Thrice-Weekly Low-Dose Rituximab Decreases CD20 Loss via Shaving and Promotes Enhanced Targeting in Chronic Lymphocytic Leukemia[1]

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Treatment of chronic lymphocytic leukemia (CLL) patients with standard dose infusion of rituximab (RTX), 375 mg/m^2, induces clearance of malignant cells from peripheral blood after infusion of 30 mg of RTX. After completion of the full RTX infusion, substantial recrudescence of CLL cells occurs, and these cells have lost >90% of CD20. To gain insight into mechanism(s) of CD20 loss, we investigated the hypothesis that thrice-weekly low-dose RTX (20 or 60 mg/m^2) treatment for CLL over 4 wk would preserve CD20 and enhance leukemic cell clearance. During initial infusions in all 12 patients, the first 30 mg of RTX promoted clearance of >75% leukemic cells. Four of six patients receiving 20 mg/m^2 RTX retained ≥50% CD20, and additional RTX infusions promoted further cell clearance. However, four of six patients receiving 60 mg/m^2 had CD20 levels <20% baseline 2 days after initial infusions, and additional RTX infusions were less effective, presumably due to epitope loss. Our results suggest that when a threshold RTX dose is exceeded, recrudesced RTX-opsonized cells are not cleared, due to saturation of the mononuclear phagocytic system, but instead are shaved of RTX-CD20 complexes by acceptor cells. Thrice-weekly low-dose RTX may promote enhanced clearance of circulating CLL cells by preserving CD20. *The Journal of Immunology,* **2006, 177: 7435–7443.**

The anti-CD20 mAb rituximab (RTX)[3] has had substantial success in the treatment of several forms of B cell lymphoma (1–7). However, RTX treatment of chronic lymphocytic leukemia (CLL) has generally shown lower response rates, and the reasons for lowered efficacy have not been clearly delineated (3, 4, 8). A possible explanation for lower efficacy may lie in the fact that malignant B lymphocytes of CLL patients have lower CD20 levels on average than tumor cells of patients with non-Hodgkin's lymphoma (NHL) (4, 9). The consequence of lower numbers of bound RTX per cell should presage decreased cytotoxicity, whether cell killing is mediated by effector cells, complement, apoptosis, or by any combination of these mechanisms (6, 7, 10–13). Moreover, any factors that decrease CD20 levels further would be expected to negatively impact RTX efficacy.

Most malignant cells targeted by RTX in NHL are not freely accessible in the bloodstream but are localized in lymph nodes and in other tissues. The standard RTX dose of 375 mg/m^2 weekly for 4 wk is intended to allow for diffusion of RTX into the site(s) of tumor growth (4, 14). However, in CLL, many malignant cells are readily available in the bloodstream, and/or cells rapidly re-equilibrate from lymph nodes, bone marrow, and spleen (15), and it is therefore possible that lower doses of RTX might be sufficient to target circulating cells. In fact, we have reported that during continuous infusion of the standard 375 mg/m^2 dose of RTX in CLL patients, the first 30 mg of RTX promotes large decreases in the number of circulating CD20$^+$ B lymphocytes (16). Moreover, 4–6 h later, immediately after RTX infusion, when high levels of the mAb are present in the circulation, we observed substantial recrudescence of malignant B lymphocytes with >90% of CD20 removed, or "shaved" from these cells, and with minimal amounts of residual bound RTX. Such a loss of tumor cell epitopes induced by infusion of therapeutic mAbs, previously called antigenic modulation (17–21), could severely compromise the potential efficacy of any mAb.

Based on these observations, we initiated a pilot clinical study for CLL patients to determine whether more frequent, lower doses of RTX could reduce shaving and still provide adequate targeting and clearance of circulating CD20$^+$ B lymphocytes. We postulated that thrice-weekly, low doses of RTX would promote slow but steady attack and continued clearance of recrudesced malignant CD20$^+$ B lymphocytes from the circulation. We chose RTX doses of 20 or 60 mg/m^2 based on our previous study, in which the first 30 mg of a 375 mg/m^2 infusion was adequate to clear circulating cells; our reported calculations indicate that a 20 mg/m^2 RTX dose should give ~2- to 3-fold molar excess of RTX over CD20 sites on circulating cells for virtually all patients (16). Because 20 mg/m^2 is approximately one-twentieth of the usual RTX dose, we were concerned that over 4 wk this dose might be too low to be effective, and therefore half of the patients received doses of 60 mg/m^2, approximately one-sixth of the usual dose.

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[3] Abbreviations used in this paper: RTX, rituximab; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; ALC, absolute lymphocyte count; AI, Alexa Fluor; MESF, molecules of equivalent soluble fluorochrome; FcγR, receptors specific for the Fc portion of IgG; MPS, mononuclear phagocytic system; HEL, hen egg white lysozyme.

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Initial effective targeting of circulating $CD20^+$ B cells was observed for both 20 and 60 mg/m^2 doses. However, in most cases the 60 mg/m^2 treatment led to much more shaving of CD20 on recrudesced-circulating cells as compared with results for the 20 mg/m^2 dose. These findings suggest a mechanism by which CLL cells may escape RTX-mediated killing, and may allow development of strategies for more effective RTX dosing for clearance of leukemic-phase B lymphocytes.

