

Rituximab reduces the number of peripheral blood B-cells *in vitro* mainly by effector cell-mediated mechanisms

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Background and Objectives. The humanized CD20 monoclonal antibody, rituximab, has significant anti-tumor activity in patients with B-cell non-Hodgkin's lymphoma and induces depletion of B-cells *in vivo*. It was the objective of this study to define the contribution of the different mechanisms of action of rituximab on primary normal and malignant B-cells.

Design and Methods. Primary human B-lymphocytes and effector cell fractions were isolated from peripheral blood of normal donors using an immunomagnetic separation technique. Blood samples from 20 patients with chronic lymphocytic leukemia (CLL) were studied and the B-lymphoblastoid Daudi cell line was used as a control. B-cells were cultured in the presence or absence of rituximab adding a secondary hyper-crosslinking antibody, serum as source of complement or different effector cell fractions. The cells were analyzed by immunofluorescence staining and flow cytometry.

Results. In contrast to the B-lymphoblastoid Daudi cell line, the number of highly purified normal peripheral blood CD19⁺ cells was only minimally affected by rituximab in the presence of autologous serum. A significant reduction in the number of B-cells was observed when mononuclear cells from peripheral blood were added back. To identify the cell type which mediates this effect, CD3⁺ T-cells, CD56⁺ cells, and CD14⁺ monocytes were added to selected CD22⁺ B-cells. A marked B-cell decrease was only observed in the presence of CD56⁺ and CD14⁺ cells in an effector to target ratio of 10:1. The experiments with mononuclear cells from patients with CLL showed a B-cell reduction by rituximab, which was significantly enhanced following addition of granulocyte-macrophage colony-stimulating factor (GM-CSF).

Interpretation and Conclusions. These data support the important role of cell-mediated mechanisms in the B-cell-depleting action of rituximab and suggest that pre-treatment with GM-CSF could improve the response to rituximab in patients with CLL.

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Key words: rituximab, mechanism, normal B-cells, CLL.

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More than 95% of normal and neoplastic B-cells express the lineage-specific antigen CD20, which is not, however, expressed on hematopoietic stem cells.¹ CD20 is an integral membrane protein, functions as a calcium-channel, can initiate intracellular signaling and modulates cell-growth and differentiation.^{2,3} Binding of mouse antibodies to CD20 does not internalize or downregulate the antigen, while various functional effects on B-cell proliferation and differentiation are induced. After binding of the CD20 antibody 1F5, progression from the G0 to the G1 phase of the cell cycle was observed,⁴ whereas the antibody B1 inhibited the mitogen-induced progression from G1 to G2 and M phases.² Extensive cross-linking of CD20 with goat anti-mouse antibodies, particularly in the presence of Fc receptor expressing cells, directly inhibited B-cell proliferation and induced nuclear DNA fragmentation and apoptosis.⁵

Rituximab (IDEC-C2B8, Hoffman La Roche, Grenzach-Whylen, Germany) is a chimeric humanized CD20 monoclonal antibody (MoAb), in which the mouse constant domains are replaced by the human γ 1 and κ domains. In contrast to the mouse antibody, the humanized Fc domain of rituximab can activate human complement, resulting in complement-dependent cell lysis.⁶

Moreover, the human Fc domain can more efficiently mediate antibody-dependent cytotoxicity than mouse Ig molecules.⁷ These characteristics favored the use of rituximab in the treatment of B-cell malignancies. Indeed, this drug has shown an effective anti-tumor efficacy in patients with follicular lymphoma.⁸⁻¹¹

Accordingly, a significant decrease in the number of B-cells was found in blood and bone marrow of treated patients.⁸⁻¹³

We added rituximab to a dose-intensified chemotherapy and observed a substantial *in vivo* depletion of t(14;18)-positive tumor cells in peripheral blood

stem cell collections from patients with follicular lymphoma.¹³

The mechanisms of B-cell toxicity of rituximab have been studied using cultured lymphoblastoid cell lines.^{5,6} The question to what extent the different mechanisms – complement lysis, antibody-dependent cytotoxicity, induction of apoptosis – contribute to the killing of primary B-cells by rituximab has not yet been solved. We compared the role of the different inhibitory pathways of rituximab on normal and neoplastic primary human B-cells.

