Binding of Submaximal C1q Promotes Complement-Dependent Cytotoxicity (CDC) of B Cells Opsonized with Anti-CD20 mAbs Ofatumumab (OFA) or Rituximab (RTX): Considerably Higher Levels of CDC Are Induced by OFA than by RTX

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*J Immunol* 2009; 183:749-758; Prepublished online 17 June 2009;
doi: 10.4049/jimmunol.0900632
http://www.jimmunol.org/content/183/1/749

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Binding of Submaximal C1q Promotes Complement-Dependent Cytotoxicity (CDC) of B Cells Opsonized with Anti-CD20 mAbs Ofatumumab (OFA) or Rituximab (RTX): Considerably Higher Levels of CDC Are Induced by OFA than by RTX1

Andrew W. Pawluczkowycz,* Frank J. Beurskens,† Paul V. Beum,* Margaret A. Lindorfer,* Jan G. J. van de Winkel,‡† Paul W. H. I. Parren,‡ and Ronald P. Taylor2*†

The CD20 mAb ofatumumab (OFA) is more effective than rituximab (RTX) in promoting complement-dependent cytotoxicity (CDC) of B cells via the classical pathway (CP) of complement. CP activation is initiated by C1q binding to cell-bound IgG. Therefore, we examined the role of C1q in the dynamics of complement activation and CDC of B cell lines and primary cells from patients with chronic lymphocytic leukemia, reacted with OFA or RTX. C1q binding, complement activation, and colocalization of C1q with cell-bound mAbs were determined by flow cytometry and high-resolution digital imaging. C1q binds avidly to OFA-opsonized Raji and Daudi cells (KD = 12–16 nM) and colocalizes substantially with cell-bound OFA. Cells opsonized with OFA undergo high levels of complement activation and CDC in C1q-depleted serum supplemented with low concentrations of C1q. Under comparable conditions, RTX-opsonized cells bind less C1q; in addition, even when higher concentrations of C1q are used to achieve comparable C1q binding to RTX-opsonized cells, less complement activation and CDC are observed. Greater CDC induced by OFA may occur because C1q is bound in close proximity and with high avidity to OFA, resulting in effective CP activation. Moreover, OFA binds to the small, extracellular CD20 loop, placing the mAb considerably closer to the cell membrane than does RTX. This may facilitate effective capture and concentration of activated complement components closer to the cell membrane, potentially shielding them from inactivation by fluid phase agents and promoting efficient generation of the membrane attack complex. *The Journal of Immunology, 2009, 183: 749–758.

The complement system plays key roles in numerous aspects of human health and disease due to its influence on both innate and adaptive immunity (1–6). Indeed, under a variety of conditions, complement activation can lead to rapid and highly effective destruction of either foreign pathogens or normal tissue. It is therefore quite reasonable that with respect to the development of mAbs in the immunotherapy of cancer, numerous paradigms have been investigated with the goal of effectively using complement to selectively destroy malignant cells (7–11). Two of the first IgG mAbs approved for the treatment of cancer, rituximab (RTX,3 anti-CD20), and alemtuzumab (anti-CD52) (12–16), have been demonstrated in vitro to be quite effective in promoting complement-dependent cytotoxicity (CDC) of target cells (17–22). Work in animal models and observational studies in the clinic also provide evidence that classical pathway (CP)-mediated complement activation promotes CDC, and this reaction is responsible for at least part of the cytotoxic mechanism of these mAbs (7, 17, 23–28).

However, a considerable body of evidence, based on clinical reports as well as animal models, argues that interaction of RTX-opsonized CD20-positive cells with effector cells that express FcγR may be principally responsible for much of the therapeutic efficacy that has been obtained with RTX treatment of B cell lymphomas (29–34). Effector cells can efficiently kill RTX-opsonized cells by both Ab-dependent cellular cytotoxicity and by phagocytosis (35–37); the relatively modest CDC mediated by RTX in vivo may be due to expression of defensive complement control proteins (CCP) on the target cells (8, 10, 17). Moreover, in the case of chronic lymphocytic leukemia (CLL), the targeted B cells may not express a sufficient number of CD20 on the cell surface to promote a high level of complement activation and CDC, explaining in part the weak activity of RTX as a single agent in CLL treatment (16, 17). Recently, a new, fully human IgG1 anti-CD20 mAb, ofatumumab (OFA), has been described, and several lines of evidence indicate that it is far more effective than RTX in promoting CDC of both cell lines and primary CLL cells (38, 39). Moreover, OFA has been shown to have substantial clinical activity as a single agent in the treatment of CLL and other B cell lymphomas (40–42).

