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Ofatumumab Is More Efficient than Rituximab in Lysing B Chronic Lymphocytic Leukemia Cells in Whole Blood and in Combination with Chemotherapy

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J Immunol 2013; 190:231-239; Prepublished online 5 December 2012;

doi: 10.4049/jimmunol.1202645

<http://www.jimmunol.org/content/190/1/231>

Supplementary Material <http://www.jimmunol.org/content/suppl/2012/12/07/jimmunol.1202645.DC1>

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Ofatumumab Is More Efficient than Rituximab in Lysing B Chronic Lymphocytic Leukemia Cells in Whole Blood and in Combination with Chemotherapy

Luca Bologna, Elisa Gotti, Fabio Da Roit, Tamara Intermesoli, Alessandro Rambaldi, Martino Introna, and Josée Golay

Ofatumumab (OFA) is a human anti-CD20 Ab approved for treatment of fludarabine-refractory B chronic lymphocytic leukemia (B-CLL). The efficacy of different immunotherapeutic strategies is best investigated in conditions that are as physiologic as possible. We have therefore compared the activity OFA and rituximab (RTX), alone or in combination with chemotherapeutic agents in unmanipulated whole blood assays, using flow cytometry. OFA (10–100 $\mu\text{g/ml}$) lysed B-CLL targets in whole blood more efficiently and with faster kinetics than RTX, with a mean 56% lysis at 24 h compared with 16%. This activity of OFA was fully complement dependent, as shown by >99% inhibition by anti-C5 Ab eculizumab and a lack of NK cell activation in whole blood. OFA-mediated NK cell activation was blocked by complement. OFA-mediated lysis could be increased an additional 15% by blocking CD55 and CD59 complement inhibitors. Interestingly, OFA-mediated lysis correlated significantly with CD20 expression levels ($r^2 = 0.79$). OFA showed overlapping dose response curves similar to those for RTX in phagocytosis assays using either human macrophages or neutrophils. However, phagocytosis was inhibited in the presence of serum or whole blood. Finally, combined treatment with mafosfamide and fludarabine showed that these therapeutic drugs are synergistic in B-CLL whole blood assays and show superior activity when combined with OFA compared with RTX. These results confirm in B-CLL samples and in physiologic conditions the superior complement mediated cytotoxicity induced by OFA alone compared with RTX, the lack of NK cell activation, and phagocytosis in these conditions and suggest effective chemoimmunotherapy strategies using this new generation anti-CD20 Ab. *The Journal of Immunology*, 2013, 190: 231–239.

The chimeric anti-CD20 mAb rituximab (RTX, MabThera) represents the gold standard for the treatment of B non-Hodgkin lymphoma (B-NHL) and other B cell neoplasias, when used in combination with chemotherapy in cyclophosphamide or fludarabine containing regimens. As a single agent, however, RTX has shown limited efficacy, especially in B chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma (MCL) (1–3). In B-CLL, RTX has therapeutic activity at the higher dose levels, but there is still need to substantially improve its activity (4).

The mechanisms of action of RTX in man include complement-mediated cytotoxicity (CDC), Ab-dependent cytotoxicity (ADCC), and phagocytosis (5). Several factors have been reported to influence its activity in vivo: high tumor burdens, Fc γ Rs polymorphisms, CD20 expression levels, modulation of CD20

expression, and complement deficiency or consumption (6–9). The knowledge about translation of preclinical findings into the clinic is, however, still incomplete, and the most important mechanisms of action of in vivo in man in different clinical contexts are still controversial, despite a number of studies performed in vitro with a variety of isolated effector and target cells and several mouse models investigated.

To further improve the therapeutic efficacy of RTX, several new or modified anti-CD20 MAbs have been developed. In comparison with RTX, these mAbs are humanized or fully human and selected for either increased or decreased complement activation capacity, ADCC, or proapoptotic property. Ofatumumab (OFA; Arzerra) is a type I, fully human Ig (IgG1k) Ab. It binds to a different epitope of the CD20 cell surface Ag, closer to the cell membrane, redistributes CD20 into lipid rafts, binds more avidly to C1q (the first component of the complement cascade), and has a slower off-rate compared with RTX (10–12). These properties are thought to be the basis for the more efficient complement activation induced by OFA after binding to a variety of target cells, including primary B-CLL cells, which express low CD20 levels and resist RTX-mediated CDC (8, 11, 13). Besides activating complement efficiently, OFA has been shown to mediate ADCC by NK cells (10, 14). Little is known about other possible mechanisms, such as phagocytosis by macrophages and polymorphonuclear cells (PMNs).

OFA is currently under evaluation in phase II–III clinical trials in B-NHL and B-CLL, as a single agent or in combination with chemotherapy (<http://www.clinicaltrials.gov>) (15–18). Overall response rates as a single agent in relapsed or refractory B-CLL are 44–58% and 73–77% in previously untreated B-CLL (17–19), which compares favorably with the response previously observed for this subgroup of patients subjected to RTX monotherapy (20).

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Received for publication September 20, 2012. Accepted for publication November 2, 2012.

This work was supported by a grant from the Associazione Italiana Ricerca contro il Cancro (to J.G.) and the Associazione Italiana Lotta alle Leucemie, Linfomi e Mieloma.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; ADCC, Ab-dependent cytotoxicity; B-CLL, B chronic lymphocytic leukemia; B-NHL, B non-Hodgkin lymphoma; CDC, complement-mediated cytotoxicity; HS, human serum; MCL, mantle cell lymphoma; MNC, mononuclear cell; OFA, ofatumumab; PMN, polymorphonuclear cell; RTX, rituximab; TRX, trastuzumab.

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Furthermore, overall response to OFA is ~43% in B-CLL patients relapsed or refractory to RTX treatment (18). These data suggest an improved clinical activity of OFA in B-CLL with respect to the gold standard anti-CD20 Ab RTX. This higher activity may be due to its higher capacity to activate complement, particularly with neoplastic cells like B-CLL, which express low levels of CD20 (13), but this still remains to be demonstrated.

To compare the activity of OFA to that of RTX and identify the major mechanisms of action of this Ab in experimental conditions as physiologic as possible, we have investigated the activity of these anti-CD20 mAbs in unmanipulated B-CLL whole blood samples drawn in lepirudin (21, 22). We show that OFA is indeed more effective than RTX through a fully complement-mediated mechanism and that it can cooperate with standard chemotherapeutic agents such as fludarabine and mafosfamide.

Materials and Methods

Cells and Abs

Peripheral blood was drawn in lepirudin (Refludan; Celgene, Summit, NJ) at 50 µg/ml final concentration. Blood was obtained from patients with B-CLL/MCL, either at diagnosis or before any treatment, or from normal donors after informed consent. All patients were diagnosed by routine immunophenotypic, morphologic, and clinical criteria. Double staining with CD19 and sIgk or sIgλ was performed to establish monoclonality and determine the percentage of neoplastic versus normal B cells present in the samples (<4% normal B cells with respect to neoplastic ones). In some cases, the mononuclear cell (MNC) fraction was also purified by standard Ficoll Hypaque gradient centrifugation (Seromed, Berlin, Germany). MNCs were then cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine (all from Euroclone, Milano Italy), and 110 µM gentamicin (PHT Pharma, Milan, Italy). The study was approved by the hospital ethics committee.

