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Anti-CD20 antibody (IDEC-C2B8, rituximab) enhances efficacy of cytotoxic drugs on neoplastic lymphocytes *in vitro*: role of cytokines, complement, and caspases

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Background and Objectives. Monoclonal antibody IDEC-C2B8 (rituximab) has been shown to be highly effective in the treatment of non-Hodgkin's lymphomas (NHL). The present study was designed to investigate relationships between the efficacy of IDEC-C2B8 and expression of CD20, presence of complement, and effects of differently acting chemotherapeutic agents used in lymphoma treatment (doxorubicin, mitoxantrone, cladribine, bendamustine).

Design and Methods. DOHH-2, WSU-NHL and Raji lymphoma cell lines and *ex vivo* cells from patients with chronic lymphocytic leukemia (CLL) (n=17) and leukemic B-cell lymphomas (n=9) were studied. Additionally, the effect of interleukin (IL)-2, IL-4, IL-6, IL-13, granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)α on expression of CD20 molecules per cell was determined.

Results. We demonstrate that 10 μg/mL rituximab saturated 80-95% of CD20 molecules per cell in all tested lymphoma samples. Although rituximab induced only a minor increase of apoptosis, combinations of rituximab with different cytotoxic drugs significantly decreased the IC_{30}- and IC_{50} dosages of the chemotherapeutic agents necessary for induction of apoptosis irrespective of addition of complement, demonstrating a chemosensitizing effect of rituximab in combination with cytotoxic drugs in the neoplastic lymphocytes. This effect seemed to be independent of the percentage of saturated CD20 molecules. After addition of caspase inhibitors to the cell lines incubated with rituximab and cytotoxic agents, caspase-7 and -8 were found, by Western blotting, to be the executioner caspases, possibly explaining the rituximab-sensitized apoptosis. Preincubation of lymphoma cells with cytokines did not alter the expression of CD20; IL-2 and IL-4 even decreased the rate of apoptosis.

Interpretation and Conclusions. We conclude that rituximab sensitizes lymphoma cells to the effect of differently acting cytotoxic drugs used in lymphoma treatment, that this effect does not require complement, and that caspase-7 and -8 may represent the main executioner caspases in chemosensitization by rituximab.

Key words: anti-CD20, complement, bendamustine, cytokines, apoptosis.

The anti-CD20 antibody (IDEC-C2B8, rituximab) has been established to be an effective single agent for the treatment of patients with low- or high-grade non-Hodgkin's lymphomas (NHL).[1] It was also successfully combined with standard chemotherapy in the first line treatment of patients with low- and high-grade NHL, improving response rates and possibly overall survival.[2-4]

The CD20 antigen is expressed in the majority of low- and high-grade B-cell lymphomas.[5] It is a 33 to 37kD phosphoprotein, expressed exclusively on the surface of B-lymphocytes. This specificity, along with its high expression during nearly all stages of B-cell development, renders CD20 an attractive target for the development of therapeutic monoclonal antibodies directed against it.[6,7] The mechanisms of action of rituximab have been extensively investigated, but the intracellular events leading to apoptosis of target cells remain to be elucidated. Complement-dependent cytotoxicity (CDC),[8-10] complement-dependent cellular cytotoxicity (CDCC), antibody-dependent cytotoxicity

(ADCC)[7,10] as well as the induction of apoptosis[11-16] have been claimed to be responsible for the efficacy of rituximab, a chimeric antibody with human IgG1 and κ constant regions, providing the Fc portion necessary for CDC or ADCC.[6] Moreover, it was hypothesized that the number of CD20-receptors on lymphatic cells determines sensitivity to rituximab, and that consequently certain cytokines may increase the efficacy of rituximab by upregulating the expression of CD20.[1] Preliminary results of clinical studies suggest that outcome in lymphoma patients is improved if cytotoxic agents are combined with rituximab. Until now it has remained to be resolved whether the increased efficacy of this combination is due to a synergistic effect or simply the result of the addition of another antineoplastic agent. This study examines the influence of different cytokines on the expression level of CD20 and defines the relationship between the expression level of CD20 and the efficacy of rituximab. This study also assesses the contribution of complement and different *classic* chemotherapeutic agents when used in combination with rituximab, and outlines effects on intracellular signaling cascades.

Design and Methods

Patients

Mononuclear cells (MNC) were separated from peripheral blood of patients with leukemic B-NHL (n= 9) and CLL (n= 17). Patients were untreated or had not been treated within the last 6 months prior to *in vitro* testing. Diagnoses were confirmed by bone marrow biopsy and immunophenotyping of leukemic cells. The malignant cells represented at least 80% of the total MNC.

