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# The Absence of CD20 Messenger RNA in Recurrent Cutaneous B-cell Lymphoma Following Rituximab Therapy

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## Abstract

**Background:** Rituximab has been used to treat relapsed low-grade or advanced non-Hodgkin's lymphoma since 1997, targeting the CD20 antigen expressed by B cells. Single-agent rituximab therapy is safe and well tolerated. Recurrences showing a loss of CD20 expression following rituximab therapy have been reported.

**Methods:** Four patients with CD20-positive cutaneous B-cell lymphoma received rituximab therapy with subsequent recurrences. The biopsies were assessed for cytoplasmic CD20 expression; CD20 messenger RNA was also assessed where tissue was available.

**Results:** Cutaneous relapses occurring within 1.5–3 months following the last dose of rituximab were CD20 negative. In three cases, subsequent relapses showed renewed expression of CD20. Those biopsies demonstrating a loss of surface and cytoplasmic CD20 by immunohistochemistry also showed no evidence of messenger RNA for CD20 using an *in situ* polymerase chain reaction-based methodology.

**Conclusions:** Rituximab may be associated with the emergence of CD20-negative B-cell clones, potentially rendering a tumor insensitive to this drug. Conversely, following cessation of the drug, a re-expression of CD20 within the neoplastic cells may occur allowing therapeutic intervention with this monoclonal antibody. The loss of CD20 expression appears to be a direct effect of the drug on CD20 messenger RNA synthesis.

Tumor-specific antigen-directed antibody as an antineoplastic tool serves to usher in a new era of immunotherapy. Monoclonal antibody (MoAb) activity in the treatment of non-Hodgkin's lymphoma (NHL) has shown significant success and less toxicity compared to aggressive approaches using conventional combination chemotherapy, radiotherapy, and stem cell transplantation. The use of MoAb-based therapy in advanced stage or relapsed low-grade NHL has succeeded where conventional approaches have failed.<sup>1</sup>

## Introduction

Rituximab [IDEC-C2B8 (RITUXAN)] is the first MoAb approved by the Federal Drug Administration (FDA) for the treatment of hematopoietic malignancies.<sup>2</sup> IDEC-C2B8 is a chimeric MoAb that avidly binds the B-cell-specific antigen CD20 expressed on neoplastic B-cells in NHL lesions.<sup>1,2</sup> Its toxic efficacy against CD20-positive cells *in vitro* has been attributed to its human IgG1 constant region that augments complement mediated lysis, antibody-dependent cell-mediated cytotoxicity, inhibition of proliferation, and induction of apoptosis.<sup>1,3</sup> No dose-limiting toxicity has been identified; the treatment is safe and shows significant clinical efficacy in patients with bulky relapsed or refractory low-grade or follicular B-cell NHL.<sup>1,4</sup> Rituximab therapy is usually well tolerated; however in select patients with high numbers of circulating CD20-positive tumor cells, complement activation and tumor necrosis factor release may play a role in causing significant first-dose side effects including fever, chills, and flushes.<sup>5,6</sup> In addition, some patients may experience myelosuppression that is refractory to granulocyte/granulocyte-macrophage colony-stimulating factor.<sup>7–9</sup>

There are increasing numbers of reports on the emergence of recurrent lymphomas with loss of CD20 expression following rituximab therapy.<sup>10–14</sup> In the context of cutaneous lymphoma, there are two case reports describing CD20-negative relapses in patients receiving rituximab therapy.<sup>15,16</sup> We present four patients with primary or secondary cutaneous B-cell lymphomas who were treated with rituximab and subsequently relapsed with CD20-negative disease. In three of these cases, CD20 re-expression occurred, one developing in the setting of continued rituximab therapy while the other two developed the CD20-positive relapses in the absence of the agent. The mechanisms leading to CD20 loss are explored, specifically assessing its potential effects on CD20 synthesis at the transcriptional level.

## Materials and methods

Four cases were encountered from the dermatopathology files of one of the authors (CM) whereby a diagnosis of CD20-positive primary or secondary cutaneous B-cell lymphoma was made. All patients were treated with rituximab at a dose of 375 mg/m<sup>2</sup> weekly. The patients developed recurrences following rituximab therapy whereby tumor cells demonstrated loss of CD20 expression. The critical question in this study was to assess the

nature of CD20 loss, specifically to determine whether this diminution in CD20 expression was due to cessation in the production of CD20 by the neoplastic lymphocyte, or an epiphenomenon reflecting the ability of rituximab to block the binding of anti-CD20 antibody to antigen expressed on the cell surface.