RTX (MabThera, chimeric IgG1κ) was obtained from Roche Italia (Monza, Italy); OFA (Arzerra, human IgG1κ) was a gift from Glaxo Smith Kline (Verona, Italy). Control Ab trastuzumab (TRX; Herceptin, humanized IgG1κ; Roche Italia) was a gift from Dr. Carlo Tondini (Oncology Department, Ospedali Riuniti, Bergamo, Italy). Anti-CD52 Alemtuzumab was obtained from Schering. Defucosylated anti-CD20 GA101 (obinutuzumab, humanized IgGκ) was a gift from Dr. C. Klein (Roche Glycart, Schlieren, Switzerland) (22). Blocking anti-C5 Ab Eculizumab (Soliris, Alexion Pharmaceuticals, Cheshire, CT) was a gift from Prof. A. Vannucchi (University of Florence, Italy).

CD20 expression levels

Whenever possible, the absolute number of CD20 molecules was measured on B-CLL/B-NHL cells using PE-labeled anti-CD20 Ab and calibrated Quantibrite beads (BD Biosciences, San Jose, CA), as described previously (8).

Complement-mediated cytotoxicity

Purified B-CLL cells were cultured at 2×10^6 /ml in medium with 20% human serum (HS), and different concentrations of mAbs were added, including TRX as a negative control. Cells were collected after 4–24 h incubation at 37°C 5% CO₂, stained with CD19-FITC and 7-aminoactinomycin D (7AAD), and analyzed by flow cytometry using a FACSCanto II instrument (BD Biosciences).

Measurement of Ab-induced cell death in whole blood

Unmanipulated peripheral blood (400 µl) of B-CLL/B-NHL patients in 50 µg/ml lepirudin (21) was plated in the presence or absence of different concentrations of RTX, OFA, or control irrelevant Ab TRX. In some cases, 200 µg/ml blocking anti-C5 mAb eculizumab (Soliris, Alexion Pharmaceuticals, Cheshire, CT), 10 µg/ml of functionally blocking anti-CD55 (BRIC216; International Blood Group Reference Laboratory [IBGRL], Bristol, U.K.) or anti-CD59 (BRIC229; IBGRL) Abs, or control irrelevant Ab was added 5 min before the lytic Abs. Whole blood samples were incubated for 2–24 h at 37°C, 5% CO₂, and then stained for 15 min at room temperature with allophycocyanin-Cy7-conjugated anti-CD-45, FITC-conjugated anti-CD19 Ab and PerCP-7AAD (BD Biosciences), as described previously (22). After incubation, samples were lysed with hypotonic lysis solution (Pharm Lyse; BD Biosciences) to eliminate platelets

and RBCs. Samples were then analyzed by double fluorescence on a FACSCanto II instrument (BD Biosciences). Cell death was measured as a decrease in CD19⁺/7AAD[−] population in treated versus control samples after gating on the CD45⁺ population.

Measurement of NK cell activation

MNCs or whole blood in lepirudin from patients or normal donors were incubated for 2 h at 37°C with 5% CO₂ with different concentrations of OFA, RTX, and GA101 as positive control (22) or TRX as negative control. Cells were then incubated with anti-CD56–Cy7 and anti-CD107a–PE for 15 min, washed in PBS and, in the case of whole blood, red cells were lysed in hypotonic lysing solution as above and analyzed using a FACSCanto II Instrument (BD Biosciences). Cells were gated on the mononuclear population and percentage CD107a in the CD56⁺ fraction was then measured. Double staining of the control samples with anti-CD56–Cy7 and anti-CD16–FITC demonstrated in all cases that more than 95% of CD56⁺ cells were CD16⁺ NK cells.

Phagocytosis assay

In vitro differentiated macrophages were obtained from purified CD14⁺ monocytes cultured in the presence of 20 ng/ml recombinant human M-CSF (R&D Systems) for 5–7 d, as described previously (23). The cells were then stained with 2 µM PKH26 (Sigma-Aldrich), washed, and incubated in culture medium overnight. Thawed B-CLL cells were stained with 0.1 µM CFSE (Molecular Probes) and then plated on the macrophages at a 1:1 ratio, in the presence or absence of therapeutic Abs. In some cases, whole blood from healthy donors was added. The plates were incubated at 37°C for 2 h, harvested, washed, stained with CD19–Cy7 (BD Biosciences), and analyzed with a flow cytometer (BD FACSCanto II, BD Biosciences). Whole blood samples were lysed and washed prior to FACS analysis. Phagocytosing cells were defined as PKH26⁺/CFSE⁺/CD19APC[−].

PMNs were purified from peripheral blood of normal donors by layering them on standard Ficoll Hypaque gradient centrifugation (Seromed, Berlin, Germany). The lowest phase was collected, and sedimentation over a 4% dextran solution for 30 min was performed. The upper phase contained >90% CD15⁺ PMNs. Thawed B-CLL cells were stained with 2 µM PKH26 and mixed with purified PMNs or whole blood in a 5:1 ratio (B-CLL:PMN), in presence or absence of Abs. In some experiments with purified PMNs, 20% HS or heat-inactivated HS was added. Cells were incubated at 37°C for 2–24 h, and then stained with anti-CD15–FITC and CD19–Cy7 (BD Biosciences), washed, and analyzed with a flow cytometer (BD FACSCanto II, BD Biosciences). Percentage phagocytosis was defined as the percentage of PKH26⁺/CD15⁺/CD19Cy7[−] cells relative to total CD15⁺ cells. In some cases, samples were centrifuged onto glass slides at 500 rpm for 5 min using a Shandon centrifuge. Slides were dried, fixed in 2% paraformaldehyde, and stained with 1.5 µg/ml DAPI in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Slides were viewed with a Nikon Eclipse E800 microscope using a Nikon 20× Plan Fluor DICM lens. At least four representative fields were photographed. The total number of PMNs (PKH26[−]/DAPI⁺/CD15FITC⁺) and the percentage of phagocytic PMN (PKH26⁺/DAPI⁺/CD15FITC⁺) were counted in a double-blind fashion using the Image J program (National Institutes of Health).

Treatment with OFA combined with chemotherapy

Purified B-CLL cells were cultured at 2×10^6 /ml in medium supplemented with mafosfamide and fludarabine for 24 h at 1 µg/ml each. After 24 h, 20% HS and different concentrations of anti-CD20 MAb were added. Cells were incubated for an additional 4 h at 37°C 5% CO₂, stained with CD19-FITC and 7AAD, and analyzed with flow cytometry using FACSCanto II.

