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B-lymphocyte reconstitution after repeated rituximab treatment in a child with steroid-dependent autoimmune hemolytic anemia

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Abstract

We report the detailed long-term reconstitution of B-lymphocyte subpopulations, immunoglobulins, and specific antibody production after two courses of rituximab in a young, previously healthy girl with steroid-dependent autoimmune hemolytic anemia. B-lymphocyte subpopulations were surprisingly normal directly after reconstitution. However, there was a slower reconstitution after the second rituximab course, especially of non-switched and switched memory B-lymphocytes, and a temporary decline in IgM below age-matched reference values.

Introduction

Rituximab is increasingly used in the treatment of various hematological malignancies and, more recently, autoimmune diseases like autoimmune hemolytic anemia (AIHA).1 Rituximab is a monoclonal antibody that reacts with the CD20 antigen, which is expressed on pre-B and mature B-lymphocytes, but not on plasma cells. B-lymphocytes play an important regulatory and potentially pathogenic role in autoimmune diseases through antibody-independent mechanisms like antigen presentation, T-lymphocyte activation, and the production of cytokines and chemokines.2,3 They are therefore likely to interfere with the complex interplay of B-lymphocytes and other immune cells. Depletion of B-lymphocytes is not yet fully understood.4

Treatment with rituximab results in a rapid and sustained depletion of B-lymphocytes. There are detailed reports on the rate and phenotype of B-lymphocyte subpopulation recovery after rituximab treatment in adults:5-12 after B-cell depletion immature B-lymphocytes reappear first, followed by naïve B-lymphocytes; CD27+ memory B-lymphocytes may remain reduced up to 2 years after rituximab treatment.5,8

It would be expected that depletion of CD20+ B-lymphocytes has direct effects on antibody production, and indirect effects on cellular immunity. Indeed, hypogammaglobulinemia requiring substitution was recently described in a child with repeated rituximab treatment for idiopathic thrombocytopenic purpura and underlying autoimmune lymphoproliferative disease.11 However, until now large numbers of serious infectious complications of rituximab treatment have not been described,4 perhaps because antibody-producing plasma cells are spared.

In this paper, we give a detailed description of the reconstitution of B-lymphocyte subpopulations, immunoglobulins (Igs), and specific antibody production after repeated treatment with rituximab in a young, previously healthy, girl with steroid-dependent AIHA.

Materials and Methods

Patient

In August 2004, a previously healthy 13-months-old girl presented in our hospital with AIHA which turned out to be steroid-dependent. Aspecific IgG autoantibodies were strongly positive. In August 2005, she was treated with rituximab (4 x 375 mg/m², 1 week apart). Human anti-chimeric antibodies (HACAs) were measured once and found negative. Subsequently, steroids could be tapered and stopped 11 months after the first rituximab treatment, in July 2006. A few weeks later, she suffered a relapse and steroids were restarted, and a second course with 4 gifts of rituximab (375 mg/m², 1 week apart) was administered. Hereafter, the steroid dose was slowly reduced and, in March 2008, at the age of 4 years, steroids were stopped completely. In 2011, at almost 8 years of age, there were still no signs of relapse. Aspecific IgG autoantibodies remained negative. She did not suffer from any significant infections. The clinical course of this girl is illustrated in Figure 1.

Preceding rituximab treatment, but while on steroids, vaccination status was optimised using Prevenar twice (Wyeth) followed by Pneumovax (Sanofi Pasteur MSD) for S. pneumoniae, and NeisVac C (Baxter) and MeningovaxACn (Sanofi Pasteur MSD) for S. meningitidis. This was considered important, because both rituximab and prolonged steroid treatment might compromise immunity. After 2.5 years, Pneumovax and Menevac ACn (Glaxo Smith Kline) vaccinations were given. Diphtheria - tetanus - inactivated poliomyelitis (DTP-NVI) vaccination was given at the age of four, according to the regular Dutch immunisation schedule (Figure 1).

