Anti-Cancer Drugs 2002, **13** (suppl 2): S3–S10

Mechanism of action of rituximab

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Rituximab, the humanized chimeric anti-CD20 monoclonal antibody, represents a powerful tool for treating B-cell malignancies and is licensed for the treatment of relapsed or chemorefractory low-grade or follicular non-Hodgkin's lymphoma (NHL). It has a unique mode of action and can induce killing of CD20$^+$ cells via multiple mechanisms. The direct effects of rituximab include complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity, and the indirect effects include structural changes, apoptosis, and sensitization of cancer cells to chemotherapy. *In vitro* studies have made a significant contribution to the understanding of these mechanisms of action and have led to the development of innovative and effective treatment strategies to optimize patient response. The most significant of these strategies is the combination of rituximab and CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone), which is proving a highly effective combination in the treatment of NHL. However, all patients do not respond equally well to rituximab, and *in vitro* studies have identified a possible mechanism of resistance involving the anti-complement inhibitors CD55 and CD59. Neutralizing antibodies to CD55 and CD59 can overcome resistance to rituximab-mediated complement-mediated cytotoxicity *in vitro*. This paper overviews our understanding of the mechanisms of action of rituximab and identifies how this knowledge could be applied in a clinical setting to maximize response in both sensitive and resistant patients. [© 2002 Lippincott Williams & Wilkins.]

Key words: rituximab, non-Hodgkin's lymphoma, apoptosis, CHOP, synergy, resistance.

Introduction

Rituximab, the humanized chimeric anti-CD20 monoclonal antibody, represents a powerful tool for treating B-cell malignancies and is licensed for the treatment of relapsed or chemorefractory low-grade or follicular non-Hodgkin's lymphoma (NHL).[1]

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Rituximab consists of human Fc constant regions and murine variable regions with antigen-binding regions specific for the CD20 antigen (Figure 1).[2] The specificity of the antibody resides in the murine regions, while the human component allows effective utilization of complement- and cell-mediated lysis mechanisms and also results in lower immunogenicity.[3]

CD20 is an ideal target for immunotherapy in NHL for several reasons. It is present on the surface of all cells of the B-cell lineage,[4] but not on stem cells, plasma cells or non-lymphoid tissues, and is expressed in over 95% of B-cell lymphomas.[5] CD20 expression is stable, without modulation or internalization on antibody binding,[6] and the antigen is not shed into the bloodstream. CD20 also appears to play a functional role in B-cell growth; certain anti-CD20'antibodies, such as the IF5 murine monoclonal antibody, stimulate cell cycle progression,[7] while the B1 monoclonal antibody (also against CD20) inhibits the cell cycle.[8] The intensity of CD20 expression may vary between different B-cell malignancies and from patient to patient. The relationship between the intensity of CD20 expression and the response to rituximab is, however, unclear.

Rituximab has a unique mode of action distinct from chemotherapy and can induce killing of CD20$^+$ cells via multiple mechanisms, which can be categorized into 'immune-mobilizing' mechanisms or 'direct' effects. Immune-mobilizing mechanisms include complement-mediated cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Direct effects mediated through binding of CD20 to the cell surface include inhibition of proliferation, induction of apoptosis, and sensitization of cancer cells to chemotherapy. Cells coated with rituximab are also targets for phagocytosis by macrophages.

While all of these mechanisms have been demonstrated independently *in vitro*,[2,9,10,11] it is not yet

ISSN 0959-4973

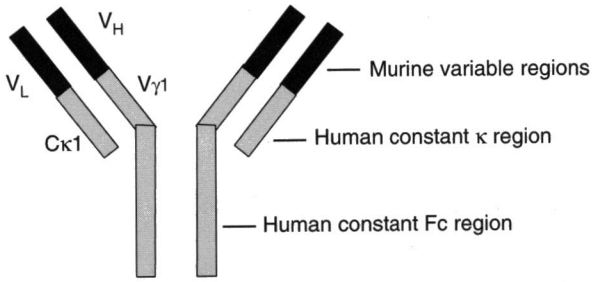

Figure 1. The structure of rituximab, a chimeric anti-CD20 monoclonal antibody. V, variable region: murine IgG1 κ anti-CD20; C, constant region: human IgG1 heavy chain and κ light chain.

clear which are the most important *in vivo*, or the extent to which the different actions may be interdependent.