Statistical analysis

The data were analyzed using paired or unpaired Student *t* tests, as appropriate. The *p* values are **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Results

OFA lyses B-CLL cells in whole blood in a fully complement-dependent manner in lepirudin drawn blood

To investigate the activity of OFA in physiologic conditions, we created an assay in unmanipulated whole blood. For this purpose, we searched for an anticoagulant that would not interfere with any

of the known immune-mediated or direct effects of therapeutic mAbs and tested in particular sodium citrate and lepirudin (21). We observed that neither sodium citrate nor lepirudin inhibited the deposition of complement fragments C3 and C9 onto the target cell membrane (Fig. 1A) or CDC (Fig. 1B). In contrast, citrate significantly inhibited CD107a induction, a marker for NK degranulation (Fig. 1C), as well as ADCC itself (Fig. 1D), whereas lepirudin had no effect on these mechanisms (Fig. 1C, 1D). Similarly, phagocytosis by macrophages was decreased by citrate, although not significantly, but was unaltered in the presence of lepirudin (Fig. 1E). Finally, citrate or lepirudin had no effect on the homotypic adhesion induced by the type II anti-CD20 mAb GA101 (Fig. 1F). We therefore chose lepirudin for all subsequent studies, because this anti-coagulant does not interfere with any of the biologic effects of therapeutic Abs tested in vitro.

To further define our experimental conditions with our test Ab OFA, we performed a dose-response curve using purified B-CLL cells and 20% HS. As shown in Fig. 2A, OFA had little activity at 1 $\mu\text{g/ml}$, but reached $>54.7\%$ lysis at 10 $\mu\text{g/ml}$ and $>70\%$ at 100 $\mu\text{g/ml}$. The highest concentration of 500 $\mu\text{g/ml}$ did not significantly increase lysis, suggesting that a plateau is reached at 100 $\mu\text{g/ml}$; therefore, this high dose was not used in further experiments.

We next used B-CLL whole blood samples drawn in lepirudin to investigate the cytotoxic activity of OFA in more physiologic conditions. Samples were incubated with 1, 10, or 100 $\mu\text{g/ml}$ OFA for 4 or 24 h. As shown in Fig. 2B, OFA-mediated cell lysis was maximal already at 4 h, reaching $\sim 55\%$ at 100 $\mu\text{g/ml}$ ($n = 4$). The dose response curve in whole blood was similar to that observed with purified B-CLL in the presence of 20% HS, with significantly higher lysis observed at 100 $\mu\text{g/ml}$ OFA compared with 10 $\mu\text{g/ml}$ ($p < 0.01$; Fig. 2B). The lowest dose (1 $\mu\text{g/ml}$) also had little activity in whole blood. The relatively high dose of mAb required suggested that the major mechanism of OFA in whole blood is CDC. Indeed, this could be demonstrated by the addition of 200 $\mu\text{g/ml}$ anti-C5 MAb eculizumab to the reaction (22, 24), which completely abolished OFA-mediated lysis of B-CLL cells in whole blood, at both 4 and 24 h (Fig. 2C).

To investigate directly whether ADCC might also have a role in the mechanism of action of OFA in whole blood, we measured induction of CD107a at 2 h as a surrogate marker of ADCC (25). We had shown previously that GA101, a defucosylated anti-CD20 Ab, can indeed induce ADCC and NK cell degranulation even in presence of HS or in whole blood (22). We therefore used the GA101 Ab as positive control in these experiments. Using three different B-CLL whole blood samples, we observed limited CD107a in-

