Oncogene (2003) 22, 7359–7368

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Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance

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Rituximab, a chimeric monoclonal antibody targeted against the pan-B-cell marker CD20, was the first monoclonal antibody to be approved for therapeutic use. Treatment with rituximab at standard weekly dosing is effective in more than 50% of patients with relapsed or refractory CD20-positive follicular non-Hodgkin's lymphoma, but is not curative. It is less effective in other subtypes of CD20-positive lymphoma and for retreatment, even with CD20 still expressed. Thus, binding of rituximab to CD20 is not sufficient to kill many lymphoma cells, indicating that there are mechanisms of resistance. Mechanisms of cell destruction that have been demonstrated to be activated by rituximab binding to CD20 include direct signaling of apoptosis, complement activation and cell-mediated cytotoxicity. The relative importance of each of these mechanisms in determining clinical response to rituximab treatment remains a matter of conjecture. Thus, the role of various resistance pathways, some documented in experimental systems and others still hypothetical, remains uncertain. Resistance could potentially be mediated by alterations in CD20 expression or signaling, elevated apoptotic threshold, modulation of complement activity or diminished cellular cytotoxicity. As the first of an expanding class of anticancer agents, lessons learned regarding the mechanism of rituximab action and resistance will be of increasing importance.
Oncogene (2003) **22,** 7359–7368. doi:10.1038/sj.onc.1206939

Keywords: antibody dependent cellular cytotoxicity; non-Hodgkin's lymphoma; B-cell receptor signaling; apoptosis

Introduction

Rituximab, a chimeric monoclonal antibody that binds to CD20, was the first monoclonal antibody to be approved for clinical use in the therapy of cancer. It is approved for use against indolent B-cell non-Hodgkin's lymphoma (NHL), although its use has expanded significantly beyond that indication to virtually any CD20-positive NHL, and more recently into other areas such as autoimmune disorders. This review will focus on efficacy of, and resistance to, rituximab in treatment of B-cell NHL.

The search for tumor-specific antigens that would serve as targets for antibody-mediated attack on cancer cells has been largely disappointing to date, although genomic and proteomic approaches hold out the promise of the future identification of such tumor-specific proteins. NHL are malignancies of lymphocytes. About 80–90% express B-cell markers and have immunoglobulin gene rearrangements indicating their clonal derivation from a B-cell progenitor, while most of the remainder express T-cell antigens and harbor clonal T-cell receptor gene rearrangements. Given the preponderance of B-cell malignancies, attention was focused on these in monoclonal antibody development. Since clonal immunoglobulin gene rearrangements translate into a clonal surface immunoglobulin specific for the NHL cell, these immunoglobulins are truly tumor-specific antigens. Thus, anti-idiotype antibodies had potential as tumor-specific therapeutic agents. While such anti-idiotype antibodies have clinical activity, the logistics of developing an individual therapeutic antibody for each patient proved insurmountable (Grillo-Lopez, 2000). B-cell-specific markers could be similarly targeted and would not require individualized development. Rituximab targets CD20, a transmembrane protein present on virtually all B cells from the stage at which they become committed to B-cell development until it is downregulated when they differentiate into antibody-secreting plasma cells (Reff *et al*., 1994). Thus, CD20 is considered a pan-B-cell antigenic marker. The role of CD20 in B-cell development remains uncertain, as CD20-deficient mice have normal B cells (O'Keefe *et al*., 1998). Similarly, CD20 is expressed on virtually all B-cell NHL, but not on most multiple myeloma cells that correspond to more mature B cells with plasma cell differentiation (Anderson *et al*., 1984). Targeting such a marker, however, would be expected to be associated with the confounding problem that it would not only kill lymphoma cells, but also normal B-cell counterparts. In fact, rituximab does clear both normal and malignant CD20-positive cells, but the absence of normal B cells for approximately 6 months has not been associated with decrease in IgG levels or significant increase in infectious risk (McLaughlin *et al*., 1998). Other major considerations in selecting CD20 as an appropriate target included that the antigen is expressed at reasonably high levels, is not downregu-

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lated after antibody binding and is not shed or secreted into the circulation, which avoids the antibody binding solely to these circulating molecules and not reaching the NHL cell (Grillo-Lopez, 2000).

NHL is an increasing problem, now being the fifth most common malignancy in the United States (Greenlee *et al*., 2000). Since the incidence rises with age, as the population ages NHL has become increasingly common, but there has also been over the past 40 years an increased incidence at any given age. The reason for the increase is not clear, but attention has focused on environmental factors. The histopathologic classification of NHL has historically been quite complex. Improved understanding of the biology of normal B-cell development, and that B-cell NHL correspond to specific stages of normal B-cell development, has led to a more rational classification of B-cell malignancy (Harris *et al*., 1994). About 1/3 of NHL have a follicular growth pattern, reminiscent of normal lymph node architecture, and these generally have an indolent clinical course, or low-grade histology. Other less common indolent B-cell NHL are small lymphocytic lymphoma, with cells indistinguishable from chronic lymphocytic leukemia, and marginal zone- or mucosa-associated NHL. Patients with these indolent NHL have a median survival of approximately 10 years (Armitage and Weisenburger, 1998), but the disease is considered incurable with current therapeutic options. The usual clinical course is a series of remissions and relapses, with different treatments used for each relapse. Thus, new treatments are needed. About 1/3 of NHL are diffuse-large-cell or immunoblastic NHL with a more aggressive clinical course. These are potentially curable with combination chemotherapy, but if not cured usually are fatal within 2–3 years (Fisher *et al*., 1993). Again, improved therapy is needed.