Peripheral blood samples were taken at multiple time points (t = 0, 8, 14, 21, 56, 110, 202, 321, 383, 396, 403, 410, 417, 446, 530, 614, 718, 816, 1085 days after the first rituximab dose, indicated with arrows in Figure 1) to establish the reconstitution of B-lymphocyte subpopulations, Ig titers and specific antibody production.

Immunophenotyping

Four-color flow cytometric immunophenotyping was performed using the lyse and wash whole-blood method. Aliquots of ethylene diamine-tetra-acetic acid (EDTA) blood (50 μL) were incubated for 15 min at room temperature in the dark with the following monoclonal antibodies: isotype control gamma 1 conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson, San Jose, CA, USA (BD)), isotype control gamma 2a conjugated to phycoerythrin (PE) (BD), CD3-FITC (Immuo Quality Products, Groningen, The Netherlands (IQP)), CD3 conjugated to allopheocyanin (APC) (BD), CD3 conjugated to PE-Cyanin5 (PE-Cy5) (Coulter Immunotech, Marseille, France (CI)), CD4-FITC (IQP), CD4-PE-Cy5 (CI), CD5-FITC (BD), CD8-FITC (IQP), CD8 conjugated to peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5) (BD), CD10-APC (BD), CD16/56-FITC (BD), CD19-FITC (BD), CD19-PE-Cy5 (CI), CD19-PerCP-Cy5.5 (BD), CD20-FITC (BD), CD21-FITC (BD), CD25-FITC (CI), CD27-FITC (BD), CD27-PE (BD), CD45RA-PE (CI), CD45RO-FITC (BD or IQP), IgG-FITC (SouthernBiotech, Birmingham, USA), and IgM-FITC (Sanofi Pasteur Marnes-La-Coquette, France). To eliminate immunoglobulins, aliquots destined for anti-IgM and anti-IgD were washed three times before staining.

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Key words: autoimmune hemolytic anemia, rituximab, child, immune reconstitution, B-lymphocytes.

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Erythrocytes were lysed using FACSLysing solution (BD) according to the manufacturer’s protocol. The cells were washed twice with 0.5% bovine serum albumin/phosphate-buffered saline. Analysis was performed using CellQuest Pro Software (BD) on a FACS Calibur flow cytometer (BD), which was calibrated according to the guidelines of Kraan et al.\textsuperscript{14} The absolute leukocyte count ($\times 10^9/L$) was determined on a Sysmex XE-2100 hematology analyzer (Sysmex, Kobe, Japan). The absolute number ($\times 10^9/L$) of a lymphocyte subpopulation was calculated by multiplying the calculated absolute lymphocyte size ($\times 10^9/L$) with the relative size of the lymphocyte subpopulation (%) within the lymphocyte gate. The results were compared to reference values in healthy children derived from our laboratory.

**Immunoglobulins**

IgG, IgA, IgM, and IgG subclasses were determined by standard nephelometric methods (BN Prospects, Dade Behring, The Netherlands). Specific antibody responses to pneumococcal vaccination were determined by ELISA, diphtheria and tetanus antitoxin antibodies with a toxin-binding inhibition assay.\textsuperscript{15,16}

**Results**

As expected, all peripheral blood B-lymphocyte subpopulations disappeared rapidly upon treatment with rituximab (Figure 2), T-lymphocyte subpopulation and NK-cell counts were not affected (Table 1). After the first rituximab course, B-cells reappeared at $t = 110$ days, after the second course they reappeared later, at $t = 614$ (218 days after the second rituximab course). The reappearing cells were mainly naive cells (CD19$^+$IgD$^+$CD27$^-$), around one third were CD5$^+$ (Figure 2). After the second course, both non-switched (CD19$^+$IgD$^+$CD27$^+$) and switched (CD19$^+$IgD$^-$CD27$^+$) memory B-lymphocytes reappeared later than after the first course ($t = 110$ days after the first course, together with the naive cells, and at $320$ days after the second course, which is 102 days after the first naive cells, at $t = 718$ days (Figure 2). However, switched memory B-lymphocytes were within the age-limits of normal from the moment they reappeared (Table 1). CD21$^+$ cells (IgM$^+$ and IgM$^-$) repopulated in the same manner as the other B-lymphocyte subpopulations. IgG, IgA, and IgG subclass levels were unaffected by the rituximab treatment (Table 1), but after the second course a temporary decrease in IgM was found (0.48 g/l at $t = 446$ days; 0.23 g/l at $t = 530$ days; 0.6 g/l at $t = 614$ days), as has been described before.\textsuperscript{17,18} After both rituximab treatments prednisone was used every other day and slowly tapered until it could be stopped. After the first treatment with rituximab the dosage of prednisone was slightly higher. During that time we didn’t see an effect on immunoglobulins or significant infections. Vaccination responses before the first rituximab course were normal; booster responses after the second course (after $t = 1085$ days) were normal as well (Table 1), despite the fact that she had been on steroids during the first round of vaccinations.