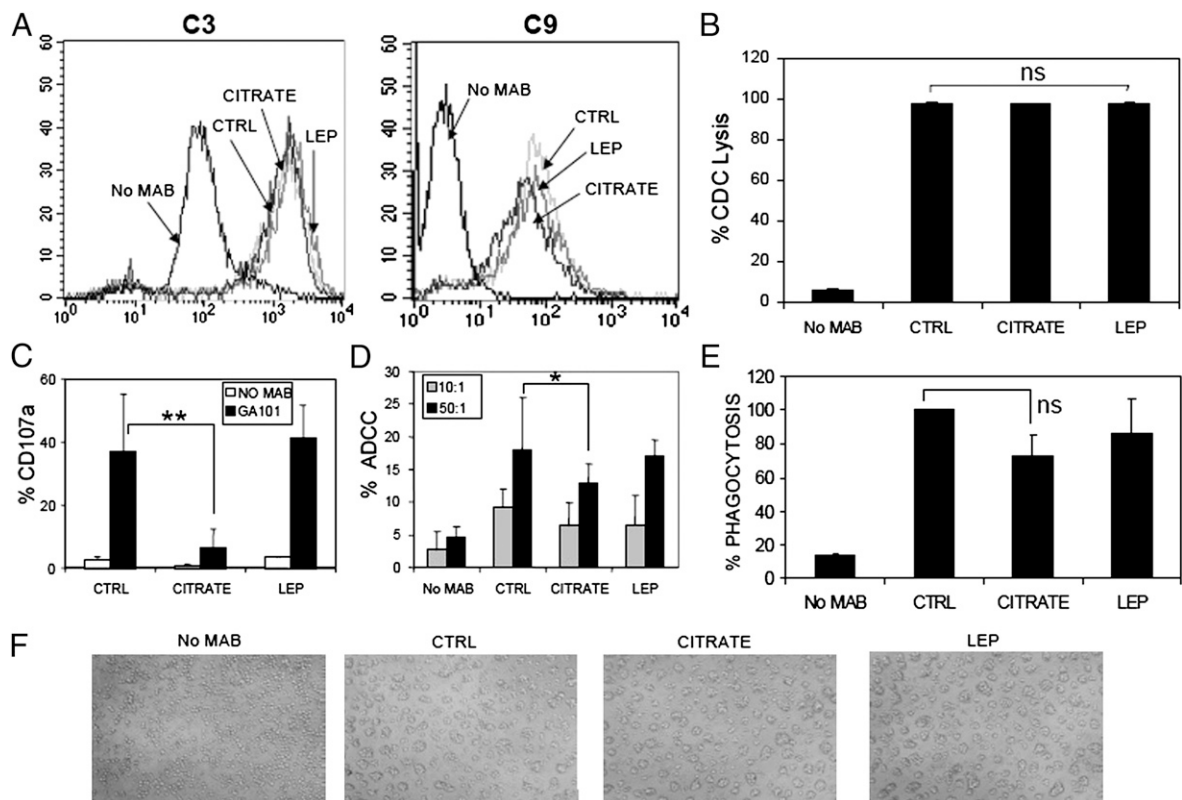


FIGURE 1. Lepirudin does not affect immune-mediated or direct mechanisms of action of therapeutic mAbs. **(A)** C3/C9 deposition: purified B-CLL cells were incubated for 1 h with 10 $\mu\text{g/ml}$ RTX and 20% HS and in the presence or absence of 0.01M Na citrate or 50 $\mu\text{g/ml}$ lepirudin. C3 or C9 deposition was then analyzed by immunofluorescence and flow cytometry. Representative histograms are shown. **(B)** CDC assay: purified B-CLL cells were incubated for 1 h with 10 $\mu\text{g/ml}$ alemtuzumab and 20% HS and in the presence or absence of 0.01 M Na citrate or 50 $\mu\text{g/ml}$ lepirudin. Cell lysis was analyzed by 7AAD staining and flow cytometry. **(C)** NK cell activation: MNCs from a normal donor were incubated with 1 $\mu\text{g/ml}$ GA101 and in the presence or absence of 0.01 M Na citrate or 50 $\mu\text{g/ml}$ lepirudin. After 4 h CD107a expression was measured by flow cytometry. **(D)** ADCC: BJAB cells were labeled with calcein AM and incubated with MNCs at a 10:1 (gray) or 50:1 (black) effector:target ratio in the presence or absence of 1 $\mu\text{g/ml}$ GA101, 0.01 M Na citrate or 50 $\mu\text{g/ml}$ lepirudin. Specific lysis was measured by standard calcein AM release after 4 h. **(E)** Phagocytosis by macrophages: phagocytosis of B-CLL targets by in vitro differentiated macrophages was analyzed in the presence of 0.1 mg/ml RTX and 0.01 M Na citrate or 50 $\mu\text{g/ml}$ lepirudin. All data are the means and standard deviations of three experiments. **(F)** Homotypic adhesion: 10 $\mu\text{g/ml}$ GA101 was added to purified B-CLL cells in the presence or absence of 0.01 M Na citrate or 50 $\mu\text{g/ml}$ lepirudin and homotypic adhesion evaluated by microscopic examination after 24 h. A representative image of each condition is shown. * $p < 0.05$, ** $p < 0.01$ compared with controls.

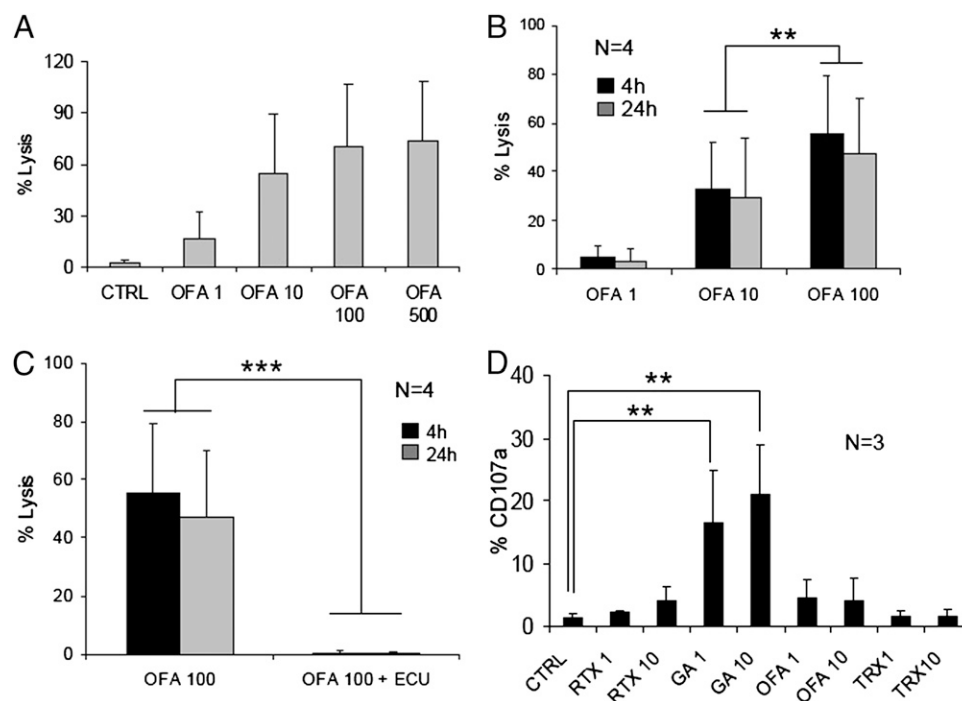


FIGURE 2. OFA kills B-CLL cells in whole blood through complement but not ADCC. (A–C) B-CLL patient's mononuclear cells (MNC) in culture medium plus 20% HS (A) or whole blood in 50 μ g/ml lepirudin (B, C) were incubated with 1, 10, or 100 μ g/ml OFA for 4 h (A), or 4 and 24 h (B, C). In some cases (C), 200 μ g/ml eculizumab (ECU) was also added. Percentage of cell death was measured as a decrease in CD19⁺ 7AAD[−] cells relative to untreated control. (D) B-CLL whole blood samples were incubated with the indicated concentrations of RTX, GA101, OFA or TRX, and CD107a expression analyzed on CD56⁺ NK after 2 h. ** $p < 0.01$, *** $p < 0.001$ compared with controls.

duction in presence of optimal concentrations of either OFA or RTX (1 or 10 μ g/ml), which reached ~4% in both cases, but was not statistically significant (Fig. 2D). In contrast, degranulation of NK cells in whole blood reached 16.4% and 21.1%, with GA101 at 1 and 10 μ g/ml, respectively (Fig. 2D; $p < 0.001$). Lack of CD107a induction by OFA in whole blood was presumably due to inhibition of CD16 binding by the Ab following complement deposition onto the Ab, as already described for RTX (26). Indeed, we could show that CD107a expression induced on purified NK cells by either RTX or OFA was inhibited by addition of 20% HS, but not heat-inactivated HS. In contrast CD107a induction by GA101 was not inhibited (Supplemental Fig. 1). These data confirm the role of complement in inhibiting NK cell activation by either RTX or OFA.

We conclude that OFA, like RTX, does not efficiently activate NK cell degranulation and ADCC in whole blood, in contrast to the defucosylated Ab GA101. Furthermore, these data confirm that the major mechanism of action of OFA in whole blood is complement-mediated lysis.

