



Rituximab is an effective and safe therapeutic alternative in adults with refractory and severe autoimmune hemolytic anemia

Francisco Javier Peñalver, Alberto Alvarez-Larrán, Jose Luis Díez-Martin, Laura Gallur, Isidro Jarque, Dolores Caballero, Joaquín Díaz-Mediavilla, Rosalía Bustelos, María Jesús Fernández-Aceñero, José Rafael Cabrera

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Abstract: Rituximab-induced B cell depletion has been proven to be a useful therapy for autoimmune hemolytic anemia (AIHA). The aim of this retrospective study was to evaluate the effectiveness of rituximab in the treatment of 36 patients with AIHA refractory to several treatments. These patients had received a median of four (1-8) previous treatments, and 13 patients had undergone splenectomy. Rituximab was administered by intravenous infusion at a dose of 375 mg/m² once weekly for 4 doses in 29 patients, and 7 patients received 1-6 doses. Overall, 28 of 36 patients (77%) achieved response. Twenty-two patients (61%) reached a complete response (CR), and 6 patients (16%) obtained a partial response (PR). All patients that reached CR (61%) were able to maintain the response during more than 6 months with a median follow-up of 14 months (1-86 months). Sixteen patients maintained response (Maintained Response; MR) for more than 1 year. The predictors of MR were achievement of CR and negative Coombs test result. Splenectomized patients showed a better response rate than those non-splenectomized. Rituximab was well tolerated, and only one patient presented a transitory rash during infusion. Rituximab induced clinical responses in multi-treated severe refractory both warm and cold AIHA patients with little toxicity and consequently, this therapy should be considered as an early therapeutic option in this setting.

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RITUXIMAB IS AN EFFECTIVE AND SAFE THERAPEUTIC ALTERNATIVE IN ADULTS WITH REFRACTORY AND SEVERE AUTOIMMUNE HEMOLYTIC ANEMIA

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Abstract

Rituximab-induced B cell depletion has been proven to be a useful therapy for autoimmune hemolytic anemia (AIHA). The aim of this retrospective study was to evaluate the effectiveness of rituximab in the treatment of 36 patients with AIHA refractory to several treatments. These patients had received a median of four (1–8) previous treatments, and 13 patients had undergone splenectomy. Rituximab was administered by intravenous infusion at a dose of 375 mg/m² once weekly for 4 doses in 29 patients, and 7 patients received 1–6 doses. Overall, 28 of 36 patients (77%) achieved response. Twenty-two patients (61%) reached a complete response (CR), and 6 patients (16%) obtained a partial response (PR). All patients that reached CR (61%) were able to maintain the response during more than 6 months with a median follow-up of 14 months (1–86 months). Sixteen patients maintained response (Maintained Response; MR) for more than 1 year. The predictors of MR were achievement of CR and negative Coombs test result. Splenectomized patients showed a better response rate than those non-splenectomized. Rituximab was well tolerated, and only one patient presented a transitory rash during infusion. Rituximab induced clinical responses in multi-treated severe refractory both warm and cold AIHA patients with little toxicity and consequently, this therapy should be considered as an early therapeutic option in this setting.

Introduction

Autoimmune haemolytic anemia (AIHA) is a disorder in which IgG and/or IgM autoantibodies are directed against autologous red blood cells (RBC) surface antigens and initiate RBC destruction by the reticuloendothelial system and complement system, respectively. About 80% of patients which suffer from AIHA have warm-reactive antibodies of the IgG isotype; the remainder exhibit cold-reactive autoantibodies. Although AIHA can be idiopathic, 20-80% of patients have a suspected secondary cause such as lymphoproliferative disorders (LD), autoimmune disorders (AD) and infections¹.

In patients with warm AIHA (WAIHA) requiring treatment, corticosteroids represent the first-line therapy, even though only 15-20% of new cases achieve a complete remission¹. For those patients initially unresponsive to corticosteroids, the second-line of therapy is usually splenectomy, which is associated with an initial response rate of approximately 50%². Treatment options for patients who fail to respond, relapse or are unable to undergo splenectomy are limited. Cytotoxic and immunosuppressant drugs, such as cyclophosphamide, azathioprine and ciclosporine A, provide a 40-60% response rate. However, these treatments may be associated with serious side effects. The effectiveness of other options, such as intravenous immunoglobulin (IVIG), plasmapheresis and danazol, is controversial^{1,2,3}.

In patients with cold AIHA (CAIHA), therapy based on corticosteroids is generally not helpful and splenectomy is not usually effective, as complement-coated RBC is mainly destroyed in the liver⁴. In cases with severe hemolysis, immunosuppression with chlorambucil or cyclophosphamide may be beneficial

1 and plasmapheresis can provide transitory improvement. Nevertheless, this
2 therapy is generally unsatisfactory^{1,2,4}.
3

