Characterization of the B cell response to *Leishmania* infection after anti-CD20 B cell depletion

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Received April 2, 2015; Accepted May 25, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: Anti-CD20 depletion therapies targeting B cells are commonly used in malignant B cell disease and autoimmune diseases. There are concerns about the ability of B cells to respond to infectious diseases acquired either before or after B cell depletion. There is evidence that the B cell response to existing or acquired viral infections is compromised during treatment, as well as the antibody response to vaccination. Our laboratory has an experimental system using co-infection of C3H mice with both *Leishmania major* and *Leishmania amazonensis* that suggests that the B cell response is important to healing infected mice. We tested if anti-CD20 treatment would completely restrict the B cell response to these intracellular pathogens. Infected mice that received anti-CD20 B cell depletion therapy had a significant decrease in CD19⁺ cells within their lymph nodes and spleens. However, splenic B cells were detected in depleted mice and an antigen-specific antibody response was produced. These results indicate that an antigen-specific B cell response towards intracellular pathogens can be generated during anti-CD20 depletion therapy.

Keywords: B cell, CD20, *Leishmania*, immunohistochemistry

Introduction

CD20 is a B-cell specific antigen that is expressed solely on B cells but not on early progenitor B cells. CD20 is present on the B cell from the pre-B cell stage until the mature B cell stage and is excluded on plasmablasts and plasma cells. CD20 is thought to be involved in B cell activation and differentiation [1, 2]. Antibodies against CD20 were developed in the late 1980s and 1990s for the treatment of follicular lymphoma, non-Hodgkin's lymphoma, and cancers of the B-cell lineage [3]. Rituximab, an anti-CD20 monoclonal antibody (Rituxan®, Genentech, Biogen IDEC), was first licensed for use in 1997 against follicular lymphoma and is now licensed for use against Non-Hodgkin's Lymphoma (NHL), Chronic Lymphocytic Leukemia (CLL), Rheumatoid Arthritis (RA), Granulomatosis with Polyangiitis (GPA or Wegener’s Granulomatosis), and Microscopic Polyangiitis (MPA). Other anti-CD20 mAbs have also been developed, including Ofatumumab, Ocrelizumab, Veltuzumab, AME-133v, PRO131921, and GA101 [3]. Anti-CD20 monoclonal antibodies are most commonly used in therapies against lymphoma and autoimmune disorders, often in combination with other immunosuppressive agents. Infections are among the most important causes of morbidity and mortality in patients suffering from cancer; however, there is a lack of research on the immunosuppression that anti-CD20 therapies may provoke and whether these therapies may exacerbate these secondary infections [4].

Although anti-CD20 therapies have a proven efficacy and safety, the question remains as to whether these therapies lead to an increased susceptibility to numerous infections. Many clinical trials have conflicting results regarding the association of anti-CD20 therapy and infections; however, one study has shown an increased incidence of infections in patients with lymphomas and rheumatoid arthritis receiving rituximab [5]. A review of recent data showed that rituximab therapy significantly increases the risk of infection in patients with...
Lymphoma or other hematological malignancies; however, this increased risk of infection was comparable to other treatments used in patients with rheumatoid arthritis [6]. Often, patients receiving anti-CD20 therapies have other complicating factors including other active infections [6]. Studies have shown that although anti-CD20 therapies induce B-cell depletion for 6 to 9 months, their immunoglobulin levels do not decrease [7]. This phenomenon may be due to the presence of long-lived plasma cells. Plasma cells can survive for periods greater than 1 year, even in the absence of a memory B cell population [8]. Therefore, most patients receiving B-cell depletion therapy do not have an increase in the number of infectious complications [7]. Other studies have shown that patients receiving anti-CD20 therapy have an impaired humoral immune response to a primary antigen but not to a recall antigen [7, 9, 10]. Although these studies show an impaired humoral immune response, many patients receiving anti-CD20 therapy still produce a measurable antibody response to vaccination and approximately 20% of the patients were seroprotected [9]. Nothing is known about the antibody response towards intracellular pathogens during anti-CD20 treatment. Although it is often thought that humoral immunity is, at best, inconsequential towards intracellular pathogens or, at worst, detrimental to immune control of intracellular pathogens there is emerging evidence that antibodies can play a role in the protective response to infection with intracellular pathogens [11-14].

