Rituximab blocks binding of radiolabeled anti-CD20 antibodies (Ab) but not radiolabeled anti-CD45 Ab

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Rituximab therapy is associated with a long in vivo persistence, yet little is known about the effect of circulating rituximab on B-cell non-Hodgkin lymphoma (B-NHL) targeting by the other available anti-CD20 monoclonal antibodies (MoAbs)131iodine-tositumomab and 90yttrium-ibritumomab tiuxetan. Therefore we assessed the impact of preexisting rituximab on the binding and efficacy of second anti-CD20 MoAbs to B-NHL and determined whether targeting an alternative lymphoma-associated antigen, CD45, could circumvent this effect. We demonstrated that rituximab concentrations as low as 5 µg/mL nearly completely blocked the binding of a second anti-CD20 MoAbs (P < .001), but had no impact on CD45 targeting (P = .89). Serum from patients with distant exposures to rituximab also blocked binding of anti-CD20 MoAbs to patient-derived rituximab-naive B-NHL at concentrations as low as 7 µg/mL, but did not affect CD45 ligation. A mouse xenograft model (Granta, FL-18, Ramos cell lines) showed that rituximab pretreatment significantly reduced B-NHL targeting and tumor control by CD20-directed radiopharmaceutical therapy (RIT), but had no impact on targeting CD45. These findings suggest that circulating rituximab impairs the clinical efficacy of CD20-directed RIT, imply that novel anti-CD20 MoAbs could also face this same limitation, and indicate that CD45 may represent an alternative target for RIT in B-NHL. (Blood. 2008;112:830-835)

Introduction

The B-cell antigen CD20 is the target of 3 US Food and Drug Administration (FDA)–approved monoclonal antibodies (MoAbs), 1 unconjugated (rituximab), 2 radiolabeled (131iodine-tositumomab and 90yttrium-ibritumomab tiuxetan), and at least 7 additional anti-CD20 antibodies that are in development (ofatumomab, ocrelizumab, PRO131921, TRU-015, veltuzumab, AME-133, and GA-anti-CD20 antibodies that are in development (ofatumomab, ocrelizumab, PRO131921, TRU-015, veltuzumab, AME-133, and GA). The anti-CD20 MoAb in this situation that131iodine-tositumomab and 90yttrium-ibritumomab tiuxetan are approved and often used.12,13 It is also anticipated that in this setting the multiple second-generation anti-CD20 MoAbs in development will also be used, even though little is known about the effect of circulating rituximab competing for the same target.7 Herein we evaluated the impact of prior rituximab on the binding of secondary anti-CD20 and anti-CD45 MoAbs to elucidate if antigen competition occurred, if it was clinically relevant, and if it could be circumvented by an alternate target.

Methods

Antibodies

The chimeric anti-CD20 MoAb C2B8 (rituximab; IgG1) was purchased from Genentech (South San Francisco, CA). The anti-CD20 MoAb tositumomab (IgG2a) was produced by GlaxoSmithKline (GSK; Philadelphia, PA). BC8 (IgG1) and HB8181 (IgG2a) were produced from the respective hybridomas using a hollow-fiber bioreactor system in the Biological Production Facility at the Fred Hutchinson Cancer Research Center (FHCRC; Seattle, WA). 1D8 (IgG1) was produced in ascites generated by pristane-primed Balb/c mice and purified by protein G immunoabsorption column chromatography and used as an isotype-matched nonbinding control. For flow cytometry experiments, tositumomab and BC8 were conjugated to fluorescein isothiocyanate using the method provided (Sigma-Aldrich, St Louis, MO). Tositumomab and BC8 were iodinated with Na131I or Na125I (Perkin Elmer, Boston, MA) by the chloramine T method as previously described.14

Cell lines

Ramos and Granta 519 cell lines were purchased from ATCC (Bethesda, MD). FL-18 was a gift from Dr Ron Levy (Stanford University, Palo Alto, CA). All cell lines were kept in log growth phase in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Carlsbad, CA).

