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Rituximab Infusion Promotes Rapid Complement Depletion and Acute CD20 Loss in Chronic Lymphocytic Leukemia[1]

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Complement plays an important role in the immunotherapeutic action of the anti-CD20 mAb rituximab, and therefore we investigated whether complement might be the limiting factor in rituximab therapy. Our in vitro studies indicate that at high cell densities, binding of rituximab to human CD20$^+$ cells leads to loss of complement activity and consumption of component C2. Infusion of rituximab in chronic lymphocytic leukemia patients also depletes complement; sera of treated patients have reduced capacity to C3b opsonize and kill CD20$^+$ cells unless supplemented with normal serum or component C2. Initiation of rituximab infusion in chronic lymphocytic leukemia patients leads to rapid clearance of CD20$^+$ cells. However, substantial numbers of B cells, with significantly reduced levels of CD20, return to the bloodstream immediately after rituximab infusion. In addition, a mAb specific for the Fc region of rituximab does not bind to these recirculating cells, suggesting that the rituximab-opsonized cells were temporarily sequestered by the mononuclear phagocytic system, and then released back into the circulation after the rituximab-CD20 complexes were removed by phagocytic cells. Western blots provide additional evidence for this escape mechanism that appears to occur as a consequence of CD20 loss. Treatment paradigms to prevent this escape, such as use of engineered or alternative anti-CD20 mAbs, may allow for more effective immunotherapy of chronic lymphocytic leukemia. ***The Journal of Immunology,*** **2004, 172: 3280–3288.**

The anti-CD20 mAb rituximab (RTX)[4] was the first mAb approved for single agent therapy for non-Hodgkin's lymphoma (NHL) (1–6). The efficacy of this mAb in indolent or follicular NHL is well documented, and it is also being examined as an immunotherapeutic agent against chronic lymphocytic leukemia (CLL) (7–10). The mechanism of antitumor activity of RTX in vivo remains a subject of some debate; preclinical studies, as well as more recent reports of animal models and clinical investigations have provided support for apoptosis (11–14), Fcγ receptor-mediated Ab-dependent cellular cytotoxicity (ADCC) (10, 15–18), and complement-dependent cytotoxicity (CDC) (19–28). In patients selected for treatment based on relatively low circulating lymphocyte counts ($<5000/\mu$l), infusion of RTX leads to rapid and prolonged depletion of normal and malignant B cells from the bloodstream (1, 4). This rapid clearance may be attributed to complement-mediated lysis and/or phagocytosis of B cells via Fcγ receptors on cells of the mononuclear phagocytic system (MPS).

We have reported that binding of RTX to CD20$^+$ cells promotes complement activation and covalent deposition of approximately half a million C3b activation fragments (C3b(i)) per cell (26). Our fluorescence microscopy experiments indicate that most deposited C3b(i) is colocalized with bound RTX on B cell lines and on primary CLL cells (26); based on studies in model systems, it is likely the C3b(i) is covalently bound to RTX (29). These findings, along with the observed RTX-mediated clearance of CD20$^+$ cells from the bloodstream, raise several important issues.

First, RTX treatment for CLL under conditions of high cell burden might consume so much complement that the ability of RTX to promote CDC could be compromised. Second, the capacity of the MPS to remove IgG-opsonized cells may be exceeded at high cell counts. Third, studies of phagocytosis reported by Griffin et al. (30) suggest that Fcγ receptor-mediated rearrangement and capping of RTX-CD20 complexes on the surface of B cells might lead to removal of the complexes by macrophages instead of whole cell phagocytosis, thus allowing the cells to escape.

We addressed these issues by studying the effects of RTX treatment on complement levels in patients with B cell lymphomas. We evaluated C3b(i) deposition and its colocalization with bound RTX on CD20$^+$ cells from RTX-treated CLL patients, taken before, during, and after RTX infusion. Our results indicate RTX infusion promotes complement consumption in CLL, and sera taken from such patients after RTX treatment have reduced capacity to lyse Raji and primary lymphoma cells due to complement depletion. We also observed acute loss of CD20 from B cells present in the circulation immediately after RTX treatment. The details of these findings, and the demonstration that cytotoxic activity can be substantially restored in RTX-rich, C-depleted CLL patient sera by

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[4] Abbreviations used in this paper: RTX, rituximab; ADCC, Ab-dependent cellular cytotoxicity; Al, Alexa dyes; APhCy, allophycocyanin; CDC, complement-dependent cytotoxicity; CH50, total complement hemolytic activity; CLL, chronic lymphocytic leukemia; MESF, molecules of equivalent soluble fluorochrome; MPS, mononuclear phagocytic system; NHL, non-Hodgkin's lymphoma; NHS, normal human serum; RT, room temperature.

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supplementation with normal human serum (NHS) or complement component C2, form the basis for this study.

Materials and Methods

Cell lines, sera, and patients

ARH77, DB, and Raji cells were obtained from American Type Culture Collection (Manassas, VA) and maintained, as described (26). Blood was obtained with written informed consent from healthy volunteers or patients diagnosed with B cell lymphomas; the University of Virginia (UVA) Institutional Review Board approved all protocols. PBMC were isolated from anticoagulated whole blood by density-gradient centrifugation with Ficoll-Paque^{Plus} (Amersham Pharmacia Biotech, Piscataway, NJ) (31). Viability defined by trypan blue exclusion was >95%. Patient and NHS were processed within 1 h after blood collection and stored at −80°C.

Patients with B cell lymphomas received one or more cycles of RTX therapy (375 mg/m^2, once per week for 4 wk (1, 2, 4)), except for CLL patient 22, who had two infusions of RTX, 5 wk apart. Other than patient 22, patients were treated as outpatients. This factor and the need to restrict the amount of blood taken limited the total number of blood samples available for analysis. Blood samples were collected immediately before and after RTX infusion and in several cases after 30 mg of RTX was infused. Complete blood counts were determined by clinical laboratories at the UVA Hospital. Of 25 patients studied, 6 had CLL, and the results for 4 of these CLL patients (patients 1, 9, 22, and 33) with respect to complement consumption and loss of CD20 are reported in detail. CLL patient 8 had low CD20 levels and a low complement titer before treatment. CLL patient 10 had normal complement levels, but low CD20, and complement was not consumed when this individual was treated with RTX.

Antibodies

IgG1 mAbs 7C12 and 3E7 (specific for C3b/iC3b), IgG2a mAb 1H8 (specific for C3b/iC3b/C3dg), and IgG1 mAb HB43 (specific for the Fc region of human IgG) have been described (26, 32). Our findings with the mAbs cited above are generally identified by their specific epitopes, e.g., mAb HB43 was used to detect the human Fc region of RTX, and is referred to as anti-RTX. RTX (IDEC Pharmaceuticals, San Diego, CA) was purchased at the hospital pharmacy. mAbs were labeled with Alexa (Al) dyes (Molecular Probes, Eugene, OR), following the manufacturer's directions. Other mAbs included (label and epitope identified first): PE anti-CD5, 5D7, IgG1 (Caltag Laboratories, Burlingame, CA); PE and allophycocyanin (APhCy) anti-CD19, SJ25-C1, IgG1 (Caltag Laboratories); PE-anti-CD20, B-Ly-1, IgG1 (DAKO, Carpinteria, CA); PerCP anti-CD45, 2D1, IgG1 (BD PharMingen, San Diego, CA); FITC anti-κ/PE anti-λ, rabbit polyclonal (DAKO). Washed blood samples were blocked with 2 mg/ml mouse IgG before probing.

