CD20 monoclonal antibody therapy for B-cell lymphoma

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Chapter 5

Rituximab treatment results in impaired secondary humoral immune responsiveness

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Abstract

In lymphoma patients, treatment with chimeric CD20 monoclonal antibodies (rituximab) results in a rapid depletion of normal B cells, persisting for 6-9 months. This sustained B-cell depletion neither leads to a decrease in immunoglobulin levels, nor to an increase in the number of infectious complications. However, the effect of rituximab treatment on the immune responsiveness is not known. In 11 relapsed low-grade non-Hodgkin's lymphoma (NHL) patients, we have investigated the effect of rituximab treatment on the humoral immune response to two primary antigens (keyhole limpet hemocyanin and hepatitis A vaccine) and two recall antigens (tetanus toxoid and poliomyelitis vaccine). We found that after rituximab treatment, the humoral immune response to the recall antigens was significantly decreased when compared to the response before treatment. Already before rituximab treatment, none of these patients was able to mount a response to the primary antigens. These findings are not only relevant as to the feasibility of rituximab in maintenance treatment, but may also offer a rationale for the treatment of antibody-mediated autoimmune diseases with rituximab.
Introduction

The chimeric CD20 monoclonal antibody (mAb) IDEC-C2B8 (rituximab) has become an important treatment modality in relapsed low-grade non-Hodgkin’s lymphoma (NHL). Its application in other CD20-positive B-cell malignancies, e.g. aggressive lymphomas, post-transplant lymphoma or CLL is rapidly expanding. Chimeric CD20 mAbs target the CD20 antigen, a nonglycosylated 33 to 37 kDa phosphoprotein, expressed on more than 95% of normal and malignant B-cells. It is present from the pre-B cell stage until the differentiation to plasma cell and is not expressed on other cells. Treatment of cynomolgous monkeys with rituximab induced a rapid depletion of B-cells from the peripheral blood (PB) (depletion >98%) and lymphoid organs (depletion 40-70%). In a clinical trial in 166 relapsed low-grade NHL patients treated with rituximab (375 mg/m² weekly x4), peripheral blood B-cell depletion occurred within 24-48 hours after the first infusion of rituximab. Recovery of B-cell counts started at 6-9 months after completion of therapy, and normal levels were obtained after 9-12 months. The prolonged period of rituximab-induced B-cell depletion might compromise the immune system. Immunoglobulin levels were reported to remain stable during this 12 month period, and the amount and severity of infectious complications was not increased when compared to relapsed low-grade lymphoma patients in general. However, no studies directly addressing the immune responsiveness of patients treated with rituximab have been published. To get more insight into the influence of rituximab treatment on the immune responsiveness, we investigated the humoral immune responses of low-grade lymphoma patients to two primary antigens (keyhole limpet hemocyanin and hepatitis A vaccine), and two recall antigens (tetanus toxoid and poliomyelitis vaccine), both before and after treatment with rituximab.

Materials and Methods

Patients

The present immunisation study was part of an ongoing phase I/II study evaluating the safety and efficacy of the combination of rituximab (375 mg/m² weekly x4) and granulocyte colony-stimulating factor (G-CSF) (5 µg/kg/day, administered on three consecutive days starting two days before each infusion). Inclusion criteria for the immunisation study were those of the phase II study: measurable progression of histologically confirmed CD20-positive B-cell lymphoma (Working Formulation A-C) after at least one and no more than three prior systemic therapies; expected survival of > 3
months; prestudy performance status of 0-2 according to the World Health Organization (WHO) scale; hemoglobin (Hb) > 5 mmol/L; white blood cell count (WBC) > 3.0 x10⁹/L; absolute granulocyte count > 1.5 x10⁹/L; platelet count (Plt) > 75 x10⁹/L; circulating tumor cells < 0.5 x10⁹/L; seronegative for human immunodeficiency virus (HIV) and hepatitis-B surface antigen (HBsAg); serum IgG > 600 mg/dL. Patients were enrolled and treated in the Academic Medical Center and the Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands. Both studies were approved by the ethics committee at each center. All patients gave written informed consent for both studies according to the rules of the institute.

Immunophenotypic analysis
FITC-labeled CD20 mAbs, FITC-labeled CD3 mAbs, PE-labeled CD4 mAbs, PE-labeled CD8 mAbs, PE-labeled CD56 mAbs and PE-labeled CD19 mAbs and the irrelevant control antibodies IgG1-FITC and IgG2a-PE were from Becton Dickinson (San Jose, CA, USA). Kappa and Lambda mAbs were from Dako (Glostrup, Denmark). The percentage of CD19-positive cells was determined by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA).

