Down-regulation of CD40 and CD80 on B cells in patients with life-threatening systemic lupus erythematosus after successful treatment with rituximab


Objectives. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoreactive T cells and polyclonally activated B cells that produce autoantibodies. Five SLE patients who failed to respond to conventional immunosuppressants were treated with anti-CD20 antibody (rituximab) and their clinical manifestations and laboratory data were evaluated, including phenotypic analysis of B cells.

Methods. Rituximab (375 mg/m²) was administered weekly for 2 weeks in five SLE patients who developed severe manifestations despite intensive treatment.

Results. Rituximab resulted in rapid improvement (within several days) in clinical manifestations such as consciousness disorder, seizures, progressive sensory disorder, haemolytic crisis, cardiac function and laboratory data. The effects lasted 20 months in one patient; other patients were in remission for more than 6 months. Flow cytometric analysis revealed down-regulation of CD40 and CD80 expression on CD19-positive B cells 1 week after infusion of rituximab, and such down-regulation was seen for more than 7 months in two patients.

Conclusions. Our pilot study provides sufficient evidence of excellent tolerability and high efficacy of rituximab therapy in refractory SLE. Rituximab not only reduced B-cell number and IgG levels but down-regulated CD40 and CD80 on B cells, suggesting possible disturbance of T-cell activation through these costimulatory molecules. Reduction of both quantity and quality of B cells suggests that rituximab could improve the disease course in patients with refractory SLE.

Key words: Rituximab, Anti-CD20 antibody, Systemic lupus erythematosus, Central nervous system lupus, Autoimmune haemolytic anaemia, CD40, CD80.

Systemic lupus erythematosus (SLE) is an autoimmune disease thought to involve disturbances in T- and B-cell functions. Immune complexes consisting of antigens and autoantibodies secreted from activated B cells cause severe inflammation on various tissues and organs. To control this inflammation, immunosuppressants such as corticosteroids, cyclosporin A (CsA) and cyclophosphamide (CY) are widely used. However, we have experience of patients with SLE who are refractory to these conventional treatments, and innovative approaches need to be developed.

CD20 is a surface molecule specific for B cells, and is expressed in most stages of B cells. Rituximab (Rituxan®; Genentech, South San Francisco, CA, USA) is a chimeric monoclonal antibody specific for human CD20, consisting of human immunoglobulin (Ig) G1x constant regions and mouse variable regions. Rituximab is known to deplete B cells by complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity [1]. This antibody has already been used and has demonstrated high effectiveness in the treatment of B-cell lymphomas. Recently, the potential efficacy of B-cell depletion therapy with rituximab has been reported in several autoimmune diseases, such as idiopathic thrombocytopenic purpura, autoimmune haemolytic anaemia (AIHA), cold-agglutinin disease, Wegener’s granulomatosis, myasthenia gravis and idiopathic membranous nephropathy [2–10]. We also reported that rituximab induced rapid recovery of life-threatening refractory SLE with renal and CNS involvement [11]. However, the underlying mechanism of the efficacy of rituximab for the long-term remission remains unknown.

The CD40/CD40L and CD28/CD80–CD86 pathways on lymphocytes are known to be up-regulated in active SLE patients and their relevance to the pathogenesis of SLE has been thoroughly investigated [12–22]. In this study, we administrated rituximab to five patients with refractory SLE and investigated the association between clinical improvement and phenotypic alteration of B cells. We found long-term effects of rituximab on down-regulation of CD40 and CD80 on B lymphocytes, suggesting that rituximab maintains long-term remission of SLE by correcting B-cell aberration. This is the first report that proposes an alternative mechanism of action of rituximab on autoimmune disease with regard to the regulation of the surface molecules on lymphocytes.

First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Kitakyushu 807-8555, Japan.

Submitted 27 June 2004; revised version accepted 17 September 2004.

Correspondence to: Y. Tanaka, First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Kitakyushu 807-8555, Japan. E-mail: tanaka@med.uoeh-u.ac.jp
Patients and methods

Patients

The study subjects were five patients with SLE who fulfilled the diagnostic criteria of the American College of Rheumatology. They represented all patients admitted to our department between 2000–2003 who had high disease activity and who failed to respond to conventional immunosuppressants, including steroid pulse therapy, intravenous CY pulse therapy (IV-CY), CsA, plasma exchange therapy (PE) and immunoadsorption (IA), over an average period of 40 months (range 3–135 months). The clinical characteristics and previous therapies of five patients are summarized in Table 1.

