

## B-lymphocytes as Targets for Therapy in Chronic Cold Agglutinin Disease

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**Abstract:** Primary chronic cold agglutinin disease (CAD) is an autoimmune hemolytic anemia induced by cold reactive autoantibodies (cold agglutinins) against erythrocyte surface antigens. Corticosteroids or alkylating agents have been used in the treatment of CAD, but the results have been disappointing.

The cold agglutinins in CAD patients are monoclonal immunoglobulins, usually of the IgM $\kappa$  type encoded by the V<sub>H</sub>4-34 gene segment. Flowcytometric assessment of lymphocytes from bone marrow aspirates and immunohistochemical assessment of biopsy samples have revealed a monoclonal CD20<sup>+</sup>κ<sup>+</sup> B lymphocyte population in 90% of the patients.

These pathogenetic features have provided a basis for novel therapies in primary CAD. Infusions of rituximab, a chimeric human-murine anti-CD20 antibody known to be effective in B-cell lymphoma, produced partial response rates of approximately 50% and occasional complete responses. Median response duration, however, was only 11 months. Complement C3 and C4 depletion in many CAD patients, as well as Fcγ-RIIIa receptor polymorphism, have been proposed as explanations for the inconstant efficacy of rituximab therapy. In order to increase response rates and response duration, we are undertaking a phase 2 study of rituximab and fludarabine combination therapy. The preliminary results are encouraging, but further studies are required in order to allow firm conclusions.

**Key Words:** B lymphocytes, cold agglutinin disease, fludarabine, hemolytic anemia, lymphoproliferative, rituximab.

### INTRODUCTION

Cold agglutinins (CA) are essential to the pathogenesis of chronic cold agglutinin disease (CAD), a subgroup of autoimmune hemolytic anemia (AIHA) [1-3]. The majority of CA are IgM proteins, and they are occasionally also found in low titers in healthy adults [3-5]. CA bind to erythrocyte surface antigens at a temperature optimum of 0-4°C. Monoclonal CA, however, often have a high thermal amplitude, which contributes to their pathogenicity at temperatures approaching 37°C [2, 6-8]. Binding of CA causes agglutination of erythrocytes [1, 2, 9], and the antigen-antibody complex induces complement activation and hemolysis [7, 10]. CAD has traditionally been classified into a primary or idiopathic type which has been regarded unrelated to lymphoma or other underlying conditions, and a secondary form associated with malignant disease, most often lymphoma [11, 12]. CA mediated AIHA complicating *Mycoplasma pneumoniae* or viral infection occurs as an acute, transient condition and will not be considered in this review.

In a recently published population-based clinical study of primary CAD in Norway, the prevalence was calculated to 16 per million and the incidence rate to 1 per million per year [5]. Little is known about possible geographic variations because previous prevalence estimations have been built upon less exact data [13]. Median age at onset of symptoms is approximately 67 years and median age of CAD pa-

tients is about 76 [5]. According to single-center series, primary CAD accounts for 13-15% of the cases of AIHA [11, 14, 15]. Cold-induced circulatory symptoms, although often not emphasized by physicians, are considered typical for CAD [9, 12, 16]. We found that more than 90% of patients with primary CAD had such symptoms [5], which may range from moderate acrocyanosis to severe Raynaud phenomena precipitated even by very slight cold exposure. The hemolytic anemia shows characteristic seasonal variations [1, 9, 17]. In contrast to the well-known worsening caused by low ambient temperatures, exacerbation may also occur during febrile illnesses and other conditions associated with an acute phase reaction [2, 5, 18, 19].

According to review articles, anemia in CAD is variable and usually not severe [1, 12]. Out of 16 patients described in an early report, however, five had minimum hemoglobin (Hb) levels below 7.0 g/dL and one below 5.0 g/dL [9]. In a series of 86 patients, we found a median Hb level of 8.9 g/dL, and one third of the patients had Hb levels at presentation ranging from 4.5 through 8.0 g/dL [5]. Approximately 50% were considered transfusion dependent at some time during the course of the disease [5].

