A longitudinal analysis of SLE patients treated with rituximab (anti-CD20): Factors associated with B lymphocyte recovery

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Abstract Identifying factors associated with B lymphocyte depletion and recovery may aid the development of individualized treatment regimens, optimizing therapy for patients with autoimmune disease. In this study, 12 patients with active SLE were monitored at baseline and monthly following treatment with rituximab. The number and phenotype of peripheral blood B lymphocytes, T lymphocytes and natural killer cells were correlated with the extent and longevity of B lymphocyte depletion. This analysis generated three candidate biomarkers for lymphocyte monitoring in patients with autoimmune disease who are treated with rituximab: circulating transitional B cells, the κ:λ ratio and natural killer cells. Further refinement of these potential biomarkers may lead to a better understanding of the role of B cells in disease pathogenesis and a more rational use of B cell depletion therapies.

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Introduction

Rituximab, a chimeric anti-CD20 monoclonal antibody, was first approved in 1997 as an effective treatment for a sub-population of CD20+ B cell non-Hodgkin’s lymphoma [1,2]. It is now also being used to treat autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus in which B cells are thought to play a pathogenic role. It has been hypothesized that elimination of pre-plasma B cells in patients with a preferential recurrence of naive cells will reestablish B cell tolerance during reconstitution [3–5].
Recent studies have described the pattern of depletion and reconstitution of the circulating B cell compartment following the treatment of patients with both rheumatoid arthritis and SLE with rituximab [6–10]. In patients with rheumatoid arthritis, depletion of B lymphocytes occurs uniformly and regeneration follows a characteristic pattern with an early presence of immature cells in addition to recirculating plasma cells [6]. The repopulation of the memory B cell compartment is delayed in these patients. In contrast, B cell depletion is highly variable among SLE patients ranging from complete and prolonged to transient to incomplete with an increase in the percentage of naive B cells after reconstitution [9].

Given the documented variability in the biological response to B-cell-targeted therapy in SLE patients, the current study employed a longitudinal analysis of B, T and NK lymphocytes in individual patients to gain further insight into the kinetics of B cell depletion and the factors associated with subsequent auto-reconstitution. Findings included (1) three out of four patients with a high proportion of circulating transitional B lymphocytes at baseline exhibited short-lived B cell depletion, suggesting that the baseline phenotype may predict the length of depletion in some patients. (2) Transitional B lymphocytes were usually the first cells to return, irrespective of the baseline phenotype. (3) A variable kappa:lambda light chain ratio (κ:λ) was observed when the B cells first returned. (4) A relative and absolute increase of circulating natural killer cells was observed during B lymphocyte depletion in 8 out of 12 patients.

Materials and methods

Patients/Clinical parameters

Patients, aged 23–56 years, who fulfilled the American College of Rheumatology criteria for SLE [11] were recruited from the University of Pennsylvania to receive rituximab as part of a multi-center phase 1, open label trial aimed to assess the safety and efficacy of rituximab in the management of SLE. All patients had SLE for at least 6 months prior to the start of the study, had failed at least one immunosuppressive drug and had active disease. Each patient underwent a 1-month washout period devoid of any immunosuppressive medications except for prednisone. Exclusion criteria included severe end-organ disease, a history of malignancy, HIV, alcohol or drug abuse, active infections and pregnancy or lactation. Detailed informed consent was obtained from all patients. These studies were performed in accordance with a protocol that was approved by the University of Pennsylvania Institutional Review Board. The present communication describes data for 12 SLE patients and 13 control subjects recruited at the University of Pennsylvania for whom we obtained detailed flow cytometric data.

Rituximab was provided by Genentech (San Francisco, CA). Ten patients received a full course with the dose approved for use in lymphoma (375 mg/m² intravenously weekly for 4 weeks). The patients were pretreated with 50 mg of oral diphenhydramine, 650 mg of oral acetaminophen, and 100 mg of intravenous methylprednisolone 30 min before each infusion. Two patients received partial doses due to the development of serum sickness (patient 20) and voluntary withdrawal (patient 24). One patient was retreated with rituximab after week 31 of the study (patient 22). The clinical response was assessed primarily by D.A.A., calculating a SLEDAI score at baseline and at weeks 4, 7, 11, 15, 19, 27, 39, and 55.