In contrast to RTX, OFA binds to the small extracellular loop of CD20 in close proximity to the cell membrane (39). This property of OFA, along with its slow off-rate, may explain the superior CDC of opsonized cells that are refractory to CDC mediated by...
RTX (38, 39). The initial step in activation of the CP is binding of C1q, the first component of complement, to Ab-Ag immune complexes (1, 23, 43–46). In the present work, we have investigated the ability of OFA and RTX to promote binding of C1q to opsonized B cells and activation of the CP. We measured the dose response and kinetics of C1q binding, C3b deposition, CDC, and the colocalization of cell-bound OFA and C1q. We find that the binding of relatively small amounts of C1q to cells opsonized with OFA rapidly activates the CP of complement and promotes high levels of C3b deposition and CDC. However, cell-bound RTX binds much lower levels of C1q, resulting in less C3b deposition and less CDC.

Materials and Methods

Cell culture and reagents

CD20+/H11001 Daudi and Raji cells were obtained from the American Type Culture Collection and cultured as previously described (20, 47). Z138C cells (48) were cultured as previously described (47). RTX was obtained at the University of Virginia hospital pharmacy (Charlottesville, VA). RTX was obtained at the University of Virginia hospital pharmacy (Charlottesville, VA). HuMab 7D8 is a somatic variant of OFA and differs at only four amino acid positions. It binds to the same small loop epitope, has similar binding characteristics, and induces CDC similarly. An IgG4 switch variant of 7D8 with a K332A C1q binding site mutation was prepared to generate a control CD20 mAb that is completely devoid of C1q binding. MAbs 7C12 and 1H8, specific for C3b/iC3b and C3b/iC3b/C3dg, respectively, have been described previously (49, 50). FITC and propidium iodide (PI) were purchased from Sigma-Aldrich, and C1q-depleted sera were obtained from Quidel. C1q, purified from pooled plasmas from more than 30 normal donors, was purchased from Complement Technologies. Polyclonal rabbit anti-C1q Ab was purchased from DakoCytomation and labeled with FITC. Anti-CD20 mAbs and purified C1q were labeled with Alexa (Al) 488 or Al647 (Invitrogen) according to the manufacturer’s instructions. Mouse IgG was from Lampire Biological Laboratories. All probing and mAb opsonization was done at 10 μg/ml mAb in 2 mg/ml mouse IgG, unless otherwise noted. Anonymous blood samples from patients with CLL were obtained at the University of Virginia Hospital. All protocols were approved by the University of Virginia Institutional Review Board, Charlottesville, VA.

Determination of C1q binding to B cells reacted with anti-CD20 mAbs

C1q and anti-CD20 mAb binding assays were conducted in complete RPMI 1640 medium (10% FBS) without added human serum. Cells were...
suspended on ice at 5 × 10^6 cells/ml. C1q was added to give final concentrations of 0–70 μg/ml, and Al647 OFA, 7D8-IgG4 (K322A), or RTX were then added and the reaction mixtures immediately transferred to a 37°C water bath. Aliquots were removed at various times (0–60 min) and quenched with 20 volumes of ice-cold BSA-PBS. Cells were then washed three times with BSA-PBS, probed with FITC anti-C1q for 30 min at room temperature and then washed with BSA-PBS and fixed in PBS containing 2% paraformaldehyde. Flow cytometry was performed on a FACS Calibur flow cytometer (BD Biosciences) and mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) using calibrated beads (Spherotech).