Understanding the mechanisms of action of rituximab is essential to optimizing therapeutic strategies and in combining this agent with other chemotherapy regimens. *In vitro* studies using $CD20^+$ cells and cell lines have contributed towards an understanding of these mechanisms. This review aims to outline some of the latest data on the mechanism of action of rituximab, in particular its ability to induce apoptosis and structural changes in the plasma membrane. The latest studies on the synergy between rituximab and chemotherapy will also be described, in addition to the latest findings on the mechanisms of resistance to rituximab.

Induction of apoptosis in NHL cells

Apoptosis is a critical element of homeostasis in the lymphoid system. Generation of genetic diversity in lymphocytes, through recombination and mutation of immunoglobulin or T-cell receptor genes, is fundamental to the breadth of the immune repertoire and requires a very high cell turnover. This, in turn, dictates the need for a highly efficient disposal mechanism to remove unwanted cells.

The relevance of apoptosis to the development of lymphoma emerged from the finding that the *bcl-2* gene, overexpressed in 85% of follicular lymphomas (FLs), suppresses apoptosis.[12] Overexpression occurs as a result of a t(14;18)(q32;q21) chromosomal translocation, in which the *bcl-2* gene on chromosome 18 comes under the control of the immunoglobulin IgH-J promoter on chromosome 14. Consequently lymphocytes, which are normally eliminated in the process of affinity maturation, become resistant to apoptosis and provide a long-lived substrate for the development of lymphoma.

Restoring the apoptotic capability of lymphoma cells is an attractive target for therapy, and there are two pathways through which this can be achieved: the 'intrinsic' and the 'extrinsic' pathways.[13] The intrinsic pathway originates in the mitochondria and is induced by cellular stress, which triggers a cascade of events involving apoptosis-promoting members of the *bcl-2* gene family, including BID, BIM and BAX.[14,15] Cellular stress induced by growth factor withdrawal, heat shock, radiation and many non-specific anti-cancer agents is known to trigger apoptosis through this pathway.[16]

The extrinsic pathway (Figure 2) is activated by cell-surface molecules including the tumor necrosis factor receptor (TNFR) family, neurotransmitters such as glutamate and dopamine, and B-cell receptors, including CD20.[13] Some cell-surface molecules carry a conserved cytosolic domain (the death domain) that, when bound by ligand, recruits adapter proteins with death effector domains (DEDs) and initiates the apoptotic cascade.[17] However, this is not the case with CD20.

Induction of apoptosis by rituximab

Rituximab binding to CD20 molecules on lymphoma cells is believed to trigger apoptosis through the extrinsic pathway. Although the exact mechanism of action remains unclear, early reports showed that, in malignant B-cells, cross-linking of CD20 with murine antibodies induced apoptosis.[18] The ability of anti-CD20 monoclonal antibodies to induce apoptosis is variable and may involve regulation of the cytokine interleukin-10 (IL-10) and the anti-apoptotic gene, *bcl-2*. In addition, studies have shown that rituximab induces apoptosis to a greater extent than the murine antibodies 1F5 and B1.[19] Cross-linking was not essential for this to occur, but it enhanced the apoptotic effect. More recently the apoptotic potential of rituximab was demonstrated in an *in vitro* study of B-lymphoma cell lines; rituximab induced apoptosis in four out of seven cell lines.[10] Evidence also suggests that rituximab sensitizes resistant lymphoma cells to apoptosis induced by chemotherapeutic agents, including cisplatin and doxorubicin.[20] This synergy will be discussed in more detail later.

