Exhaustion of Cytotoxic Effector Systems May Limit Monoclonal Antibody-Based Immunotherapy in Cancer Patients

Frank J. Beurskens, Margaret A. Lindorfer, Mohammed Farooqui, Paul V. Beum, Patrick Engelberts, Wendy J. M. Mackus, Paul W. H. I. Parren, Adrian Wiestner and Ronald P. Taylor

J Immunol 2012; 188:3532-3541; Prepublished online 24 February 2012;
doi: 10.4049/jimmunol.1103693
http://www.jimmunol.org/content/188/7/3532

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/02/24/jimmunol.1103693.DC1

References
This article cites 50 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/188/7/3532.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Exhaustion of Cytotoxic Effector Systems May Limit Monoclonal Antibody-Based Immunotherapy in Cancer Patients

Frank J. Beurskens,*−1 Margaret A. Lindorfer,†1 Mohammed Faroqui,‡
Paul V. Beum, † Patrick Engelberts,* Wendy J. M. Mackus,*2 Paul W. H. I. Parren,*
Adrian Wiestner, ‡ and Ronald P. Taylor†

The CD20 mAb ofatumumab (OFA) induces complement-mediated lysis of B cells. In an investigator-initiated phase II trial of OFA plus chemotherapy for chronic lymphocytic leukemia (CLL), OFA treatment promoted partial CLL B cell depletion that coincided with reduced complement titers. Remaining CLL B cells circulated with bound OFA and covalently bound complement breakdown product C3d, indicative of ongoing complement activation. Presumably, neither complement- nor effector cell-based mechanisms were sufficiently robust to clear these remaining B cells. Instead, almost all of the bound OFA and CD20 was removed from the cells, in accordance with previous clinical studies that demonstrated comparable loss of CD20 from B cells after treatment of CLL patients with rituximab. In vitro experiments with OFA and rituximab addressing these observations suggest that host effector mechanisms that support mAb-mediated lysis and tumor cell clearance are finite, and they can be saturated or exhausted at high B cell burdens, particularly at high mAb concentrations. Interestingly, only a fraction of available complement was required to kill cells with CD20 mAbs, and killing could be tuned by titrating the mAb concentration. Consequently, maximal B cell killing of an initial and secondary B cell challenge was achieved with intermediate mAb concentrations, whereas high concentrations promoted lower overall killing. Therefore, mAb therapies that rely substantially on effector mechanisms subject to exhaustion, including complement, may benefit from lower, more frequent dosing schemes optimized to sustain and maximize killing by cytotoxic immune effector systems. The Journal of Immunology, 2012, 188: 3532–3541.

The B cell-targeting CD20 mAbs, rituximab (RTX) and ofatumumab (OFA), achieve the high levels of cytotoxicity necessary for effective cancer treatment by using effector mechanisms of the body’s innate immune system (1–11). These mechanisms include complement-dependent cytotoxicity (CDC), Ab-dependent cell-mediated cytotoxicity (ADCC), and phagocytosis. In CDC, mAb-targeted cells activate the classical pathway of complement by which they are covalently tagged with activated complement protein fragments C4b and C3b, and are then lysed because of generation of membrane attack complexes (12–14). However, the increased understanding of immunotherapeutic mAb cytotoxic mechanisms, including that of alemtuzumab (ALM), which also kills targeted cells by CDC (15, 16), has not yet led to scientifically formulated fundamental approaches to dosing regimens. Indeed, most modifications of dosing strategies have been empirical, with the unstated presumption that for CD20 mAbs, the usual weekly 375 mg/m² RTX treatment is likely to be close to an optimal dosage (17–19). For the CD52 mAb ALM, dosing has been set at 10–30 mg three times weekly.