Multispectral imaging of B cells reacted with C1q and anti-CD20 mAbs

B cells were suspended in complete RPMI 1640 medium and varying amounts of Al488 C1q were added. Al647 OFA, 7D8-IgG4 (K322A), or RTX were then added to the reaction mixtures to give final mAb concentrations of 10 μg/ml and final cell concentrations of 8 × 10^6/ml. The mixtures were incubated at 37°C for 60 min, washed three times with cold BSA-PBS, and resuspended in 2% paraformaldehyde. The suspensions were filtered through nylon mesh, centrifuged, aspirated to 100-μl final volume, and stored in the dark at 4°C before analysis 16 h later. A total of 10,000 cells were analyzed for each sample using the ImageStream multispectral imaging flow cytometer system (Amnis). Following data collection, images were analyzed using Amnis IDEAS software. Cells were gated based on light scattering (to exclude small particulates and cell aggregates) and the upper 50% most focused cells were included in the final analyses. The degree of colocalization of C1q with bound mAbs was calculated with the bright detail similarity score feature (51).

Assays for CDC and C3b deposition of cells reacted with anti-CD20 mAbs in C1q-depleted serum supplemented with exogenous C1q

Cells were suspended in complete RPMI 1640 medium on ice. C1q-depleted serum, supplemented with 1 mM MgCl2 and 1 mM CaCl2, was added to give a final serum concentration of 50%. Following the addition of C1q and anti-CD20 mAbs, the reaction mixtures were incubated at 37°C and aliquots were removed and quenched in cold BSA-PBS. The cells were washed three times, aliquoted, and then probed with FITC anti-C1q, Al488 mAbs 7C12, or 1H8 (to monitor C3b deposition) for 30 min at room temperature. The mixtures were then washed and resuspended in BSA-PBS containing 1 μg/ml PI and immediately analyzed.

All experiments were performed independently at least two times. In some flow cytometry experiments, certain individual points were run in duplicate and in these cases the means and SD are displayed, thus providing a representative estimate of the uncertainties in the measurements. The determination of C1q colocalization with OFA or with RTX bound to Daudi cells was performed three times. In all cases the results of replicate experiments were in good agreement, although different lots of reagent preparations shifted absolute fluorescence values. In the absence of mAbs, background killing of B cells in C1q-depleted serum supplemented with C1q averaged 10%, with a range of 2 to 15%. Paired t tests were accomplished with SigmaStat software (SPSS).

CLL patient samples

Similar protocols were followed to examine CLL cells isolated from patient samples as previously described (47). Cells were reacted with unlabeled mAbs as described above. The percentage of dead cells was determined by uptake of TOPRO-3 (47).

Results

C1q binds to, and colocalizes with OFA on B cells

The experiments illustrated in Fig. 1, A and B, reveal that in medium, binding of C1q to CD20^+ Raji or Daudi cells saturated with

FIGURE 2. C1q colocalizes with bound OFA on Daudi cells. Daudi cells were incubated with 10 μg/ml Al647 mAb and 1.6 μg/ml Al488 C1q in medium for 60 min at 37°C. Samples were washed, fixed, concentrated, and then analyzed by multispectral high resolution digital imaging. A, Fluorescence intensity of Al488 C1q and Al647 mAbs bound to Daudi cells.
OFA or with RTX is clearly demonstrable for relatively high input concentrations of Clq. Clq binding to OFA-opsonized cells appears to reach saturation; analyses of the binding isotherms gave $K_D$ for binding of Clq to OFA-opsonized Raji cells and Daudi cells of 12 and 16 nM, respectively. However, considerably less Clq is bound to RTX-opsonized cells; binding does not appear to reach saturation and a $K_D$ could not be calculated. It is noted that the differences in Clq binding with respect to OFA opsonization vs RTX opsonization are quite pronounced for Clq input concentrations of 10 $\mu$g/ml or less. Binding of Clq to the RTX-opsonized cells does appear to be specific, because this level of binding is greater than the Clq binding observed for either unopsonized cells or for cells opsonized with an IgG4 control CD20 mAb (7D8), which contains a K322A Clq binding site mutation to completely abrogate Clq binding. B cells from most CLL patients express considerably less CD20 than is seen on B cell lines (16, 17, 20) and, as illustrated in Fig. 1C, there is a lower level of binding of Clq to OFA- or to RTX-opsonized B cells from a representative CLL patient, whose cells express $\sim 1/15$ of the amount of CD20 found on Daudi cells. Clq binding to the OFA-opsonized CLL cells can be fit to a binding isotherm, but the avidity of binding is lower ($K_D = 106$ nM) than to the OFA-opsonized B cell lines, likely reflecting lower numbers of multimers of IgG OFA on the cell surface that are required to promote high avidity Clq binding. Finally, in agreement with the studies with B cell lines, there is less binding of Clq to the RTX-opsonized CLL cells than to the OFA-opsonized CLL cells, and the results for RTX cannot be fit to a binding isotherm.