Patients with indolent NHL were considered a suitable population in which to test antibody-based therapy initially, because there is often no urgent need for therapy. Since antibody-based therapy might be slow to act, an indolent disease would also permit a safe period of observation. Rituximab is an active therapy as a single agent against indolent NHL. The success of rituximab likely relates to a number of factors, one of which is that it is a chimeric rather than a murine antibody (Grillo-Lopez, 2000). Rituximab retains the murine CD20-binding Fab regions, but uses a human Fc portion (Figure 1). This has at least two major effects. First, with less anti-mouse antibody (HAMA) development, the $t_{1/2}$ of the antibody is prolonged by the presence of the human Fc portion. Second, the Fc portion contains the effector aspects of the molecule, including complement activation and attraction of cytotoxic cells, so that the human Fc is more effective in activating these effectors in patients.

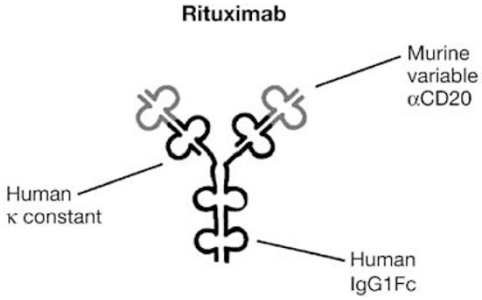

Figure 1 Rituximab chimeric structure. Binding regions from original murine anti-human CD20, consisting of variable regions of immunoglobulin heavy and light chains, are fused to human IgG1 heavy-chain and human kappa light-chain constant regions. Fc portion from human IgG1 was selected for its ability to fix complement and activate antibody-dependent cellular cytotoxicity

Rituximab efficacy and scope of clinical resistance

Rituximab is approved for use in refractory or relapsed indolent NHL, based on a pivotal trial demonstrating a response rate of over 50% in such patients (McLaughlin *et al*., 1998). The responses in this study lasted just over 1 year. The standard dose and schedule for rituximab, used in this trial, is 375 mg/m^2 given once weekly for 4 weeks. In phase I trials, no true maximum tolerated dose was reached due to lack of toxicity, so the dose and schedule were selected somewhat empirically and limited by practical considerations about rituximab availability. Thus, higher doses, more frequent administration and/or additional doses are now possible. Owing to its efficacy and tolerability, trials of rituximab as induction therapy are underway and preliminary results suggest higher responses than in relapsed patients (Colombat *et al*., 2001; Hainsworth *et al*., 2002), although ultimately improved survival is the critical end point. While rituximab is an effective addition to therapy for CD20-expressing NHL, it is active in patients with relapsed or refractory disease in only 50–60% of follicular lymphoma and 10–15% of small lymphocytic lymphoma (McLaughlin *et al*., 1998), despite CD20 expression in all cases treated. Furthermore, patients with indolent NHL treated with rituximab rarely develop CD20-negative disease. Yet, of those who respond to rituximab and then relapse more than 6 months later, only 40% will respond again when retreated with rituximab (Davis *et al*., 2000). Thus, there must be mechanisms of resistance independent of CD20 expression.

Evidence for multiple mechanisms of rituximab action has been reported, and it remains unclear which is/are most important in patients. Therefore, it is difficult to know the relative importance of potential mechanisms of resistance. What is known about the steps that lead to rituximab activity, as well as potential levels of resistance, is reviewed here, with the view that this schema will serve as a framework to consider potential resistance mechanisms (Table 1) and to highlight specific areas that require attention in clinical trials. The steps are divided into those that lead up to CD20 binding to the surface of the NHL cell, that is, pre-CD20 binding, and the results of such binding that lead, hopefully, to NHL cell elimination, that is, post-CD20 binding. Many

Table 1 Summary of potential mechanisms of rituximab action, corresponding means of resistance and conceptual approaches that might address these resistance mechanisms. These issues are discussed further in the text

Mechanism of action	*Examples of resistance mechanisms*	*Potential means to overcome resistance*
CD20 binding	↓/absent CD20 expression	Higher/more frequent rituximab doses Cytokines to ↑ CD20 expression
	↑ accessible CD20 Soluble CD20 ↑ tumor burden	Cytoreductive therapy + rituximab
	↑ Ab metabolism	Administer with IVIg
	Poor tumor penetration	Alter affinity, charge or size of Ab
Post-CD20 binding		
B-cell signaling	Membrane rafts/clustering	
	Altered PLC	Small molecule
	Altered tyrosine kinase	Kinase activators/inhibitors
Direct apoptosis	Elevated bcl-2	Antisense bcl-2
Complement activation	Complement inhibitors	Fludarabine to ↓ CD55/CD59 Antibodies to CD55/CD59
ADCC	FcγRIIIA polymorphism	Ab engineered to bind to FcγRIIIA F158
	↓ Effector cells (e.g. after chemotherapy)	Restore effector cells (e.g. cell transfer or cytokine expansion)
	Inhibit perforin/granzyme	Small molecules to block inhibitors

of the pre-CD20-binding considerations are similar to traditional drugs, while others depend on the specificity of binding and the distribution of target sites. Post-CD20 binding effects may include receptor signaling, calcium flux, direct apoptosis, complement activation or attraction of antibody-dependent effector cells. Potential exists for resistance at any one of these steps.

Events up to CD20 binding

Many events occur prior to rituximab binding to tumor cell surface CD20. These events determine whether rituximab reaches a sufficient percentage of the lymphoma cells in sufficient quantities to lead to cytotoxicity. Sufficient, however, remains at best empirically defined.

