To the editor:

Apparent modulation of CD20 by rituximab: an alternative explanation

We read with interest the paper from Jilani et al.1 In which rituximab treatment appeared to down-modulate CD20 expression through a combination of internalization and RNA regulation. The result is unexpected because previous studies had shown that CD20 is not modulated by monoclonal antibody (mAb) treatment.2-4 Even in vivo.5 The study by Jilani et al used an anti–mouse immunoglobulin polyclonal antibody (Ab) that binds to the mouse V regions in rituximab. This reagent will bind to rituximab, while the chimeric antibody is coated onto CD20/H11001 cells. We are concerned that the apparent loss of binding reported may be due to factors other than loss of CD20 expression—specifically blocking by normal immunoglobulin.

As part of our ongoing CD20 studies, we have developed a mouse anti-idiotype (Id) mAb (2A4) specific for the V regions of rituximab and its parent Ab 2B8 (M.S.C. and M.C.B., manuscript submitted, March 2004). This reagent binds rituximab or 2B8 while it is coated onto CD20+ cells (Figure 1A). Using this highly specific reagent we can find no evidence that CD20 is down-modulated on malignant B cells in either the presence or absence of plasma (Figure 1B). Using the anti-Id mAb (MB2A4), together with a human Fcγ-specific mAb, we examined the ability of rituximab and its murine counterpart 2B8 to bind to CD20 on Raji cells and fresh B-cell chronic lymphocytic leukemia (B-CLL) cells with and without plasma. In these experiments, cells were incubated for 2 hours at 37°C as described by Jilani et al, before the cells were washed twice by centrifugation and stained for surface CD20 mAb. Interestingly, when using fluorescein isothiocyanate (FITC)–conjugated anti-Fcγ mAb, our

Figure 1. Lack of CD20 modulation on malignant B cells following rituximab treatment in vitro in the presence or absence of plasma. (A) Specificity of the mouse anti-rituximab idiotype (anti-Id) mAb, MB2A4. Following binding of various mouse/human chimeric mAbs (10 μg/mL) to EHRB or Raji cells for 15 minutes at room temperature (mAb [target]; 2B8 [mouse Ab against CD20]; rituximab [CD20]; chAT80 [CD20]; chWR17 [CD37]; chAT13/5 [CD38]; and chLOB7-4/7-6 [CD40]), cells were washed twice in phosphate-buffered saline/bovine serum albumin (BSA)/Azide before being stained with FITC-labeled mAb MB2A4 (10 μg/mL) for 15 minutes at room temperature and being analyzed by flow cytometry. As can be seen, binding of 2A4 was observed only on cells coated with rituximab or its parent mAb 2B8, demonstrating both the ability of the mAb to detect rituximab when it was bound to cells and its specificity for the rituximab V regions. (B) Typical dot-plots generated by staining B-CLL cells incubated with either rituximab or 2B8 in the presence or absence of 33% plasma for 2 hours at 37°C, followed by washing, as indicated in panel A, and staining with either FITC-labeled anti-Id (MB2A4) or FITC-labeled anti-human Fcγ (Hu Fcγ, SB2H2) for 30 minutes at room temperature and analysis by flow cytometry. The anti-Id mAb was able to detect both mAbs equally well in the presence or absence of plasma, whereas the Hu Fcγ mAb could not detect rituximab after incubation in the presence of plasma. (C) The importance of wash steps in the detection of rituximab. After binding and incubation as in panel B, B-CLL cells were washed 1, 2, or 3 times by standard centrifugation prior to staining with either FITC-labeled anti-Id (MB2A4) or FITC-labeled Hu Fcγ as described in panel B. Flow cytometry data are expressed as the percent of FITC staining measured in the presence, compared with the absence, of 33% plasma. These data show that the detection of rituximab with anti-Hu Fcγ mAb was extremely sensitive to the number of wash steps used and that no apparent modulation of rituximab was observed when sufficiently washed. Conversely, the anti-Id mAb was unaffected by the number of wash steps. This effect of wash steps was replicated in 7 different experiments, using either Raji or B-CLL cells. (D) IgG in plasma blocks the detection of mouse/human chimeric mAbs. Raji cells were incubated with either rituximab or other chimeric human Fc-containing mAbs directed to CD20 (chAT80) or CD37 (chWR17), or with the parent mouse mAbs (2B8, AT80, or WR17). Cells were incubated for 2 hours at 37°C in the presence of media alone (NT), plasma, or plasma depleted of IgG (using protein A column chromatography), before washing twice and staining with FITC-labeled anti-human or anti-mouse Fcγ. These data show that all mAbs with a human Fcγ domain showed apparent down-modulation in the presence of plasma, but that this “modulation” is not apparent when plasma was depleted of IgG or when the murine counterparts were used in the presence of human plasma.
results closely mirrored those seen by Jilani et al, with rituximab staining being lost (Figure 1B, right panel). However, when our FITC-conjugated anti-Id mAb was used, minimal loss of rituximab was observed (Figure 1B, left panel).