Because of low CD20 expression on chronic lymphocytic leukemia (CLL) cells together with high tumor burden, the efficiency of OFA-mediated CDC is particularly relevant for CLL treatment (6, 8, 10, 12–22). As part of a phase II trial in CLL (NCT01145209) combining i.v. OFA infusion with chemotherapy, we investigated the in vivo consequences of OFA treatment on circulating B cells, and evaluated absolute lymphocyte counts (ALC), complement consumption, C3 fragment deposition on cells, and levels of B cell-associated CD20 and bound OFA.

At the trial start, patients had high burdens of circulating B cells that were significantly reduced by day 29. In addition, large reductions in complement titers were observed, most notably after the first OFA infusion. Intriguingly, nondepleted cells included B cells with substantial amounts of deposited complement C3 breakdown fragment C3d; these cells could continue circulating for extended time periods. Based on these findings, we conducted parallel quantitative in vitro investigations comparing OFA and RTX with respect to their potential to activate and consume complement, and to promote CDC upon binding to CD20+ cells. In vitro studies demonstrated the ability of OFA to induce robust CDC in which only a fraction of available complement components.

The online version of this article contains supplemental material.

†European Medicines Agency, Amsterdam, The Netherlands.
‡Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908; and 3Hematology Oncology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication December 19, 2011. Accepted for publication February 1, 2012.

This work was supported by GlaxoSmithKline, which supported the collaborative studies and provided ofatumumab for the trial, and by Genmab, which supported the in vitro studies. The clinical trial was funded by the National Heart, Lung and Blood Institute, National Institutes of Health.

Address correspondence and reprint requests to Dr. Ronald P. Taylor, University of Virginia, Department of Biochemistry and Molecular Genetics, P.O. Box 800733, Charlottesville, VA 22908. E-mail address: rtp@virginia.edu


Abbreviations used in this article: ADCC, Ab-dependent cell-mediated cytotoxicity; AL, Alexa; ALC, absolute lymphocyte count; ALM, alemtuzumab; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukemia; EA, Ab-opsonized sheep erythrocyte; FCO, ofatumumab and fludarabine/cyclophosphamide; FO, ofatumumab and fludarabine; MESF, molecules of equivalent soluble fluorochrome; NHS, normal human serum; OFA, ofatumumab; RTX, rituximab; UVA, University of Virginia.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103693
were required to effect cell killing. Using high cell burden conditions, we demonstrated that complement could be severely depleted, leading to inadequate killing of a second target cell challenge. Significantly, we were able to reduce complement consumption and retain killing capability by reducing OFA concentrations. Our studies suggest that standard doses of CD20 mAb, in contrast with current dogma, may be excessive, resulting in wasteful complement consumption that depletes the body’s complement reservoir and cytotoxic capacity. This insight provides a framework for the design of mAb-based immunotherapy regimens that preserve complement, as well as other effector functions, thus leading to increased overall tumor cell killing and potentially enhanced efficacy.

Materials and Methods

National Institutes of Health clinical trial NCT01145209

During cycle 1, patients received i.v. OFA (day 1, 300 mg; days 8, 1 g) and either fludarabine (FO; 25 mg/m² days 2–6) or fludarabine 25 mg/m² and cyclophosphamide 250 mg/m²/d (FCO; days 2–4). During cycle 2 (day 29), patients received 1 g OFA plus chemotherapy. Blood samples from patients N1 to N9 (obtained with informed consent) were drawn immediately before starting OFA infusions and several times after infusions started. ALC were determined at the National Institutes of Health clinical laboratory. Analyses of B cells were based on flow cytometry protocols described in previous studies (23, 24). B cells were identified by light scattering and development with PE CD19 and PERCP CD45. CD20 was determined based on staining with TO-PRO 3 (FACSCalibur; BD) and cellular deposition of C3b and C4b determined according to published procedures (23, 26). To assess cellular C4b deposition, we reacted saturating concentrations of mAbs with B cells for 15 min at room temperature. C5-deficient serum was added (final concentration of 20%) followed by incubation at 37°C for 45 min. After washing, FITC-conjugated polyclonal Abs against C4c (FPC109) (Dako, Glostrup, Denmark) and C4d (Dako) were added (1:100) and samples incubated for 30 min at 4°C and analyzed by flow cytometry, with readouts being mean fluorescent intensities.