Assessment

Clinical and laboratory assessments were performed before treatment and weekly for 1 month after the initial infusion. Patients were evaluated for clinical manifestations of SLE and any adverse effects of therapy. Laboratory measurements included full blood count, erythrocyte sedimentation rate (ESR), renal and liver serum function tests, serum complement, serum Ig levels, anti-double-stranded (ds) DNA levels, and CD markers on lymphocytes using flow cytometry. The SLE Disease Activity Index (SLEDAI) [23] was used for individual organ system assessment. Treatment efficacy was evaluated on the basis of improvement in both clinical and laboratory indices of active disease.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Lymphocyte Separation Medium (LSM; ICN/Cappel Pharmaceuticals, Aurora, OH, USA), and then washed twice with phosphate-buffered saline (PBS). PBMCs (2 x 10⁶) were resuspended in blocking buffer [0.25% human globulin/0.5% human albumin (Yoshitomi, Osaka, Japan)/0.1% NaCl] in PBS) in 96-well plates for 15 min at 4°C. Fluorescein isothiocyanate-conjugated monoclonal antibodies (mAb) against human CD40, CD86 (PharMingen, San Diego, CA, USA) and CD80 (Chemicon Europe, Chandlers Ford, UK), CyChrome-conjugated mAb against human CD19 (PharMingen) was added to PBMC in 100 μl FACS medium (0.5% human albumin/0.1% NaCl) in PBS) for 30 min at 4°C. Cells were washed three times in FACS medium and analysed with FACSScan and CellQuest software (Becton-Dickinson, San Jose, CA, USA). Quantification of the cell surface antigens on one cell was performed using Qifkit (Dako Japan, Kyoto, Japan). Treatment efficacy was tested statistically by Student’s t-test. A P value less than 0.05 denoted a statistically significant difference.

Results

Clinical outcome

The clinical condition significantly improved in each patient after the administration of rituximab (Table 2). Among five patients, cases 1 and 2 showed marked decreases of SLEDAI on day 28 (Fig. 1). SLEDAI decreased from a median of 24.4 (range, 2–49) at baseline to a median of 10.2 (range 0–16) after 28 days.

Case 1 was diagnosed with SLE in 1991. She had had many episodes of refractory lupus nephritis since 1995. The disease relapsed with leucocytopenia, increased ESR and anti-dsDNA level, a low level of complement, and nephrotic syndrome (urinary protein >8 g/day) in May 2002. Steroid pulse therapy, IV-CY and IA therapy with high-dose oral prednisolone failed to improve the clinical symptoms and signs. In addition, consciousness disorder appeared in June and progressed rapidly. Despite PE six times, her consciousness level worsened to a Glasgow Coma Scale (GCS) score of 7–11. After the first administration of rituximab, she became alert (GCS score of 15) on day 5. Proteinuria improved

Table 1. Characteristics of five patients with SLE at study entry

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Disease duration</th>
<th>Previous therapy</th>
<th>Disease manifestations</th>
<th>Laboratory data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>19 yr</td>
<td>CS, IV-CY, CSA, MTX, VCR, PE</td>
<td>Consciousness disorder (disorientation, soliloquy, abnormal behaviour, GCS 6–8), convulsion, fever, fatigue, oedema</td>
<td>Leucocytopenia, anaemia, ESR ↑, anti-dsDNA ↑</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>3 months</td>
<td>CS, IV-CY, PE, IA</td>
<td>Consciousness disorder (unconsciousness, drowsy, GCS 3), convulsion</td>
<td>Leucocytopenia, thrombocytopenia, anaemia, ESR ↑, proteinuria, AIH IgG ↑, anti-dsDNA ↑</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>25 yr</td>
<td>CS, CY, PE</td>
<td>Sensory disorder (paraesthesia of fingers)</td>
<td>Severe AIHA, reticulocytes ↑, d-Coombs 4+, anti-dsDNA ↑</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>1 yr</td>
<td>CS, CSA</td>
<td>Fever, malar rash, depilation, fatigue, muscular pain, headache, sensory disorder (left axilla precordia)</td>
<td>Cardiomyopathy (ejection fraction ↓), leucocytopenia, thrombocytopenia, ESR ↑, C4 ↓, anti-dsDNA ↑, IgG index 0.92</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>3 yr</td>
<td>MZ, CS, IV-CY</td>
<td>Sensory disorder (paraesthesia of fingers, toes left precordia left back), photosensitivity, mouth ulcer</td>
<td>Lymphocytopenia, C4 ↓, IgG index 0.85</td>
</tr>
</tbody>
</table>

Disease activity in the five patients was high and they had not responded to conventional immunosuppressants. Cases 1, 2 and 3 had life-threatening refractory SLE before they received rituximab.

