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Effector mechanisms of anti-CD20 monoclonal antibodies in B cell malignancies.

Abbreviated title: Effectors of anti-CD20 therapies.

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Abstract

Activation of the complement system by tumor cells was long believed to act only for benefit of the host. Overexpression of complement inhibitors by many tumor cell types and results obtained in several experimental animal models were all in agreement with this hypothesis. However, recent reports imply that the situation is more complex than initially believed and that under certain circumstances tumor cells may use complement to their own advantage, e.g. by recruitment of suppressor T cells or promoting local angiogenesis. Such a dual role of complement may also be apparent when considering the effect of therapeutic monoclonal antibodies (mAb) used to successfully treat B cell malignancies, such as CD20 mAbs. Some argue that besides direct tumor cell killing by mAbs, two main immune effector mechanisms, complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC), may be competing with each other. Experiments aiming at answering the question whether complement is our friend or foe in mAb therapy ended up with seemingly contradictory conclusions. Herein, we revisit the existing knowledge on this pivotal issue based on rituximab and other anti-CD20 mAb as a model of therapeutic agents.

Keywords: CD20, antibodies, immunotherapy, lymphoma, CLL
**Brief introduction to the complement system**

Nowadays there are three generally acknowledged complement pathways: classical, alternative and lectin, which differ in initiating mechanisms and molecules participating in the early stages of cascade activation. Additionally, there are two more mechanisms distinguished recently, i.e. direct processing of the C3 molecule by proteases, and the properdin-driven pathway. The formation of the C3 and C5 convertase enzyme complexes are key events in complement activation, and also the stage at which all pathways converge. These convertases activate C3 and C5, causing the release of chemoattractants (C5a and C3a), opsonization of surfaces with C3b, and cell lysis due to assembly of the membrane-attack complex (MAC) built on the platform of cell-associated C5b molecule. This system may be efficient enough to remove all the complement-activating cells, unless membrane bound or soluble complement inhibitors interfere with the process.

**Tumor cells activate the complement system**

Transformation from normal to malignant phenotype is often reflected in cell membrane composition due to accompanying metabolic changes. There are reports showing that altered glycosylation patterns, changed proportions of phospholipids, lipid peroxidation or exposure of novel tumor epitopes distinguish cancer cells from their normal counterparts and make them visible to the immune system, including complement. Indeed, complement activation via the classical, lectin and alternative pathways by tumors of different origin was described already 30 years ago. However, spontaneous complement activation by tumor cells has usually little or no therapeutic importance, since tumor cells often overexpress complement inhibitors and tumor-specific antibodies are present at low titer or have low affinity. Nevertheless, the fact that tumor cells usually overexpress one or more membrane-bound complement inhibitors, produce substantial amounts of soluble complement inhibitors or develop strategies to counterattack complement activation suggest that controlling complement activation is pivotal for tumorigenesis. More to this end, an oncogenic virus
Kaposi’s sacroma herpesvirus (KSHV/HHV8), which is an etiologic factor responsible for Kaposi’s sarcoma and certain lymphoproliferative malignancies, encodes its own complement inhibitor - KCP. On the other hand, there are reports showing that tumor cells may benefit from triggering complement activation, by mechanisms such as the C5a-dependent recruitment of myeloid-derived suppressor cells or induction of proangiogenic factors. Also, under hypoxia some tumor cells seem to purposely give up their mechanisms of protection from complement, which is in sharp contrast to endothelial cells, which increase the expression of complement inhibitors under hypoxia. Interest in complement as a significant effector mechanism of the immune response has been greatly renewed since mAb therapeutics were introduced, and the vulnerability of tumor cells to CDC was classified as one of the predictors of efficacy of mAb-based cancer therapy. Also, a promising outlook for the experimental application of bispecific antibodies interacting with both tumor antigens and complement inhibitors on tumor cell surfaces has also turned attention to complement in the context of cancer.