To distinguish between C4c deposition on anti-B-cell mAbs or on B-cell membranes, an acidic wash (RPMI 1640 + 0.3% BSA, pH 2.5) was implemented (28, 32). mAb binding was determined based on staining with FITC-F(ab’)2 fragments of goat anti-human IgG, Fab-specific.

CH50 assays

Complement hemolytic assays (CH50) were performed according to standard procedures (33, 34). In brief, washed sheep E (Lampire) were reacted with anti-sheep E stroma (hemolsyn; Sigma), washed, and reconstituted to a hematocrit of 2% Ab-opsinized sheep erythrocytes (EA) in gelatin veronal buffer supplemented with Ca²⁺ and Mg²⁺ (GVB⁺). Test and standard sera were first diluted 10-fold in GVB⁺ and then further diluted sequentially by factors of 2 across 6 wells of a conical 96-well plate. The hemolytic reaction was initiated by addition of 25 μl of 2% EA to 25 μl diluted serum. Assay plates were incubated for 60 min at 37°C. The reaction was stopped by addition of 200 μl ice-cold GVB⁺ to each well. The lysed EA were pelleted by centrifugation at 1800 × g for 3 min, the supernatant removed to a flat-bottom plate, and the optical densities measured at 405 nm. CH50 titers were reported relative to a NHS pool. Assays were performed in triplicate.

Statistics

All experiments were conducted independently two or more times, and representative results are presented. Where replicates measurements were performed, means and SD are provided. Unpaired two-tailed t tests (SigmaStat) were used to compare selected results in Fig. 2.

Results

OFA infusion in CLL patients rapidly exhausts complement and induces loss of CD20 from the tumor cells

A first group of nine CLL patients (N1–N9, NCT0145209) was treated with OFA (300 mg for the first dose [day 1] and 1 g for all doses thereafter [days 8 and 29]) in combination with chemotherapy (Table I). Blood samples were collected before, during, and after OFA infusions on days 1, 8, and 29. Measurements included ALC, deposition of C3 fragments on target cells, complement titters, and binding of OFA to circulating cells.

Significant B cell depletion in peripheral blood was achieved with the first cycle (Fig. 1A). However, six of eight patients had ALC in excess of 2000 cells/μl on day 29 (one patient withdrew on day 10). The results of our measurements on blood samples...
Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age (y)/Sex</th>
<th>Rai Stage</th>
<th>Cytogenetics</th>
<th>Treatment</th>
<th>OFA MESS (MESF) average ± SD</th>
<th>Maximum Bound OFA (MESF) average ± SD</th>
<th>Maximum Bound C3d (MESF) average ± SD</th>
<th>Pre-Rel. C5b-9 (1000/m$^3$) average ± SD</th>
<th>Pre-ALC (1000/m$^3$) average ± SD</th>
<th>Pre-CD20 (1000/m$^3$) average ± SD</th>
<th>% Saturation</th>
<th>% Saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>26/M</td>
<td>II</td>
<td>11q</td>
<td>FCO</td>
<td>11</td>
<td>4 ± 1</td>
<td>10,300 ± 100</td>
<td>15,400 ± 900</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N2</td>
<td>49/F</td>
<td>III</td>
<td>13q</td>
<td>FO</td>
<td>11</td>
<td>68 ± 9</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N3</td>
<td>61/F</td>
<td>II</td>
<td>17p, 11q, 13q</td>
<td>FCO</td>
<td>16</td>
<td>95 ± 12</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N4</td>
<td>56/F</td>
<td>III</td>
<td>T12</td>
<td>FO</td>
<td>16</td>
<td>136 ± 10</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N5</td>
<td>47/M</td>
<td>III</td>
<td>17p, T12, 13q</td>
<td>FO</td>
<td>24</td>
<td>100</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N6</td>
<td>50/M</td>
<td>II</td>
<td>11q, 13q</td>
<td>FCO</td>
<td>16.5</td>
<td>273 ± 15</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N7</td>
<td>66/F</td>
<td>II</td>
<td>17p</td>
<td>FCO</td>
<td>22.5</td>
<td>273 ± 15</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
<tr>
<td>N8</td>
<td>78/M</td>
<td>III</td>
<td>Normal</td>
<td>FO</td>
<td>16</td>
<td>253 ± 15</td>
<td>15,400 ± 200</td>
<td>7,300 ± 200</td>
<td>3,900 ± 300</td>
<td>3,900 ± 300</td>
<td>100</td>
<td>93–98%</td>
</tr>
</tbody>
</table>