AZA, azathioprine; MTX, methotrexate; VCR, vincristine; MZ, mizoribine; AIH, autoimmune hepatitis; d-Coombs, direct Coombs; C, complement.
Case 2 was diagnosed with SLE with renal dysfunction, pancytopenia, positive anti-dsDNA and consciousness disorder (GCS score of 3) in April 2003. Consciousness disorder and renal dysfunction progressed and abnormal data persisted despite various therapies, which included oral steroid, IV-CY, IA and PE. Finally, she received rituximab in July 2003; her consciousness improved (GCS score of 14) on day 2 and convulsion and proteinuria disappeared completely. She showed marked and rapid responses to rituximab treatment.

Case 3 was treated for SLE with oral steroid since 1978. She developed AIHA after surgery for carcinoma of the uterine cervix in 2001. IV-CY seven times followed by oral steroid improved AIHA, but she could not continue the treatment because of fungal infection. AIHA deteriorated to a haemoglobin (Hb) level of about 7–8 g/dl and a reticulocyte count of about 200% with 2 mg of betamethasone. This steroid dosage could not control haemolytic attacks, and Hb dropped to about 5 g/dl and thrombosis was observed due to red blood cell (RBC) coagulation. Accordingly, all treatment was stopped and she was started on rituximab in April 2003. RBC coagulation disappeared 3 months later and her Hb level recovered up to 10–11 g/dl without blood transfusion.

In case 4, SLE had started with malar rash and arthritis in 2002. In September 2003 the clinical condition worsened with sensory deficit on the area extending from the left axilla to the left precardia, severe headache (IgG index 0.92), myocardial dysfunction due to SLE (ejection fraction, 44% on ultrasonic cardiography), leucocytopenia, thrombocytopenia, increased ESR and anti-dsDNA, and low-level complement. Since these manifestations worsened rapidly despite the combination therapy with high doses of oral steroids and CsA for a month, rituximab was administered in November 2003. This treatment gradually reduced the sensory deficit and headache, and ejection fraction in ultrasonic cardiography increased to 72.1%.

Case 5 developed arthritis, photosensitivity, dryness of the mouth, positive anti-dsDNA and lymphocytopenia, and was diagnosed with SLE and Sjögren’s syndrome in 2000. Sensory disorder on the left half of the body and deficit of the visual field appeared in 2001, and paraesthesia on the lower part of the chest appeared in 2003. T2-weighted magnetic resonance imaging (MRI) showed a high-intensity lesion in lower segment of the medulla oblongata and cervical spinal cord and the white matter of the cortex. Steroid, mizoribine for 2 yr and IV-CY eight times were not effective for these manifestations, and a new high-intensity lesion was detected on MRI in 2004. After administration of rituximab, these disorders diminished and the abnormal lesion on MRI disappeared, along with the resolution of skin and mucous lesions.

The above five patients were in remission more than 6 months after the commencement of rituximab therapy. Their oral daily steroid doses have been decreased and immunosuppressants have been discontinued in all patients because of remission of the clinical state.

Clinical manifestations and laboratory data were improved significantly after rituximab injection. Steroid could have been reduced and immunosuppressants were discontinued in all patients. Data for case 1 refer to 19 months after treatment; data for case 2 after 8 months; data for case 3 after 7 months; data for case 4 after 7 months; data for case 5 after 3 months.

PSL, prednisolone; m-PSL, methylprednisolone; WBC, white blood cells; Hb, haemoglobin; U protein, urinary protein.
Adverse effects

No infusion-related serious adverse effects of rituximab were observed. In case 1, the serum IgG level decreased significantly to about 200–300 mg/dl 1 month after treatment, and she was treated with infusion of Ig when her serum IgG level was <300 mg/dl. During this period, she had infection with herpes zoster localized at temporal, which appeared on day 25, but it improved smoothly with Ig therapy and acyclovir. Other patients had no severe infections.