Although counseling on cold avoidance is often recommended as the treatment of choice for most patients with primary CAD [12, 20, 21], a systematic evaluation showed that in more than 70% of cases, the physician and/or the patient did not perceive such measures as sufficient [5]. As in other immune mediated diseases, corticosteroid treatment still has some popularity among physicians. We found that 32 (37%) of 86 CAD patients had received corticosteroids

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for shorter or longer periods [5]. The response rate, however, was only 14%, and three of the five responders required high doses that were unacceptable for long-term use [5]. Poor efficacy of corticosteroid therapy has also been reported from several small series and case studies [16, 20]. Monotherapy with alkylating agents has been recommended for decades, mainly based on observations of improvement of laboratory parameters [22, 23]. However, the clinical benefit has been shown to be poor [5, 16, 20]. Single agent therapies with interferon- $\alpha$  and cladribine have also failed to produce encouraging results [24, 25]. Furthermore, theoretical considerations and clinical experience do not support the use of splenectomy as a therapeutic procedure [5, 20].

Considerable progress has been made during the last years in the knowledge of cellular immunology, pathogenesis, candidate targets for therapy, and more efficient therapeutic measures in this challenging disorder. We will review relevant findings by our group and others on pathogenetic features of primary CAD. Based on these results, we will then provide an overview of more recent therapeutic options and give some suggestions for further studies.

#### CHARACTERISTICS AND SYNTHESIS OF COLD AGGLUTININS

CA can be determined by their ability to agglutinate erythrocytes at 4°C [2]. The concept of CA should not be confused with that of cryoglobulin, although obvious similarities do exist between primary CAD and cryoglobulinemia type I and II [26]. Immunoglobulins have occasionally been described that possess both CA and cryoglobulin properties [3, 16, 27]. In the great majority of CAD patients, CA are specific for the erythrocyte surface carbohydrate antigen termed I [2, 3]. Specificities other than anti-I have been described, in particular anti-Pr and anti-P [3, 28]. The red cell destruction induced by the antigen-antibody binding may in some situations partly occur as an intravascular hemolysis mediated by triggering of the classical and lytic complement pathway. Studies have shown, however, that phagocytosis of complement-opsonized cells by reticulo-endothelial phagocytes in the liver is responsible for most of the removal of erythrocytes in stable CAD patients. [8, 10, 29, 30].

The thermal amplitude, defined as the temperature interval at which the antibody will react with the antigen, appears to be more important than the titer for the pathogenicity of CA [7, 8, 14]. The CA found in some healthy individuals are usually present in titers below 10, and titers in excess of 256 are very uncommon in this group [3, 31]. The thermal amplitudes of these normal cold-reactive autoantibodies do not exceed 15-20°C and, therefore, they are of no clinical significance [3].

Already in 1957, Christenson and co-authors found that CA in CAD may sometimes be seen as an abnormal peak in the  $\gamma$  region by electrophoretic separation of serum proteins on cellulose columns [32]. During the 1960's, Harboe and co-workers characterized this immunoglobulin as monoclonal IgM $\kappa$  [4, 33, 34]. In a study of sera from 172 patients with

monoclonal IgM associated with a variety of clinical disorders, CA were identified in 10 sera (8.5%) [35]. In our population-based descriptive study of primary CAD, a monoclonal immunoglobulin was detected by electrophoresis and immunofixation in sera from 79 (94%) of 84 patients with available data [5]. The monoclonal immunoglobulin was of the IgM class in 71 patients (90%), IgA and IgG in three (3.5%) each, while two patients (2.5%) had clonal bands of both IgG and IgM. The light chain restriction was  $\kappa$  in 74 patients (94%),  $\lambda$  in two (2.5%) and unknown in three (3.5%) [5].

Pentameric as well as significant levels of hexameric IgM have been found in samples of purified CA from CAD patients [36]. Absence of J chains seems to enhance the formation of hexameric IgM and has been interpreted as a deleterious feature of IgM-mediated disorders, resulting in a higher ability to activate the complement cascade and thereby in a higher lytic efficiency of IgM [36, 37].