Complement opsonization and analyses of patient whole blood

EDTA anticoagulated patient blood was processed as follows: ~0.5 ml of whole blood was washed three times by addition of 4 ml of BSA-PBS, followed by centrifugation at 1260 × *g* at room temperature (RT) in a swinging bucket centrifuge. The supernatant was then carefully aspirated to spare the buffy coat. The washed cell pellet was reconstituted to its original volume in BSA-PBS. Autologous patient or ABO blood type-matched serum was added, along with RTX and BSA-PBS, to give a final serum concentration of 25% and a final RTX concentration of 10–25 μg/ml. Alternatively, patient sera containing infused RTX, but low in complement titer, were supplemented with autologous or matched sera with full complement activity. One volume of reconstituted washed cell pellet was mixed with one-half volume of patient serum containing RTX, and then either one-half volume of BSA-PBS (25% serum final) or one-half volume of serum with normal complement titer (50% serum final) was added. After incubation for 30 min at 37°C, samples were washed three times, blocked with mouse IgG, and probed with a mixture of PE anti-CD19, PerCP anti-CD45, Al488 anti-C3b(i), and Al633 anti-RTX. After incubation for 30 min at RT, E were lysed, and the samples were washed, fixed in 1% paraformaldehyde PBS, and analyzed by flow cytometry. The combination of CD19, CD45, and side scattering identified B cells, which were analyzed for bound RTX and C3b(i). Calibrated fluorescent beads (Spherotech, Libertyville, IL) were used to convert fluorescence intensities to molecules of equivalent soluble fluorochrome (MESF) (26).

Immunophenotyping

Washed blood cell pellets, reconstituted in 2 mg/ml mouse IgG in BSA-PBS, were incubated for 30 min at 37°C with or without unlabeled RTX (50 μg/ml). Samples were washed and probed with a mixture of APhCy anti-CD19, PerCP anti-CD45, and one of the following: Al488 anti-RTX; Al488 RTX (anti-CD20) + PE-anti-CD5; Al488 anti-C3b(i) + PE anti-CD20; FITC anti-κ + PE anti-λ; Al488 IgG1 isotype control; Al488 IgG2a isotype control; or no addition.

Fluorescence microscopy

Washed blood cell pellets reconstituted in 2 mg/ml mouse IgG in BSA-PBS were incubated for 30 min at RT with a mixture of Al488 anti-C3b(i) and Al594 anti-RTX, processed, and examined with a BX40 fluorescent microscope (Olympus, Melville, NY), equipped with a Magnafire digital camera.

Killing assays

Raji cells or isolated patient PBMC were adjusted to 10^6 cells/ml in medium (RPMI 1640, antibiotics, and FBS) (26), and 100 μl mixed with 25–50 μl of patient sera and additional reagents, including RTX, complement component C2 (Advanced Research Technologies, La Jolla, CA), NHS, and mAb 3E7. Mixtures were incubated for varying periods at 37°C in 5% CO_2, and after two washes stained with FITC annexin V and propidium iodide and analyzed by flow cytometry (26). Alternatively, sera deficient in individual complement components (Quidel, San Diego, CA) were examined (10% final concentration) ± the missing complement protein (Advanced Research Technologies) ± RTX (10 μg/ml) to determine the role of individual components of the complement pathway in RTX-mediated CDC of Raji cells.

Total complement hemolytic activity (CH50) assays

Sheep E were opsonized with rabbit anti-sheep hemolysin (Sigma-Aldrich, St. Louis, MO). NHS or patient sera were serially diluted, and equal volumes of diluted sera were incubated with opsonized sheep E for 1 h at 37°C. Serum dilutions were examined in duplicate or triplicate, and the complete hemolysis titration curve was used to calculate complement titer (33). An NHS pool was used as a standard (absolute CH50 titer of 280; clinical laboratories at the UVA Hospital) and was normalized to a titer of 100 for each day's experiment. Data were evaluated for significance by Student's *t* test (Sigmastat; Jandel, San Rafael, CA). C2 titers of selected patient sera were determined (compared with a purified C2 standard) by measuring their ability to promote hemolysis of sensitized sheep E in the presence of C2-deficient serum (33).

RTX assays

The RTX concentration in patient sera and plasmas was determined by ELISA or flow cytometry; detailed procedures will be reported separately. In brief, the ELISA was based on capture and development of RTX with rabbit anti-mouse IgG and peroxidase-labeled goat anti-mouse IgG, respectively. Alternatively, Raji cells were incubated with RTX-containing samples or prepared standards, and then washed and developed with Al488 anti-RTX to determine RTX levels.

Western blotting

Isolated patient mononuclear cells were lysed in 1% Triton X-100 buffer on ice (34) and centrifuged at 14,000 × *g*, and the detergent-soluble fraction was subjected to SDS-PAGE on 4–15% gradient gels and transferred onto nitrocellulose. The membranes were blocked with 5% milk, incubated with a 200-fold dilution of rabbit polyclonal anti-CD20 (C terminus specific; LabVision, Fremont, CA) or mouse mAb anti-CD20 (N terminus specific; Santa Cruz Biotechnology, Santa Cruz, CA), washed, and developed using a 10,000-fold dilution of donkey anti-rabbit IgG HRP or sheep anti-mouse IgG HRP, respectively, with ECL-plus as substrate (Amersham Pharmacia Biotech). Finally, the pellets that were insoluble in 1% Triton X-100 were solubilized in 3% Triton X-100 and also examined by Western blotting.

Results

Binding of RTX to CD20$^+$ cells consumes complement at high cell burdens in vitro and in vivo

Cells from three CD20$^+$ lines were incubated with varying concentrations of RTX in 50% NHS at a concentration of 1 × 10^5 cells/μl for 1 h at 37°C. Cells were pelleted, and the complement titer of the supernatants was measured (Fig. 1). Previous investigations have demonstrated that binding of RTX to CD20$^+$ cells promotes complement activation, as demonstrated by C3b(i) capture (10, 26, 27). The present results indicate that CD20-RTX im-

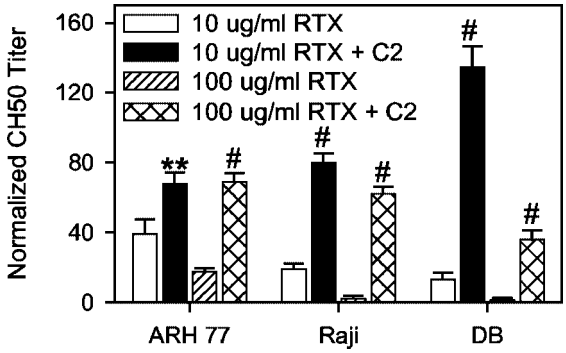

FIGURE 1. Binding of RTX to $CD20^+$ cells depletes complement at high cell densities, and addition of C2 restores complement activity. Human B cell lines were incubated in 50% NHS with RTX at 1×10^5 cells/μl for 1 h at 37°C. The supernatants ± C2 were assayed for CH50. Mock incubations without RTX, or without cells, gave no decrease in CH50 (CH50 = 100). Averages and SD of three determinations. The reduction in CH50 upon addition of RTX to sera plus cells was highly significant, $p <$ 0.001 in all cases. **, $p < 0.01$; #, $p < 0.001$ for C2 addition vs the comparable control. Representative of two similar experiments.

mune complexes formed on the cells also consume complement at cell densities that are often seen in CLL (7–9) (also, see Table I). At cell densities of 2×10^4 cells/μl, we observed moderate complement consumption (~50% reduction in CH50; data not shown). Complement component C2 is the component at lowest concentration in serum, and is likely to be the first component dissipated during complement activation (35). In fact, addition of C2 alone to these C-depleted sera led to substantial restoration of complement activity (Fig. 1).

Based on these in vitro findings, we measured the complement titer in sera of CLL patients before and after RTX treatment. In four CLL patients with circulating $CD20^+$ lymphoma cells, treatment with RTX led to profound loss of complement activity. Individual patients manifested distinct phenotypes with respect to the dynamics of complement depletion and recovery. For example, there was loss of complement activity in serum of patient 1 after the RTX infusion; thereafter, his complement levels did not fully recover until after the 4-wk cycle was completed (Fig. 2*A*). This pattern of complement depletion was repeated for patient 1 during two additional 4-wk cycles of RTX treatment. Serum of patient 9 lost complement activity after RTX infusion, but complement was almost completely restored 1 wk later (Fig. 2*B*), and similar patterns were observed for patient 33 (data not shown). Serum of patient 22 also lost complement activity after RTX infusion, but 4 days later, when RTX was no longer demonstrable in the circulation (data not shown), complement levels were restored (Fig. 2*C*).