**CD20 and CD20 mAb**

CD20 is a suitable target for immunotherapy due to its presence in high numbers on most (but not all) individual tumor B cells, limited internalization and long persistence on the surface after being bound by antibodies. Also, it is expressed at the majority of B cell developmental stages. So far there is no ligand for CD20 identified and CD20 knockout mice appear to have no discernible B cell defects but display changes in calcium signaling upon activation and reduced IgM expression on the surface. Nonetheless, the role of CD20 in proliferation, activation and survival of B cell has been suggested based on the effects of specific antibodies as well as the role in proper generation of B cell responses based on a case report of a patient lacking surface CD20 expression. Binding of CD20 by mAb can result in different effector mechanisms such as CDC, which stems from direct lysis due to MAC formation, antibody dependent cellular cytotoxicity (ADCC) employing immune cells bearing Fc-receptors, or direct induction of apoptosis. The outcome depends partially, besides the class of antibody used, on the epitope recognized by a given antibody and its proximity to the cell.
membrane, but also on the ability to relocalize and cluster CD20 molecules into lipid rafts. Generally, anti-CD20 antibodies are classified into two types: type I, which clusters CD20 into lipid microdomains and potently activate complement but do not directly induce apoptosis, and type II, which does not relocate CD20 and are therefore weak CDC activators, but are strong inducers of apoptosis (or caspase-independent cell death, as described for tositumomab and reactive oxygen species–dependent cell death described for obinutuzumab). Importantly, both classes retain the ability to activate ADCC. The possible downstream effects of anti-CD20 antibodies bound to the surface of B cells are described in Fig. 1. An important question emerging at this point is which of the given mechanisms is the most important for the in vivo therapeutic effect. Rituximab is a class I antibody and since approval as a treatment for patients with CD20+B cell lymphomas, it has contributed to a 50% reduction of mortality of patients with diffuse large B cell lymphomas, which is the biggest success in lymphoma treatment in the last half century. This, along with the beneficial effects of rituximab in low-grade NHL and CLL (if combined with chemotherapy), is why there is much interest in elucidating the main effector mechanisms of anti-CD20 reagents, as well as other anticancer mAbs, due to the potentials for increasing efficacy by improvement of effector mechanisms through antibody selection or engineering.

Are CDC and ADCC direct competitors?

In spite of the fact that CDC and ADCC lead to the same end point, i.e. cell lysis and death, they do not always seem to work in a synergistic manner. In fact CDC and ADCC may compete with each other, most probably due to steric hindrance caused by early complement component deposition on the surface of target cells. Addition of serum but not heat-inactivated serum to rituximab-coated cells inhibited activation of NK cells and this effect was dependent on the presence of C1q and C3 in serum, but not C5. The same phenomenon was found when authors replaced serum with transudative pleural fluid or nonmalignant ascites in order to establish a model for extravascular fluids, where most B cell lymphoma cells appear to reside (e.g. in lymph nodes). The occurrence of CDC and ADCC depends on a certain threshold of CD20 molecules
present on the surface of target cells. However, CDC requires a much higher expression of specific antigen and the correlation between cell-surface CD20 and CDC is described by a sigmoidal curve \(^{41}\). Interestingly, maximal ADCC (analyzed at a 1:10 target / effector cell ratio) was achieved at an absolute number of CD20 molecules lower than that necessary for saturation of CDC (measured using 50% serum). The authors concluded that ADCC and CDC act in a cooperative manner since cells resistant to one killing mechanism were sensitive to the other and vice versa. However, there are several potential reasons for the resistance of individual cells to killing, and the observed results do not exclude the previous hypothesis of direct competition between CDC and ADCC upon rituximab treatment. Recently this hypothesis was further confirmed by a study using immortalized NK cells as effectors for ADCC \(^{42}\). Such a model, which allows reproducible cytotoxic activity omits the problem of variation of ADCC efficiency between different patients and even the same patient examined at different time points. However, the hypothesis of antagonism between CDC and ADCC was not tested for CD20 antibodies other than rituximab, and as related to steric hindrance, may not hold for different monoclonal reagents such as ofatumumab, which bind to alternative epitopes on CD20.