4 The aetiology of autoantibody production is not well understood, but is thought
5 to involve a complex dysfunction of the immune system and immune surveillance
6 mechanism⁵. Despite the complex pathogenic mechanism of AIHA, B cells play
7 a crucial role in the pathogenesis of these disorders⁶.
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10 Rituximab is a humanized chimeric anti-CD20 monoclonal antibody specific for
11 the CD20 antigen expressed on all normal and malignant mature B-
12 lymphocytes. After introduction of rituximab as a powerful therapeutic agent in
13 B-cell non-Hodgkin's Lymphoma, numerous attempts have been made to use B-
14 lymphocyte depletion induced by rituximab for the treatment of both non-
15 haematological and haematological autoimmune diseases^{4, 6,7,8}.
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29 Similar to other autoimmune cytopenias, AIHA is a rare disease, which
30 estimated incidence is 1-3 cases per 100.000 per year¹, making it difficult to
31 perform randomized trials. Up to now, non-randomized prospective studies are
32 relatively few, and retrospective series of consecutive patients are often small⁹.
33
34 An overview of studies including five or more patients reveals that rituximab is
35 effective in treating warm and cold AIHA, with an overall response rate ranging
36 from 40 to 100% (median 60%), and with patients of all ages responding⁴.
37
38 Therefore, controlled studies that include larger numbers of patients that have
39 received several treatments in different clinical situations (associated diseases,
40 splenectomy or not) are necessary to assess the role of rituximab in the
41 treatment of adult patients with refractory and severe AIHA.
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In the present study, we show the results of a retrospective multicentric Spanish study designed to asses the therapeutic efficacy and toxicity of rituximab in refractory and severe AIHA patients unresponsive to several treatments.

Design and methods

Our group has performed a retrospective multicentric analysis of adult patients with AIHA treated with rituximab between April 2001 and June 2007 in 21 centres in Spain.

Inclusion criteria

In the present study, we have included adult patients with AIHA, both idiopathic AIHA and associated with LD and AD.

Diagnosis of AIHA was based on laboratory evidence of hemolysis (increased reticulocyte count with increased indirect bilirubin, lactate dehydrogenase, or absent haptoglobin levels) and a positive Coombs test.

Refractory and severe AIHA patients were eligible for treatment with rituximab. We defined as refractory AIHA those patients with hemoglobin levels < 11 g/dL prior to rituximab treatment who had received at least two therapy lines and patients with hemoglobin levels > 11 g/dL prior to rituximab who had received at least three therapy lines and with treatment dependence to maintain this level of hemoglobin before rituximab initiation. All patients were negative for hepatitis B virus (HBV) and hepatitis C virus (HCV). All patients gave informed consent to receive the rituximab under off-label conditions. This study was approved by the local ethics committee, and informed consent for analysing patients' data was obtained.

Treatment

Patients received a weekly dose of 375 mg/m² rituximab (Mabthera; Roche Farma, Spain) intravenously for 4 consecutive weeks. During the initial period, 26 multitransfused patients with severe anemia and/or requiring a maintenance dose of therapy received rituximab associated with other therapies as follows:

1 corticosteroids (9 of 26 patients), corticosteroids and cyclophosphamide (6 of
2 26 patients), corticosteroids and other treatments they were previously receiving
3 (8 of 26 patients) and other treatments (2 of 26 patients).
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6 Response criteria

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9 The response criteria were defined as follows: complete response (CR),
10 nontransfused hemoglobin level > 12 g/dL and hemoglobin increase of at least
11 > 2 g/dL above their pretreatment hemoglobin level with the discontinuation of
12 concomitant immunosuppressive therapies; partial response (PR),
13 nontransfused hemoglobin level >10 g/dL and hemoglobin increase of at least 2
14 g/dL above their pretreatment hemoglobin levels. Maintained response (MR)
15 was defined as the maintenance of response for more than 6 months after the
16 beginning of treatment and with the discontinuation of concomitant
17 immunosuppressive therapy.
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31 Statistical analysis

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33 Analysis of the data was primarily descriptive, using percentages, mean values
34 and standard deviations, medians and ranges as required. Chi-squared test
35 was used to compare proportions and Student's *t* test to compare means. We
36 have also represented the results with graph bars including means and 95%
37 confidence intervals (CI) and box plots with percentiles and extreme values.
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45 A Kaplan-Meier analysis was performed to represent probability of maintaining
46 response in patients and in order to estimate the median and mean of MR. The
47 event considerate to perform this analysis was losing MR and we censored
48 those patients that had reached the endpoint of maintenance of response. We
49 use the Kaplan-Meier program contained in SPSS ver 13.0.
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Results

We analyzed 36 adult patients with AIHA, both idiopathic AIHA and associated with LD and AD.

Pretreatment characteristics

Patients' demographics and clinical characteristics are summarized in Table 1.

Prior to rituximab, 31 patients had received three or more treatments, with a median of 4 (range 1-8). The previous treatment included corticosteroids (97%); cyclophosphamide (47%); IVIG (42 %); cyclosporine (28%), azathioprine (14%) and chlorambucil (11%).

Splenectomized patients had received a median of 5 treatments before rituximab. 77.8 % of the patients (28 out of 36) had been transfused.

We have also included 5 multitransfused patients with severe AIHA, that showed hemoglobin levels < 8 g/dL (median hemoglobin levels of 6 g/dL, range 4-8 g/dL), prior to rituximab, and who had received one therapy line, either corticosteroids (including high dose, 4 patients) or cyclosporine before receiving rituximab.

Overall, 80.5% (29 out of 36) of patients received four doses of rituximab, and 19.5 % (7 of 36) of patients received between one and six doses of rituximab (one patient, one dose, five doses and six doses each; two patients, two doses and three doses each).

Response to treatment and its characteristics

The overall response rate for patients receiving rituximab was 77%. Twenty-two patients (61%) achieved CR and 6 patients (16%) obtained PR (**Figure 1**).

