ABSTRACT

Rituximab is a chimeric antibody with human γ and κ constant regions and murine variable regions. It recognizes the CD20 antigen, a pan B-cell marker. Therapeutic trials in patients with B-cell non-Hodgkin’s lymphoma (NHL) have shown significant efficacy with a primary response rate of 50%, and a secondary response rate of 44% after repeat treatments in prior responders. The selection for proliferating tumor cells that no longer express CD20 may compromise repeated treatment. We have identified a patient who developed a transformed NHL that lost CD20 protein expression after two courses of therapy with rituximab. In a pretreatment lymph node biopsy, 83% of B cells (as defined by CD19 and surface immunoglobulin) expressed surface CD20. A biopsy from the recurrent tumor after two courses of rituximab revealed a diffuse large cell NHL where 0% of B cells expressed CD20 with no evidence of bound rituximab. Cytoplasmic staining showed no CD20 protein. Sequencing of immunoglobulin heavy chain cDNA identified identical variable sequences in the initial and recurrent lymphomas, confirming the association between the two tumors. Literature and database review suggests that approximately 98% of diffuse large cell lymphomas express CD20, which suggests that these tumors rarely survive without CD20. This is the first identified case of loss of CD20 expression in a lymphoma that has relapsed after rituximab therapy, although several other cases have since been identified. Considering the significant number of patients treated with anti-CD20 antibodies, this may occur only rarely and is unlikely to preclude recurrent therapy with anti-CD20 antibodies in the majority of patients. However, because many patients have relapsed after anti-CD20 antibody therapy and have not been biopsied to identify clones with down-regulated CD20 antigen, we do not currently know the true frequency of this phenomenon. When possible, patients should undergo evaluation for CD20 expression before repeated courses of anti-CD20 therapy.

INTRODUCTION

The recent FDA approval of rituximab, a chimeric anti-CD20 monoclonal antibody, has ushered in a new era of immunotherapy for cancer. Rituximab’s limited toxicity, single-agent efficacy (50% response rates; Refs. 1–5) and potential for combination with standard therapies predict an increased usage over the coming years. Early data on repeated usage of this agent for recurrent disease suggests persistent efficacy in patients who responded to initial therapy without an increase in side effects, and three sequential treatments have been given without complication. Recurrent or maintenance therapy may be appropriate for some patients, provided that their relapsing lymphoma continues to express the antibody’s target, the membrane protein CD20 (6).

The reality of all current antineoplastic therapies, however, includes tumor escape, wherein clonal tumor cells develop a mechanism by which they can resist specific therapies. Therapeutic agents place selective pressure on the tumor, and malignant clones that have developed resistance to the therapeutic agent are able to proliferate. Targeting of specific molecules on a tumor cell surface by monoclonal antibodies can select for clones that are not recognized by the antibody or are not affected by its binding. Anti-idiotype antibodies used therapeutically to target the immunoglobulin on the surface of malignant B cells can select for clones with mutated idiotypes that are no longer recognized by the therapeutic antibodies (7, 8). Approximately 20% of patients treated with anti-idiotype antibodies relapsed with mutated idiotypes. Of course, clonal B cells spontaneously mutate the idiotypic region of their immunoglobulin. This high mutation rate makes them prone to escape under anti-idiotypic antibody selection. Approximately 95% of B-cell lymphomas express the CD20 antigen. The fact that some tumors do not express CD20 indicates that CD20 is not critical for B-cell survival. This is supported by data from a CD20 knockout mouse that shows normal B-cell development and function. It is, therefore, possible that treatment with rituximab or other anti-CD20 antibodies could select for clones that no longer require the expression of CD20 on their surface. We present the first identified case of a patient who, after two courses of therapy with rituximab, developed a transformed lymphoma that no longer expressed CD20.