Patient serum and cell samples

Human lymphoma cells and serum samples were provided by patients following human subject review board approval and informed consent obtained in accordance with the Declaration of Helsinki. The malignant lymphocytes were isolated from whole blood using Ficoll-Hypaque separation and stored at −80°C in 5% dimethyl sulfoxide before use. Serum rituximab concentrations were determined as previously published.8

In vitro studies

Cells (10 × 106/mL in 2% FBS–phosphate-buffered saline [PBS]) were incubated with the presumptive blocking (eg, rituximab) or control
Granta (1.2/H11003 FHCRC Institutional Animal Care and Use Committee. Ramos (107), IN) and were housed and cared for according to protocols approved by the

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For all mouse studies, female BALB/c nu/nu mice, aged 6 to 8 weeks, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were housed and cared for according to protocols approved by the FHCRC Institutional Animal Care and Use Committee. Ramos (107), Granta (1.2 × 10⁷), and FL-18 (10⁷) cells were injected subcutaneously in the right flank, and palpable xenografts developed within approximately 10 days. Groups of 5 or more mice were injected intraperitoneally with 400 µg rituximab or 1D8 24 hours before a tail-vein injection of 200 µg BC8-680 or tositumomab-680. Mice were then anesthetized with isoflurane, and images were captured using an IVIS 100 Imaging System (Xenogen). Imaging and quantification were done in accordance with the IVIS 100 Imaging System manual. Briefly, 2 images were captured, one captured with the emission set to Cy5, and the second image (background) captured using the emission set to Cy5 background. The Cy5 setting excites at 615 to 665 nm, and emission is between 695 to 770 nm. A region of interest was placed on the negative control mouse in the background image to allow determination of the total fluorescence for each image. The Cy5 background total was divided by the Cy5 total to calculate “K.” A new image was then generated using the formula a − b × K, where “a” equals the Cy5 image and “b” equals the Cy5 background image.

Therapy. B-NHL xenografts were established as described. BC8 and tositumomab were labeled with fluoromer-680 as described by the manufacturer (Xenogen, Hopkinton, MA). When tumors were measurable (approximately 100 mm³), groups of at least 5 mice each were injected intraperitoneally with 400 µg rituximab or 1D8 24 hours before a tail-vein injection of 200 µg BC8-680 or tositumomab-680. Mice were then anesthetized with isoflurane, and images were captured using an IVIS 100 Imaging System (Xenogen). Imaging and quantification were done in accordance with the IVIS 100 Imaging System manual. Briefly, 2 images were captured, one captured with the emission set to Cy5, and the second image (background) captured using the emission set to Cy5 background. The Cy5 setting excites at 615 to 665 nm, and emission is between 695 to 770 nm. A region of interest was placed on the negative control mouse in the background image to allow determination of the total fluorescence for each image. The Cy5 background total was divided by the Cy5 total to calculate “K.” A new image was then generated using the formula a − b × K, where “a” equals the Cy5 image and “b” equals the Cy5 background image.

Statistical considerations

Continuous variables with normal distributions were compared using the Student t test. Simple proportions were evaluated using the chi-square or Fisher exact test as appropriate.

All studies were approved to collect patient samples for research purposes by the FHCRC Institutional Review Board.

Results

In vitro studies

We first evaluated the effect of rituximab (anti-CD20) pretreatment on the ability of tositumomab (anti-CD20) and BC8 (anti-CD45) to

Figure 1. Rituximab blocks CD20 binding, but not CD45. (A) Rituximab blocks CD20 binding to Ramos cells. (B) but has no impact on CD45 binding by BC8.

