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Induced Resistance to Ofatumumab-Mediated Cell Clearance Mechanisms, Including Complement-Dependent Cytotoxicity, in Chronic Lymphocytic Leukemia

Nisar A. Baig,* Ronald P. Taylor,† Margaret A. Lindorfer,† Amy K. Church,* Betsy R. LaPlant,‡ Adam M. Pettinger,‡ Tait D. Shanafelt,* Grzegorz S. Nowakowski,* and Clive S. Zent*

Ofatumumab (OFA), a human CD20-targeting mAb, kills B lymphocytes using the innate immune system including complement-dependent cytotoxicity (CDC). The efficacy of OFA in patients with chronic lymphocytic leukemia (CLL) is limited by drug resistance, which is not well characterized. To better understand mechanisms of resistance, we prospectively studied CLL cells isolated from blood samples collected before and after in vivo exposure to the initial dose of OFA therapy in 25 patients undergoing their first treatment for progressive CLL. As previously reported, OFA therapy rapidly decreased the absolute lymphocyte count, CD20 expression by CLL cells, and serum complement levels. We now show that after administration of the first dose of OFA, there was a modest rebound in the absolute lymphocyte count and serum complement levels, but substantial ongoing loss of CD20 expression by CLL cells. These post-OFA treatment CLL cells were highly resistant to OFA-mediated CDC but retained sensitivity to alemtuzumab-mediated CDC in vitro. Posttherapy serum OFA levels correlated inversely with both the amount of pretreatment circulating cell-bound CD20 and with the decrease in this value following treatment. In vitro OFA-mediated CDC did not predict clinical responses, and the patients with first-dose reactions to OFA did not have markers of increased complement activation in vivo. We propose that optimal efficacy of CD20-targeted therapy for CLL requires determining an mAb dose size and frequency that optimizes CLL killing without exceeding the cytotoxic mechanisms and thus minimizes loss of CD20 expression in the surviving CLL cells. The Journal of Immunology, 2014, 192: 1620–1629.

C hemoimmunotherapy combining the type I anti-CD20 mAb rituximab (RTX), purine analogs, and alkylating agents has markedly improved treatment responses and overall survival for patients with chronic lymphocytic leukemia (CLL) (1–3). However, the mechanisms by which RTX improves treatment efficacy, and the reasons why this therapy is noncurative, remain obscure. Unconjugated mAb can mediate cytotoxicity of CLL cells using effector functions of the innate immune system. These include complement-dependent cytotoxicity (CDC), Ab-dependent cellular cytotoxicity, and phagocytosis (4). In CLL, CDC is an important mechanism of action for alemtuzumab (ALM), a humanized rat anti-CD52 mAb that is particularly effective at eliminating circulating blood CLL cells, which have high expression of CD52 (5, 6). In contrast, RTX is considerably less effective at promoting CDC of CLL cells, which have lower cell membrane CD20 expression. The modest CDC mediated by RTX has led to a search for more effective anti-CD20 targeting Abs.

Ofatumumab (OFA) is a human anti-CD20 mAb that is Food and Drug Administration–approved for the treatment of fludarabine- and ALM-resistant CLL. OFA binds to a CD20 epitope that is different from the RTX-binding site and is closer to the cell membrane, thereby allowing for more effective complement activation (7, 8). Indeed, in vitro studies have shown that OFA is substantially better than RTX at inducing CDC of CLL cells (9–11). Monotherapy of CLL with OFA resulted in clinical responses in patients who were refractory to purine analogs and ALM (12), including those who were previously treated with RTX (13). However, there are no published reports of clinical trials directly comparing OFA and RTX therapy either as monotherapy or in combination therapy for CLL. Treatment of CLL with OFA monotherapy rarely achieves complete remissions (12, 14). Phase II clinical trials using OFA with purine analogs and cyclophosphamide have reported high overall and complete response (CR) rates but are not curative (15, 16). The mechanisms of resistance of CLL cells to OFA are also not well defined.

