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Loss of CD20 and Bound CD20 Antibody from Opsonized B Cells Occurs More Rapidly Because of Trogocytosis Mediated by Fc Receptor-Expressing Effector Cells Than Direct Internalization by the B Cells

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We previously reported that 1 h after infusion of CD20 mAb rituximab in patients with chronic lymphocytic leukemia (CLL), >80% of CD20 was removed from circulating B cells, and we replicated this finding, based on in vitro models. This reaction occurs via an endocytic process called shaving/trogocytosis, mediated by FcyR on effector cells including monocytes/macrophages, which remove and internalize rituximab–CD20 immune complexes from B cells. Beers et al. reported that CD20 mAb-induced antigenic modulation occurs as a result of internalization of B cell-bound mAb–CD20 complexes by the B cells themselves, with internalization of ∼40% observed after 2 h at 37°C. These findings raise fundamental questions regarding the relative importance of shaving versus internalization in promoting CD20 loss and have substantial implications for the design of mAb-based cancer therapies. Therefore, we performed direct comparisons, based on flow cytometry, to determine the relative rates and extent of shaving versus internalization. B cells, from cell lines, from patients with CLL, and from normal donors, were opsonized with CD20 mAbs rituximab or ofatumumab and incubated for varying times and then reacted with acceptor THP-1 monocytes to promote shaving. We find that shaving induces considerably greater loss of CD20 and bound mAb from opsonized B cells in much shorter time periods (75–90% in <45 min) than is observed for internalization. Both shaving/trogocytosis and internalization could contribute to CD20 loss when CLL patients receive rituximab therapy, but shaving should occur more rapidly and is most likely to be the key mechanism of CD20 loss. The Journal of Immunology, 2011, 187: 3438–3447.

In 2004, we first reported that infusion of the usual 375 mg/m² dose of rituximab (RTX) into the bloodstream of patients with chronic lymphocytic leukemia (CLL) led to considerable loss of CD20 on circulating B cells several hours after initiation of the RTX infusion. We later found that infusion of even limited amounts of RTX (20–60 mg/m²) in CLL patients also promoted substantial loss of CD20 from circulating B cells in just 1 h (1, 2). We developed in vitro paradigms and a mouse model that demonstrated that shaving of CD20 is due to an endocytic process, trogocytosis, that is mediated by effector cells that express FcyR, such as monocytes, macrophages, or NK cells. Upon formation of an immunologic synapse that allows close juxtaposition and partial merging of the cell membranes of the RTX-opsonized B cells and the FcyR-expressing acceptor cells, there is transfer to and internalization by the acceptor cells of the RTX–CD20 immune complex along with fragments of the B cell membrane; moreover, FcyRI, which mediates trogocytosis, is also internalized by acceptor monocytes, again consistent with the definition of trogocytosis as a receptor-mediated process (3–5).

Our in vitro investigations revealed that this process goes to completion in <1 h, and studies in a SCID mouse xenograft model also demonstrated that infusion of RTX led, within 1 h, to loss of >90% of CD20 from human Z138 cells isolated from the lungs of SCID mice (6).

Beers et al. (7) recently reported, based in part on studies in FcyR-knockout mice, that type I anti-CD20 mAb-mediated antigenic modulation can occur as a result of internalization of mAb–CD20 complexes by B cells. Human CD20 transgenic mouse B cells were opsonized with Rit m2a, a mouse IgG2a type I mAb with RTX activity, and internalization of the bound mAb as well as CD20 loss because of “rapid CD20 internalization” was demonstrable, but this reaction was not observed when a type II mAb was tested under comparable conditions. They also reported in vitro experiments indicating that after 2 h at 37°C, on average, 40% of surface CD20, along with cell-bound RTX, was internalized by RTX-opsonized CLL cells (7). The authors concluded that trogocytosis or shaving mediated by cells expressing FcyR could not explain the differences they observed between in vivo activities of type I or type II mAbs in their mouse models. Their studies, taken in the context of our reports, raise important and interesting questions regarding the biological mechanisms
responsible for CD20 loss from B cells associated with RTX infusions in CLL patients. An understanding of the relative contributions of trogocytosis or internalization to loss of CD20 will have an important impact on the design of improved treatment paradigms.

To study these questions, we have conducted an extensive series of in vitro experiments to evaluate kinetically and quantitatively shaving versus internalization. Murine 38C13 B cells stably transfected with human CD20 were opsonized with either RTX or ofatumumab (OFA) and were then incubated in the absence or presence of acceptor cell THP-1 monocytes. We also examined these phenomena using primary CLL B cells and normal B cells, and we have confirmed that there can be a variable amount of internalization of cell-bound anti-CD20 mAb after it binds to the B cells. However, our results, based on direct comparisons, indicate that the shaving reaction occurs much more rapidly and promotes considerably more loss of bound mAb and CD20 from the B cell and transfer to acceptor cells than does direct internalization into the B cells.