During B-lymphocyte maturation, each cell constructs its specific immunoglobulin heavy chain by assembly of coding sequences from the variable ( $V_H$ ), diversity (D), and joining ( $J_H$ ) gene segments. The diversity created by this recombination process is further increased by enzymatic modification at the cut ends of the gene segments, followed by the event of somatic hypermutation, typically occurring in the hypervariable segments of  $V_H$  genes. Pascual, Thorpe, Stevenson and others have shown that anti-I CA encoded by the  $V_H4-34$  gene segment, formerly termed  $V_H4.21$ , are preferentially found in serum samples from patients with primary CAD [38, 39]. This gene segment appears to be overrepresented among the coding unit repertoire, although it accounts for a very small fraction of normal circulating immunoglobulins [38, 40]. In order to assess the frequency of  $V_H4-34$  gene expression, we tested sera from 11 CAD patients with hemagglutination inhibition assay using the rat monoclonal anti-idiotypic antibody 9G4, which is specific for  $V_H4-34$  encoded protein. All patient sera were confirmed to be idiotope positive [2]. In contrast, "naturally" occurring CA in healthy individuals as well as CA artificially induced by Rhesus (D) immunization are often derived from  $V_H$  gene segments other than  $V_H4-34$  [40, 41].

#### COMPLEMENT CONSUMPTION AND "PARADOXICAL" EXACERBATION OF CAD

In 1998 Ulvestad described a CAD patient who, with advancing disease, experienced that the initial cold-induced exacerbations were substituted for "paradoxically" enhanced hemolytic anemia at elevated body temperatures [18]. The patient had decreasing complement protein C4 levels which eventually became undetectable, and the *in vitro* hemolytic activity of serum (CH50) declined to zero. Reduced complement component levels in CAD had previously been reported by Jonsen and co-authors [42].

In order to further explore these phenomena, we measured complement protein levels in 15 CAD patients and found reduced levels of C3 in nine and C4 in 11 patients, six of whom had low CH50 [2]. Five of these patients had experienced exacerbation of hemolysis during febrile illnesses.

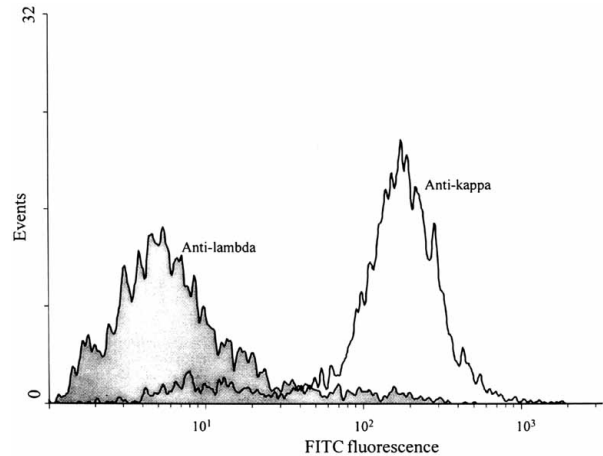
We also undertook a longitudinal, prospective, 12 month follow-up study of one single patient with “paradoxical” exacerbations of hemolysis [19]. In the absence of any acute events, low C3 and undetectable C4 levels were confirmed. We observed a non-functional classical complement pathway and a normal alternative pathway. Exacerbation of hemolytic anemia occurred during pneumonia and then again following a hip fracture with subsequent surgery, and was paralleled by increased CRP levels. During each of these acute events the serum IgM levels were temporarily reduced, and after the hip fracture we recorded increased C3 levels, detectable C4, significantly increased levels of the pro-inflammatory cytokines interleukin-6, tumor necrosis factor- $\alpha$  and interferon- $\gamma$ , and slightly increased interleukin-1 $\beta$  [19]. In our most recent study, data on exacerbation during febrile illnesses were available for 68 of 86 patients with primary CAD [5]. Fifty patients (64% of those with available data) had experienced exacerbation during febrile illnesses.