## Immunohistochemistry

We employed an immunohistochemical methodology that specifically assessed for the presence of CD20 within the cytoplasm of the neoplastic B lymphocyte, as opposed to an antibody which exclusively recognized the surface expression of CD20. The antibody used was to clone L26 (Dako, Carpinteria, CA, USA).

Paraffin-embedded tissue was cut at 4 µm and placed on positively charged slides. Slides with specimens were then placed in a 60°C oven for 1 h and cooled, then deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase.

Antigen retrieval was performed by a heat method in which the specimens were placed in a citric acid solution (pH 6.1) for 30 min at 94°C using a vegetable steamer. Slides were then placed on a Dako Autostainer, immunostaining system. Primary antibody is in ready-to-use form, no dilution necessary. The detection system used was Labeled streptavidin–biotin Complex. This method is based on the consecutive application of a primary antibody against the antigen to be localized, biotinylated linking antibody, enzyme-conjugated streptavidin, and substrate chromogen (diaminobenzidine). Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions, and coverslipped.

## Reverse transcriptase *in situ* polymerase chain reaction studies

Next we employed specific primers to determine the presence of CD20 messenger RNA within the neoplastic lymphocyte using reverse transcriptase (RT) *in situ* polymerase chain reaction (PCR) (RTISPCR). Our methodology for RTISPCR has been previously published. Briefly, optimal protease digestion time was determined using non-specific incorporation of the reporter nucleotide digoxigenin dUTP. Optimal protease digestion was followed by overnight incubation in Rnase-free DNase (10 U per sample; Boehringer Mannheim, Indianapolis, IN, USA) and one step RT/PCR using the rT<sup>th</sup> system and digoxigenin dUTP. Primers specific for CD20 transcript were as follows: 5' CD20 5' GGG GGC TGT CCA GAT TAT GA 3' and 3' CD20 5' ACG ATG CCA GCT ATT ACA AGT TCC 3'. The chromogen was nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP) with nuclear fast red as the counter stain.<sup>17</sup>

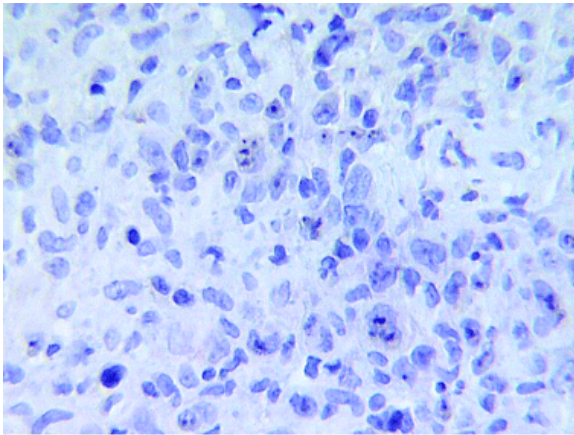
## Results (Table 1)

**Table 1.** Summary of clinical and pathology data

| Case number | Age/sex | Clinical presentation                | Type of lymphoma                                | Multi-organ disease              | Date of initial diagnosis | Rituximab treatment | First post-rituximab recurrence                              | CD20 RT in situ PCR | Additional recurrences  | Outcome                             | CD20 |
|-------------|---------|--------------------------------------|---|----------------------------------|---------------------------|---------------------|--|---------------------|---|-------------------------------------|------|
| 1           | 77/F    | Cutaneous nodules: upper extremities | CD30 <sup>+</sup> diffuse large B-cell lymphoma | Lung concurrently with skin      | February 2002             | Single agent        | 6 weeks after completing treatment                           | –/–                 | Lost to follow up   | Lost to follow up                   | N/A  |
| 2           | 37/M    | Subcutaneous nodules: back           | Marginal zone lymphoma                          | No                               | November 1991             | Single agent        | Multiple recurrences in 1999/2000 despite being on rituximab | –/–                 | >5 recurrences since 1998; 24 treatments with rituximab thru 2002 | Alive                               | +    |
| 3           | 51/M    | Testicular mass                      | Mantle-cell lymphoma                            | Lymph nodes and skin             | April 2000                | Multiagent          | April 2002   | –/–                 | 4 recurrences; continued on rituximab May 2002                    | Dead                                | +    |
| 4           | 86/F    | Pneumonia and nodules on legs        | Primary cutaneous leg lymphoma                  | Later developed gastric lymphoma | June 2000                 | Single agent        | 3 months after finishing treatment                           | –/–                 | Off rituximab; 13 months later developed a gastric lymphoma       | Alive as of 2002; Lost to follow up | +    |