Materials and Methods

Cells, reagents, and Abs

Murine 38C13 B cells stably transfected with human CD20 (38C13-CD20) were a gift from Dr. J. Golay (Ospedali Riformati di Bergamo, Bergamo, Italy) (8). THP-1 cells were cultured as previously described and were activated by incubation with 1 μM PMA or with 1 μM all-trans-retinoic acid (RA) 1–3 d prior to the shaving experiment (3). Mononuclear cells were isolated from CLL patient blood samples (CLL B cells) using Ficoll-Paque as previously described (3), washed, and reconstituted in RPMI 1640 medium. On the basis of the complete blood counts for the CLL blood samples reported by the clinical laboratory (absolute lymphocyte count 75,000–200,000 cells/μl), B lymphocytes constituted the vast majority of the mononuclear cells in these blood samples. For example, in one CLL patient, 84% of the WBCs were lymphocytes, and 4% were monocytes. Several isolated mononuclear cell preparations were examined by flow cytometry, and >90% of the cells stained positive for CD19 and CD45 (data not shown). B cells were isolated from the blood of normal volunteers as previously described, based on centrifugation through Ficoll-Paque, followed by negative selection using a B cell isolation kit (Miltenyi Biotec (9)). More than 80% of the isolated cells were CD20 positive, based on staining with FITC-labeled OFA. The University of Virginia Institutional Review Board approved all protocols.

PKH26, PMA, and RA were obtained from Sigma-Aldrich, and human IgG and mouse IgG were obtained from Lampire Biological Laboratories. RTX was obtained from the University of Virginia hospital pharmacy, and OFA was provided by Genmab. F(ab')2 fragments were prepared from OFA and RTX as described previously (10). Anti-human IgG Fc-specific mouse mAb HB43 and mouse anti-Ai488 mAb 4A9 have been previously described (1, 10), and Ai633 goat anti-human IgG, H and L chain specific, was obtained from Molecular Probes. mAbs were labeled with FITC (11) or with Alexa (Ai)488 or Ai647 (Invitrogen) or with N-hydroxysuccinimide-biotin (Pierce), according to the manufacturer’s instructions. All probing and mAb opsonizations were performed at a final concentration of 10 μg/ml mAb.

Separate or simultaneous tests for internalization versus shaving

In experiments in which only internalization was examined, unlabeled B cells were incubated with Ai488 RTX or Ai488 OFA for periods varying between 10 min and 6 h at 37°C, washed, and then examined for internalization by as many as four separate criteria, as described in the next section. In experiments in which both internalization and shaving were evaluated on mixtures of B cells with THP-1 cells, the donor B cells or acceptor THP-1 cells were first labeled with PKH26 (12). The donor B cells were then opsonized with Ai488 RTX or OFA for varying periods of time, and then, separate aliquots of the B cells were either directly analyzed for internalization or combined with THP-1 cells to allow for shaving.

Analyses of shaving

The donor 38C13-CD20 B cells or CLL B cells were stained with PKH26 to distinguish them from the THP-1 cells. The PKH26-stained B cells were then opsonized with Ai488 RTX or Ai488 OFA in RPMI 1640 medium at 37°C for 10 min, followed by 50 min on ice (to minimize internalization), or they were opsonized at 37°C for varying periods up to 6 h (to allow for internalization). The B cells were then washed with cold BSA/PBS and reconstituted in RPMI 1640 medium. To begin the shaving reaction, 2 × 10⁵ RTX- or OFA-opsonized, PKH26-dyed donor cells in 400 μl RPMI 1640 medium were added to an empty 24-well plate well (0-minute control) or to a well containing 1 × 10⁵ adherent PMA-treated THP-1 cells. The plates were then centrifuged briefly to bring the cells into close proximity and incubated for 45 min at 37°C. In some experiments, the mAb-opsonized donor cells were held on ice for 45 min or were added to wells of a plate containing THP-1 acceptor cells, and to prevent shaving, these samples were immediately diluted and quenched by addition of cold BSA–PBS and then processed. Following the incubations, donor cells were separated from THP-1 cells by gentle aspiration; an aliquot of donor cells was probed with Ai647-labeled mAb HB43 (specific for the Fc region of human IgG and thus able to bind to cell surface RTX or OFA) for 45 min on ice, allowing independent determinations of residual B cell-bound Ai488 RTX or Ai488 OFA. A second aliquot was probed on ice for 45 min with Ai488 RTX or Ai488 OFA to measure total CD20 remaining on the surface of the cells, which were then analyzed by flow cytometry; alternatively, the cells were not secondarily probed but directly examined by flow cytometry for the decrease in the Ai488 mAb signal because of shaving. The percent shaving was calculated as described previously (3). In additional experiments, the cells were first opsonized with FITC-labeled mAbs and then probed with Ai633 goat anti-human IgG (H + L specific). As we have reported, the three independent measures of shaving are in good agreement (3, 4).