These observations are best explained by assuming that during steady state of CAD, a majority of patients have low levels of C3 and especially C4 because of a continuous consumption. Complement factors, in particular low C4 availability, seem to be rate-limiting for hemolysis. During acute phase reactions, C3 and C4 levels increase due to an enhanced production, resulting in an accentuation of hemolysis.

Clinicians should be aware of the potential practical implications of the complement consumption and depletion in many CAD patients. First, administration of complement-containing plasma products should probably be avoided. Second, these findings provide an explanation for the “paradoxical” exacerbation of hemolysis during conditions associated with acute phase reactions. Third, a non-functional classical complement pathway may have implications for the therapeutic potential of monoclonal antibodies in CAD [43-45].

#### MONOCLONAL B-LYMPHOCYTES IN PRIMARY CAD

The findings of monoclonal IgM $\kappa$  CA in serum of patients with CAD [4, 33, 34] and the demonstration that monoclonal immunoglobulin is present in most if not all patients [5, 16] provided indirect evidence for the existence of a clonal B-cell expansion even in the primary form of CAD. Flowcytometric investigations by Silberstein and co-workers disclosed B-cell clones in at least some patients [46]. In 1995 we reported the findings of lymphoplasmacytic lymphoma in the bone marrow of three consecutive patients otherwise classified as having primary CAD [47]. In a subsequent study by our group, CAD patients with no clinical or radiological evidence of an underlying lymphoma were examined by flow-cytometric immunophenotyping of bone marrow aspirates as well as morphological and immunohistochemical assessment of trephine biopsies [16]. A clonal lymphoproliferative bone marrow disease characterized by the CD19<sup>+</sup>,CD20<sup>+</sup>, $\kappa$ <sup>+</sup> phenotype was detected in 10 of 11 patients (Fig. 1).



**Fig. (1).** Flow cytometric analysis of light chain expression on B-cells in a patient with primary CAD. Cells from bone marrow aspirate were analyzed in a dual-parameter cytogram of orthogonal and forward light scatter. An acquisition gate containing lymphocytes was drawn and cells were analyzed by two-color fluorescence for CD19 and  $\lambda/\kappa$  chains. 93% of CD19<sup>+</sup> cells were  $\kappa$ <sup>+</sup> and 0.5% were  $\lambda$ <sup>+</sup>. Cells stained with FITC- and PE-labeled irrelevant antibodies served as negative control. (Reproduced with permission from APMIS [16]).

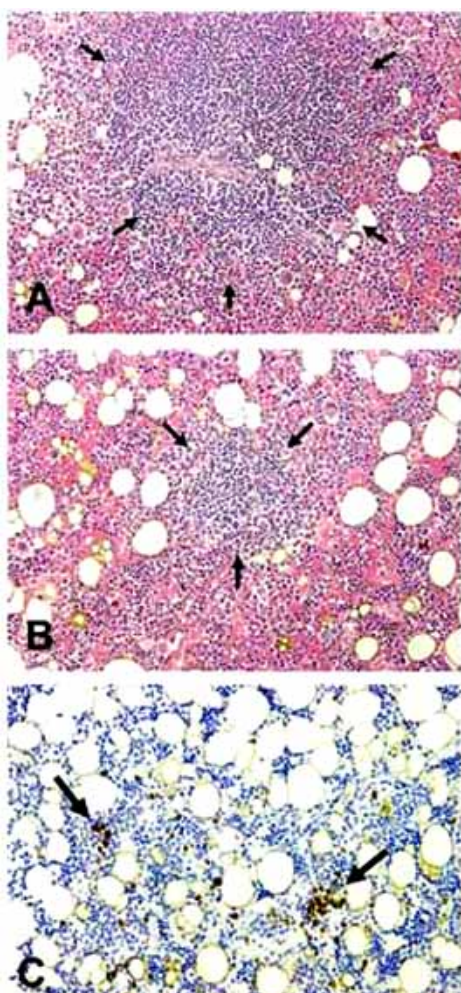
More recently, we examined the records of 86 patients otherwise classified as having primary CAD with regard to the presence of a clonal lymphoproliferative bone marrow disorder [5]. Again, monoclonal CD20<sup>+</sup> $\kappa$ <sup>+</sup> lymphocytes were found in the bone marrow of most patients in whom a flow-cytometric assessment had been performed. Based on previous published data [16, 48], a cellular  $\kappa/\lambda$  ratio > 3.5 was considered strongly indicative of a clonal lymphoproliferative B-cell disorder. The median  $\kappa/\lambda$  ratio was 7.8 (range 0.9-186), and a ratio higher than 3.5 was found in 36 (90%) of 40 patients with available data. Information on bone marrow histology was available for 66 patients as shown in Table 1. Morphologic and immunohistochemical signs of non-Hodgkin's B-cell lymphoma (Fig. 2) were found in 50 patients (76%). Applying the WHO classification [49], 33 patients had lymphoplasmacytic lymphoma (50% of patients with available histology data and 66% of those with a demonstrable clonal lymphoproliferative bone marrow disorder). According to recent criteria, Waldenström's macroglobulinemia (WM) is defined as lymphoplasmacytic lymphoma of the bone marrow combined with monoclonal IgM at any serum concentration [50]. When these criteria were applied, 33 CAD patients (50% of those with available histology data) met the diagnostic criteria for both primary CAD and WM [5]. On the other hand, we have observed an occasional patient with CAD and a monoclonal IgM $\kappa$  for more than 20 years without any demonstrable clonal B-cell population as repeatedly assessed by flow cytometry and immunohistochemistry.