Cell lines

Two CD20-positive follicular lymphoma cell lines (DOHH-2, WSU-NHL, DSMZ, Braunschweig, Germany) and one CD20-positive Burkitt's lymphoma cell line (Raji, DSMZ) were used. Cell preparation and incubation with rituximab ± other drugs were carried out as follows. Peripheral MNC were isolated by Ficoll-density gradient sedimentation. Cells were incubated with drugs, at the concentrations specified below, in RPMI medium (LifeTechnology, Paisly, Scotland) supplemented with 10% inactivated fetal calf serum (Greiner, Frickenhausen, Germany), 2% L-glutamine (Life Technology), and 1% penicillin/streptomycin (Bio Whittaker, Verners, Belgium). Samples of 1×10^6 cells/mL medium were incubated with rituximab and the various cytotoxic drugs for 24 and 48 hours as described previously.[17] Complement rabbit serum was purchased as a standardized lyophilisate from Calbiochem-Novabiochem Corporation (San Diego, USA).

Drug concentrations

Rituximab (Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) was used in dosages between 0.01 μg/mL-100 μg/mL, bendamustine hydrochloride (Ribosepharm, Muenchen, Germany) in the range of 0.1 μg/mL-200 μg/mL, cladribine (2-CdA) (Janssen-Cilag GmbH, Neuss, Germany) in the range of 0.001 μg/mL-20 μg/mL, doxorubicin (Pharmacia & Upjohn GmbH, Erlangen, Germany) in the range of 0.001 μg/mL-5 μg/mL, and mitoxantrone (Lederle Arzneimittel GmbH, Wolfratshausen, Germany) in the range of 0.001 μg/mL-5 μg/mL. Subsequently drug dosages were chosen according to the IC30, 50, 75 and 95 established in the experiments.

Measurement of CD20 antibody binding capacity (ABC)

For determination of the expression of the CD20 antigen, flow cytometric analysis of geo-mean fluorescence intensity (MFI) of *ex vivo* lymphocytes and cell lines was performed. These relative MFI values were translated into absolute antibody binding capacity (ABC) units using the Quantum Simply Cellular Kit (Flowcytometry Standards Corp., San Juan, USA),[18] which contains a mixture of four highly uniform microbead populations and a blank population. Each population has a different binding capacity for mouse monoclonal IgG, creating a set of standards for calibrating the instrument's response to that antibody. By entering data into QuickCal 2.0 Sample Report software,[19] any MFI value yields a corresponding number of ABC units. Briefly, 50 μL of microbead suspension were added to a test tube, followed by 50 μL phosphate buffered saline (PBS) and 20 μL of mouse IgG diagnostic phycoerythrin (PE)-labeled anti-CD20 antibody (Becton Dickinson), agitated and incubated for 30-60 minutes. The stained microbeads were washed twice with 2 mL PBS, centrifuged and resuspended in 500 μL PBS. The geo-mean MFI values of each microbead population were entered into QuickCal software to create a set of standards. To prepare the samples, 100-300 μL of the cell suspension were washed with PBS and resuspended in 100 μL PBS. After adding 20 μL of anti-CD20 antibody, samples were stained for 15 minutes, washed with PBS, and measured.

Cytokines

TNFα (Cell Concepts GmbH, Umkirch, Germany; 2 and 10 ng/mL), IL-2 (Strathmann AG, Hamburg, Germany; 200 and 400 U/mL), IL-4 (Strathmann AG;

200 and 400 U/mL), IL-6 (Strathmann AG, 1000 and 2000 U/mL), IL-13 (Biochrom KG, Berlin, Germany; 1 and 10 ng/mL) and GM-CSF (Biochrom KG; 500 and 1000 U/mL) were used. Analysis of antibody binding capacity and apoptosis was performed after 24, 48, 72 and 96 hours.

Analysis of apoptosis/cytotoxicity by flow cytometry

Apoptotic cell death was analyzed by a FACScan flow cytometer with the CellQuest software package (Becton Dickinson, Heidelberg, Germany). Apoptosis was detected with the Annexin V-Kit (Bender MedSystems, Vienna, Austria/ Alexis Corporation, Lausen, Switzerland). Assays were performed in triplicate. Populations of leukemic cells were gated in a forward side scatter/side side scatter dot plot. The percentage of apoptotic and necrotic/lytic cells was defined by their distribution in a fluorescence (caused by annexin/propidium iodide) dot plot (WinMdi, Vers. 2.8, Scripps Research Institute, San Diego, USA). Only samples with a spontaneous apoptosis rate less than 25% were accepted in the experiments when rituximab was combined with the cytotoxic drugs or complement.