Leishmaniasis is a commonly used immunologic model of chronic infectious disease. Leishmania species are obligate intracellular protozoan parasites transmitted by the bite of a sand fly. L. major and L. amazonensis cause cutaneous leishmaniasis in many mammalian species, and L. amazonensis can lead to nonhealing lesions [15]. Leishmaniasis is prevalent in 98 countries in the tropics and subtropics and is considered a neglected tropical disease. Multiple mouse models of leishmaniasis are commonly used to study host-pathogen dynamics. C3HeB/FeJ (C3H) mice infected with L. major will resolve cutaneous lesions within 8 to 12 weeks whereas the same mouse strain infected with L. amazonensis develops nonhealing cutaneous lesions. However, mice coinfected with L. major and L. amazonensis resolve their lesions. Our lab has discovered that CD4+ T cells and CD19+ B cells from L. major-infected C3H mice are necessary to kill L. amazonensis within infected macrophages in an in vitro assay [12, 16, 17]. We wanted to test the ability of anti-CD20 administration to prevent a detectable Leishmania-specific B cell response to determine if we could use this model in our experimental system. Treatment with monoclonal anti-CD20 antibodies have been associated with approximately 99% depletion of normal B cells in peripheral blood [18]. However, we show that C3H mice coinfected with L. major and L. amazonensis and treated with anti-CD20 mAb still have a B cell response to these intracellular parasites. Although the depleted mice had significantly less CD19+ cells in the lymph nodes and spleen they still had some germinal center formation and detectable antibodies via immunoblotting. In this report we determine the ability of the mouse to mount an effective immune response to an intracellular infection during monoclonal anti-CD20 treatments.

Materials and methods

Mice

C3HeB/FeJ (C3H) mice (8-10 weeks of age) were obtained from an in-house breeding colony and maintained in a specific pathogen-free facility. Mice were infected with either 5 × 10^6 stationary phase L. major, 5 × 10^6 stationary phase L. amazonensis or 2.5 × 10^6 L. major (LM) and 2.5 × 10^6 L. amazonensis (LA) promastigotes in 50 μL of PBS in the left hind footpad. In the first experiment there were a total of 25 mice with 5 mice per treatment group: 1) LA infected mice 2) LM infected mice 3) LM infected and anti-CD20 treated 4) coinfected, and 5) coinfected and anti-CD20 treated. In the second experiment there were 20 mice total with 5 mice per treatment group: 1) uninfected 2) uninfected and anti-CD20 treated 3) coinfected and 4) coinfected and anti-CD20 treated. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University. Lesion size was monitored and the results were expressed as the difference between the footpad thickness for the uninfect ed foot and the footpad thickness for the infected foot.
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**Parasites and antigens**

*L. amazonensis* (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace’s medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA) and prepared to a concentration of $1 \times 10^8$ parasites/ml. Freeze-thawed *Leishmania* antigen was obtained from stationary phase promastigotes as previously described [20].

**Flow cytometry**

For flow cytometry analysis of surface molecule expression, $1 \times 10^6$ total draining lymph node cells or splenocytes were washed in 2 ml of fluorescence-activated cell sorting buffer (FACS, 0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fcy receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) in 1 mg/ml rat IgG for 20 minutes at 4°C to prevent non-specific binding. Cells were then incubated with the appropriate antibody or isotype control for 30 minutes on ice in the dark. The antibodies used include phycoerythrin-labeled CD19 and phycoerythrin-labeled rat IgG2a isotype control. Antibodies were purchased from BD Pharmingen (San Diego, CA). Following staining, cells were washed in 2 ml of FACS buffer and fixed in 200 μl of 1% paraformaldehyde and stored at 4°C until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA), and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Lymph node and spleen histopathology and immunohistochemistry**

