients can be efficiently protected against meningococci.[70] Modulation at the C3 level may carry a much greater risk because efficient inhibition of C3 completely blocks complement activation beyond this level, whether initiated by the classical, alternative, or lectin pathway.[66,69]

Interestingly, the still more proximal blockade at the C1 level achieved by TNT003 selectively affects the classical pathway as required for control of hemolysis in CAD, whereas the lectin and alternative pathways remain intact. Probably, therefore, these pathways will still enable the system to generate anaphylotoxins C3a and C5a in response to microbial stimuli, even though the production of these anaphylotoxins induced by the classical pathway will be blocked.[30] Although this selectivity may, theoretically, reduce the risk of infection, careful studies are required to address this issue. Finally, temporary complement inhibition in acute situations (eg, acute phase reaction–induced exacerbation or preoperative management) may be expected to be less dangerous than long-term therapy.

SECONDARY COLD AGGLUTININ SYNDROME

Mycoplasma Infection

Mycoplasma pneumoniae pneumonia is the most frequent cause of secondary CAS and has been reported to account for 8% of the cases of AIHA.[2] Patients with *M pneumoniae* infections produce CA as part of the physiologic immune response, but most of them do not develop hemolytic anemia. Occasionally, however, production of high-titer, high-thermal amplitude CA can result in hemolysis.[71–73] Among 25 patients with *Mycoplasma* infection admitted to a referral center, 6 (25%) had hemolysis, which was severe in 2 patients and mild to moderate in 4.[73] In general hospitals and the community, the frequency of hemolytic complications is obviously much lower.

In CAS caused by *Mycoplasma*, the CA are polyclonal, anti-I–specific and almost invariably of the IgM class; and titers usually range between 512 and 32,000.[72] DAT is always positive for C3d. The onset of anemia typically occurs during the second or third week after the febrile infection has started. Although the hemolytic anemia can be severe, the prognosis is generally good and the complication is self-remitting within 4 to 6 weeks.[3,72] A lethal course has been described in 1 patient.[74]

Epstein–Barr Virus Infection

CA-mediated AIHA, usually but not invariably mild, occasionally complicates infectious mononucleosis with confirmed Epstein–Barr virus (EBV) etiology.[3,75–77]

Compared with *M pneumoniae* pneumonia, EBV is an infrequent cause of AIHA, accounting for approximately 1% of cases.[1,2] Conversely, the frequency of clinically significant hemolysis in EBV infections is unknown, but probably low.[3,76] The CA are polyclonal and almost invariably specific for the i antigen.[75,77]

Other Infections

Several series and case reports have described transient CAS mediated by anti-i autoantibodies after cytomegalovirus infection.[77,78] Rarely, CA-mediated hemolysis has been reported in adenovirus infections, influenza A, varicella, rubella, *Legionella pneumophilica* pneumonia, listeriosis, and pneumonia caused by *Chlamydia* species. Severe CAS with a prolonged course and cryoglobulin activity of the CA has been observed following *Escherichia coli* lung infection. Autoantibody specificities in these rare cases have included anti-I, anti-I, and anti-Pr.[3]

Aggressive Lymphoma

Among 295 consecutive individuals with AIHA described by Dacie,[2] 7 patients (2.4%) were classified as having CAS secondary to malignancies. Thus, CAS secondary to overt malignant disease is far more uncommon than primary CAD. The best documentation for a clinically malignant disease resulting in CAS has been provided in aggressive non-Hodgkin lymphoma.[79–81] CA found in such patients are usually monoclonal and anti-I–specific but, unlike CA in CAD, the light chain restriction can be lambda as well as kappa.[79]

Nonlymphatic Malignancies

Systematic series as well as case reports have described secondary CAS in a variety of other malignancies, including carcinomas, sarcomas, metastatic melanoma, and myeloproliferative disorders.[1–3] Some of these associations are probably real, whereas in other examples the documentation and association may be questioned, as extensively discussed elsewhere.[3]

Therapy for Secondary Cold Agglutinin Syndrome

For obvious reasons, treatment of the underlying disease is important when possible. In curable malignancies such as aggressive lymphoma, achieving complete remission is usually accompanied by resolution of the hemolytic anemia.[3,81] *M pneumoniae* pneumonia should be treated according to existing guidelines, although, owing to the time of onset of hemolysis, antibiotic therapy will often have been initiated before the hemolytic anemia manifests itself.[72] In some viral infections, causal therapy is not possible; in others, antiviral therapy is not necessary because the CAS is slight and self-remitting.

No evidence-based therapy exists for secondary CAS per se.[3] Prospective studies or well-designed retrospective series have not been published, and recommendations have been based on clinical experience, case reports, and theoretic considerations. Improvement after the administration of corticosteroids has been described in several cases.[82,83] Because spontaneous remission occurs eventually in nearly all patients; however, guidelines cannot be built on case reports. Erythrocyte transfusions can safely be given provided the same precautions are observed as in primary CAD.[3,38,42] Plasmapheresis should be considered in selected, extreme cases.[42,84]

The possibility of intervention by complement modulation is very interesting in secondary CAS, but clinical documentation is currently nonexistent. The same options as discussed for CAD may be considered for investigation.[30,34,64,65,68,69] Unlike CAD, infection-associated acute CAS only requires temporary complement inhibition during the most severe hemolysis or until spontaneous resolution occurs. In theory, therefore,

the safety issues discussed for CAD may turn out to be less problematic in CAS. Ideally, the efficacy and safety of complement modulation in severe CAS should be explored in systematic series or prospective uncontrolled trials, which will have to be done at a very large, international scale to collect statistically solid data.

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