PLATELETS AND THROMBOPOIESIS

The effect of rituximab on vaccine responses in patients with immune thrombocytopenia

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Key Points

- After treatment with rituximab, immunological responses to both polysaccharide and conjugated vaccines are impaired in patients with ITP.
- Splenectomized patients who received rituximab may be at increased risk of infection because of compromised immune responses to vaccines.

B-cell depletion may impair vaccine responses and increase infection risk in patients with immune thrombocytopenia (ITP). We investigated the effects of rituximab on antibody and cellular responses to Streptococcus pneumoniae polysaccharide and Haemophilus influenzae type b (Hib) vaccines in ITP patients. Of 60 patients in the main trial, 24 patients received both vaccines 6 months after rituximab (n = 17) or placebo (n = 7). Among 20 evaluable patients, 3 of 14 (21%) in the rituximab group and 4 of 6 (67%) in the placebo group achieved a fourfold increase in anti-pneumococcal antibodies (P = .12). For anti-Hib antibodies, 4 of 14 (29%) and 5 of 6 (83%), respectively, achieved a fourfold increase (P < .05). Fewer patients in the rituximab group demonstrated Hib killing (2 of 14 [14%), 5 of 6 [83%], P < .05). Three of 14 rituximab-treated patients failed to respond to vaccines by any criteria. After vaccinations, preplasma cell blasts and interferon-γ-secreting T cells were reduced in rituximab-treated patients. Antibody responses were impaired for at least 6 months after rituximab. Cellular immunity was reduced in parallel with depleted B-cell pools. These findings have implications for the timing of vaccinations and the mechanism of infection after rituximab in ITP patients. (Blood. 2013;122(11):1946-1953)

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by anti-platelet autoantibodies, thrombocytopenia, and bleeding. Approximately 80% of adult patients will develop a chronic form of ITP and many will require treatment; but oftentimes, the disease runs a benign course. B-cell depletion therapy with the monoclonal anti-CD20 antibody rituximab, is commonly used to induce remission in patients with ITP.1,2 In a systematic review, we reported that rituximab was associated with an initial overall response rate of ~60% and a median response duration of 10 months. Additional observational data suggest that long-term remissions occur in ~20% of patients.3-5 Our review also identified a potential signal of harm with rituximab: of 303 treated patients in published reports, 7 (2.3%) developed serious infections 4 of which were fatal.

Many patients with ITP including those who fail rituximab will ultimately require splenectomy, which improves platelet counts in 60% to 70% of patients.6 Splenectomy itself poses a risk of infection, mainly with encapsulated bacteria; thus, vaccination against Streptococcus pneumoniae, Haemophilus influenzae type b (Hib), and Neisseria meningitidis are recommended for all patients before splenectomy.7,8 Immune responses to these vaccines may be impaired in patients who have previously been exposed to rituximab because of its effect on antibody-producing cells.9-17 This raises particular concerns for ITP patients who have received rituximab and who eventually require splenectomy since the protection from presplenectomy vaccines may be compromised.

We designed a sub-study of a multicentered, randomized, placebo-controlled trial of nonsplenectomized patients with ITP to determine the impact of rituximab on vaccine responses. All participants received the pneumococcal polysaccharide vaccine and the Hib conjugate vaccine 6 months after rituximab or placebo infusions. We measured antibody responses after vaccinations and correlated the results with the frequency of T- and B-cell subsets. We found significant impairments in the ability of rituximab-treated patients to mount a specific antibody response with some patients demonstrating altered T-cell function. The data may explain how rituximab may predispose to infection, and provide a rationale for appropriate timing of vaccinations in this patient group.
Methods

Study design
We conducted a prospective cohort study in a subgroup of patients who were enrolled in a multicentered, randomized, double-blind, placebo-controlled trial of rituximab for nonsplenectomized patients with ITP receiving standard treatment. Three of 7 centers in Canada participated in this study. Six months after rituximab or placebo infusion, patients received both the 23-valent pneumococcal polysaccharide vaccine (Pneumovax-23; Merck) and the Hib conjugate vaccine (ActHIB; Aventis). The pneumococcal vaccine was administered by subcutaneous injection and contained a mixture of purified pneumococcal capsular polysaccharides from the 23 most common serotypes associated with invasive pneumococcal infection. The Hib vaccine was administered by intramuscular injection and contained the capsular polysaccharide polyribosyl-ribitol phosphate antigen, the major virulence factor, conjugated to tetanus toxoid. Blood samples were collected immediately before rituximab or placebo infusion, immediately before vaccinations (6 months after rituximab), and 1 week, 4 weeks, and 6 months after vaccinations. Serum samples were frozen and batched for antibody testing. Whole blood samples for B- and T-cell quantification were shipped fresh to the central laboratory for immediate processing and testing.