No patient had bulky disease (>5 cm).

% Saturation = 100 × maximum bound OFA/maximum bound C3d.

*The Pre-Rel. C5b-9 values are based on measurements on blood samples taken just before the first OFA infusion; n = 2 for CD20 determinations.

Maximum Bound OFA was observed in blood samples taken 2 h after the first infusion for all patients except patient N3; in this patient, maximum bound OFA was observed at 6 h (n = 2).

The “Pre” values are based on measurements on blood samples taken just before the first infusion on ALC, except patient N3 in this patient, maximum bound OFA was observed at 6 h (n = 2).

Maximum bound C3d was observed in the 2-h samples (day 1) for patients N2, N4–6, and N8–9. For patients N3 and N7, C3d binding peaked at the 6-h point. Apparently because of low initial CH50 titer in patient N1, C3d binding did not peak until day 29 (n = 2).

OFA promoted deposition of C3d fragments on CLL B cells (Fig. 1E). C3d, a degradation product derived from active C3b during complement activation, remains covalently attached to circulating cells that are targeted by complement-fixing Abs but are not lysed (23, 30). In most cases, circulating cells with deposited C3d fragments were demonstrable 24 h after OFA infusion. We also found that neither C3b nor iC3b were demonstrable on circulating cells (not shown), indicating these fragments are converted to C3d. A particularly interesting finding centers on the increased levels of C3d deposition on circulating cells of patients 1, 2, and 6 observed between day 9 (24 h after the second OFA infusion) and day 29, before the third OFA infusion. The most likely explanation is that after day 9, as CD20 was reexpressed by circulating CLL B cells, the cells were opsonized by circulating OFA, leading to C3b deposition followed by its degradation to C3d. CD20 levels were modestly restored on B cells of patient 6 by day 29 (Fig. 1B). It is also noteworthy that after the start of the OFA infusion on day 29, there was substantial C3d deposition on cells of several of the patients, reflecting binding of OFA and subsequent complement activation, because of partially restored levels of CD20.

In vitro studies

To investigate the basis of our in vivo findings, we studied the interplay of complement with OFA-opsonized cells using various in vitro experimental designs. As demonstrated in Supplementary Figs. 1–3, cell lines opsonized with OFA demonstrate substantial deposition of C4b directly on the cell surface and are effectively lysed by CDC in the presence of relatively low concentrations of complement components C2 or C3. Indeed, maximal OFA-induced CDC required only 10–100 μg/ml C3, which represents...
10% of the amount present in normal plasma. Nevertheless, killing of high tumor cell loads required high serum concentrations (i.e., 50% NHS; Supplemental Fig. 4). OFA induced substantially more CDC of Daudi and Z138 cells than did RTX.