1 A large proportion of responses took place between the first and third week of
2 treatment (82% of the responders, 18 patients). The median time to have a
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4 response was 3 weeks (range 1-8 weeks). We also found late responses in five
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6 patients between 6-8 weeks (**Figure 2**).
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9 Maximum response was achieved after a median time of 10 weeks (range 4-35
10 weeks), with median hemoglobin levels of 13 g/dL (range 10-15.6 g/dL) The
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12 increase of hemoglobin levels since pre-treatment conditions was statistically
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14 significant ($p < 0.001$) (**Figure 3**).
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18 All patients with PR showed an increase of hemoglobin > 3 g/dL above their
19 pre-treatment hemoglobin levels, with a median increase of hemoglobin post-
20 treatment of 5 g/dL (range 3-7.5 g/dL), a median hemoglobin post-treatment of
21 10.8 g/dL (range 10-11.7 g/dL) and a median duration of response of 4 months
22 (range 1-9 months).
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30 Response maintenance

31 Overall, response was maintained in all the patients that had reached CR (61
32 %, 22 out of 36), with a median duration of response of 19 months (range 6-86
33 months), and 16 patients were able to maintain the response even more than a
34 year with a median duration of 23 months (**Figure 1**).
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42 In Kaplan-Meier analysis, the estimated mean of MR over the study period was
43 65.9 months (95% confidence interval [CI]: 52.9-78.9). The estimated probability
44 of maintaining response between 6 and 83 months was estimated around 75%.
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50 At the end of the study period, median of MR was not achieved. We censored
51 those patients that had maintained response. It must be taking in account that
52 the estimated mean of MR obtained by Kaplan-Meier must be assumed with
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caution as patients who had maintained their response for the longest periods of time were censored (**Figure 4**).

Factors associated with response

There were no differences in the response rate between patients with warm (CR and MR 61.5%; 16 out of 26) or cold AIHA (CR and MR 66.7%; 6 out of 9) ($p=0.9$). Moreover, the duration of MR was not significantly different between warm AIHA patients (median 15 months, range 6-86 months) and cold AIHA patients (median 21 months, range 17-36 months) ($p=0.62$).

We did not find noteworthy statistical differences in CR and MR rates and duration of response between patients with idiopathic AIHA or those with AIHA associated to LD, AD, allogenic stem cell and liver transplant. Fifty percent (7 out of 14) of patients with idiopathic AIHA and 69% (11 out of 16) of patients with AIHA associated to LD experienced CR. We found non-significant differences between both groups in CR and MR ($p=0.5$) and duration of response (21 and 14 months; idiopathic disease and AIHA associated to LD respectively; $p=0.73$). Two out of 4 patients with cold AIHA associated to LD reached CR and all of them were able to maintain it with a median duration of response of 19 and 29 months respectively.

Most of patients that had maintained response reached a negative result from Coombs test (83.3%; 15 out of 18). The frequency of patients that achieved a MR and showed a negative Coombs test was significantly higher than the percentage of patients with a MR but whose result from Coombs test was positive ($p=0.017$). On the other hand, we did not find significant differences when we compared the duration of response between these subpopulations of

1 patients (15 and 28 months, negative and positive Coomb test respectively;
2 $p=0.65$) (**Figure 5**).
3

4 Splenectomized patients with warm AIHA showed a trend to an enhanced
5 response rate comparing to non-splenectomized patients, but this difference
6 was not statistically significant ($p=0.3$). Seventy-five percent of splenectomized
7 patients (9 out of 13) achieved a CR that was maintained for a median of 21
8 months and 54% (13 out of 24) of non-splenectomized patients showed a CR
9 that was maintained during a median of 19 months. No differences were found
10 in the length of MR regarding splenectomy ($p=0.67$). Moreover, we did not
11 found remarkable differences either in CR or MR when we analyzed the
12 responses to rituximab in splenectomized or non splenectomized patients with
13 cold AIHA. Nevertheless, it is worth noting that all splenectomized patients with
14 cold AIHA treated with rituximab achieved CR in relation to less than a half of
15 non-splenectomized (100% and 50%; splenectomized and non-splenectomized
16 patients respectively; $p=0.53$). Furthermore, no differences were found in
17 duration of MR between these groups.
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19 Rituximab therapy alone achieved a CR rate similar to that showed by rituximab
20 in combination with other therapies (60% and 58%; monotherapy and combined
21 therapy respectively; $p=0.8$). Moreover, duration of MR between this subsets of
22 patients was comparable (18 and 20 months respectively; $p=0.6$). Six patients
23 with warm AIHA received rituximab in combination with cyclophosphamide and
24 steroids. Five of these patients suffered from AIHA associated LD and they had
25 also received corticosteroids. Four of these patients (80%) achieved CR and
26 were able to maintain this response with a median of duration of 13 months
27 (range 6-19 months).
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1 Heavily treated patients before rituximab therapy (more than four treatments,
2 including any corticosteroid) did not present significant differences in terms of
3 response rate than those receiving fewer treatments (CR and MR 54.5% and
4 64.3%; $p=0.81$). Similarly, both groups maintained the response during a
5 median of 19 months.
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10 The median time from diagnosis of AIHA to rituximab treatment was 8.7
11 months. Our findings showed no significant differences in the CR and the
12 maintenance of response between the patients with longer duration of AIHA (>
13 8 months) and patients with AIHA lasting less than 8 months before rituximab
14 therapy (55.6% and 61% respectively; $p=1$). The median duration of response
15 between these groups did not showed notable differences (20 and 15.5 months
16 respectively; $p=0.7$).
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28 Age and gender did not seem to influence response to rituximab therapy (Data
29 not shown).
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33 Toxicity, side effects and infectious complications

34 The treatment was generally well tolerated. All patients were pretreated with
35 acetaminophen, diphenhydramine and intravenous hydrocortisone. Only one
36 patient presented a transitory rash during infusion. No infectious complications
37 were comunicated.
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Discussion

This study aimed to describe the characteristics of response to rituximab in 36 patients with refractory AIHA both idiopathic and associated with AD or LD. Up to now, rituximab had been shown to be effective for the treatment of refractory AIHA in children and in a small number of adults^{10,11}. Our findings show that rituximab induces a clinically significant response in patients with AIHA who are unresponsive to other therapeutic options. Overall, response rate for patients receiving rituximab was 77%. Sixty-one percent of patients achieved CR and all of them were able to maintain it. Moreover, this response is not influenced either by gender or age of the patient or by hemoglobin levels prior to treatment in contrast to other reports^{12,13}.