Materials and Methods

Patients and protocols

Criteria for inclusion in the study included previously untreated or relapsed CLL, demonstrable B cell expression of CD20, and an absolute lymphocyte count (ALC) >5000/μl. The treatment protocol was approved by the University of Virginia Cancer Center Protocol Review Committee and by the Institutional Review Board. Twelve consecutive patients who met the criteria and signed an informed consent were enrolled; the first three and last three patients (1–3, 10–12) received RTX 20 mg/m^2 thrice weekly (Monday, Wednesday, and Friday), and patients 4–9 received RTX 60 mg/m^2 on the same schedule. Pretreatment medications included oral or i.v. diphenhydramine, famotidine, and oral acetaminophen; meperidine and promethazine were used for infusion-related chills or rigors. A second i.v. catheter was placed to obtain blood samples. RTX (1 mg/ml) was infused at 50 ml/hour, with blood samples taken at the following times: 0, 24, 36, 50 (after completion of infusion), 108, and 170 min (2 h after completion of infusion) for the 20 mg/m^2 group; and 0, 24, 36, 108, 135 (after completion of infusion), and 255 min (2 h after completion) for the 60 mg/m^2 group. Several patients experienced grade 1–2 infusion reactions, and in these cases infusions were temporarily halted, but were later continued when symptoms resolved; the times at which blood samples were taken were adjusted and are reported accordingly. In only one case (patient 11, first infusion, 24 mg infused) was a patient unable to tolerate the targeted RTX dose. Treatment included an initial 2-wk cycle (six infusions), with an option for each patient to continue thrice-weekly therapy at the same dose for two or three more weeks per attending physician discretion. Eight patients completed 4 wk of therapy, three patients completed 5 wk, and patient 9 was treated for 2 wk. During the second 2 wk of therapy, only three blood samples were collected per treatment (preinfusion, after 30 mg of RTX, and immediately after completion of the infusion).

mAbs

mAb 3E7 (specific for C3b/iC3b), mAb 1H8 (specific for C3b/iC3b/C3dg), and mAb HB43 have been described previously (16). RTX (IDEC Pharmaceuticals) was purchased at the University of Virginia (UVA) hospital pharmacy. mAbs were labeled with Alexa Fluor (Al) dyes (Molecular Probes) following the manufacturer's directions. Other mAbs included (label, epitope, clone) the following: PE anti-CD5, 5D7 (Caltag Laboratories); allophycocyanin anti-CD19, SJ25-C1 (Caltag Laboratories); PE anti-CD20, B-Ly1 (DakoCytomation); PerCP anti-CD45, 2D1 (BD Pharmingen); Al488 anti-CD55, HD1A (provided by Profs. P. Morgan and C. Harris, University of Wales, Cardiff, England); PE anti-CD59, MEM-43 (Caltag Laboratories); and FITC anti-κ/PE anti-λ, rabbit polyclonal (DakoCytomation). Washed blood samples were blocked with 2 mg/ml mouse IgG before probing.

Laboratory analyses

Complete blood counts were obtained on all blood samples. Cell phenotyping was performed on a BD FACSCalibur (BD Biosciences), based on previous protocols (16, 22). Lymphocytes from whole blood were identified based on a CD45/side-scattering gate, and B lymphocytes were further identified by probing with an anti-CD19 mAb. Before the first infusion, and at the start of later infusions when leukocyte counts were >20,000/μl, positively identified B cells constituted $\geq$90% of lymphocytes. All analytical measurements on B lymphocytes were based on probes labeled with either FITC, Al488, or PE. Isotype controls for specific mAbs established thresholds for positive events. During the third and fourth week of therapy, phenotyping was limited to CD20 determinations. No phenotyping was performed for the three patients who had a fifth week of treatment.

Total available CD20 on cells was determined with an indirect and rigorous quantitative assay, described previously (16). The assay is based on reacting cells with excess unlabeled RTX followed by washes and secondary development with Al488 mAb HB43, specific for the Fc region of RTX. In a similar experiment, the percentage of saturation of CD20 sites by infused, bound RTX was evaluated by using Al488 mAb HB43 to probe for bound RTX on samples that were washed but not first reacted with unlabeled excess RTX. Raji cells, either naive or opsonized with excess RTX in medium, were used as negative and positive controls. Mean fluorescence intensities are reported as molecules of equivalent soluble fluorochrome, or MESF units, based on use of calibrated beads (16, 22). RTX concentrations in plasma were measured by ELISA (23). Complement titers were measured by CH50 assay, based on lysis of sensitized sheep erythrocytes (16).

Statistics

Comparison of results for the two treatment groups was based on Student's *t* tests, using Sigma Stat version 3.1 (Jandel Scientific).

Results

Preliminary findings in two CLL patients at intermediate RTX doses

Before the pilot study, we evaluated the effects of reduced doses of RTX on lymphocyte counts and cellular CD20 levels in two CLL patients (Fig. 1). Due to a late afternoon start, CLL patient 49-P received half of the standard dose (350 mg) on the first treatment day and an additional 350 mg dose the next morning. RTX treatment on the first day appeared to be quite effective in terms of reducing lymphocyte counts (Fig. 1*A*). The following day, lymphocyte levels had rebounded substantially; however, RTX treatment on the second day had little effect, presumably because the first RTX treatment promoted considerable loss of CD20 on B lymphocytes (Fig. 1*B*). CLL patient 39-P received an initial dose of 100 mg of RTX, which was followed by a standard RTX dose on the second day, according to the approach reported by Byrd et al. (24). Although the first 100 mg RTX dose led to a reduction in lymphocyte counts (Fig. 1*C*), by the next day recrudescence was evident, and just as for patient 49-P, we found that the previous RTX dose induced loss of CD20 from B cells in the circulation (Fig. 1*D*). Thus, the second dose of RTX had no impact on the number of circulating lymphocytes for either patient, presumably because the B cells had been shaved of CD20.