Dose-response tests: CLL cells

It has been reported that OFA promotes high levels of CDC and kills CLL B cells more effectively than RTX (6, 8, 10, 20–22). To evaluate this phenomenon quantitatively, we labeled OFA and RTX with fluorophore Al488 (which does not affect CDC potency) (10, 21) and evaluated CDC, cell binding, and C3b deposition on CLL cells from 10 patients (seen at the UVA Hospital) not in the trial (V1–V10; Fig. 2). To model physiological conditions, we used 50% NHS and varying cell densities typical for CLL (i.e., 10^7 and 10^8 cells/ml). The results, using B cells from two representative CLL patients, reveal the following: saturation of OFA binding to cells in media is achieved at 10 μg/ml OFA (Fig. 2B, 2C), and substantial CDC is observed at this concentration in 50% NHS for both cell burdens. At cell inputs of 10^8 cells/ml, OFA concentrations of 100 μg/ml and 50% NHS maximize CDC and C3b deposition (Fig. 2D–G); less OFA-mediated CDC occurs if less complement (lower % NHS) is available. Finally, even at RTX concentrations of 100 μg/ml, there was little CDC, but complement activation was evident, as demonstrated by C3b deposition; thus, RTX would appear to be less efficient in making use of complement to kill cells.

Complement depletion

We reported that CD20+ B cell lines opsonized with RTX (100 μg/ml) at high cell burdens (10^9 cells/ml) activate and deplete complement in vitro (30). The present correlative results for CLL patients treated with OFA demonstrate complement consumption in vivo (Fig. 1D). We extended our in vitro measurements to include ALM, with Wien cells as targets because these cells can be bound by OFA.

FIGURE 1. Correlative studies on patients for the first 30 d of the trial. OFA was infused on days 1 (300 mg), 8 (1 g), and 29 (1 g). Blood samples were obtained immediately before and 2, 6, and 24 h after starting OFA infusions. Results are normalized to pretreatment values for ALC, CD20, and CH50. Bound OFA is normalized to the 2-h mark (first infusion), usually the maximum amount bound; values for bound C3d are normalized to maximum amount bound, observed at 2 or 6 h. Absolute values for these parameters are provided in Table I, along with representative uncertainties (SD). (A) ALC; (B) B cell surface levels of CD20; (C) cell-bound OFA; (D) complement titers (CH50 determinations); (E) C3d deposition on B cells. The complement titer of patient N1 was low throughout the study (1–4 d; 7–8 d 29) and is not plotted in (D). Results in (B), (C), and (E) are based on duplicate determinations; CH50 titers were determined in triplicate.
killed in NHS via CDC induced with either RTX, OFA, or ALM. We varied target cell burdens with mAb concentrations held at 100 μg/ml, and measured CDC and complement depletion. In intact 50% NHS, all three mAbs promote CDC, but complement was depleted at high cell burdens (10⁸ cells/ml; Fig. 3A). Similar results for CDC and complement depletion were obtained when OFA and RTX were tested with Daudi or Raji cells (not shown).

We next used varying concentrations of CLL cells from six additional UVA CLL patients (V11–V16) and evaluated CDC and complement consumption mediated by OFA, RTX, and ALM in 50% NHS. Representative results from two patients are shown in Fig. 3B and 3C. OFA and ALM induced CDC at high cell burdens (10⁸/ml), and complement was consumed. RTX promoted modest CDC of the B cells of patient V11 (Fig. 3B), and some complement was consumed. However, RTX-opsonized cells of patient V12 were not killed by CDC, and less complement was consumed for this RTX-treated sample (Fig. 3C).