To our knowledge, this study includes the largest series reported to date about the use of rituximab in adults with severe refractory AIHA⁴. Similar findings were obtained in a recent retrospective study including 27 patients with idiopathic or secondary warm AIHA¹⁴, in which rituximab led to an initial response in 25 (93%) cases, with a CR in 8 (30%) and PR in 17 (63%) patients. Three patients were retreated with rituximab, and the same pattern of response (1CR, 2PR) was obtained. In our series, only one patient was retreated after maintaining the response during 11 months. This patient reached a new CR with 2 doses of rituximab, which was sustained with rituximab every 3 months.

The therapeutic objective of rituximab therapy in refractory AIHA must be achieve CR, and furthermore the CR could be consider as a predictor of MR seeing that all the patients with a CR were able to maintain their response. Although to date, no laboratory parameters predictive of response have been identified^{15,16}, our results show that a negative result from Coombs test after

1 rituximab administration could be a factor associated to the maintenance of
2 response. The negative result from Coombs test after treatment seem to be an
3 adequate laboratory predictor of maintenance of response seeing that 83.3% of
4 patients that had achieved CR showed a negative result from Coombs test and
5 they were able to maintain the response for a median of 15 months.
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10 Regarding splenectomy, both splenectomized and non-splenectomized patients
11 have obtained a remarkable response, with a trend to a higher response rate in
12 splenectomized patients with warm AIHA. It has been previously reported that
13 about 60% of patients who undergo splenectomy achieve a complete
14 remission^{3,17,18}. Patients with cold AIHA behaved equally in terms of response
15 whether or not they had been previously splenectomized. Besides, it is worth
16 noting that all splenectomized patients with cold AIHA treated with rituximab
17 achieved CR in relation to less than a half of those non-splenectomized that
18 were able to experience a CR. However, splenectomy did not show an effect on
19 response maintenance either in warm or cold AIHA patients in our study. These
20 findings could support the hypothesis that an intact spleen is not necessary to
21 achieve a response when patients are treated with rituximab in contrast to other
22 studies particularly in ITP¹⁹. Moreover, previous splenectomy in AIHA patients
23 could favour the response to immunosuppressant agents. In fact, rituximab has
24 demonstrated to be more effective in patients with AIHA refractory to
25 splenectomy, providing a therapeutic alternative to the prolonged administration
26 of immunosuppressant agents.
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53 The aetiology of autoantibodies of AIHA did not influence response to treatment
54 seeing that there were no statistical differences in response rate and duration of
55 response between patients with warm and cold AIHA. Although MR rates
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1 obtained in treatment of cold AIHA have been generally lower than those
2 reported for the management of warm-antibody disease²⁰, the present study
3 shows that rituximab could be effective in obtaining MR in patients with cold
4 AIHA, offering an additional treatment choice to this group with limited options.
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9 The present study also includes cases of refractory AIHA secondary to a range
10 of conditions including AD, LD, allogenic stem cell and liver transplant, which
11 have been excluded in other reports^{21,12}. We did not find statistically significant
12 differences in CR, MR rate and duration of response between these patients
13 and those with idiopathic refractory AIHA. This data suggests that rituximab
14 could behave as an effective immunosuppressor in refractory AIHA despite the
15 cause of this autoantibody mediated disease as it can be seen in other
16 analyses^{22,23}.
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19 Rituximab induced rapid responses, most of which took place between the first
20 and third week of infusion. The early response obtained could be attributed to
21 rituximab since a temporal relationship between rituximab infusion and
22 response has been noted. Moreover, these responses seemed to be unaffected
23 by the fact that 86% of patients received other drugs together with rituximab,
24 mainly corticosteroids, therefore suggesting that rituximab could be the main
25 factor responsible for the therapeutic benefit. Reported therapeutic mechanisms
26 of rituximab include antibody-dependent cell-mediated cytotoxicity (ADCC),
27 complement-dependent cytotoxicity (CDC), and direct apoptosis signalling that
28 alter B cell homeostasis and disturb T-cell activation^{24,25}. Although the
29 mechanism of action of rituximab may be related to depletion of auto-reactive B
30 cells, early responses in our study could suggest a saturation of Fc receptors of
31 the monocyte-macrophage system by opsonized B cells as an additional
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1 mechanism preventing the immune-mediated destruction of the autoantibody
2 coated erythrocyte as previously described¹³.
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4 We have found that the response rate in patients treated with rituximab in
5 combination with other therapies was not different from that shown in patients
6 with rituximab therapy alone. However, in patients with underlying
7 lymphoproliferative disorders in particular, treatment with a combination of
8 rituximab and other agents may offer additional benefits^{11,13}. Our findings
9 demonstrate that administration of rituximab along with cyclophosphamide and
10 steroids in patients with AIHA and underlying lymphoproliferative disorders may
11 result in an optimal approach with an effective and durable immunosuppressive
12 response without significant toxicity. Accordingly, it has been reported that
13 rituximab, cyclophosphamide and steroids combination is very effective in
14 treating immune cytopenic diseases (ITP, AIHA) associated with chronic
15 lymphoproliferative syndromes^{10,13}.
16