We investigated both the kinetics and dose-response aspects of Clq binding to 3 different B cell lines, in the presence of OFA or RTX (Fig. 1D–F). The rate of binding of Clq to the cells is slower than the rate at which the mAbs bind (Fig. 1G–I), and Clq binds more slowly to RTX-opsonized cells than to the OFA-opsonized cells. Also of note, at longer times binding of Clq to OFA-opsonized cells is evident at input Clq concentrations as low as 1 $\mu$g/ml; however, in most cases, under these conditions Clq binding to RTX-opsonized cells is only marginally above background.

To further examine the interaction of Clq with cell-bound mAbs, we used multispectral imaging technology (51) to examine binding of Al488 Clq to Daudi cells opsonized with Al647-labeled mAbs. This technique is based on high resolution digital imaging of thousands of cells in a flow cytometry environment, and allows for quantitative determination of both the binding of different fluorescent probes as well as the degree of colocalization of the probes on individual cells. The results in Fig. 2A and Table I, in which the geometric mean fluorescence intensities of binding are displayed, are in good agreement with the flow cytometry experiments. All three Al647 CD20 mAbs bind well to Daudi cells after a 1-h incubation at 37°C (striped bars), but only in the case of OFA-opsonized cells is a high level of binding of Clq evident (black bars). We also found that Al488 Clq is indeed bound to Daudi cells in close proximity to Al647 OFA, as is seen for representative individual cells displayed in Fig. 2B. To test for colocalization quantitatively, double-positive cells (Fig. 2C) were analyzed for colocalization based on an algorithm that calculates the bright detail similarity score (51). Values of 3–3.5 indicate substantial colocalization, and values of 2.5–3 indicate moderate colocalization. The results of three independent experiments (Table I) reveal that Al488 Clq bound to Al647 OFA-opsonized cells is indeed colocalized with bound OFA. However, little colocalization of Clq with RTX is observed, most likely because the absolute level of binding of Clq to RTX-opsonized cells is quite low, thus precluding a rigorous quantitative analysis due to a low signal to noise ratio.

Small amounts of cell-bound Clq are adequate to promote complement activation and CDC on OFA-opsonized B cells

The results of the Clq-binding isotherm experiments and colocalization studies suggest that Clq and OFA may interact avidly when they are bound together on a cell. As it is the binding of Clq to several molecules of cell-bound IgG that activates the CP of complement (1, 44 – 46), we next examined whether cell-bound OFA or RTX could promote CDC with limited amounts of Clq. To address this question, Daudi cells were reacted with OFA or with RTX in Clq-depleted normal human serum (NHS) supplemented with different concentrations of exogenous Clq. The results in Fig. 3A reveal that in Clq-depleted NHS, exogenous Clq can promote CDC of OFA- or of RTX-opsonized Daudi cells after a 15 min incubation at 37°C. Moreover, a higher level of CDC is achieved in the presence of OFA, and the maximum level of killing (in effect, saturation of CDC) is obtained at lower Clq concentrations for OFA than for RTX.

We next examined the Clq-concentration dependence and kinetics of CDC of OFA-opsonized Raji and Z138 cells in Clq-depleted serum. The results, illustrated in Fig. 3, B–C, show that CDC of these cells can be induced at relatively low concentrations of Clq. Moderate to substantial levels of CDC are evident after incubations of just 4 min, indicating that the OFA-opsonized cells are able to activate the CP and are killed with small amounts of Clq bound, far below saturating levels. We also measured binding of Clq to the cells under the same conditions and found that when the mAb-opsonized cells are killed in Clq-depleted serum supplemented with Clq, very little Clq appears to be bound to the cells (open triangles, Fig. 3, D and E). In contrast, when complement activation is prevented by addition of EDTA to the Clq-depleted serum, Clq binding to the cells is demonstrable and comparable to the binding obtained in medium. Indeed, in medium or in Clq-depleted serum containing EDTA, Clq binding to Raji or Z138 cells is evident at final Clq concentrations of either 35 $\mu$g/ml (top panels, filled triangles and open circles, Fig. 3, D and E, respectively) or 1 $\mu$g/ml (bottom panels). In fact, we have found that as cells are killed by CDC, substantial amounts of both bound Clq and bound mAb dissociate from the cells (our unpublished observations), thus precluding a rigorous measurement of actual Clq