After RTX treatment, concentrations of hemolytically active C2 for CLL patients 1, 9, and 33 averaged less than 2 μg/ml. C2 concentrations for these CLL patients before RTX treatment averaged 25 μg/ml. In analogy to our results obtained with C-depleted serum supernatants from cell lines, addition of complement component C2 to C-depleted sera substantially restores complement activity (Fig. 2, *B* and *C*). In addition, there was virtually no loss (≤33% decrease) of complement activity after RTX treatment in a total of 76 non-CLL patient sera, representing 19 patients. These results suggest that in 4 CLL patients, RTX binds rapidly to accessible $CD20^+$ target cells in the bloodstream (and perhaps in the spleen), resulting in rapid complement activation and consumption.

Reduced RTX-mediated cytotoxicity in complement component-depleted or in low complement titer patient sera is restored by addition of complement components or NHS

In view of the reduction in CH50 and depletion of C2 after RTX infusion, we examined the importance of individual complement components in killing Raji cells (Fig. 3*A*). RTX-mediated killing is modest in sera lacking single complement components; addition of the missing complement component to sera containing RTX markedly increases killing. No additional killing was observed when these components were added to sera in the absence of RTX (data not shown).

We next examined the ability of sera of CLL patients treated with RTX to kill Raji cells. Before treatment, killing by naive patient sera was modest and comparable to that of NHS (10–15%; data not shown). After RTX infusion, sera of CLL patients 1, 8, and 9 had lower cytotoxic activity compared with post-RTX sera of control patients 5, 6, and 10 (Fig. 3*B*) (NHL, low grade B cell lymphoma, low CD20 CLL, respectively). In these control post-RTX sera, there was no loss of complement activity, and a very high level of Raji cell killing was evident. These sera killed >90% of Raji cells in a 24-h incubation period, and killing was demonstrable immediately after RTX infusion (serum RTX >200 μg/ml), and 1 wk later (serum RTX >150 μg/ml), before the next infusion.

Although the levels of RTX were lower in CLL patient sera 1, 8, and 9 1 wk after infusion (13 ± 8, 91 ± 36, 3 ± 1 μg/ml, respectively), the reduced Raji cell killing potential of the sera, especially immediately after RTX infusion (>133 μg/ml average levels), might be due to reduced complement levels. To test this hypothesis, we supplemented post-RTX CLL patient sera with NHS, or with complement component C2. The results demonstrate that addition of NHS or C2 substantially enhances the efficacy of RTX-mediated killing by CLL sera (Fig. 3*C*). However, in CLL patient 8, addition of C2 was not as effective; sera of this patient had low complement titers before treatment, and although we have not identified the origin of the defect, our results suggest that it was

Table I. *Temporary sequestration of lymphocytes during RTX infusion*[a]

	Pretreatment		During 30 mg of RTX		Immediately Posttreatment	
Pat. No.	WBC/μl	% Lymphocytes	WBC/μl	% Lymphocytes	WBC/μl	% Lymphocytes
1[b]	17,300	75	4,000	37	7,400	59
1[b]	8,000	57	2,700	20	4,500	26
22	138,000	75	51,000	90	170,000	93
33[c]	25,300	82	6,800	64	18,000	67
33[c]	23,100	75	8,400	49	12,600	57

[a] Other data on these samples are presented in Fig. 7; WBC, white blood cells.
[b] Patient 1, two different treatment cycles, 3 mo apart.
[c] Patient 33, two consecutive treatments, 1 wk apart.

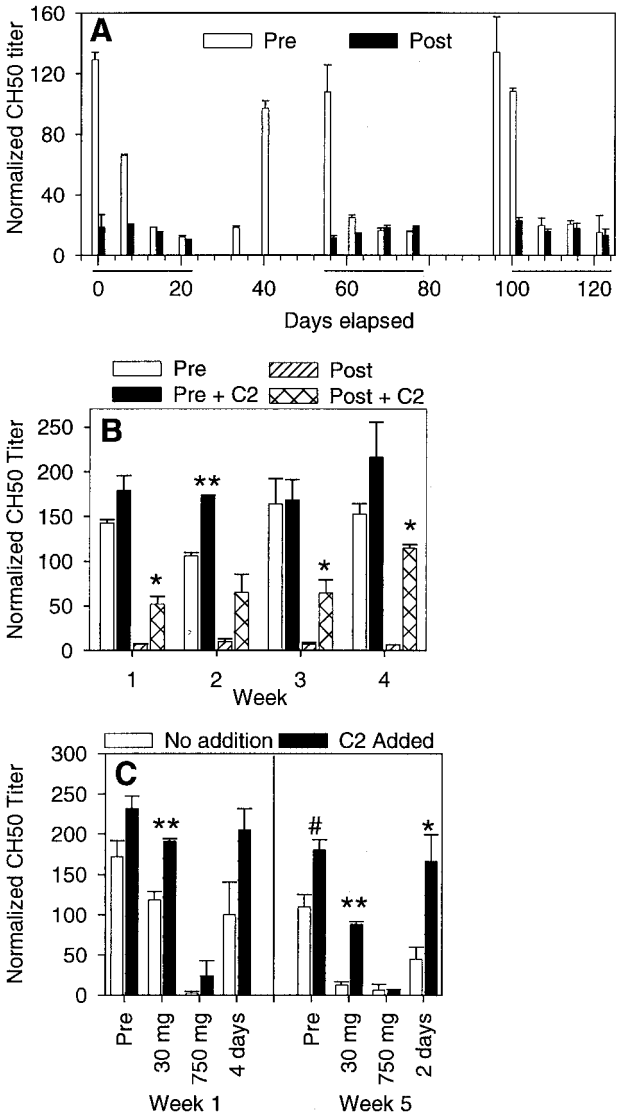

FIGURE 2. Complement is consumed in the bloodstream of CLL patients during RTX treatment. *A*, CH50 of sera from patient 1 before (Pre) and after (Post) RTX treatments. The horizontal lines denote three treatment cycles. CH50 values reported on days 34, 41, and 97 were not associated with RTX treatments. Averages and SD of three replicates. Representative of three independent determinations. *B*, CH50 of sera from patient 9 before (Pre, □) and after (Post, ▨) RTX treatment ± C2 (■ and ▨, respectively). Averages and SD of two replicates, performed in duplicate. *C*, CH50 of sera from patient 22 (□) before, during (30 mg RTX administered), after (750 mg administered), and 4 days (week 1) or 2 days (week 5) posttreatment with RTX for the first and second treatment, 5 wk later. The CH50 was also determined after supplementation with C2 (■). Averages and SD of two replicates, performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$, for C2 addition vs the comparable control. *A–C*, Compared with naive serum, the reduction in CH50 immediately after RTX infusion was highly significant ($p < 0.001$).