Resistance of CLL cells to the cytotoxic effects of type I anti-CD20 Abs is likely to be multifactorial. The characteristic low levels of CD20 expression of CLL cells could result in lower levels of mAb binding compared with binding obtained in most other B cell malignancies and with normal B cells. Moreover, after initiation of therapy with RTX or OFA, there is substantial and rapid loss of expression of CD20 on CLL cells by trogocytosis and, to a lesser extent, by B cell internalization (11, 17–23). Several studies have demonstrated that trogocytosis is mediated by acceptor
cells, such as monocytes and macrophages, which express FcγRs. During this process, both the B cell–bound mAb as well as CD20 are removed from the B cells and taken up and internalized by the acceptor cells. This additional loss of CD20 expression could further decrease the efficacy of CD20 targeting mAb. CLL cells treated with complement-activating mAb have also been shown to include subpopulations that are resistant to activated complement despite the generation of membrane attack complexes (24). Possible additional reasons for failure of CDC include depletion of circulating complement, high levels of expression of complement control proteins on the targeted cells, and inadequate concentrations of mAb. Efforts to improve the treatment of CLL patients with type I anti-CD20 mAb will require a better understanding of the relative contributions of each of these potential mechanisms of resistance to mAb-mediated killing of targeted cells.

To determine the mechanisms of resistance of circulating CLL cells to OFA-mediated CDC, we examined CLL cells isolated from blood samples collected before and after the initial dose of OFA therapy in patients undergoing their first treatment for progressive CLL. We hypothesized that OFA would fail to clear all the circulating CLL cells and that the mechanisms of this resistance to therapy could include loss of expression of CD20, saturation of the cytotoxic capacity of the effector mechanisms, and intrinsic resistance of some CLL cells to CDC. The results of this study support these hypotheses. We observed marked decreases in expression of CD20 on cells collected after the first dose of OFA. These surviving CLL cells have evidence of nonlethal complement activation and are resistant to in vitro OFA-mediated CDC, but most retain full sensitivity to ALM-mediated CDC. The first dose of OFA also resulted in a rapid, profound, and sustained decrease in serum complement levels.

Materials and Methods

Patients

The study was conducted at Mayo Clinic Rochester with Institutional Review Board approval according to the guidelines of the Declaration of Helsinki. Patients with previously untreated progressive CLL (25, 26) received pentostatin, cyclophosphamide, and OFA in a phase II clinical trial (MC0983, ClinicalTrials.gov, NCT01024010) (16). The plan was to treat all patients with six cycles of therapy. On day 1 of the first cycle, patients were scheduled to receive a 300-mg infusion of OFA over ∼8 h. This was followed on the same day with the first doses of pentostatin and cyclophosphamide. The following day, patients received a 1000-mg infusion of OFA. CLL prognostic markers and responses to treatment were evaluated using published methods (25, 27–30) (Table I). We studied the first 25 patients treated at Mayo Clinic Rochester on this clinical trial who had all scheduled blood specimens collected and adequate CLL cells for analysis at each time point (absolute lymphocyte count ≥6 × 10^9/l).

Specimen collection

Peripheral blood specimens were collected immediately before starting the first 300-mg dose of i.v. OFA (0 h), immediately after the completion of the OFA infusion (8 h), and at 24 h after the initiation of the first infusion of OFA just before the start of administration of the second dose of OFA (24 h). PBMCs were isolated using Ficoll and the percentage of CD5+CD19+ CLL cells determined by flow cytometry as previously described (24). In 66 of 75 samples, the percentage of CLL cells (CD5+CD19+) was ≥90%, and these specimens were analyzed without any further manipulation. The nine samples with <90% CLL cells were purified to ≥90% before being studied.

Table I. Patient characteristics

<table>
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<th>Characteristic</th>
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<tr>
<td>Stage (modified Rai)</td>
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<tr>
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<td>1 (4)</td>
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</table>

Data are N (%) unless otherwise noted.

^aDohner classification (27).

^bDose administered: 100 mg (n = 1), 120 mg (n = 1), 150 mg (n = 4), 175 mg (n = 2).

^cInternational Workshop on Chronic Lymphocytic Leukemia—modified National Cancer Institute criteria (25).

FISH, Fluorescence in situ hybridization; IGHV, Ig H chain V region.

Samples with >90% cell viability were incubated for 4 h under sterile conditions at 3 × 10^9 cells/ml in AIM-V medium at 37 °C with 95% humidity and 5% CO₂. Cells were then checked for viability by flow cytometry using Annexin V and propidium iodide (PI; BD Biosciences, San Jose, CA) as previously described (5) to ensure viability ≥85% before being studied.

Serum hemolytic complement assay and OFA concentration

Hemolytic complement assays (CH50) were performed as previously described (11). A flow cytometric method was used to quantitate immunologically active OFA in patient sera as previously described for RTX, with minor modifications (31). OFA standards were prepared by serial dilutions of a commercial sample of OFA (GlaxoSmithKline, Research Triangle Park, NC) into pooled normal human serum (NHS). Patient sera and OFA standards were diluted 400-fold with BSA/PBS and then incubated with Daudi cells; the cells were washed and then secondarily probed with Alexa 488 (Life Technologies, Grand Island, NY) mAb HB43 (anti-human Fc) for bound OFA.