Design and Methods

Cell preparation

The human Burkitt lymphoma cell line Daudi (DSMZ, Braunschweig, Germany), which expresses CD19, CD20 and CD22, was cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS). Peripheral blood (PB) samples were collected from healthy volunteers, aged between 20 and 40 years. Chronic lymphocytic leukemia (CLL) samples were collected from 20 patients, aged between 46 and 77 years (median 65 years). The patients' characteristics are detailed in Table 1. The median time from diagnosis was 60 months (range 3-156 months) and the white blood cell count varied between 10.3 and $157.9 \times 10^9/L$ (median: $29.1 \times 10^9/L$).

After separation of plasma, erythrocytes and cell debris were removed by density centrifugation at 400 g and 4°C, for 20 minutes, using the lymphocyte separation medium Lymphoprep (Nycomed Pharma, Oslo, Norway). Mononuclear cells (MNC) were then washed twice with phosphate-buffered saline (PBS, GIBCO, Karlsruhe, Germany) at 4°C and 400g for 7 minutes. CD22⁺ B-cells, CD14⁺ monocytes, CD16⁺ natural killer cells, CD56⁺ cells and CD3⁺ T-cells were selected using the MiniMacs immunomagnetic separation system (Miltenyi Biotech, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. Beads directly conjugated with murine monoclonal antibodies were used for enrichment of all cell populations (isotypes: CD22, IgG1; CD14, IgG2a; CD16, IgM; CD56, IgG1; CD3, IgG2a). PB-mononuclear fraction of normal donors contained $3.27 \pm 1.1\%$ CD19⁺ B-cells and a $94.8 \pm 0.72\%$ purity was achieved following selection. MNC mainly contained $16.1 \pm 2.4\%$ CD14⁺, $23.2 \pm 4.5\%$ CD3⁺ and $6.6 \pm 0.8\%$ CD56⁺ cells. Following immunomagnetic selection a purity of $84.3 \pm 1.5\%$ CD14⁺ cells, $92.1 \pm 2\%$ CD3⁺ cells and $60.9 \pm 6\%$ CD56⁺ cells was achieved.

Table 1. Chronic lymphocytic leukemia samples.

| | | |
|---------------------------------|------------------|----------------|
| Patients (n) | | 20 |
| Rai stage (n) | 0 | 6 |
| | I | 1 |
| | II | 10 |
| | III | 1 |
| | IV | 2 |
| Steroid treatment | yes | 3 |
| | no | 17 |
| % of PB-MNC (mean \pm SEM) | CD19 | 72.6 \pm 4.1 |
| | CD20 | 70.4 \pm 6.0 |
| | CD14 | 5.3 \pm 0.8 |
| | CD3 | 11.7 \pm 1.9 |
| | CD19-/CD3-/CD14- | 10.8 \pm 2.3 |

Cell culture assays

Daudi cells were cultured in RPMI-1640-medium (Sigma, München, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 IU/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine (all purchased from Biochrom KG, Berlin, Germany). This medium will be designated in the following as *culture medium*. As indicated, autologous serum was added to the culture at a final concentration of 10%, native or heat inactivated at 56°C for 30 min. Cells were incubated in the culture medium at a concentration of $1 \times 10^6/mL$, at 37°C and 5% CO₂, for 24 to 48 hours, in the presence or absence of rituximab at a final concentration of 100 μ g/mL. This concentration was chosen for two reasons: the mean of the maximum serum concentrations of rituximab after a single standard dose (375 mg/m²) was determined to be 205 μ g/mL, and after repeated administration reached 460 μ g/mL.¹⁴ Moreover, we observed a plateau of B-cell inhibitory effect at the concentration of 100 μ g/mL for Daudi cells in dose-response experiments with rituximab concentrations of 1, 10, 100 and 1000 μ g/mL (*data not shown*). A direct relation between rituximab concentration and proportion of normal B-cells was observed at concentrations up to 1000 μ g/mL. We, therefore, feel that by choosing a high rituximab concentration, which is reached *in vivo*, we had optimized the *in vitro* conditions for inhibition by rituximab with respect to antibody concentration. In some experiments, cells coated with rituximab were further cross-linked by adding 50 μ g/mL of a secondary rabbit anti-human IgG antibody (DAKO, Hamburg, Germany).