Peripheral blood lymphocyte immunophenotyping

Flow cytometric analysis of T and B lymphocytes and natural killer (NK) cells was performed at baseline for each patient and at 4- to 8-week intervals following rituximab therapy for an average of 54 weeks (range 41–60). Flow cytometry was not performed at the following time points: subject 4 (5 months), subject 5 (6 months), subject 7 (4 and 10 months), subject 9 (3 and 12 months), subject 15 (4 months), subject 22 (3 and 10 months), subject 23 (10 months) and subject 24 (3, 5, 10, 11 and 12 months; this subject withdrew from the study.) A similar analysis was performed at a single time point for 13 apparently healthy normal controls. Monoclonal antibodies were added directly to 150 μl aliquots of fresh whole blood (anticoagulated in sodium EDTA) and incubated at room temperature for 20 min. Red blood cells were lysed in ammonium chloride (BD Pharm Lyse) and the remaining mononuclear cells were washed in Dulbecco’s PBS with 5% fetal calf serum and sodium azide. Cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences). Only fresh whole blood specimens (<24 h old) were analyzed in this study. A seven-tube, four-color panel with antibodies against the following cell surface markers (clone) was used: CD3 (HIT3a), CD4 (SK3), CD8 (SK1), CD19 (SJ25C1 or HIB19), CD27 (MT271), CD38 (HIT2), IgM (G20–127), IgD (IA6-2), kappa (G20–193), lambda (JDC-12), CD56 (B159) and CD16 (clone 3G8) (BD Pharmingen San Jose, CA). Analysis was performed on a FACSCalibur flow cytometer with CellQuest software (Version 5.2.1, Becton Dickinson, San Jose, CA). After gating on lymphocytes utilizing forward scatter and side scatter, T cells were identified based on CD3 expression and analyzed for CD4 and CD8 expression. B cells were identified based on CD19 expression and analyzed for CD27, CD38, IgM, IgD, kappa and lambda expression. NK cells were identified as CD3-negative, CD56 and/or CD16 positive lymphocytes. Negative populations were determined using anti-mouse isotype controls. We collected either 10,000 CD3+ or CD19+ events per tube or the entire sample if this number of CD19+ cells was not reached. Absolute cell numbers were calculated using the white blood cell count and the percentage of lymphocytes from a complete blood count (CBC) usually obtained on the same day (or at most within 1 week) of the immunophenotyping sample.

Statistical analysis

Mean values, standard deviation and standard error of the mean were calculated for clinically significant groups. Statistical significance between patients and controls was determined using a two-sided Student’s t-test with a p value of <0.05, with the caveat that the subject size is small and that small differences between groups will be missed in this analysis. Correlation coefficients were calculated for ranked and unranked
data were calculated using regression analysis in Microsoft Excel.

Results

Overview of the study design

Patients with SLE tend to be heterogeneous with respect to disease activity and their immunologic profiles, including the composition of their lymphocyte subsets [12–14]. Therefore, this study utilized a longitudinal analysis of peripheral blood lymphocytes in 12 patients with SLE in which a baseline sample from each patient was compared to all subsequent samples from the same patient. In addition, the baseline phenotype of the SLE subjects was compared to the phenotype of 13 control subjects. Demographic information and clinical responses are summarized for the study subjects in Table 1. There was a statistically significant improvement in the SLEDAI at the exit time point compared to baseline \((p < 0.007, \text{ Wilcoxon signed rank test})\). The full clinical description of these patients will be detailed elsewhere.

Full B cell depletion obtained in 11 of the 12 SLE patients

The B cell count data for each subject are shown in Table 2. Full depletion, defined as a B cell count less than 5 cells/\(\mu l\),
was achieved in 10 of the 12 patients by week 7 and in an eleventh patient by week 15. These subjects had an average B cell count of 0.5 (0–2.2) cells/μl at their nadir, a 97–100% decrease from baseline. One patient (subject 15) experienced only partial depletion, reaching her nadir at week 7 with a B cell count of 65 cells/μl. The B cell count at the nadir correlated inversely with the timing of B cell recovery ($r_s = -0.8$, $r^2 = 0.64$, $p = 0.006$, Spearman rank order correlation coefficient).

A predominance of transitional cells at baseline is associated with early B lymphocyte recovery

CD27 and CD38 expression was used to differentiate between naive transitional B cells (CD27+, CD38+), naive mature B cells (CD27+, CD38−) and activated mature B cells (CD27−, CD38+), as described by Sim and colleagues [15]. Analysis of the 11 SLE patients treated with rituximab. The timing of B lymphocyte recovery is depicted for 11 SLE patients who achieved B lymphocyte depletion to levels below 5 B cells/μl whole blood. The number of CD19+ lymphocytes per microliter of whole blood is plotted as a function of the time point following rituximab therapy. The baseline measurement is made at a screening visit that occurred anywhere from the week of the first infusion to 8 weeks before the first rituximab dose (rituximab treatment spans weeks 1–4). Black bars represent patients in whom circulating B lymphocytes return to >5 B cells/μl before week 24 (early reconstitution). Gray bars represent patients in whom circulating B lymphocytes return to >5 B cells/μl after week 24 (late reconstitution). Error bars represent the standard error of the mean.