Given the high frequency of bone marrow lymphoma and diagnostic overlap with WM, one may argue that most cases of primary CAD should be re-classified as secondary or that

**Table 1. Bone Marrow Histology**

	<i>n</i>	%
<i>Normal findings or reactive lymphocytosis</i>	7	11
<i>Irregular lymphoid hyperplasia</i>	9	13
<i>Non-Hodgkin's B cell lymphoma</i>	50	76
Lymphoplasmacytic lymphoma	33	50
Marginal zone lymphoma	5	8
Small lymphocytic lymphoma/ Chronic lymphocytic leukemia	4	6
Clonal lymphocytosis/ Other small B cell lymphoma	8	12
<i>Total</i>	66	100

the distinction between primary and secondary CAD should be abandoned. However, CAD patients diagnosed by us to



**Fig. (2).** Bone marrow trephine biopsy. B-cell infiltrates (arrows) may be large (A), small (B) or so discrete (C) that they are only discovered by means of immunohistology. All images are taken at identical magnification (objective x20) to allow comparison of infiltrate size. A and B: HE-stain. C: Immunohistological staining with monoclonal anti-CD20 antibody, visualized with horse radish peroxidase and diaminobenzidine. Courtesy of Dr. Klaus Beiske.

have a low-grade lymphoproliferative bone marrow disorder undoubtedly represent the same majority that used to be classified as having primary CAD [3, 16, 43]. Except in the uncommon event of transformation, these lymphoproliferative disorders seldom, if ever, show features of a clinically overt lymphoma even after decades [5]. Furthermore, most of the rare patients traditionally classified as having secondary CAD suffer from a readily demonstrable lymphoma, often of an aggressive type, that can be associated with IgM $\lambda$  as well as IgM $\kappa$  CA [51, 52]. Therefore, we have continued to apply the term primary CAD in patients not showing the classical features of the secondary form.

#### ANTI-CD20 THERAPY IN CAD

Based on the recognition of primary CAD as a clonal lymphoproliferative B-cell disease, targets can be defined for development and trials of new therapeutic modalities. The monoclonal, chimeric, human-murine anti-CD20 antibody rituximab has been used successfully for the treatment of several other CD20<sup>+</sup> lymphoproliferative diseases [53, 54]. Compared with most cytotoxic drugs, the adverse effects of rituximab are different and less severe, and the B lymphocyte elimination is not cell cycle dependent [53, 54]. Remission of CAD after the administration of rituximab was first described in a case report by Lee and Kueck [55]. Since then, one small [56] and two somewhat larger [43, 57] phase 2 trials have been published in addition to a number of case reports. In the first 16 published case reports, all patients improved after rituximab therapy, and a high proportion of the responses were classified by the authors as complete [58, 59]. This probably reflects the fact that response rates estimated from case reports are likely to have been influenced by publication bias, lack of strict disease definitions, and heterogeneous or lacking response criteria.