Antigen
Keyhole limpet hemocyanin (KLH, Calbiochem Corp., San Diego, CA, USA) was dissolved in NaCl (final concentration 0.58 mg/mL) as described previously. 15 1000 mg of KLH was administered subcutaneously (sc). Hepatitis A vaccine (1440 IE of inactivated hepatitis A virus (HAV) strain HM-175; Havrix®) was obtained from SmithKline Beecham (Rijswijk, The Netherlands) and administered intramuscularly (im). Tetanus toxoid (TT) (0.5 mL tetanusvaccin containing 10 Lf tetanus toxoid per mL) and inactivated poliomyelitis vaccine (consisting of poliomyelitis vaccine type 1: at least 30 DE, type 2: at least 6 DE, type 3 at least 24 DE; further referred to as PV1, PV2 and PV3 respectively) were obtained from the RIVM (Utrecht, The Netherlands), and administered im and sc respectively.

Immunisation schedule
Each patient received two primary antigens and two recall antigens. One of each was administered two weeks before rituximab treatment and the other two were administered 4 weeks after completion of rituximab treatment (table 1). Four different immunisation schedules were developed. Patients were sequentially assigned to one of the four groups. Before immunisation and two weeks after immunisation, serum samples were collected and stored at -20°C until use.
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Table 1. Immunisation schedule

<table>
<thead>
<tr>
<th>patient group</th>
<th>2 weeks before rituximab treatment</th>
<th>4 weeks after rituximab treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>primary ag</td>
<td>recall ag</td>
</tr>
<tr>
<td>1 A</td>
<td>KLH</td>
<td>TT</td>
</tr>
<tr>
<td>1 B</td>
<td>KLH</td>
<td>PV</td>
</tr>
<tr>
<td>2 A</td>
<td>HAV</td>
<td>TT</td>
</tr>
<tr>
<td>2 B</td>
<td>HAV</td>
<td>PV</td>
</tr>
</tbody>
</table>

Patients were sequentially assigned to one of the four groups. Abbreviations: ag = antigen; KLH = keyhole limpet hemocyanin; TT = tetanus toxoid; PV = poliomyelitis vaccine; HAV = Hepatitis A vaccine

Measurement of antibody titres

Anti-KLH specific immunoglobulin levels (IgG) and anti-TT antibodies were measured with an ELISA as described previously by Korver et al. Anti-HAV specific immunoglobulin levels (IgG) were measured by ELISA (HAVAB 2.0 Quantitative, ABBOTT Laboratories, Diagnostics Division Abbott Park, Illinois). Titres of neutralizing antibodies against PV1, PV2 and PV3 were measured by poliovirus neutralizing antibody test as described previously by Albrecht et al. For KLH, a positive response was defined as a ratio of IgG antibodies (post-immunization versus pre-immunization) of >1.25. Seroconversion to HAV was defined as an increase in HAV titres to >20 mIU/mL.

Statistics

Antibody responses are expressed as the ratio between the antibody titres 14 days after immunization and before immunization. The responses to the two different primary antigens (KLH and HAV) and recall antigens (TT and PV) were compared within one patient. For this purpose, for each vaccine the ratios of antibody responses were ranked within the group. The rank a patient obtained before rituximab (e.g. for TT) and the rank that this same patient obtained after rituximab (for PV 1, 2 or 3, or vice versa) formed pairs. The pairs of ranks achieved by all patients were analyzed using the Wilcoxon Signed Rank test. A P-value of <0.05 was considered significant.

Results

Patients

Eleven out of the 14 patients included in the phase II part of the study evaluating safety and efficacy of rituximab and G-CSF agreed to participate in the immunisation-study.
In 2 patients, the post-rituximab immunizations were not performed either due to non-study-related disease (patient E; would have received HAV/PV) or to logistic problems (patient H; would have received KLH/TT). Patient characteristics are listed in table 2. All patients had progressive disease at time of immunisation. Median age was 53 years (range 27-70) and the median number of prior chemotherapy regimens was 1 (range 1-3). Chemotherapy regimes for each patient, as well as the time between last chemotherapeutic treatment and immunization are listed in table 2.