B cells were then examined with THP-1 cells in suspension. In this case, RA-treated THP-1 acceptor cells were labeled with PKH26, and then, donor Ai488 mAb-opsonized B cells and the THP-1 cells were incubated together at varying ratios for 45 min at 37°C. Aliquots of the cell mixtures were then probed with Ai647 mAb HB43 or with the respective Ai488 mAbs, and then, the mixtures of donor and acceptor cells were examined by flow cytometry, thus allowing for a simultaneous determination of the loss of the CD20 mAbs (and CD20) from the donor cells as well as the uptake of the anti-CD20 mAbs by the acceptor THP-1 cells.

Analyses of internalization

Acid wash. Cells were reconstituted for 5 min in RPMI 1640 medium, pH 2.5 containing 10% FBS, a condition that promotes release of mAbs bound to the cell surface but does not induce release of internalized Ai488 mAbs (4, 13). The cells (as well as cells in control samples not subjected to the acid wash) were then washed twice with PBS and analyzed by flow cytometry for the residual Ai488 signal, which reflects internalized Ai488 mAb. In selected experiments (Supplemental Fig. 1), after the acid wash, B cells from CLL patients were also probed with the Ai647 anti-CD20 mAb to provide an alternative measure of CD20 on the cell surface. Quenching of the Ai488 fluorescence signal associated with mAb bound to the cell surface. An aliquot of Ai488 mAb-opsonized donor cells was reacted with rabbit polyclonal anti-Ai488 or with anti-Ai488 mAb 4A9 and then analyzed by flow cytometry for quenching of the Ai488 fluorescence. These anti-Ai488 Abs are only able to interact with and quench the fluorescence of mAb-associated Ai488 bound to the cell surface, not Ai488 mAb that has been internalized. This difference in quenching thus provides a measure of the percentage of the Ai488 mAb signal that has been internalized (7, 14). The Ai488 fluorescence signal (molecules of equivalent soluble fluorophore [MESF] units, see below) of cells briefly opsonized with the Ai488 mAbs (and then washed) was measured in the absence and presence of the quenching mAb to set parameters for no quenching and maximum quenching. The same measurements were then made on cells that were opsonized for several hours to allow time for internalization to occur. The relative decrease in quenching for the Ai488 signal was then used in the equations developed by Austin to estimate the degree of internalization of the Ai488 mAbs.

Accessibility of cell-bound mAbs to development with mAb HB43. The opsonized cells were probed with Ai647 mAb HB43; the amount of Ai647 mAb HB43 that binds to the cells will decrease in direct proportion to the degree to which the anti-CD20 mAbs are internalized by the B cells. Accessibility of biotinylated RTX or OFA to Ai488 streptavidin (SA). The principle of this assay is similar to that of the assay described above for the mAb HB43; in this case, if the biotinylated mAb is internalized, the signal based on probing with Ai488 SA (1 μg/ml) will decrease in proportion to the degree of internalization.
Flow cytometry

Flow cytometry was performed using a dual laser FACS Calibur cytometer (BD Biosciences). Mean fluorescence intensities were converted to MESF using standard fluorescent beads (Spherotech) (1). In some cases, modest differences in absolute MESF values for cells for different experiments were noted. Experiments were conducted over a period of >1 y, and in addition, several different preparations of the Al488 mAbs were used, likely explaining these differences. However, all CLL cell samples were examined with the same Al488 mAb preparations to provide a relative estimate of CD20 expression. On the basis of these determinations, CD20 levels (MESF units, Al488 OFA) were as follows: CLL cells, 2,000–37,000 MESF units (seven patients); and 38C13-CD20⁺ cells, 320,000 MESF units.

Statistics

All reported experiments are representative of two or three independent determinations. For most experiments, individual points were usually run in duplicate, but several experiments are based on three or more determinations as noted, and means and SD are displayed. Differences in experimental results were tested for significance on the basis of unpaired, two-tailed t tests (SigmaStat). *p < 0.05, **p < 0.01, or ***p < 0.001 are noted in the figures or figure legends.

Results

Modest internalization is demonstrable for murine 38C13-CD20⁺ cells opsonized with CD20 mAbs

We first evaluated the rate and extent of internalization of OFA and RTX that bound to murine 38C13-CD20⁺ B cells. The 38C13-CD20⁺ cells were chosen to allow analysis of cells similar to the transgenic murine B cells used by Beers et al. (7). We used four independent criteria to evaluate quantitatively whether incubation of 38C13-CD20⁺ B cells with RTX or OFA (labeled with either Al488 or biotin) for up to 2 h at 37°C could lead to demonstrable internalization of the cell-bound mAbs. After incubation with the anti-CD20 mAbs, the cells were washed, and separate aliquots were examined as follows: 1) one aliquot was probed with Al487 mAb HB43 to measure cell surface-bound RTX or OFA (Fig. 1A); 2) another aliquot was subjected to an acid wash to remove surface-bound mAb and then evaluated by flow cytometry for the residual Al488 signal (Fig. 1B); 3) a third aliquot was reacted with mAb 4A9 or a polyclonal Ab preparation, each of which is specific for Al488, and substantially quenches the fluorescence of cell surface-bound Al488 but cannot quench internalized Al488 (Fig. 1C); and 4) the fourth aliquot of cells, opsonized with biotinylated RTX or OFA, was probed with either Al647 mAb HB43 or with Al488 streptavidin (SA), specific for the mAb-bound biotin (Fig. 1D. 1E). The representative experiments in Fig. 1 reveal that over a period of 30 min to 2 h at 37°C, internalization of RTX by the 38C13-CD20⁺ cells is clearly demonstrable, varying between 3 and 20%, depending on which index of internalization is used. Internalization of OFA tended to be somewhat higher, reaching 20–30% at longer incubation times. The kinetic analyses for internalization of RTX and OFA indicate a relatively slow internalization process; in most instances, there was ∼≤10% internalization after 1 h and ∼≤25% after 2 h.