CD20 and CD52 expression

CLL cells were treated with either a mouse anti-human CD20-FITC Ab (2H7; BD Biosciences) or mouse anti-human CD52-FITC Ab (HI186; Pierce Abs, Thermo Fisher Scientific, Rockford, IL). Target Ag expression was quantified by flow cytometry using molecules of equivalent soluble fluorochrome (MESF) with the Quantum FITC-5 MESF Premix kit (Bangs Laboratories, Fishers, IN) and QuickCal v2.3 software units as previously described (32). The change (δ) in MESF (dMESF) was calculated by subtracting the baseline control value (IgG2b-FITC; BD Biosciences) from that obtained after the experimental intervention.

Measurement of mAb binding to CLL cells

We used flow cytometry and the anti-human Fc FITC-conjugated mAb HB43 to measure the binding of OFA to CLL cells in vivo and binding of
were performed as previously described (5, 24). In brief, multiplication by 100.

each sample by dividing the absolute viable (PI-negative) counts of the measures the sum of lysed cells and intact dead cells, was determined for the control specimen (10% NHS). Total CDC (percent cytotoxicity), which detected by flow cytometry) or remain intact with PI-permeable mem-

branes (intact dead cells). Cell lysis was determined for each sample by dividing the absolute cell count of the experimental specimens by that of the control specimen (10% NHS). Total CDC (percent cytotoxicity), which measures the sum of lysed cells and intact dead cells, was determined for each sample by dividing the absolute viable (PI-negative) counts of the experimental specimens by that of the control specimen followed by multiplication by 100.

Measurement of complement activation

We used flow cytometry to measure covalent deposition of C3 fragments on CLL cells (complement activation). C3 fragment deposition was measured with the anti-C3b/iC3b/C3d FITC-conjugated mAb 1H8 (34). As noted above, CDC can cause complete disintegration of target cells (cell lysis), which is most likely to occur in the cells with the highest levels of complement activation. Therefore, to ensure that our studies measured C3 activation in the entire CLL population in vitro, these measurements were also conducted using C5-depleted serum in which complement activation does not extend to formation of the membrane attack complex responsible for cell lysis, but C3 fragment deposition occurs freely. For all of our experiments, samples were incubated with the complement-specific Abs in the presence of 2 mg/ml purified mouse IgG (Lampire Biological Laboratories, Pipersville, PA) to minimize nonspecific binding. Quantitation of binding was measured as median dMESF compared with the specimens not reacted with mAbs but simply treated with 10% NHS or 10% C5-depleted serum only as appropriate.

Measurement of expression of complement regulatory proteins

The expression of CD59 and CD55 was measured by flow cytometry using anti-CD59 FITC (BD Biosciences) and the anti-human CD55 FITC-conjugated HD1A Ab (35).

Statistical analysis

The Wilcoxon signed-rank test was used to evaluate the relationship between paired values. Continuous measures were compared between groups using the Wilcoxon rank sum test. Relationships between continuous variables were evaluated using Spearman rank correlation coefficient. All tests were two-sided, and statistical significance was defined as p < 0.05.

Results

Patient characteristics and response to therapy with pentostatin, cyclophosphamide, and OFA are summarized in Table I. With a median follow-up time of 27.6 mo, the median progression-free survival, time to next treatment, and overall survival have not been reached. The eight patients who had a first-dose reaction to OFA that limited the dose of their first infusion had a similar rate of CR/CR with incomplete recovery of blood counts (CRi) compared with the 17 patients who received the full dose of 300 mg (38 versus 29%; p = 1.0).

OFA therapy substantially decreases the absolute lymphocyte count and serum complement levels

The first dose of OFA resulted in a 45% decrease in the median absolute lymphocyte count from 129.4 × 10^9/l to 68.5 × 10^9/l (p < 0.0001) at 8 h (Fig. 1A, 1B). The median absolute lymphocyte count then increased between 8 and 24 h to 87.0 × 10^9/l (p = 0.0002) despite interim administration of pentostatin and cyclophosphamide. Administration of OFA was associated with an 86% decrease in the median serum complement level from a baseline CH50 of 96 to 13.8% (p < 0.0001) at 8 h (Fig. 1C). The median CH50 then increased modestly to 25.3% (p = 0.05) between 8 and 24 h. Because levels of complement <5% can be limiting for CDC at even low cell burdens in vitro (6), we determined the number of patients whose complement level was <5% of baseline after the OFA infusion. The 8-h CH50 was <5% of baseline in five patients but was >5% of baseline in all patients at 24 h.