Alternative mAb dosing strategies better preserve complement activity for more effective CDC

Our findings raise fundamental questions with respect to mAb dosing paradigms in cancer immunotherapy. If a mAb depends on host innate effector systems (CDC, ADCC, or phagocytosis) to mediate cytotoxicity of malignant cells, it would seem important to determine the most effective immunotherapeutic doses, and their optimal timing, rather than to base treatment on maximum...
Circulating cell burdens in CLL, readily accessible to infused mAbs, can be quite high (23, 24, 37, 38). The infused mAb may thus promote clearance/destruction of cells at the cost of available effector mechanisms. Therefore, several hours after mAb infusion, as more cells reequilibrate to the bloodstream from other compartments, effector functions such as complement may be saturated or exhausted for periods of days or weeks. Under these conditions, clearance/killing of the next wave of malignant cells may be severely compromised (11, 23, 39). To examine this potential problem, we established a two-step model to approximate likely scenarios with respect to infusion of mAbs for CLL treatment, based on treatment paradigms currently used for RTX and OFA.

In step 1, varying amounts of OFA were added to multiple identical aliquots of Daudi cells in 50% NHS; after a 1-h incubation at 37˚C, cell death and C3b deposition were determined. In step 2, an equal number of naive PKH26-labeled Daudi cells were added along with additional mAb. These PKH26-labeled cells represent CLL cells reequilibrating into the bloodstream. The mixtures were incubated for an additional 1 h at 37˚C, and CDC and C3b deposition on the PKH26 cell population was measured. The results for OFA and RTX are shown in Fig. 4A–H and 4I–K, respectively.

This two-step experiment demonstrates that use of moderate to high concentrations of OFA in step 1 (4–100 µg/ml) promotes high levels of CDC of unlabeled Daudi cells (95% CDC) in 50% NHS (Fig. 4A, open circles; UNL). However, PKH26-labeled cells added in step 2 (along with an additional 100 µg/ml OFA) are effectively lysed only in samples that contained relatively low concentrations of OFA in step 1 (<4 µg/ml; Fig. 4B, filled circles, PKH). That is, although “excess” additional OFA (100 µg/ml) was added in step 2, only a fraction of PKH26-labeled Daudi cells were killed when added to samples exposed to greater OFA concentrations in step 1 (39 and 23% CDC, for samples reacted with 10 and 100 µg/ml OFA, respectively). The bell-shaped curve in Fig. 4C represents the sum of CDC achieved for both populations in this two-step experiment (% CDC of unlabeled cells in step 1 plus % CDC of PKH26-labeled cells in step 2). Thus, most effective killing is achieved with an OFA concentration (4 µg/ml) just sufficient to achieve maximal killing in step 1 (Fig. 4A). Presumably, complement activity is preserved under these conditions, and the second wave of B cells is effectively killed in step 2, whereas greater OFA concentrations in step 1 do not markedly increase killing of the first wave of cells but promote exaggerated complement activation and consumption. This interpretation is supported by examining C3b deposition in our two-step paradigm (Fig. 4D, 4E). C3b deposition for unlabeled cells modestly increases as the OFA input increases from 10 to 100 µg/ml in step 1 (Fig. 4D), indicating increasing complement activation and deposition even though maximal cell killing is already achieved. In contrast, C3b opsonization of the PKH26-labeled cells (after addition of OFA at
FIGURE 4. Two-step experiment: Lower mAb concentrations in step 1 are more effective in promoting overall CDC of the combined cell populations. (A–C) Step 1: Unlabeled Daudi cells (1.8 × 10⁷ cells/ml in 50% NHS) were reacted (1 h at 37°C) with OFA (0–100 μg/ml). In step 2, an equal number of PKH26-labeled Daudi cells was added, plus 100 μg/ml additional OFA, and after incubation, CDC and C3b deposition was determined. (A and B) The % CDC of unlabeled cells after step 1 (open circles) and of PKH26-labeled cells after step 2 (filled circles) are provided. (C) The summation of % CDC for unlabeled cells after step 1 plus % CDC for PKH26 cells in step 2 is a bell-shaped curve. The decrease in CDC at high OFA concentrations is not due to final high OFA concentrations (200 μg/ml). In a separate one-step control, varying amounts of OFA were added to Daudi cells (3.6 × 10⁵ cells/ml in 50% NHS); CDC was 85% for OFA concentrations of 100–400 μg/ml. (D and E) C3b deposition on unlabeled cells after step 1 or on PKH26-labeled cells after step 2. (F–H) Similar experiment, except OFA added in step 2 was 100 or 20 μg/ml. Close to maximal C3b deposition and CDC is achieved for OFA concentrations of 4 μg/ml in step 1 and 20 μg/ml in step 2. (I–K) Similar to (A)–(C), except for RTX. Means and SD (n = 2) are displayed; usually SD are smaller than symbols. Each figure represents two or more experiments.