**Concluding “per analogiam” is not possible**

Currently there are more than ten agents targeting CD20, which are clinically approved or in clinical testing (originally reviewed in \(^{43,44,29,35,45}\) and listed here in Table 1, which is compiled from these references, updated and modified). Accordantly, there are a number of published studies aiming to identify the molecular basis of the exerted clinical effects. However, the main translational problem is that in spite of non-contradictory results (e.g. these obtained for ofatumumab in CLL \(^{46,47,48}\)) these antibodies recognize different epitopes of CD20, localized at various distances from the cell membrane. This variable appears to be of major importance for efficacy, as described in terms of T cell mediated lysis \(^{49}\) or in terms of complement activation and CDC \(^{50,34,51}\). Moreover, different antibodies may induce differing mobility and further segregation of Ab-Ag complexes into membrane rafts, with potential importance for complement
activation, as shown for the panel of mAbs recognizing CD20 antigen. Ofatumumab serves as good example of a similar immunotherapeutic, whose mechanism of action should not be translated directly to e.g. rituximab. Although both are class I anti-CD20 antibodies, ofatumumab binds an epitope located more proximal to the cell surface, which is more conducive to triggering CDC and significantly lowers the threshold of surface-exposed CD20 molecules necessary to allow cell lysis. Recently, ofatumumab was shown to exert clinical responses in rituximab–refractory NHL patients as well as in fludarabine / alemtuzumab–refractory CLL. Similarly, some of the clinically tested anti-CD20 mAbs were engineered to enhance ADCC, but these modifications and their effects could not distinguish either CDC or ADCC as a crucial effector mechanism for the parental compound. An important lesson comes from veltuzumab, a humanized anti-CD20 IgG1 mAb containing the same complementarity determining regions (CDRs) as rituximab, but with a single aa substitution in the CDR3 region (Asp101 to Asn), and with a Fc domain derived from another immunotherapeutic, epratuzumab. Although epratuzumab does not exhibit any CDC activity, veltuzumab triggers CDC even more efficiently than rituximab when tested on Daudi cells. Therefore, results and conclusions obtained for different anti-CD20 antibodies cannot be directly extrapolated to each other, as overall efficacy is determined by multiple factors of the antibody structure and the experimental setting. Another problem in assessing antibody suitability is associated with differences in human and murine complement, since many studies are performed in mouse models. In addition to the presence of unique inhibitors (Crry) or altered functionality of other common inhibitors (C4b-binding protein, C4BP), serum from most laboratory mouse strains shows considerably weaker lytic activity comparing to other species including humans, as reported by Ong and Mattes. Further, it was demonstrated that the classical complement pathway in mice exists mainly at an initiation level because of critical changes in C4 structure, which disable further classical C5 convertase activity.

Are the data from animal models really contradictory?
Knowing the above limitations regarding interspecies and inter-antibody extrapolation, one can consider the seemingly contradictory results obtained from different animal studies and ex vivo assays. Controversies over the most important effector mechanism mainly concern rituximab, whereas for other CD20 antibodies existing results give unanimous conclusions. For example, the \textit{in vivo} therapeutic effect of tositumomab is not affected by decomplementation by cobra venom factor (CVF) \textsuperscript{64}. Ofatumumab appeared more effective in controlling lymphoma xenograft growth than rituximab and this feature correlated with superior CDC capabilities \textit{in vitro} \textsuperscript{65}. However, one mouse model showed that ADCC may play an important role in ofatumumab’s therapeutic effect \textsuperscript{66}. Nonetheless, this model did not judge the relative contributions of CDC and ADCC. Depletion of NK cells and neutrophils totally abrogates veltuzumab-mediated prolonged survival of SCID mice injected with Raji cells and thus underlines the importance of ADCC for this antibody \textsuperscript{58}. Returning to controversies, a 38C13 murine lymphoma model and MS11G6 anti-lymphoma mAbs were used to mimic the human model of leukemia treatment by rituximab. In such a system, depletion of C3 from mouse serum prolonged survival and increased NK-dependent ADCC compared to mAb alone \textsuperscript{40}. \textit{Uchida et al.} studied depletion of B cells in mice upon injection of different mouse anti-mouse CD20 antibodies and concluded that elimination was dependent on FcR\textsubscript{\gamma} receptors and was performed mainly by monocytes/macrophages and was not facilitated by complement, as demonstrated in C3 and C4 deficient animals \textsuperscript{67}. In contrast to these studies, Di Gaetano \textit{et al.} showed, using the mouse lymphoma cell line EL4 stably expressing human CD20 (EL4-CD20), that rituximab’s therapeutic effect is not dependent on NK cells and neutrophils, and is also possible in athymic mice. In addition, C1q-deficient mice were not protected from tumor burden by rituximab, showing that complement plays a role in rituximab’s effect \textsuperscript{68}. Having differing studies, where first one seems to dismiss the role of complement in therapeutic effects whereas the latter underlines the importance of complement, one may ask which of these two models properly reflects the action of this mAb in B cell malignancies. To make the question even more complex, another study performed on the same EL4-CD20 model concluded that ADCC and CDC have different impacts on rituximab’s therapeutic effect depending on the local tumor burden \textsuperscript{69}. Authors inoculated wild type or FcR\textsubscript{\gamma} receptor deficient
mouse intraperitoneally with labeled tumor cells followed by rituximab, ofatumumab or PBS injection 16 hours later and finally assessed the number of tumor cells washed out from peritoneal cavity after another 24 hours. When inoculated with a low initial number of EL4-CD20 cells, fewer cells were recovered from wild-type and FcRγ⁻ animals treated with rituximab or ofatumumab compared to PBS–treated mice. However, application of anti-CD20 mAb was ineffective in FcRγ– deficient mice when ten times more tumor cells were applied. Notably, to combat such high numbers of tumor cells both functional complement and FcRγ receptors were necessary. Imai and colleagues used EL4 cells but a different target (gangliozyde GD2) and showed that complement may be irrelevant or has supplementary role in therapeutic effect depending on the concentration of sensitizing antibody \(^70\). Taken together, different lymphoma cells and their number together with different mouse strains and different anti-CD20 antibodies may generate too many variables making a unanimous conclusion impossible to draw. Instead, the picture from animal studies seems to be model-dependent and thus conclusions are resistant to generalization.