Structural changes induced by rituximab

Treatment of $CD20^+$ lymphoma cells with rituximab results in multiple cell-damaging effects, including

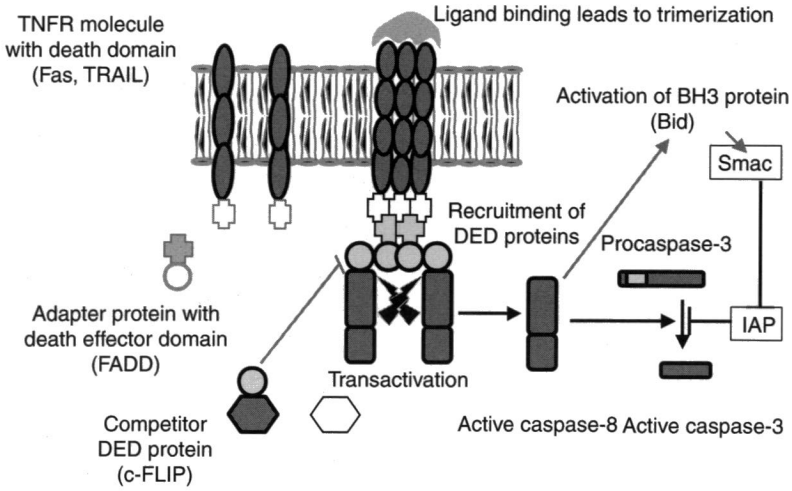

Figure 2. The extrinsic pathway of apoptosis. DED, death effector domain; TNFR, tumor necrosis factor receptor; c-FLIP, cellular-Flice-Like Inhibitory Protein; FADD, Fas associated protein with death domain; IAP, inhibitor of apoptosis protein; TRAIL, TNF related apoptosis inducing ligand; Smac, second mitochondrial activator of caspases.

CDC, ADCC and induction of apoptosis. Observations of the intracellular effects of CD20 ligation include rapid calcium flux, translocation of cell surface phospholipids, activation of serine/threonine protein kinases, caspase activation, inhibition of DNA synthesis and cell cycle arrest.[19,21] The precise sequence of events and the signaling pathways have yet to be established, but recent studies suggest that changes in the membrane organization of CD20 following rituximab exposure may be important.

CD20 redistribution in the plasma membrane

The view of the plasma membrane has changed in recent years and it is now known that parts of it contain specialized microdomains or 'rafts' of sphingolipids and cholesterol[22] (Figure 3). These rafts serve as signaling platforms in B-lymphocytes and facilitate transmembrane propagation of receptor-mediated extracellular signals. Incubation of $CD20^+$ cells with rituximab results in CD20 molecules, which are normally distributed diffusely throughout the membrane, redistributing and accumulating in these rafts.[23] In a study of two B-lymphoma cell lines, RAJI and DOHH-2, this redistribution was accompanied by decreased raft-associated protein tyrosine kinase activity,[24] while in the non-tumor lines EBV13 and SKW, there was no significant change in the level of phosphorylation. The decrease predominantly affected the *lyn* kinase and its substrate, phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) – a major phosphoprotein of rafts and regulator of transmembrane signaling.[25]

An additional consequence of CD20 accumulation in rafts is increased sensitivity of glycolipid-anchored CD55 – a complement inhibitor – to cleavage by phospholipases. Cells lacking CD55 have increased sensitivity to complement-mediated lysis,[26] and recent studies have shown that rituximab treatment of RAJI cells renders CD55 sensitive to cleavage with either human or bacterial phospholipases.[24]

In summary, although the accumulation of CD20 in rafts is not acutely toxic to lymphoma cells, such modifications to raft structure and function are likely to cause long-lasting perturbations of the lymphoma cell physiology, modify transmembrane signaling, and enhance the death of rituximab-targeted cells.