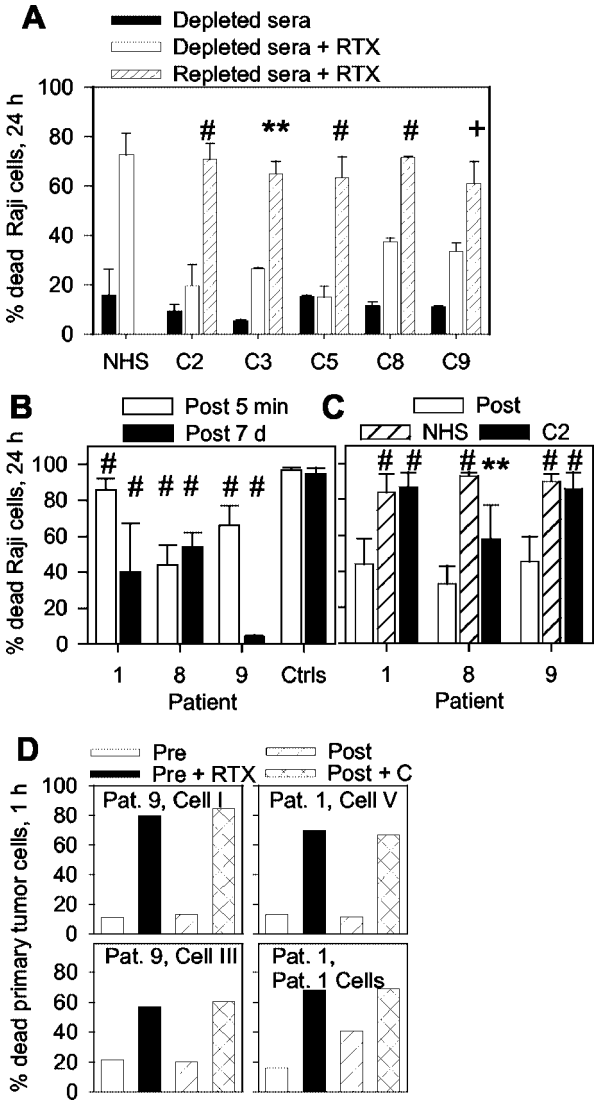

FIGURE 3. Complement promotes RTX-mediated killing of Raji cells and primary CLL cells. *A*, Raji cells were incubated in 10% sera (complement-replete NHS, or human sera depleted of the indicated complement components, ■), or 10% sera + 10 μg/ml RTX (□), or 10% depleted sera replenished with the deficient component and 10 μg/ml RTX (▨). Cell viability was determined by flow cytometry. Averages and SD of two or three independent determinations. **, $p < 0.01$; #, $p < 0.001$; +, $p = 0.056$, for depleted serum + RTX vs repleted serum + RTX. *B*, Raji cells were incubated in 33% sera from CLL patients treated with RTX immediately after infusion (□) and 7 days later (■). In *B* and *C* (below), for a given patient, between three and nine different serum samples, obtained during one or more cycles of treatment, were analyzed and averaged. Sera from control patients 5, 6, and 10 retained the ability to kill Raji cells and maintained CH50 titers 5 min and 1 wk after RTX treatment. #, $p < 0.001$ for patients 1, 8, and 9 vs controls. *C*, Raji cells were incubated in sera (16%) from patients 1 ($n = 13$), 8 ($n = 8$), and 9 ($n = 6$), taken immediately after RTX treatment (□) or supplemented with either 16% NHS (▨) or purified C2 (■). **, $p < 0.01$; #, $p < 0.001$ for additional NHS or C2 vs control. *D*, Isolated primary tumor cells from three untreated CLL patients and one previously treated with RTX were incubated for 1 h at 37°C with 16% pre- or post-TX treatment patient sera, ± RTX ± complement, respectively, and assayed for viability by flow cytometry. Pat., Patient.

not due to lack of C2. Finally, dose-response experiments indicated that supplementation with 10% NHS was sufficient to recover the maximum killing potential of C-depleted patient sera containing RTX (data not shown).

The in vitro binding of RTX to CLL cells in NHS promotes C3b(i) deposition on these cells (26), and we have observed RTX-induced complement activation in the bloodstream of CLL patients (Fig. 2). However, RTX is less effective therapeutically in CLL than in NHL (8, 10, 23), probably due to at least two factors: the cells express lower levels of CD20 and they may also up-regulate complement control proteins (8, 10, 23, 36, 37). We have found that a minority (<20%) of CLL patients seen at UVA have B cells that are killed by RTX in NHS in vitro. These cells, which tend to

express higher levels of CD20, are also killed by C-replete naive patient sera upon addition of RTX (Fig. 3*D*, Pre + RTX). Moreover, supplementation of RTX-rich, but C-depleted, sera of RTX-treated CLL patients with a complement source increases their killing of these primary CLL cells (Fig. 3*D*, Post + C).

mAb 3E7, most specific for C3b(i) covalently deposited on cell surfaces, binds avidly to RTX-opsonized cells in NHS, and can enhance RTX activity in killing cells and in suppressing growth (26). We examined the potential of this mAb to enhance RTX-mediated killing of Raji cells by sera of patients with adequate complement levels. As noted above, in the 24-h killing assay, >90% of Raji cells are killed. Therefore, to allow for clear demonstration of the effects of mAb 3E7, we report the percentage of live Raji cells at 24 h for RTX-containing serum samples examined ± mAb 3E7 (Fig. 4). In four independent experiments with several different sera, mAb 3E7 clearly enhances the action of RTX in both promoting killing and suppressing growth.

Reduced RTX-mediated C3b(i) opsonization is evident in low complement titer patient sera

The mechanism of action of RTX in vivo may include ADCC, which can be mediated by Fcγ receptors acting in concert with receptors for cell-bound C3b(i) activation products (38, 39). Therefore, in view of the reduction in complement titer observed in CLL patients' sera after RTX treatment, we investigated whether in vitro C3b(i) opsonization of the patient's own cells was compromised. We determined the efficacy of a patient's serum containing RTX to opsonize their cells, based on development with mAbs specific for C3b(i) or for RTX. Addition of RTX to complement-replete sera (either NHS, or pretreatment patient serum) leads to deposition of C3b(i) activation fragments (Fig. 5, Pre, RTX). When sera taken after RTX infusion and subsequent complement consumption are used, although RTX binds to the cells, C3b(i) opsonization is low (Fig. 5, Post). Supplementation of these sera with NHS or with the patient's own pretreatment serum (high in complement activity) fully restores deposition of C3b(i) fragments mediated by RTX (Fig. 5, Post, Pre).

Infused RTX can opsonize cells in the bloodstream

In view of the activation of complement observed after RTX infusion in CLL patients, we examined residual circulating B cells

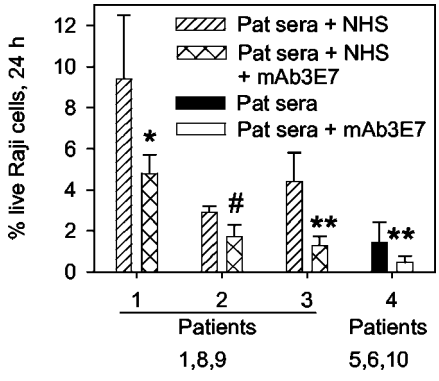

FIGURE 4. mAb 3E7 enhances RTX-mediated killing of Raji cells. Raji cells were incubated in 16% sera from CLL patients 1, 8, and 9, taken immediately after RTX treatment and supplemented with either 16% NHS (▨) or 16% NHS + 10 μg/ml mAb 3E7 (▩). Sera (16%) from patients 5, 6, and 10 were either not supplemented (■) or supplemented with 10 μg/ml mAb 3E7 only (□). In each experiment, 4–10 determinations were conducted with sera selected from patients 1, 8, and 9 (experiments 1–3) or patients 5, 6, and 10 (experiment 4). *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$ for treatment with mAb 3E7 vs the comparable control. Pat., Patient.

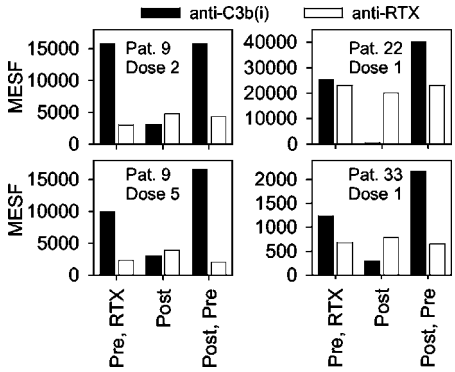

FIGURE 5. Supplementation of patient sera with complement restores RTX-mediated C3b(i) opsonization of autologous B cells. Primary tumor cells from three CLL patients, obtained immediately before RTX treatment, were opsonized for 30 min at 37°C with: autologous pretreatment serum (no RTX, normal complement) + RTX (50 μg/ml); posttreatment serum (typically >100 μg/ml RTX, but low in complement); or posttreatment serum (RTX, low complement) + pretreatment serum (no RTX, normal complement). Opsonized cells were washed and probed to reveal bound RTX and deposited C3b(i), and analyzed by flow cytometry. The values for anti-C3b(i) are divided by 10. Background binding for anti-RTX (no RTX added) averaged less than 100 MESF. Pat., Patient.

for bound RTX and/or bound C3b(i) fragments. Based on our previous in vitro studies, we expected that a fraction of these cells would contain RTX colocalized with deposited C3b(i). As infusion of RTX promotes rapid clearance of both normal and malignant B cells 24 h postinfusion (1–6, 19, 40, 41), we analyzed blood samples collected before, 5 min after completion of RTX infusion, and in several cases during RTX infusion, after 30 mg was infused.