Serum levels of rituximab

As a targeted monoclonal antibody, rituximab behaves differently from standard chemotherapeutic agents, in that its pharmacokinetics are influenced not only by the characteristics of an antibody molecule, but also by the presence of the target, and whether the target is circulating or cellular. The half-life of a monoclonal antibody in human circulation is determined, in part at least, by the Fc portion of the immunoglobulin molecule. The actual level, however, depends upon the total amount of accessible CD20, a value that reflects circulating CD20 and both the number of CD20-positive cells and the density of CD20 per cell. Levels of rituximab tend to increase with repeated doses (Berinstein *et al*., 1998) as the number of CD20 molecules decreases. Serum rituximab levels have been reported to be higher in responders than in nonresponders (Berinstein *et al*., 1998). However, whether the higher levels lead to a better response rate or whether responders have fewer CD20 molecules due to lower tumor burden and therefore higher circulating antibody levels have not been determined. Nonetheless, rapid metabolism of rituximab, due either to high numbers of accessible CD20 molecules and/or to alterations in host antibody metabolism, could be a basis for resistance. Attempts to circumvent this possibility with repeated dosing have been pursued to raise antibody levels and therefore to try to improve response rates. Use of 8 weekly doses rather than the standard 4 weeks in a nonrandomized trial suggested the possibility of a higher response rate in relapsed/refractory low-grade lymphoma (Piro *et al*., 1999). In CLL, a disease in which the cells have low CD20 levels and a low response rate to standard dose and schedule rituximab ($375\,mg/m^2$ weekly $\times$ 4), higher and repeated doses did lead to higher response rates, although these tend to be short lived (Byrd *et al*., 2001; Keating *et al*., 2002b). Another approach would be to individualize dosing to maintain rituximab levels above a yet to be determined threshold for prolonged periods. In fact, a randomized trial has shown a benefit for additional rituximab doses every 2 months for four doses after the initial 4-week cycle (Ghielmini *et al*., 2002). Alternatively, since normal B cells begin to recover about 6 months after rituximab administration, a schedule of 4 weeks of rituximab every 6 months has been used (Hainsworth *et al*., 2002). Development of anti-chimeric antibodies (HACA) that might rapidly reduce rituximab levels has been an uncommon finding in clinical trials, likely reflecting the human Fc portion as well as the generally immunosuppressed status of the patients.

Rituximab distribution

Antibody distribution in various compartments within the body, as well as within a malignant lymph node or extranodal tumor mass, may affect drug sensitivity or resistance. This concept is most clearly illustrated by data for the monoclonal anti-CD52 antibody Campath-

1H, found to be much more efficient at clearing NHL cells from blood and marrow than from lymph nodes, leading to studies of this agent in CLL (Osterborg *et al*., 1997). In the CLL studies, again responses are higher in blood and marrow than in nodes, although with not as marked a difference as in NHL (Osterborg *et al*., 1997; Keating *et al*., 2002a). Similarly, encouraging reports of the ability of rituximab therapy to clear blood and marrow of lymphoma cells, even as assayed by sensitive PCR methods for IgH gene rearrangements or *t*(14;18), were followed by the realization that even patients with molecular remissions continue to relapse, indicating persistence of lymphoma cells (Foran *et al*., 2000; Pichert *et al*., 2001). While more complete responses may translate into longer disease-free intervals, patients are not cured (Czuczman *et al*., 2001). Thus, antibodies may be more successful in removing cells from blood and marrow than from the rest of the body. Such findings could reflect differential access to antibody or to effector mechanisms (see below). Little data exist on the access of rituximab or other therapeutic antibodies to traditional chemotherapy sanctuary sites, such as brain or testis, which are protected from toxins by physiologic barriers, although one case report detected only very low levels of rituximab in the CSF of a patient with CNS involvement of systemic NHL (Harjunpaa *et al*., 2001).

Other factors need to be considered regarding distribution of antibody within lymph nodes and lymphoma. IgG molecules move readily out of the vessels into the tissue. The distance from a vessel reached by effective concentrations of antibody depends on the diffusion characteristics of the antibody molecule, in turn largely determined by size and charge, as well as the affinity of the antibody for its target and the target concentration in tissue (Figure 2). Rituximab has an apparent affinity constant of 5.2 nM for CD20 on human SB cells (Reff *et al*., 1994). Thus, while it might seem reasonable to strive for the highest affinity when engineering antibodies, too high an affinity and high numbers of target molecules can lead to antibody being bound only to targets close to the vessel, but not penetrating more deeply into the tissue. Too low an affinity and binding may be inadequate to have an effect. Smaller constructs, such as single-chain F_v fragments, have been designed to improve tissue penetration, but these need to be optimized based on affinity as well.

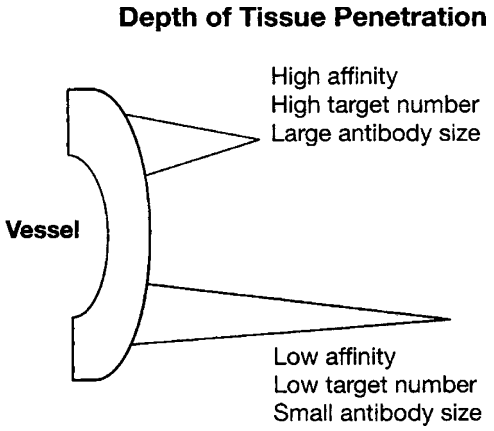

Figure 2 Potential effects of antibody characteristics and target antigen distribution on delivery of antibody as it diffuses from the vessel into the tissue. Tissue penetration of antibody depends on target density, antibody affinity and diffusion characteristics of the antibody. The latter depends on physical factors such as size, charge and aggregation. The width of the triangles indicates concentration of the antibody at variable depth of penetration from the blood vessel. The optimal balance for sufficient antibody delivery to an adequate depth of penetration is not certain