Therapeutic OFA levels are achieved in all patients

OFA levels were >5 µg/ml, a level that has previously been shown to saturate CD20 binding in vitro (36) in all patients at 8 h (median 33.7 µg/ml) and in 22 (88%) patients at 24 h (median 28.8 µg/ml) (Fig. 1D). Median OFA levels in patients who received the 300-mg OFA dose (n = 17) (Fig. 1D, closed circles) were higher than those who received lower OFA doses (n = 8) (Fig. 1D, open circles) at either 8 h (48.5 versus 18.2 µg/ml; p = 0.048) or 24 h (28.8 versus 19.1 µg/ml; p = 0.16). Two of the eight patients who received a lower dose of OFA had OFA levels at 24 h that were <5 µg/ml (175-mg dose with level of 0.5 µg/ml and 150-mg dose with level of 0.8 µg/ml). Among patients receiving the full 300-mg dose of OFA, we tested for correlations between OFA levels and several parameters, including the pretreatment absolute lymphocyte count, change in absolute lymphocyte count, total cell-associated CD20 in the circulation, trough complement levels and change in complement levels, and clinical outcome. Total cell-associated CD20 in the circulation was estimated using the product of the absolute lymphocyte count and CD20 expression.

These correlations showed that 8-h OFA levels were inversely correlated with pretreatment total cell-associated CD20 (r = −0.64; p = 0.0054) (Fig. 2A) and with the change in the total cell-associated CD20 between the 0- and 8-h specimens (r = −0.70; p = 0.002) (Fig. 2B). This finding suggests that the amount of available CD20 target on circulating cells could determine the amount of OFA consumption and thus the posttreatment serum OFA levels. OFA levels did not correlate with clinical stage, pretreatment absolute lymphocyte count, decreases in absolute lymphocyte count, serum complement levels after treatment, or clinical response to therapy.

OFA therapy decreases CD20 expression on circulating CLL cells

Pretreatment (0 h) circulating CLL cells were tested for CD20 expression by both reaction with a mouse anti-CD20-FITC Ab and reaction with 10 µg/ml of OFA followed by secondary development with an anti-human Fc FITC Ab HB43. These assays resulted in similar results (median dMESF 10968 versus 9918; r = 0.97; p < 0.0001). In contrast, as shown in Fig. 3A, circulating CLL cells collected from patients after their first dose of OFA (and then probed with FITC mAb HB43) had substantially lower amounts of bound OFA with a median dMESF of 2077 at 8 h (bound in vivo 8 h; p < 0.0001) and 258 at 24 h (bound in vivo 24 h; p < 0.0001). The OFA concentrations in the bloodstream...
should have been adequate to saturate CD20 on the cells. However, to ensure saturation of all available CD20 sites, we then reacted the CLL cell samples with 10 mg/ml of OFA in vitro and retested for OFA binding by probing with FITC mAb HB43. Compared to the 0-h sample, the in vitro binding of OFA to the CLL cells (maximum binding) from the 8-h sample was 69% lower (median dMESF, 3113; p = 0.0001) and then rebounded slightly to 20% of baseline at 24 h (p = 0.05). Median OFA levels were 33.7 μg/ml at 8 h and 28.8 μg/ml at 24 h. These scatter plots show the ALC (B), CH50 (C), and OFA (D) values for each patient. Solid circles represent patients receiving a 300-mg first dose of OFA on day 1, and open circles represent those patients who received <300 mg of OFA. The bars represent the median of the values.

To determine if the size of the first dose of OFA influenced subsequent in vitro OFA binding to CLL cells, we compared median OFA dMESF in patients who received 300 mg (n = 17, closed circles) to those who received a lower dose (n = 8, open circles) (Fig. 3A). There were no significant differences between in vitro OFA binding of CLL cells sampled before initiation of therapy (maximum binding) from patients who received full or reduced doses of OFA. This finding suggests that pretherapy levels of CD20 expression did not influence the risk of a first-dose effect from OFA that limited the size of the clinically tolerable initial dose.
dose of this drug. To determine if the size of the OFA doses used in this study correlated with subsequent decreases in CD20 expression by circulating CLL cells, we compared the in vitro binding of OFA in both the 8- (maximum binding 8 h) and 24-h samples (maximum binding 24 h). We found that within the dose range administered in this clinical trial, lower doses of OFA did not limit the loss of expression of CD20 and suggest that all of the doses administered resulted in substantial loss of expression of CD20 in vivo.