Spleens and popliteal lymph nodes from the left hind leg draining the site of infection were harvested and placed in cassettes in 10% neutral buffered formalin for histological and immunohistochemical analyses. Histological examination was performed on paraffin-embedded tissues cut at 5-μm thickness onto positively charged slides and stained with H&E. For immunohistochemistry, slides were de-paraffinized and blocked with 20% normal rabbit serum. The sections were then incubated with either a rat anti-mouse B220/CD45R antibody (BD Pharmingen, San Diego, CA) at a concentration of 1:50 or biotin-labeled PNA (Vector Laboratories, Burlingame, CA) at a concentration of 1:100 in 10% normal rabbit serum. The slides were rinsed with PBS and then incubated with biotin-labeled goat anti-rat IgG (KPL, Gaithersburg, MD) at a concentration of 1:100 in 10% normal rabbit serum. The slides were stained with H&E. For immunohistochemistry, sections were de-paraffinized and blocked with 20% normal rabbit serum. The sections were then incubated with either a rat anti-mouse B220/CD45R antibody (BD Pharmingen, San Diego, CA) at a concentration of 1:50 or biotin-labeled PNA (Vector Laboratories, Burlingame, CA) at a concentration of 1:100 in 10% normal rabbit serum. The slides were rinsed with PBS and then incubated with biotin-labeled goat anti-rat IgG (KPL, Gaithersburg, MD) at a concentration of 1:500 in TBS for the B220/CD45R labeled slides. Slides were washed and incubated with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) for 15 minutes. After 2 PBS washes, the color was developed with Nova Red (KPL, Gaithersburg, MD) and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).
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Gaithersburg, MD). The slides were then counterstained with Harris’ hematoxylin, then dehydrated and mounted with coverslips.

**Immunoblot analysis**

Protein content of all cell extracts was determined via BCA protein assay (Pierce, Rockford, IL) according to manufacturer’s recommendations, and all samples were normalized to 2 mg/ml using distilled water. Samples (20 to 30 μg of protein) were heated for 4 minutes at 95°C in 4x loading buffer and electrophoresis was performed on a 12% SDS-polyacrylamide electrophoresis gel. Gels were transferred onto polyvinylidene fluoride membranes, blocked with 5% dry milk, and probed with pooled serum from mice. Signals were detected with horse-radish-peroxidase-conjugated goat anti-rabbit antibodies (1:20,000) (Jackson ImmunoResearch, West Grove, PA) using the SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL) and signal was detected with radiography film (Midsci, St. Louis, MO).

**Statistics**

Statistical analysis was performed with Prism5 (Graph-Pad Software Inc., La Jolla, CA). Differences between groups were determined using unpaired t-tests or a Mann-Whitney U-test when appropriate. P values < 0.05 were considered statistically significant.

**Results**

*C3H mice co-infected with L. major and L. amazonensis resolve cutaneous lesions following B cell depletion*

We have previously demonstrated that C3H mice will heal a co-infection with *L. major* and

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*Figure 2. Mice co-infected with Leishmania major (LM) and L. amazonensis (LA) that received anti-CD20 monoclonal antibody therapy had significantly less CD19+ cells and significantly fewer total lymphocytes within the draining lymph nodes as compared to the isotype control. Total draining lymph node cells and splenocytes were harvested at 10 weeks post-infection. Cells were analyzed via surface expression of CD19. A-C. Data are the mean +/- SEM of two separate experiments. A, B. *P < 0.0001, C. *P=0.0048 (t-test).*
CD20 depleted C3HeB/FeJ mice have significantly fewer CD19+ cells present in draining lymph nodes and spleens

In order to determine how the anti-CD20 antibody treatment affected B cells, we analyzed draining lymph node and spleen cell homogenates at 10 weeks post-infection by flow cytometry with anti-CD19 to identify B cells. We used anti-CD19 instead of anti-CD20 or anti-B220 to avoid antibody interference and to allow us to differentiate B cells from plasmacytoid dendritic cells, which also express B220 [21]. CD19, in conjunction with CD21 and CD81, is part of the B cell co-receptor [22]. CD19, like CD20 is present on B cells throughout the developmental stages, excluding plasmablasts and plasma cells [23]. At 10 weeks the percentage of CD19+ B cells from the draining lymph nodes or spleen of anti-CD20-treated mice was significantly less as compared to isotype-treated mice (Figure 2A and 2B). Overall, the percentage of CD19+ B cells in the spleen and draining lymph node of isotype-treated mice were similar, 40% and 50% respectively. However, post-treatment, we observed a 97.5% reduction of CD19+ B cells in the draining lymph node of anti-CD20 treated mice, but only an 80% reduction in the percentage of CD19+ B cells in the spleen of treated mice (Figure 2A and 2B). These data would suggest that anti-CD20 antibody treatment differentially affects B cell depletion, with the spleen being less affected by the treatment. Similar results were found in L. major infected mice with more depletion in the lymph nodes as compared to the spleen (data not shown).