SLE patients with the longest depletion time have the greatest improvement in their SLEDAI score. Improvement in the SLEDAI score (SLEDAI score at baseline minus the SLEDAI score at study exit) for all patients with full depletion and documented recovery is plotted as a function of the length of B cell depletion. Patient 6 is an outlier with a long duration of depletion associated with a worsening of the SLEDAI score.
patients that achieved full depletion revealed two groups that differed in the percentage of circulating transitional B cells present at baseline (Fig. 1a). When the proportion of transitional B cells at baseline is plotted as a function of the week following rituximab, SLE patients also appeared to fall into two groups. The first group consisted of 3 patients with elevated transitional B cell levels who experienced early recovery with the B cell count returning before 24 weeks. The second group consisted of 6 patients with low transitional B cell levels who experienced late recovery with the B cell count returning after 24 weeks (Fig. 1b). One subject (number 3) did not fit this pattern, having a high transitional B cell fraction at baseline (59%) and a long period of B cell depletion (over 40 weeks). Subject 22 was excluded from the analysis in Fig. 1b because this subject was re-treated with rituximab at week 31 of the trial.

SLE patients exhibit different patterns of auto-reconstitution

The absolute number of B cells and the subset composition were analyzed in the ten SLE patients who achieved full depletion and were not re-treated with rituximab during the follow-up period. The analysis of absolute B cell numbers (Fig. 2) revealed two groups of SLE patients as described above. Three SLE patients reconstituted their B cells before week 24 and had two waves of B cell recovery occurring between weeks 13 and 24 and after week 24. Seven SLE patients exhibited delayed B cell reconstitution and had only one wave of B cell recovery during the study period. Further analysis comparing the length of depletion to the change in the SLEDAI score suggests that those patients with the longest depletion time have the greatest improvement (Fig. 3).

Figure 4  SLE patients exhibit variable patterns of B lymphocyte reconstitution following rituximab therapy. Immunophenotyping was used to classify circulating B lymphocytes (CD19+) into 5 different subpopulations: transitional (CD27−, CD38+), naive mature (CD27−, CD38−), double negative (CD27 CD38−), activated mature (CD27+CD38+) and memory (CD27+CD38−). Lymphocyte numbers per microliter of whole blood are plotted as a function of the time point in the study (week number). Four patients, each exhibiting a different profile, are shown BL = baseline.
A longitudinal analysis of the expression of CD27 and CD38 on B cells revealed the manner of lymphocyte auto-reconstitution following treatment with rituximab. Examples of lymphocyte reconstitution profiles are shown for single subjects in Fig. 4. Reconstitution profiles of all of the other study subjects are provided in the Supplementary data. In eight out of the ten SLE patients who achieved full depletion without re-treatment, transitional cells accounted for the majority of the B lymphocytes during the 1-year follow-up period. Two of these eight patients (subjects 5 and 20) experienced early B cell recovery. These patients had mature activated B cells present during the early reconstitution period and trended toward their baseline phenotype by week 55 (Fig. 4a and supplement). Six of these eight patients (subjects 3, 4, 6, 9, 18 and 23) experienced late recovery, with an increase in both the relative and absolute number of transitional cells over baseline levels (Fig. 4b and supplement). In three of the six patients with late recovery (subjects 3, 9 and 18), the B cell count at the end of the study period (41–60 weeks) was greater than the baseline B cell count. In the other three (subjects 4, 6 and 23), the B cell count had not reached the baseline value by the end of the study period.

The other two SLE patients (subjects 7 and 24) who achieved full depletion without re-treatment exhibited a predominance of naive mature and mature activated cells early in the reconstitution period (Figs. 4c, d and supplement). Subject 7 went on to develop a transitional B cell predominance 16 weeks into reconstitution. The B cell recovery of subject 24 was delayed until week 41 (Fig. 4d) with a predominance of circulating mature activated B cells. These cells were also present during the two previous time points when the B cell count was only 4 cells/μL. Interestingly, this patient received only a single dose of 375 mg/m² of rituximab.