We reported on 37 courses of rituximab single agent therapy administered prospectively to 27 patients with primary CAD [43]. Table 2 shows the inclusion criteria and Table 3 summarizes the response criteria. Each eligible patient received a course of rituximab at a dose of 375 mg/m<sup>2</sup> on day 1, 8, 15 and 22. Re-treatment was allowed in patients who responded but later relapsed. Fourteen of 27 patients responded to their first course of rituximab, and six of ten

**Table 2. Inclusion and Exclusion Criteria for Therapeutic Trials<sup>1</sup>**

Inclusion criteria	Exclusion criteria
<ol style="list-style-type: none"> <li>1. CAD diagnosis defined by the combination of –                             <ol style="list-style-type: none"> <li>a. Chronic hemolysis</li> <li>b. Cold agglutinin titer <math>\geq 64</math></li> <li>c. Positive direct antiglobulin test when performed with polyspecific antiserum, negative (or only weakly positive) with anti-IgG, and strongly positive with anti-C3d</li> </ol> </li> <li>2. The presence of a clonal B-cell lymphoproliferative disorder defined by –                             <ol style="list-style-type: none"> <li>a. Monoclonal IgM<math>\kappa</math> band by serum electrophoresis and immunofixation, and</li> <li>b. Lymphocyte phenotype with <math>\kappa/\lambda</math>-ratio <math>&gt; 3.5</math> and CD20<sup>+</sup>,<math>\kappa</math>+ co-expression, using flowcytometric immunophenotyping of bone marrow aspirates</li> </ol> </li> <li>3. Clinical symptoms requiring treatment, such as anemia or Raynaud-like symptoms</li> <li>4. Informed consent</li> </ol>	<ol style="list-style-type: none"> <li>1. An aggressive lymphoma</li> <li>2. Blood lymphocyte count <math>&gt; 50 \cdot 10^9/L</math></li> <li>3. Non-lymphatic malignant disease other than basal cell carcinoma</li> <li>4. Contra-indications to therapy with study drug</li> <li>5. Inability to cooperate</li> </ol>

<sup>1</sup>as used in trials of therapy for CAD with rituximab, purine analogues or combinations [25, 43, 56, 79, 80].

Inclusion criteria 2a-b (clonality) and 3 were not required for the descriptive studies [2, 5, 16].

responded to re-treatment. In both groups combined, responses were achieved after 20 of 37 courses, resulting in an overall response rate of 54%. We observed one complete and 19 partial responses. Responders achieved a median increase in Hb levels of 4.0 g/dL and a median decrease in IgM levels by 54%. Clinical and laboratory data indicated a benefit even in some patients classified as non-responders. Median time to response was 1.5 months (range, 0.5-4.0) and median observed response duration was 11 months (range, 2-42). No serious adverse effects occurred with the rituximab therapy.

The results of a subsequent similar study of 20 patients by Schöllkopf and co-workers fit in very well with our findings, although they reported a shorter response duration [57]. Some minor discrepancies between the results of the two studies can probably be explained by slightly different inclusion and response criteria. In our retrospective, population

based study that included patients treated within as well as outside study protocols, 40 patients had received one or more courses of rituximab single agent therapy [5]. According to the criteria shown in Table 3, complete response had been achieved in two patients (5%) and partial response in 21 (53%), giving an overall response rate of 58%. Despite the retrospective design, these figures provide further support to the results of the prospective trials.