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2 To whom requests for reprints should be addressed, at Stanford University, Department of Medicine, Division of Oncology, SUMC M207, Stanford, California 94305-5306.
3 T. F. Tedder, personal communication.
Therapy with Anti-CD20 Can Result in the Loss of CD20 Expression

<table>
<thead>
<tr>
<th>Date</th>
<th>Histology</th>
<th>CD20 Expressed</th>
<th>Therapy</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>FSC</td>
<td>No</td>
<td>None</td>
<td>Spontaneous regression</td>
</tr>
<tr>
<td>1987</td>
<td>FM with foci of DLC</td>
<td>Yes</td>
<td>XRT</td>
<td>Complete response</td>
</tr>
<tr>
<td>1993</td>
<td>FM</td>
<td>Yes</td>
<td>Rituximab 375 mg/m² × 4</td>
<td>Partial response for 7 mo</td>
</tr>
<tr>
<td>12/95</td>
<td>M (FNA)</td>
<td>Yes</td>
<td>CHOP</td>
<td></td>
</tr>
<tr>
<td>8/96</td>
<td>M (FNA)</td>
<td>Yes</td>
<td>CHOP</td>
<td></td>
</tr>
<tr>
<td>11/96</td>
<td>DLC</td>
<td>No</td>
<td>CHOP</td>
<td></td>
</tr>
</tbody>
</table>

* FSC, follicular small cleaved cell; FM, follicular mixed cell; M, mixed cell; DLC, diffuse large cell; CEOP, cyclophosphamide, etoposide, vincristine, and prednisone; CHAD, cis-platinum, ara-C, and dexamethasone; M-BACOD, methotrexate, bleomycin, Adriamycin, cyclophosphamide, vincristine, and dexamethasone; XRT, X-ray therapy; CHOP, cyclophosphamide, Adriamycin, vincristine, and prednisone.

**MATERIALS AND METHODS**

**Immunohistochemistry.** Immunohistochemical studies were performed on paraffin sections using an indirect biotin-avidin method on a Ventana 320 automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ). Sections were cut at 4 μm and microwaved for 10 min at 750 W in citrate buffer (pH 6.0) before staining. The automated method used primary antibody incubation at a temperature of 37°C for 32 min with the mouse monoclonal anti-CD20 antibody (Ref. 9; clone L26, Dako, Carpinteria, CA). The Ventana DAB Detection System (Ventana Medical Systems) was used for detection of antibody reactivity. Tissues known to express the determinants of interest were used as positive controls.

**Flow Cytometry.** Cells from lymph node biopsies or FNAs were manipulated into single-cell suspensions. Viable mononuclear cells were isolated by centrifugation in a Ficoll-Paque gradient and then washed. Cells were stained in two colors with the following fluorochrome conjugated antibodies: (a) goat antimouse γ1 and γ2 (negative controls), anti-CD3 (T cells), and anti-CD19 (B cells) from Becton Dickinson Immunocytometry Systems (San Jose, CA); (b) anti-α and α Fab ′ fragments from Southern Biotech Associates (Birmingham, AL); and (c) anti-IgM and anti-IgG antibodies from Biosource International (Camarillo, CA). Surface CD20 detection was performed with Leu16 from Becton Dickinson Immunocytometry Systems. Samples were analyzed using the Becton Dickinson FACScan.

**Immunoglobulin Sequencing.** Ten million cells from tumor biopsies were lysed in RNAzol total RNA purification (TEL test “B”, Inc., Friendswood, TX) to produce RNA. cDNA was made with random hexamer priming and reverse transcriptase (Superscript II, Life Technologies, Inc.). Five percent by volume was taken for PCR amplification using Taq polymerase (Life Technologies, Inc.). VH leader and constant region primers specific for the immunoglobulin heavy chain gene from the original lymph node biopsy were used (10). A simultaneous amplification for β2-microglobulin was performed to confirm the quality of the cDNA preparation, and the reagent solution was amplified separately without template to detect possible contamination. Amplification products were identified after electrophoretic separation in 2% agarose containing ethidium bromide. Clonal bands were sequenced from both priming ends using fluorescein-labeled nucleotides.

**Patient History.** Patient SS initially presented in 1982 with progressive peripheral lymphadenopathy and was diagnosed with stage IIIA, follicular small cleaved cell non-Hodgkin’s lymphoma (see Table 1). He did not require immediate therapy and subsequently experienced a spontaneous tumor regression. In 1987, he developed progressive disease in the supraclavicular fossa. A biopsy showed follicular mixed small and large cell lymphoma with foci of diffuse large cells. He again experienced a spontaneous regression and did not receive initial therapy until 1989, when he received five courses of cyclophosphamide, etoposide, vincristine, and prednisone with an initial response but subsequent kinetic failure, which prompted a switch to Adriamycin, cis-platinum, ara-C, and dexamethasone. His disease responded well to two cycles, but he was switched to M-BACOD after severe nausea and vomiting prevented further platinum therapy. After four cycles of M-BACOD, only minimal residual disease remained, and cytotoxic therapy was discontinued. He relapsed in 1993 with localized disease, and a repeat biopsy revealed only follicular mixed small and large cell lymphoma. He was treated with radiation therapy in a mini-mantle distribution, for a total dose of 3060 cGy. When he again relapsed in 1995, a FNA confirmed a population of small and large lymphocytes. All of the B cells present in the sample expressed CD20. He was enrolled on a phase II clinical trial evaluating the safety and efficacy of rituximab. He received 375 mg/m² weekly for four doses with little symptomatic toxicity but transient self-limited neutropenia. Tumor measurements confirmed a partial response at 3 months posttherapy that lasted for 7 months. His course was complicated by fatigue and fevers, atypical for his lymphoma, but extensive work-up could not identify any infectious or rheumatological source.