(HB8181) antibody for 30 minutes at 4°C and washed with 2% FBS-PBS. This was followed by a second incubation with the desired binding (BC8 fluorescein isothiocyanate conjugation [BC8-FITC] or tositumomab-FITC) or control (horse anti–mouse-FITC [for HB8181 antibody]) antibody for 30 minutes at 4°C. After a second washing, the cells were put in PBS and immediately analyzed in a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA). Fifty microliters containing peripheral blood lymphocytes were collected from rituximab-naive lymphoma patients with documented circulating tumor (20 × 10⁶ cells/mL in 2% FBS-PBS) were similarly incubated for 30 minutes, at 4°C with 50 µL serum from rituximab-pretreated patients and washed with 2% FBS-PBS. Next, the cells were then incubated for 30 minutes, at 4°C with BC8-FITC or tositumomab-FITC. Ten microliters phycoerythrin (PE)-Cy-5–conjugated mouse anti–human CD-20 (BD Pharmingen, San Jose, CA) was included to identify the B cells from other CD45-bearing lymphocytes. The cells from each experiment were washed, put in PBS, and immediately analyzed in a FACS Caliber flow cytometer (Becton Dickinson).

In vivo studies

Biodistribution. For all mouse studies, female BALB/c nu/nu mice, aged 6 to 8 weeks, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were housed and cared for according to protocols approved by the FHCRC Institutional Animal Care and Use Committee. Ramos (10⁷), Granta (1.2 × 10⁷), or FL-18 (10⁷) cells were injected subcutaneously in the right flank, and palpable xenografts developed within approximately 10 days. Groups of 5 or more mice were injected intraperitoneally with 400 µg rituximab or 1D8 24 hours before a tail-vein injection of 200 µg BC8-680 or tositumomab-680. Mice were then anesthetized with isoflurane, and images were captured using an IVIS 100 Imaging System (Xenogen). Imaging and quantification were done in accordance with the IVIS 100 Imaging System manual. Briefly, 2 images were captured, one captured with the emission set to Cy5, and the second image (background) captured using the emission set to Cy5 background. The Cy5 setting excites at 615 to 665 nm, and emission is between 695 to 770 nm. A region of interest was placed on the negative control mouse in the background image to allow determination of the total fluorescence for each image. The Cy5 background total was divided by the Cy5 total to calculate “K.” A new image was then generated using the formula a − b × K, where “a” equals the Cy5 image and “b” equals the Cy5 background image.

Therapy. B-NHL xenografts were established as described. When tumors were measurable (approximately 100 mm³), groups of at least 9 mice each were injected intraperitoneally with 400 µg rituximab or 1D8 24 hours before a tail-vein injection of 200 µg BC8-680 or tositumomab-680. Mice were then anesthetized when they experienced weight loss greater than 20% or tumor growth greater than 12 mm × 12 mm × 6 mm per animal health guidelines.

Statistical considerations

Continuous variables with normal distributions were compared using the Student t test. Simple proportions were evaluated using the chi-square or Fisher exact test as appropriate.

All studies were approved to collect patient samples for research purposes by the FHCRC Institutional Review Board.

Figure 2. Patient-derived serum blocks CD20 binding, but not CD45. A comparison of the mean fluorescence intensity of tositumomab and BC8 binding to patient-derived malignant B cells after incubation with rituximab-treated patient-derived serum.
bind their respective targets. Malignant B-NHL cells were preincubated with increasing concentrations of rituximab (0-2000 μg/mL), washed, and then incubated with fluoresceinated tositumomab (anti-CD20) or fluoresceinated BC8 (anti-CD45) and evaluated by flow cytometry for antibody binding. Fluoresceinated isotype-matched MoAb, HB8181, was used as a nonbinding negative control antibody. Prior rituximab exposure reduced binding of tositumomab to B-NHL cells (Ramos) by 86% at concentrations as low as 5 μg/mL (P < .001) and fully blocked ligation at concentrations of 50 μg/mL or greater (P < .001; Figure 1A). Increasing concentrations of rituximab had no impact on CD45 binding (P = .89; Figure 1B). Similar results were observed using the B-NHL lines FL-18 and Granta (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