In our prospective trial, a clinical response to rituximab therapy was accompanied by a histologic response in a majority of cases [43]. However, we observed patients achieving a partial response without a significant histologic regression of the bone marrow disorder, and in other patients we found a significant improvement of bone marrow histology without any clinical improvement. Moreover, we observed an almost complete elimination of CD20<sup>+</sup> cells from bone

**Table 3. Response Criteria<sup>1</sup>**

Complete response	Absence of anemia No signs of hemolysis Disappearance of clinical symptoms of CAD Undetectable monoclonal serum protein No signs of clonal lymphoproliferation as assessed by bone marrow histology, immunohistochemistry and flow cytometry
Partial response	A stable increase in hemoglobin levels of at least 2.0 g/dL or to the normal range A reduction of serum IgM concentrations by at least 50% of the initial level or to the normal range Improvement of clinical symptoms Transfusion independence
No response	Failure to achieve complete or partial response

<sup>1</sup>as defined in prospective studies on therapy for CAD [25, 43, 56, 79, 80].

In order to qualify for any given response level, all criteria had to be present

marrow aspirates following most courses of rituximab in responders as well as non-responders. This effect was not caused by blocking of the antigen, since antigen blocking should result in CD19/20 discrepancy. The histologic and flow-cytometric findings suggest, therefore, that some mechanism other than elimination of clonal CD20<sup>+</sup> cells may contribute to the therapeutic effect of rituximab in CAD in some patients. Rituximab is also known to eliminate polyclonal B cells, as manifested clinically by its beneficial effect in several polyclonal autoimmune diseases including warm antibody AIHA and autoimmune thrombocytopenia [60, 61]. We do not yet know whether this polyclonal B cell depletion may contribute to the therapeutic effect of rituximab in CAD.

Since response duration is relatively short in many patients and re-treatment with rituximab may be effective twice or even several times [5, 43], severe B cell immunodeficiency or other long-term adverse effects might constitute possible concerns. In follicular lymphoma, however, prolonged therapy with rituximab has not resulted in significantly increased incidence of adverse events [62]. Moreover, although peripheral B-lymphocytes are initially virtually absent after weekly administration of four doses of rituximab, naïve and memory B-cells can usually be detected again after 4-6 months [63]. In our experience with CAD patients, even repeated treatment with rituximab has been well tolerated.

#### POTENTIAL FOR IMPROVING ON RESULTS OF THERAPY

Although well-documented, the benefit achieved by rituximab single agent therapy in CAD is limited by the rather high failure rate (45-50%) and the relatively short response duration [5, 43, 57]. These limitations should warrant further studies in order to explain the variable effect of rituximab therapy, identify possible predictors, and improve on response rates and duration.

Patients in whom CD20<sup>+</sup>κ<sup>+</sup> lymphocyte clones can merely be detected and monoclonal IgM is present at low levels, can still have very high CA titers and rather severe clinical disease [2, 16]. Small B-cell clones that produce deleterious proteins are well-known from several monoclonal gammopathies that are difficult to treat [64, 65]. Thus, one explanation for the difficulties in achieving remissions may be that in most cases, small cell clones produce biologically highly active antibodies that may have to be nearly eradicated in order to achieve clinical improvement. A low proliferation rate of the clonal B cells may be another explanation [16]. One would expect this limitation to apply to conventional cytotoxic drugs more than to rituximab and other targeted therapeutic agents.

As for therapy with rituximab, limitations may also relate to its mechanisms of action. Rituximab is assumed to kill CD20<sup>+</sup> lymphocytes by at least three mechanisms; complement-dependent cytotoxicity (CDC), antibody-directed cellular cytotoxicity (ADCC), and induction of apoptosis by direct intracellular signaling [44, 45, 66]. Some *in vitro* and *in*

*vivo* data indicate that CDC is an essential mechanism of action and, therefore, the reduced availability of complement proteins in many patients with CAD may turn out to be of clinical importance [44, 45]. In our prospective trial, however, we found no association between C3 or C4 levels and response to rituximab therapy [43].