Peripheral blood mononuclear cells or immunomagnetically selected effector cells were added to B-cells at effector to target ratios between 1:10 and 10:1, and cultured for 24 hours. In some experiments GM-CSF (10 ng/mL) was added to the culture 4 hours prior to the addition of rituximab.¹⁵

Controls without antibodies were included in all experiments. At the end of culture, the number of viable cells was enumerated using the trypan-blue exclusion method, and the proportion of CD19⁺ B-cells was determined by flow cytometry. The effect of rituximab was assessed by calculating the absolute CD19⁺ cell count at the end of the cell culture with rituximab and dividing this number by the CD19⁺ cell number at the end of a cell culture with identical conditions but without rituximab. All results are expressed as percent of control.

Immunofluorescence staining and flow cytometry

Following culture, viable cells were counted under an inverted microscope, using trypan-blue staining. The proportion of the different cell populations was calculated using fluorescence activated cell sorting (FACS). For immunofluorescence analysis, at least 5×10^5 cells were incubated for 30 min at 4°C in the presence of CD19 (4G7, mIgG1), CD3 (SK7, mIgG1), CD14 (Mf9, mIgG2b), or CD56 (My31, mIgG1) phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies, or isotype-identical control antibodies (Becton-Dickinson, BD, Heidelberg, Germany). In order to avoid interference of rituximab with B-cell fluorescence staining, the CD19 monoclonal antibody was used to determine the proportion of B-cells. Ten thousand cells were analyzed with a Becton-Dickinson FACScan and the CellQuest computer program (BD).

Statistical analysis

All experiments were repeated at least 4 times, as indicated. Results are expressed as mean \pm standard error of the mean (SEM). Laboratory data were evaluated according to standard statistical methods and commercially available computer programs (Microsoft Excel 2000, GraphPad Prism software). Statistical differences were studied using the Student's t-test. A significance level of $p < 0.05$ was chosen.

Results

Mechanisms of the anti-proliferative effect of rituximab on B-lymphoblastoid cells

CD20 MoAbs can inhibit the proliferation of malignant human B-cell lines. Using the B-lymphoblastoid Daudi cell line, we compared the dif-

ferent mechanisms by which rituximab exerts its B-cell depleting effect: direct inhibition, complement lysis and effector cell-mediated inhibition. Following 24 hours of incubation in the presence of rituximab at a saturating concentration of 100 μ g/mL, the proportion of viable cells was calculated by trypan blue exclusion. The growth of Daudi cells was reduced to $76.1 \pm 3.5\%$ of the control ($n=13$ experiments, $p=0.001$) (Figure 1A). The addition of human serum as source of complement to the culture medium led to a further decrease ($35.0 \pm 4.2\%$ of the control, $n=9$, $p=0.005$). To control for this effect, we used human serum that had been heat-inactivated at 56°C for 30 minutes. Heat-inactivation abolished the serum-related effect (Figure 1A).

We next examined whether effector cells from peripheral blood were capable of increasing the effects of rituximab. MNC were cultured with Daudi cells at a 10:1 effector/target (E/T) cell ratio (Figure 1B). After 24 hours of culture in the presence of rituximab, the number of CD19⁺ lymphoblastoid cells decreased to $26.1 \pm 8.7\%$ of the control ($n=6$, $p=0.02$) (Figure 1B). The addition of human serum had no further effect ($30.1 \pm 8.4\%$, $n=6$, $p=0.7$). These results indicate that effector cells significantly increase the B-cell inhibitory efficacy of rituximab, and that effector-cell and complement-mediated mechanisms contribute equally to this effect without apparent synergism.