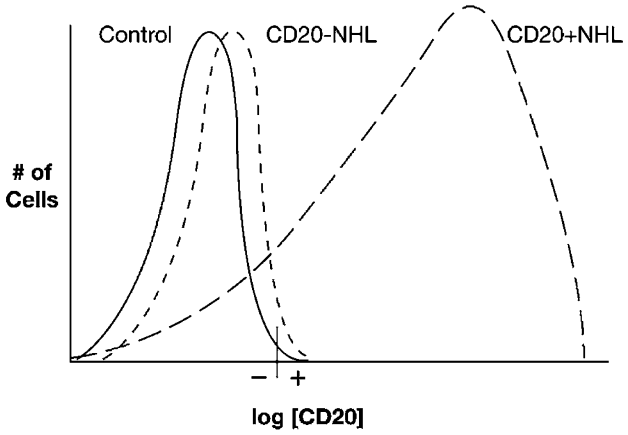

Figure 3 Flow cytometric analysis of CD20. Schematic histogram demonstrates that surface marker expression has a normal distribution. Some cells will have low expression with a signal that overlaps that of negative cells. The demarcation point between positive *vs* negative depends on the characteristics of the negative control and positive cell populations and is somewhat arbitrary, as is the number of cells that must be above this level to call a specimen positive. CLL/small lymphocytic lymphoma cells typically have dim CD20 expression, so that, while they may be clearly positive by visual inspection of the profiles, they may be considered negative. Note that the abcissa is a log scale, so that there is a wide range of levels of expression for individual cells within a given sample

Surface CD20 expression

CD20 expression is quite heterogeneous in different lymphoma types, as well as among cells of an individual tumor sample. This can be visualized when examining the distribution in a flow cytometric histogram of a suspension of lymphoma cells stained with anti-CD20 (Figure 3). A positive sample is typically determined as having $>30\%$ of cells above the cutoff, which varies with the staining of the isotype control antibody. Thus, even in a CD20-positive lymphoma, many cells may have low or background staining, and the intensity of the positive cells may vary over a log range. Typically, CLL and small lymphocytic lymphoma have dim CD20 staining, and a corresponding lower rituximab response rate than follicular lymphoma, at least for previously treated disease (McLaughlin *et al*., 1998). Mantle-cell and diffuse-large-cell lymphoma, however, have similar or even higher CD20 expression than follicular lymphoma,

but lower response rates, so that staining intensity alone does not predict for response. Furthermore, if CD20 expression alone determined rituximab responsiveness, then one would expect development of CD20-negative relapses after rituximab treatment, and this circumstance has rarely been reported, at least for indolent lymphoma (Davis *et al*., 2000). Recent reports are perhaps suggestive that CD20-negative relapses may develop in aggressive lymphomas (Davis *et al*., 1999; Foran *et al*., 2001; Kennedy *et al*., 2002), but care must be taken to differentiate true CD20 negativity from false-negative results attributable to blocking of labeled CD20 antibody by bound rituximab. There have been attempts to upregulate CD20 to try to enhance rituximab efficacy, such as with G- or GM-CSF, but mechanisms other than CD20 upregulation may account for any enhanced effect (Ravetch and Lanier, 2000; van der Kolk *et al*., 2002).

Events after CD20 binding

The events that lead to cell killing following antibody binding to CD20 may be multifactorial. These events undoubtedly influence the cytotoxicity of rituximab and resistance mechanisms in that there are tumor cells that bind rituximab but are not killed. Thus, there must be postbinding mechanisms of resistance.

CD20 binding as a B-cell signal

While the exact role of the transmembrane CD20 molecule is not known, it is involved in B-cell differentiation and activation (Golay *et al*., 1985; Tedder *et al*., 1985), although CD20 knockout mice have normal B-cell number and development (O'Keefe *et al*., 1998). CD20 can act as a calcium channel (Bubien *et al*., 1993), either directly or by binding to or activating a calcium channel. Binding by rituximab, especially if crosslinked, initiates a cascade of intracellular signals (Figure 4). These signals may play a role, at least in part, in rituximab-mediated cell killing. CD20 is associated with a number of protein tyrosine kinases, including lyn, fyn, lck and p75/85 kinase (Deans *et al*., 1995). CD20 engagement leads to activation of phospholipase C_γ (Deans *et al*., 1993). Inhibitor studies indicate that this occurs via src-family kinases (Shan *et al*., 2000). The pleiotropic effects of PLC-γ including MAP kinase activation and specifically JNK, ERK and p38MAPK have been implicated in rituximab signaling (Pedersen *et al*., 2002). PLC-γ also cleaves PIP_3, generating inositol triphosphate and a resultant calcium flux, the latter being implicated in CD20-mediated apoptosis by the observation that intracellular and extracellular calcium chelation each inhibited apoptosis induced by anti-CD20 antibody in RAMOS cells (Hofmeister *et al*., 2000). Calcium flux could also relate to the direct effects of CD20 as a calcium channel. PLC-γ cleavage of PIP_3 also generates diacylglycerol with resultant protein kinase C (PKC) activation, although this pathway has not yet been explored as a potential mechanism for anti-

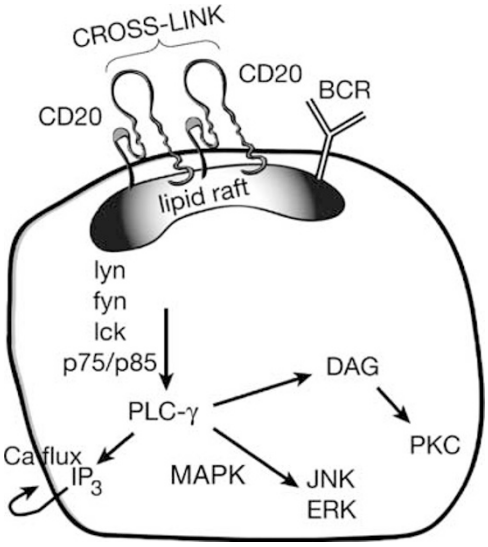

Figure 4 Outline of intracellular signaling pathways reported to be activated by crosslinking of CD20. Listed enzymes are those for which data have been published, although clearly an array of other intersecting pathways could be involved and need to be explored. Note the similarities of CD20 signaling to BCR signaling pathways, each of which involves lipid rafts