17 The optimal rituximab dosing scheme in autoimmune diseases has not been
18 established. In all reported series, the typical dosing regimen for refractory AIHA
19 patients is a weekly dose of 375 mg/m² of rituximab for 2 or 4 weeks². In our
20 series, most of patients received 4 doses of rituximab and 7 patients received
21 between 1–6 doses of rituximab without clear differences in their responses.
22

23 Rituximab may probably be less effective in patients previously treated for long
24 periods of time with different immunosuppressive drugs, besides causing
25 damage in these heavily treated patients²⁶. Nevertheless, our results indicate
26 that rituximab could induce excellent responses even in heavily treated
27 refractory AIHA patients and accordingly, this agent could be a fine option as a
28 second line of therapy before splenectomy.
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1 Treatment toxicity was minimal in our study, and no infectious processes were
2 reported. None of the patients developed neutropenia. In similar studies, the
3 doses of rituximab used in the present analysis were also well tolerated^{27,28}.
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5 Rituximab has demonstrated a well-established safety profile through its use in
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7 hemato-oncological indications for over 10 years²⁹ and it has been used for
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9 treatment of AD in children, adolescents and old patients with a good
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11 tolerability^{11,28,30}. However, minor treatable infections have been common in
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13 patients with B cell depletion, whereas severe infections occurred in a small
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15 minority²⁹. In a recent review³¹ regarding the use the rituximab in AD, an
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17 incidence of serious infections of 12.5% was reported. In AIHA patients (5
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19 studies, 76 patients), 5 serious infections were reported and 2 patients died of
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21 septicemia. The frequency of infections in patients with AD treated with
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23 rituximab depends on several factors. Therefore, concomitant
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25 immunosuppressive therapies (T cell immunosuppressive agents), which also
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27 enhance or contribute to susceptibility to infection, age of patient and the
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29 presence of other medical problems have been involved. Rituximab has been
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31 associated with viral infections including hepatitis B, cytomegalovirus, herpes
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33 simplex virus, varicella zoster virus, West Nile virus and JC virus³². Recently, 57
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35 patients with progressive multifocal leucoencephalopathy (PML) after rituximab
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37 treatment have been reported³³. Fifty-two patients suffered from LD and 5
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39 patients had AD. One patient with AIHA developed PML after treatment with
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41 corticosteroids and rituximab.
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53 In conclusion, rituximab induces a clinical effect not seen with other therapies in
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55 AIHA patients refractory to several treatments with little or tolerable associated
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57 toxicity. Therefore, this therapy should be considered as a potential early
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1 treatment that may achieve a rapid and MR in this subset of patients.
2 Achievement of CR must be the therapeutic objective of rituximab in these
3 patients. Furthermore, we could consider a negative Coombs test after
4 treatment as a predictor of maintenance of response. The clinical effect of
5 rituximab and its minimal toxicity could reduce the need for immunosuppressive
6 agents avoiding their cytotoxic effects. Due to the excellent responses
7 associated to rituximab in combination with cyclophosphamide and steroids, this
8 therapy should be considered as a potential treatment of AIHA associated to
9 LD. Additional large studies are required to answer key questions regarding
10 dosage of rituximab, its optimal position in the therapeutic armamentarium of
11 AIHA and the role of retreatment and combination therapy in order to enhance
12 its therapeutical effect and duration of response.
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Figure legends

Figure 1. Response to rituximab therapy. 22 patients achieved CR (61%) and 6 patients obtained PR (16%). The response was maintained (MR) in all patients that achieved CR, with a median duration of 19 months.

Figure 2. Timing of response to rituximab therapy. Most of the responses took place between the first and third week, (18 patients; 82% of the responders). Only five patients responded between 6-8 weeks.

Figure 3. Evolution of hemoglobin levels. Median of hemoglobin level prior to treatment was 7 g/dL, and at maximum response, median hemoglobin level was 13 g/dL. There was a significant increase of hemoglobin levels since pre-treatment conditions ($p<0.001$).

Figure 4. Kaplan-Meier analysis of MR. The estimated mean of MR along the study period was 65.9 months (95% confidence interval [CI]: 52.9-78.9).

Figure 5. Relation between Coombs test and MR. 15 out of 18 patients with MR reached a negative result from Coomb's test, and the number of these patients was significantly higher in relation to those that reached MR but whose result from Coomb's test was positive ($p<0.001$). CT indicates Coombs test.

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Table 1

Number of patients	36
Sex	
Female	21 (58%)
Male	15 (42%)
Age (years)	
Median	64
Range	20-86
Warm AIHA	27 (75%)
Cold AIHA	9 (25%)
Duration of AIHA to rituximab therapy (months)	
Median	8.7
Range	1-138
Transfused patients	28 (77.8%)
Splenectomy	13 (36.1%)
Number of previous treatments	
Median	4
Range	1-8
Pretreatment haemoglobin level (g/dL) (range)	
All patients	6.95 (3.3-12.7)
Warm AIHA	6.85 (3.3-12.7)
Cold AIHA	7.6 (4-10.8)
Associated disease (total/warm)	
Idiopathic	15 (41.7%)/ 10 (37%)
LD	16 (44.4%)/ 12 (44%)
AD	2 (5.6%)/ 2 (7.4%)
ASCT	2 (5.6%)/ 2 (7.4%)
LT	1 (2.7%)/1 (4%)

Table 1. Patients' clinical characteristics. The present table exhibits basal patients' characteristics. LD indicates lymphoproliferative disorders; AD, autoimmune disease; ASCT, allogenic stem cell transplant; LT, liver transplant.