Patients
Patients who were eligible for the main trial were adults with ITP who had a platelet count below 30 × 10^9/L and required treatment. Exclusions were secondary causes of thrombocytopenia, splenectomy, previous rituximab treatment, significant cardiac or pulmonary disease, active infection or known allergies to vaccines. Patients provided separate consent to participate in the vaccine substudy in accordance with the Declaration of Helsinki, which was approved by the research ethics board at each participating site.

Antibody response to vaccines
The antibody response to vaccines was measured using quantitative and functional methods. We measured anti-pneumococcal capsular polysaccharide antibodies (specific to S. pneumoniae) and anti-polyribosyl-ribitol phosphate antibodies (specific to Hib) by enzyme-linked immunosorbent assays (The Binding Site: Vacczyme Anti-PCP Immunoglobulin G [IgG] Enzyme Immunoassay; and Vacczyme Human Anti-Hib Enzyme Immunoassay). IgG-specific anti-pneumococcal antibodies were directed against a mixture of the capsular polysaccharide serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F, which are contained in the 23-valent vaccine. Interfering antibodies against the pneumococcal cellular antigen were absorbed in a preincubation step. Samples were added to precoated wells, incubated for 30 minutes and washed with PBS. Captured IFN-γ was detected with an enzyme-conjugated substrate and peroxidase substrate, enumerated (Bioreader 5000 ELISPOT reader; Bio-Sys GmbH) and expressed as spots per 5 × 10^4 well.

A serum bactericidal assay was used to assess the functional effect of anti-Hib antibodies on bacterial growth. For the test, twofold serial dilutions of test sera were incubated with 10,000 bacterial cells per well of Hib (strain GB3292) with 10 μL of Hanks buffer with Ca^2+ and Mg^2+ (Life Technologies), 2% Fildef enriched broth (BBL; Becton Dickinson and Co.), and complement (sterile serum from 3- to 4-week-old baby rabbits; Pel-Freez). Bacterial activity was assessed using AlamarBlue, a fluorescent metabolic indicator (Life Technologies). Bacterial titers were reported as the reciprocal of the serum dilution capable of killing 50% of bacterial cells compared with complement controls.

An adequate response to either the pneumococcal vaccine or the Hib vaccine was defined according to previously established criteria, as a fourfold increase in antibody concentration from baseline within the first month after vaccinations. For Hib, a specific IgG titer of 1 μg/mL or higher was considered protective. For the Hib bactericidal assay, a fourfold increase in bacterial killing within the first month after vaccinations was considered an adequate response.

Results

Patient demographics
Of the 60 patients (from 7 centers) enrolled in the main trial, 24 patients (from 3 centers) participated in this vaccine substudy. Seventeen patients had received rituximab and 7 patients had received placebo (Table 1). Median age was 40 years, and 17 of 24...
were females. At the time of vaccinations, median platelet count was $115 \times 10^9/L$ in the rituximab group and $42 \times 10^9/L$ in the placebo group. Three patients in the rituximab group had received other ITP treatments during the 4 weeks before vaccinations (intravenous immune globulin, n = 1; corticosteroids, n = 1; and danazol, n = 1); and 1 patient in the placebo group had received danazol before vaccinations (Table 1).

Antibody response to vaccines

Antibody responses were assessed in 14 patients in the rituximab group, and 6 patients in the placebo group (4 patients were not evaluable because of missing baseline samples). Within 1 month of receiving the pneumococcal and Hib vaccines, 3 of 14 (21%) patients who had received rituximab 6 months earlier achieved a fourfold increase in specific anti-pneumococcal antibody titers compared with 4 of 6 (67%) patients in the placebo group ($P = .12$; difference in proportions = 46%; 95% CI, 2%-89%). For anti-Hib, 4 of 14 (29%) patients in the rituximab group and 5 of 6 (83%) patients in the placebo group achieved a fourfold increase in antibody titers ($P < .05$; difference in proportions = 54%; 95% CI, 17%-93%) (Figure 1).