Comparison of internalization versus shaving for 38C13-CD20⁺ cells opsonized with CD20 mAbs

As there was demonstrable internalization of bound mAb by the 38C13-CD20⁺ cells after a 2-h incubation, we next investigated how this internalization might influence the susceptibility of the cells to shaving when they were subsequently reacted with adhered THP-1 acceptor cells. The B cells were opsonized with Al488-labeled anti-CD20 mAbs RTX or OFA either at room temperature (RT) for 15 min or at 37°C for 2 h. They were then reacted with adhered THP-1 acceptor cells for 45 min at 37°C. The B cells were then removed from the wells and probed with Al647 anti-human Fc-specific mAb HB43 to measure residual cell-bound RTX or OFA (1, 3). Alternatively, after removal from the well, the cells were reacted again with Al488 RTX or OFA to provide a measure of total CD20 on the B cell surface (Fig. 2A). Control samples were held on ice or at 37°C without exposure to THP-1 cells (Fig. 2A, 2B), or the B cells were mixed with THP-1 cells and immediately quenched (Fig. 2C, 2D). These paradigms closely follow the procedures we previously used to evaluate THP-1 cell-mediated shaving of RTX-opsonized human B cells (3).

The results, based on either the Al488 signal (Fig. 2A, 2C) or the signals associated with Al647 mAb HB43 (Fig. 2B, 2D), indicate that even with a 2-h preincubation to allow internalization to occur, reaction with THP-1 cells for 45 min promotes substantial removal of Al488 RTX and Al488 OFA (>80%) and CD20 (Fig. 2A) from the B cells. Aliquots of the B cells that were not reacted with THP-1 cells were also examined by probing with the polyclonal anti-Al488 Abs to test for internalization and the associated resistance to quenching of the Al488 fluorescence. On the basis of this assay, after 2 h at 37°C, 11 and 20% of the Al488 RTX and OFA, respectively, were internalized before reaction with the THP-1 cells.

To provide an alternative analysis of shaving, independent of the Al488 and Al647 fluorochromes, 38C13-CD20⁺ cells were opsonized with FITC-labeled RTX and OFA and, after a wash, were reacted with THP-1 cells. After a 45-min incubation, the 38C13-CD20⁺ cells were secondarily probed with Al633 goat anti-human IgG (H and L chain specific) to provide an alternative measurement of the loss of cell-bound mAb. The results (Fig. 2E, 2F), based on the FITC signals as well as the secondary Al633 probes for human IgG, demonstrate that within 45 min at 37°C, >90% of the RTX or OFA associated with the 38C13-CD20⁺ cells was removed upon interaction with THP-1 cells. Furthermore, representative two-color dot plots shown in Fig. 2G verify that the signals due to both fluorochromes are substantially reduced after the shaving reaction. Thus, in the presence of FcγR-expressing effector cells in this model system, most bound RTX or OFA on the target cells is removed by the effector cells before the target cells can internalize the mAbs.

To further evaluate internalization versus shaving, we tested for shaving with THP-1 cells in suspension. 38C13-CD20⁺ cells were either briefly opsonized (10 min) at 37°C and then held on ice or incubated for 2 h at 37°C with Al488 RTX or OFA to allow for internalization. Both sets of opsonized cells were washed and reacted for 45 min with RA-treated PKH26-dyed acceptor THP-1 cells at varying acceptor cell-to-donor cell ratios. After this step, the cell mixtures were reopsonized with additional Al488 RTX or OFA to measure total available CD20 on the B cells. The results of these assays indicate that at a ratio of 3 THP-1 cells for each donor B cell, ∼50% of CD20 and associated Al488 RTX or Al488 OFA was removed from the cells (3x THP), based on the reduced Al488 signals compared with values obtained for 38C13-CD20⁺ cells held on ice in the absence of acceptor THP-1 cells (media/ice) (Fig. 3A, 3B, B cells, left side). We also found that shaving of cells opsonized for only 10 min (data not shown) was quite comparable to shaving of cells opsonized for 2 h. The Al488 RTX and OFA are indeed removed from the B cells and are taken up by the acceptor THP-1 cells during the shaving reaction (Fig. 3A, 3B, right side, THP-1 cells). Moreover, as we reported previously for shaving of donor Z138 cells mediated by acceptor THP-1 cells (3), the largest amount of shaving of the donor 38C13-CD20⁺ cells occurs when the acceptor THP-1 cells are in excess (3:1). When the relative number of THP-1 acceptor cells is reduced (1:3), then the absolute degree of shaving of the donor cells is reduced, but...
The use of the polyclonal anti-Al488 preparation. Results in the internalization of RTX or OFA were obtained, based on the ability of anti-Al488 Abs (mAb 4A9 or a polyclonal preparation) to quench the Al488 signal as a result of shaving for B cells that were opsonized at 37°C for either 120 or 10 min (data not shown). Finally, the measurements on THP-1 cells based on probing with Al647 mAb HB43 (Fig. 3C, 3D, right side) must be considered only semiquantitative, because a significant portion of the RTX and OFA will be internalized by the THP-1 cells, thereby precluding their detection by mAb HB43 (3). Thus, the experiments to this point demonstrate that although 20–35% of RTX or OFA can be internalized by 38C13-CD20+ cells after incubation in media for 2 h at 37°C, the shaving reaction, both in suspension and with adherent acceptor THP-1 cells, is much faster and promotes much greater loss of bound mAb and CD20 from the donor cells.