To investigate possible blocking by normal immunoglobulin, we repeated the experiments, but used 1, 2, or 3 wash steps to remove plasma immunoglobulin G (IgG). As shown in Figure 1C, as the level of washing was increased so the capacity to detect bound rituximab with anti-human Fcγ was restored. We also confirmed that staining with the anti-id mAb was not influenced by washing (Figure 1C). Further analysis, using a panel of murine or chimeric human Fc-bearing mAbs (anti-CD20, -CD37, and -CD38 [Figure 1D]) and detecting their binding using either FITC-conjugated anti-human or anti-mouse Fc reagents, revealed that only mAbs bearing a human Fc domain were affected by the presence of plasma. The phenomenon was not restricted to CD20 mAbs, and critical to these observations, the parent murine mAb of rituximab, 2B8, was not affected—indicating a specificity-independent phenomenon. A similar lack of modulation was seen using plasma that had been depleted of human IgG using protein A column chromatography (Figure 1D) and from patients with naturally low levels of IgG (not shown). We repeated these experiments using different B-cell lines as well as ex vivo, fresh B-CLL cells and observed the same phenomenon, namely that loss of anti-CD20 binding could not be detected with MB2A4 or with anti-Fcγ reagents after sufficient washing. These data suggest that similar blocking phenomena might provide an explanation for the observations made by Jilani et al and underline the importance of using anti-Id mAbs to detect therapeutic mAbs in plasma-containing clinical samples.

Note added in proof. In support of our observations that CD20 is not modulated in vitro, Kennedy et al. have recently documented minimal modulation of CD20 on CLL cells with rituximab and 50% serum. Interestingly, however, they do support the notion that CD20 is reduced on CLL cells following in vivo treatment and suggest that CD20 expression is “modulated,” not in the conventional sense, but through removal of CD20/rituximab/C3b complexes from the membrane via their interactions with phagocytes.

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Response:

Anti-idiotype versus anti-mouse Ig for detecting rituximab

Cragg et al used completely different reagents and a different approach from those used by our group, and they report different findings and conclusions. Cragg et al used antihuman Fc-γ antibodies to detect the rituximab on the surface of B cells. They demonstrate that the binding of the antihuman Fc-γ antibodies to the surface of cells can be blocked by normal human immunoglobulin G (IgG). This is expected because human IgG binds to the antihuman Fc-γ. In fact, antihuman Fc-γ antibodies have been previously reported to bind to Raji cells without the presence of rituximab. Appropriate controls in their experiments (Figure 1B-D) should include the binding of antihuman Fc-γ antibodies to cells before adding rituximab. The antihuman Fc-γ antibodies should bind to all B cells that show expression of surface IgG and at the same time can be blocked by free IgG. To specifically detect rituximab on the surface of cells, it is crucial to use anti-mouse immunoglobulin antibodies that do not cross-react with the human IgG, otherwise human IgG (free or surface bound) would block this binding. As for the use of anti-idiotype, it is the experience of many investigators that anti-idiotype antibodies cross-react with other antibodies, frequently with low affinity. For this reason, we used polyclonal anti-mouse immunoglobulin antibodies that were absorbed against the human immunoglobulin to prevent potential cross-reactivity to human IgG. Unfortunately the data as presented in the letter by Cragg et al do not allow us to evaluate the specificity of the anti-idiotype antibody. The possibility that the anti-idiotype antibody can bind to other human immunoglobulins on the surface of cells in specific conditions should be investigated and ruled out with proper controls. It is also important to study patients being treated with rituximab at various stages of therapy and monitor the detection of rituximab or lack of it on the surface of cells in a fashion similar to that reported by us. As we reported, rituximab can be detected at an early stage of therapy in some patients and can disappear with time without CD20 expression. This is difficult to explain based on cross-binding to IgG, especially since the anti-mouse immunoglobulin antibodies used to detect the rituximab do not cross-react with the human IgG.

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To the editor:

Severe hypochromic microcytic anemia caused by a congenital defect of the iron transport pathway in erythroid cells

We report a patient, a product of a consanguineous union, with liver hemosiderosis and severe congenital microcytic anemia due to defective erythroid iron use. Iron is an essential element indispensable for the synthesis and the function of hemoglobin (Hb) and for normal red blood cell development. Iron deficiency can lead to decreased production of Hb and to a microcytic, hypochromic anemia. Erythroid iron uptake occurs via a receptor-mediated endocytosis of diferric transferrin (Tf) bound to the Tf receptor (TfR). Following acidification of internalized endosomes, iron is released from Tf and transported through the endosomal membrane by the transmembrane iron transporter divalent metal transporter 1 (DMT1), also known as natural resistance-associated macrophage protein-2 (Nramp2). Subsequent intracellular iron transport from DMT1 to mitochondria for heme synthesis is not completely understood.