Table 2. Patient characteristics

<table>
<thead>
<tr>
<th>patient</th>
<th>sex/age</th>
<th>prior regimens</th>
<th>months since last therapy</th>
<th>response to rituximab</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>m/44</td>
<td>CVP</td>
<td>13</td>
<td>CR</td>
</tr>
<tr>
<td>B</td>
<td>f/53</td>
<td>CVP</td>
<td>5</td>
<td>PR</td>
</tr>
<tr>
<td>C</td>
<td>m/39</td>
<td>CVP</td>
<td>2</td>
<td>SD</td>
</tr>
<tr>
<td>D</td>
<td>m/59</td>
<td>CVP, F</td>
<td>2</td>
<td>SD</td>
</tr>
<tr>
<td>E</td>
<td>m/66</td>
<td>CVP,CHOP</td>
<td>6</td>
<td>SD</td>
</tr>
<tr>
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<td>m/49</td>
<td>CVP</td>
<td>48</td>
<td>CR</td>
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<td>f/51</td>
<td>CVP</td>
<td>11</td>
<td>PD</td>
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<tr>
<td>H</td>
<td>m/55</td>
<td>CVP, CHVMP,Chl</td>
<td>2</td>
<td>SD</td>
</tr>
<tr>
<td>I</td>
<td>f/70</td>
<td>Chl</td>
<td>29</td>
<td>PR</td>
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<tr>
<td>J</td>
<td>f/27</td>
<td>F</td>
<td>38</td>
<td>SD</td>
</tr>
</tbody>
</table>

Abbreviations: CVP: cyclophosphamide, vincristine, prednison; CHOP: cyclophosphamide, adriamycin, vincristine, prednison; CHVMP: cyclophosphamide, adriamycin, VM-26, prednison; F: fludarabin; Chl: chlorambucil; CR: complete remission; PR: partial remission; SD: stable disease; PD: progressive disease

Effect of rituximab treatment on peripheral blood B-cell counts and immunoglobulin levels

In all patients, a complete depletion of B-cells from the peripheral blood was observed 72 hours after the first infusion (fig. 1). One month after last rituximab treatment (i.e. at time of second immunisation), B-cells were still absent in all but one patient (patient H). Immunoglobulin levels (IgG, IgA and IgM) remained stable during treatment and follow-up period (fig. 2). One patient (D) had an immunocytoma with an IgM M-protein. In this patient, upon rituximab treatment IgM levels decreased from 22.2 mg/dL at study entry to 13 mg/dL at 3 months follow up. This patient was not included in fig. 2.
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Fig. 1 Number of B-cells during treatment with rituximab
The absolute number of peripheral blood B-cells was measured throughout the study period at the following time points: entry; +72 hrs: 72 hours after the first infusion of rituximab (375 mg/m²); month 1, 2 and 3: follow up at one, two and three months respectively after the last rituximab infusion. Arrows indicate the timepoints of first and second immunisation. Values are depicted as mean ± SEM of 11 patients. * indicates a significant difference as compared to pretreatment value (P<0.05).

Fig. 2 Immunoglobulin levels did not change upon treatment with rituximab
Levels of IgG, IgA and IgM were measured throughout the study period at time points: entry; follow up at one, two and three months respectively after last rituximab infusion. Values are depicted as mean ± SEM of 11 patients.
* indicates a significant difference as compared to entry value (P<0.05).

Response to recall antigens
Tetanus toxoid (TT) and poliomyelitis vaccine (PV1, PV2 and PV3) were used as recall antigens. Nine patients were immunized with both TT and PV. Antibody responses, expressed as the ratio of titres post/pre-immunization, are shown in fig. 3 and table 3.
To allow for comparison of the responses to TT and PV within one patient, ratios to TT and ratios to PV were ranked. The pairs of ranks obtained by each patient were analyzed using the Wilcoxon signed rank test (n=9 paired samples). Responses to PV1, PV2 and PV3 are separately documented and analyzed in combination with TT. The response to recall antigens after rituximab treatment was significantly lower than the responses before treatment (P = 0.078, P = 0.013 and P = 0.012 for TT in combination with PV1, PV2 and PV3 respectively).
Table 3. Immune responses to recall antigens