Lysis of B-CLL in whole blood is more efficient with OFA than with RTX but still depends on CD20 expression levels

It is well known that the relatively low level of CD20 expressed by B-CLL is one important factor of resistance of these cells to RTX treatment, in vitro and in vivo. We therefore compared the activity of OFA or RTX in whole blood and investigated the role of CD20 levels in this lysis. Thirteen untreated B-CLL/MCL whole blood samples were drawn in lepirudin, and increasing concentrations of Abs were added. Lysis was measured at both 4 and 24 h. OFA at 10 and 100 μ g/ml showed superior activity compared with RTX at 4 h (data not shown) and 24 h (Fig. 3A). Lysis was 10.5% and 16% with RTX at 24 h and 40% and 56% with OFA at 10 and 100 μ g/ml, respectively ($p < 0.001$). OFA was therefore ~3.5–4 fold superior versus RTX at these concentrations (Fig. 3A); 100 μ g/ml

OFA was again more effective than 10 μ g/ml of the same Ab ($p < 0.01$, Fig. 3A). We noted, however, that lysis with OFA was highly variable between samples, ranging from 15–99% at 100 μ g/ml Ab (Fig. 3B), which could depend on CD20 expression levels. Indeed, as shown in Fig. 3C, lysis induced by either OFA or RTX correlated directly with the number of CD20 expressed on the cells, except that the level of lysis was in all cases superior with OFA compared with RTX. More in detail, OFA induced measurable CDC lysis (12–35%) even in the presence of B-CLL targets expressing <5000 CD20 molecules per cell. Lysis was higher (59–79%), with expression between 5000 and 10,000 molecules/cell; finally, it was highly efficient (>90%), with CD20 expression of 15,000 and greater. In contrast, RTX-mediated lysis was low (generally <10%) at <10,000 CD20 molecules/cell and reached only 35–50% in cases expressing at least 15,000 CD20 molecules/cell (Fig. 3C).

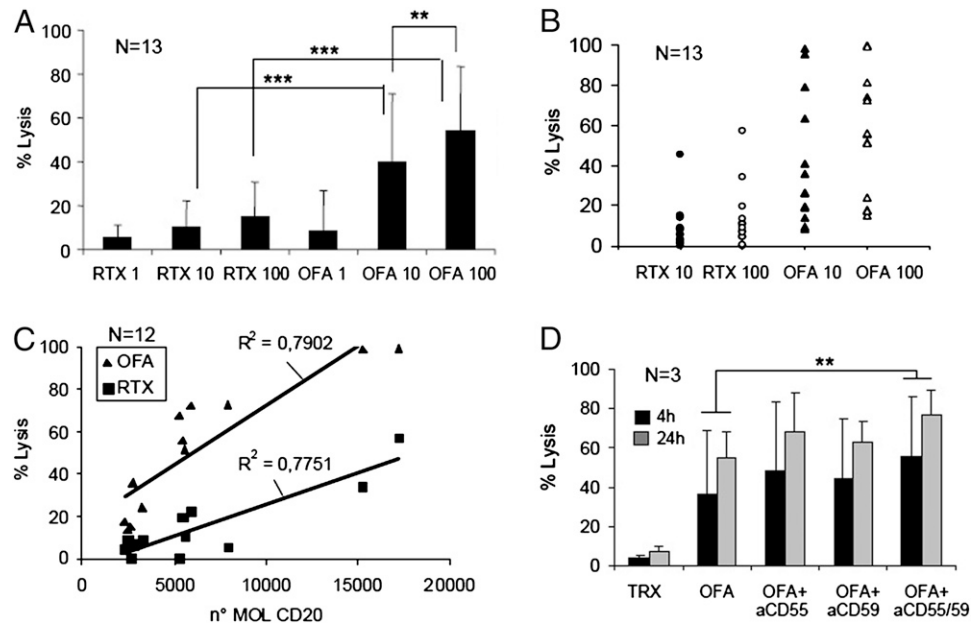
Given the dependence of both OFA and RTX on CD20 expression levels and the fact that these Abs bind to different CD20 epitopes, we investigated whether these Abs could show increased efficacy when combined. We could not, however, observe any additive or synergistic effects of the anti-CD20 when combined compared with the single mAbs (data not shown).

We conclude that OFA-mediated lysis in whole blood is more efficient than that of RTX, but it is still dependent on CD20 expression levels. Furthermore, OFA and RTX do not synergize in these conditions.

OFA-mediated lysis can be enhanced by blocking anti-CD55 or anti-CD59

Because OFA does not induce 100% lysis of B-CLL cells, even after 24 h, we searched for means of increasing this activity. Functional blocking of complement inhibitor proteins CD55 and CD59 levels have an important role in regulating complement-dependent cytotoxicity and can enhance RTX-mediated cytotox-

FIGURE 3. OFA lyses B-CLL more efficiently than RTX in whole blood. (A–C) Thirteen B-CLL–MCL whole blood samples were incubated with 1, 10, or 100 $\mu\text{g/ml}$ OFA or RTX (A) or only 100 $\mu\text{g/ml}$ OFA or RTX (B, C) for 24 h and percentage cell death of neoplastic B cells were measured by flow cytometry. (A) Means and SD for the 13 samples, whereas (B) plots the percentage lysis for each sample. (C) Percentage lysis at 100 $\mu\text{g/ml}$ RTX or OFA was plotted against the measured number of CD20 molecules/cell ($n = 12$). (D) B-CLL–MCL whole blood samples were incubated for 4 (black bars) or 24 h (gray bars) with 10 $\mu\text{g/ml}$ OFA or RTX in the presence or absence of 10 $\mu\text{g/ml}$ blocking anti-CD55, anti-CD59, or both Abs. The results are the means and SD of three different experiments with different donors. $**p < 0.01$ compared with controls.



icity (27). We therefore investigated whether this was also true for OFA in whole blood. Blocking anti-CD55 and CD59 mAbs (10 $\mu\text{g/ml}$), alone or in combination, were added to OFA in B-CLL whole blood and lysis measured at 4 and 24 h. Blocking anti-CD55 and anti-CD59 alone at both 4 and 24 h induced a 10% and 7% increase in OFA-mediated lysis, respectively, but this was not statistically significant. In contrast, in the presence of both blocking Abs, B cell lysis increased from 36 to 56% at 4 h and from 54 to 77% at 24 h, with a net increase of 20% and 23% at 4 and 24 h, respectively ($p < 0.01$). Control Ab TRX had no effect (Fig. 3D). These data show that although OFA is more effective than RTX at activating complement, this activity is still partially held in check by membrane complement inhibitors CD55 and CD59 in whole blood.

Both OFA and RTX induce phagocytosis by macrophages and neutrophils, but this is inhibited in whole blood

To investigate other possible mechanisms of action of OFA that might occur in vivo, we compared the activity of RTX and OFA in phagocytosis assays using in vitro differentiated macrophages and purified B-CLL cells as targets. As shown in Fig. 4A, RTX and OFA showed overlapping dose-response curves in standard phagocytosis assays in culture medium, suggesting similar efficacy of the two mAbs through this mechanism. Because we have shown previously that excess Igs or whole blood strongly inhibit phagocytosis, we also analyzed phagocytosis in the presence of whole blood. Whole blood inhibited phagocytosis by ~90%, whether RTX or OFA were used (Fig. 4B; $p < 0.001$). We conclude that macrophage-mediated phagocytosis is not significantly different in the presence of OFA compared with RTX and that both are inhibited in whole blood.