Disruption of mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was measured using a specific fluorescent probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Alexis Biochemicals, Gruenberg, Germany), used at a concentration of 5 μ/mL for 20 min. After incubation with JC-1, cells were analyzed by FACScan using fluorescence channels FL1 and FL2. JC-1 emits a red fluorescence when sequestered as an aggregate in the mitochondrial membrane of healthy cells, whereas release of JC-1 into the cytoplasmic compartment of the cell promotes the monomeric state, thus emitting a green fluorescence.[20] At depolarized (-100 mV) membrane potentials the JC-1 green monomer emission peaks are at approximately 527nm. Upon hyperpolarization of membrane potentials (-140 mV) emission of the JC-1 aggregates shifts towards 590 nm.[21-23]

Western blot analysis

For this analysis 1×10^6 cells were pelleted and fractionated by SDS-page (12-15% gradient gels) and proteins were transferred to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Hercules, CA, USA) and standard protocols. The loading of equal amounts of protein was verified by Ponceau staining of the nitrocellulose membranes and by Coomassie staining of the polyacrylamide gels. The membrane was blocked with 5% non-fat, dry milk for 1 hour and subsequently incubated with the primary antibody at a dilution of 1:1000 (PARP, Boehringer Mannheim GmbH, Mannheim, Germany; pro-caspase-3, Transduction Laboratories, Lexington, USA; activated-caspase-7 and -8, BD PharMingen, Becton Dickenson GmbH, Heidelberg, Germany; p53, Santa Cruz, Heidelberg, Germany) for 2 hours at room temperature. Unbound antibody was removed by washing with Tris buffered saline (pH 7.2) containing 0.5% Tween 20. The membrane was then incubated with the secondary antibody (alkaline-phosphatase-conjugated antibody, Sigma, Deisenhofen, Germany) for two hours at room temperature. After extensive washing with Tris-buffered saline, proteins were detected upon addition of the staining substrates (BCIP: 5-bromo-4-chloro-3-indolyl-phosphate, Boehringer Mannheim, Mannheim, Germany, NBT: 4-nitro-blue-tetrazolium-chloride, Boehringer Mannheim, Indianapolis, IN, USA).[24]

Inhibition of caspase activity

The specific caspase-3 inhibitor Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-CHO, Bachem, Heidelberg, Germany) and the general caspase-inhibitor ZVAD-fmk (benzyloxyoxycarbonyl-valine-alanine-aspartate-(O-methyl)-fluoromethylketone, Bachem) were used in concentrations between 50 μmol/L-400 μmol/L applied one hour prior to rituximab/chemotherapeutic agents. Previously conducted experiments showed a sufficient inhibition of caspase-3-activity (Ac-DEVD-CHO) and of general caspase-activity (ZVAD-fmk) at a dose of 50 μmol/L (*Boehrer S, unpublished data*).

Statistical analysis

To determine synergistic, additive and antagonistic effects the combination index method (CI) with median effect plot or isobologram analysis were established.[25] Rituximab used as a single agent did not follow an adequate dose-response curve, so neither CI-calculations or isobologram analysis could be applied. We, therefore, assessed the changes in the IC_{30} and IC_{50} (inhibiting concentration of a drug when 30% or 50% of its effect are achieved) of the chemotherapeutic agents with and without the presence of rituximab. A two-sided Wilcoxon-test was used to test the statistical significance of results. Results were considered to be significant when $p<0.05$. To calculate the IC_{30} or IC_{50} of the drug combinations, Calcusyn software (Biosoft, Cambridge, UK) was applied.[26]

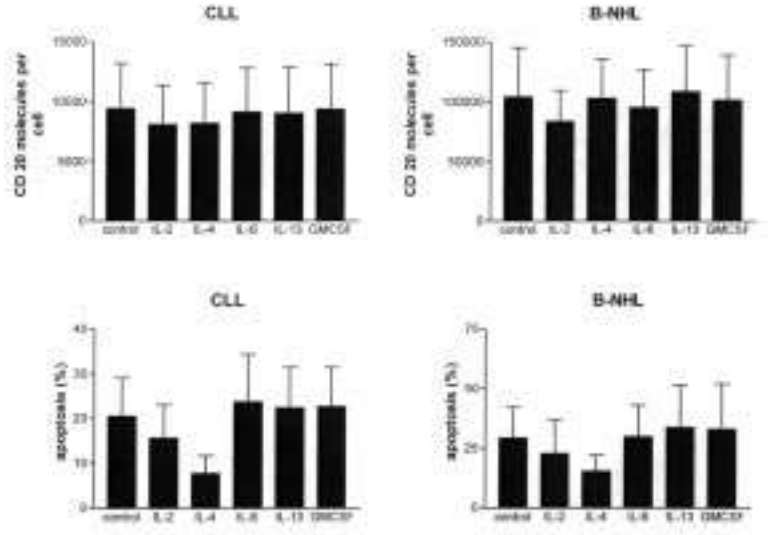

Figure 1. Effect of different cytokines on CD20 expression and the rate of apoptosis. Graphs show the effects of various cytokines on the number of CD20 molecules per cell and the corresponding rate of apoptosis in CLL cells (n=11) and cells from patients with B-cell lymphomas (n=9) after 48 hours incubation. Concentrations of cytokines: IL-2 (400 U/mL), IL-4 (400 U/mL), IL-6 (2000 U/mL), IL-13 (10 ng/mL), GM-CSF (1000 U/mL).