Circulating CLL cells obtained from patients after i.v. OFA therapy have modest levels of membrane-bound activated complement fragments, and these cells also have a decreased ability to activate complement after OFA treatment in vitro

Complement fragments bound to the membranes of circulating CLL cells were measured on cells of patients before and after the first dose of OFA (Fig. 3B). Pretreatment CLL cells incubated in vitro with 10 μg/ml OFA and 10% C5-depleted serum (maximum binding 0 h) had a median activated complement C3 dMESF of 40,667 (based on probing with FITC mAb 1H8). In contrast, CLL cells collected at 8 h after starting OFA therapy had considerably lower levels of activated complement C3 (bound in vivo 8 h) with a median dMESF of 7150 (p < 0.0001), which decreased further at 24 h (bound in vivo 24 h; median dMESF, 2476) (p < 0.0001). To measure maximum possible C3 fragment deposition, we reacted the CLL cells ex vivo with 10 μg/ml of OFA and 10% C5-depleted serum. This only modestly increased the median dMESF of the 8- and 24-h CLL samples (maximum binding) to 8027 and 5325, respectively. Of note, these values were considerably lower than those achieved in treatment of CLL cells not exposed to OFA in vivo (40,667; p < 0.0001). Thus, CLL cells in the peripheral blood of patients treated with OFA sampled at either 8 or 24 h after starting therapy have low levels of activated complement C3 fragment deposition and a limited capacity for additional complement activation by OFA in vitro.

To evaluate the relationship between the size of the first dose of OFA and complement activation, we compared activated complement C3 fragment deposition in patients treated with standard (solid circles) versus lower doses of OFA (open circles) as shown in Fig. 3B. There were no significant differences in C3 fragment deposition based on the size of the initial dose of OFA. These data suggest that circulating CLL cells from patients unable to tolerate the full initial dose of OFA because of a first-dose reaction do not have significantly increased complement activation.

The effects of decreased CD20 expression on complement activation and CDC

We conducted in vitro titration experiments to determine the OFA concentration resulting in binding equivalent to that of the circulating CLL cells in patients 8 h after the initiation of OFA therapy (median dMESF 2077). In pretreatment CLL specimens from five study patients, this OFA concentration was determined to be ∼0.025 μg/ml (dMESF median 2471; data not shown). These naive CLL cells were then incubated in 10% NHS with OFA concentrations of 0.025, 0.050, or 0.100 μg/ml to determine the relationship among OFA concentration, C3 fragment deposition, and CDC. Results of these experiments are reported as the percentage of those achieved using a saturating concentration (10 μg/ml) of OFA in the same CLL cells. As shown in Fig. 4, there was a progressive increase in OFA binding as the OFA concentration was increased from 0.025 to 0.100 μg/ml. However, this resulted in minimal C3 fragment deposition (0–3%) and CDC (0–3%; data not shown). These results suggest that decreased binding of OFA to CLL cells in circulation following the first standard dose of OFA therapy for CLL is a major mechanism of acquired resistance to OFA-mediated CDC.
**FIGURE 3.** Circulating CLL cells sampled after the first dose of OFA have low levels of CD20 expression, decreased OFA binding capacity, and complement fragment deposition after in vitro incubation with OFA and C5-deficient serum. (A) Median OFA binding to CLL cells (dMESF OFA, based on probing with mAb HB43 specific for the Fc region of OFA) sampled from patients before initiation of treatment was measured after incubation of cells with 10 μg/ml of OFA and was found to be 9,918 (range 1,786–39,694) (max binding 0 h). Median OFA binding in cells from patients sampled 8 h after the initiation of OFA treatment was 2077 (range 96–5769) (bound in vivo 8 h) and decreased to 258 (range 0–2542) at 24 h (bound in vivo 24 h). CLL cells sampled at 8 and 24 h were then incubated in vitro with 10 μg/ml of OFA. Compared to the 0-h sample (max binding 0 h), the OFA binding to the CLL cells from the 8-h sample was significantly lower at 8 h (max binding 8 h; median dMESF, 3113; range 276–7951; \( p < 0.0001 \)) and at 24 h (max binding 24 h; median dMESF, 1124; range 0–6870; \( p < 0.0001 \)). There were no significant differences in median dMESF OFA in patients who received an initial OFA dose <300 mg (open circles; \( n = 8 \)) compared with those who received a dose of 300 mg of OFA (closed circles; \( n = 17 \)). (B) Median C3 fragment deposition (dMESF C3, based on probing with FITC mAb 1H8 specific for C3 fragments) in CLL cells collected before the initiation of therapy and incubated in vitro with 10 μg/ml OFA and 10% C5-depleted serum was 40,667 (range 2,914–89,828) (max binding 0 h). dMESF C3 in CLL cells collected at 8 h after starting OFA therapy (bound in vivo 8 h) was lower at 7,150 (range 757–35,809) and decreased further at 24 h (bound in vivo 24 h) to 2,476 (range 0–24,139). Compared to the 0-h sample, CLL cells collected at 8 h after initiation of OFA therapy and then incubated with 10 μg/ml of OFA and 10% C5-depleted serum in vitro (max binding 8 h) had a significantly lower median dMESF C3 of 8,027 (range 1,225–44,856; \( p < 0.0001 \)), which decreased further in CLL cells sampled at 24 h (max binding 24 h) to 5,325 (range 927–25,940) (\( p < 0.0001 \)). dMESF C3 was not significantly different in CLL cells from patients who received lower (<300 mg) (open circles) compared with standard 300-mg (closed circles) initial doses of OFA.