Effects of rituximab on normal human peripheral blood B-cells

We then studied whether normal human B-lymphocytes were also susceptible to the inhibitory effects of rituximab, as observed with Daudi cells. B-cells were enriched from peripheral blood using the MoAb CD22 and immunomagnetic selection. In the presence of fetal calf serum, without any other stimulation of proliferation, the number of B-lymphocytes remained constant over the 24-hour culture period. The addition of rituximab alone had a modest, but significant suppressive effect on freshly isolated B-cells ($68.3 \pm 3.7\%$, $n=10$, $p < 0.001$) (Figure 2A). Hyper-crosslinking of rituximab-targeted CD20 with a rabbit-anti-human-IgG did not enhance the effect of rituximab alone ($59.7 \pm 5.7\%$, $n=8$, $p=0.12$), nor did the addition of autologous serum add to the effect of rituximab ($66.8 \pm 8.0\%$, $n=7$, $p=0.8$) (Figure 2A). The rabbit-anti-human-IgG alone had no effect.

A significant reduction in the number of B-cells in the presence of rituximab was observed when mononuclear cells were added back to selected B-

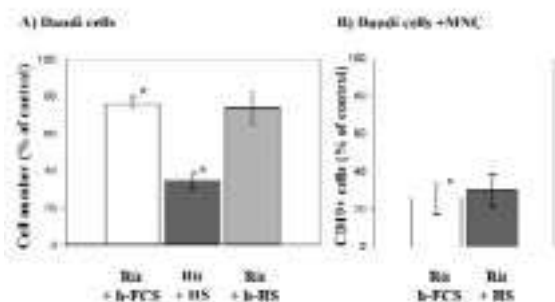


Figure 1. Effector cell- and complement-mediated mechanisms contribute equally to the inhibitory effect of rituximab on the human lymphoblastoid Daudi cell line. **A)** Daudi cells were incubated for 24 hrs in culture medium containing 10% heat-inactivated fetal calf serum (h-FCS), or 10% human serum (HS), or 10% heat-inactivated human serum (h-HS) and 100 $\mu\text{g}/\text{mL}$ rituximab (Rit). Identical culture conditions without rituximab were used as individual controls. Viable cells were enumerated at the end of the culture period by trypan blue exclusion and the cell number was expressed as percent of the cell number in the culture without rituximab. The mean and standard error of the mean (SEM) are given. *The reduction of Daudi cell growth by rituximab in heat-inactivated FCS was statistically significant ($p=0.001$), as was the additional inhibition in human serum in comparison to in FCS ($p=0.005$). **B)** Daudi cells were cultured for 24 hrs in the presence of mononuclear cells from peripheral blood at a 10:1 effector/target cell ratio in medium containing 10% heat-inactivated fetal calf serum (h-FCS) or 10% human serum (HS) and 100 $\mu\text{g}/\text{mL}$ rituximab (Rit). At the end of culture, viable cells were counted and the proportions of CD19⁺ lymphoblastoid cells were determined by FACS. The absolute CD19⁺ cell number was calculated, divided by the CD19⁺ cell number after culture under identical conditions but without rituximab, and expressed as percent of control. The mean and standard error of the mean (SEM) of 6 experiments are given. *Addition of rituximab to effector cells from peripheral blood induced a significant depletion of Daudi cells in comparison to addition of rituximab alone (Figure 1A, Rit + h-FCS, $p=0.002$). The addition of human serum had no synergistic effect (Rit+HS).