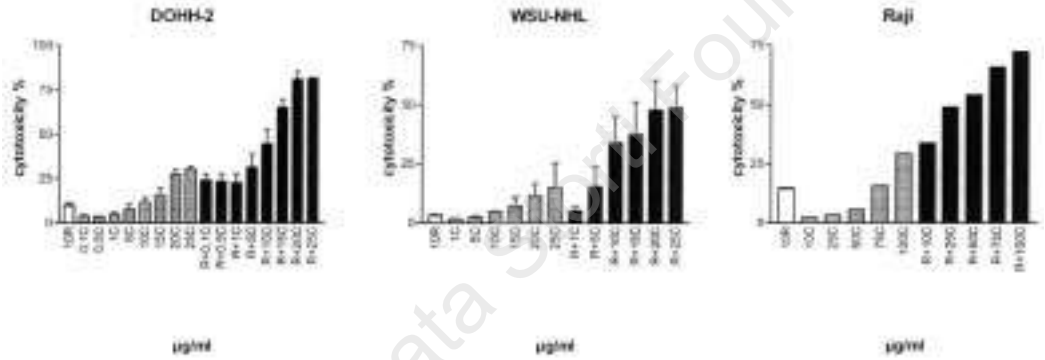

Figure 2. Intrinsic lytic activity of complement ± rituximab. Cytotoxic effects of complement alone or in combination with rituximab (10 µg/mL) on lymphatic cell lines. R=rituximab, C=complement, R+C=rituximab+complement.

Results

Incubation of *ex vivo* cells from patients with B-cell NHLs with IL-2, -4, -6, -13, GM-CSF and TNFα does not increase expression of CD20 or the rate of apoptosis. Since it was postulated that certain cytokines are able to upregulate expression of the CD20 antigen we first assessed changes in the expression of CD20 after incubation with different dosages of IL-2, IL-4, IL-6, IL-13, GM-CSF (Figure 1) and TNFα (data not shown). As shown in Figure 1 none of the cytokines was able to upregulate the expression of the CD20 receptor in *ex vivo* cells of patients suffering from CLL or leukemic B-cell lymphomas. The obtained results were independent of incubation period (24-96 hours) and cytokine concentrations (data for lower concentrations are not shown). Moreover, the percentage of CD20-positive cells in relation to total cell number remained unaltered (data not shown). To assess a direct effect of the cytokines on the tumor cells the rate of apoptosis under the different cytokines, dosages and incubation periods was measured. *Spontaneous* apoptosis, i.e. apoptosis of tumor cells observed *in vitro* without specific treatment after 48 hours, was in the range of 8.1-45.3% in CLL cells, and between 11.7-67.3% in cells of B-NHLs (Figure 1). Incubation with IL-6, IL-13, GM-CSF (Figure 1) and TNF α (data not shown) did not have any effect on the rate of apoptosis independently of the dosage and the duration of the incubation period. In con-