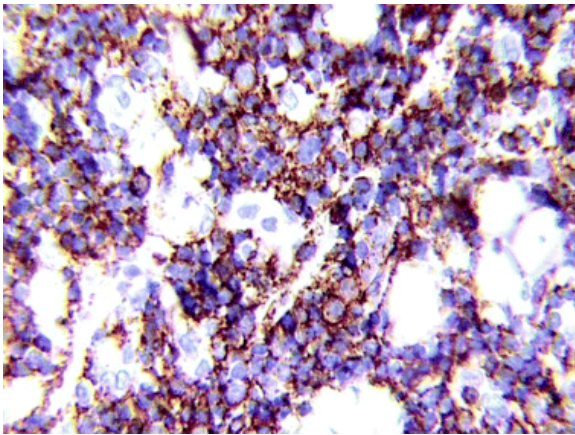
The patient population comprised two women and two men ranging in age from 36 to 86 years (mean age 52 years). Three of the patients had developed primary cutaneous B-cell lymphoma, while one patient had multinodal mantle-cell lymphoma with secondary skin involvement. Each of the cases will be detailed separately.

Patient 1 was a 77-year-old female who had been taking methotrexate for 15 years for treatment of rheumatoid arthritis. She had a sudden onset of subcutaneous nodules on her upper extremities, and over the course of approximately 1 week, she noted rapid development of multiple nodules across her upper extremities and torso. She was diagnosed with a cutaneous CD20-positive B-cell lymphoma for which she received a rituximab eight-dose schedule single-agent chemotherapy leading to a resolution of lesions. Six weeks after cessation of this treatment, she had a recurrence of her lymphoma limited mainly to her upper and lower extremities, which was compatible with a CD30-positive, CD20-negative diffuse large B-cell lymphoma that also coexpressed Epstein–Barr virus (**Fig. 1**).

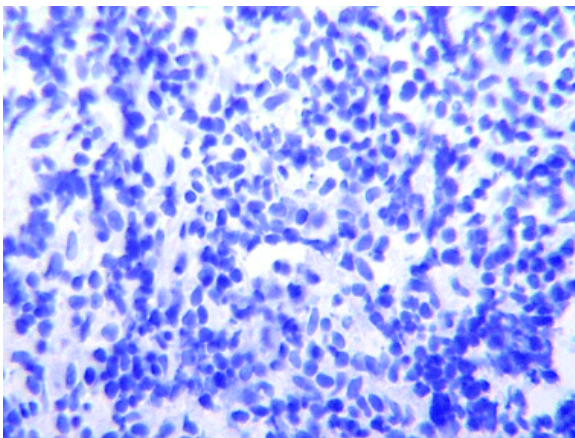


**Figure 1** Following rituximab therapy, this patient developed a recurrent CD30-positive Epstein–Barr virus-associated diffuse, large B-cell lymphoma. The tumor cells show no CD20 expression (Patient 1) (×40).

Patient 2, a 36-year-old male presented with multiple subcutaneous nodules on his back, a recurrence of his primary cutaneous marginal zone lymphoma 5 years after the initial diagnosis. The relapsed disease involved multiple cutaneous nodules across the chest, back, and forehead. The cutaneous marginal zone lymphoma was CD20 positive (**Fig. 2**). At that time, he received a single-agent four-dose therapy of rituximab that led to lesional resolution. He developed multiple relapses between 1999 and 2000, although was still continued on rituximab for a total of 24 treatments. The recurrence from 2000 showed a loss of CD20, and the infiltrate showed greater pleomorphism compared to the original lymphoma (**Fig. 3**). Rituximab was discontinued, and the patient's treatment was confined to involved-field radiation that led to resolution of his skin lesions. In October 2001, he developed a CD20-positive recurrence on his back; he was then treated with eight cycles of rituximab. The most recent recurrence arose in March 2004, although the patient has not yet been treated. The tumor, again on the patient's back, was CD20 positive. His last treatment with rituximab was in January 2002.



**Figure 2** This patient developed a recurrence of his marginal zone lymphoma. The initial recurrence was CD20 positive (Patient 2) (×40).