These results, along with our previous report (16), indicate that although single low RTX doses ($\sim$30 mg) can promote immediate B cell clearance, it appears that doses of 100 mg or more induce

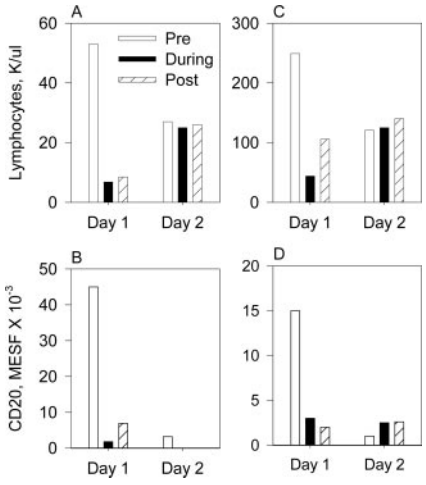

FIGURE 1. CD20 is shaved from CLL patient B cells even when RTX doses are reduced. Two CLL patients received RTX infusions on two consecutive days. Patient 49-P (*A* and *B*), 350 mg each infusion; Patient 39-P (*C* and *D*), 100 mg followed by 750 mg. ALCs (*A* and *C*) and CD20 levels per B lymphocyte (*B* and *D*) are illustrated. Pre, Prior to RTX infusion; during, after 30 mg of RTX infused; post, immediately after completion of the RTX infusion. On the second treatment day CD20 is reduced for both patients, and the second dose of RTX has little effect on lymphocyte levels.

Table I. *Patient demographics and pretreatment status*[a]

Patient	Age/Sex	Prior Rx's	Time (mo) from Dx	Rai Stage	CD38 (%)	FISH
1	78/M	C, R, FR	63	1	4	−17p13
2	71/F	C, CP, P, F	160	2	95	−17p13[b]
3	64/M	F, P, C, PcR	111	4	89	−13q14
10	77/F	None	26	1	3	Tri 12
11	82/F	C	183	3	82	−13q14
12	59/F	F, C	30	4	20	−17p, −13q
4	52/M	None	13	1	3	−17p, −13q
5	62/M	C	45	4	2	−17p, −13q
6	68/M	F	108	1	31	−13q14
7	43/F	F	65	1	1	−13q[c]
8	82/M	C	7	4	8	−17p, −13q
9	83/M	None	141	0	4	−13q[c]

[a] C, Chlorambucil; F, fludarabine; P, prednisone; R, rituximab; PcR, pentostatin, cyclophosphamide, and rituximab; FISH, fluorescent in situ hybridization.
[b] Patient 2 had both −17p and −13q at progression.
[c] Biallelic.

substantial shaving of recrudesced cells. Therefore, in an attempt to promote clearance and reduce shaving, we treated CLL patients with either 20 or 60 mg/m^2 RTX, thrice weekly.

Patient characteristics, treatment response, and toxicity

Twelve patients were randomized, six to each dose group. Patient demographics and pretreatment status are presented in Table I. The average time from diagnosis was 79 mo (range 7–183). Grade 1–2 cytokine release syndrome consisting of chills, fever, and/or hypotension occurred during 19 infusions at the 20 mg/m^2 dose and during 5 in the 60 mg/m^2 dose group. Twenty-one of the 24 episodes occurred in three patients, including the two patients with the highest CD20 density. One patient had grade 1 nausea; otherwise no nonhematologic toxicity was observed. Transient grade 3 neutropenia or thrombocytopenia occurred during infusions at both dose levels, with recovery of granulocyte and platelet counts by 2 h postinfusion.

Both low RTX doses promote lymphocyte clearance during the first infusion

Infusion of 20–30 mg of RTX on the first treatment day led to large decreases (>75%) in circulating lymphocytes for both the 20 and 60 mg/m^2 doses (Figs. 2, *A–D*, and 3, *A–D*). Two hours after completion of the infusion lymphocyte levels modestly increased, but substantial recrudescence of malignant B cells was evident 48 h later. During the first infusion, average CD20 densities on cells remaining in the circulation decreased (Figs. 2, *E–H*, and 3, *E–H*); this acute effect may be due to preferential clearance of higher CD20-expressing cells as well as shaving (see below). After 20–30 mg of RTX was infused, circulating B cells had peak levels of demonstrable bound RTX corresponding to 43 ± 13% (SD) of total CD20 for 10 of 12 patients. In the two patients with the highest initial CD20 densities (patients 2 and 8; Table II), the peak level of bound RTX corresponded to ~80% saturation of available CD20 binding sites.

Thrice-weekly RTX doses of 20 mg/m^2 better preserve the CD20 target

We next focused on how additional treatments with RTX, starting 48 h after the first treatment, would impact recrudesced, circulating B cells. Representative findings for both patient groups are illustrated in Figs. 2 and 3. In the 20 mg/m^2 group, additional RTX infusions continued to manifest efficacy, as defined by the ability of infused RTX to clear circulating cells (Fig. 2, *A–D*); it is noteworthy that cellular CD20 densities for patients 2, 3, 10, and 11 (and patient 1; data not shown) were reasonably well-conserved at the start of each new infusion (≥50% pretreatment values; Fig. 2, *E–H*, and Table II) throughout the first 2 wk of treatment. Finally,

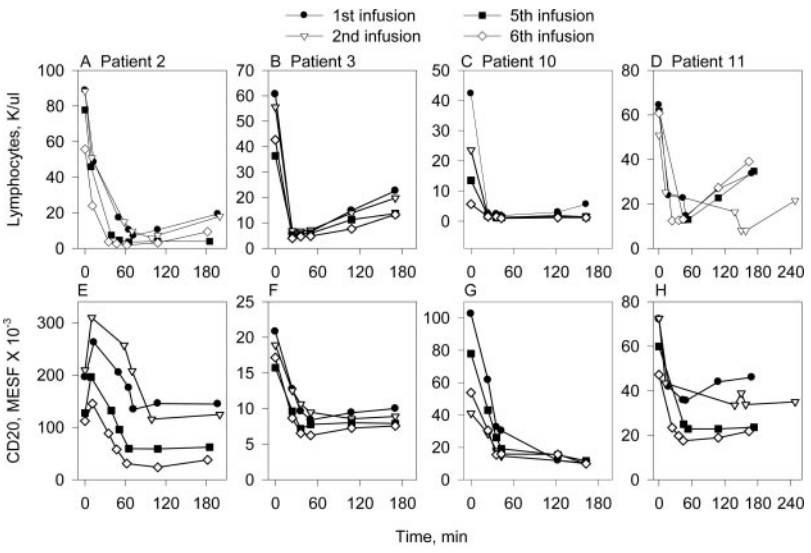

FIGURE 2. Thrice-weekly infusions of 20 mg/m^2 RTX in CLL patients incrementally clear leukemic B lymphocytes, and RTX-promoted shaving of CD20 of recrudesced cells is modest. ALCs (*A–D*) and CD20 levels per B lymphocyte (*E–H*) during and up to 2 h after the first, second, fifth, and sixth infusion are illustrated for four out of six patients in this group. Based on MESF units, the percentage of CD20 expressed on patient cells (all but patient 12), at the start of day 2, compared to the start of day 1 averaged 86 ± 27%.