CD20 activity. Many of these signaling pathways have been found to be quite similar to signaling after physiologic engagement of the B-cell receptor (BCR) (Mathas *et al*., 2000). The result of stimulation of these complex signaling pathways, that is, cell cycle arrest or apoptosis, appears to be cell line specific. One can certainly envision that multiple factors including, but not limited to, the growth state of the cell, presence of costimulatory surface molecules and their ligands and simultaneous signals would determine whether the response of a cell to CD20 binding would be none, growth arrest, growth stimulation or apoptosis. Resistance to rituximab killing, therefore, might be due to as yet undefined alterations in intracellular signals induced by CD20 binding.

The extent of intracellular signal transduction, at least in some systems, can also be affected by the clustering of CD20 (Hofmeister *et al*., 2000). This may be dependent on the density of CD20 and the amount of antibody present. Another means of clustering is the finding that after CD20 is bound, it rapidly redistributes into a cell membrane fraction called a lipid raft (Deans *et al*., 1998, 2002), which may aid in signal activation through the src-family tyrosine kinases. These membrane changes may be long lived, and are one of several potential explanations for late responses to antibody therapy. Of interest is that CD20 associates with the BCR in these lipid rafts (Petrie and Deans, 2002), suggesting that the observed similarities in BCR and CD20 signaling may have a structural basis.

Clustering of CD20 has been more directly related to crosslinking, that is, crosslinking of the antibody molecules by several different mechanisms amplifies signaling. Thus, whereas slight tyrosine kinase activa-

tion and calcium flux may be observed after soluble anti-CD20 binds to cells, quantitatively more pronounced signals develop when homodimerized antibody is used (Ghetie *et al*., 2001) or when antibody is crosslinked by secondary antibody (Cragg *et al*., 2003) or by cells expressing Fcγ receptors (Shan *et al*., 1998). The potential role of activating and inhibitory receptors of the Fcγ family is discussed below; however, the inhibitory FcγRIIB (CD32) is the Fcγ receptor on B cells. Binding of the Fc portion of IgG to CD16 blocks simultaneous BCR-mediated signaling (Ravetch and Lanier, 2000). Given the similarities between BCR and CD20 signaling noted above, complex signaling effects downstream of rituximab binding to CD20 may occur. Also of interest is that CD22 is also a B-cell specific inhibitory signaling receptor, suggesting a possible mechanism of interaction between rituximab and anti-CD22 monoclonal antibody, a combination that has shown promising activity in clinical trials (Leonard and Link, 2002). Adjacent lymphoma cells themselves may act as crosslinking agents if they express CD32 or other Fc receptors. The degree of *in vivo* crosslinking and its importance to rituximab activity remains to be elucidated.

CD20 binding induces apoptosis

While cell- and complement-mediated cell death is discussed later, there is evidence, at least in some experimental systems, that cell death is mediated by antibody through apoptotic pathways (Figure 5). Rituximab-mediated apoptosis is thought to be a consequence of caspase-3 activation, and recent data from patients with CLL support this concept (Byrd *et al*., 2002), whereas the FAS ligand/FAS death pathway does not appear to be necessary. Thus, the mitochondrial-dependent pathway would be likely to be important. However, the role of bcl-2-dependent pathways remains unclear. In the EBV-positive 2F7 cell line derived from AIDS-associated Burkitt's lymphoma, rituximab binding led to decreased bcl-2 expression and sensitization to chemotherapy acting via mitochondrial pathways (Alas *et al*., 2002). This was shown to be due to down-regulation of IL-10, resulting in decreased activation of STAT3 through an IL-10/IL-10R autocrine loop. In contrast, EBV-infected bcl-2-expressing RAMOS-AW and the EBV-negative bcl-2-nonexpressing parent RAMOS are equally sensitive to anti-CD20 apoptosis (Shan *et al*., 2000), and no significant alterations in bcl-2 levels were seen after anti-CD20 treatment. One consideration for the relative insensitivity of CLL to rituximab, in addition to dim CD20 expression, is that bcl-2 is overexpressed in these patients raising the apoptosis threshold. However, follicular NHL in which bcl-2 is dysregulated by t(14;18), is very sensitive to rituximab, so that complex factors must regulate resistance *vs* sensitivity. Recent data suggest that down-regulation of bcl-2 by antisense oligonucleotides does enhance rituximab efficacy (Smith *et al*., submitted), but whether this involves the two agents affecting the same or parallel pathways needs to be further elucidated.

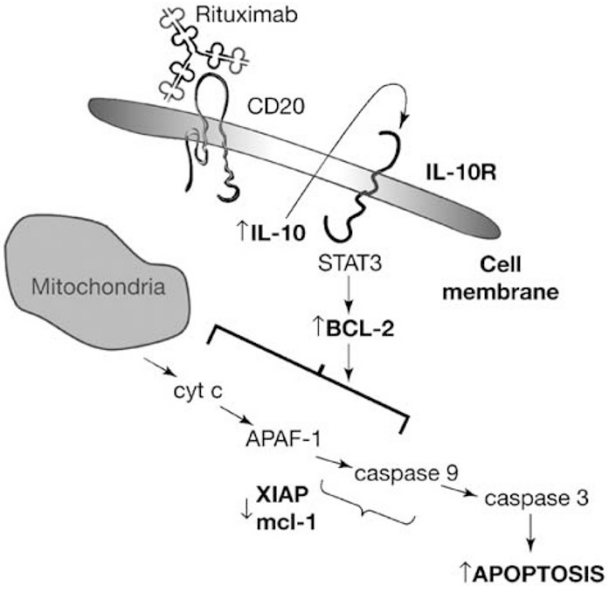

Figure 5 Apoptotic control pathways involved in rituximab-induced apoptosis. The IL-10 autocrine loop has been identified in the 2F7 cell line, but not in others, as downregulating bcl-2. XIAP and mcl-1 have been reported to play a role in the control of rituximab-induced apoptosis in patients with CLL. The FAS ligand/TNF/TRAIL pathway does not appear to be involved in CD20-induced cell death. Any alterations in signals that affect pro- and antiapoptotic balance could potentially alter rituximab sensitivity