Phenotypic analysis of SLE B cells

Costimulatory molecules such as CD40–CD40L and CD28–CD80/CD86 are known to be prerequisites for the activation of autoreactive T cells, and increases in these molecules are reported in active SLE lymphocytes [12–22]. To examine the effects of rituximab on refractory SLE, we assessed the changes in costimulatory molecules on B cells in our patients before treatment and weekly for 1 month after the initial infusion by using flow cytometry. Rituximab therapy markedly reduced CD40 on B cells. In four cases (cases 2, 3, 4 and 5), the mean expression level of CD40 on B cells was 1668 ± 657 (S.D.) molecules/cell before treatment (day 0). CD19⁺ cells significantly decreased within the first week after the administration of rituximab (data not shown). CD40 expression was also down-regulated within 1 week in all cases, and remained low for the first month (Fig. 3); on day 28 it was significantly lower than that on day 0 (664 ± 336 molecules/cell). Furthermore, CD80 was also down-regulated with time after treatment and had decreased significantly by day 28 (Fig. 4) (7834 ± 1775 vs 1396 ± 637 molecules/cell).

The expression levels of CD40 and CD80 were followed for 7 months in case 3 and case 4. The down-regulation of CD40 and CD80 among CD19⁺ cells lasted for 7 months after rituximab therapy in these two patients and a marked change in the histogram of CD80 expression on CD19⁺ cells was observed in case 4 (Fig. 4 and data not shown). Case 3 was still in clinical remission 1 yr after rituximab therapy, although the B-cell count increased to 6.6%. The percentages of CD40⁺ and CD80⁺ cells among her CD19⁺ cells were 94.6 and 97.2% respectively before treatment, but had decreased to 55.5 and 70.8% respectively 1 yr after treatment (data not shown). These results suggest that rituximab normalizes the expression of surface molecules or autoreactivity of B cells, and may have long-term stabilizing effects on the immune system in SLE.

Discussion

In the present study, rituximab was administrated to five patients with SLE who had showed progressive disease and had failed to respond to conventional immunosuppressive therapy. At the start of rituximab treatment, patients received low to moderate dose of corticosteroid, corresponding to 15–40 mg of prednisolone. Rituximab treatment resulted in improvement of their clinical condition and had no significant adverse effects. Though the five patients had various manifestations, such as CNS lupus (cases 1–5), peripheral nervous system disorder (cases 4 and 5), lupus nephritis (cases 1 and 4), haemolytic crisis (cases 1 and 4), and myopathy (case 4), the clinical symptoms and signs and laboratory data rapidly improved in all five patients, as evidenced by reduction in the SLEDAI score. In particular, case 1 became fully alert on day 5, and a similar effect was noted in case 2 on day 2, indicating that the rapid effect of rituximab (a few days) on life-threatening CNS disorder.

The clinical manifestations appear to improve rapidly in parallel with the deletion of B cells from peripheral blood after

FIG. 2. Case 1. Clinical course and response to treatment. The patient was treated with immunosuppressive therapies, including PE (arrows), IA (open arrows), IV-CY (open triangles), m-PSL pulse therapy (closed triangles), betamethasone (open squares), CsA (striped squares), azathioprine (AZA; dotted squares) and methotrexate (MTX; closed squares). Rituximab was administrated at 375 mg/m² once weekly (indicated by dotted arrows).
administration of rituximab. However, autoantibodies will remain in the serum at least for a few weeks and could be produced from CD20 plasma cells, since rituximab does not work on these cells [24]. Furthermore, rituximab was effective in three patients who did not improve with PE and IA, which are thought to eliminate immune complexes and/or cytokines. These results suggest that some of the clinical manifestations, including the CNS disorder observed in these SLE patients, may be largely elicited by cell-mediated immunity rather than autoantibodies.

Rituximab was originally designed to deplete B cells. In our five SLE patients, the quantity of B cells diminished rapidly after treatment. Interestingly, since the B-cell count returned to normal levels, all patients did not relapse and they continue to be in remission. For instance, case 1 received rituximab in July 2002 and the CD19-positive B-cell count was 2.5% of PBMC before treatment and decreased to 0.4% 1 week after the first administration of rituximab. In August 2003, she remained in remission despite the recovery of CD19- and CD20-positive cells to 5.1 and 180, respectively. The CD80 expression on CD19+ cells was analyzed before and at the 7th month after the administration in case 4.

**Fig. 3.** Serial changes in expression level of CD40 on CD19+ cells in four patients (cases 2, 3, 4 and 5) analyzed by FACScan. Data are the amounts of cell surface antigen measured on single CD19+ cells, calculated using Qifkit.