**Patient-derived specimens**

To further evaluate the clinical relevance of these findings, we assessed whether this phenomenon could be replicated in patient-derived specimens. Serum was obtained from patients with B-NHL 4 weeks or more after the last rituximab therapy. The median rituximab concentration in these specimens was measured at 23.4 μg/mL (range, 14.6-82.2 μg/mL). Dilutions (1:1) of serum from 3 patients treated with rituximab were preincubated with malignant B-NHL cells obtained from a rituximab-naive patient, washed, incubated with fluoresceinated tositumomab (Figure S2) or fluoresceinated BC8 (Figure S3), then evaluated for the ability of the fluoresceinated MoAbs to bind to the CD20 and CD45 antigens, respectively, as assessed by flow cytometry. Figure 2 illustrates a direct comparison of the mean fluorescences of tositumomab and BC8 binding to patient-derived cells in the presence of serum from patients treated with rituximab. These data indicate that serum from patients with a remote exposure to rituximab fully blocks the binding of tositumomab to CD20 at concentrations at least as low as 7.3 μg/mL, but has no effect on CD45 ligation.

**Mouse models of biodistribution**

We then determined if the observed in vitro findings truly affected the potential to deliver radioimmunotherapy (RIT) to tumor sites in vivo. B-NHL-bearing mice were injected either with rituximab or nonbinding isotype-matched control antibody (1D8), followed by coinjection with 131I-tositumomab and 125I-BC8. The retained

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**Figure 3. Rituximab pretreatment impairs anti-CD20 tumor targeting, but not anti-CD45 targeting in Ramos xenografts.** Effect of rituximab pretreatment on percent injected radioactivity dose per gram (%ID/g) of normal and tumor tissue in Ramos xenograft-bearing mice treated with (A) 125I-BC8 and (B) 131I-tositumomab. Tumor-to–normal organ ratios of delivered radioactivity after pretreatment with rituximab or 1D8 followed by (C) 125I-BC8 or (D) 131I-tositumomab. Error bars represent SE.
radioactivity measured as a percentage injected dose per gram of normal tissue and tumor tissue was determined at 24 and 48 hours after RIT. Tumor sites in mice with human B-NHL (Ramos) xenografts received comparable radioisotope doses when treated with $^{125}$I-BC8 in the presence or absence of prior rituximab (Figure 3A). In contrast, rituximab pretreatment reduced the tumor uptake of $^{131}$I-tositumomab by 55% compared with mice treated with a nonbinding control MoAb ($P < .001; \text{Figure~3B}$). Importantly, the estimated ratio of radiation delivered to tumor sites compared with nonhematopoietic tissues was greater than 1 with both $^{125}$I-BC8 (Figure 3C) and $^{131}$I-tositumomab (Figure 3D) when no prior rituximab was used. However, the impact of rituximab pretreatment reduced the predicted tumor-to–nonhematopoietic target ratios of delivered radiation to 1:1 or less when CD20 was targeted, but had no appreciable effect on ratios when CD45 was targeted using the identical tumor models. For example, at 24 hours the mean predicted tumor-kidney ratio of delivered radiation using $^{125}$I-BC8 was 2.7:1 after prior rituximab and 2.6:1 without prior rituximab ($P = .7$). In contrast, the predicted tumor-kidney ratio using $^{131}$I-tositumomab in the absence of rituximab was 2.5:1, but fell to 1.1:1 when mice were pretreated with rituximab ($P < .001$). In the absence of rituximab, tumor retention of $^{131}$I-tositumomab/g of tissue was 30% more than the lungs (the critical nonhematopoietic organ with the highest radiation exposure), whereas after rituximab pretreatment, $^{131}$I-tositumomab/g uptake in the lungs was double that of tumor sites ($P = .007$). These results were replicated with the Granta and FL-18 B-NHL lines (Figure S4).

Real-time imaging studies using fluoresceinated tositumomab and fluoresceinated BC8 corroborated the previously quantified biodistribution results (Figure 4). Mice pretreated with rituximab followed by fluoresceinated tositumomab demonstrated inferior tumor uptake and retention compared with those pretreated with a nonspecific binding control antibody (1D8). Rituximab pretreatment had no apparent impact on CD45 targeting by BC8.