### Table I. Binding of Al488-labeled Clq to mAb-opsonized Daudi cells and colocalization of Clq with mAb

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<td>Al647 mAb (GMF)</td>
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<td>Al647 OFA</td>
<td>181,000</td>
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<td>Al647 RTX</td>
<td>186,000</td>
<td>7,500</td>
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<td>Al647 7D8</td>
<td>133,000</td>
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* Different Al488 Clq preparation.
* IgG4 (K322A).
* BDSS, Bright detail similarity score; GMF, geometric mean fluorescence.
supplemented with 1, 2, or 35 cells were incubated at 37°C for 1–20 min in 50% C1q-depleted NHS. OFA-opsonized cells are killed very rapidly. OFA-opsonized Raji (C) or Z138 (C) are killed more effectively in C1q-depleted serum supplemented with low C1q concentrations than are RTX-opsonized cells. In contrast, binding to cells in medium (<10% killed after 10 min) corresponded to 24,000 MESF and background C1q binding (no C1q added) was 1,800 MESF. Additional details of these observations, including analyses of the mechanisms responsible for dissociation of bound mAbs and C1q from dead cells, will be reported separately.

**Comparison of OFA to RTX with respect to C1q binding and CDC of three cell lines**

In view of the relatively high efficacy of OFA in promoting CDC in the presence of small amounts of C1q, we next conducted a systematic comparison of OFA with RTX to determine how much C1q binding to cells is adequate to promote rapid C3b deposition and CDC in C1q-depleted serum. For the reasons noted above, C1q binding to the cells was determined in medium to provide a reasonable estimate of the maximum amount of C1q that could have been bound to the cells before initiation of CDC. The results for Raji cells are presented in Fig. 4, and similar results for Daudi cells and Z138 cells are presented in Figs. 5 and 6, respectively. As noted previously, relatively small amounts of C1q are bound to the cells at C1q concentrations of 1–2 μg/ml (Figs. 4A, 5A, and 6A), but this small amount of C1q binding promotes both C3b deposition and CDC (Figs. 4, B and C, 5, B and C; and 6, B and C). C1q concentrations of 0.1–0.33 μg/ml gave less than 30% CDC (not shown). The results for OFA and RTX are qualitatively similar, but it is noteworthy that much more C3b deposition and CDC were obtained for OFA-opsonized cells. In most cases, after 24 min, 1 μg/ml C1q promoted more C3b deposition and CDC on OFA-opsonized cells than 35 μg/ml C1q with RTX-opsonized cells. We note that after 24 min, modest amounts of C3b deposition and CDC are observed in C1q-depleted serum that was not supplemented with C1q for both Raji cells and Daudi cells, but not for Z138 cells. These results may be due to trace amounts of residual C1q in the depleted serum, or could be due low-level activation of the alternative pathway of complement by the OFA-opsonized cells. For example, in one prototype experiment in C1q-depleted serum, the killing of OFA-opsonized Raji cells was 16% with no C1q added and increased to 85% after addition of C1q to a concentration of 10 μg/ml. In the presence of Mg-EGTA, only permissive for the alternative pathway, killing was reduced to 9% for both conditions, which was higher than background (3%, no mAb added).

We next normalized all of the data obtained in the C1q binding, C3b deposition, and CDC experiments (Figs. 4, A–C, 5, A–C, and 6, A–C) to estimate, relative to approximate saturation of binding by C1q (60 min, 35 μg/ml C1q, OFA-opsonized cells), how much C1q must be bound to the mAb-opsonized cells to maximize C3b deposition and CDC. The results (Figs. 4, D–G, 5, D–G; and 6, D–G) indicate that maximum C3b deposition and CDC are obtained when ~10–20% of the potential C1q binding sites on the OFA-opsonized cells are occupied. Comparison of the curves for OFA- vs RTX-opsonized cells reveals that maximum activity is reached at lower C1q concentrations for cells reacted with OFA, and the maximum amount of CDC and C3b deposition promoted by C1q on RTX-opsonized cells is considerably less than that observed for OFA-opsonized cells.