cells at a MNC to B-cell ratio of 10:1 ($24.3 \pm 10.8\%$, $n=5$, $p=0.05$) (Figure 2B). The use of autologous serum as a source of complement did not increase this effect ($30.2 \pm 6.2\%$, $n=8$, $p=0.18$, Figure 2B). We next added rituximab to the total peripheral blood mononuclear cell fraction of normal donors to exclude that the manipulation of B-cells during immunomagnetic selection contributed to their depletion. MNC fractions contained $3.8 \pm 1.8\%$ CD19⁺ cells. After 24-hour culture in the presence of rituximab, the number of B-cells was reduced to $52.6 \pm 12.9\%$ of the control without rituximab (mean \pm SEM of 13 experiments; $p=0.007$). The addition of human serum did not enhance this effect ($49.1 \pm 6.9\%$, $n=9$).

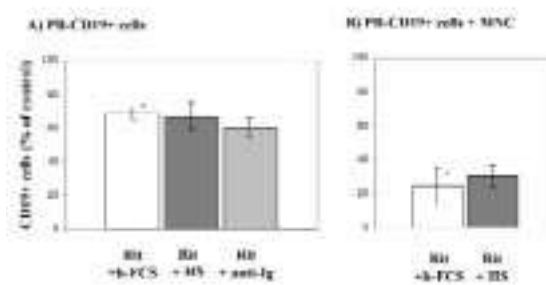


Figure 2. The inhibition of selected normal B-cells by rituximab is significantly enhanced in the presence of effector cells, but not by autologous human serum. **A)** B-cells were selected from peripheral blood using immunomagnetic separation with CD22 antibody-coupled beads. Mean purity was 94.8% as assessed by CD19 staining. Selected B-cells were cultured for 24 hrs in the presence of 10% heat-inactivated fetal calf serum (h-FCS) or 10% human serum (HS), 50 $\mu\text{g}/\text{mL}$ anti-human IgG antibody (anti-Ig) and 100 $\mu\text{g}/\text{mL}$ rituximab (Rit), as indicated. At the end of culture, viable cells were counted and the proportion of CD19⁺ cells was determined by FACS. The absolute CD19⁺ cell number was calculated, divided by the CD19⁺ cell number after culture under identical conditions but without rituximab, and expressed as percent of control. The mean and standard error of the mean (SEM) of at least 7 experiments are given. *The addition of rituximab reduced the number of freshly isolated B-cells after 24-hr culture modestly, but significantly ($p<0.001$). Hyper-crosslinking of rituximab-targeted CD20 with a rabbit-anti-human-IgG or the addition of autologous serum did not induce a further reduction of B-cells. **B)** CD22-selected B-cells with a mean purity of 94.8% were cultured in the presence of autologous mononuclear cells at 10:1 MNC to B-cell ratio for 24 hrs in medium containing 10% heat-inactivated fetal calf serum (h-FCS) or 10% human serum (HS) and 100 $\mu\text{g}/\text{mL}$ rituximab. At the end of culture, viable cells were counted and the proportion of CD19⁺ cells was determined by FACS. The absolute CD19⁺ cell number was calculated, divided by the CD19⁺ cell number after culture under identical conditions but without rituximab, and expressed as percent of control. *The reduction of CD19⁺ cells by rituximab in the presence of MNC was significantly enhanced in comparison to the effect of rituximab without MNC ($p=0.05$). The use of autologous serum as a source of complement did not add to this effect (Rit+HS).

Cell-mediated inhibition

To identify the cell type that mediates the inhibitory effect of rituximab on normal B-cells, CD3⁺ T-cells, CD16⁺, CD56⁺ natural killer cells and CD14⁺ monocytes isolated through immunomagnetic selection were added to enriched CD22⁺ B-cells.

Rituximab reduced the number of CD19⁺ cells in the presence of CD3⁺ T-cells, at an effector to target cell ratio of 10:1, to $79.7\% \pm 5.1\%$, which is not different from the effect of rituximab alone on B-cells ($n=6$, $p=0.41$) (Figure 3). Selected CD56⁺ cells including natural killer cells, incubated as described for CD3⁺ cells, induced a significant depletion of CD19⁺ cells ($28.4 \pm 8.5\%$, $n=6$, $p=0.03$) (Figure 3).