Considerable levels of internalization are demonstrable for CLL cells opsonized with CD20 mAbs

We next evaluated internalization using a more clinically relevant model: primary CLL cells. The cells were opsonized with Al488 RTX or OFA for 10 min, 2 h, or 6 h at 37°C and then washed and tested for internalization, based on the paradigms presented in Fig. 1. The results, illustrated in Fig. 4A and 4B, indicate that after a 2- or 6-h opsonization period, substantially less Al488 RTX and Al488 OFA could be removed from CLL cells by the acid-wash procedure than after a 10-min period of opsonization. Likewise, the ability of the anti-Al488 Ab to quench the Al488 signal associated with the cells was reduced considerably for cells opsonized for 2 or 6 h than for cells opsonized for 10 min (Fig. 4A, 4B). Moreover, as seen in Fig. 4C and 4D, the Al647 mAb HB43 probe detected less RTX and OFA on the surface of the cells, which were opsonized for 2 or 6 h compared with cells opsonized for 10 min. These results indicate that the CLL cells internalized substantial amounts of the Al488 mAbs over incubation periods of 2 and 6 h, consistent with the observations of Beers et al. (7). The amount of internalization that occurred, calculated using the data shown in Fig. 4A–D, differed somewhat for the three different methods used to assess internalization, but in all cases, a considerable amount of mAb is internalized, and in general, Al488 OFA appears to be internalized more readily than Al488 RTX (Fig. 4E, 4F).

To provide an alternative determination of CD20 on the cell surface after partial internalization of bound mAbs, B cells from four other CLL patients were opsonized for up to 2 h at 37°C with Al488 RTX or OFA and then subjected to the acid-wash procedure. The cells were then re-equilibrated in PBS and analyzed for residual Al488 signal, or the cells were probed with Al647 OFA

The amount of Al488 mAb taken up by the individual acceptor cells is increased, because under these latter conditions, there is more donor cell Al488 mAb available for shaving per acceptor THP-1 cell.
or RTX to measure available CD20. The opsonized cells were not incubated with THP-1 cells in this experiment because the objective was simply to determine the amount of RTX, OFA, and CD20, which remained on the cell surface, and the acid-wash procedure provides this information. The results, illustrated in Supplemental Fig. 1, again show that substantial amounts of RTX and OFA for 15 min at RT or for 2 h at 37˚C. After a wash, the cells were added to adhered THP-1 cells and immediately quenched and isolated. E and F. Similar to C and D, except the cells were opsonized for 2 h at 37˚C with FITC-labeled RTX or OFA, and the final probing step was with Al633 goat anti-human IgG (H + L). In this case, the control is based on cells held for 45 min at 37˚C in the absence of THP-1 cells. G. Dot plots from the experiments illustrated in E and F. In A–F, the substantial reduction in signals (compare open bars to filled bars; average reduction: 92%) indicate almost all cell-bound mAb was removed by the adhered THP-1 cells. Thus, substantial amounts of RTX and OFA could be removed from the CLL B cells upon reaction with adhered THP-1 cells, suggesting that at least this much of the Al488 RTX remained bound to the surface of the cells. In most cases, less OFA could be removed than RTX under comparable conditions, likely reflecting a greater degree of prior internalization of OFA than RTX. The CLL B cells were also probed with Al647 mAb HB43, and two trends are evident: first, in control samples (immediately quenched), less mAb HB43 bound to cells incubated for longer periods (compare the open bars for the three different incubation times; Fig. 5C), thus indicating some internalization had occurred. Second, after reaction of the opsonized CLL cells with THP-1 cells, the ability of Al647 mAb HB43 to bind to the CLL B cells decreased substantially in all cases (Fig. 5C, 5D; compare open and filled bars for each incubation time, Fig. 5C), demonstrating loss of RTX and OFA from the CLL B cell surface as a result of shaving mediated by the THP-1 cells. Thus, substantial amounts of RTX and OFA could still be recognized by FcγR on the THP-1 cells after 2 or 6 h of opsonization, and these mAbs on the cell surface were indeed subject to shaving.