Polymorphonuclear neutrophils (PMN) are a major blood component that can mediate phagocytosis of Ab-opsonized targets, although this has been seldom investigated with anti-cancer Abs. We therefore determined whether PMN could mediate phagocytosis of B-CLL in the presence of increasing concentrations of OFA or RTX. Phagocytosis was measured by flow cytometry after 2 or 24 h of incubation of opsonized targets with purified PMN. Phagocytosis with either Ab was maximal at 24 h and 10 $\mu\text{g/ml}$ Ab. We observed a mean 60% and 64% phagocytosis in the presence of 10 $\mu\text{g/ml}$ RTX or OFA Abs, respectively (Fig. 5A). To

confirm by a more direct method that phagocytosis was indeed occurring in these conditions, in some experiments we performed cyto-spin at the end of culture and visualized the PMN that had engulfed the B-CLL targets, seen as double-positive cells (Fig. 5B). At 24 h, the double-blind manual count of these events confirmed the flow cytometry data, with equivalent activity of both Abs (Fig. 5B and data not shown).

We then wanted to determine whether phagocytosis by PMN could take place in whole blood. We observed that PMN did not mediate significant phagocytosis of labeled B-CLL targets in whole

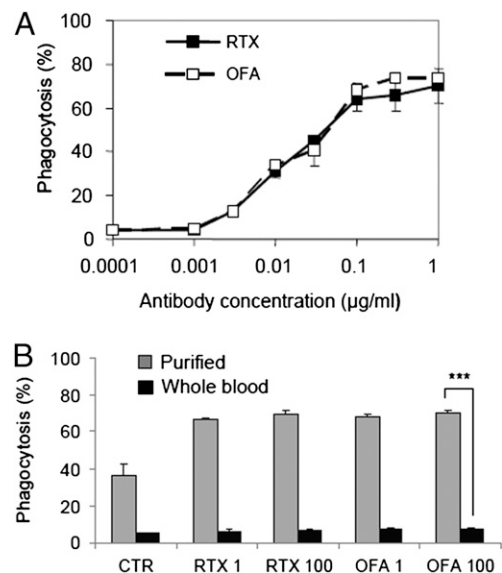


FIGURE 4. In vitro-differentiated macrophages mediate phagocytosis in the presence of OFA or RTX, but not in whole blood. (A) Purified CFSE-labeled B-CLL cells were incubated for 2 h with PKH26-stained in vitro differentiated macrophages, in the presence or absence of the indicated concentration of RTX or OFA, and phagocytosis measured. (B) Whole blood from a normal donor was also added (black bars). Percentage phagocytosis was measured by flow cytometry as percentage PKH26⁺/CFSE⁺/CD19APC⁺ with respect to total PKH26⁺ cells. The results are the mean and SD of three independent experiments. The effect of whole blood was significant in all cases ($p < 0.001$).

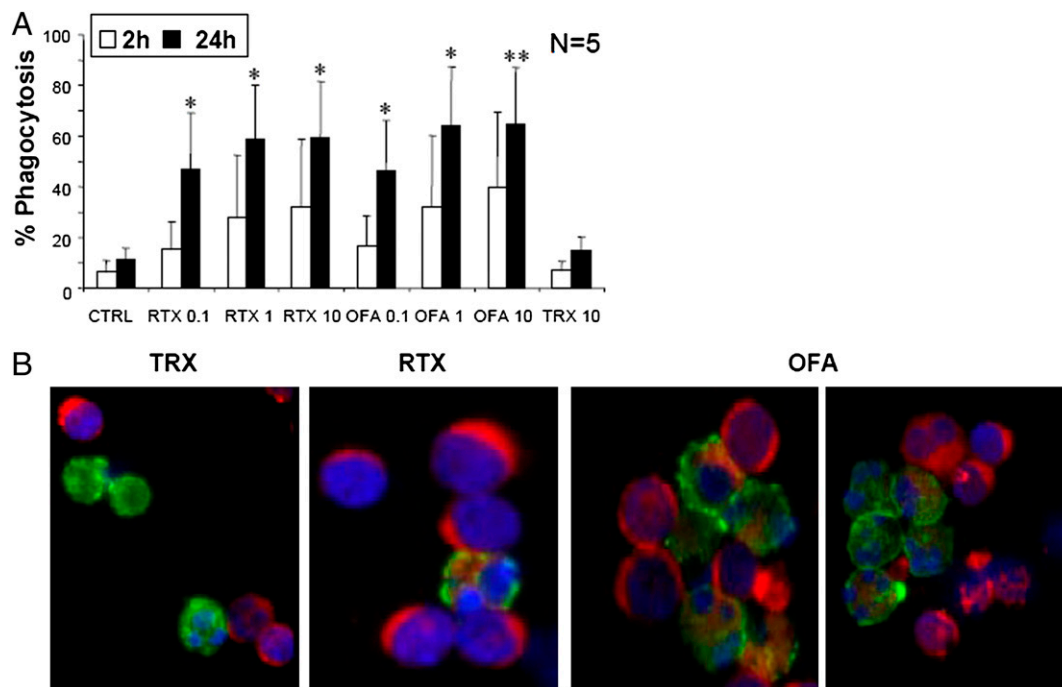


FIGURE 5. PMNs mediate B-CLL phagocytosis in the presence of OFA or RTX. Purified PMNs were incubated with PKH26-stained B-CLL cells and increasing concentrations of RTX or OFA. After 2 (white bars) or 24 h incubation (black bars), the PMNs were stained with anti-CD15-FITC. **(A)** Percentage phagocytosis measured by flow cytometry as double fluorescent cells. The results are the means and SD of three experiments. **(B)** After 24 h, stained cells were cytopspun and fixed, and the nuclei were stained with DAPI. Representative images of cells ($1 \times$ TRX, $1 \times$ RTX, and $2 \times$ OFA) were taken at $\times 20$ magnification. Green indicates CD15⁺ granulocytes; red indicates PKH26⁺ B-CLL cells. * $p < 0.05$; ** $p > 0.01$ compared with controls.

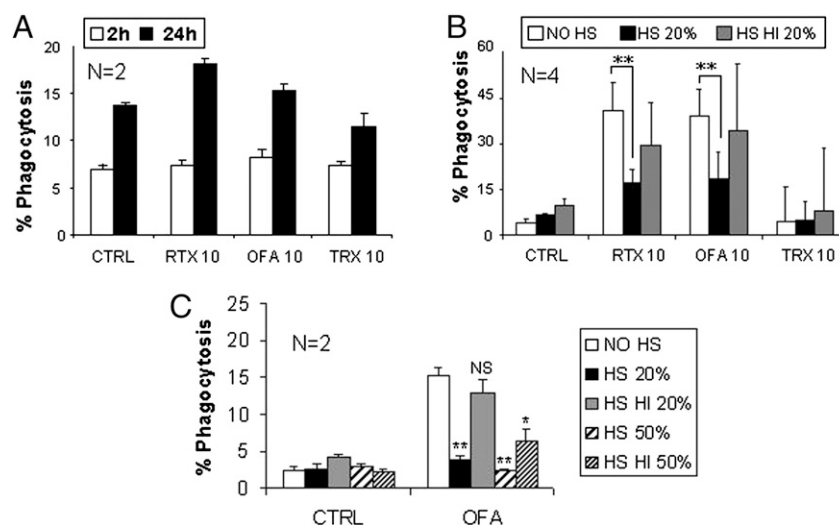
blood (Fig. 6A), whereas the same purified PMN used in parallel at the same effector:target ratio were active (Fig. 5A). This finding suggested that phagocytosis by PMN may be inhibited by complement activation. Indeed we could show that the addition of 20% serum, but not heat-inactivated serum, inhibited phagocytosis mediated by purified PMN and either RTX or OFA (Fig. 6B; $p < 0.01$). The same experiment was also repeated with 50% fresh or heat-inactivated HS, a concentration that is more similar to that present in whole blood. As expected, the addition of 50% HS, like 20% HS, strongly inhibited phagocytosis ($p < 0.01$). Interestingly, 50% heat-inactivated serum also inhibited phagocytosis, although to a lower extent than untreated 50% HS (Fig. 6C). These data suggest that in whole blood, phagocytosis may be inhibited by both complement activation and the excess IgG present in plasma.