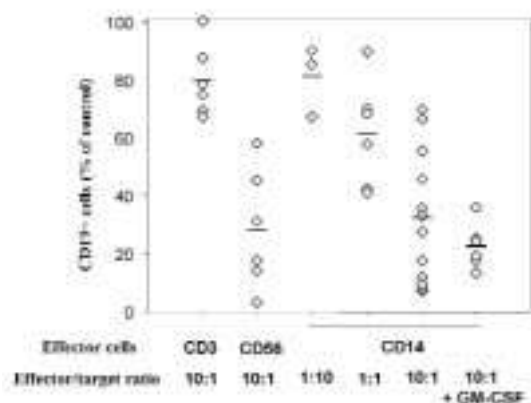


Figure 3. CD56⁺ and CD14⁺ cells mediate the rituximab-dependent effector-cell inhibitory effect on selected normal B-cells at an effector/target ratio of 10:1. The different cell fractions were enriched using immunomagnetic selection and incubated in the presence of CD22-selected B-cells at the indicated E/T cell ratios. CD14⁺ cells were pre-incubated with 10 ng/mL GM-CSF where indicated (+GM-CSF). Cells were cultured for 24 hrs in the presence of 100 µg/mL rituximab. At the end of culture, viable cells were counted and the proportion of CD19⁺ cells was determined by FACS. The absolute CD19⁺ cell number was calculated, divided by the CD19⁺ cell number after culture under identical conditions but without rituximab, and expressed as percent of control. The mean is indicated by a bar. *Reduction of CD19⁺ cells was significant with respect to the reduction following culture with rituximab alone for CD56⁺ and CD14⁺ effector cells at a 10:1 E/T ratio ($p=0.03$ and $p=0.04$). Pre-incubation of CD14⁺ cells with GM-CSF did not result in a further reduction of B-cells with respect to non-primed CD14⁺ cells.

On the other hand, effector cells selected using a CD16 antibody recognizing and interfering with the function of the Fc-γ receptor RIII did not exert any significant inhibition on B-cells in the presence of rituximab (*data not shown*).

To study the contribution of monocytes, CD14⁺ cells were added to B-cells at different effector to target ratios (E/T) (Figure 3). No effects were observed at an effector to target ratio of 1:10, while only a modest inhibition was observed at a 1:1 E/T cell ratio. At a 10:1 E/T ratio, CD14⁺ monocytes reduced the number of CD19⁺ cells to 32.4±6.6% of the control without rituximab ($n=12$, $p=0.04$).

We next pre-incubated the CD14⁺ cells with 10 ng/mL GM-CSF prior to the addition of rituximab and B-cells. The proportion of B-cells decreased at 24 hours to 22.6±3.2% of the control without rituximab, which was not statistically significant different from the effect of rituximab and CD14⁺ cells without GM-CSF ($n=6$, $p=0.42$) (Figure 3).

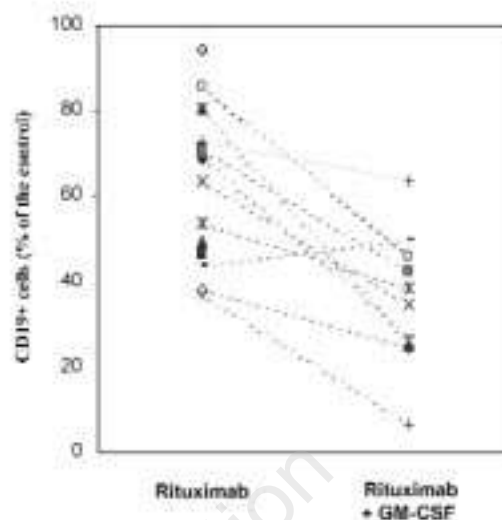


Figure 4. B-cells from patients with CLL are sensitive to the inhibitory effect of rituximab, particularly in the presence of GM-CSF. PB-MNC were cultured in the presence of 100 µg/mL rituximab + 10 ng/mL GM-CSF for 24 hrs. Viable cells were counted at the end of culture, and the proportion of CD19⁺ cells was determined. The absolute CD19⁺ cell number was calculated, divided by the CD19⁺ cell number after culture under identical conditions but without rituximab, and expressed as percent of control. Dotted lines indicate paired samples.