He relapsed with retroperitoneal disease. A computed tomography-guided FNA of these nodes showed recurrent mixed cellularity lymphoma with ampl CD20 expression. He was, therefore, enrolled in a second protocol that provided repeated therapy with rituximab for patients who had responded to initial therapy. He received an additional four doses of 375 mg/m² over...
a period of 4 weeks. The infusions were complicated by severe retroperitoneal pain requiring potent narcotic analgesia. A computed tomography scan performed 1 month posttherapy showed a mixed tumor response, but a scan performed 3 months post-treatment documented tumor progression. He simultaneously developed peripheral lymphadenopathy in an epitrochlear node. An open biopsy of the epitrochlear node was performed. The histology from this specimen showed diffuse large cell lymphoma with no evidence of cytoplasmic CD20 expression by repeated L26 antibody staining. He was subsequently treated with multiple regimens of combination chemotherapy but experienced only transient responses and died from complications of lymphoma.

### RESULTS

To fulfill enrollment criteria, a FNA of an involved lymph node was performed before therapy and at relapse to ascertain the level of CD20 expression in tumor cells. At relapse after the second treatment with rituximab, a tumor biopsy was evaluated by both immunoperoxidase staining and flow cytometry. Immunoperoxidase staining of histological samples before the first course of rituximab showed strong CD20 expression, but staining of the final biopsy after two courses of rituximab showed no stainable CD20. The antibody L26 was used for paraffin staining. He was subsequently treated with multiple regimens of combination chemotherapy but experienced only transient responses and died from complications of lymphoma.

### Table 2: Tumor FACS analysis

<table>
<thead>
<tr>
<th>Time point</th>
<th>CD3+ (%)</th>
<th>CD19+ (%)</th>
<th>CD20+ (%)</th>
<th>% B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-rituximab</td>
<td>63</td>
<td>30</td>
<td>25</td>
<td>83</td>
</tr>
<tr>
<td>First relapse</td>
<td>76</td>
<td>8.6</td>
<td>9.9</td>
<td>100</td>
</tr>
<tr>
<td>Second relapse</td>
<td>23</td>
<td>71</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Fig. 1

CD20 expression in lymph node biopsies from before (A) and after (B) rituximab therapy. Two color FACS analyses staining for CD19 (B cells) and CD20.

### Fig. 2

FACS analysis of CD20 tumor. Viable mononuclear cells from the lymph node biopsy performed in November of 1996 were stained with fluorescence-labeled antibodies. Samples were gated on the lymphocytic population. A compares B cells (CD19+) with T cells (CD3+). B identifies B cells with CD19 staining, and shows no CD20 binding. In C, B cells identified by CD19 all express IgM, the immunoglobulin isotype expressed by the tumor, and D identifies malignant cells (IgM-positive) that do not bind IgG, the isotype of rituximab.

### DISCUSSION

Selective pressures provided by antigen-specific therapies can isolate tumor cells that have mutated such that they are no

![Fig. 1](image1.png)  
![Fig. 2](image2.png)
longer recognized by the targeting agents, with resultant tumor resistance to further treatments with those therapies. This is particularly true with antibody therapies that selectively target individual amino acid sequences on specific proteins. After therapeutic treatments with anti-idiotype antibodies for low-grade B-cell lymphoma, approximately 20% of patients developed tumor relapses that expressed idiotype molecules that were not recognized by the original therapeutic antibodies. In several cases, alternate anti-idiotype antibodies that did recognize the mutated idiotype were able to provide additional tumor regression, with an ongoing remission in one patient that now exceeds 10 years (11).