While little data yet exist to prove or disprove the hypothesis, alterations in the set-point for apoptosis, perhaps by changes in the levels of bcl-2 or its related family of proteins, apoptosome components such as APAF-1, or other regulators of apoptosis sensitivity could lead to rituximab resistance allowing rituximab-coated cells to escape cell death.

In general, there has been wide variation in the amount of direct apoptosis observed, and this may reflect differences in cell lines, growth conditions, source and Ig subclass of anti-CD20 antibody, and different time points of analysis. The question of the relevance of studies of rituximab effects on Burkitt's lymphoma cell lines must be kept in mind, as this is not the clinical situation in which the drug has been tested. Little data on indolent lymphoma cell lines, corresponding to the predominant clinical use, have been reported, although transformed lymphoma cell lines have been tested and some are quite sensitive to direct apoptosis. Recent data from patients with CLL treated with rituximab do, however, support a role for intravascular apoptosis through the mitochondrial pathway, that is, activation of caspases 3 and 9, but not caspase 8, and down-regulation of XIAP and mcl-1 (Byrd *et al*., 2002).

Role of complement activation in CD20 efficacy

Complement activation by the Fc portion of the antibody leading to cell lysis, or complement-dependent cytotoxicity (CDC), is another postulated mechanism of

action of monoclonal anti-CD20 (Figure 6). As for other potential mechanisms of antibody activity, *in vitro* evidence confirms that this phenomenon exists, but there is significant variability in sensitivity among B-cell lymphoma cell lines. *In vitro* testing of cells from patients has correlated CD20 levels with sensitivity to rituximab-mediated CDC in some reports (Golay *et al*., 2001; Bellosillo *et al*., 2001), but not others (Manches *et al*., 2003). *In vitro* CDC, however, did not correlate with clinical response (Weng and Levy, 2001).

Complement lysis is controlled not only by the degree of activation, but also regulated by a series of complement inhibitory proteins, especially CD35 (complement receptor type 1, CR1), CD46 (membrane cofactor protein), CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis, protectin) (Charles and Foerster, 1999). CD59 was identified on rituximab-bound persistent cells in patients (Treon *et al*., 2001), suggesting a possible role in acquired resistance. While evaluation of a panel of cell lines, many of which were myeloma cells, suggested that CD59 expression correlated with CDC (Treon *et al*., 2001), expression of these proteins has not been found in patient samples to predict *in vitro* CDC (Golay *et al*., 2001) or clinical response (Weng and Levy, 2001). Nevertheless, blockade of CD55 and CD59 has been reported to enhance rituximab-mediated CDC (Golay *et al*., 2000, 2001). Also, the nucleoside analog fludarabine, clinically active against CLL and indolent NHL, has been reported to downregulate CD55 and CD59 (Di Gaetano *et al*., 2001) and this is a potential means by which fludarabine + rituximab may have beneficial clinical efficacy. A recent report suggests, based on data with nonsaturating levels of anti-CD20, that an overall assessment of the levels of both CD20 and the complement inhibitory proteins can predict *in vitro* sensitivity to rituximab-mediated CDC, and that this may correlate with clinical response (Manches *et al*., 2003).

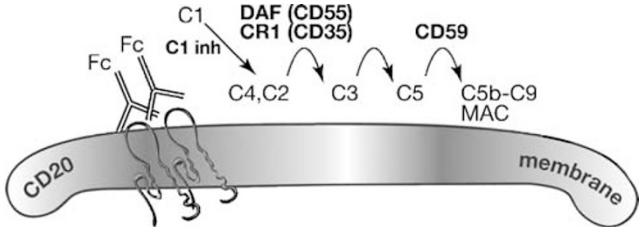

Figure 6 Mechanism of antibody-dependent complement lysis and potential resistance mechanisms. The complement cascade involves a series of zymogens that are sequentially activated, as well as a series of inhibitors (indicated in bold). Two closely approximated immunoglobulin Fc portions initiate the classical pathway by activating C1, which in turn activates C4 and C2. This reaction is closely controlled by circulating C1 inhibitor. The C1, C4 and C2 complex, known as C3 convertase, activates C3 leaving a fragment, C3b, on the cell surface. C3b associating with the plasma membrane becomes C5 convertase. Membrane-associated decay accelerating factor (DAF, CD55) and complement receptor 1 (CR1, CD35) inhibit both C3 and C5 convertase. C5b, C6, C7, C8 and C9 then associate to form the MAC. This activity is inhibited by the membrane inhibitor of reactive lysis (CD59). Note that CD55 and CD59 are linked to the membrane by glycosyl phosphotidylinositol or GPI. Red blood cells deficient in GPI-linked molecules are exquisitely sensitive to complement-mediated lysis in the disease paroxysmal nocturnal hemoglobinuria (PNH), demonstrating that complement sensitivity of cells can be controlled by modulating the cell surface. Furthermore, cells opsonized by IgG and C3b molecules remaining on the surface are targets for phagocytosis via Fc receptors or the C3b receptors CR1 (CD35). It is interesting to note that red cells targeted by high-titer IgG are largely cleared extravascularly by the reticuloendothelial system rather than by intravascular direct complement lysis. Clearly, there are many levels of potential control of cell destruction after antibody binding