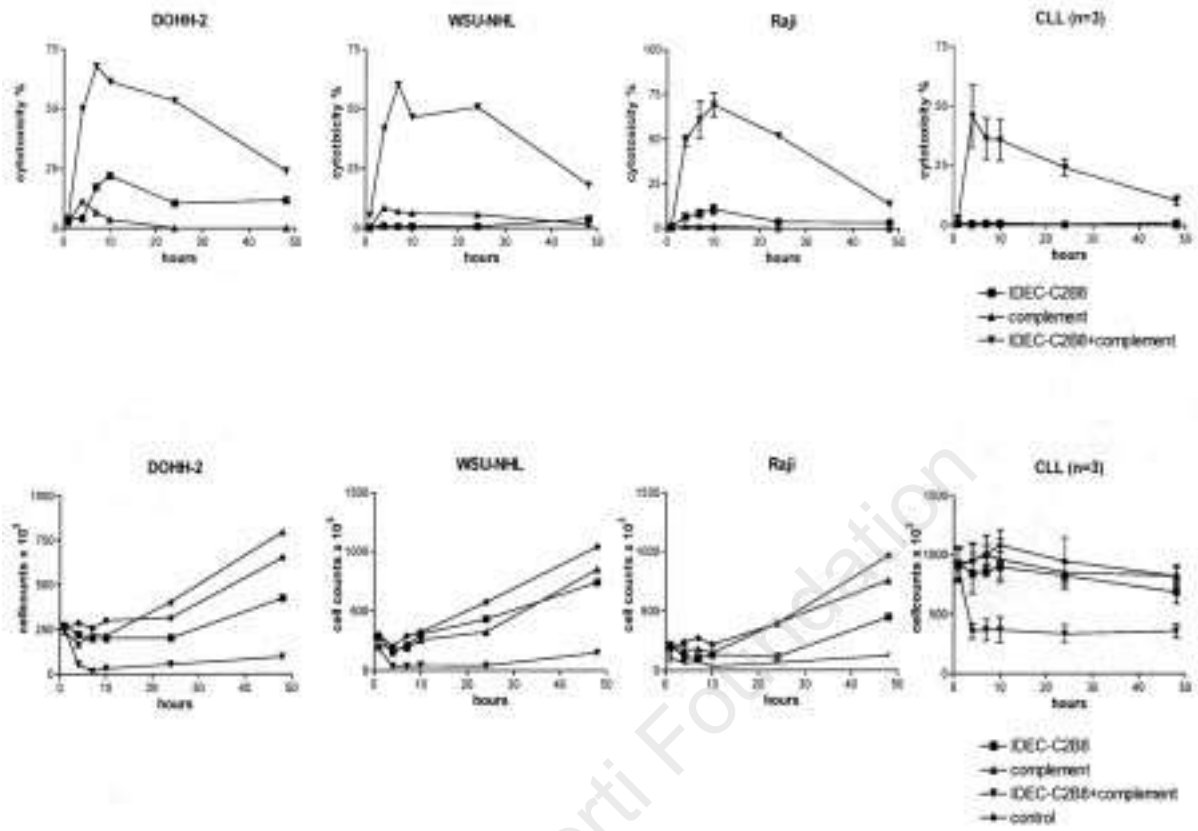

Figure 3. Kinetics of rituximab + complement. A series of kinetic experiments examining the effects of combining 10 μg/mL of rituximab with 25 μL of complement on various cell lines and CLL cells. The cytotoxicity obtained by the rituximab or complement dose used did not differ significantly from that of the controls in all tested cells. The maximum cytotoxicity was achieved within the first 10 hours. The graphs below show the corresponding numbers of vital cells.

trast, incubation with IL-2 led to a moderate decrease in apoptosis of *ex vivo* cells from patients with CLL as well as other leukemic B-cell lymphomas (Figure 1, not significant) and the decrease in the rate of apoptosis observed upon incubation with IL-4 was statistically significant in CLL cells ($p< 0.05$), as well as in NHL leukemic cells ($p<0.05$).

CD20-binding capacity of rituximab is dose-dependent

The ability of the CD20 antibody to bind to the CD20 receptor (antibody-binding capacity, ABC) was evaluated using the cell lines DOHH-2, WSU-NHL and Raji, as well as *ex vivo* cells from three patients with CLL and one patient with hairy cell leukemia (HCL). The expression of the CD20 antigen per tumor cell was considerably higher in the lymphoma cell lines and in *ex vivo* cells of HCL patients, than in the *ex vivo* MNC of CLL patients (data not shown). Upon incubation with rituximab antibody-binding capacity was observed to be dose-dependent, i.e. increasing dosages of rituximab were able to block increasing numbers of CD20 receptors per tumor cell. In the lymphoma cell lines as well as in the *ex vivo* cells of HCL patients the majority of CD20 receptors (i.e. more than 95%) per tumor cell were saturated upon incubation with 10 μg/mL rituximab (data not shown). It is noteworthy that the observed dose-dependency of rituximab's antigen-binding capacity was independent of the absolute number of CD20-molecules expressed on the tumor cells. The binding of rituximab remained stable over at least 96 hours (data not shown).

Complement causes cytotoxicity in lymphoma cell lines and increases cytotoxicity upon addition to rituximab

In order to assess intrinsic lytic activity the lymphatic cell lines DOHH-1, WSU-NHL and Raji were incubated with increasing concentrations of complement rabbit serum. As shown in Figure 2

increasing concentrations of complement led to increased cytotoxicity after 4 hours of incubation. In Raji cells the concentration of complement leading to cytotoxicity in the range of 20-30% was about four times higher than in the cell lines DOHH-2 and WSU-NHL (100 μg/mL versus 25 μg/mL, Figure 2). When the same cells were incubated with 10 μg/mL of rituximab and increasing concentrations of complement, enhanced cytotoxicity against the tumor cells was found (Figure 2). Furthermore, these experiments revealed the subtoxic concentrations (i.e. no lysis of the cells by intrinsic activity) of complement (0.1-1μL for the DOHH-2, 1-5μL for the WSU-NHL and 10-25 μL for the Raji cell lines; Figure 2).