<table>
<thead>
<tr>
<th>patient</th>
<th>before rituximab treatment</th>
<th>after rituximab treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PV1</td>
<td>PV2</td>
</tr>
<tr>
<td>A</td>
<td>PV*</td>
<td>32*</td>
</tr>
<tr>
<td>F</td>
<td>PV</td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>PV</td>
<td>0.5</td>
</tr>
<tr>
<td>K</td>
<td>PV</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>TT</td>
<td>2.8</td>
</tr>
<tr>
<td>C</td>
<td>TT</td>
<td>4.1</td>
</tr>
<tr>
<td>D</td>
<td>TT</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>TT</td>
<td>3.8</td>
</tr>
<tr>
<td>J</td>
<td>TT</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*ratios to PV1, PV2 and PV3 are listed separately; **antibody responses to TT and PV1, 2 and 3 in 9 patients immunized before and after rituximab treatment are expressed as ratios of post/pre-immunization titres. Ratios to TT, and ratios to PV1, 2 and 3 were ranked. Paired ranks, consisting of the rank obtained by each patient before and after rituximab treatment, were analyzed by Wilcoxon signed rank test.

Fig. 3 Immune response to recall antigens before and after treatment with rituximab

Responses to the recall antigens TT and PV1, PV2 and PV3 are depicted as the ratio of post- and pre-immunization titres. The ratios obtained 2 weeks before (black figures) and 1 month after (open figures) rituximab treatment are demonstrated, the median of the ratios is marked (—).

Primary immune responses

Keyhole limpet hemocyanin (KLH) and hepatitis A vaccine (HAV) were used as primary antigens. In 8 patients (from 11), the primary immune response before rituximab treatment could be evaluated (KLH n=3; HAV n=5). Three of these patients were found to have high anti-HAV antibody titres (see below), indicating that these patients had either experienced
hepatitis A infection or had been immunized with hepatitis A before. Therefore, for only 2 patients the HAV was a primary antigen. None of the 5 patients developed a response to KLH or HAV. Ratios of anti-KLH antibody titres post/pre-immunization were 0.84, 0.87 and 0.96 respectively; post-immunization titres to HAV remained below 10 mlU/mL (n=2).

In 8 patients, response to KLH or HAV was measured after rituximab treatment (KLH n=4; HAV n=4). Again, in 2 of these patients anti-HAV antibody titres were already high (see below), leaving 2 patients for whom HAV was a primary antigen. After rituximab treatment, ratios of anti-KLH antibody titres post/pre-immunization were 0.98, 0.8, 0.71 and 1.09 respectively, and post-immunization titres to HAV remained below 10 mlU/mL (n=2). Thus, none of the patients developed a humoral immune response to either primary antigen, neither before, nor after rituximab treatment.

As mentioned, 5 patients were found to be seropositive for anti-HAV antibodies. The GMT of anti-HAV antibody titres in these 5 patients was 19,952 mlU/mL, supporting the assumption that these patients had experienced a hepatitis A infection. Upon vaccination of these patients with HAV, anti-HAV antibody titres did not increase. This might be explained by the fact that titres generated after a natural HAV-infection are far higher than titres obtained after immunization.20,21 Thus, the increase in antibody levels after vaccination with HAV may be relatively low and therefore not detectable in HAV-seropositive patients.

Discussion

In the present study, the influence of rituximab treatment on the humoral immune responsiveness was investigated in 11 low-grade NHL patients. Major observations made in this study are 1) the humoral immune response to recall antigens after treatment with rituximab was significantly decreased when compared to the response before treatment, and 2) none of the patients was able to mount a primary humoral immune response to the primary antigens used, neither before nor after rituximab treatment.

Based on the literature22-25, it was expected that this group of pretreated low-grade lymphoma patients would have an impaired immune responsiveness when compared to healthy volunteers. Therefore, comparison of the immune response after rituximab treatment with the response obtained in the same patient group before treatment, was judged to be methodologically superior to comparison of the response after rituximab treatment with responses obtained in healthy volunteers. Rather surprisingly, whereas all patients responded to recall antigens, none of the patients responded to the primary antigens, neither before, nor after rituximab treatment. Therefore, our data do not allow conclusions as to the influence
of rituximab on the primary immune response.
Although several groups have investigated the immune responsiveness of lymphoma patients, the majority of these studies focussed on the cellular immune response.\textsuperscript{22-24,26} To our knowledge, only one study has directly evaluated the primary humoral immune response in NHL patients. In this study, the response to KLH was found to be slightly but not significantly decreased when compared to healthy controls.\textsuperscript{24} However, data on the humoral immune responsiveness of lymphoma patients might be derived from a study by Hsu et al.\textsuperscript{27}, in which lymphoma patients were vaccinated with tumor Ig-protein (coupled to 0.5 mg KLH carrier protein). In this study, only half of the patients developed a response to the idiotype of the tumor Ig. The responsiveness to the idiotype was significantly related to the disease status at time of vaccination, i.e. 15 of 21 patients in clinical remission at time of vaccination responded to the idiotype, whereas only 5 of 20 patients with residual disease at time of vaccination responded. No other factors, such as number and type of prior therapies or the time since last chemotherapy regimen, were found to influence the responsiveness to idiotype. These results are in line with the data of van Rijswijk et al\textsuperscript{26}, who demonstrated that the impaired (cellular) immune response of previously untreated patients with Hodgkin’s disease improved after treatment.