**Clonal dynamics: skewing of the κ:λ ratio when B cells first return**

To investigate the possibility that the circulating B cell repertoire is oligoclonal during early B cell reconstitution, we evaluated the expression of κ and λ antibody light chains at baseline and at different times following depletion (Fig. 5). If the repertoire derives from a small number of peripherally expanding clones, one might expect the κ:λ ratio to fluctuate dramatically as the B cells first return. In normal individuals approximately 60% of circulating B lymphocytes express κ light chains and 40% express λ light chains [16]. The 95% confidence interval of the κ:λ ratio (1.3–1.9) in 5 control subjects is denoted by the shaded horizontal bar in Fig. 5. The baseline κ:λ ratio for all 12 SLE patients was similar at 1.6 ± 0.3. The κ:λ ratio was followed longitudinally in the 10 SLE patients who achieved full depletion and had documented recovery. Fig. 5 shows the baseline κ:λ ratio and the ratios after B cell recovery has begun, normalized for the onset of B cell recovery. The early B cell repertoire during auto-reconstitution exhibited a skewed κ:λ ratio (range 0.6–5.6 with 7 out of 10 patients falling outside of the 95% confidence interval). The κ:λ ratio then trended toward baseline over the remainder of the follow-up period. The number of B cells analyzed for κ and λ expression at the earliest reconstitution time point ranged from 26 to 814 and did not correlate with the magnitude of the κ:λ ratio (data not shown). These results are consistent with the hypothesis that the peripheral B lymphocyte repertoire is oligoclonal during early B cell reconstitution. Molecular studies will be required to confirm this hypothesis.

**Shifts in natural killer cells**

We also looked for shifts in the number of T cells and NK cells. Among T cells, there were no consistent changes in this small series of patients. However, both the relative and absolute count of natural killer cells (CD3⁺, CD56⁺ or CD16⁺)

**Figure 5**  Shifts in the ratio of kappa to lambda light chain usage during B lymphocyte reconstitution in SLE patients. The κ:λ light chain ratio for each patient is depicted as a function of weeks normalized for timing of recovery (the first time point at which the circulating B cell count is above 5 cells/μL whole blood is listed as week 1–4 for all subjects). The shaded horizontal bar indicates the mean κ:λ ratio ± 2 SD for 5 normal control subjects. Each SLE patient is represented by a different color. The patients with early recovery are represented by circles. The patients with late recovery are represented by squares. The single patient with partial depletion is represented by a circle BL = baseline.
increased in 8 of the 12 of SLE patients after treatment with rituximab (Fig. 6). During B cell depletion, the NK cell count consistently rose in these patients and the increase did not correlate with changes in the CD3+ T cell count. Interestingly, the NK cell count continued to rise even after the B cells had returned (after 36 weeks.) This later (>36 weeks) increase in the NK cell count was strongly correlated with the CD19 count and the CD3 count (r=1.0 and 0.9, respectively; data not shown).

Discussion

This study suggests that the biological response to rituximab in patients with SLE is positively correlated with the length and depth of B cell depletion. However, alterations in the B cell compartment varied between patients. Based on these data, three factors were identified that may serve as candidate biomarkers in the setting of B cell depletion: the proportion of transitional B cells at baseline may predict the longevity of depletion, the $k:\lambda$ ratio may give insight into clonal expansion and repertoire dynamics and the number of NK cells increases in some patients.

B cell phenotype at baseline as a potential predictor of B cell depletion

In this study, we found that an SLE patient’s baseline circulating B cell phenotype may predict the length of B cell depletion. SLE patients with the greatest proportion of circulating transitional cells (CD27+CD38−) experienced the earliest B cell recovery. The mechanistic basis for the correlation between transitional cell predominance and early B cell recovery is unknown. We speculate that it may be due to an increase in the synthetic capacity of the bone marrow that leads to both an elevated proportion of transitional cells and early reconstitution. Alternatively, or in addition, a subset of SLE patients could have B cells (not necessarily transitional cells per se) that are more resistant to depletion or apoptosis.

Anolik and others [9,12,17] have also reported a "naive lymphopenia" in SLE patients. Our analysis of CD27− vs. CD27+ B lymphocyte subsets did not reveal a significant difference between SLE patients and normal controls: the average percentage of CD27− B cells was 78% for SLE subjects and 77% for control subjects. We doubt that a technical difference in sample preparation or CD27 staining explains this discrepancy because the absolute level of CD27− cells in our controls is similar to the control subjects reported by the other groups [9,12,17]. Part of the difference may be due to the ages of the research and control subjects in these studies. In our series, there appears to be a decline in the proportion of the CD27− B cells in SLE subjects between the ages of 30 and 40 years. The average percentage of circulating CD27− B cells in the three SLE subjects younger than 30 is 90%, whereas the average percentage of CD27− B cells in four subjects aged 40 and above is 76%. In addition, other studies have reported that the proportion of CD27− B cells is higher in children than it is in adults [18] and mouse models have shown that the bone marrow production of immature and transitional B cells decreases with advancing age [19].