Cytotoxic effect of rituximab in combination with complement reaches a maximum within the first hours of incubation

To determine the kinetics of cytotoxicity caused by rituximab, complement and the combination of both, the percentage of lysed tumor cells was measured after 0, 4, 7, 10, 20, 30, 40 and 50 hours following incubation with 10 μg/mL rituximab and 25 μg/mL complement. At the same time points the number of vital cells was counted using a Neubauer chamber. Figure 3 demonstrates that independently of the type of lymphatic cell the maximum lysis caused by a combination of rituximab and complement was reached within the first 10 hours of incubation. It is noteworthy that the percentage of lysed cells in the cell lines was higher than in the *ex vivo* cells from patients with CLL. Although about 46.5% of the CLL tumor cells already exhibited lysis after four hours, there was no further increase after this time point and the number of lysed cells steadily declined (Figure 3). These results are in accordance with the number of vital cells counted in each population. Thus, an initial decrease in the number of vital cells was observed concomitantly with the maximum cytotoxic effect of rituximab and complement (Figure 3).

Combination of rituximab and chemotherapeutic agents synergistically affects apoptosis, whereas addition of complement remains without effect

To define the effects on apoptosis of tumor cells caused by a combination of chemotherapeutic agents and rituximab in the absence and in the presence of complement, the MNC of the cell lines DOHH-2 and WSU-CLL as well as *ex vivo* MNC of CLL were used. Standard chemotherapeutic agents established in the treatment of malignant lymphoma were used: bendamustine, cladribine, doxorubicin and mitoxantrone. Complement was added at a sublytic dosage in the range of 1-5 μL, this dosage determined by dose-finding studies prior to each experiment, as demonstrated in Figure 2. Results were evaluated using the IC_{30} and IC_{50} for each chemotherapeutic agent (defined as the dosage of drug necessary to cause apoptosis of 30% and 50% of tumor cells, respectively). As demonstrated in Figure 4 the IC_{30} and the IC_{50} of all chemotherapeutic agents was considerably decreased by addition of 10 μg/mL rituximab. Comparable results were obtained in the DOHH-2 and WSU-NHL cell lines (Figure 4 and 11). The addition of complement decreased neither the IC_{30} nor the IC_{50} of any of the tested chemotherapeutic agents. In accordance with the results obtained with the cell lines, incubation of CLL cells with rituximab was accompanied by a decrease of the IC_{30} and the IC_{50} of all chemotherapeutic agents applied (Figure 5). In contrast to the data obtained using the cell lines, the addition of complement led to an increase in the IC_{30} and the IC_{50} independently of the chemotherapeutic drug used (Figure 5).

To investigate whether the observed synergistic

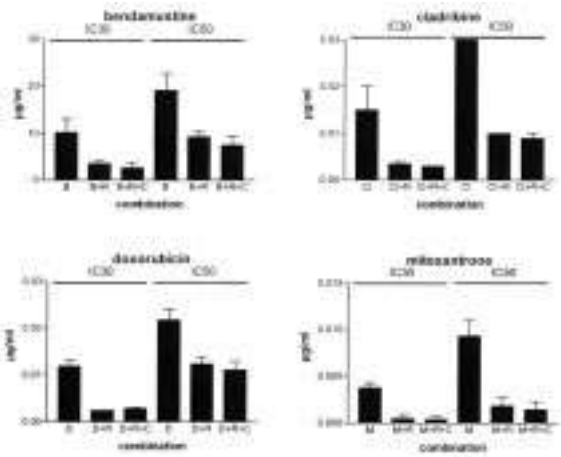

Figure 4. Synergistic effects of rituximab + cytotoxic agents after 24 hours. Effect of rituximab (10 μg/mL) in combination with standard and new drugs (cladribine, doxorubicin, mitoxantrone and bendamustine) and complement (sublytic dose in the range 1-5 μL as determined in previously conducted experiments, data not shown) on the IC30 and IC50 dose of apoptosis on DOHH-2. C=complement, R=rituximab, c=cladribine, b=bendamustine, d=doxorubicin, m=mitoxantrone.