Table II. *Effects of low-dose RTX on lymphocyte counts and CD20 levels*

Patient	RTX (mg/m^2)	ALC[a]			CD20[b]		
		Pre-Rx	Post-Rx	Percentage of reduction[c]	Start	Finish	Percentage of shaved[d]
1	20	60	8	87	27	12	56
2	20	89	2	98	196	NDB[e]	
3	20	61	20	67	21	13	38
10	20	42	4	90	102	74	27
11	20	64	52	19	72	48	33
12	20	7	4	43	14	3	79
4	60	86	21	76	19	2	89
5	60	64	41	36	42	6	86
6	60	10	3	70	32	2	94
7	60	97	17	82	23	4	83
8	60	12	0.8	93	554	NDB[e]	
9	60	21	17	19	26	4	85

[a] One thousand per microliter prior to first, and at the start of twelfth RTX infusion (11 of 12 patients) or sixth infusion (patient 9). NBD, No detectable B cells.

[b] CD20 levels prior to first, and at the start of twelfth RTX infusion (11 of 12 patients) or sixth infusion (patient 9) (MESF $\times 10^{-3}$).

[c] Mean and SD for 20 and 60 mg/m^2 groups, respectively; 67 ± 31 and $63 \pm 29\%$.

[d] Mean and SD for 20 and 60 mg/m^2 groups, respectively; 47 ± 21 and $87 \pm 4\%$ ($p = 0.0026$). If patient 12 is omitted, the value for the 20 mg/m^2 groups is $39 \pm 13\%$ ($p < 0.001$).

[e] Patients 2 and 8 were not included in the calculation of percentage of shaved.

patient 12 had very low CD20 density, and even at the 20 mg/m^2 RTX dose this patient's cells demonstrated CD20 shaving (Table II). For this reason, results for patient 12 were not included in the statistical tests presented in the captions of Figs. 2 and 3.

In the 60 mg/m^2 group, additional RTX infusions were less effective at reducing lymphocyte levels in patients 4, 7, and 9 (Fig. 3) and patient 6 (data not shown). This result is likely a consequence of reduced densities of CD20 relative to pretreatment levels (Fig. 3, *E*, *G*, and *H*, and Table II). The results for patient 5 gave an intermediate pattern (Fig. 3, *B* and *F*, and Table II); additional RTX infusions promoted cell clearance, although the cumulative effect of the infusions led to shaving of CD20 by the start of the sixth treatment. Finally, although additional treatments of patient 8 led to shaving of CD20, circulating B lymphocytes were reduced with each infusion. It is important to note that this patient initially had much higher CD20 densities than the other 11 patients (Table II).

Fig. 4 summarizes changes in lymphocyte counts after infusion of 30 mg of RTX for the first and sixth infusion for all patients. During the first infusion, lymphocyte clearance was equally effective in both groups (Fig. 4, *A* and *C*). For the sixth infusion, patients 6, 8, and 12 had low initial lymphocyte counts and were excluded from analysis; these three patients also began treatment with the lowest lymphocyte levels (Table II). In the remaining patients, there is a clear distinction between the 20 and 60 mg/m^2 group (Fig. 4, *B* and *D*). The sixth treatment continued to remove lymphocytes in the 20 mg/m^2 group ($77 \pm 17\%$ removed), but this treatment had very little effect on lymphocyte counts for three of four patients in the 60 mg/m^2 group ($8 \pm 4\%$ removed). This finding can be readily understood in terms of the reduced CD20 densities of the patients' B lymphocytes as a consequence of the intervening 60 mg/m^2 RTX treatments.

Cumulative effects of RTX on shaving and lymphocyte counts

CD20 levels and lymphocyte counts during the complete treatment for all patients are illustrated in Fig. 5. Although too few patients were enrolled to establish definitive trends, the results reveal that considerably more shaving occurs in the 60 mg/m^2 group than in the 20 mg/m^2 group (Table II), and this shaving is manifest after the first RTX infusion. With respect to reduction in circulating lymphocytes, three patients are noteworthy. In patient 2, each infusion led to clearance of >75% of circulating lymphocytes during

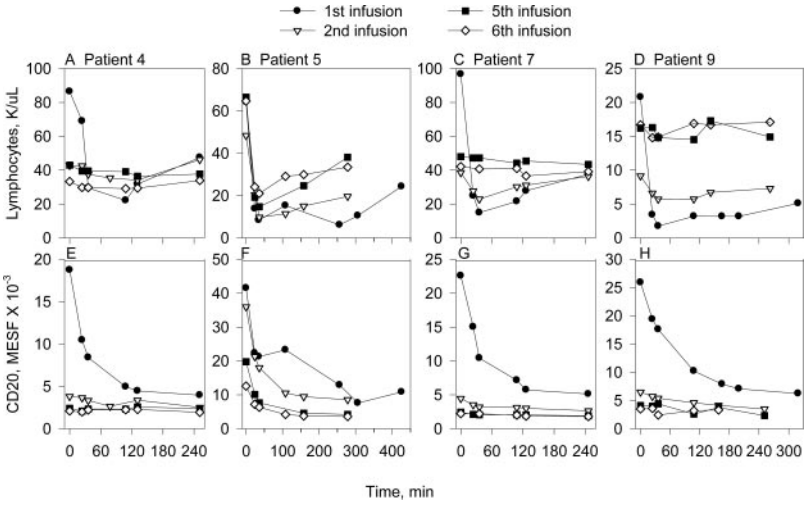

FIGURE 3. Thrice-weekly infusions of 60 mg/m^2 RTX in CLL patients lose effectiveness in some patients, due to RTX-promoted shaving of CD20. Absolute lymphocyte counts (*A–D*) and total CD20 per B lymphocyte (*E–H*) during and up to 2 h after the first, second, fifth, and sixth infusion are illustrated for four out of six patients in this group. Note that in several patients CD20 levels are reduced after the first or second RTX infusion, and under these conditions clearance of B lymphocytes is compromised. The percentage of CD20 expressed on patient cells (all six patients), at the start of day 2, compared to the start of day 1 averaged $36 \pm 28\%$. $p = 0.015$ compared to the 20 mg/m^2 group.