In the case we present here, after a single course of rituximab with partial tumor regression, the lymphoma progressed without significant mutation. After a second course of rituximab therapy, however, the lymphoma no longer expressed CD20 either on the membrane or in the cytoplasm. The lack of staining with labeled anti-CD20 antibodies may have been caused by previously bound rituximab antibodies. However, immunoperoxidase staining with L26 antibody would not be blocked by bound rituximab because this antibody recognizes an entirely different portion of the CD20 molecule. For this analysis, it was fortunate that the parental tumor expressed an IgM immunoglobulin receptor although rituximab is an IgG molecule. Surface staining for heavy or light chains was negative (Fig. 2), which also shows that no residual rituximab antibody was present on the relapsed tumor cells, ruling out the possibility of blocking by residual rituximab.

The patient had not experienced significant tumor reduction after this second course of antibody. The bulk of his disease was located in the abdomen, and the residual lymphoma could not be biopsied without invasive procedures. The relapse site that was biopsied was a new site of disease. Thus, it is possible that the residual intra-abdominal tumor, which was resistant to rituximab therapy, may still have retained CD20 expression. A tumor cell that had changed in several ways, including the loss of CD20 expression and transformation to a higher grade lymphoma, was able to proliferate at this new and distant site. This tumor cell also developed some of the characteristics of the more differentiated plasma cell (m1 and cytoplasmic light chain staining). Plasma cells do not express significant amounts of CD20. Thus, we cannot determine whether the presence of rituximab induced a clonal cell to forego its CD20 expression, or whether the rituximab selected a CD20variant for outgrowth.

### Table 3 CD20 expression by B-cell lymphoma subtypes

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Stanford database</th>
<th>Chang a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large cell</td>
<td>95%</td>
<td>98%</td>
</tr>
<tr>
<td>Follicular</td>
<td>98%</td>
<td>97%</td>
</tr>
<tr>
<td>Small lymphocytic</td>
<td>96%</td>
<td>100%</td>
</tr>
<tr>
<td>Myeloma</td>
<td>NA b</td>
<td>10–20%</td>
</tr>
<tr>
<td>High-grade</td>
<td>NA</td>
<td>90–100%</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>NA</td>
<td>50%</td>
</tr>
</tbody>
</table>

a Chang et al., literature review (12).

b NA, not available.

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![Fig. 3 Immunoglobulin cDNA sequence.](clincancerres.aacrjournals.org) The sequence of the immunoglobulin heavy chain variable regions of pre-rituximab tumor and the CD20+ relapse are compared. Mismatches are bold without asterisk.
However, the immunoglobulin heavy-chain cDNA sequence data, supported by the bcl-2 staining in the transformed lymphoma, confirmed that the transformed lymphoma did originate from the primary malignant clone.

To assess the frequency of CD20− B-cell lymphomas, we performed a review of the literature. A comparative review by Chang et al. (12) indicates that a high percentage of B-cell lymphomas, both follicular and diffuse, express CD20. A review of our database of biopsies containing non-Hodgkin’s lymphoma and evaluated by flow cytometry similarly showed that only a small percentage of both follicular and diffuse lymphomas do not express surface CD20 (Table 3). Recognizing that a small percentage of B-cell lymphomas proliferate without expressing CD20, this molecule cannot be essential for lymphocyte survival. It is likely, therefore, that selective pressures from anti-CD20 antibodies could isolate CD20− tumors. This is the first documented case in 60 relapsed patients who underwent biopsy of their relapsed tumors before repeated therapy with rituximab. More than 300 patients have been treated on protocol at sites within the United States, and many more have been treated with alternate anti-CD20 antibodies. Because many patients have relapsed after anti-CD20 antibody therapy and have not been biopsied to identify clones with down-regulated CD20 antigen, we do not currently know the true frequency of this phenomenon. As more patients receive multiple or prolonged courses of therapy, CD20− B-cell lymphoma may become more common. When possible, patients should undergo evaluation for CD20 expression before repeated courses of anti-CD20 therapy. However, current experience suggests that the loss of CD20 expression will not preclude the repeated use of anti-CD20 antibody-based therapies in the majority of patients with B-cell lymphoma.

REFERENCES


Therapy of B-Cell Lymphoma with Anti-CD20 Antibodies Can Result in the Loss of CD20 Antigen Expression

Thomas A. Davis, Debra K. Czerwinski and Ronald Levy