**Discussion**

We followed a girl with steroid-dependent AIHA who was treated with 4 gifts of rituximab twice. She repopulated mainly with naive B-lymphocytes, around one third CD5$^+$, as could be expected for her age. Interestingly, her B-lymphocytes returned to normal faster than described before in both adults\textsuperscript{17-19} and children,\textsuperscript{17,18} but these children had underlying diseases that may have influenced the reconstitution. She did not suffer any significant infections, and responded well to vaccination, despite her prolonged steroid use.

During repopulation memory B-lymphocytes peaked just before the relapse in our patient. Increasingly, early relapse of autoimmune diseases like rheumatoid arthritis or SLE are reported in adult patients with a higher number of memory B-lymphocytes in early reconstitution in comparison to patients with later or no relapse.\textsuperscript{5,8,18} Our case suggests that this relation may also exist in children.

After the second rituximab treatment, the reconstitution was somewhat slower in our patient. This has been described by others,\textsuperscript{4,13} suggesting that multiple rituximab courses are necessary.

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**Table 1. Absolute values of lymphocyte subpopulations in comparison with control values from healthy age-matched children.**

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>$t=0$</th>
<th>$t=110$</th>
<th>$t=396$</th>
<th>$t=614$</th>
<th>$t=718$</th>
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<tbody>
<tr>
<td>CD19$^+$ total B-lymphocytes</td>
<td>0.84*</td>
<td>0.54</td>
<td>0.54</td>
<td>0.54</td>
<td>0.54</td>
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<tr>
<td></td>
<td>(0.3-2.8)**</td>
<td>(0.3-2.8)</td>
<td>(0.3-2.8)</td>
<td>(0.3-2.8)</td>
<td>(0.3-2.8)</td>
</tr>
<tr>
<td>CD19$^+$CD5$^+$ CD5$^+$ B-lymphocytes</td>
<td>0.54</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>(0.1-1.3)</td>
<td>(0.1-1.3)</td>
<td>(0.1-1.3)</td>
<td>(0.1-1.3)</td>
<td>(0.1-1.3)</td>
</tr>
<tr>
<td>CD19$^+$CD10$^+$ Immature B-lymphocytes</td>
<td>0.24 (0.09-0.87)</td>
<td>0.24 (0.09-0.87)</td>
<td>0.12 (0.04-0.25)</td>
<td>0.12 (0.04-0.25)</td>
<td>0.12 (0.04-0.25)</td>
</tr>
<tr>
<td>CD19$^+$IgD$^+$CD27$^+$ naive B-lymphocytes</td>
<td>0.74</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>(0.24-2.61)</td>
<td>(0.24-2.61)</td>
<td>(0.24-2.61)</td>
<td>(0.24-2.61)</td>
<td>(0.24-2.61)</td>
</tr>
<tr>
<td>CD19$^+$IgD$^+$CD27$^+$ non-switched memory B-lymphocytes</td>
<td>0.06</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>(0.01-0.12)</td>
<td>(0.01-0.12)</td>
<td>(0.01-0.12)</td>
<td>(0.01-0.12)</td>
<td>(0.01-0.12)</td>
</tr>
<tr>
<td>CD19$^+$IgD$^+$CD27$^+$ switched memory B-lymphocytes</td>
<td>0.00 (0.005-0.03)</td>
<td>0.00 (0.005-0.03)</td>
<td>0.00 (0.005-0.03)</td>
<td>0.00 (0.005-0.03)</td>
<td>0.00 (0.005-0.03)</td>
</tr>
<tr>
<td>CD3$^+$ cells (T lymphocytes)</td>
<td>2.79</td>
<td>2.64</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
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<tr>