Normal B cells opsonized with RTX or OFA are also subject to internalization, but the shaving reaction is considerably faster

B cells isolated from the blood of several normal individuals were opsonized with either FITC-labeled intact or F(ab')2 fragments of RTX and OFA for 15 min at RT or for 2 h at 37˚C. After a wash, the cells were subjected to the acid-wash procedure to test for internalization. The results, illustrated for the 2-h incubation (Fig. 6A), indicate that moderate amounts of intact RTX and OFA are internalized (~10–25%) and that less internalization occurs when the F(ab')2 fragments are bound to the cells. In contrast, after the 15-min incubation, the degree of internalization was <4% in all cases (data not shown).

We next determined whether mAb-opsonized normal B cells are subject to the shaving reaction. The cells were opsonized with the FITC-labeled intact or F(ab')2 mAbs for 2 h at 37˚C, washed, and incubated with adhered THP-1 cells, following the same general protocols used in the experiments described in Figs. 2, 4, and 5. The results, illustrated in Fig. 6C and 6D, reveal that within 45
FIGURE 3. 38C13-CD20+ cells opsonized with RTX or OFA are subject to the shaving reaction mediated by THP-1 cells in suspension. A–D. Cells were opsonized with RTX or OFA for 2 h, washed, and then reacted with THP-1 cells in suspension for 45 min or held on ice. The mixtures were then reopsonized with the same Al488 mAb (to measure total available CD20; A, B) or probed with Al647 mAb HB43 (to measure bound mAb; C, D). The results for B cells are on the left of each lettered figure (A, B), and the results for THP-1 cells are on the right (C, D). Compared with samples held on ice, the reaction of opsonized B cells with THP-1 cells promoted substantial loss of CD20 and bound mAb, and this effect was most pronounced at a THP-1/38C13-CD20+ cell ratio of 3:1. Conversely, the largest amounts of Al488 mAb were taken up by the THP-1 cells at THP-1/38C13-CD20+ cell ratios of 1:3. Means and SD for RTX (phase and subsequently probed with Al647 HB43 are displayed, based on the percent reduction in Al647 HB43 binding because of shaving mediated by

Effect of continued presence of opsonizing CD20 mAb on internalization and shaving

In the experiments reported in Figs. 2, 3, 5, and 6, the mAb-opsonized B cells were washed to remove unbound CD20 mAb before reacting with THP-1 cells. We next investigated whether the continued presence of opsonizing mAbs in the medium would affect the relative efficacy of internalization versus shaving. 38C13-CD20+ cells or CLL B cells were opsonized at 37°C with Al488 RTX for 10 min, 1 h, or 2 h, and then, without washing out excess unbound mAb, the B cells were immediately brought into contact with THP-1 cells (or media) in suspension (Fig. 7A, 7B, 38C13-CD20+ donor cells) or with THP-1 cells adhered to wells in 24-well plates (Fig. 7C, 7D, CLL donor cells), and the cell mixtures were incubated for 60 min at 37°C. The cells were then washed and probed with Al647 mAb HB43. In other samples, after opsonization of the B cells with Al488 RTX, the cells were not reacted with THP-1 cells but were instead subjected to the acid-wash procedure to remove residual surface-bound mAbs.

The general trends for both 38C13-CD20+ and CLL B cells were quite similar to those shown in Figs. 2, 3, 5, and 6 in which the opsonized B cells were washed prior to the shaving reaction. First, the acid-wash procedure removed the vast majority of the cell-associated Al488 RTX (compare filled bars and striped bars in Fig. 7A, 7C). For longer incubation times, more Al488 RTX remained bound to the cells after the acid wash, and this trend was more pronounced for the CLL B cells, likely reflecting a greater amount of RTX internalization by CLL B cells than by 38C13-CD20+ cells. Incubation of the Al488 RTX-opsonized cells with THP-1 cells promoted removal of the majority of RTX from the B cells (compare filled bars and open bars in Fig. 7A, 7C). As was the case for the results based on the acid-wash paradigm, THP-1 cells removed less RTX if the cells had first been incubated with Al488 RTX for 2 h rather than for only 10 or 60 min. This indicates that some internalization had occurred during the prior 2-h incubation, and again, this trend was more pronounced for the CLL B cells, likely reflecting a greater amount of RTX internalization by CLL B cells than by 38C13-CD20+ cells. It is noteworthy that the values of the residual Al488 signals on the cells after exposure to THP-1 cells or after the acid washes were quite similar, pre-
sumably reflecting in each case the presence of Al488 RTX in the same compartment (i.e., inside the cell rather than on the surface) that could neither be shaved nor released by an acid wash. In control samples in which B cells were not reacted with THP-1 cells, probing with Al647 mAb HB43 revealed a gradual reduction in cell surface-bound RTX for longer incubation times (filled bars; Fig. 7B,7D), again consistent with internalization. However, when these cells were reacted with THP-1 cells to promote shaving, the ability of Al647 HB43 to bind to the cell was nearly abolished, demonstrating that virtually all of the RTX associated with the cell was removed (open bars; Fig. 7B,7D). This clearly indicates that the majority of cell-bound RTX had not been internalized and was still on the surface of the cell, available to be removed by the THP-1 cells.