It appears that the ability of CD20 to move into lipid rafts is a requirement for CDC to occur (Cragg *et al*., 2003). Whether this is necessary to activate the early stages of the complement pathway or somehow increases the sensitivity to the membrane attack complex (MAC) needs additional study. In addition, a role has been reported for the protein kinases PKC, PKA and MEK in complement resistance of some tumor cells (Donin *et al*., 2003). Thus, inhibitors of these kinases sensitized the carcinoma cells to complement lysis. Perhaps, we can also obtain insight into complement-associated resistance mechanisms from parasites that have evolved the capacity to evade this host system (Sacks and Sher, 2002).

It is clear that complement activation occurs in patients treated with rituximab and, in fact, seems to be related to many of the infusional toxicity reactions associated with this agent. Protein fragments indicative of complement activation have been measured in patients. The relative importance of this as a clearance mechanism, however, remains controversial. Further, complement activation has other effects besides lysis by the final MAC, such as depositing C3, C3b and additional C3b breakdown products on the cell surface. Membrane-attached C3 and C3 fragments could be targets of C3 receptor on effector cells, enhancing cell killing by antibody-dependent cellular cytotoxicity ADCC (see below) (Kennedy *et al*., 2003). Thus, the precise role of complement activation and potential as a mechanism of rituximab resistance requires additional investigation.

Antibody-dependent cellular cytotoxicity

ADCC in the presence of rituximab represents killing of lymphoma cells by effector cells that are activated by binding to the Fc portion of the chimeric anti-CD20 molecule coating the lymphoma cell (Figure 7). Members of the Fcγ receptor family are expressed on monocytes, macrophages and granulocytes and include the activating high-affinity FcγRI (CD64) and low-affinity FcγRIIIA (CD16), as well as the inhibitory low-affinity FcγRIIB (CD32). The activating FcγRIIIA (CD16) is also present on NK cells, while the inhibitory FcγRIIB (CD32) is a key regulator on B lymphocytes (Clynes *et al*., 2000; Ravetch and Lanier, 2000). Two recent avenues of investigation have pointed to ADCC as an important *in vivo* mechanism of rituximab action, as well as other antibodies. First, knockout mice deficient in the inhibitory FcγRIIB are hypersensitive to antibody-mediated tumor suppression (Clynes *et al*.,

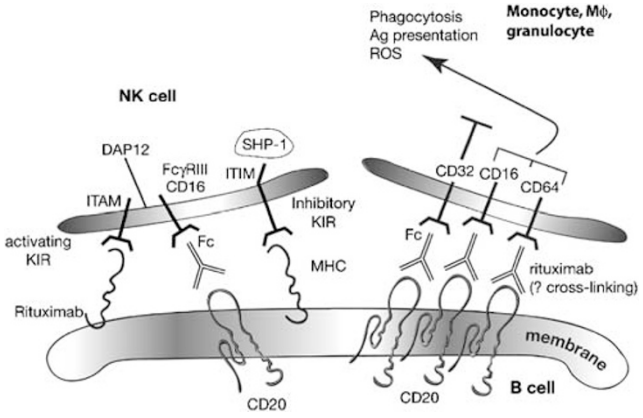

Figure 7 Cellular cytotoxicity mechanisms and controls after rituximab binds CD20 on B-cell surface. NK cell cytotoxicity and cytokine secretion in response to a rituximab-coated B cell is determined by the balance of activating and inhibiting signals through HLA-restricted receptors. Inhibitory signals are via ITIM recruiting SHP-1. Activation is via an adapter, DAP12, that contains ITAM. For myeloid cells, the balance of signals through activating Fcγ receptors CD16 and CD64 and inhibitory Fcγ receptor CD32 determines whether various cytotoxic mechanisms are induced when the cell encounters a rituximab-coated B cell

2000). In mice with the common γ chain knocked out, which are deficient in both activating receptors (FcγRI and FcγRIII), antibodies were no longer effective in inhibiting tumor growth. Further, antibodies engineered to have reduced ability to interact with the FcγR were unable to suppress tumor growth. These data indicate that, at least in nude mouse xenograft models, antibody control of tumor growth requires FcγR interactions, and that the balance of activating and inhibitory receptor signaling is important. Second, evidence that FcγR plays a direct role in efficacy in patients treated with rituximab comes from investigations of FcγRIIIA genetic polymorphisms. A dimorphism exists in which residue 158 of FcγRIIIA can be either valine, with stronger binding to IgG1, or phenylalanine with weaker binding (Koene *et al*., 1997; Wu *et al*., 1997). In a small group of patients with previously untreated follicular lymphoma who received rituximab, response rates and molecular complete remission rates were higher in 10 of the 49 patients homozygous for the FcγRIIIA with V158, than those who had either one or two copies of FcγRIIIA with F158 (Cartron *et al*., 2002). These data not only indicate a key role for FcγRIIIA in rituximab activity, but also suggest that response may be predicted and more effective antibody constructs engineered to overcome this basis of resistance.