Fluorescence microscopy analyses indicate that RTX activated complement and promoted deposition of C3b(i) on circulating cells (Fig. 6). Blood samples of patient 1, taken during and immediately after RTX infusion, had numerous examples of cells and cell aggregates with debris, portions of which showed coincident staining for RTX and C3b(i). Patient 1 was treated again with RTX 1 year later, and we again observed cellular colocalization of RTX and C3b(i). Similar patterns, indicative of uptake of RTX and C3b(i), were evident when the patient's B cells were analyzed by flow cytometry (data not shown). In the case of patient 22, intact cells had discrete sites showing colocalization of C3b(i) and RTX. No such staining was found in comparable samples from either patient before RTX infusion (data not shown).

Treatment with RTX acutely reduces CD20 on circulating cells

The CLL patients had varying, but high levels of lymphocyte counts (Table I), ranging from ~20,000 to >100,000 white blood cells/μl, of which at least 75% were lymphocytes. The other patients with B cell lymphomas had few circulating malignant cells and averaged <6,000 white blood cells/μl, and of these cells only ~20% were lymphocytes. Although in CLL patients 1, 9, 22, and 33 we generally observed reduction in lymphocyte levels after completion of RTX infusion, final cell counts were sufficiently high that we could examine CD20 levels of residual circulating B cells. In the case of patient 22, although the B cell count decreased after infusion of 30 mg of RTX, there was marked recrudescence of cell counts by the end of the 7-h infusion. Similar patterns were observed for other patient samples, although the levels of recrudescence were lower (Table I). Washed patient bloods were assayed for available CD20 based on probing with either Al488 RTX or another PE anti-CD20 mAb. The results, expressed as the

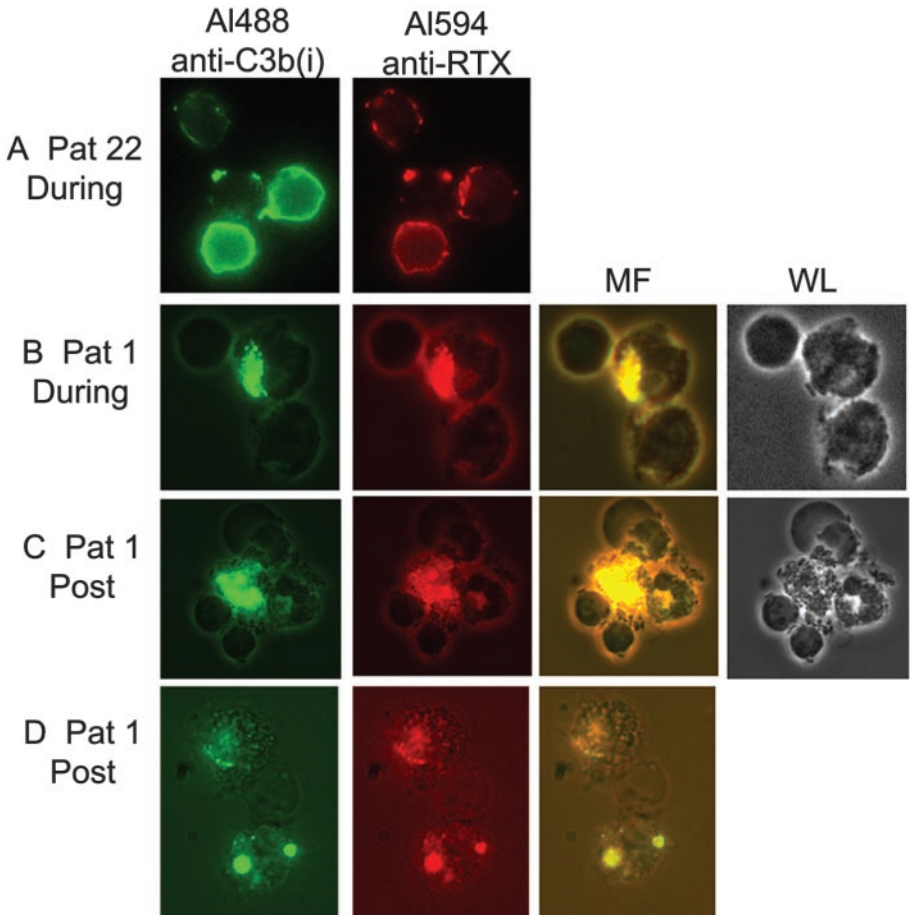

FIGURE 6. RTX and C3b(i) are colocalized on patient B cells isolated during or immediately after RTX infusion. Washed patient cells were probed with Al594 anti-RTX, and Al488 anti-C3b(i) using either mAb 1H8 (patient 22, *A*) or mAb 3E7 (patient 1, *B–D*). Patient 1 cells were also probed after the initial RTX treatment 1 year earlier (*C* and *D*). MF, mixed function (merged); WL, white light. Original objective magnification ×100. Pat., Patient.

MESF ratio for the post-RTX sample vs the pre-RTX sample, indicate that after completion of the RTX infusions, B cells remaining in the circulation have considerably reduced levels of CD20 (Fig. 7*A*, RTX and anti-CD20). Although these results could be interpreted to indicate that CD20 is simply blocked by bound RTX, additional experiments on these blood samples reveal that this is not the case: we reacted washed blood cell pellets, taken before and immediately after RTX treatment, with BSA-PBS (background), or with the patient's post-RTX sera, which typically had final RTX concentrations of >100 μg/ml. After incubation, cells were washed and probed with anti-RTX, and the results are again reported as the ratio of MESF bound to post-RTX cells divided by MESF for pre-RTX cells (Fig. 7*A*, ■). The results indicate that RTX binds well to B cells in whole blood taken before RTX infusion; however, RTX binding to post-RTX-treated samples is reduced considerably. This reduced binding cannot be explained by occupancy of CD20 sites by previously bound RTX, as the background signal for bound RTX was much lower in all cases; the levels of RTX bound to washed patient cells after RTX infusion were only slightly higher than the background levels for washed cells taken before RTX treatment. Moreover, after subtraction of this background signal, the anti-RTX signal for washed patient cells after RTX infusion averaged only 5% of the signal for pretreatment cells reacted with autologous RTX-rich serum. Thus, little residual RTX was bound to the circulating B cells after RTX infusion. Finally, the profound and acute loss of CD20 immediately after the completion of RTX infusion was confirmed by analyses of representative samples by the clinical laboratories at the UVA Hospital (Fig. 7*B*).

The reduction in CD20 levels may be explained by a process in which RTX-CD20 complexes are removed from opsonized B cells by fixed tissue macrophages (see *Discussion* below). To determine whether CD20 is simply released or internalized due to prolonged reaction with RTX, we incubated primary CLL CD20$^+$ cells in washed whole blood reconstituted in 50% autologous serum ± 25 μg/ml RTX. After 7 h at 37°C (thus modeling the patient infusion paradigm), samples incubated with RTX had modest loss of CD20; compared with controls incubated in the absence of RTX, 86 ± 28% of the signal was preserved after 7 h ($n = 6$).

Finally, Western blots performed on 1% Triton X-100 extracts of mononuclear cells taken before and after RTX treatment (patient 33) argue that internalization or steric hindrance cannot explain loss of CD20, as posttreatment samples showed a large loss of CD20 (Fig. 7*C*). When the 1% Triton X-100 pellets were solubilized with 3% Triton X-100, very little CD20 (much less than seen in Fig. 7*C*) was found, even after prolonged development (data not shown).