Different profiles of B cell reconstitution in SLE

The pattern of B cell recovery varied in our 12 SLE patients following B cell depletion with rituximab. Transitional B cells predominated during the initial reconstitution period in eight patients. In mouse models, transitional B cells appear to be more susceptible to negative selection and tolerance induction [20]. The predominance of this B cell subset in SLE patients recovering form B cell depletion, at least in the peripheral blood, could favor the reestablishment of self-tolerance. On the other hand, the skewed $k:\lambda$ ratio is consistent with oligoclonal expansion or an altered threshold of B cell selection. In the setting of lymphopenia, newly emerging B cell clones may be able to undergo homeostatic proliferation due to decreased competition for survival factors such as BlyS [21–23]. Furthermore, BlyS levels can be elevated in patients with SLE [24, 25]. Additional studies will be needed to determine if homeostatic proliferation with clonal expansion of B cells is occurring in SLE patients treated with rituximab. If clonal expansion is demonstrated, it will be of interest to determine the origin of these clones, which may arise either from the newly forming repertoire or from B cells that were resistant to depletion.

Another interesting finding is the presence of mature activated B cells (CD27+CD38−) during depletion and early reconstitution in a minority of subjects. The early emergence of mature cells could be due to resistance to depletion. One of the patients with an early return of mature activated B cells received only a single dose of rituximab (subject 24). This patient had low levels of circulating B cells (~9/μl) for 41 weeks, but nearly all of the B cells that initially returned to the circulation had a mature phenotype. Thus, a single dose of rituximab was sufficient to produce long-lasting depletion in this patient but may not have successfully eliminated some of the mature lymphocyte pool.

In three patients (subjects 5, 6 and 9) additional immunosuppressive medications were re-started following treatment with rituximab. In all three cases, the initiation of treatment with additional immunsuppressives preceded the return of significant numbers of B cells to the circulation. It is possible that some of these immunosuppressives contributed to a longer period of B cell depletion. Additional effects of immunosuppressives on B cell phenotype and function could not be rigorously evaluated in this study.

Expansion of NK cells following B cell depletion

A fourth feature of our data set is the increase in both absolute and relative numbers of NK cells after B cell depletion in 8 out of 12 SLE patients. NK cells increased during B cell lymphopenia and continued to increase as B cells returned. A role for T and B lymphocytes in the homeostasis of NK cells has been proposed [26]. Mature NK cells undergo homeostatic proliferation when transferred to irradiated mice [27]. In the present study, the average lymphocyte count at baseline is lower in the 8 patients experiencing an increase in the NK cell count when compared to the other 4 patients (920 cells/μl vs. 1544 cells/μl). Alternatively or in addition, NK cell production may be ramped up during rituximab therapy due to increased consumption of NK cells in the ADCC of B cells [28]. As rituximab is cleared, the levels of
ADCC and NK cell consumption likely decrease, which may result in increased NK cell longevity. NK cell longevity may be modulated further by shifts in the number and maturation state of B and T cells. It is interesting in this regard that the NK cell number and the number of CD3+ T cells closely parallel one another after B cell recovery (data not shown). Finally, the NK cell number may be altered by a reduction in disease activity. Reductions in NK cell function and numbers have been associated with SLE[29].

Summary and future directions

The clinical response of SLE subjects to B cell depletion therapy, while encouraging, appears to be variable [30]. Since B cell depletion therapy has some risks and is expensive, it would be helpful to find a way of identifying patients that are most likely to benefit. In this study, the baseline B cell phenotype appears to predict the longevity of B cell depletion. At a minimum, this finding is relevant to the projected re-dosing schedule. Despite the small numbers in our study, the data suggest that patients with a longer duration of depletion have the greatest improvement in their clinical score. Clearly, though, resolution of this issue will require larger clinical trials. Another important question is whether the B cell repertoire is re-set following B cell depletion. The ordered appearance of immature, followed by more mature B cell subsets in the circulation is suggestive of auto-reconstitution from early precursors. Alternatively, B cell clones that resist depletion may dominate the peripheral repertoire. A detailed molecular analysis of expanded B cell clones, if present, will help to address this issue. Overall, the biology of B cells in the pathogenesis of SLE is complex. Studying the immunologic response of SLE patients to specific B-cell-targeted therapies will help elucidate the biology and allow for improved predictors of the therapeutic value of B-cell-targeted monotherapy or combination therapy to achieve more profound and long-lived clinical efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2007.11.012.

References