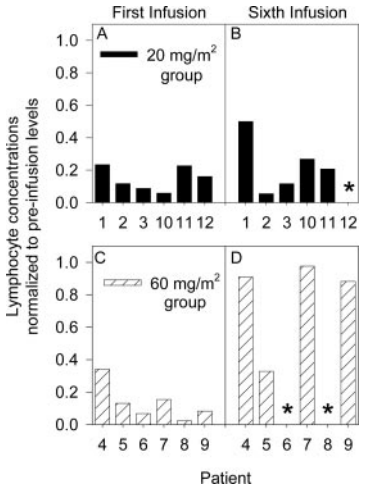

FIGURE 4. Lymphocyte levels after infusion of 30 mg of RTX, normalized to preinfusion values. The response to RTX is similar in both groups during the first infusion (*A* and *C*). However, by the sixth infusion lymphocytes in five of six patients in the 20 mg/m^2 group continue to decrease during the infusion (*B*), but three patients in the 60 mg/m^2 group are refractory to RTX treatment (*D*). ∗, Preinfusion lymphocyte counts were <3000/μl. On the first treatment day, normalized lymphocyte concentrations averaged 0.15 ± 0.07 (20 mg/m^2 group) and 0.13 ± 0.11 (60 mg/m^2 group); $p = 0.80$. On the sixth treatment day (patients 6, 8, and 12 omitted due to low counts), the corresponding values were 0.23 ± 0.17 and 0.77 ± 0.30; $p = 0.011$.

the first 2 wk, but substantial recrudescence was evident 2 days later (Figs. 2*A* and 5*B*). However, by the fourth week of treatment no circulating B lymphocytes were detectable. Patient 8 was discussed in the preceding section. Patient 11 had bulky adenopathy, and although each RTX infusion initially cleared cells in the bloodstream (Fig. 2*D*), 4 wk of treatment at this dose had little impact on the steady-state level of circulating B cells (Table II and Fig. 5*E*).

Most patients showed moderate to substantial reductions in lymphocyte counts after 11 treatments (average of ~65% in both groups; Table II). This finding can be compared with our previous observations in CLL patients who received the usual weekly dose of 375 mg/m^2 RTX and in whom considerable shaving of CD20 was demonstrable (16). Although three of these patients had an average reduction in lymphocyte counts of 59 ± 22% (SD) at the start of the fourth RTX infusion, in two other patients who received doses of 375 mg/m^2, lymphocyte counts actually increased by 33 and 123%.

RTX infusions induce moderate innocent bystander loss of some epitopes in the 60 mg/m^2 group

We extended phenotyping studies to several B lymphocyte CLL markers, including CD5, Ig L chain, CD55, and CD59, to determine whether RTX-promoted shaving might affect other cell surface proteins. For five patients in the 20 mg/m^2 group, in whom overall loss of CD20 was <50%, other cell surface proteins tested showed little or no change (≤20%) over the first 2 wk of treatment. However, in patient 12 and in all six patients in the 60 mg/m^2 group, in whom CD20 shaving was demonstrable (>75% overall loss of CD20), we observed 30–40% decreased Ig L chain in all, 30–50% decreased CD55 in four patients, and 30–75% decreased CD59 in three patients.