Discussion

Complement and RTX

Binding of RTX to B cells activates complement, and increasing evidence, based on both clinical investigations and a mouse model, indicates that complement may play an important role in the in vivo action of RTX (16, 19–28). The present findings reinforce and extend these observations to CLL. Our experiments reveal that at cell levels of $1 \times 10^5/\mu$l in 50% NHS, formation of RTX-CD20 immune complexes on several CD20$^+$ cell lines depletes complement, and this reaction consumes complement component C2, consistent with activation of the classical complement pathway by these complexes (Fig. 1). We extended this paradigm to RTX treatment of CLL patients with comparable levels of circulating

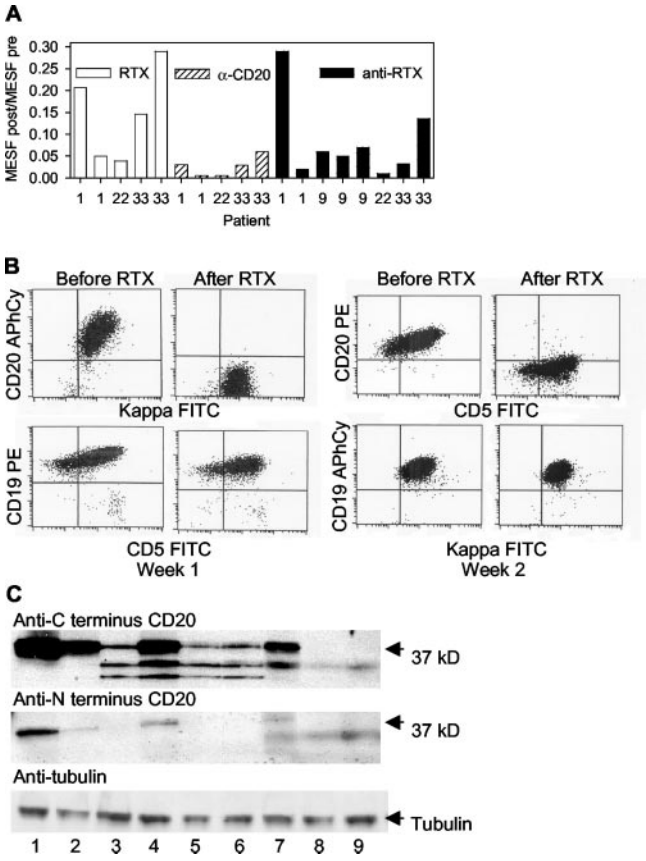

FIGURE 7. Post-RTX treatment patient B cells have substantially reduced CD20. *A*, Washed whole bloods were probed with either Al488 RTX (□) or PE anti-CD20 (▨) and PerCP CD45 and APhCy CD19. The levels of CD20 on $CD45^+$, $CD19^+$ cells are presented as the MESF signal for posttreatment cells divided by the MESF signal for pretreatment cells. Alternatively, the washed cell pellet was reconstituted in post-RTX treatment autologous patient sera as a source of RTX, incubated at 37°C for 30 min, processed as before, and probed with Al633 anti-RTX, PerCP CD45, and PE CD19 (■). Absolute values for binding to pretreatment cells by Al488 RTX ranged from 35,000 to 540,000 MESF; for PE anti-CD20, from 12,000 to 54,000 MESF. Background binding of Al633 anti-RTX to pre- or posttreatment cells averaged less than 300 MESF. After opsonization with RTX, MESF values for Al633 anti-RTX ranged from 500 to 15,000. Multiple determinations for patients 1, 9, and 33 were at different times during treatment. Blood samples for patient 9 were only obtained pre- and postinfusion. White blood cell counts (per μl) for 3 consecutive wk of treatment of patient 9 were 25,000, 22,000, and 14,000; the corresponding post-samples were 5,000, 6,000, and 3,200, respectively. *B*, Blood samples from patient 33, before and after RTX infusion, were analyzed by the clinical laboratories at the UVA hospital. The PerCP $CD45^+$ cells were κ^+, $CD5^+$, and $CD19^+$ before and after RTX treatment, but CD20 was completely lost immediately after RTX treatment. Representative of three other determinations on other patients. *C*, Immunoblots of 1% Triton X-100 cell extracts (50 μg protein/lane) from 3 consecutive wk of treatment (patient 33). Pre-RTX, *lanes 1*, *4*, and *7*; after 30 mg of RTX, *lanes 2*, *5*, and *8*; immediately post-RTX, *lanes 3*, *6*, and *9*; each set corresponds to weeks 1, 2, and 3, respectively. The bands below 35 kDa in the *top* and *middle panels* may correspond to partial digestion products of CD20. The C terminus and N terminus Western blots were performed a total of four and two times, respectively, with very similar results.

$CD20^+$ B cells ($\sim 2 \times 10^4$ to $>1 \times 10^5/\mu$l), and we find that RTX infusion promotes complement consumption and depletion of C2 (Fig. 2).

Although in one case (patient 22, week 2) complement was consumed after infusion of only 30 mg of RTX, in most cases we did not observe complement consumption until after the infusion was complete, when a total of ~700 mg had been infused. It is likely that complement consumption in the other patients would have been demonstrable before completion of the infusion, but study design limited the number of blood samples available for analysis. The amount of RTX infused should have been more than sufficient to saturate CD20 sites on the circulating cells and promote complement activation. For example, after infusion of 70 mg of RTX (~1/10 of a full infusion), the initial concentration of RTX within an intravascular volume of 4 L would be 17 μg/ml. If the cell count were $1 \times 10^5/\mu$l, this would correspond to a ratio of >600,000 RTX molecules per cell, which would be more than enough to bind to all CD20 molecules on the CLL cells, which vary from <5,000 to ~100,000 per cell (10, 23, 27, 37). Moreover, dose-response binding experiments have demonstrated that the affinity of RTX for CD20 is sufficiently high that 10 μg/ml RTX will saturate binding (24, 27, 42). Finally, we note that in the in vitro model system (Fig. 1) we observed consumption of complement at RTX concentrations of 10 and 100 μg/ml, for cell inputs of 1×10^5 cells/μl.

The entire classical complement cascade appears to be required for full cytotoxic activity of RTX against Raji cells (Fig. 3*A*), and addition of C2 to complement-depleted patient sera restores complement activity as defined by the CH50 assay. Furthermore, addition of C2, pre-RTX patient serum, or NHS to complement-depleted CLL patient sera containing RTX restores B cell-killing activity (Fig. 3, *B* and *C*). Although most of the reconstitution-killing assays were performed with Raji cells, we were able to show that addition of a complement source to complement-depleted patient sera containing RTX enhanced RTX-mediated killing of primary CLL cells (Fig. 3*D*). Therefore, we suggest that if complement is required to promote killing of RTX-opsonized cells, then use of C2, or compatible fresh frozen plasma as a complement source, may enhance the action of RTX in patients with reduced or depleted complement levels.

We used a similar approach to demonstrate that RTX-mediated in vitro deposition of C3b(i) fragments on patients' $CD20^+$ cells could be restored by supplementation of their RTX-containing serum with either NHS or the patients' complement-replete sera, taken just before RTX infusion (Fig. 5). Restoration of deposition of C3b(i) on target cells may increase the immunotherapeutic action of RTX, even when complement-mediated lysis does not occur. Several lines of evidence suggest that recognition of RTX-opsonized cells by Fcγ receptors on phagocytic cells promotes ADCC and contributes to RTX immunotherapeutic action (10, 15–18). Opsonization of IgG-containing target cells with C3b activation products enhances Fcγ receptor-mediated phagocytosis of cells by both neutrophils and monocytes (38, 39). This enhancement is based on synergistic interaction between complement and Fcγ receptors on the phagocytic cell. Czuczman et al. (43) reported, in a mouse lymphoma model, that up-regulation of CD11b (a subunit of CR3, specific for iC3b) enhanced RTX-mediated ADCC, and we suggest that deposition of C3b activation products on RTX-opsonized cells will enhance ADCC. Thus, with respect to the continuing controversy regarding the in vivo mechanism of action of RTX, it appears that complement-promoted lysis, and cellular cytoxicity, mediated by both complement receptors as well as Fcγ receptors, play important roles.