Synergy between rituximab and chemotherapy

The potential synergy between rituximab and chemotherapy was first highlighted by Demidem *et al.*,[9,27] who showed that rituximab pretreatment sensitized chemoresistant $CD20^+$ lymphoma cell lines to chemotherapeutic agents *in vitro*. At the time the mechanism involved was unclear, but it has since been shown in one Epstein–Barr virus (EBV)-associated malignant B-cell line, derived from a patient with human immunodeficiency virus (HIV), that the binding of rituximab $CD20^+$ cells

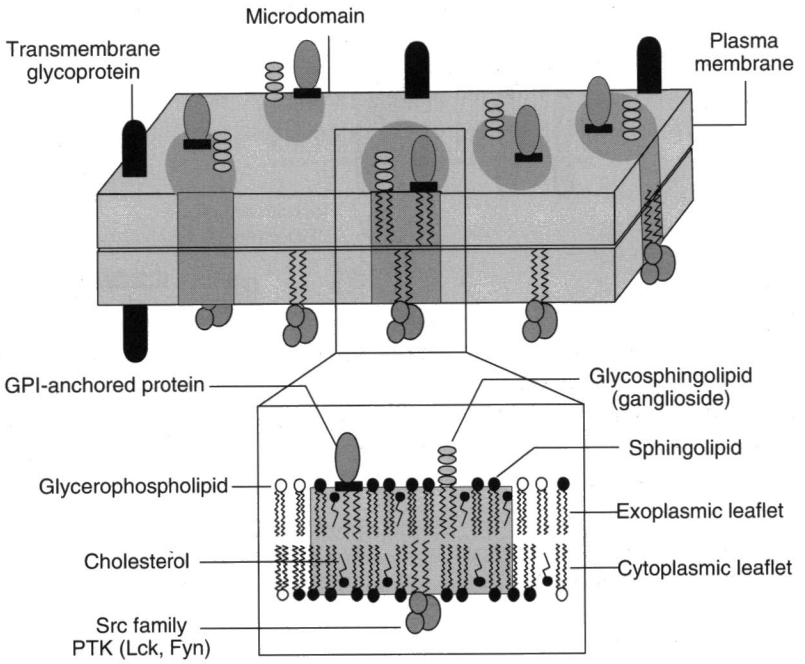

Figure 3. Schematic representation of a raft, a specialized plasma membrane microdomain. Fyn and Lck are two kinase proteins. GPI, glycosylphosphatidylinositol; Src, signalling protein; PTK, protein tyrosine kinase. Reproduced with permission from Ilangumaran *et al.*[37]

downregulates IL-10, a cytokine that regulates *bcl-2* through an autocrine loop involving STAT-3. This in turn results in decreased levels of bcl-2 protein and sensitizes cells to apoptosis.[28] However, this mechanism was not reproduced in other NHL B-cell lines.

Another mechanism of synergy between rituximab and chemotherapeutic agents was described in a study of the FL cell line Karpas 422, which, although $CD20^+$, is resistant to complement-mediated lysis by rituximab. Karpas 422 cells were exposed to various chemotherapeutic agents for different time periods and then exposed to rituximab and human complement. Pretreatment with doxorubicin, idarubicin, cisplatin and paclitaxel resulted in increased lysis, but the effect was only additive, since the cytotoxic drugs alone caused lysis. In contrast, however, pretreatment with fludarabine produced a synergistic effect. Cell lysis increased from 10–20% using fludarabine or rituximab and complement alone to 70% with both cytotoxic agents (Figure 4).[29] A possible mechanism was revealed through further analysis. Fludarabine exposure downregulated the anti-complement antigen CD55, rendering the cells more susceptible to complement-mediated lysis, while the CD20 expression remained unchanged.