**Conclusions from observations in man.**

In spite of translational problems described above, animal studies may give us a hint of what can happen when CD20 mAbs are used in humans. However, *in vitro* and *ex vivo* studies performed on cells isolated from patients and their subsequent correlation with clinical parameters seem to be more adherent to the real, clinical situation. Nonetheless, these approaches have their own limitations. Some methods used to measure the activity of immune cells or to indicate cell death may generate artefacts and these problems were recently described in review by Golay and Introna \(^71\). Also, the multifactorial nature of immune system-mediated killing mechanisms requires consideration of every single parameter as a variable influencing the total readout, before one merges data from different studies. Otherwise, as with the use of differing animal models, one may conclude that obtained results are contradictory. For example, effectiveness of CDC, ADCC and complement dependent cellular cytotoxicity (CDCC) in *in vitro* killing of two human non-Hodgkin lymphoma (NHL) cell lines Raji and HF-
1.3.4 were compared after incubation with rituximab. CDC at 25% serum concentration killed 50% and 20% of HF-1.3.4 and Raji cells, respectively and this effect could be increased to 80% by simultaneous neutralization of membrane bound complement inhibitors, whereas ADCC and CDCC (at a 1:10 target to effector ratio) eliminated only 10 or 15% of cells. Addition of N-formylmethionylleucylphenylalanine or PMA increased the efficiency of these mechanisms to only 25%. Susceptibility of primary cultures from patients suffering from follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphomas and small lymphocytic lymphomas to CDC, ADCC, antibody mediated phagocytosis and induced apoptosis have also been studied. All cell types were equally sensitive to all killing mechanisms except for CDC, which was dependent on the combination of expression levels of CD20 and membrane bound complement inhibitors. Follicular lymphomas exhibited the most efficient lysis due to CDC while small lymphocytic lymphomas were the most resistant type, which followed the clinical data describing the response to rituximab. However, another study showed that lymphocytes isolated from lymph nodes of patients subsequently treated with rituximab did not significantly differ in expression of complement inhibitors or CDC susceptibility when patients were classified into responder, partial responder and non-responder groups. Authors also eliminated CD20 as a single parameter influencing the outcome, since there were no significant differences in its expression between the groups, but data were not related to the absolute number of CD20 per cell and one can speculate whether the expression was not already in the range of saturation of the sigmoidal curve described by van Meerten et al in their in vitro studies. Trying to reconcile these results, it was suggested that supracellular factors (including tumor burden, which would be in agreement with mouse model experiments) are responsible for the overall effect and CDC could still play an important role as a part of the whole machinery, which fuels the other components. Such a scenario appears reasonable if one considers the existence of a delayed response to mAb therapy in some patients, which cannot originate from CDC due to its rapid kinetics. Initiation of the complement cascade leads to both CDC and CDCC and the latter may have been lost or underappreciated in all the experiments with relatively short time frame like minutes or a few hours. For example, the beneficial effects of fresh-frozen plasma therapy administrated simultaneously to
rituximab in CLL patients were proposed 77 and reported 78,79. Importantly, fresh-frozen plasma may serve as a source of all the components of complement, and thereafter they may be used in CDC or CDCC, and since none of the patients were deficient in certain type of immune cells or had any other kind of diagnosed immunodeficiency, one cannot exclude any of these two effector mechanisms. Nonetheless, CLL is characterized by a much lower level of CD20 expression compared to NHL 80, and therefore any compensation or modulation of complement activity would certainly cause different outcomes. There are reports showing that some CLL patients either deficient or had altered expression of one or more complement components 81. Moreover, symptoms of acquired C1 inhibitor deficiency are found in an appreciable percentage of CLL patients and as such may interfere with the effectiveness of CDC 9. Another possibility for the underestimation of CDC’s role in short-time experiments lies in sublytic MAC deposition on the cell surface. Direct lysis will only be achieved when sufficient number of MAC complexes is inserted into the target membrane 82 but cells bearing the number of MAC under this threshold experience a variety of responses including both further resistance due to intracellular signaling and kinase activity, or sensitization (reviewed in 83). Sublytic MAC insertion results in DNA fragmentation dependent on serum DNase I in a number of malignant B cell derived cell lines 84.