In conclusion, the experiments on normal peripheral blood B-cells demonstrated the dominant role of effector-cell mediated mechanisms in the inhibitory action of rituximab.

Effects of rituximab on peripheral blood B-cells of patients with B-CLL

We next studied rituximab-dependent effector-cell mediated mechanisms on malignant B-cells from 20 patients with B-CLL. Peripheral blood MNC contained 72.6±4.1% CD19⁺ B-cells, 70.4±6% CD20⁺ cells, 5.3±0.8% CD14⁺ cells and 11.7±1.9% CD3⁺ cells (Table 1). The addition of rituximab resulted in a significant depletion of CD19⁺ cells to 63.7±4.3% of the control without rituximab ($p=0.005$) (Figure 4). This effect was more pronounced following addition of 10 ng/mL GM-CSF (depletion of CD19⁺ cells to 35.8±5.6%, $p=0.002$). We found no correlations between degree of inhibition by rituximab and patients' characteristics, such as stage, duration of the disease, previous

treatment, proportion of CD20⁺ B-cells in the samples and mean CD20 expression, which was low in all samples (CD20 mean fluorescence intensity 25.2±1.8). There was also no correlation with the number of CD3⁺ T-cells, CD14⁺ monocytes and the CD19⁻/CD3⁻/CD14⁻ cell fraction containing the NK cells. These data indicate a role for cell-mediated mechanisms in the inhibitory action of rituximab and suggest that pre-treatment of patients with GM-CSF may improve the therapeutic outcome of patients with CLL.

Discussion

Several mechanisms have been proposed for the depletion of normal and malignant B-cells *in vivo* by rituximab, including (a) activation of intracellular signaling and apoptosis, (b) complement-mediated cell lysis and (c) effector-cell mediated cytotoxicity.^{5,6} These effects of rituximab were mainly examined on cell lines. We evaluated the rituximab-related effects on freshly isolated B-cells from normal donors and found that effector cell-mediated inhibition plays the major role. Furthermore, we could enhance the *in vitro* B-cell depletion in patients with B-cell chronic lymphocytic leukemia by the addition of GM-CSF.

We observed a direct inhibition of the proliferation of Daudi lymphoma cells by rituximab to 76% of the control. In the same line, the number of immunomagnetically purified normal B-cells from peripheral blood was reduced to 68% of the control. Direct inhibition of B-lymphoma cell lines by murine CD20 antibodies has been described.^{2,5} It has been reported that the proliferation of normal tonsillar B-cells induced by some but not all mitogenic signals can be very efficiently inhibited by rituximab suggesting a cell-cycle-dependent effect.¹⁶ B-cells in peripheral blood from normal donors are non-cycling, and B-cells were cultured without growth stimulation which might explain the moderate effects that we observed in this cell population.

We next determined the contribution of complement-mediated mechanisms of inhibition. Using normal human serum as a complement source, Daudi cells could be efficiently reduced by rituximab. In contrast, the addition of autologous serum to normal peripheral blood B-cells did not have significant effects. Differences in the sensitivity to complement-mediated cell lysis have been attributed to differences in the expression of the complement inhibitors CD55 and CD59.¹⁶ Peripheral blood B-cells express CD55 and CD59.¹⁷⁻¹⁹ The addition of antibodies blocking CD55 and CD59 can

induce a significant lysis of otherwise complement-resistant CLL B-cells by rituximab.^{20,21} On the other hand, a recent study did not find any correlation between expression of complement inhibitors on tumor cells and clinical outcome in follicular non-Hodgkin's lymphoma questioning the role of complement-lysis in the effects of rituximab *in vivo*.²²