Finally, two-color dot plots (Fig. 7E) confirm that shaving occurred, as shown by the reduction in both the Al488 RTX signal and the binding of Al647 mAb HB43. For the sample opsonized for 10 min and then reacted with THP-1 cells, there is a substantial decrease in both the Al488 signal attributable to cell-bound Al488 RTX, and in the binding of secondary probe Al647 mAb HB43.

**FIGURE 4.** Reaction of CLL cells for up to 6 h at 37˚C with either RTX or OFA leads to internalization of cell-bound mAbs. Representative results for CLL cells (patient 1 [A, C, E] and patient 2 [B, D, F]) are given. After opsonization for the indicated times, the washed cells were evaluated for internalization, based on either an acid wash (striped bars) or by reaction with a quenching Ab specific for Al488 (gray bars: A, polyclonal; B, mAb 4A9), and the Al488 signals for these cells were compared with signals for cells that were opsonized only (Al488, open bars: A, B). Alternatively, the cells were probed with Al647 mAb HB43 (C, D). The 10/50 control bar refers to cells reacted for 10 min at 37˚C, followed by 50 min on ice. Means and SD (n = 2) are displayed. The calculated degree of internalization for the different protocols is presented in E and F. For the sake of clarity, statistical tests are not illustrated for the data presented in E and F. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 5.** CLL cells (patient 2) that are opsonized with RTX or OFA for up to 6 h and then washed are still subject to the shaving reaction. After opsonization under the indicated conditions, the CLL cells were reacted for 45 min with adhered THP-1 cells (black bars) and then analyzed for shaving, based on direct measurement of residual-bound Al488 mAb (A) or based on probing with Al647 mAb HB43 (C). The controls (C, 0 min, immediately quenched) are as in Fig. 2C, 2D. Means and SD (n = 2) are displayed. The percentage of Al488 removed (B) or the percent reduction in Al647 HB43 binding (D) were significantly different from 0, as noted. *p < 0.05, **p < 0.01, ***p < 0.001.
(specific for surface-bound RTX) after shaving. However, in the sample that was exposed to THP-1 cells after a 120-min opsonization period (which allows time for internalization to occur), more Al488 signal remains associated with the cells (Fig. 7E, lower left panel, 43% of the cells modestly positive for Al488), likely reflecting internalized Al488 RTX that was not subject to shaving. These results suggest that some internalization of RTX
did occur during the 2-h incubation period, but they also confirm that even after the 120-min incubation, most Al488 RTX was still on the cell surface and available to be removed by shaving. These results are in agreement with our experiments that made use of opsonized and washed 38C13-CD20⁺ cells and CLL B cells (Figs. 1–6) and indicate that shaving is a relatively fast process and occurs more quickly than direct internalization by the B cells.

**Discussion**

The use of anti-CD20 mAbs constitutes one of the real successes in the immunotherapy of B cell lymphomas, and more recently, high levels of therapeutic efficacy with anti-CD20 mAbs have also been demonstrated for several other indications (15–21). However, many patients are refractory to these therapies, and resistance often develops in initially responsive patients who relapse (17, 22–28). One of the many mechanisms that can compromise these therapies is the loss or downregulation of CD20 on targeted B cells (7, 27). In the present investigation, we have performed experiments designed to provide an in vitro evaluation and comparison of the kinetics and relative efficacy of shaving versus internalization as mechanisms that promote loss of CD20 from the surface of B cells.

In agreement with our earlier work in which we used mouse macrophages as acceptor cells (5), we confirmed that murine 38C13-CD20⁺ B cells are subject to shaving by acceptor THP-1 cells (Figs. 2, 3, 7) when these B cells are opsonized with either RTX or OFA. We also found that 38C13-CD20⁺ cells and normal human B cells can internalize bound RTX or OFA (Figs. 1, 6). The amount of internalization we observed for the 38C13-CD20⁺ cells after a 2-h incubation was somewhat lower than that reported by Beers et al. (7) for a different transgenic cell line, suggesting that this reaction may be model dependent. Indeed, Beers et al. (7) examined internalization of mAb–CD20 complexes on several cell lines and reported large variations in the amount of internalization of RTX seen for different types of cells. Of more importance, we have found that the internalization process is considerably slower than shaving. Thus, even if the 38C13-CD20⁺ cells or B cells from normal donors are first allowed to internalize the bound mAbs for 2 h, they very rapidly give up substantial amounts (60–90%) of surface-bound RTX or OFA on reaction with acceptor THP-1 cells in just 45–60 min (Figs. 2, 3, 5–7). As stated earlier, we also reported in a mouse model that human Z138 cells in the lungs of SCID mice were completely stripped of CD20 just 1 h after RTX infusion (presumably by lung macrophages) (6).