We conclude that purified neutrophils can phagocytose opsonized B-CLL targets in the presence of either OFA or RTX, but that this mechanism is not significant in whole blood because inhibition by complement and excess IgG.

OFA in combination with mafosfamide and fludarabine

Unconjugated therapeutic mAbs are best combined with chemotherapeutic agents for optimal activity in vivo, and the development of methods that allow the rapid identification of the best combinations is warranted. In this context, we have analyzed both in whole blood assays and using purified mononuclear cells from B-CLL patients receiving the combination of OFA or RTX with fludarabine or mafosfamide (or both), two drugs commonly used in B-CLL (28–30). The exposure of CLL cells for 28 h to the combination of fludarabine and mafosfamide at $1 \mu\text{g/ml}$ resulted in

FIGURE 6. Phagocytosis by PMNs is inhibited by human serum or in whole blood. **(A)** PKH26-stained B-CLL cells were added to whole blood in the presence or absence of $10 \mu\text{g/ml}$ Abs. After 2 (white bars) or 24 h incubation (black bars), the PMNs were stained with anti-CD15-FITC, and percentage phagocytosis was measured by flow cytometry as double fluorescent cells. **(B and C)** Phagocytosis by purified PMNs was performed for 24 h with $10 \mu\text{g/ml}$ RTX or OFA in absence (white bars) or in the presence of 20% HS (black bars), 20% heat inactivated HS (HS HI, gray bars), 50% HS (thick-striped bars) or 50% HS HI (fine-striped bars). The results are the means and SD of four experiments (B) and two experiments (C). * $p < 0.05$, ** $p > 0.01$. NS, Not significant (with respect to samples in absence of HS).



the expected synergistic cytotoxicity compared with either drug alone (Fig. 7A, 7B). Using isolated B-CLL, either drug induced ~10% cytotoxicity and >40% when combined (Fig. 7A, bar 9 compared with 3 and 6). Similarly, using B-CLL whole blood samples (Fig. 7B), the cytotoxicity of the compounds combined (38%, bar 9) was significantly more than the sum of the cytotoxicities of each compound (~25% adding bars 3 and 6). As expected, OFA alone at 10 μ g/ml was more cytotoxic than RTX when using either MNC (in presence of HS) or whole blood from B-CLL patients, with 20–25% mean lysis with OFA against less than 5% with RTX (Fig. 7A and B, bars 1 and 2). Lysis by OFA was approximately additive over that observed with fludarabine or mafosfamide alone or both compounds together (bars 5 versus 3, 8 versus 6, and 11 versus 9), so that maximal lysis with both chemotherapeutic agents plus OFA reached 53–56% with either MNC or whole blood (Fig. 7A, 7B, bar 11). The combination of the three drugs was stronger using OFA than RTX by ~15%, reflecting the greater complement-mediated cytotoxicity of OFA compared with RTX (Fig. 7A, 7B, bars 5, 8, and 11 compared with bars 4, 7, and 10).

We conclude that OFA can be combined with fludarabine and mafosfamide and has an additive effect with respect to these compounds, which is stronger than that observed with RTX. Synergistic and additive effects can be observed using both purified MNCs in the presence of HS or whole blood from B-CLL patients, suggesting that the whole blood assay can be a rapid *in vitro* assay to test the activity of different immunotherapeutic strategies.

Discussion

In this report, we have compared the activity of new and old generation anti-CD20 Abs OFA and RTX in whole blood assays, which are likely to better represent the *in vivo* condition in the circulation. Furthermore, we have investigated the mechanism of

action of OFA, showing that complement is the major mechanism of OFA in these conditions. Finally, we have measured the efficacy of the Abs in combination with chemotherapeutic agents, showing that OFA in whole blood has an additive effect over fludarabine- and mafosfamide-mediated cytotoxicity.

Lysis of B-CLL targets by OFA alone required at least 10 μ g/ml Abs and was maximal at 100 μ g/ml. Lysis was variable, ranging from 15% to >99% (mean 55%) at 100 μ g/ml. Lysis by OFA was more rapid and 3–6-fold higher than that observed with the same doses of RTX (range, 0–58% lysis; mean, 14% at 100 μ g/ml). Furthermore, we could demonstrate in the whole blood assays that lysis by OFA was fully complement dependent, because it was completely inhibited by excess blocking anti-C5 Ab eculizumab. The higher CDC activity of OFA compared with RTX is expected because the Ab was selected for its high complement-activating capacity, and this property has already been demonstrated using cell lines or purified B-CLL cells as targets (10–13, 31, 32). However, this mechanism had not been characterized previously in unmanipulated whole blood (i.e., in the presence of all blood components, platelets, RBCs, granulocytes, plasma, all of which express soluble or membrane bound complement inhibitors that could affect the ultimate outcome) and at cell concentrations that reflect the *in vivo* conditions. Several detailed studies have shown that the major reasons for higher CDC by OFA in regard to RTX are its high-avidity binding to C1q, the first complement component, its recognition of a CD20 epitope close to the plasma membrane, and its slower off-rate in regard to RTX (10–12, 31, 33).

Interestingly, the efficacy of B-CLL–MCL lysis by OFA correlated significantly with the CD20 expression levels on the target, similar to that observed with RTX *in vitro* and *in vivo* (8, 34) and with OFA using purified neoplastic targets (32). However, OFA-mediated lysis was in all cases higher than that observed with RTX. We could confirm significant CDC even of low CD20 expressing B-CLL by OFA, in contrast to virtually no lysis of these targets by RTX. This finding is in agreement with previous data showing that RTX-resistant cells may be sensitive to OFA (13, 31). Furthermore, we could show that neoplastic samples expressing intermediate to high CD20 showed relatively high lysis ranging from 50–99%. Nonetheless, membrane-bound complement inhibitor was holding the complement cascade in check, even in the presence of OFA, because lysis in whole blood could be increased by an additional 15% in the presence of blocking anti-CD59 and anti-CD55 Abs. This finding indicates that strategies to block complement inhibitors simultaneously to OFA, as previously suggested also for RTX, could further improve the anti-CD20 Ab activity *in vivo* (32, 35).