Figure 1
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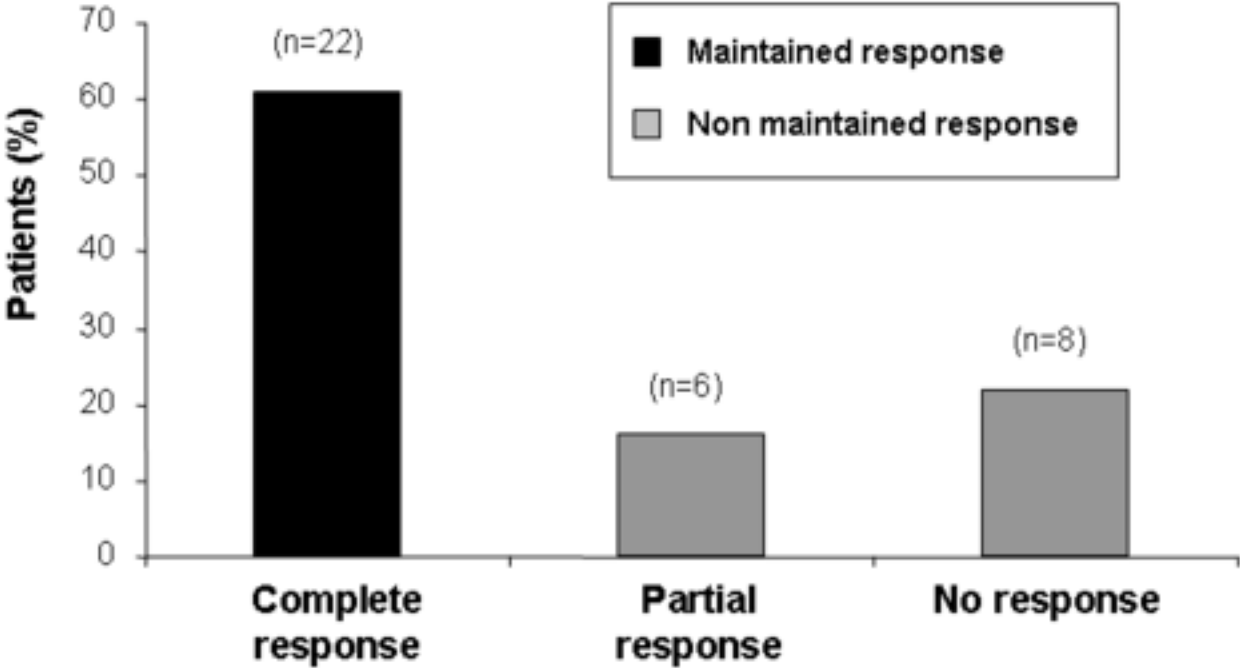


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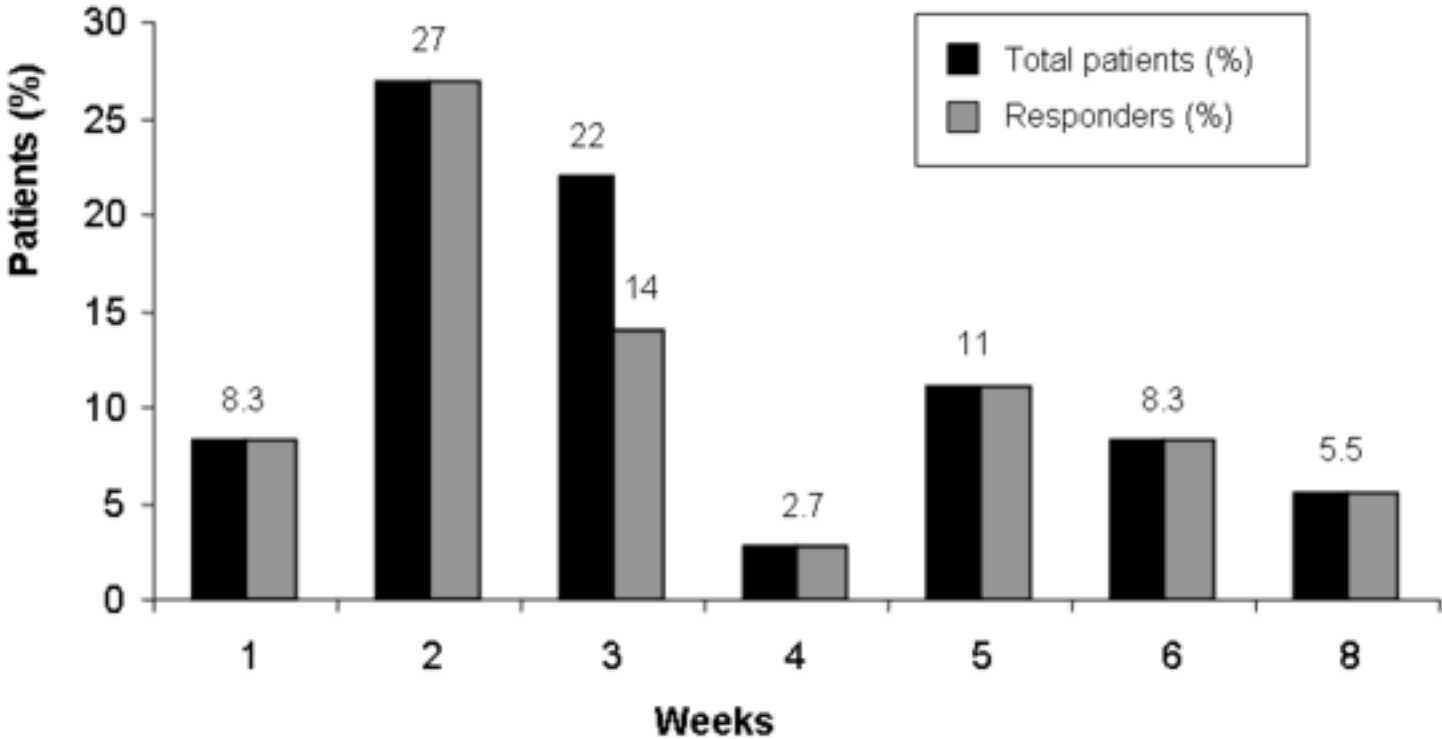