Outwardly contradictory results are also presented regarding CLL and obinutuzumab, which is class II humanized IgG1 antibody, engineered in Fc portion 85. Patz et al showed, that CDC is not involved in killing of isolated CLL cells 86 whereas Bologna et al. postulated moderate effect of CDC in obinutuzumab cytoidal effect, since full blood killing by this mAb was reduced c.a. 60% by C5 –blocking Ab eculizumab 87. However, experiments were performed at different CD20 mAb concentrations, 10 µg/ml and 100 µg/ml, respectively and also different cell number (4x 10^5 and 1x 10^6 cells/ml, respectively) thus making a direct comparison difficult. Another problem is the usage of DNA-binding dye 7-AAD as a marker of CDC in flow cytometry. Since it cannot pass intact cell membrane, negative result would mean no CDC but any positive result will inherit a risk of false positive readout due to e.g. secondary necrosis caused by direct effect of mAb 88. Interestingly, importance of tumor cell number in ADCC assays was confirmed by the fact that blocking of CD16 on NK cells significantly reduced
obinutuzumab–mediated depletion of B cells in full blood from healthy individuals but not from CLL patients. This phenomenon could be explained by different ratio of target to effector cells.

There is some evidence that individual polymorphisms in Fcγ receptors may be associated with clinical responses to rituximab as a single marker and these data favor ADCC as an important mechanism of therapeutic effect. However, when tumor cells taken before therapy from responders and non-responders from the same study were examined for in vitro sensitivity to ADCC, no significant differences were found between the groups. Thus, similarly to CDC, some important aspects may be underestimated in relatively rapid in vitro assays for ADCC. Fresh, purified lymphocytes were taken from biopsies of patients with follicular lymphomas and diffuse large B cell lymphomas and tested for CDC sensitivity in vitro. Imaging propidium iodide uptake by such cells treated with 20% serum for 10 minutes revealed strong correlation between positive response and cell damage by complement. Comparing this study with that by Weng and Levy, who obtained opposing results, several differences in methodological and technical aspects must be pointed out. Most importantly Mishima et al. studied patients receiving rituximab not as a single agent but with chemotherapy. Then, other important inconsistencies include cryopreservation of the cells after biopsy and purification of CD19 expressing cells in order to eliminate potential effector cells (e.g. T lymphocytes, NK cells or leukocytes).

Work by Beurskens et al. extends the hypothesis of tumor burden as a critical parameter for the effectiveness of killing mechanisms from mouse models to human systems, while addressing ofatumumab, but also underlines the importance of optimal dosing of therapeutic antibody. Interestingly, increasing the dose of ofatumumab resulted in higher C3b deposition on CLL cells but did not increase CDC. Instead, complement became exhausted, and when the first wave of cells was treated with antibody concentrations exceeding the CDC saturation level, subsequently introduced CLL cells with ofatumumab could not be killed. Since elimination of CD20-positive cells from the bloodstream promotes the re-equilibration of these cells from other compartments, such experiments correspond to the in vivo situation, and reveal that tumor cell load, antibody
concentrations and schedule of administration are important variables which should be taken into account when comparing experimental data.