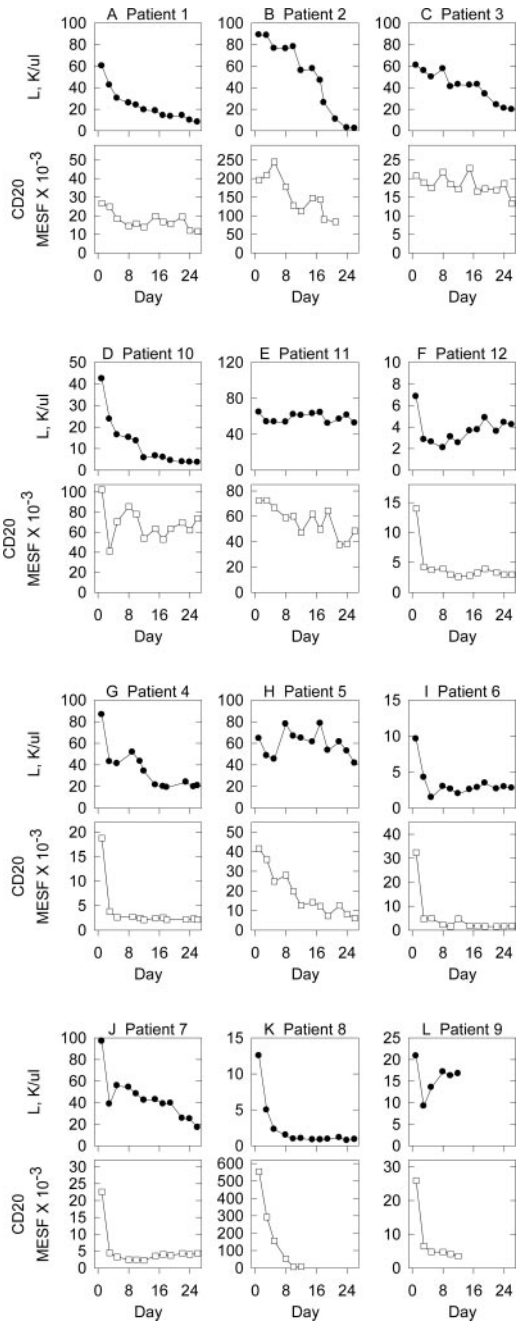

FIGURE 5. Over 4 wk CD20 was largely preserved on B lymphocytes in five of six patients in the 20 mg/m^2 group (*A–F*), but CD20 was shaved from B lymphocytes in most patients in the 60 mg/m^2 group (*G–L*). Results for patients in the 20 mg/m^2 group (1–3, 10–12) are displayed in the *top half of the figure*, and results for patients in the 60 mg/m^2 group (4–9) are in the *lower half*. ALCs of patients in both groups during the study are also displayed. The greatest reduction in lymphocyte counts, for patients 2 and 8 over the limited 4-wk treatment period, may be related to the very high initial CD20 levels on their lymphocytes (196,000 and 554,000 MESF units, respectively).

RTX was demonstrable in the bloodstream, and complement was usually not consumed

RTX concentrations and complement hemolytic titers were measured over the first six treatments. In the 20 mg/m^2 group, plasma RTX levels for five of six patients (patient 12 excluded) remained low during the first 2 wk of therapy. RTX was in the range of 1–4

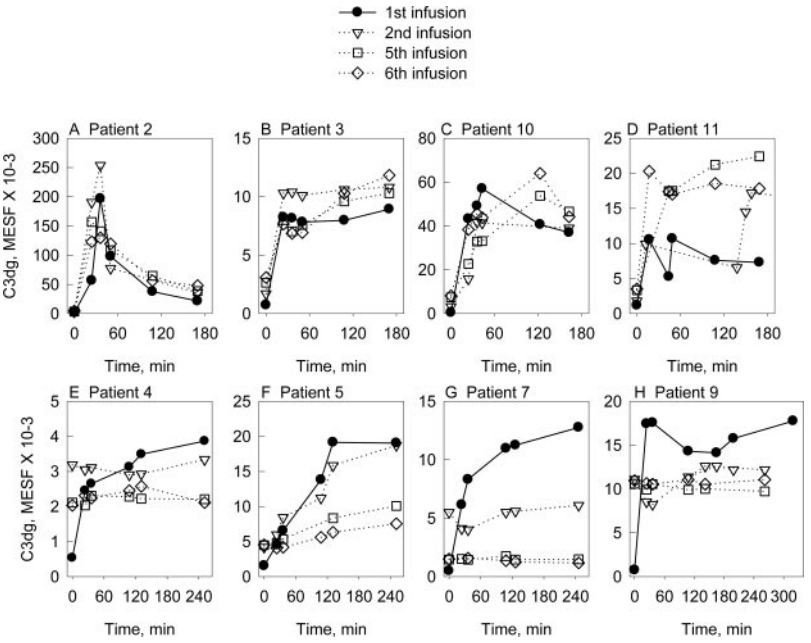

FIGURE 6. C3dg serves as a molecular marker for shaved B lymphocytes after RTX infusion. Representative results for both groups are displayed. Although the time scale shown extends over only 3–5 h, the results provide information ~2 days after treatment one (time zero for second infusion) and ~2 days after treatment five (time zero for sixth infusion), respectively. In the 20 mg/m^2 group, and in patient 5 (*A–D* and *F*), C3dg-tagged 36 cells were cleared by the start of the next infusion 2 days later. In three of four patients from the 60 mg/m^2 group (*E*, *G*, and *H*), these tagged cells persisted in the circulation over several days. Based on MESF units, the percentage of residual C3dg found on patient cells at the start of the second infusion day, compared to the maximum observed during treatment on day 1 (~2 days earlier), was 12 ± 18% for the 20 mg/m^2 group and 51 ± 30% for the 60 mg/m^2 group ($p = 0.033$).

μg/ml during and immediately after infusion, and returned to baseline levels (<1 μg/ml) 2 days later. RTX concentrations for four patients (4, 6, 7, and 9) in the 60 mg/m^2 group were considerably higher: mean values at the start of the third and sixth infusions were 10 ± 3 and 23 ± 6 μg/ml (SD), respectively, and reached 44 ± 9 μg/ml at the end of the sixth infusion. Mean RTX concentrations at the start of infusions for patients 5 and 8 were lower, but peaked at 14 and 20 μg/ml after the sixth infusion. Finally, plasma RTX levels in patient 12, whose cells were also shaved (possibly due to very low initial CD20; Table II), were 3 and 6 μg/ml, respectively, at the start of infusions 3 and 6. Overall, these results suggest that the lower RTX dose of 20 mg/m^2 is adequate to opsonize and clear circulating cells, but that at the higher dose there is sufficient leftover RTX that it can bind to recrudesced cells and promote shaving.

During the first 2 wk of treatment, complement levels in the patients generally remained within a factor of 2 of baseline values (normal range) during the study, with two exceptions: In patient 5, complement titers fell 10-fold during each infusion, but returned to ≥50% of baseline 2 days later. A similar pattern was observed for patient 8 over the first three infusions, when B cells were demonstrable in the bloodstream (Fig. 5*B*). In no patients were complement levels suppressed for extended periods, in contrast to our observations in some CLL patients treated with 375 mg/m^2 doses of RTX, once per week for 4 wk (16).