**CLL cells from patients treated with OFA are resistant to in vitro OFA CDC**

CLL cells obtained from patients at 8 and 24 h were tested for in vitro OFA-mediated CDC using saturating doses of OFA and complement. Compared to OFA-mediated CDC in pretreatment specimens (median 37% cytotoxicity), there was considerably less OFA CDC for cells obtained at 8 h (median 0%; \( p < 0.0001 \)) and at 24 h (median 0%; \( p < 0.0001 \)) (Fig. 5). These data suggest that the availability of OFA and complement are not major factors limiting CDC in these cells. To determine if these CLL cells were resistant to the lytic effects of activated complement, they were treated with ALM and NHS. As shown in Fig. 5, ALM-mediated CDC does not decrease after in vivo exposure of CLL cells to OFA, showing that there is no appreciable selection for CLL cells resistant to activated complement or acquired resistance to CDC. The marked susceptibility of CLL cells previously treated with OFA in vivo, to in vitro ALM-mediated CDC, suggests that the resistance of CLL cells in the 8- and 24-h specimens to OFA-mediated CDC is largely due to the decreased expression of CD20 (Fig. 5).

**Levels of complement regulatory proteins CD55 and CD59 do not predict response to treatment**

The expression of CD55 (\( n = 14 \)) and CD59 (\( n = 25 \)) was evaluated using dMFI and showed no correlation with the magnitude of the decrease in absolute lymphocyte count, clinical response, or in vitro CDC (data not shown). This result is similar to previous reports that measurement of CD55 and CD59 levels on CLL cells does not correlate well with CDC (24), even though inhibition of complement control protein activity can increase CDC (37).

**Evaluation of the relationship between the in vitro measurement of OFA CDC and CD20 expression and clinical outcome**

In vitro OFA CDC in the pretreatment (0 h) specimens was compared with the response of patients to treatment. For the purposes of this analysis, clinical responses were categorized as CR/CRi (\( n = 8 \)) versus non-CR/CRi (\( n = 17 \)). Patients with a CR/CRi had similar in vitro OFA CDC compared with those did not achieve a CR/CRi (median 33 versus 37%; \( p = 0.29 \)). We then compared clinical response with the median total pretreatment CDC cell–associated circulating CD20 and the decrease in this value after the initial dose of 300 mg of OFA. There was no difference between patients who did or did not achieve a CR/CRi (total CD20 expression \( 10^{13.0} \times 10^{12}/l \) versus \( 1191.8 \times 10^{12}/l \), \( p = 0.84 \); and decrease in expression \( 412.5 \times 10^{12}/l \) versus \( 800.4 \times 10^{12}/l \), \( p = 0.54 \)).