Recently, it has been shown that the microcytic anemia (mk) mouse and the Belgrade rat carry the same missense mutation (G185R) in DMT1. Homozygous animals demonstrate diminished intestinal iron absorption and a severe deficit in erythroid iron use. The reduced rate of heme synthesis in reticulocytes from Belgrade rats can be corrected by the iron chelate salicylaldoxime saturated with iron (Fe-SIH), which is known to bypass the TfR/DMT1 cycle of iron transport. These results indicate that DMT1 plays a crucial role in erythroid iron uptake, since the most prominent consequence of impaired DMT1 function is disturbed erythropoiesis. Even though the residual activity of this transporter in affected rodents allows the animals to remain viable, it is not sufficient to ensure appropriate rate of iron supply and subsequent heme synthesis in the erythroid cells.

The Czech proband is a 20-year-old female who is suffering from a severe congenital hypochromic microcytic anemia with high serum iron, normal total iron-binding capacity (TIBC), slightly elevated ferritin, and highly increased serum TfR (sTfR) values (Table 1). She is a child of a consanguineous union of second-degree relatives. Her Hb varied around 70 g/L, and she required the first blood transfusion after birth and 8 transfusions during infancy. Later she received transfusions only when her Hb levels dropped to less than 70 g/L; however, these averaged less than one per year (hence, the patient received < 4 g of iron, via transfusions, in her lifetime). She has had erythroid hyperplasia with defective hemoglobinization of intermediate and late normoblasts and absence of sideroblasts in the bone marrow. By the age of 19 years, she had gradually developed liver hemosiderosis with the liver iron content of 16 286 µg/g dry weight.

We analyzed isolated erythroid progenitors from peripheral blood and bone marrow of the patient using an in vitro colony-forming assay. The ethics committee of the Palacky University Hospital approved the study and the informed consent. In brief, we plated 3 × 10^5 light-density mononuclear cells to the methylcellulose media (H4531; StemCell Technologies, Vancouver, BC) with addition of 1 U or 2 U erythropoietin (Epo) per milliliter of media. The numbers and the size of the patient’s erythroid colonies were smaller compared with the healthy controls and exhibited low cell content and abnormal morphology (Figure 1A-B). Based on the clinical observations and biochemical measurements, we postulated that addition of iron chelates to the cultures might correct the poor growth of the patient’s erythroid colonies. Fe-SIH was added to the Epo-containing cultures at the final concentration of 10 µM. Indeed, addition of the iron chelate to the cultures rescued the numbers, cellularity, and defective hemoglobinization of the patient’s erythroid burst-forming units (BFU-Es; Figure 1C). This result is congruent with impaired TfR/DMT1-dependent iron trafficking in erythroid precursors in this patient.

To further elucidate this defect at the protein level, we harvested erythroid colonies using cytopin, followed by immunofluorescence analysis. In addition, we isolated the patient’s and 3 healthy volunteers’ erythroid progenitors from a monoclonal fraction of bone marrow cells using labeling with

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glycophorin A antibody and magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequent immunofluorescence analyses were performed with TIR antibody (CD71), DMT1/Nramp2 antibody (N-20; both from Santa Cruz Biotechnology, Santa Cruz, CA), and early endosomal antigen 1 (EEA1; Alexis, Montreal, QC, Canada) antibody. TIR expression in the patient’s erythroblasts was normal (Figure 1E) and comparable with that seen in healthy controls (not shown). We thus hypothesized that the defect in this patient was most likely due to defective expression of DMT1. This was further supported by immunofluorescence analysis of glycophorin-positive erythroid cells. As shown in Figure 1F-G, the patient’s erythroblasts showed decreased DMT1 expression, with the protein being localized within the cytoplasmic vesicles and on the surface of the blasts.

We conclude that the propositus was born with a homozygosity for an autosomal recessive defect of iron transport/use in erythroid cells. We hypothesize that this defect is caused by a malfunction of the endosomal iron transporter DMT1. In mk mice and Belgrade rats such a defect leads to a decrease in intestinal iron absorption. In contrast, the patient reported here has signs of iron overload that we explain by increased absorption of heme-derived iron in response to anemia. mk mice or Belgrade rats are unlikely to develop such a compensatory mechanism, since rodents have a low ability to use heme iron compared with inorganic iron; moreover, neither laboratory mice nor laboratory rats have heme in their diets. The absorption of heme iron is poorly understood, but it likely involves heme binding to specific, yet-uncharacterized high-affinity binding sites in the mucosal brush border. It is plausible that a pathway involved in heme iron absorption is up-regulated in our patient.

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