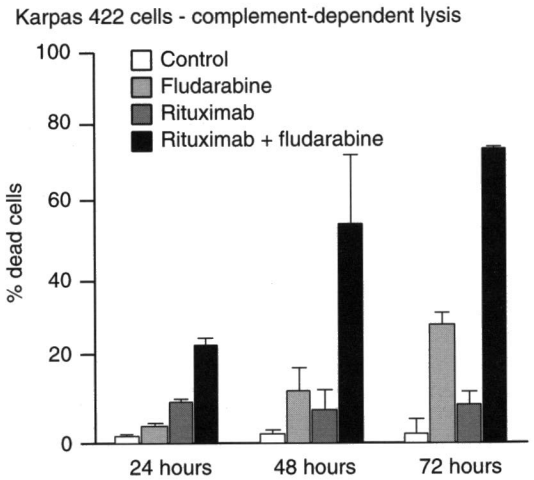

Figure 4. Fludarabine sensitizes rituximab-resistant Karpas 422 cells to complement-mediated cytotoxicity following rituximab treatment. Reproduced with permission from Di Gaetano *et al.*[29]

Synergy between rituximab and CHOP chemotherapy

Several studies now indicate that rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) is a highly effective combination in

NHL.[30–32] Indeed, a randomized study by the Groupe d'Etude des Lymphomes d'Adulte (GELA) showed that rituximab plus CHOP was superior to CHOP alone in elderly patients (60–80 years) with diffuse large B-cell lymphoma (DLCL).[31] The GELA study was the first in over 20 years to show a clinically significant improvement over CHOP.

The possible synergy between rituximab and CHOP has been investigated by combining each component of CHOP with rituximab in *in vitro* assays of growth inhibition and cytotoxicity.[10] Cyclophosphamide, doxorubicin, vincristine or dexamethasone was added to cultures of lymphoma cell lines along with rituximab, and growth inhibition measured. The addition of rituximab to dexamethasone, a glucocorticoid, increased growth inhibition by 13–30%. This was not true, however, for cyclophosphamide, doxorubicin or vincristine. The effect of combining rituximab with dexamethasone was also analyzed using *in vitro* assays for apoptosis, CDC and ADCC. In Tab cells, the combination of rituximab and dexamethasone induced a high level of apoptosis (59%) compared with either drug used alone (dexamethasone alone, 9%; rituximab alone, 8%). In the CDC assay, dexamethasone pretreatment significantly enhanced rituximab-induced cell lysis in three of five lymphoma lines tested, namely DHL-4, FL-18 and Tab ($p < 0.05$). Conversely, ADCC was reduced with dexamethasone pretreatment, while concurrent treatment with rituximab and dexamethasone had no significant effect.

Understanding the mechanism of synergy between rituximab and chemotherapeutic agents requires further *in vitro* analysis. These data, however, suggest that the steroid component of CHOP regimens is involved in achieving synergy with rituximab, and that the timing of steroid administration may be important for optimal benefit.

Mechanisms of resistance to rituximab

Not all patients respond equally well to rituximab, and the reasons for this are unknown. Davis *et al.*[33] studied the effect of retreatment with rituximab in patients who had an initial response. Those who responded to retreatment often had responses of longer duration than to the previous course of rituximab. Others, however, failed to respond to rituximab retreatment, despite responding to a prior course.

FL cell lines are lysed very effectively by rituximab and human complement, although a high degree of heterogeneity in the response is observed. In a study of NHL cell lines, lysis ranged from almost 100% in the sensitive DHL-4 cells to less than 10% in the resistant Karpas cell line.[11]