**Fig. 4.** Serial changes in expression level of CD80 on CD19+ cells in three patients (cases 3, 4 and 5) analyzed by FACScan. Data are the amounts of cell surface antigen measured on CD19+ cells, calculated using Qifkit. Histograms of CD80 on CD19+ cells at day 0 and at the 7th month after the administration in case 4 are shown.
5.8% respectively. The anti-dsDNA titre, complement, ESR, and white blood cells were still within the normal range and proteinuria was not detected at that stage. These cases suggest that the clinical effects of rituximab can be explained by mechanisms other than its effect on B-cell count.

Leandro et al. [25] reported that treatment with rituximab caused repopulation of naive B cells in RA and SLE. Our results indicate that rituximab not only reduces the number of B-cell and IgG levels but down-regulates CD40 and CD80 on B cells, indicating that mutual T-cell activation through these costimulatory molecules may be disturbed. It has been reported that the CD40–CD40L and CD28–CD80 pathways are necessary for the activation of autoreactive T cells and for polyclonal B-cell activation. In fact, CD40L–CD40 interaction correlated with the CD40–CD40L and CD28–CD80 pathways are necessary for the activation of autoreactive T cells and for polyclonal B-cell activation.

Thus, the facts that clinical manifestations improved rapidly in all five patients, remission lasted for 20 months in one patient, and changes in both the quantity and the quality of B cells were observed, suggests that rituximab treatment could improve the disease course in patients with SLE. The long-term effects of rituximab on disease activity may be due to ‘resetting’ of the whole autoimmune system through the down-regulation of B-cell activity and the repopulation of inactive B cell clones. This pilot study, therefore, provides sufficient evidence of the excellent tolerability and high efficacy of anti-CD20 rituximab therapy in refractory SLE that is resistant to conventional treatments, and a formal clinical trial is justified.

### Key messages

- Anti-CD20 rituximab therapy provided excellent tolerability and high efficacy in five refractory SLE patients who were resistant to conventional treatments, in a pilot study.
- Rituximab resulted in rapid improvement in clinical manifestations and long-term remission for more than 6 months.
- Rituximab down-regulated CD40 and CD80 on B cells, suggesting that rituximab maintains long-term remission of SLE by correcting B-cell aberration.

#### References


do:10.1093/rheumatology/keh266

Clinical Vignette

Diffuse skeletal hyperostosis and pseudohypoparathyroidism

A 44-yr-old Chinese woman enjoying good health presented with progressive low back pain for 8 yr. Physical examination revealed tenderness at the lumbar spinal processes but not at the sacroiliac joints. Movements of the lumbar spine were limited in all planes. There were no skin lesions, peripheral arthritis or enthesitis. Rheumatoid factor and HLA-B27 were negative.

Plain X-rays of her lumbar and thoracic spine demonstrated undulating calcifications of the anterior longitudinal ligaments involving at least four consecutive vertebral bodies. Intervertebral disc height was relatively preserved. Ectopic calcifications of the iliolumbar ligaments and hip joints were also present. An MRI did not show features of sacroilitis.

Incidentally, she was found to have hypocalcaemia (adjusted serum calcium level 1.45 mmol/l; normal range 2.10–2.55 mmol/l) and hyperphosphataemia (1.81 mmol/l; normal range 0.91–1.55 mmol/l). The serum parathyroid hormone (PTH) level was grossly elevated (18.3 pmol/l; normal range 1.6–6.9 pmol/l). A CAT scan of the brain revealed calcification of the basal ganglia.

Diffuse idiopathic skeletal hyperostosis (DISH) is characterized by hyperostosing and ossifying enthesopathies involving the spine and extra-axial structures [1]. DISH usually affects older individuals and common presenting features are spinal rigidity and polyenthesopathies, simulating the inflammatory spondyloarthopathies. In our patient, the radiological differential diagnoses include DISH, ankylosing spondylitis (AS) and degenerative spondylosis. The absence of true syndesmophytes, apophyseal joint ankylosis, sacroilitis and a negative HLA-B27 virtually excludes AS. The relatively young age and the absence of degenerative changes in the intervertebral discs make lumbar spondylosis unlikely.

Pseudohypoparathyroidism (PHP) is a hereditary disorder characterized by a defective end-organ response to PTH. Chronic hyperphosphataemia may induce ectopic calcifications of the skeletal and soft tissues, mimicking the radiological appearance of DISH. However, these calcifications are often incidental. The long history of back pain in our patient raises the possibility of the co-existence of DISH and PHP, which has hitherto been unreported.

The authors have declared no conflicts of interest.

A. MAK, C. C. MOK

Correspondence to: C. C. Mok. E-mail: ccmok2005@yahoo.com