100 μg/ml decreases continuously for samples in which greater OFA concentrations were used in step 1 (Fig. 4E), indicating that complement was consumed in step 1, thus limiting C3b opsonization of the PKH26 cells in step 2.

We next performed a more comprehensive experiment in which OFA doses in steps 1 and 2 were varied (Fig. 4F–H), and the findings are consistent with the earlier observations. Close to the maximum sum of CDC for the two populations was achieved at OFA inputs of only 4 μg/ml in step 1 and 20 μg/ml in step 2 (Fig. 4H). Similar patterns were evident based on evaluation of C3b deposition (Fig. 4F–G); opsonization with 4 μg/ml OFA in step 1 and 20 μg/ml OFA in step 2 gave more C3b deposition on PKH26-labeled cells than when 100 μg/ml OFA was used in both steps. Finally, we replicated these findings for RTX in similar experiments in Fig. 4I–K, and these findings are generalized in Fig. 5.

Discussion

The most important finding in this study is that the potential of the complement cascade to support mAb-mediated CDC of tumor cells is finite and saturable, and the degree of CDC is strongly influenced by the nature of the opsonizing mAb and its concentration. Our findings in the CLL clinical study demonstrate that at high initial cell burdens, infusion of OFA activates and consumes complement, and this was also made evident by the appearance of C3d-tagged circulating cells (Fig. 1D, 1E). Apparently, after an initial wave of clearance/killing, some complement activation may continue, but complement as well as the mononuclear phagocytic system and NK cell-mediated ADCC are insufficient to deplete the cells (Fig. 1A). The increase in net C3d found on cells of three patients by day 29, relative to day 9, suggests that as CD20 is reexpressed by cells, the infused OFA may promote chronic low-level complement activation as it targets CD20 on cells.

Our findings of the rapid loss of CD20 caused by infusion of OFA are most likely mediated by trogocytosis, but direct internalization of OFA by the CLL B cells may also play a role in loss of CD20 (40, 41). However, we have directly compared the relative rates and extent of loss of CD20 for trogocytosis versus internalization, and these experiments demonstrate that trogocytosis reduces CD20 expression more strongly and in a shorter time frame (42).

We compared ALM, OFA, and RTX with respect to their potential to use and activate individual components of the classical pathway of complement activation. OFA is more effective in promoting C4b deposition on opsonized Wien cells than RTX or ALM (Supplemental Fig. 1). The results are not due to higher densities of cell-bound OFA; binding of RTX and OFA to Wien cells was comparable and OFA binding was lower than ALM binding. Less cell-associated C4b was released from OFA-opsonized cells via acid wash than from RTX- or ALM-opsonized cells, indicating more deposited C4b was covalently bound to the cell membrane; this finding may be because OFA binds close to the cell membrane (6, 8, 10, 20–22). CDC studies in sera depleted of C2 or C3 provide additional support for the concept that cell-bound OFA can use limited amounts of complement to lyse cells, and that complete serum therefore initially contains a relatively large excess of complement components required for cell killing (Supplementary Figs. 2, 3).
Indeed, because of downstream amplification in the complement cascade, small amounts of early components are adequate to allow for deposition of large amounts of C3 activation fragments on cells (12–14). However, complement component C2, at lowest concentration in the plasma, is often the limiting factor that is first depleted during classical pathway activation. Based on our previous observations of C2 consumption in CLL patients who received RTX therapy (25, 30), it is reasonable to predict that C2 was also first depleted in these studies. These considerations, taken in context with the work of Ziccardi (43), may also explain the sharp drop in CDC observed for serum concentrations of 25% at the higher cell densities (Supplemental Fig. 4). Under these conditions, the potential of C1 inhibitor to control C1 activation may be abrogated, thus leading to uncontrolled activation of C1 followed by complete consumption of C2.