CD20: level and intensity of expression

One possible reason for a lack of response to rituximab could be the level of CD20 expression. An *in vitro* study of clinical samples from patients with mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL) and prolymphocytic leukemia (PLL) examined the relationship between sensitivity to CDC following rituximab treatment and both the level and intensity of expression of CD20.[34] There was no correlation between the level of expression of CD20 (i.e. the percentage of $CD20^+$ cells) and susceptibility to CDC following rituximab treatment. The intensity of expression measured by standard immunofluorescence or using calibrated beads was predictive of CDC. Samples with CD20 expression $\geqslant 400$ mean fluorescence intensity (MFI) were more susceptible to CDC following rituximab than those with expression < 400 MFI (Figure 5). These results suggest CD20 intensity may influence the *in vivo* effectiveness of rituximab. In contrast, Byrd *et al.*[35] showed no relationship between CD20 density on tumor cells and response to rituximab in patients with CLL. It is worth noting, however, that CD20 expression is lower in CLL than in other NHLs.[36]

CD55 and CD59 in rituximab resistance

The cell surface molecules CD55 and CD59 function as complement inhibitors. *In vitro* studies have

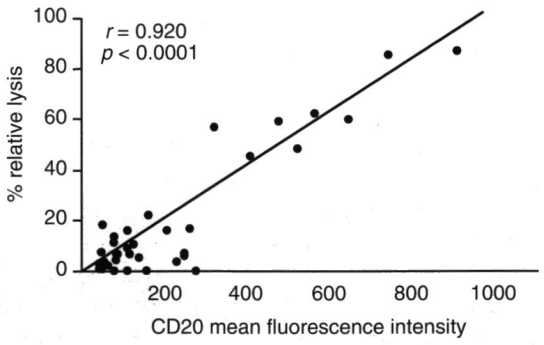

Figure 5. Rituximab-induced CDC correlates with CD20 expression on isolated malignant cells from 38 patients with chronic lymphocyte leukemia (CLL) and prolymphocyte leukemia (PLL). Reproduced with permission from Golay *et al.*[34]

demonstrated that resistance of FL cell lines to lysis by CDC following rituximab treatment may in part be due to expression of CD55 and CD59.[11] Exposure of resistant Karpas 422 cells to neutralizing antibodies to CD55 and CD59 significantly increased rituximab-mediated cell lysis (Figure 6). Evidence for the role of CD55 in mediating the response to rituximab came from a study of the interaction between rituximab and fludarabine on Karpas 422 cells.[29] As described earlier, exposure of the cells to fludarabine followed by rituximab and complement led to a synergistic cytotoxic effect, which further analysis revealed was mediated by downregulation of CD55.

As a follow-up to the cell line studies, Golay *et al.*[34] used the clinical samples from patients with MCL, CLL and PLL, as described above, to investigate the