**Discussion**

Initial i.v. OFA therapy of progressive CLL with an intended dose of 300 mg achieved high serum OFA levels, a moderate decrease in
The emergence of a population of circulating CLL cells that express very low levels of CD20 following initial OFA treatment should be a major consideration in planning of CD20 targeted therapies. This loss of CD20 expression in CLL cells following anti-CD20 mAb therapy is well described (11, 17) and attributed primarily to trogocytosis (18) and to a lesser extent to internalization of the CD20–mAb complexes (19, 20). Our finding, that the first OFA infusion, at doses as low as 100 mg, causes rapid decreases in CD20 expression in circulating CLL cells was previously reported for RTX (38) and suggests that extensive trogocytosis occurs at these doses. OFA doses <100 mg will thus need to be tested for their effect on CD20 expression in vivo. CD20 expression in circulating cells at 24 h was significantly lower than at 8 h; therefore, the second dose of 1000 mg of OFA given immediately after collection of the 24 h specimen on day 2 of therapy is likely to be ineffective and possibly even detrimental to patient care. Absolute lymphocyte counts increased between 8 and 24 h when most patients had OFA levels within the range predicted to saturate CD20 binding sites on circulating CLL cells. Because circulating CLL cells do not divide, the increase in absolute lymphocyte counts is most likely due to mobilization of CLL cells from tissue sites including the lymph nodes, spleen, and bone marrow (39, 40). There is some uncertainty concerning the CD20 expression levels in CLL cells in the bone marrow (41, 42) and lymph nodes, and mobilization of CLL cells into the circulation after the first dose of OFA might have contributed to the decrease in CD20 levels in circulating cells at 8 and 24 h. However, the median level of CD20 expression decreased to 21% of baseline at 8 h and to 3% of baseline at 24 h, which suggests that there was substantial CD20 loss even by CLL cells mobilized into the circulation, and this loss is very well validated by in vitro demonstraions of trogocytosis (18, 20). Thus, our study reveals that i.v. OFA administration decreases CD20 expression both in CLL cells in circulation and in those CLL cells mobilized into the circulation after the completion of the infusion. We propose that optimal efficacy of CD20-targeted therapy for CLL requires determining an mAb dose and frequency that optimizes CLL killing without exceeding the capacity of the cytotoxic mechanisms and thus minimizes loss of CD20 expression in the surviving CLL cells (43). Although we did not investigate loss of other markers from CLL cells, we note that previous investigations have demonstrated that mAb-mediated trogocytosis of CD20 and of CD22 can promote innocent bystander decreases in B cell proteins likely to be closely associated with these proteins (18, 44, 45).
OFA is effective at complement activation in vitro, and the rapid decrease in absolute lymphocyte counts and complement levels after the first OFA dose suggests that CDC is important in reducing circulating CLL cell counts in patients with CLL. Moreover, at high CLL cell burdens, higher levels of complement activity are likely to be required to promote robust CDC (11). The 86% decrease in the median serum complement level at 8 h, with 20% of patients having a level <5% of baseline, suggests that serum complement deficiency could have contributed to suboptimal OFA cytotoxicity. However, our in vitro experiments confirmed that under standard conditions, CLL cells isolated 8 or 24 h after OFA infusion could no longer be killed by OFA-mediated CDC (Fig. 5). This suggests that the reduced OFA cytotoxicity was primarily limited by low CD20 expression in the circulating cells rather than by low levels of serum complement or OFA.

In this study, we examined circulating CLL cells sampled from patients after OFA treatment to investigate mechanisms of resistance to this mAb. Although acquired loss of CD20 expression is a major cause of resistance to OFA cytotoxicity, we also determined whether CLL cells survived stochastically or if there were populations more likely to have survived in vivo OFA exposure. We assessed complement activation in vivo by measuring covalent binding of complement C3 activation products to CLL cells collected at 8 and 24 h after initiation of OFA therapy and compared these values to those in pretreatment CLL cells exposed to saturating amounts of OFA and complement in vitro (Fig. 3). Compared to pretreatment CLL cells, cells collected at 8 h had considerably lower levels of C3 fragment deposition (18% of pretreatment cells), which decreased further in CLL cells sampled at 24 h. This finding of decreased complement activation correlated well with decreases in CD20 expression in CLL cells at 8 and 24 h. To further determine the role of low CD20 expression in limiting complement activation, we conducted experiments using pretreatment CLL cells incubated in vitro with OFA at a concentration resulting in OFA binding equivalent to that observed for cells evaluated at 8 h after starting OFA therapy (Fig. 4). These experiments showed that at these levels of OFA binding (17–28% of maximal binding achieved with 10 μg/ml of OFA), there was minimal complement activation and CDC. This finding extends and generalizes the results of dose-response experiments reported by Bleecker et al. (36), who demonstrated that very high levels of OFA binding occupancy of CD20 in Daudi cells (>50% saturation) were required to promote CDC. We conclude that the major mechanism of resistance to OFA-induced CDC in circulating CLL cells after the first OFA dose is loss of CD20 expression by surviving CLL cells.