Dynamics of B cell opsonization and clearance

Previously, we reported, based on in vitro studies and a monkey model, that complement activation induced by binding of RTX to

$CD20^+$ cells promotes deposition of large numbers of C3b activation products colocalized with cell-bound RTX (26). We endeavored to replicate this finding in RTX-treated patients, by isolating and identifying RTX-opsonized cells containing bound C3b(i). In most cases, we found few RTX-opsonized cells in the circulation after RTX treatment. However, we were able to identify cells opsonized with RTX and C3b(i) in the circulation of two RTX-treated CLL patients (Fig. 6). The fact that RTX and C3b activation products were colocalized on the cells or cellular debris is consistent with our previous observations and provides additional evidence that cell-bound RTX is an important site for capture of nascent C3b in vivo (26).

A proposed mechanism for acute RTX-mediated loss of CD20 in CLL

RTX treatment led to reduction in lymphocyte counts, and the reduction in lymphocytes was demonstrable after infusion of only 30 mg of RTX (Table I). However, after RTX infusion was completed (4–7 h later), often there was an increase in lymphocyte counts (compared with the number observed after 30 mg of RTX), and at this time B cells had considerably reduced levels of CD20 (Fig. 7). These observations, taken in the context of work reported by Schreiber and Frank (44) and Griffin et al. (30), suggest a mechanism by which the RTX-opsonized cells were processed.

Studies of the clearance of IgM/C3b-opsonized ^{51}Cr-labeled E revealed that these cells are rapidly removed from the bloodstream by liver macrophages that have receptors specific for C3b or iC3b (44). However, a sizeable fraction of opsonized E is later released back into the circulation after C3b fragments bound to the cells are degraded to C3dg. Thus, there is precedence for temporary sequestration of opsonized cells, followed by proteolytic processing steps that allow cells to be released. In studying phagocytosis of IgG-opsonized lymphocytes, Griffin et al. (30) reported that capping of IgG on targeted lymphocytes could lead to removal of the cap and thus "prevent the destruction of these cells by macrophages." Binding of RTX to $CD20^+$ cells may induce rearrangement and cross-linking of CD20, and these RTX-CD20 complexes can be clustered by action of macrophage Fcγ receptors (11). Griffin et al. postulated that "phagocytic cells may clear" (Ab-opsonized) "abnormal surface determinants from these (neoplastic) cells, leaving them no longer recognizable as abnormal and thus able to proliferate within the host" (30).

We believe that the CD20-depleted B cells, which we observed in the circulation of CLL patients after RTX treatment, are lymphoma cells that had previously bound RTX and C3b(i). These cells had reduced CD20 and contained very little bound RTX (Fig. 7*A*). Western blotting experiments confirmed loss of CD20 (Fig. 7*C*), and our findings and other reports indicate it is unlikely the cells spontaneously internalized and/or shed CD20 after ligation by RTX (1, 11, 45). Thus, our results suggest that these cells were temporarily sequestered by phagocytic cells in the liver and/or spleen, where the complexes of CD20, RTX, and C3b(i) were removed, thus allowing the CD20-depleted lymphocytes to return to the circulation. Foran et al. (46) reported the case of a mantle cell lymphoma patient who died of splenic rupture following RTX treatment. The patient had a high burden of circulating cells, and these cells lost CD20 after RTX treatment. We suggest that the mechanism we have proposed may have led to acute CD20 loss in this patient.

Studies in monkey models and more recent reports in humans suggest that normal B cells are rapidly depleted from the circulation upon treatment with RTX (19, 26, 40, 41, 47). We are unaware of any reports suggesting loss of CD20 from normal cells and/or recrudescence of the cells after RTX treatment, and the reason may simply be that the cell burden is considerably lower than that found in CLL.

Therapeutic implications

It is generally believed that one of the reasons for the efficacy of RTX as an immunotherapeutic agent is its stable binding to CD20 on B cells with little internalization or release (1, 11, 45). Although the mechanism of action of RTX is still under investigation, its Fc region is required to promote complement activation and/or ADCC in the immunotherapy of NHL (19). Our experiments suggest that in CLL, processing of RTX-opsonized cells in the circulation allows for more complex mechanisms to operate. In particular, removal of RTX-CD20 complexes by the MPS may allow CLL cells to escape and nullify the RTX immunotherapeutic potential. Use of RTX as a single agent has been less effective in CLL than in the lymphomas (8, 10, 23), and this escape mechanism may be an important underlying factor. Use of other anti-CD20 mAbs, which can kill B cells as $F(ab')_2$, may provide an approach for targeting circulating $CD20^+$ cells in CLL (42). Alternatively, it should be possible to engineer RTX to activate complement, but not bind to Fcγ receptors (48). Although such an engineered molecule may not be appropriate for the treatment of NHL, it may have therapeutic efficacy in CLL.

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References

1. McLaughlin, P., A. J. Grillo-Lopez, B. K. Link, R. Levy, M. S. Czuczman, M. E. Williams, M. R. Heyman, I. Bence-Bruckler, C. A. White, F. Cabanillas, et al. 1998. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J. Clin. Oncol. 16:2825*.
2. Grillo-Lopez, A., C. White, C. Varns, A. Wei, A. McClure, and B. Dallaire. 1999. Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. *Semin. Oncol. 26:66*.
3. Czuczman, M. S., A. Grillo-Lopez, W. Saleh, L. Gordon, A. LoBuglio, D. Klippenstein, B. Dallaire, and C. Varns. 1999. Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD20 monoclonal antibody and CHOP chemotherapy. *J. Clin. Oncol. 17:268*.
4. Maloney, D. G. 1999. Preclinical and phase I and II trials of rituximab. *Semin. Oncol. 26:74*.
5. Colombat, P., G. Salles, N. Brousse, P. Effekhari, P. Soubeyran, V. Delwail, E. Deconinck, C. Hajoun, C. Foussard, A. Stamatoullas, et al. 2001. Rituximab (anti-CD20 monoclonal antibody) as single first-line therapy for patients with follicular lymphoma with a low tumor burden: clinical and molecular evaluation. *Blood 97:101*.
6. Hainsworth, J. D., S. Litchy, H. A. Burris, D. C. Scullin, S. W. Corso, D. A. Yardley, L. Morrissey, and F. A. Greco. 2002. Rituximab as first-line and maintenance therapy for patients with indolent non-Hodgkin's lymphoma. *J. Clin. Oncol. 20:4261*.
7. Byrd, J., T. Murphy, R. Howard, M. Lucas, A. Goodrich, K. Park, M. Pearson, J. Waselenko, G. Ling, M. Grever, et al. 2001. Rituximab using a thrice weekly dosing schedule in B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma demonstrates clinical activity and acceptable toxicity. *J. Clin. Oncol. 19:2153*.
8. Huhn, D., C. von Schilling, M. Wilhelm, A. Ho, M. Hallek, R. Kuse, W. Knauf, U. Riedel, A. Hinke, S. Srock, et al. 2001. Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. *Blood 98:1326*.
9. O'Brien, S., H. Kantarjian, D. Thomas, F. Giles, E. Freireich, J. Cortes, S. Lerner, and M. Keating. 2001. Rituximab dose-escalation trial in chronic lymphocytic leukemia. *J. Clin. Oncol. 19:2165*.
10. Golay, J., M. Lazzari, V. Facchinetti, S. Bernasconi, G. Borleri, T. Barbui, A. Rambaldi, and M. Introna. 2001. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood 98:3383*.
11. Gopal, A. K., and O. W. Press. 1999. Clinical applications of anti-CD20 antibodies. *J. Lab. Clin. Med. 134:445*.
12. Byrd, J. C., S. Kitada, I. W. Flinn, J. L. Aron, M. Pearson, D. Lucas, and J. C. Reed. 2002. The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood 99:1038*.
13. Deans, J. P., H. Li, and M. J. Polyak. 2002. CD20-mediated apoptosis: signalling through lipid rafts. *Immunology 107:176*.