![FIGURE 3. OFA-opsonized cells are killed more effectively in C1q-depleted serum supplemented with low C1q concentrations than are RTX-opsonized cells. A, Daudi cells were combined with OFA, RTX, or with no mAb in 50% C1q-depleted NHS supplemented with varying amounts of C1q. After a 15-min incubation at 37°C, the cells were washed and probed with PI to determine CDC. B and C, In the presence of C1q, OFA-opsonized cells are killed very rapidly. OFA-opsonized Raji (B) or Z138 (C) cells were incubated at 37°C for 1–20 min in 50% C1q-depleted NHS supplemented with 1, 2, or 35 μg/ml C1q. D and E, In C1q-depleted NHS supplemented with either 35 or 1 μg/ml C1q, binding of C1q to OFA-opsonized Raji or Z138 cells is quite weak (open triangles). Addition of 10 mM EDTA to block CDC restores C1q binding (open circles) to levels close to those observed in medium (closed triangles). Background binding for cells reacted with OFA and C1q-depleted serum (no C1q added) was 1800 MESF (Raji cells) and 1500 MESF (Z138 cells). Representative of two similar experiments shown. In addition, very similar results were obtained for Daudi cells (data not shown).](http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/ by guest on September 15, 2017)
Extension of the paradigm to CLL cells

To further extend these observations, we evaluated the reactivity of C1q with OFA- or RTX-opsonized primary CLL cells in medium and in C1q-depleted NHS. Representative results for one patient are shown in Fig. 7, A–C. As with the B cell lines, we found that more C1q bound to OFA-opsonized primary CLL cells than to RTX-opsonized cells (Fig. 7A). Consistent with this observation, in C1q-depleted NHS, OFA was able to promote considerably more C3b deposition and CDC on the CLL cells upon addition of...
small amounts of C1q (Fig. 7, B and C). Indeed, at C1q concentrations of 2 μg/ml, we observed more C3b deposition and CDC for OPA-opsonized cells than for RTX-opsonized cells at 35 μg/ml C1q. The absolute levels of CDC for the OPA-opsonized CLL cells in C1q-depleted NHS supplemented with C1q were less than those obtained with the cell lines, likely due to a combination of lower levels of CD20 on the CLL cells as well as up-regulation of CCP on the cells. We also evaluated C3b deposition and CDC in 50% NHS. After a 1-h incubation, C3b deposition corresponded to 4100 ± 200 and 1200 ± 15 MESF for cells reacted with OPA or with RTX, respectively; CDC was 63 ± 2% and 18 ± 1%. These results are in agreement with the reports of Beum et al. (28) and Teeling et al. (38, 39) that OPA is far more effective in mediating C3b deposition and CDC than RTX in complete NHS. It is also interesting to note that in C1q-depleted NHS, C3b deposition on OPA-opsonized cells in the presence of 2 μg/ml C1q (1390 ± 15 MESF units) is slightly higher than C3b deposition for RTX-opsonized cells in intact 50% NHS. Finally, in similar experiments we compared OPA with RTX, with respect to C1q binding, C3b deposition, and CDC for B cells from 7 CLL patients, for C1q concentrations of 35 μg/ml (Fig. 7D) and incubations of 60 min. The results indicate that OPA that was bound to the CLL cells was significantly more effective than RTX in promoting C1q binding, complement activation, and CDC for all seven patient samples.
Discussion