Our results suggest a major role for effector-cell mediated mechanisms. Mononuclear cells from peripheral blood induced a reduction in the number of B-cells in the presence of rituximab to 24%. Purification of different cell populations of PB-MNC showed that CD14⁺ monocytes and CD56⁺ natural killer cells were equally effective in mediating the inhibition by rituximab, while CD3⁺ T-cells were not involved. The activity depended on the effector/target ratio, with a significant effect observed at a 10:1 E/T ratio.

Monocytes and NK cells are the main effector cells of antibody-dependent cellular cytotoxicity (ADCC), which requires the presence of activating Fc receptors on the effector cells. In this line, effector cells enriched using an antibody binding and blocking CD16, a receptor for the IgG Fc portion, did not mediate a reduction of B-cells by rituximab *in vitro*. The predominant role of effector-cell mediated mechanisms of B-cell inhibition by rituximab is also supported by animal studies. An IgG4 anti-CD20 antibody, which does not activate ADCC and complement, failed to deplete B-cells in monkeys.²³ More importantly, in nude mice deficient for the activation component of Fc receptors, rituximab failed to inhibit human B-cell lymphoma growth.²⁴ These data show that the *in vivo* activity of rituximab against lymphoma cells depends on the engagement of Fc receptors on effector cells.

We then studied whether we could augment the cell-mediated inhibitory activity of rituximab by stimulation of monocytic effector cells with GM-CSF, a cytokine approved for clinical use. GM-CSF promotes the differentiation of monocytes to macrophages increasing the lysis of antibody-targeted tumor cells.^{15,25} A stronger decrease of the tumor growth rate of large human B-cell tumors in nude mice has been reported for the combination of a CD20 monoclonal antibody with recombinant murine GM-CSF in comparison to the antibody alone.²⁶ We therefore pre-incubated purified monocytes with GM-CSF and then cultured the stimulated monocytes with purified B-cells in a 10:1 E/T ratio. B-cells were depleted to 22 %, when compared to a control without rituximab, although this reduction was not significantly different from

the depletion by non-stimulated monocytes.

We examined whether this approach could be promising for patients with CLL. The addition of rituximab to the PB-MNC of patients with B-CLL reduced the number of B-cells to 63% of the control. Addition of GM-CSF significantly enhanced the inhibitory effect of rituximab resulting in a decrease of B-cells to 34.8% of the control.

In conclusion, these data could help improve the treatment of B-cell malignancies with rituximab, by including GM-CSF, as responses to rituximab in patients with CLL are variable and the duration of remission is only transient.²⁷ Some caution must be expressed as patients with CLL and high lymphocyte counts treated with rituximab are at risk of tumor-lysis and cytokine-release syndromes.²⁸

Contributions and Acknowledgments

MTV: design, supervision, writing the paper and its revision; GP: experiments; SR: FACS analysis; MW: experiments; Fd'A: experiments; RU: cell preparation; GL: critical revision; RH: conception and revision; SH: conception, writing the paper and its revision.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

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What is already known on this topic

Several modes of action for rituximab, a monoclonal antibody against B-cells, have been described *in vitro* but little is known of what happens *in vivo*. Several studies were performed on cell lines or in animal models.

What this study adds

This study was performed on freshly isolated normal peripheral B-cells and B-CLL cells. The results further support the idea that antibody-mediated cellular cytotoxicity (ADCC) is an important mechanism of action of rituximab on circulating B-cells.

Potential implications for clinical practice

The modulation of ADCC through cytokines (IL-2, IL-12, G-CSF, GM-CSF) that increase NK-cell and macrophage functions may reinforce the therapeutic action of rituximab, as suggested by the authors using GM-CSF.

Gilles Salles, Associate Editor