14. Bannerji, R., S. Kitada, I. W. Flinn, M. Pearson, D. Young, J. C. Reed, and J. C. Byrd. 2003. Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to in vivo rituximab resistance. *J. Clin. Oncol. 21:1466*.
15. Weng, W. K., and R. Levy. 2003. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J. Clin. Oncol. 21:3940*.
16. Stockmeyer, B., M. Dechant, M. van Egmond, A. L. Tutt, K. Sundarapandiyan, R. F. Graziano, R. Repp, J. R. Kalden, M. Gramatzki, M. J. Glennie, et al. 2000. Triggering Fcα-receptor I (CD89) recruits neutrophils as effector cells for CD20-directed antibody therapy. *J. Immunol. 165:5954*.
17. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med. 6:443*.
18. Cartron, G., L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat, and H. Watier. 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIa gene. *Blood 99:754*.
19. Reff, M. E., K. Carner, K. S. Chambers, P. C. Chinn, J. E. Leonard, R. Raab, R. A. Newman, N. Hanna, and D. R. Anderson. 1994. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood 83:435*.
20. Flieger, D., S. Renoth, I. Beier, T. Sauerbrunch, and I. Schmidt-Wolf. 2000. Mechanism of cytotoxicity induced by chimeric mouse human monoclonal antibody IDEC-C2B8 in CD20-expressing lymphoma cell lines. *Cell. Immunol. 204:55*.
21. Golay, J., L. Zaffaroni, T. Vaccari, M. Lazari, G. Borleri, S. Bernasconi, F. Tedesco, A. Rambaldi, and M. Introna. 2000. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement mediated cell lysis. *Blood 95:3900*.
22. Harjunpää, A., S. Junnikkala, and S. Meri. 2000. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. *Scand. J. Immunol. 51:634*.
23. Bellosillo, B., N. Villamor, A. Lopez-Guillermo, S. Marce, J. Esteve, E. Campo, D. Colomer, and E. Montserrat. 2001. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood 98:2771*.
24. Golay, J., R. Gramigna, V. Facchinetti, D. Capello, G. Gaidano, and M. Introna. 2002. Acquired immunodeficiency syndrome-associated lymphomas are efficiently lysed through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity by rituximab. *Br. J. Haematol. 119:923*.
25. Manches, O., G. Lui, L. Chaperot, R. Gressin, J. P. Molens, M. C. Jacob, J. J. Sotto, D. Leroux, J. C. Bensa, and J. Plumas. 2002. In vitro mechanisms of action of rituximab on primary non-Hodgkin's lymphomas. *Blood 101:949*.
26. Kennedy, A. D., M. D. Solga, T. A. Schuman, A. W. Chi, M. A. Lindorfer, W. M. Sutherland, P. L. Foley, and R. P. Taylor. 2003. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of $CD20^+$ cells by rituximab. *Blood 101:1071*.
27. Cragg, M. S., S. M. Morgan, H. T. C. Chan, B. P. Morgan, A. V. Filatov, P. W. M. Johnson, R. R. French, and M. J. Glennie. 2003. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid "rafts." *Blood 101:1045*.
28. Gaetno, N. D., E. Cittera, R. Nota, A. Vecchi, V. Grieco, E. Scanziani, M. Botto, M. Introna, and J. Golay. 2003. Complement activation determines the therapeutic activity of rituximab in vivo. *J. Immunol. 171:1581*.
29. Vivanco, F., E. Munoz, L. Vidarte, and C. Pastor. 1999. The covalent interaction of C3 with IgG immune complexes. *Mol. Immunol. 36:843*.
30. Griffin, F. M., J. A. Griffin, and S. C. Silverstein. 1976. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. *J. Exp. Med. 144:788*.
31. Kuhn, S. E., A. Nardin, P. E. Klebba, and R. P. Taylor. 1998. *E. coli* bound to the primate erythrocyte complement receptor via bispecific monoclonal antibodies are transferred to and phagocytosed by human monocytes in an in vitro model. *J. Immunol. 160:5088*.
32. Lindorfer, M. A., H. B. Jinivizian, P. L. Foley, A. D. Kennedy, M. D. Solga, and R. P. Taylor. 2003. The B cell complement receptor 2 transfer reaction. *J. Immunol. 170:3671*.
33. Whaley, K., and J. North. 1997. Haemolytic assays for whole complement activity and individual components. In *Complement: A Practical Approach*. A. W. Dodds and R. B. Sim. IRL Press at Oxford University Press, Oxford, p. 19.
34. Deans, J. P., S. M. Robbins, M. J. Polyak, and J. A. Savage. 1998. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. *J. Biol. Chem. 273:344*.
35. Morley, B. J., and M. J. Walport. 2000. *The Complement Facts Book*. B. J. Morley and M. J. Walport, eds. Academic Press, San Diego.
36. Almasri, N. M., R. E. Duque, J. Iturraspe, E. Everett, and R. C. Braylan. 1992. Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukemia. *Am. J. Hematol. 40:259*.
37. Ginaldi, L., M. De Martinis, E. Matutes, N. Farahat, R. Morilla, and D. Catovsky. 1998. Levels of expression of CD19 and CD20 in chronic B cell leukemias. *J. Clin. Pathol. 51:364*.
38. Ehlenberger, A. G., and V. Nussenzweig. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. *J. Exp. Med. 145:357*.
39. Fries, L. F., S. A. Siwik, A. Malbran, and M. M. Frank. 1987. Phagocytosis of target particles bearing C3b-IgG covalent complexes by human monocytes and polymorphonuclear leukocytes. *Immunology 62:45*.
40. Schroder, C., A. M. Azimzadeh, G. Wu, J. O. Price, J. B. Atkinson, and R. N. Pierson. 2003. Anti-CD20 treatment depletes B-cells in blood and lymphatic tissue of cynomolgus monkeys. *Transplant Immunol. 12:19*.
41. Sawada, T., S. Fuchinoue, and S. Teraoka. 2002. Successful A1-to-O ABO-incompatible kidney transplantation after a preconditioning regimen consisting of anti-CD20 monoclonal antibody infusions, splenectomy, and double-filtration plasmapheresis. *Transplantation 74:1207*.
42. Cardarelli, P. M., M. Quinn, D. Buckman, Y. Fang, D. Colcher, D. King, C. Bebbington, and G. Yarranton. 2002. Binding to CD20 by anti-B1 antibody or $F(ab')_2$ is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol. Immunother. 51:15*.
43. Czuczman, M. S., S. Reising, R. Repp, and F. J. Hernandez-Ilizaliturri. 2003. Concurrent administration of G-CSF or GM-CSF enhances rituximab's biological activity and up-regulates CD11b in a severe combined immunodeficiency SCID mouse lymphoma model. *Blood 100:157a*.
44. Schreiber, A. D., and M. M. Frank. 1972. Role of antibody and complement in the immune clearance and destruction of erythrocytes: in vivo effects of IgG and IgM complement fixing sites. *J. Clin. Invest. 51:575*.
45. Johnson, P., and M. Glennie. 2003. The mechanisms of action of rituximab in the elimination of tumor cells. *Semin. Oncol. 30:3*.
46. Foran, J. M., A. J. Norton, I. N. M. Micallef, D. C. Taussig, J. A. L. Amess, A. Z. S. Rohatiner, and T. A. Lister. 2001. Loss of CD20 expression following treatment with rituximab (chimeric monoclonal anti-CD20): a retrospective cohort analysis. *Br. J. Haematol. 114:881*.
47. Weide, R., J. Heymanns, A. Pandorf, and H. Koppler. 2003. Successful long-term treatment of systemic lupus erythematosus with rituximab maintenance therapy. *Lupus 12:779*.
48. Idusogie, E. E., P. Y. Wong, L. G. Presta, H. Gazzano-Santoro, K. Totpal, M. Ultsch, and M. G. Mulkerrin. 2001. Engineered antibodies with increased activity to recruit complement. *J. Immunol. 166:2571*.