The principal finding of this paper is that very small amounts of C1q can rapidly bind to OFA-opsonized cells and effectively activate complement and promote C3b deposition and CDC. The binding isotherms for C1q binding to OFA-opsonized Raji and Daudi cells indicate that binding is of relatively high avidity, characterized by a $K_D$ of 12–16 nM, which is almost as strong as the avidity of binding of RTX or OFA to CD20 on B cells ($K_D = 5$ nM) (39, 52). Based on comparable measurements (Fig. 1, A and B), the avidity of binding of C1q to RTX-opsonized cells must be considerably lower, and binding does not reach saturation even after a 1-h incubation. During the first 4–10 min of reaction of cells with these mAbs in medium (Figs. 3–7) or in C1q-depleted serum supplemented with C1q (Fig. 3), the amount of C1q bound to OFA- or to RTX-opsonized cells is modest and corresponds to less than 10% of the maximum amount of C1q that can be bound to OFA-opsonized cells in medium. However, although C1q binding is low under these conditions, enough C1q is bound to promote substantial C3b deposition and CDC for cells opsonized with OFA (Figs. 3–7).

Indeed, in C1q-depleted serum, C1q concentrations of 1 $\mu$g/ml promote complement activation and CDC of OFA-opsonized cells (Figs. 4–6). Wouters et al. have reported that C1q concentrations of 5 ng/ml are sufficient to induce lysis of Ab-opsonized sheep erythrocytes (53). The requirement for a higher threshold in the present study is reasonable, as nucleated human cells express several CCP and use other mechanisms that increase the cells’ resistance to CDC (8–11, 17, 54, 55). Thus, multiple sites on the cells must bind enough OFA or RTX so that large numbers of membrane attack complexes can be generated. However, our results indicate that cells do not have to be saturated with C1q to activate complement and generate lethal hits. It appears that close to maximum complement activation and CDC is reached at a threshold for Clq binding, which corresponds to less than 20% of saturation; binding of more C1q does not lead to higher levels of C3b deposition or CDC. In our calculations of the degree of saturation of Clq binding, we have presumed that the number of Clq binding sites should be the same for OFA- or for RTX-opsonized cells, as both anti-CD20 mAbs are human IgG1 and bind to cells with comparable affinities (38). Wouters et al. reported that during complement activation, complexes consisting of C1q covalently cross-linked with either C4d or C3d are generated, and the covalently modified Clq had decreased hemolytic activity (53). Although we did not examine the present system for such complexes, OFA-opsonized nucleated cells were killed with high efficacy at low Clq concentrations, suggesting that if such complexes were formed, they did not inhibit CDC.

Binding of RTX to large numbers of CD20 molecules on B cells is necessary to promote CDC, and the number required may be governed in part by expression of CCP on the cell (8, 10, 17). If CCP are highly expressed, then RTX-mediated CDC may not occur unless the cell also has high levels of CD20. Van Meerten et al. have demonstrated that in a T cell line, transfection with at least 25,000 CD20 molecules per cell is required to obtain even low levels of RTX-mediated CDC; at least 60,000 CD20 per cell are required to achieve RTX-mediated CDC of the majority of the cells (56). Our results, taken with the reports of Teeling et al. (38, 39), suggest that in comparing OFA with RTX, the threshold level for effective Clq binding leading to CDC is reduced considerably, likely due to enhanced interaction of Clq with cell-bound OFA. This enhanced interaction is demonstrable based on the binding and colocalization studies (Figs. 1–2, Table I). Saturation of binding of either RTX or OFA to CD20-positive cells is achieved at mAb concentrations of 10 $\mu$g/ml (23, 38, 56, 57). Approximate saturation of Clq binding to OFA-opsonized cells is reached at Clq concentrations of 35 $\mu$g/ml, whereas RTX-opsonized cells do not reach saturation at 75 $\mu$g/ml Clq (Fig. 1).

Experiments with primary CLL cells revealed similar differences in Clq binding, C3b deposition, and CDC reactivity between OFA and RTX-opsonized cells (Figs. 1C and 7, A–C). However, the avidity of binding and amount of Clq bound to OFA-opsonized CLL cells is less than the Clq binding observed for OFA-opsonized B cell lines, which express considerably higher levels of CD20. In principle up to six closely associated cell-bound IgG molecules can bind a single Clq molecule with high avidity (43–46). The reduction in Clq binding strength for OFA-opsonized CLL cells compared with OFA-opsonized B cell lines suggests that at saturation the oligomerization level of cell-associated OFA IgG multimers is lower for CLL cells. CD20 appears to be organized on cells in small patches composed of at least tetramers, and at high CD20 densities the CD20 molecules may be further cross-linked after chelation by the anti-CD20 mAbs (16). The maximal organization of Clq into hexamers may not be achieved at low CD20 expression levels. Notably, the avidity of Clq binding to RTX-opsonized cells is considerably reduced relative to OFA-opsonized cells and could not be accurately quantified in our experiments. Thus, even at comparable CD20 expression levels, RTX may organize into lower level oligomers than OFA.