C4 levels may be raised by the administration of interferon-α [67], and this cytokine also up-regulates CD20 expression [68, 69]. When studying the potential of rituximab therapy, it was our intention to evaluate whether combining rituximab and interferon-α could improve on efficacy [43]. However, patient and/or physician preferences resulted in only five patients receiving the combination, and it was impossible to put forward any firm statements on the efficacy of combining rituximab with interferon-α in CAD.

Killing of CD20<sup>+</sup> cells by anti-CD20 induced ADCC requires binding of the Fc-domain of the CD20-bound antibody to the Fc-receptor of effector cells, and polymorphism in the IgG Fcγreceptor IIIa (FcγRIIIa) gene has been proposed to affect the depletion of B lymphocytes by rituximab [70, 71]. Although the consequences of such genetic variations remain to be proven in CAD, clinical studies have suggested that FcγRIIIa polymorphism may explain variability in the response to rituximab therapy in WM [72].

The purine analogues, fludarabine and cladribine, have shown a remarkable efficacy in low-grade lymphoproliferative diseases [73, 74]. They terminate DNA synthesis by acting as competitors for deoxyadenosine triphosphate at the A sites of the elongating DNA strand, resulting in induction of apoptosis [73]. Even though purine analogues do not seem very promising in CAD when administered as monotherapy [25], remission has been reported in two patients after the administration of cladribine and fludarabine, respectively [5, 75]. In a small, prospective study, cladribine was shown to reduce the number of clonal cells although not resulting in any significant clinical improvement [25]. A synergistic effect of fludarabine and rituximab have been shown on a follicular lymphoma B cell line resistant to the cytotoxic activity of either drug alone, probably mediated through a down-modulation of membrane CD55 [76]. In WM, purine analogue and rituximab combination therapy has resulted in higher response rates and more prolonged remissions as compared to purine analogue single agent therapy [77]. Fludarabine may induce autoimmune hemolytic anemia in patients with chronic lymphocytic leukemia, but such events have not been reported in classical primary CAD. Moreover, recent observations may indicate that the addition of rituximab will further reduce the risk of AIHA associated with fludarabine therapy [78].

Based on these considerations, we are now running a phase 2 study on the safety and efficacy of rituximab and fludarabine combination therapy in primary CAD [79], still using the response criteria listed in Table 3. Eight patients have been treated so far, aged median 72.5 years (range, 59-85) [80]. Six had previously received rituximab single agent therapy, resulting in one complete response and one partial

response, while four had been non-responders. After the combination therapy, circulatory symptoms resolved completely in four patients and improved in three. Hemoglobin levels increased by more than 2.0 g/dL in four of six anemic patients. Overall, three patients achieved a complete response, three achieved a partial response and two were non-responders. Hematologic toxicity was observed in four patients (grade 2, 3 and 4, respectively) and infection grade 2, nausea and dermatitis in one each. As a preliminary conclusion, rituximab and fludarabine combination therapy is feasible even in elderly patients with CAD. Response rates are promising and suggestive of a higher efficacy of combination therapy, but superiority over rituximab single agent therapy remains to be proven.

Because the hemolytic activity of CA is dependent upon complement fixation and activation, interfering with the complement cascade might, in theory, provide a way of achieving control over hemolysis in CAD. The chimeric, humanized monoclonal anti-complement C5 antibody eculizumab has recently shown a remarkable clinical effect in paroxysmal nocturnal hemoglobinuria [81, 82]. No studies or reports have been published on the use of eculizumab in CAD. Since most removal of complement-opsonized erythrocytes takes place in the liver without binding of C5, however, one would not expect a marked clinical effect in stable CAD patients.

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#### ABBREVIATIONS

ADCC	=	Antibody-directed cellular cytotoxicity
AIHA	=	Autoimmune hemolytic anemia
C3, C4	=	Complement protein C3 and C4, respectively
CA	=	Cold agglutinin(s)
CAD	=	Chronic cold agglutinin disease
CDC	=	Complement-dependent cytotoxicity
CH <sub>50</sub>	=	Complement hemolytic activity of serum
CRP	=	C-reactive protein
Hb	=	Hemoglobin
Ig	=	Immunoglobulin
WM	=	Waldenström's macroglobulinemia

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