We examined CLL cells at 8 and 24 h for susceptibility to CDC using ALM. These studies showed that exposure of CLL cells to OFA in vivo did not significantly alter sensitivity to ALM CDC in vitro. That is, these surviving OFA-exposed CLL cells were not intrinsically resistant to CDC and further support the conclusion that CD20 loss is the principal factor limiting treatment efficacy.

OFA therapy of CLL can result in a first-dose effect, which can cause considerable toxicity and is only partially controlled by the use of premedications. We investigated the characteristics of the eight (32%) patients who had first-dose effects that prevented them from receiving the full planned dose of 300 mg of OFA. Our analysis showed that infusion reactions limiting tolerance to the first OFA dose were not associated with significantly higher levels of baseline CD20 expression, pretreatment CD20 binding sites, or changes in CD20 binding sites 8 h after treatment. The first-dose effect was not associated with higher levels of activated complement C3 fragment deposition on circulating CLL cells or lower levels of serum complement after therapy with OFA. Thus, the first-dose reactions in patients receiving OFA are unlikely to be caused by increased complement activation or higher levels of CD20 expression.

Serum OFA levels after therapy in patients who tolerated the 300-mg initial dose were inversely proportional to the number of pretreatment circulating CD20 binding sites (absolute lymphocyte count > dimesf CD20) and the magnitude of the decrease in CD20 binding sites at 8 h after starting therapy (Fig. 2). This finding suggests that OFA binding to CD20 on CLL cells results in consumption of OFA. Indeed, based on mouse models as well as the classic studies of Frank and others (11, 46–50), who demonstrated that IgG-opsonized erythrocyte substrates are cleared from the circulation by fixed tissue macrophages in the liver and spleen, it is quite likely that a similar cytotoxic mechanism (in addition to CDC) obtains for OFA-opsonized cells. However, although macrophage clearance mechanism can be saturated or exhausted, trogocytosis and loss of CD20 on circulating CLL cells can continue unabated (23, 51–53). Because the 8- and 24-h posttreatment circulating CLL cells have very low levels of CD20 expression, this phenomenon argues against use of higher OFA doses to improve therapeutic outcome.

This study combined prospective clinical data with correlative measurements on blood specimens collected in 25 CLL patients meeting strict criteria for treatment. To the best of our knowledge, this is the first study to measure serum OFA levels and find a correlation between OFA serum levels and the amount of circulating cell-associated CD20. We also demonstrated in vitro resistance to OFA-mediated CDC but not ALM-mediated CDC; this OFA resistance was induced by in vivo exposure of CLL cells to OFA. The results of our measurements on cells isolated after OFA infusion strongly support and extend the findings and conclusions of Beurskens et al. (11), who first reported that treatment of patients with CLL with OFA resulted in loss of CD20 expression by CLL cells and that higher concentrations of OFA caused exhaustion of effector mechanisms and thus decreased OFA cytotoxicity. We did not perform in vitro studies of mAb-dependent cellular cytotoxicity on cells exposed to OFA in vivo. However, based on previously published studies of cellular cytotoxicity (10, 36), the marked decrease in CD20 expression on the CLL cells would be expected to considerably reduce OFA-mediated cellular cytotoxicity. This would be compatible with the observed increases in absolute lymphocyte counts after completion of the OFA infusions. Our study did not show any correlation between the in vitro measurement of OFA CDC and CD20 expression by CLL cells and clinical outcome. These data need to be interpreted with caution because of the relatively small number of patients studied and the inability to directly measure the cytotoxic effect of therapy in patients using clinical response evaluations.

We conclude that OFA activates complement in the circulation of CLL patients and promotes lysis and elimination of opsonized cells, resulting in substantial decreases in circulating CLL cell counts in most patients. This is followed by a rebound in the absolute lymphocyte counts caused by mobilization of CLL cells from the noncirculating compartment. After administration of the initial large dose of OFA, surviving circulating CLL are subjected to trogocytosis, and as a consequence, these cells have very low levels of CD20 expression, which is the principal reason for their failure to respond to adequate concentrations of OFA. This suggests that the first dose of OFA has exceeded the maximum capacity of mAb-mediated cytotoxic mechanisms and that a lower dose of OFA administered more frequently should be evaluated in future clinical trials to optimize the therapeutic effect of this valuable drug (43).

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