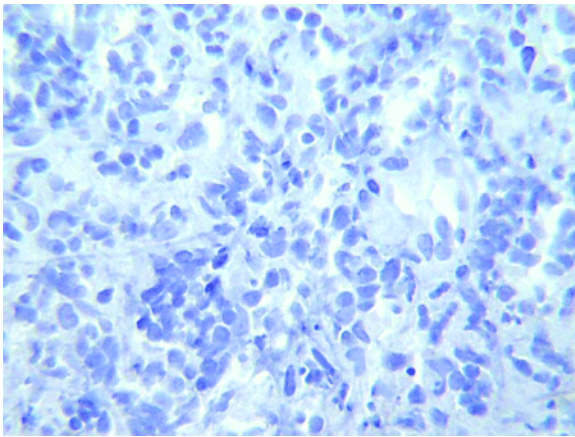


**Figure 3** Despite continued rituximab, the patient continued to develop recurrent tumors, which became CD20 negative (Patient 2) (×40).

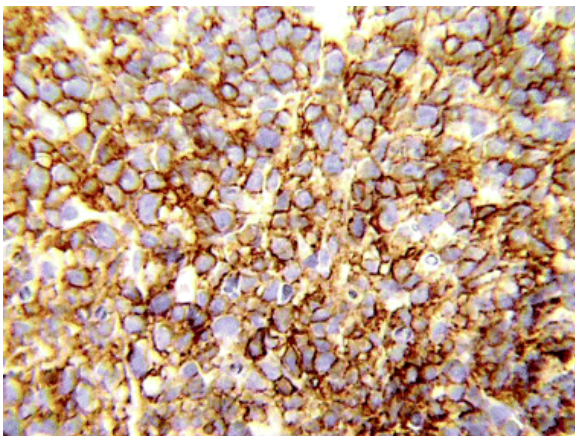
Patient 3, a 49-year-old male, was diagnosed with testicular mantle-cell lymphoma in April 2000. After eight cycles of cyclophosphamide doxorubicin Oncovin and prednisone (CHOP), he developed a cervical lymph node recurrence which led to further chemotherapy and an autologous bone marrow transplant in April 2001. In October 2001, he developed cutaneous nodules on his right calf and right leg above the knee compatible with blastic mantle-cell lymphoma. Rituximab was initiated in February 2002. In April 2002, there was a recurrence of his skin tumor on his right thigh that was now CD20 negative. In May 2002, another recurrence developed on his left forearm and back, manifesting a similar morphology although now the tumor was CD20 positive. During the entire period up to this recurrence in May 2002, he was continued on rituximab. He died shortly thereafter.

Patient 4, an 86-year-old female, presented with pneumonia and multiple cutaneous nodules on her lower extremities. A diagnosis was rendered of primary cutaneous large B-cell lymphoma of the legs, leading to therapy with single-agent rituximab with which complete lesional resolution occurred. Three months after completion of her chemotherapy, a recurrence developed on the lateral aspect of her left leg below the knee, which showed loss of CD20 expression (**Fig. 4**). The tumor eventually resolved within 3 months without treatment. However, 5 months later, she subsequently developed a diffuse, large B-cell lymphoma of the stomach that manifested CD20 expression (**Fig. 5**).





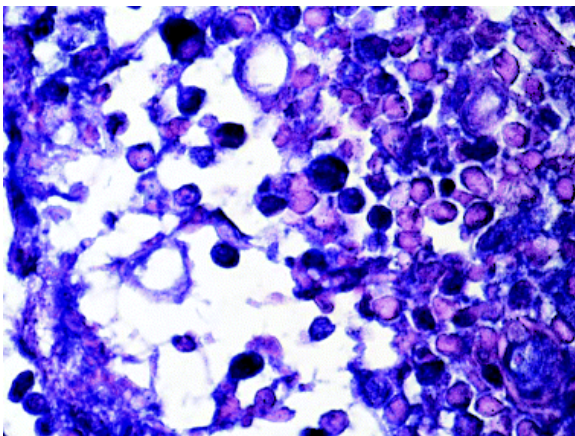
**Figure 4** The patient received rituximab which initially resulted in lesional resolution. However, a subsequent recurrence developed which failed to show expression of CD20 (Patient 4) (×40).



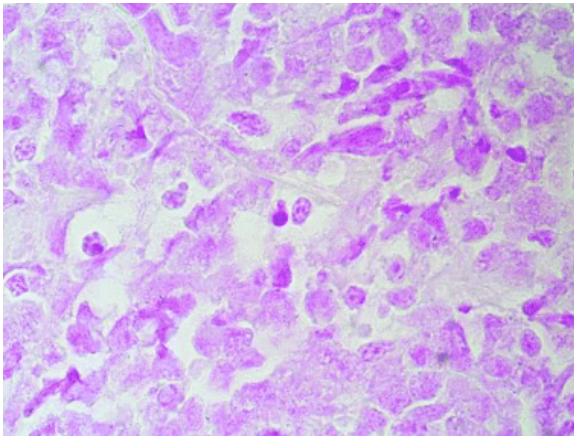
**Figure 5** A recurrence (gastric lymphoma) following cessation of the rituximab was CD20 positive (Patient 4) (×40).