Receptors other than FcγR may also be important. NK cells are normally inhibited from attacking MHC-1 identical cells, that is, 'self', by coligation of MHC-1 with inhibitory killer Ig-like receptors or KIR. Additional activating and inhibitory coligating signals exist and the balance determines NK activity (Campbell and Colonna, 2001). These signals are transmitted from the membrane into the cell by transmembrane proteins containing immunoreceptor tyrosine-based activation or inhibitory motifs (ITAM and ITIM). The inhibitory FcγRIIB and KIR signal via ITIM domains that are substrates for src-family tyrosine kinases and that in turn activate PIP_3 phosphatase (SHIP) for FcγRIIB or tyrosine phosphatase (SHP-1, -2) for KIR (Ravetch and Lanier, 2000). Further, ADCC can kill by direct lytic action of enzymes such as granzyme and perforin. Resistance to such enzymatic activity is another potential avenue by which a tumor may evade killing usually triggered by CD20 binding. Expression of protease inhibitor 9 has now documented the existence of this resistance mechanism (Bladergroen *et al*., 2002). Thus, the final decision of an effector cell to kill a target or not can be affected by a complex interplay of signals, including the nature of the interaction of the antibody with FcγR that could be altered by either the antibody or the FcγR, the presence of other activating or inhibitory receptors and their ligands, or perturbation of the downstream intracellular signals. With novel signal inhibitors being studied, this latter possibility becomes a testable method for indirectly overcoming a number of potential antibody resistance mechanisms by amplifying a weak antibody signal from the lymphoma cell surface.

The preceding data indicate that FcγR are important in antibody efficacy in mice and in patients, but do not identify which cells are involved. NK cells appear to be important, but would account only for the FcγRIIIA data. The FcγRIIB data suggest a role for myeloid cells and/or B cells. The myeloid growth factors G-CSF and GM-CSF alter FcγR expression patterns and in some systems enhance ADCC. Neutrophils could act by the production of oxygen radicals, as well. The precise contributions of the various effector cells to ADCC in patients remain to be determined. This may be of critical importance since the effector cell populations in patients with lymphoma, especially after various treatment modalities, may be variably depleted. Altered repertoires of effector cells may lead to rituximab resistance, and may account, for instance, for the improved response to rituximab in patients with the small lymphocytic subtype of lymphoma when rituximab is administered as initial therapy (Hainsworth *et al*., 2002), in contrast to the resistance observed in patients with this tumor who are treated in the relapsed setting (McLaughlin *et al*., 1998). The ability to restore the effector cell compartments with cytokines such as interleukins (ILs) 2, 12 or 15 and myeloid growth factors, or potentially with cellular replacement therapy, may also enhance therapeutic antibody activity, as suggested by preclinical data demonstrating that IL-2 can promote NK cell development and enhance rituximab activity (Hooijberg *et al*., 1995). Additional clinical trials are in progress (Friedberg *et al*., 2002). Similarly, myeloid growth factors in combination with rituximab are being explored. Alternative approaches such as enhanced effector cell activity induced by CpG oligonucleotides are also being investigated (Warren *et al*., 2000).

The observation that some patients treated with rituximab have either delayed responses or responses that become more pronounced over a period of months has led to several considerations. A cell cycle kinetic

explanation is possible in that cells affected by antibody binding may die only when they attempt to divide. Persistent perturbations in cell membrane signaling by alterations in lipid rafts are also theoretically possible (discussed above). Altered immune function, however, is also a possibility. Dying cells may release antigens recognized by adaptive immune cells, leading to an antilymphoma response. *In vitro* studies support such a crosspriming effect (Selenko *et al*., 2002).

What can we learn from radioimmunotherapy results?

Radioactive anti-CD20 antibody therapy has been approved for use in relapsed or refractory low-grade or transformed lymphoma. In a randomized trial, this agent, which consists of ^{90}Yttrium bound to the murine antibody parent of rituximab, administered along with rituximab to help saturate blood and spleen CD20 sites, had a higher rate of overall and complete responses compared with rituximab alone (Witzig *et al*., 2002b). Somewhat surprisingly, the time to disease progression was not increased, suggesting intrinsic resistance of at least a subpopulation of NHL cells. Also, since relapses continue to occur following radioimmunotherapy, cure is not generally attained and therefore resistance must develop. In patients refractory to rituximab, overall response rate to the radioimmunotherapy was similar to that of patients who had not previously received rituximab (Witzig *et al*., 2002a). Similar response rates have been reported for a ^{131}I-labeled murine CD20 antibody (Vose *et al*., 2000). The response to radioimmunotherapy in rituximab refractory patients suggests that the radioimmunoconjugate is acting by a different mechanism, that is, targeted radiation therapy, rather than the antibody mechanisms discussed above. This suggests that resistance to radioimmunotherapy is not due to aberrant CD20 signaling, or to deficiencies of ADCC or complement activation. Resistance to radioimmunotherapy is not likely to be due to poor access of the agent to lymphoma cells, since the path length of the ^{90}Y beta particle is about 10 mm. A possible unifying basis for resistance to radiation-induced cell damage and to rituximab could be intrinsic lymphoma cell resistance to apoptosis, for example, altered apoptotic threshold. The paradox remains that despite the initial response of most indolent lymphomas to treatment, inevitably recurrence, implying a resistant fraction of cells, occurs.

Summary

While therapy of lymphoma with rituxiamb is an important addition to our therapeutic armamentarium, not all patients respond and relapses are common. Until we know the relative importance of the various potential mechanisms of antibody-induced cell killing in patients, it will be difficult to focus attention on the most clinically important reasons for resistance. Ongoing investigation into each of these areas will, therefore, continue to be of importance, especially as it appears likely that multiple mechanisms of action and of resistance are operative, indicating that a multipronged attack on resistance will be required. It will be important to study patients serially, prior to and after rituximab treatment and at the time of relapse, with regard to properties of their lymphoma cells, looking for intrinsic changes to account for resistance. In addition, parallel studies of the innate and adaptive immune systems are needed. The identification of resistance mechanisms should lead to improved treatment, perhaps by antibody engineering or cytokine or cellular therapies designed to enhance effector actions.

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