In our whole blood assays, we could also show that OFA does not induce significant NK cells activation, seen as CD107a induction (<5%), in contrast to the defucosylated GA101 Ab, which activated up to 25% of NK cells. Lack of NK cell activation parallels what has been observed with RTX and is likely due to inhibition of Fc γ RIIIA binding by complement fragments deposited onto the cell-bound Ab (26, 36). This result supports a fully complement dependent mechanism of target cell killing in whole blood. Whether NK cell activations takes place in tissues where complement levels may be lower is unclear and should be investigated further.

We also investigated whether OFA mediated phagocytosis by *in vitro* differentiated macrophages or by neutrophils. Phagocytosis by macrophages showed overlapping dose-response curves with OFA and RTX using purified B-CLL as targets. The uptake of targets by macrophages was likely true phagocytosis, and not the previously described trogocytosis (37), because our assay used a triple fluorescence method, which detects targets effectively

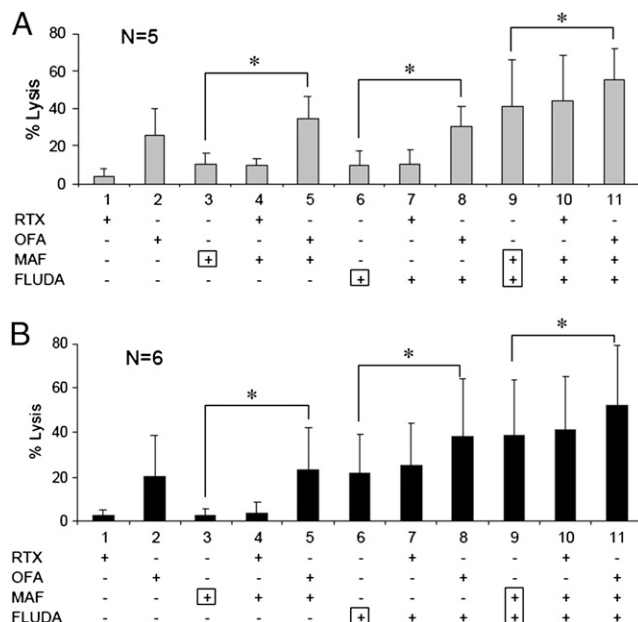


FIGURE 7. OFA in combination with mafosfamide and fludarabine is superior to RTX. MNCs (A) and whole blood (B) from B-CLL patients were incubated for 24 h with 1 μ g/ml fludarabine or mafosfamide, or both, followed by 10 μ g/ml OFA or RTX (A, B) and 20% HS (A). After an additional 4 h incubation, cell lysis was measured by flow cytometry as a decrease in CD19⁺/7AAD⁻ cells relative to untreated control. The data are the means and SD of five (A) or six independent experiments (B), using different patients samples. **p* < 0.05.

engulfed by effector cells. As expected in whole blood, macrophage phagocytosis was not significant because of IgG or complement competition, or both, as demonstrated previously for RTX (22, 38). Similarly, PMNs were able to phagocytose both RTX or OFA opsonized targets with similar dose-response curves, although this mechanism was relatively slow, being maximal at 24 h and at relatively high Ab concentrations (10 $\mu\text{g/ml}$). To our knowledge, the PMN-mediated phagocytosis of anti-CD20 opsonized targets has not been demonstrated previously. Interestingly, PMN-mediated phagocytosis was inhibited by serum complement and in part by excess IgG, and it was undetectable in whole blood. Therefore, the lack of PMN-mediated phagocytosis in whole blood is likely due to inhibition of CD16B binding by complement fragments deposited onto the anti-CD20 Ab, as shown previously for CD16A on NK cells, as well as to competition between IgG complexes in serum and opsonized targets for binding to this same Fc γ R. We could show that CD16B is required for phagocytosis by PMN (L. Bologna and F. Da Roit, unpublished observations and manuscript in preparation). We conclude that phagocytosis by PMNs is unlikely to be a significant mechanism of action of OFA or RTX in the circulation, in agreement with the fully complement dependent lysis observed in whole blood, as discussed above. Whether this mechanism takes place in tumor tissues remains to be determined.

Analysis of the effect of OFA with fludarabine and mafosfamide showed the expected synergism between these two standard chemotherapeutic agents in whole blood, previously demonstrated with purified cells, and a clear additive effect of OFA at 10 $\mu\text{g/ml}$, which added ~25% lysis, that is more than that obtained with RTX (5% increased lysis). Therefore, the combination of OFA with chemotherapeutics may effectively achieve a high level of target lysis with relatively low doses of drugs, perhaps diminishing the toxicity and the complement consumption observed at high doses of Abs (39). We believe that our whole blood system may be a useful screening assay in physiologic conditions for the best combinations of unconjugated Abs with chemotherapeutic agents. Furthermore, other biological effects of Abs could also be investigated in the same conditions in the future, such as the potentially toxic release of cytokines.

Our data are significant in light of current and published phase I–II studies with OFA in B-CLL. These studies use high OFA doses, generally 2000 mg/infusion, ~4-fold higher than the standard RTX dose (375 mg/m²). This dose is in part based on previous studies with RTX (6, 40) and on pharmacokinetic studies in monkeys by Bleeker et al. suggesting the need to reach peak levels of OFA in plasma of at least 100 $\mu\text{g/ml}$ and maintain 5–10 $\mu\text{g/ml}$ for long periods for best circulating B cell depletion (14). Our data confirm in physiologic conditions that relatively high doses of OFA are required for significant activity in whole blood (10–100 $\mu\text{g/ml}$). This finding is particularly true considering the sink effect of high-burden disease (6, 34). Nonetheless, high dosing will also need to consider the resulting depletion of rate-limiting complement components or loss of CD20, as suggested by the studies of Dr. R. Taylor's group, which suggested that an initial lower dosing, followed by higher dosing, may achieve the best lysis (9, 39).

To conclude, we believe that the whole blood assay used here is an easy and relevant tool to screen the efficacy, mechanism of action, factors of resistance, and required plasma concentrations of new therapeutic Abs. In addition, it is useful when performing direct comparison of new and old generation Abs and designing optimal combination strategies with chemotherapeutic agents in physiologic conditions, as demonstrated here with the new generation anti-CD20 Ab OFA.

Acknowledgments

We thank Dr. Bonifazio (Glaxo Smith Kline, Verona, Italy) for providing the OFA Ab for these studies, the patients and normal donors who agreed on multiple occasions to provide blood samples, and the nurses of the hematology division for constant and kind help.

Disclosures

The authors have no financial conflicts of interest.

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