In patients with CLL, circulating B cells in excess of 10^8/ml are commonly observed (23, 24, 37–39), and we reported that infusion of the usual RTX dose can deplete complement in CLL patients for a week or longer (30). We extended these findings and show that at these physiologically relevant cell burdens, complement activity in 50% NHS can be severely depleted (reduced 20-fold) when cells are reacted with mAbs such as ALM or OFA and are killed by CDC (Fig. 3). Indeed, under our standard assay conditions, 50% NHS is required to promote effective CDC at 10^8 cells/ml, and CDC and C3b deposition are substantially reduced if the NHS concentration is reduced only 2-fold to 25% (Fig. 2, Supplemental Fig. 4). Moreover, our two-step experiments described in Fig. 4 provide dramatic evidence that at moderate to high cell burdens, large amounts of mAb indeed promote effective CDC of CLL cells, but if more mAb is added than essentially required, this leads to exhaustion of complement activity and, therefore, additional CDC is severely compromised upon challenge with additional cells, even if adequate mAb concentrations are present. These results, taken along with our previous findings for CLL patients treated with RTX (30), indicate that after the standard (high) mAb doses, patients’ complement levels can be depleted in vivo for periods of several days to >1 wk. It would seem reasonable that mAb dosing strategies that take into account patient complement titers as well as other indices of immune function would help provide guidance for more efficacious, individualized therapies.

On the basis of accumulated evidence, high tumor burdens combined with high mAb concentrations may exhaust effector functions of the body’s innate immune system, including mAb-mediated CDC (Fig. 5). Our results specifically relate to prominent therapeutic mAbs that are highly dependent on Fc-mediated effector functions for target cell cytotoxicity, such as type I CD20 mAbs. Provocative results in uncontrolled trials with RTX suggest that infusion of fresh-frozen plasma to increase or restore complement activity may increase clinical efficacy of single-agent treatment in CLL, in support of our findings (30, 44–46). Our results support this concept with respect to maximizing the potential CDC that can be achieved by normal complement levels in the bloodstream. It is possible, based on findings in several reports, that other effector functions important in mAb-based immunotherapy of cancer, such as ADCC, are similarly subject to exhaustion, likely as a consequence of downregulation of CD16 and depletion of NK cell-associated perforin and granzymes (47–51). Moreover, the substantial and protracted loss of CD20 mediated by infusions of large doses of mAb, coupled with the loss of potency of cytotoxic effector systems, would generate a “perfect storm” that can allow malignant cells to continue to proliferate for extended time periods, even after infusion of gram quantities of mAb.

We hypothesize that our results not only apply to OFA or RTX treatment, but impact all therapeutic mAbs that use Fc- or complement-mediated effector functions as part of their cytotoxic mechanisms. Reformulation of mAb dosing schedules based on more frequent treatments (several times per week) with lower doses may allow for recovery and/or preservation of effector functions between doses (23, 24) and should be investigated. Our study emphasizes that the time-honored approach to use drugs at maximum tolerated doses as derived from the chemotherapy field may not necessarily be optimal for mAb-based immunotherapy of cancer.

**Acknowledgments**

We thank Marleen Voorhorst for technical assistance and Joost Bakker for graphic design. We also thank the patients for participation in the trial.
Disclosures

F.J.B., P.E., and P.W.H.I.P. are employees of Genmab. W.J.M. is a former employee of Genmab. R.P.T. has received research funding from Genmab. The other authors have no financial conflicts of interest.

References