RTX infusion promotes deposition of C3 fragments on targeted cells

We next investigated whether binding of RTX would activate complement and promote deposition of C3 activation fragments on circulating cells at these low RTX doses. Cells were probed with either mAb 3E7, specific for C3b/iC3b, or with mAb, 1H8, specific for all three fragments, C3b/iC3b/C3dg. Flow cytometric analyses (Fig. 6) revealed that, as a consequence of RTX infusion, varying amounts of C3dg, but not the earlier activation products C3b/iC3b, were demonstrable on targeted B cells of all patients during the first infusion. Because complement opsonization requires initial deposition of C3b, the finding of only C3dg on the cells indicates that C3b was rapidly degraded to iC3b and then to C3dg (25–28). We detected an interesting difference between the two treatment groups in the kinetics of clearance of C3dg-tagged cells. In all six patients in the 20 mg/m^2 group, C3dg-tagged cells were generated as a consequence of each RTX infusion; however, 2 days later little C3dg was demonstrable on circulating cells in four of the six patients (Fig. 6, *A–D*), suggesting that the previously tagged cells were cleared. In contrast, in the 60 mg/m^2 group, C3dg-tagged cells were still present in the circulation of patients 4, 6 (data not shown), 7, and 9, 2 days after the initial infusion (Fig. 6, *E–H*). Additional infusions of RTX had only modest effects on cellular C3dg levels for these patients, consistent with the observation that these cells had been shaved of CD20. Under these conditions additional RTX could not bind to the cells, and C3 fragment deposition would therefore be largely precluded.

Discussion

The most important observation from this pilot trial is that very low doses (20 mg/m^2) of RTX can promote substantial and rapid clearance of circulating CD20$^+$ cells in patients with CLL (Figs. 2–5). The results for the 60 mg/m^2 group (Figs. 3–5), along with our findings on patients 39-P and 49-P (Fig. 1), confirm and extend our previous investigations demonstrating that moderate to standard doses of RTX induce substantial loss of CD20 in patients with CLL (16), thus leading to a condition in which additional RTX infusions demonstrate little efficacy at clearing circulating malignant cells. In contrast, in five of six patients in the 20 mg/m^2 group, additional RTX infusions continued to demonstrate clearance efficacy. Because CD20 levels were preserved within a factor of two on the malignant cells of these patients, the additional RTX could bind to these cells and continue to promote their rapid clearance and eventual destruction, presumably in the liver and spleen, by FcγR (receptors specific for the Fc portion of IgG)-containing cells of the mononuclear phagocytic system (MPS) (29–31).

In most patients, considerable recrudescence of B cells was demonstrable 2 days after each RTX treatment. There is precedent for these observations. Treatment of CLL and other hematologic malignancies by leukapheresis also leads to recrudescence due to re-equilibration into the bloodstream of malignant cells from other sites including lymph nodes, spleen, and bone marrow (15). The number of B cells cleared by the low-dose RTX infusions is quite

comparable to the number removed in a single leukapheresis procedure. CD20 was substantially preserved on recrudesced cells of patients in the 20 mg/m^2 group; this finding suggests that there may not be differential expression of CD20 on cells in the various tissue compartments, in contrast to the report by Huh et al. (32).

Infusion of only 20 mg of RTX on the first treatment day, corresponding to ~30 nM RTX in the blood, promoted clearance of >75% of B cells (Figs. 2 and 3). High levels of free CD20 (~700 nM) have been reported in CLL (33), and such levels would be expected to inhibit the action of RTX. However, our findings indicate that there is not any free circulating CD20 to compete for infused RTX. This result reinforces our previous report, based on in vitro assays, which also found no evidence for free CD20 in the plasma of CLL patients (23).

Our results suggest that recrudesced B cells, exposed to even moderate concentrations of circulating RTX (>10 μg/ml), lose CD20 over the next 16–48 h (Figs. 1 and 3–5). A phenomenon similar to shaving was first reported more than 20 years ago under the rubric of antigenic modulation (17–19). Although low doses of certain therapeutic mAbs promoted clearance of opsonized malignant cells in the bloodstream, higher doses also induced loss of targeted epitopes on recrudesced cells found in the circulation several hours after the mAbs were infused. Such rapid antigenic modulation does not occur when RTX is used to treat NHL or diseases such as systemic lupus erythematosus or rheumatoid arthritis in which the level of B lymphocytes in the bloodstream is comparatively low (34–36).

In all patients, the first 20–30 mg of RTX induces rapid clearance of the majority of circulating malignant cells (Figs. 2 and 3). Based on previous studies of the handling of IgG-opsonized cells in the bloodstream, we suggest that cells with bound RTX are taken up and destroyed by liver and splenic macrophages (29–31). The capacity of the MPS is likely to be temporarily saturated by this large burden of opsonized cells (>10^{11} cells) (37, 38). Then, as additional cells re-equilibrate into the bloodstream they are immediately opsonized with RTX, but due to MPS saturation these cells are shaved instead of cleared. Both clearance and shaving may occur simultaneously, and even in the 20 mg/m^2 group we observed decreases in CD20 on surviving cells in the circulation during most infusions (Fig. 2). We therefore suggest that effective RTX immunotherapy for CLL must achieve a balance that promotes effective clearance and killing of malignant cells while minimizing shaving.

The site(s) of shaving remain to be defined. FcγR-positive cells, such as macrophages or liver endothelial cells (39, 40), may promote shaving and uptake of cell-bound RTX and CD20 in an endocytic reaction called trogocytosis (41–43). This process takes place at an immunological synapse between two cells in which receptors on the endocytic or acceptor cell (e.g., FcγR on a macrophage) recognize ligands on the target cell (e.g., RTX-opsonized B lymphocyte), and then RTX and Ag (e.g., CD20), along with target cell membrane fragments, are taken up and internalized by the acceptor cell. Recently, we demonstrated, in an in vitro model, that THP-1 cells (a monocytic cell line) as well as isolated human PBMC can shave RTX-opsonized cells in a process mediated by FcγR (44). This shaving reaction, in which RTX and CD20 are removed from B cells, is reasonably specific for CD20, because most other epitopes we examined were preserved or showed modest decreases. Likewise, in the present clinical study some innocent bystander epitope loss (Ig L chain, CD55, and CD59) was demonstrable in shaved cells; this loss was not observed in cells in which CD20 was spared. Some of these cell surface molecules may be in close proximity to CD20, and thus could be "trapped" in the immunological synapse, and be taken up during the shaving reaction. It is possible that segregation of RTX-CD20 complexes in lipid rafts enhances their removal by the MPS (11). It is unlikely that CLL cells themselves function as acceptor cells, because incubation of CLL cells with bound RTX for up to 7 h at 37°C leads to little loss of CD20 in the absence of effector cells (16).