<td></td>
<td>(2.5 (0.7-8.8)</td>
<td>(2.5 (0.7-8.8)</td>
<td>(2.5 (0.7-8.8)</td>
<td>(2.5 (0.7-8.8)</td>
<td>(2.5 (0.7-8.8)</td>
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<tr>
<td>NK cells</td>
<td>0.28</td>
<td>1.18</td>
<td>1.18</td>
<td>1.18</td>
<td>1.18</td>
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<tr>
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<td>(0.47 (0.05-4.0)</td>
<td>(0.47 (0.05-4.0)</td>
<td>(0.47 (0.05-4.0)</td>
<td>(0.47 (0.05-4.0)</td>
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<tr>
<td>IgG***</td>
<td>5.8</td>
<td>6.2</td>
<td>8.6</td>
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<td>(5.2 (0.8-12)</td>
<td>(5.2 (0.8-12)</td>
<td>(5.2 (0.8-12)</td>
<td>(5.2 (0.8-12)</td>
<td>(5.2 (0.8-12)</td>
</tr>
<tr>
<td>IgA***</td>
<td>0.52</td>
<td>0.68</td>
<td>0.92</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>(0.47 (0.4-0.6)</td>
<td>(0.47 (0.4-0.6)</td>
<td>(0.47 (0.4-0.6)</td>
<td>(0.47 (0.4-0.6)</td>
<td>(0.47 (0.4-0.6)</td>
</tr>
<tr>
<td>IgM***</td>
<td>1.07</td>
<td>0.65</td>
<td>1.56</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>(0.80 (0.5-2.8)</td>
<td>(0.80 (0.5-2.8)</td>
<td>(0.80 (0.5-2.8)</td>
<td>(0.80 (0.5-2.8)</td>
<td>(0.80 (0.5-2.8)</td>
</tr>
</tbody>
</table>

*Value in patient in 10^9/L. **Median (range) of healthy age-matched controls in 10^9/L, t = 0: start of first rituximab course; t = 396: start of second rituximab course; t = 614: start of recovery after second rituximab course; t = 718: full recovery of all B-lymphocyte subpopulations. ***Immunoglobulins in g/L.
Figure 1. Time schedule of patient’s history. Dotted line: hemoglobin (mmol/L). Prednisone (mg/kg/day) uninterrupted line; prednisone (mg/kg every other day) interrupted line. Arrows: *rituximab treatment; ** blood sample (resp t = 0, 8, 14, 21, 56, 110, 202, 321, 383, 396, 403, 410, 418, 446, 530, 614, 718, 817, 1085 days after the first rituximab dose); # vaccination. a: first presentation with autoimmune hemolytic anemia (August 2004); b: first treatment with rituximab (August 2005); c: recovery of B-lymphocytes (day 110 after first rituximab treatment); d: relapse and second treatment with rituximab (July 2006); e: recovery of B-lymphocytes (day 218 after 2nd rituximab treatment). Timeline stops in July 2007 when steroids are stopped.

Figure 2. B-lymphocyte subpopulations. Timeline starts in August 2005, directly after first rituximab treatment, shows depletion after 2nd rituximab treatment and continues until roughly 2.5 years remission. Total B-lymphocytes (CD19+) bold line; naive B-lymphocytes (CD19-IgD-CD27-) narrow line. Switched memory B-lymphocytes (CD19-IgD+CD27+) score-dot-dot; non-switched memory B-lymphocytes (CD19-IgD+CD27-) interrupted line; CD5+ B-lymphocytes dotted line. Reference values determined in healthy children in our laboratory are shown as vertical lines.

may eventually lead to secondary antibody deficiency and infectious complications. Future studies in larger numbers of children are needed to analyze this further.

References