The role of complement in CD20 immunotherapy is still being discussed despite a large body of evidence from in vitro and in vivo studies, as well as observations in the clinic supporting its contribution (7, 17, 23–28). In our in vitro comparisons between OFA and RTX, we have shown that a main differentiator between these two CD20 mAbs is the strong ability of OFA to engage complement (Ref. 28, 38, 39, and this manuscript). For CLL cells, which express low levels of CD20, in particular, we have observed potent complement-mediated killing in vitro with OFA, whereas these cells are relatively resistant to RTX (28, 38). Single-agent RTX has very limited activity in relapsed/refractory CLL compared with other low-grade B cell malignancies (58–60). Fludarabine-refractory CLL specifically is associated with low response rates, even when treated with high-dose RTX regimens (60). The excellent single-agent activity of OFA in fludarabine-refractory CLL is therefore of high interest (42). Significantly, these observations indicate that the effective killing of CLL tumor cells via complement by OFA but not RTX may translate into clinical benefit. This observation supports the notion that effective complement activation is an important component of the in vivo activity of CD20 Abs.

Racila et al. reported that polymorphisms in $ClqA_{276A/G}$ impacted on responses to RTX in therapy of follicular lymphoma (61). Patients with the G allele had a poorer outcome, yet this form of the protein is associated with more complement activity, which may bring the role of complement into question, at least for RTX in this indication. However, the mechanism by which the $ClqA_{276A/G}$ polymorphism affects RTX treatment is not understood. Perhaps $ClqA_{276A}$ is activated more readily, resulting in faster complement consumption. Our measurements were made with a commercial pooled source of Clq added to Clq-depleted NHS. In the future it will be interesting to compare the activities of the two Clq allotypes in our system. That is, how well do they activate complement and promote C3b deposition and CDC of B cells in Clq-depleted serum in the presence of OFA or RTX. The ability of OFA to maintain CDC activity at low concentrations of both Clq allotypes will be of particular interest.
OFAs and RTX rely on the body’s effector mechanisms, e.g., CDC, Ab-dependent cellular cytotoxicity by NK cells, and clearance/phagocytosis by fixed tissue macrophages, to kill targeted CD20⁺ cells, and at high cell burdens these mechanisms may be temporarily saturated due to exhaustion of killing mechanisms (16, 62). We reported that in certain CLL patients, infusion of RTX leads to consumption of greater than 90% of the patients’ hemo-lytic complement activities for days to weeks, thus potentially limiting CDC activity promoted by RTX (20). We suggested that use of fresh frozen plasma could enhance or restore complement titers and RTX therapeutic activity in CLL patients (20, 63); anecdotal results reported in selected patients have provided some support for this idea (64). These findings, taken together with the present results, suggest that for certain B cell lymphomas, OFA may have a higher level of therapeutic activity than RTX, and the differences in activity between the mAbs may be particularly important under conditions of high tumor burden where complement is more likely to be consumed and potentially exhausted.

The concentration of C1q in human plasma is 70 μg/ml (43), and it is remarkable that C1q concentrations as low as 1 μg/ml can support substantial complement activation and CDC by OFA-opsonized cells. Presumably, the highly avidity binding of C1q to an oligomer of OFA molecules in close proximity to the cell membrane (39) allows for generation of a new cascade of complement activation in a site protected from fluid phase agents that would otherwise down-regulate the complement cascade. Generation of the membrane attack complex in such close juxtaposition to the cell membrane may also allow for more effective CDC. Our work suggests that a particularly fruitful area of investigation could be based on the presence of OFA-opsonized cells in conditions of high tumor burden where complement is more likely to be consumed and potentially exhausted.

References


Disclosures

F.J.B., J.G.J.W., and P.W.H.I.P. are employees of Gennmab. R.P.T. received a research grant from Gennmab.

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