Mouse models of therapy

Although the biodistribution studies indicate that circulating rituximab abrogates tumor targeting of CD20-directed therapy, it is unknown if this effect translates into reduced clinical efficacy. We thus determined if the inferior tumor targeting of the anti-CD20 radiolabeled MoAb due to rituximab pretreatment translated into inferior tumor control in mice bearing palpable human B-NHL xenografts. Mice received therapeutic doses (14.8 MBq [400 µCi]) of $^{131}$I-BC8 or $^{131}$I-tositumomab after being pretreated with either rituximab or an isotype-matched nonbinding control MoAb. Without rituximab pretreatment, tumor progression in both the $^{131}$I-BC8 or $^{131}$I-tositumomab groups was similarly delayed compared with untreated controls (Figure 5A). In contrast, after rituximab therapy, tumor control in the $^{131}$I-tositumomab group paralleled the control groups and was impaired relative to the $^{131}$I-BC8 cohort (Figure 5B).

Discussion

In these series of experiments, we demonstrate that the sequential use of anti-CD20 MoAbs results in almost complete inhibition of binding of the second anti-CD20 MoAb, but does not affect targeting of alternate tumor antigens such as CD45. This effect was observed in vitro using both cell lines and patient-derived specimens and in vivo in human lymphoma xenograft models. Importantly, this phenomenon directly translated into inferior tumor control when competing MoAbs blocked binding of the radioimmunoconjugates to target sites. Such findings should have an immediate clinical impact, as patients currently receiving the FDA-approved radiolabeled antibodies $^{131}$I-tositumomab and $^{90}$Y-ibritumomab tiuxetan are...
often treated after progression on rituximab maintenance regimens or as consolidation after rituximab-chemotherapy combinations and undoubtedly have significantly elevated serum rituximab concentrations at the time of RIT administration. In the clinic, however, it is unlikely that circulating rituximab completely abrogates the antitumor effect of RIT, because studies have shown efficacy in the setting of rituximab refractory disease where many patients likely had measurable serum rituximab just before RIT administration. Furthermore, initial studies of anti-CD20 RIT in rituximab-naive patients with measurable B-NHL demonstrated that a limited dose of unlabeled antibody, delivered just before or concurrent with the radiolabeled dose, helped improve delivery of the conjugate to lymph nodes by blocking uptake in the spleen. Nevertheless, Witzig and colleagues were able to demonstrate inferior response rates in patients with no detectable B cells (a potential surrogate for recent rituximab therapy; 47% vs 88%, P = .003) and a trend toward inferior responses in patients with measurable serum rituximab before RIT (66% vs 93%, P = .078). Taken together, these data suggest that there may be an “optimal” cold dose for every given patient depending on tumor burden, CD20 antigen density, splenomegaly, and serum rituximab concentrations. Ultimately, these hypotheses will need to be confirmed in the clinic with dosimetry studies paralleling our mouse models.

In our model, targeting an alternate antigen such as CD45 could circumvent this effect, but such strategies are not readily available in the clinic, leaving only a lengthy washout period from the last rituximab as an option. Furthermore, the panhematopoietic expression of CD45 would likely require that stem cell support be used after its administration. This same feature of CD45, however, could also be advantageous to enhance the crossfire targeting of minimal disease sites within hematopoietic tissues. Our future clinical trials plan to target CD45 with high-dose RIT in patients with lymphoid malignancies by building our group’s experience using 131I-BC8 for the treatment of acute myeloid leukemia (AML).

Our findings may also affect unconjugated antibodies. Due to the past success of CD20-directed therapies, extensive development of novel anti-CD20 MoAbs is ongoing, but these agents may also face the same limitations as we observed in our studies. MoAb design using epitope-mapping strategies initially intended to optimize antibody function may be able to alleviate some of these concerns, though functional testing of these agents in clinically representative settings of circulating rituximab remains critical.

Our results support the hypothesis that excess rituximab can impair the efficacy of CD20-directed RIT. Moreover, these findings also broadly illustrate that one can no longer develop an antigen-targeted therapy in isolation without considering the potential impact of competing MoAbs for the same target. Future preclinical and human evaluations of such drugs should reflect the likely clinical scenario whereby one or more agents may be vying for the identical epitope within a given patient.

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