Hib bactericidal activity was reduced for rituximab-treated patients compared with placebo especially in the first week after vaccinations (Figure 2A). Two of 14 (14%) patients in the rituximab group achieved a fourfold increase in bactericidal activity from baseline values compared with 5 of 6 (83%) patients in the placebo group ($P < .05$; difference in proportions = 69%; 95% CI, 34%-100%) (Figure 2B). Two patients in the rituximab group who had achieved a fourfold increase in anti-Hib antibody titer by enzyme-linked immunosorbent assay and who had antibody concentrations above the threshold that is considered protective (1 mg/mL) did not demonstrate bactericidal activity. In contrast, all patients in the placebo group who achieved a fourfold increase in anti-Hib titers also demonstrated a significant increase in bactericidal activity. Results of antibody responses to pneumococcal and Hib vaccines did not change when the 4 patients who had received ITP therapy within 4 weeks before vaccinations were removed from the analysis.

B-cell mobilization after vaccines

Enumeration of B and T cells was performed in all patients (rituximab, n = 17; placebo, n = 7). Peripheral blood CD19+ B cells were rapidly depleted by rituximab and remained depleted 12 months after treatment; whereas CD3+ T-cell levels were unaffected (data not shown). Naïve B cells (CD19+CD27−) were slightly reduced 1 month after rituximab and recovered to baseline levels by 1 year (Figure 3A). In contrast, resting memory B cells (CD19+CD27+CD38−/low) were significantly lower than baseline
values after rituximab treatment and remained 80% depleted 1 year later ($P < .05$) (Figure 3B). In rituximab-treated patients, preplasma cell blasts (CD19$^-$CD27$^+$CD38$^{hi}$) were at near-normal levels 6 months after the infusion (Figure 3C); however, a high degree of between-patient variability was observed. Absolute numbers of preplasma cell blasts were evaluable in a subset of patients: 4 of 5 patients in the placebo group demonstrated a sharp burst 1 week after vaccinations as expected$^{22,23}$; whereas, only 1 of 4 evaluable patients in the rituximab group demonstrated a rise in preplasma cell blasts immediately after vaccinations (Figure 4).

**Figure 2. Serum bactericidal activity against Hib.** (A) Titer (reciprocal of the serum dilution capable of killing 50% of bacterial cells compared with complement controls) of serum bactericidal activity against Hib post vaccinations in ITP patients treated with rituximab ($n = 14$) or placebo ($n = 6$) (*$P < .05$; bars represent SEM). (B) Vaccine responders, defined as patients who achieved a fourfold increase in functional Hib killing in the first month after vaccinations.

T-cell responses after vaccinations

Functional T-cell responses were assessed in 17 patients in the rituximab group and 6 patients in the placebo group (one patient on placebo could not be analyzed due to missing samples). Following vaccinations, the mean number of IFN-γ–producing T cells was reduced in patients who had received rituximab compared with placebo at 1 week (38 cells vs 93 cells per $5 \times 10^5$ total cells) and 1 month (14 cells vs 43 cells per $5 \times 10^5$ total cells) (Figure 5).

Vaccine failures after rituximab

Three of 14 evaluable patients in the rituximab group failed to respond to both vaccines by all of the following established criteria: (1) They did not achieve a fourfold increase in specific antibodies against *S pneumoniae* and Hib; (2) the concentration of anti-Hib antibodies was below protective levels after vaccinations; and (3) they did not demonstrate an increase in bactericidal activity against Hib. No vaccine failures were observed among the 6 evaluable patients on placebo.

**Discussion**

Rituximab results in a platelet count response in $\sim 60\%$ of ITP patients$^3$ and 20% of patients will achieve long-term remissions.$^5$ The increasing use of rituximab in ITP, an autoimmune disorder that often runs a benign course, calls for an urgent need to better understand the potential harms in this patient population. Most concerning is the possibility of prolonged alterations in normal immunity, which may predispose to infection or impair vaccine responsiveness. This is the first analysis of the impact of rituximab on vaccine responses in patients with ITP. We found that specific antibody responses to both the pneumococcal polysaccharide vaccine (representing a T-cell independent response) and the Hib conjugate vaccine (representing a T-cell dependent response) were significantly impaired for at least 6 months after rituximab treatment. We also demonstrated a reduction in bacterial killing using a functional assay, which was often less pronounced than specific IgG levels. The dissociation between quantitative and functional immunity has been previously reported after Hib and *N meningitidis* immunizations$^{24-26}$ and may be attributable to differences in antibody avidity$^{26}$ or the presence of other isotypes, such as IgM. Immunophenotypic analysis...
indicated that, while preplasma cell blasts were relatively resistant to
depletion by rituximab since they express weak or no CD20, the “burst” in preplasma cell blasts that is expected after vaccina-
tions was frequently lost.