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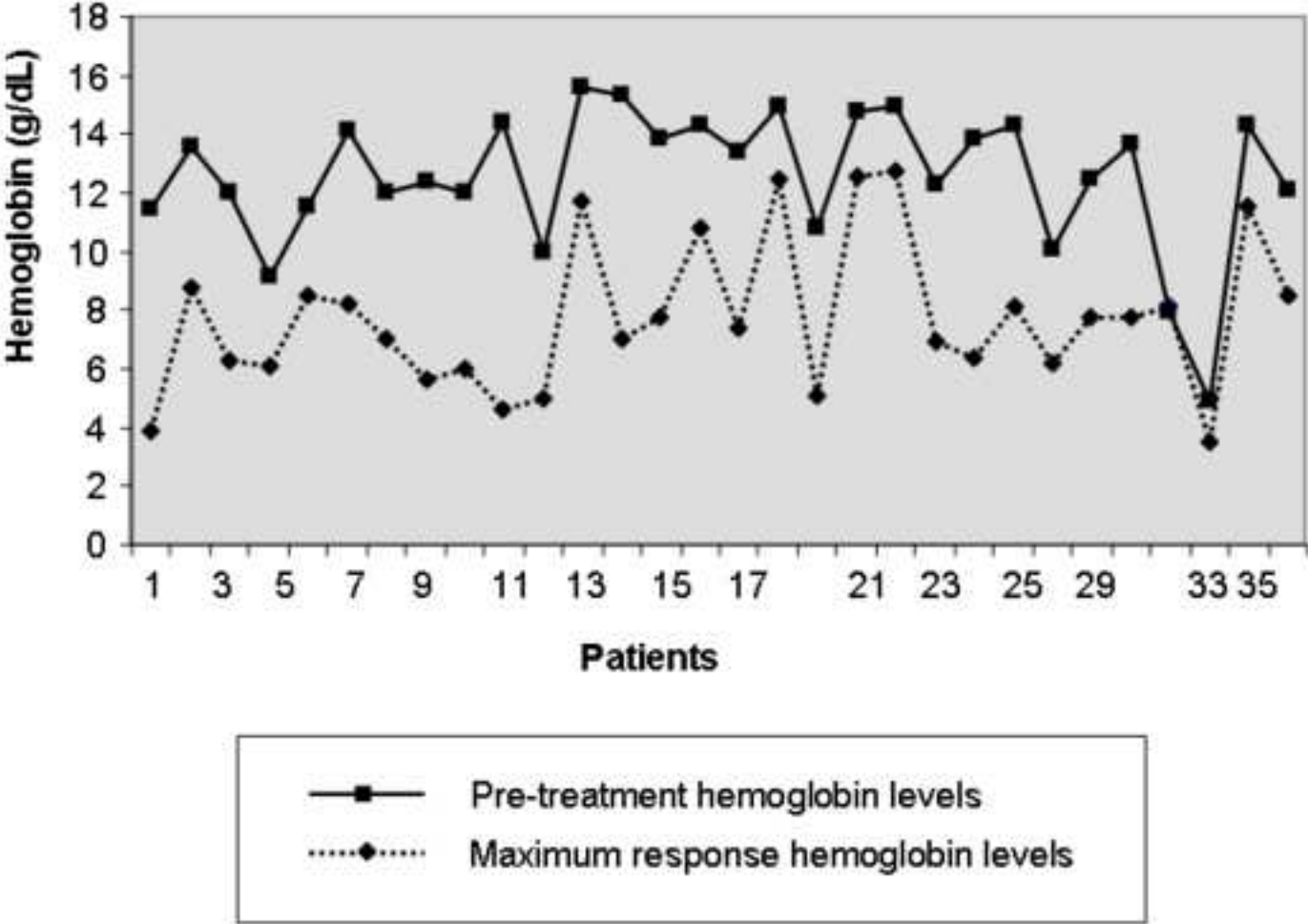