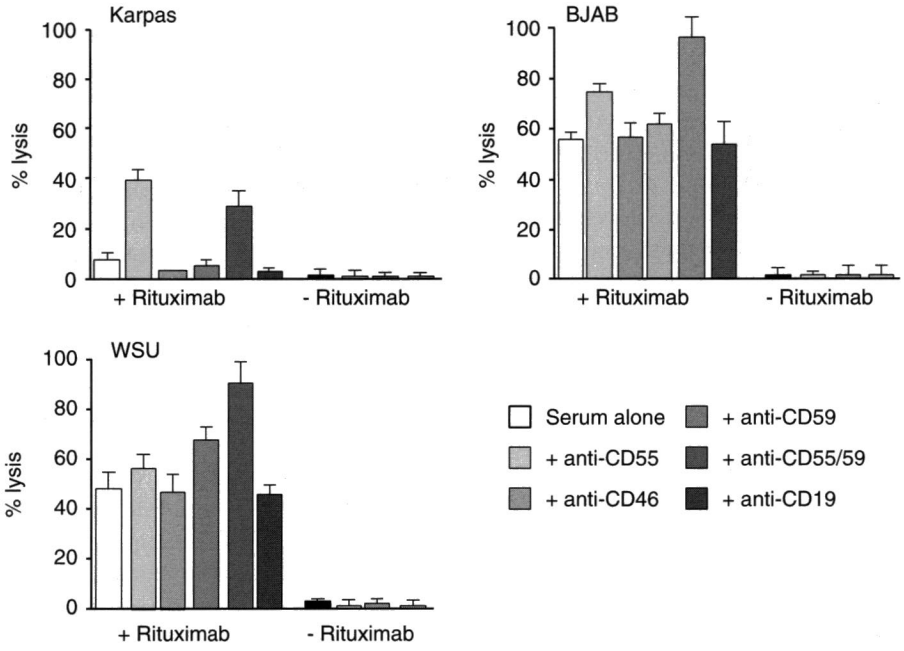

Figure 6. Treatment with neutralizing antibodies to CD55 increases rituximab-induced CDC in the resistant Karpas 422 cell line. Reproduced with permission from Golay *et al.*[11]

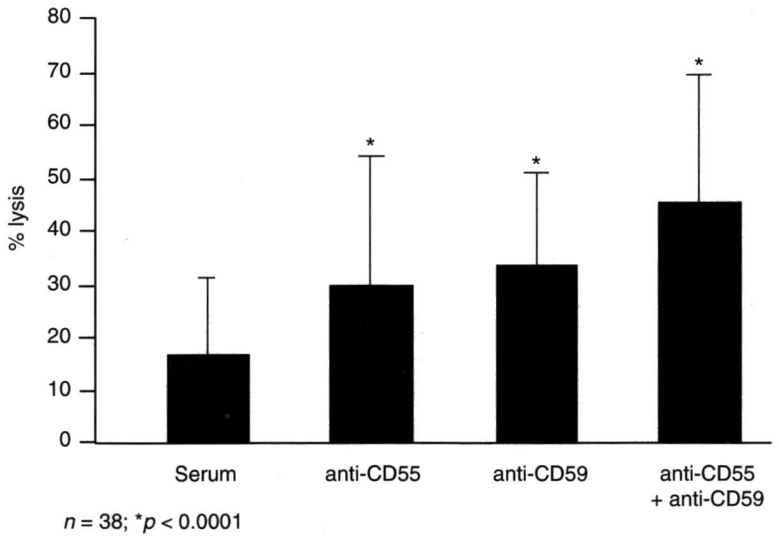

Figure 7. Effect of blocking CD55/CD59 on rituximab-induced complement-mediated cytotoxicity measured *in vitro* on clinical samples from 38 patients with chronic lymphocyte leukemia and prolymphocyte leukemia. Reproduced with permission from Golay *et al.*[34]

role of CD55 and CD59 in regulating CDC by rituximab. Expression of CD55 and CD59, measured as MFI, was shown to be heterogeneous and not predictive of CDC activity. However, inhibition of either of these antigens using anti-CD55 or anti-CD59 antibodies increased CDC five- to six-fold in cells initially resistant to rituximab (Figure 7). These data suggest that CD55/59 may be important in mediating resistance to cell killing by rituximab. Inhibition of these antigens may therefore offer a potential mechanism to improve the response to rituximab in the clinical setting.

Discussion

The distinct array of anti-cancer mechanisms offered by rituximab makes it an attractive treatment option for NHL. Cell kill via the indirect effects of CDC and ADCC provides an effective anti-cancer mechanism with minimal toxicity. The ability of rituximab to induce apoptosis and synergize with other chemotherapeutic agents also adds to its potential as part of a combination regimen. In addition, knowledge of the mechanisms of resistance to rituximab offers ways to overcome this and enhance the efficacy.

Now that our knowledge of the mechanisms of rituximab is increasing through *in vitro* studies, the challenge is to apply this knowledge to the development of optimal treatment regimens that can be applied in the clinical setting. Rituximab is used both as monotherapy and in combination with a wide range of chemotherapeutic agents, radiotherapy or radionuclides. Novel approaches such as combining rituximab with cytokines in order to augment immune effector mechanisms (ADCC) or other antibodies (i.e. anti-CD22) are also promising. Understanding the synergistic effects of rituximab combined with chemotherapy increases our ability to tailor treatment regimens to individual patients and to optimize response.

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