Infection, although rare, is a serious concern with rituximab in
patients with ITP and other autoimmune diseases. Splenec-

omy itself is associated with a risk of infection that is ~2.6-fold higher than nonsplenectomized ITP patients in the first 90 days after splenec-
tomy, and 1.4-fold higher beyond 1 year. In a pooled analysis of
splenectomized ITP patients (n = 484; median follow-up for the
entire cohort, 6.9 years), 2.1% developed invasive infection, and
1.2% died. Encapsulated bacteria have been implicated in over-
whelming postsplenectomy infections, thus routine vaccination
against S pneumoniae, H influenzae, and N meningitidis is recom-
mended before splenectomy. In longitudinal studies, vaccination
generally resulted in a protective antibody response in most patients; however, antibody levels are not routinely measured in practice. Splenectomized patients who have received rituximab may be at an even greater risk of infection because the protection afforded by vaccines may be compromised.

The impact of rituximab on the immune response to vaccination has been examined in patients with malignancy and rheumatic disease. In patients with lymphoma previously treated with high-dose chemotherapy, rituximab was associated with an inadequate antibody response to pneumococcal vaccine but a preserved response to tetanus toxoid and Hib vaccines. The addition of rituximab to chemotherapy in 11 patients with hematological malignancies

antibody, the frequency of hepatitis B reactivation was 3 times higher in patients who received rituximab compared with standard chemotherapy (2.7% vs 0.8%). HBV reactivation is characterized by rapid viral replication due to a loss of immune surveillance, and a loss of CD4+ T-helper and CD8+ cytotoxic T-cell activity. Rituximab has also been associated with rare cases of progressive multifocal leukoencephalopathy, a lethal neurological condition caused by reactivation of latent JC virus, which prompted a safety warning by the FDA. Our data may offer some insight into the mechanism of viral reactivation: We documented a significant impairment in T-cell function, which was likely the result of a reduced pool of antigen presenting B cells. An indirect effect of rituximab on T cells is consistent with other data showing an improvement of T-cell dysregulation after treatment.

A significant proportion of adults with chronic ITP will eventually undergo splenectomy to control their disease. Splenectomy itself is associated with a risk of infection that is ~2.6-fold higher than nonsplenectomized ITP patients in the first 90 days after splenectomy, and 1.4-fold higher beyond 1 year. In a pooled analysis of splenectomized ITP patients (n = 484; median follow-up for the entire cohort, 6.9 years), 2.1% developed invasive infection, and 1.2% died. Encapsulated bacteria have been implicated in overwhelming postsplenectomy infections, thus routine vaccination against S pneumoniae, H influenzae, and N meningitidis is recommended before splenectomy. In longitudinal studies, vaccination generally resulted in a protective antibody response in most patients; however, antibody levels are not routinely measured in practice. Splenectomized patients who have received rituximab may be at an even greater risk of infection because the protection afforded by vaccines may be compromised.

Figure 3. Effect of rituximab on peripheral blood B-cell subsets. (A) Naive B cells (CD19+CD27+); (B) resting memory B cells (CD19+CD27+CD38low); and (C) preplasma cell blasts (CD19+CD27+CD38hi) expressed as percentage of total CD19+ cells (*P < .05; bars represent SEM). Rituximab (n = 17) or placebo (n = 7) was administered at time 0; S pneumoniae and Hib vaccines were administered on week 24.

Figure 4. Preplasma cell blasts before and after vaccinations. Absolute cell counts of peripheral blood preplasma cell blasts in patients with ITP who received rituximab (n = 4) or placebo (n = 5) 6 months earlier. All patients received vaccinations with S pneumoniae and Hib vaccines.
HBV infection before receiving rituximab, given the alterations in cellular immunity and the risk of viral reactivation. Other investigators have made similar recommendations about vaccinations before rituximab in ITP.\(^49\) and rheumatic disease.\(^50\)

In summary, rituximab was associated with an impaired antibody response to pneumococcal and Hib vaccines during the period of B-cell depletion. Antibody levels against Hib did not indicate functional bactericidal activity for all patients. B-cell depletion resulted in a loss of the “burst” in preplasma cell blasts that is expected after vaccinations, which may explain why specific antibody production was reduced. The overall reduction in antigen presenting cells may result in impaired T-cell function. Our findings provide insight into the mechanisms of humoral and cellular immune suppression caused by rituximab in patients with ITP.

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Authorship

Contribution: I.N. designed and performed the research, analyzed the data, and wrote the manuscript; J.G.K., M.L., and D.P.S. designed the research, analyzed the data, and edited and approved the manuscript; N.M.H., M.A.C., and R.J.C. conceived the research, analyzed the data, and edited and approved the manuscript; D.M.A. conceived the research, designed the research, analyzed the data, and wrote the manuscript; and A.T.T. and J.M. collected the data and edited and approved the final manuscript.

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