In the current study, to have a well-controlled and uniform model, we have focused on using THP-1 monocytes as the acceptor cells to study the kinetics of shaving of RTX–CD20 complexes mediated by cells that express FcγR; however, we have also found that primary human monocytes and NK cells can efficiently mediate rapid shaving of mAb-opsonized B cells (4). Moreover, several other groups have recently reported that primary human monocytes can mediate rapid trogocytosis/shaving of RTX–CD20 complexes bound to B cells. Pedersen et al. (29) reported that human monocytes promote shaving of type II CD20 mAbs from B cells as well. Also of note, Pham et al. (30) examined trogocytosis mediated by mouse RAW macrophages on RTX-opsonized Ramos cells, and their measurements revealed that the half-life for uptake of the RTX–CD20 complexes by the macrophages was ~20 min; this is in good agreement with our previous studies. Additional evidence suggests that transfer of cell surface-bound mAb–Ag complexes to acceptor cells that express FcγR may be a more general phenomenon; Iwasaki et al. (31) reported that human monocytes could take up CD8 molecules from T lymphocytes that were opsonized with anti-CD8 mAbs.

Ultimately, it is important to ascertain how effectively shaving or internalization can occur on primary human tumor cells in vivo. We have previously reported the loss of CD20 in vivo upon infusion of RTX; 1 h after infusion of 35–100 mg RTX, levels of CD20 on circulating CLL B cells were reduced by ≥80%. The results suggest that the B cells that had not been cleared by either the mononuclear phagocytic system (32, 33) or by other effector mechanisms (34, 35) had been subjected to shaving, likely because of multiple passes through the liver and spleen, allowing exposure to fixed cells expressing FcγR that can mediate either clearance or shaving. The report by Pham et al. (30) provides additional evidence suggesting that RTX–CD20 complexes bound to circulating B cells can indeed be removed by fixed tissue macrophages. Our present in vitro results confirm the findings reported by Beers et al. (7), namely that substantial amounts of bound RTX can be internalized by CLL B cells within 2 h of binding the mAb (Fig. 4). However, subsequent reaction of these opsonized cells with acceptor THP-1 cells still leads to nearly complete removal of RTX and CD20 from the cell surface in only 45–60 min (Figs. 5, 7).

It is likely therefore that shaving is the primary mechanism responsible for the substantial and rapid loss of CD20 observed in vivo after infusion of the standard dose (375 mg/m²) of RTX. This large dose tends to promote exhaustion of effector mechanisms that remove and kill the CLL cells, thus allowing persistence of RTX-opsonized cells in the bloodstream that are subject to shaving (27, 35). In our model system, we use an excess of THP-1 acceptor cells, which should mimic the in vivo situation in which RTX-opsonized CLL B cells are likely to make many passes through tissues such as the liver and spleen, which contain large numbers of fixed cells that can mediate shaving. We therefore submit that the in vitro model we have tested is quite reasonable and replicates the clinical findings. We note that we have demonstrated in a pilot study that treatments, based on much lower but more frequent doses of RTX, may better preserve effector function and also reduce shaving of CD20 (1, 2).

We have also previously demonstrated that several other Food and Drug Administration-approved mAbs, including trastuzumab and cetuximab, promote rapid shaving of opsonized cells in the presence of acceptor THP-1 cells (4). Moreover, ~30 y ago, there were reports of several examples of “antigenic modulation” (36–39). In these studies, opsonized tumor cells were found to lose bound mAb and target Ag, and in vitro investigations confirmed that these reactions required the action of acceptor cells that expressed FcγR. It is quite likely that these reactions were also mediated by shaving/trogocytosis.

In summary, downmodulation of CD20 may impact the efficacy of anti-CD20 mAb-based therapies, depending on the level of CD20 expression required to promote killing of the target cells. In this study, we directly compared two different mechanisms that can promote downmodulation of CD20 on B cells. Internalization is mediated directly by the B cells themselves, whereas shaving or trogocytosis is mediated by acceptor cells, based on recognition of the mAb-CD20 immune complexes by FcγR on the acceptor cells. Our experiments indicate that shaving induces considerably more loss of CD20 from the opsonized B cells in a shorter period of time than observed for internalization, and therefore, shaving/trogocytosis is most likely responsible for the acute loss of CD20 observed when CLL patients are treated with RTX. The reported Ag loss from mAb-targeted cells that can occur as a consequence of mAb-based therapies in other systems may also be mediated by the shaving reaction. It would seem reasonable that additional studies should be conducted in the future that evaluate...
directly whether other tumor-associated Ags targeted by immuno-
therapeutic mAbs are also subject to shaving/trogocytosis.

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F.J.B., P.J.E., P.W.H.I.P., and J.G.J.v.W. are employees of Genmab and have stock holdings. The other authors have no financial conflicts of interest.

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