### Molecular results

All biopsies with a loss of CD20 expression following rituximab therapy were assessed for CD20 mRNA by RTISPCR and protein by immunohistochemistry (IHC). The positive controls were a reactive lymph node and a case of nodal follicular lymphoma manifesting strong CD20 expression both by IHC and RTISPCR (**Fig. 6**). In all of the study cases that were negative for CD20 using IHC, the tumor cells were also negative for CD20 mRNA (**Fig. 7**). There were rare positive-staining cells in these lesions likely representing a small population of normal reactive B cells.



**Figure 6** A positive control for CD20 mRNA showing striking positivity of the tumor cells in this case of CD20-positive follicular lymphoma ( $\times 100$ ).



**Figure 7** This patient had a mantle-cell lymphoma which was refractory to various treatment modalities. The administration of rituximab was associated with continued tumor recurrences; however, the tumor cells now showed both an absence of CD20 expression and no discernible CD20 mRNA expression in any of the cells. The photomicrograph shows the absence of CD20 mRNA (Patient 3) ( $\times 100$ ).

## Discussion

We have presented four patients with CD20-positive cutaneous B-cell lymphoma who were treated with rituximab, all in whom a CD20-negative recurrence developed with subsequent recurrences following the cessation of rituximab as early as 1 month off the drug manifesting a re-expression of CD20. Through *in situ* PCR studies, we showed that the loss of CD20 was due to the cessation of synthesis of the CD20 molecule at the mRNA level. Extra-cutaneous lymphoma developed in two patients with primary cutaneous B-cell lymphoma and one patient died, suggesting the development of CD20-negative recurrent tumors in the setting of rituximab may portend a worse prognosis.

The loss of CD20 expression in the setting of rituximab therapy has been previously described; however, the focus in this article was to establish the basis for the loss. A favored hypothesis is one reflecting the apparent intended mechanism of action of this agent, which includes surface binding to the CD20 antigen. Such binding, while effectively evoking cell lysis through mechanisms of complement activation, also masks the detection of CD20 by flow cytometry and routine IHC, basically defining an epiphenomenon without specific pathophysiologic significance with reference to the biology of the tumor *per se*.<sup>18</sup> A second hypothesis centers on the fact that not all B-cell lymphoma cells express CD20; there will always be a minor cell population that will show a deletion of CD20 expression. Rituximab, while effective against the CD20-positive cells, would have no effect on those neoplastic cells that do not express CD20. Hence, the latter cell population would continue to proliferate especially if only a single-agent approach is used to treat the lymphoma. Indeed, three of the four patients in this series were on single-agent rituximab. The growth advantage may be given to a more aggressive clone of B cells, namely those that are sufficiently aberrant to escape the normal production of CD20, possibly explaining the tendency for a poorer outcome following the development of CD20-negative tumors.

The third mechanism, defining a novel observation in tissue sections, is the absence of CD20 messenger RNA production in B lymphocytes failing to express CD20 negatively by IHC. Although rituximab binds surface CD20, until recently, it was not known to have any effect on the messenger RNA that controls CD20 production. The re-acquisition of CD20 expression suggests that the agent may directly interfere with the production of CD20 transcript. There are two prior reports describing the down-modulation of CD20 mRNA as a potential

mechanism of CD20 loss. The first study was by Pickartz et al. whereby leukemic cells isolated from patients with chronic lymphocytic leukemia (CLL) down-modulated CD20 mRNA after exposure to rituximab; after cessation of the rituximab, a CD20-positive clone emerged.<sup>19</sup> The most recent study demonstrated that exposure of viable B cells to rituximab led to the down-modulation of CD20 expression, a phenomenon that was transient, with re-acquisition of CD20 expression within 24 h.<sup>20</sup>

In conclusion, the basis of the development of CD20-negative recurrent tumors in the setting of rituximab therapy may be multifactorial. The mechanisms by which the drug exerts direct inhibitory effect of the drug on CD20 mRNA synthesis have yet to be elucidated. Whether or not CD20-negative recurrent disease may define an adverse prognostic variable possibly due to selective growth advantage studies requires further study.

## Acknowledgement

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