In the present study, at time of vaccination all patients had progressive disease requiring treatment. In line with the studies mentioned, the disease status at time of vaccination might explain the strongly impaired immune responses to primary antigens observed in our patients. Still, the exact mechanism underlying the impaired immune responsiveness of lymphoma patients remains unclear.

Rituximab induces a depletion of normal B-cells from the peripheral blood for 6-9 months. Clinically, the sustained B-cell depletion after rituximab treatment was not reported to be accompanied by an increase in infectious complications. This might be explained by the stable levels of immunoglobulins during the period of B-cell depletion.\textsuperscript{1} In contrast to (memory) B-cells, plasma cells are CD20-negative and thus are not depleted after rituximab treatment. Conventional models suggested that plasma cells are short-lived, i.e. have a half-life of a few weeks only.\textsuperscript{28,29} Since the half-life of immunoglobulins is 1-3 weeks, in these models a continuous development of memory B-cells into plasma cells would be required in order to maintain stable immunoglobulin levels. Recent studies in mice however showed that plasma cells may survive for > 1 year (i.e. more than half of the lifetime of mice) in the absence of any detectable memory B-cells.\textsuperscript{30,31} Thus, the presence of long-lived plasma cells may very well offer the explanation for the stable immunoglobulin levels during the period of B-cell depletion induced by rituximab treatment.

In the present study, we observed a significant decreased response to recall antigens after rituximab treatment, when compared to the response generated before treatment. This
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might be due to a decrease in the amount of memory B-cells after rituximab treatment. It is conceivable, that prolonged treatment with rituximab eventually will lead to a decrease in the number of plasma cells due to a diminished replenishment from the pool of (memory) B-cells. Recently, studies have been started evaluating rituximab in maintenance treatment of low-grade lymphoma in clinical remission. It will be important to follow the immunoglobulin levels in these patients, not only in order to protect these patients from infectious complications in case of decreasing immunoglobulin levels, but also because these data might contribute to our knowledge regarding the life-span of plasma cells in men.

Our data provide a rationale for the treatment of antibody-mediated diseases with rituximab.\textsuperscript{32,33} Recently, preliminary data were published on 10 patients with chronic idiopathic thrombocytopenic purpura (ITP) treated with rituximab 375 mg/m\textsuperscript{2} weekly x4. Six of these patients responded and the duration of response varied from > 3 months to > 14 months.\textsuperscript{34} Rituximab treatment was also reported to be successful in a patient with Evans syndrome and a patient with immune mediated thrombocytopenia in chronic graft-versus-host disease.\textsuperscript{32,35} One might speculate, that the observed activity of rituximab is mediated by a mechanism similar to that responsible for the effect of intravenous immunoglobulins (IVlg) in autoimmune diseases.\textsuperscript{36-38} However, since some responses were ongoing for over 9-14 months\textsuperscript{34}, whereas the mean serum half-life of rituximab (after the 4th infusion) was found to be only \textasciitilde 200 hours\textsuperscript{1}, we believe that other mechanisms must contribute to the observed efficacy of rituximab in treatment of antibody-mediated auto-immune diseases.

We suggest that rituximab, by depleting (memory) B-cells, interrupts the ongoing humoral autoimmune response. Although plasma cells, surviving rituximab treatment, may continue to produce (auto-)antibodies for a certain period of time, deletion of the autoreactive B-cell clone will eventually lead to a decrease in antibody production.

In conclusion, we have demonstrated that the humoral immune response to recall antigens decreased significantly upon rituximab treatment. Furthermore, the immune response to primary antigens was severely impaired in our group of relapsed low-grade lymphoma patients, which might (in part) be due to disease progression at time of vaccination. Our data might have implications for studies investigating the feasibility of rituximab as maintenance therapy. Furthermore, these findings provide a rationale for treatment of antibody-mediated autoimmune diseases with rituximab.

**Acknowledgements:**

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Reference List


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