To further assess the B cell response, we also compared total lymphocyte counts from lymph nodes and spleens of co-infected mice with and without anti-CD20 treatment. Co-infected mice treated with anti-CD20 had significantly less lymph node cells and splenocytes compared to the isotype controls (Figure 2C). This was also true for L. major-infected mice treated with anti-CD20 (data not shown).

C3HeB/FeJ mice depleted with anti-CD20 have smaller lymph nodes and altered lymph node histomorphology

Next we characterized the histomorphology of draining lymph nodes and spleens from anti-CD20 and isotype control-treated mice 10 weeks post-infection with L. major and L. amazonensis. Lymph nodes from co-infected mice had reactive lymphoid hyperplasia with a sinus histiocytosis. There was diffuse expansion of the cortex due to marked proliferation of lymphocytes within the paracortex, follicles, and medullary cords. There was also hypercellularity of the medullary sinuses composed of infiltrates of inflammatory cells consisting mainly of macrophages with fewer lymphocytes, plasma cells, and neutrophils (Figure 3).

Draining lymph nodes from anti-CD20 treated mice were significantly smaller as compared to the isotype-treated controls (Figure 3). Anti-CD20 treatment also resulted in a loss of normal lymph node architecture and a lack of follicular organization. There was accentuation of the lymph node stroma, including numerous fibroblasts and adipose cells admixed with lymphocytes, plasma cells, and fewer neutrophils (Figure 3).

C3HeB/FeJ mice depleted with anti-CD20 have significantly less B220/CD45R positive immunostaining and a significant difference in the number of germinal centers in the draining lymph node

Anti-B220/CD45R and biotin peanut agglutinin (PNA) immunohistochemistries were performed to compare B cell populations and germinal

center formation, respectively between the B cell depleted and isotype-treated groups of co-infected mice. Mice that were B cell depleted had significantly less B220/CD45R immunostaining as compared to the isotype-treated groups (Figure 4). B220/CD45R is predominantly expressed on all B lymphocytes, including pro, mature, and activated B cells, and most B220+ cells are also CD19+.

Biotin peanut agglutinin (PNA) is generally used to identify mature lymphocytes in germinal centers. The germinal center score was significantly different between the isotype control and anti-CD20 treated draining lymph nodes. There were few small PNA positive germinal centers throughout the isotype control (Figure 5A), but no germinal centers were seen in the anti-CD20 treated draining lymph nodes (Figure 5B). However, there were no significant differences in the germinal center scores of the spleens between co-infected mice that had been treated with anti-CD20 or an isotype-control antibody. There were very low numbers of germinal centers in the isotype control spleens at 10 weeks post-infection, despite finding only one germinal center in an anti-CD20 treated mouse spleen.

C3HeB/FeJ mice treated with anti-CD20 mAb produce a specific antibody response

Based on our data thus far, anti-CD20 treatment resulted in depletion of B cells from both
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The draining lymph node and spleen, with the spleen containing readily detectable populations of B cells and a rare germinal center. We hypothesized that perhaps, despite the observed B cell depletion, there was still a detectable antigen-specific antibody response. In order to test this, we performed an immunoblot with serum collected from the co-infected mice treated with anti-CD20 or isotype control antibody, at 10 weeks post-infection. The immunoblot was performed for total IgG, IgG2a, and IgG1. The depleted mice still produced *L. major* and *L. amazonensis* specific antibodies (Figure 6).

**Discussion**

The work presented in this manuscript demonstrates that mice co-infected with *L. major* and *L. amazonensis* followed by treatment with monoclonal anti-CD20 antibodies resolved cutaneous lesions with normal kinetics. Anti-CD20 antibody treatment resulted in a 97.5% reduction of CD19+ B cells in draining lymph node and an 80% reduction in the spleen compared to isotype controls. Anti-CD20 treatment led to significantly less total lymphocytes, significantly smaller lymph nodes, significantly less B220+ immunostaining, and significantly
fewer germinal centers within draining lymph nodes. However, treated mice still had detectable antigen-specific antibodies to *L. major* and *L. amazonensis* antigens. Given these results, we have demonstrated that mice treated with anti-CD20 depletion antibodies at 2 weeks post-infection retain a population of responsive B cells and are capable of developing a detectable antibody response to the intracellular pathogen.