**Concluding remarks**

There is still no conclusive evidence for the pivotal role of CDC, ADCC, apoptosis or non-apoptotic mechanisms as the predominant therapeutic effectors of various types of CD20 mAbs. Existing limitations concerning the translation of in vivo mouse experiments, together with technical issues of *ex vivo* assays, lead to as yet inconclusive answers. Heterogeneity among patients, tumor burden and treatment regimens add further to this problem. In spite of probable direct competition on the surface of single cell, the various mechanisms may cooperate in terms of whole populations of tumor cells. Identification of strong, single factors predicting the outcome of therapy would be valuable, but they must be assessed individually for the different narrowly classified lymphomas, as they differ in composition of membrane proteins or kinase activity, which influence vulnerability to mAb therapy and sensitivity to complement and the other effector functions. It seems that in most cases such a single predicting parameter has been extremely hard to identify because of too many variables crucial for therapy, or due to the generation of possible artefacts by assay methods. For that reason, one way to proceed is to identify tests, which will better discriminate between particular effector mechanisms, such as CDC and CDCC or CDC and direct apoptotic / non-apoptotic killing of tumor cells. Having a clear picture of which effector mechanism is crucial for a given lymphoma type and tumor cells with given parameters, one could attempt to further modulate the existing anti-CD20 immunotherapeutics in order to maximize the favorable killing system at the possible expense of others which may be less important. Following the clinical approval of rituximab and ofatumumab, next generation CD20 mAbs have been introduced in clinical trials, and there are also attempts to introduce additional modifications resulting in multifunctional activities\(^93,94\). Understanding the relative contribution of particular effector mechanisms are very likely to contribute to further clinical improvement of these therapeutics.
Conflict of interest statement

AÖ has received honoraria for lectures and advisory boards by Glaxo-Smith Kline and Sanofi.

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**Table 1. Anti-CD20 antibodies in clinical trials and clinically approved.**

Data were compiled from references \(^{43,44,29,35,45}\) and updated from www.clinicaltrials.gov \(^{131}\), § - bound to Y\(^{90}\), n.s. – not specified
Figure legend

Fig. 1 Possible actions of anti-CD20 mAbs.

Binding of anti-CD20 mAbs to B cells can exert different effects: CDC (1), ADCC (2) or direct effects (3) all resulting in subsequent cell death. CDC (1) takes place when a certain threshold of CD20 molecules is available for mAbs and C1q crosslinks several Fc domains bound in close vicinity. C1r/C1s proteases then cleave serum components C2 and C4 to C2a / C2b and C4a / C4b, respectively. C4b2a acts as classical C3 convertase – an enzymatic complex capable of cleaving C3 to C3a and C3b. C3b binds to C4b2a thus switching its specificity to the C5 component (C4b2aC3b is the classical C5 convertase). Alternatively, C3b binds to the cell surface, where it forms a novel platform for alternative C3/C5 convertases (C3bBb or C3bBbC3b) acting as an amplification loop of the classical complement pathway. The cleavage product of C5, C5b, is inserted into the cell membrane and initiates the assembly of MAC together with C6, C7, C8 and several C9 molecules, leading to osmotic cell lysis. ADCC (2) needs a moderate, number of CD20-mAb complexes compared to CDC, but seems to act in a competitive manner with CDC. The first step is the recognition of the antibody Fc portion by Fc receptors on effector cells (mainly NK cells but also neutrophils or eosinophils). Then, effector cells release the content of specific granules containing pore-forming and cytotoxic compounds, which target the B cell and lead to its programmed or spontaneous cell death. Direct effects (3) cause cell death without additional effector cells or serum proteins but by binding of CD20 by mAbs alone. Aggregation of B cells by anti-CD20 Abs may precede caspase –independent cell death, as described for tositumomab \(^{36}\), reactive oxygen species –dependent cell death (described recently for obinutuzumab \(^{37}\)) or apoptotic cell death \(^{95,96}\).