A process similar to shaving has been documented in a mouse model. Zimring et (45) al. reported that infusion of E bearing a transmembrane form of hens egg white lysozyme (HEL), into immunized mice with circulating anti-HEL IgG Abs, led to clearance of approximately half of the infused E in 6 h. However, over the next 72 h HEL was removed from surviving E in a reaction mediated by FcγR. The HEL Ag was apparently removed from E subsequent to binding of IgG anti-HEL Abs. The remaining cells, shaved of HEL, persisted in the circulation. This phenomenon, called Ag suppression, may follow a similar mechanism to that of shaving. That is, a bolus of IgG-opsonized cells is rapidly cleared, but after saturation of the primary clearance pathway, additional IgG-opsonized cells suffer Ag loss (and presumably bound IgG loss) without destruction of the cells.

Important questions center on whether CD20$^+$ malignant cells in CLL that re-equilibrate into the bloodstream can be effectively targeted by frequent low-dose RTX for destruction by cells of the MPS, as well as whether the rate of cell killing per RTX infusion cycle is greater than the rate at which cells proliferate. Based on the nadir in cell counts during the first infusion (Figs. 2 and 3), it would appear that ~35 mg of RTX can remove as many as 2–3 × 10^{11} malignant B cells from the bloodstream in a single treatment. The 35 mg RTX dose corresponds to an input stoichiometry of ~400,000 RTX molecules per cleared cell, substantially in excess of cellular CD20 levels in all B cell lymphomas, including CLL (46, 47). Messmer et al. (48) estimate that B cells in CLL are regenerated at ~10^{10} cells per day; therefore, if a single 35 mg treatment can remove 20–30 times as many cells, then multiple periodic low doses of RTX may be sufficient to achieve long-term reduction in malignant cells; in fact, based on the present study, doses as low as 20 mg/day may also be effective. RTX-mediated clearance followed by recrudescence and additional clearance is most readily illustrated for patient 2 (Figs. 2*A* and 5*B*).

Most CLL cells have insufficient numbers of CD20 molecules to promote complement-mediated destruction (9, 16), and it is very unlikely that the rapid clearance we observed in all patients during the first infusion (Figs. 2 and 3) can be explained by complement-mediated lysis. However, we did observe opsonization of CD20$^+$ cells with C3 fragments upon infusion of RTX. The cell-associated C3dg served as a molecular marker to identify cells targeted by RTX (Fig. 6). Moreover, in several patients in the 60 mg/m^2 group, shaved cells previously targeted by RTX remained in the circulation and could be identified due to deposited C3dg. Because C3dg is not present on normal tissue, tumor-associated C3dg could be exploited in a combination immunotherapy with RTX and a mAb specific for C3dg on cell surfaces. Finally, in common with RTX, most immunotherapeutic mAbs used to target cancer cells are human IgG1 isotypes and capable of fixing complement (49); it is likely that malignant cells not cleared or destroyed by such mAbs will also have deposited C3 activation fragments.

The focus of this pilot study was to evaluate the efficacy of lymphocyte clearance and the degree of CD20 shaving with low-dose RTX. Future clinical trials, based on the present paradigm but with a much longer treatment course and/or with supplementation with cytokines such as GM-CSF to enhance Ab-dependent cell-mediated cytotoxicity (50, 51), should allow for definitive clinical evaluations of efficacy. The thrice-weekly regimen tested in this study for RTX in CLL finds a precedent in the usual course of

therapy for CLL with another mAb, Campath. This mAb is administered i.v. in thrice-weekly doses of 30 mg for 12 wk (52). Recently, Campath has also been demonstrated to be effective when administered s.c. (53). It is unknown whether s.c. RTX is safe or therapeutically effective, but low doses of RTX, comparable to or slightly lower than those examined in our study, could be tested in a trial based on s.c. administration.

Finally, the study of O'Brien et al. (54) suggests that very high weekly doses of RTX (2250 mg/m^2) may be more effective clinically, a result that appears counterintuitive to the hypothesis and data we report. These findings may be reconciled due to the potential of very high levels of circulating monomeric RTX (IgG1 isotype) to mediate blockade of FcγRI, the receptor that is principally responsible for shaving (44). Under these conditions shaving could be minimized, and over an extended period effector cells which express FcγRII and FcγRIII would be able to clear and destroy more cells with bound RTX.

In summary, we find that although moderate doses of RTX (60 mg/m^2), approximately one-sixth of the usual infusion dose, can promote clearance of circulating CD20$^+$ cells in CLL, these doses may also lead to substantial shaving (antigenic modulation) of CD20 from cells found in the circulation 2 days later. These results, taken in the context of our previous report documenting shaving at the standard, higher RTX doses, suggest that RTX-opsonized cells not rapidly cleared from the bloodstream by the MPS must be processed by an alternative mechanism that removes CD20 and bound RTX. However, infusion of lower doses of RTX (20 mg/m^2), almost 20-fold less than the standard dose used in the treatment of CLL, promotes rapid and efficient clearance of CD20$^+$ malignant cells but leads to much less shaving of cells that enter the bloodstream within the next 48 h. Therefore, these cells can be targeted and cleared by additional thrice-weekly low doses of RTX. In terms of future therapies, periodic low-dose infusions of RTX, perhaps even on a daily schedule and administered by other routes (e.g., s.c.) may provide new and improved treatment regimens for RTX in the treatment of CLL. Antigenic modulation has been reported for several other mAbs, and therefore our findings may have important implications for the use of other mAbs in the immunotherapy of hematologic malignancies.

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