In this study mice were treated with anti-CD20 antibody therapy at 2 weeks post-infection to ensure a proper CD4+ T cell response. Other studies have shown that B cells regulate the initial proliferation of CD4+ T cells after encounter with antigen, influence the maintenance of CD4+ T cells, have roles in CD4+ T cell memory responses, and can influence regulatory T cell numbers and function [24-26]. Therefore, we did not begin B cell depletion therapy before infection, to ensure the functionality of the effector CD4+ T cell populations that are needed to resolve cutaneous leishmaniasis.

It is possible that the mice were able to mount an effective humoral immune response to the *Leishmania* antigens within the first 2 weeks.
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Other possible explanations for the lack of total depletion of functional B cells include the presence of CD20\(^+\) B cells that are resistant to anti-CD20 therapies, such as marginal zone B lymphocytes, which have been suggested to be resistant to rituximab depletion [30]. The site at which the B cells are located may also play a large role in the ability of the anti-CD20 depletion therapies. These differences by location may be due to the body's ability to clear antibody targeted cells or the lack of FcR\(^+\) cells that are able to clear the targeted cells [18].

Another less likely theory that could account for the development of antibodies includes the presence of a CD20\(^-\) or CD20\(^{low}\) population of B cells that are not depleted by conventional B cell depletion therapies with anti-CD20 antibodies that are still able to produce an antibody response. This may explain the different pattern of immunoglobulins seen on the immunoblots (Figure 6). Regardless, our results support other studies that have shown that despite B cell depletion therapy patients are still able to mount a humoral immune response to a primary antigen, although that response may be impaired [7, 9, 10, 31]. In our experimental infection we show that mice were able to produce a demonstrable B cell response to an intracellular pathogen despite treatment with anti-CD20 antibodies. These results precluded our ability to definitively test the role of B cells during Leishmania co-infection.

To our knowledge, the work presented here describes, for the first time, the parameters of the B cell response to Leishmania infection after anti-CD20 B cell depletion. We showed that despite B cell depletion, the infected mouse is still able to mount a pathogen-specific humoral immune response. Since B cell depletion therapy is common in people and is often used in conjunction with other immunosuppressive therapies, further research is necessary to determine the risks of B cell depletion before depletion therapy began. This may have lead to the development of long-lived antigen-specific plasma cells that would not be affected by the anti-CD20 therapy. The anti-CD20 dose we used was comparable to other murine studies that demonstrated B cell depletion with some studies depleting essentially all splenic B cells [27, 28]. Our experimental system resulted in only an 80% reduction of B cells in the spleen. In addition, our data differs from some articles that have shown that bone marrow and spleen are more easily depleted than lymph nodes [1, 28, 29]. We expected a more robust depletion and although we did not have an opportunity to test it, a reasonable hypothesis is that the decreased efficiency in depletion may be because of the ongoing chronic intracellular infection. It would be interesting to determine if splenic B cells are relatively resistant to anti-CD20 depletion during a chronic infection, in contrast to draining lymph node B cells which had a 97.5% reduction in B cells. Many factors can influence depletion, including the drug dose administered, distribution to tissues, B-cell intrinsic and microenvironment factors affecting recruitment of effector mechanisms and antigen and effector modulation [1, 29].

**Figure 6.** Co-infected mice treated with anti-CD20 still produce parasite-specific antibodies. Western blot analysis of parasite-specific production of total IgG and isotypes IgG1 and IgG2a were performed at 10 weeks post-infection. Freeze-thawed *Leishmania major* (LM) and *L. amazonensis* (LA) antigen were separated on a polyacrylamide gel and protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were subsequently hybridized with mouse serum (1:25 dilution) pooled from 5 C3H control mice and 5 C3H B-cell depleted mice that were co-infected with *L. major* (LM) and *L. amazonensis* (LA). Following serum hybridization the membranes were probed with goat anti-mouse antibodies to total IgG, IgG1, or IgG2a. Results are from one experiment at 10 weeks post-infection.
therapy in patients with simultaneous infections and the possible differential susceptibility of B cell subpopulations to depletion during chronic infection. Therefore, further understanding of this model could better reflect some real life scenarios, in which those needing B cell therapy also have concurrent infections or established infections prior to treatment.

Acknowledgements

The authors would like to thank Biogen IDEC for providing murine anti-CD20 antibodies and Deb Moore for her help with immunohistochemistry.

Disclosure of conflict of interest

None.

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