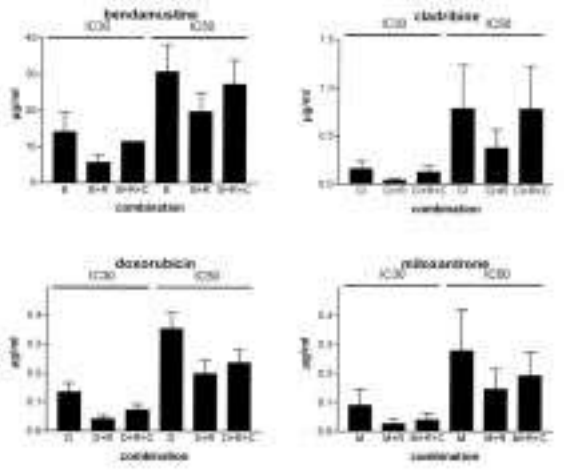

Figure 5. Synergistic effects of rituximab + cytotoxic agents after 48 hours. Effect of rituximab + drugs ± complement on CLL cells (n=5). According to the tested cell lines there was a significant decrease of IC_{30} and IC_{50} of apoptosis drug dosages when the drug was combined with rituximab. In contrast to the cell lines the addition of the sublytic complement dose yielded a protection from cytotoxicity when cladribine, doxorubicin or bendamustine was used. C=complement, R=rituximab, c=cladribine, b=bendamustine, d=doxorubicin, m=mitoxantrone.

effects of rituximab in combination with the chemotherapeutic agents are dependent on the dosage of rituximab necessary to saturate the CD20 antigen (10 µg/mL) or whether they may also be observed at a lower dosage, experiments were repeated using rituximab at a decreased dosage of 0.1 µg/mL. As demonstrated above, this dose is able to bind to 50-60% of CD20 molecules per cell. Remarkably, effects on the IC_{30} and the IC_{50} on MNC of the cell lines as well as CLL cells were comparable to the results obtained in the experiments with the higher rituximab dose (data not shown).

Mechanisms of apoptosis induced by chemotherapeutic agents combined with rituximab

In a further series of experiments the influence of rituximab, mitoxantrone and complement by themselves and in combination on the main events of apoptosis were determined. Figure 6 demonstrates that the efficacy of the agents on the morphologic changes during apoptosis (annexin V) and the disruption of the mitochondrial membrane potential (MMP by JC-1) were nearly identical. The *in vitro* activity on apoptosis was not significantly decreased when the specific caspase-3 inhibitor was added (Figure 6). Pro-caspase-3 was only activated when the drugs (e.g. mitoxantrone) were added or applied as single agents (Figure 6). After incubation with caspase-3 inhibitor, pro-caspase-3 was not activated in any of the tested samples (Figure 6). These findings were corroborated by experiments determining the expression of cleaved PARP. PARP cleavage was observed mainly under the influence of mitoxantrone, independently of the addition of caspase-3-inhibitor, supporting the findings assessed by annexin V or JC-1 (Figure 6). Figure 7 demonstrates the activation of executioner caspase-7, initiator/effector caspase-8 and the upregulation of p53. All events were mainly achieved when mitoxantrone (as an example of the drugs) was applied to the tested samples. The activation of caspase-7 and –8 was not influenced by addition of Ac-DEVD-CHO, but nearly completely blocked by ZVAD-fmk (Figure 6). p53 upregulation was independent of addition of the caspase inhibitors. It is noteworthy that rituximab itself did not activate or upregulate any of the tested proteins (Figures 6 and 7).

Discussion

Preliminary clinical studies with the monoclonal antibody rituximab in the treatment of low- and high-grade lymphomas demonstrate a high efficacy of the antibody used as a single agent as well as in combination with standard chemotherapy.[2, 3] The aims of this study were, first, to evaluate a possible contribution of different cytokines and complement to the effects caused by rituximab. Particular interest was given to the role of the expression level of the CD20 receptor and the impact of altered antibody binding capacities of rituximab. Finally, changes in the IC_{30}/IC_{50} as well as intracellular changes attributable to the combination of rituximab with differently acting chemotherapeutic agents were assessed. Another goal of our study was to improve the efficacy of rituximab on CD20 positive tumor cells from patients with CLL or leukemic B-cell lymphomas by preincubation of the cells with various cytokines. We hypothesized that response to rituximab (IDEC-C2B8) would potentially be increased if upregulation of CD20 were possible, and that a combination of cytokine priming and subsequent administration of CD20 antibodies may lead to increased efficacy especially in CLL, a lymphoma entity with usually low CD20 surface expression. However, neither upregulation of CD20 nor a change of the proportion of CD20 positive cells was observed. These results are in contrast with those of other groups demonstrating a significant upregulation of CD20 expres-