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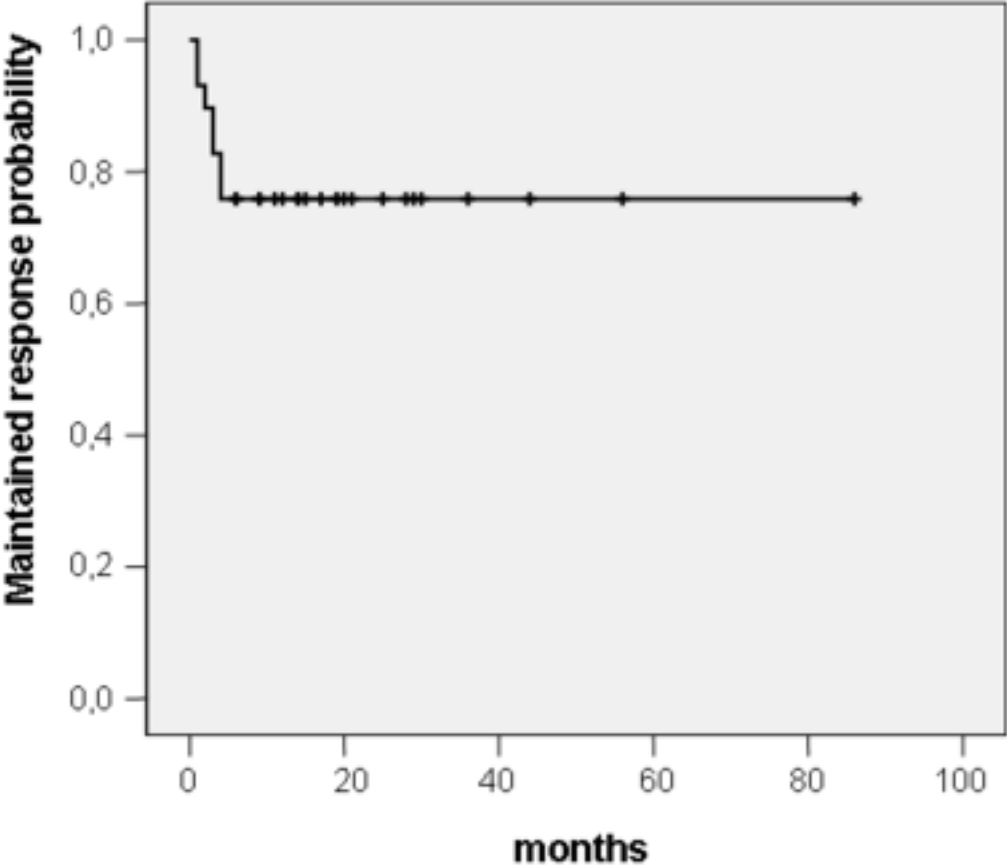
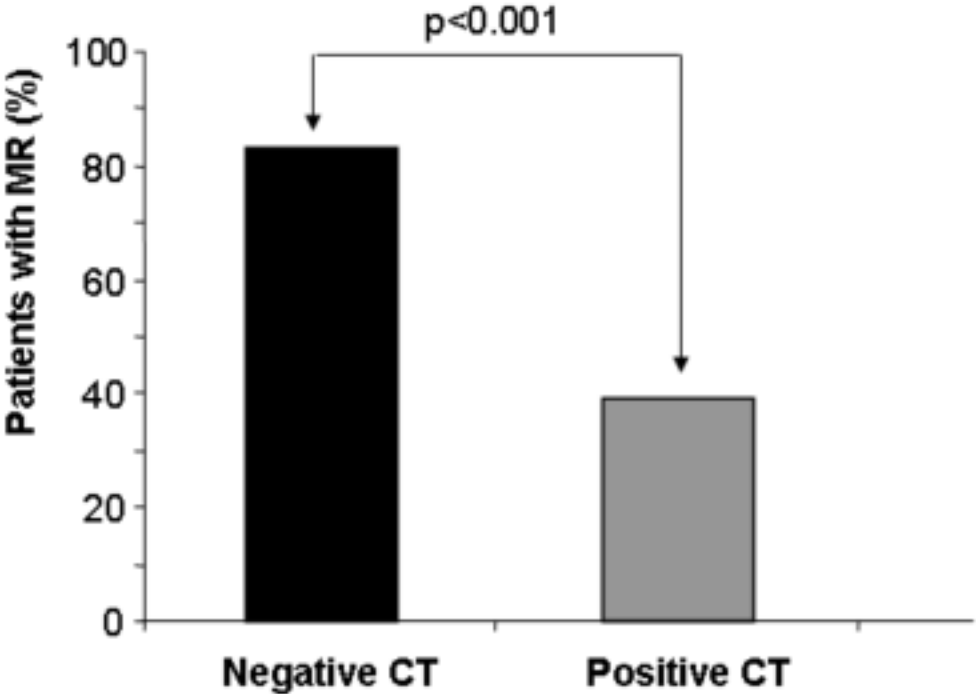


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RITUXIMAB IS AN EFFECTIVE AND SAFE THERAPEUTIC ALTERNATIVE IN ADULTS WITH REFRACTORY AND SEVERE AUTOIMMUNE HEMOLYTIC ANEMIA

Reviewers' comments:

Reviewer #2: All of my concerns have been addressed by the authors.

The paper needs editing by a native English speaker.

Discussion page 17:

- Rows 2 to 12. This paragraph does not sound well
- Row 17: infections
- Row 19: autoimmune

[The manuscript has been carefully revised in order to improve the language and style and some grammatical errors have been correct as advised by the reviewers.](#)

Discussion page 17:

- Rows 2 to 12. This paragraph does not sound well

[This paragraph has been re-written in order to clarify the content.](#)

- Row 17: infections
- Row 19: autoimmune

[We have corrected the grammatical errors regarding these words in the Discussion section.](#)