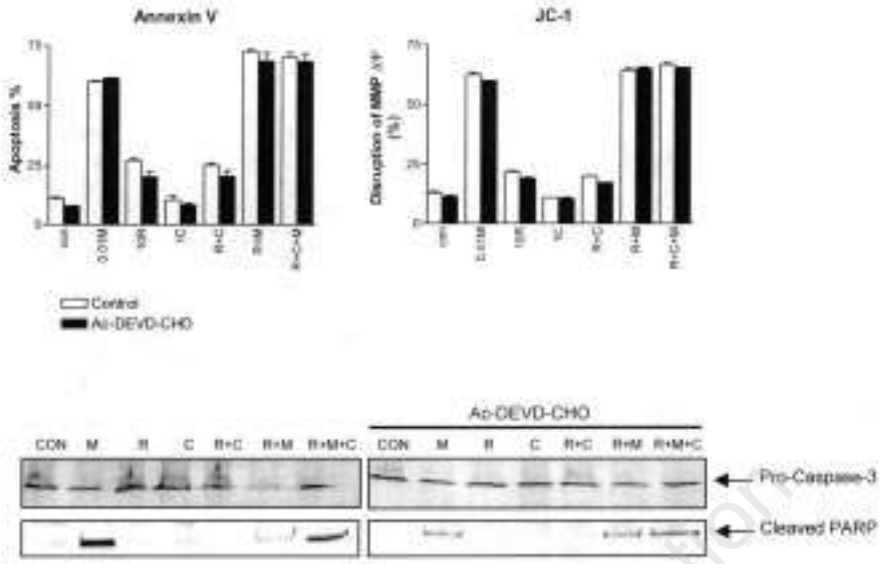

Figure 6. Mechanisms of apoptosis influenced by rituximab sensitization. Efficacy of rituximab, mitoxantrone and complement on morphologic changes during apoptosis (annexin V), disruption of mitochondrial membrane potential (MMP by JC-1) and expression of pro-caspase-3 and cleaved PARP + specific caspase-3-inhibitor Ac-DEVD-CHO in DOHH-2 cells after 24 hours. Con=control (without addition of drugs), M=mitoxantrone, R=rituximab, C=complement. Concentration of the drugs in μg/mL.

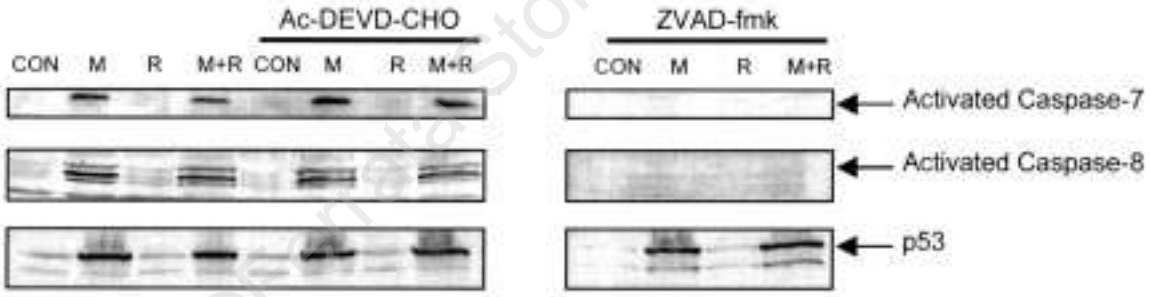

Figure 7. Mechanisms of apoptosis influenced by rituximab sensitization. Activation of executioner caspase-7, initiator/effector caspase-8 and upregulation of p53 during apoptosis under the influence of mitoxantrone and rituximab ± specific caspase-3-inhibitor Ac-DEVD-CHO or general caspase-inhibitor ZVAD-fmk. Con=control (without addition of drugs) in DOHH-2 cells after 24 hours, M= 0.01 μg/mL mitoxantrone, R= 10 μg/mL rituximab.

sion by IL-4, GM-CSF and TNFα.[1] It is possible that other strategies to evaluate the data led to these different results. In our hands the proposed increase of the effect of rituximab following pre-treatment of patients with cytokines is questionable.

It is noteworthy that in the same series of experiments a significant decrease of apoptosis by IL-4 and a not significant trend of apoptosis protection by IL-2 were achieved. As described by other authors, certain cytokines (i.e. IL-4, IL-10) are capable of decreasing the rate of apoptosis.[14,27] This decrease of apoptosis under the influence of cytokines may be an explanation for the increased drug dosages required in CLL cells to reach the IC30 or IC50 upon addition of complement. This effect was only observed in *ex vivo* CLL cells, but not in the lymphoma cell lines cultured in complement-free media, indicating that

cytokines contained in rabbit serum may be responsible for the described effect.

The mechanism of action of rituximab has been widely investigated.[7-14, 16, 28] The contribution of complement in the cytotoxic activity of rituximab is not fully understood. In our hands both compounds combined resulted in a sufficient dose response of cytotoxicity, but the application of complement had no effect on sensitization of drug-induced apoptosis by rituximab. These findings support those of other authors[9,10] who demonstrated that rituximab itself exhibits its efficacy mainly due to an interaction with complement. Data from others[13,14] and our data demonstrate that rituximab induces some degree of apoptosis. The mechanisms by which the antibody causes apoptosis or sensitizes lymphoma cells towards apoptosis by cytotoxic drugs are, however, largely unknown. In this study we focused on effects observable when the majority of CD20 molecules on each cell were bound by rituximab. Thus saturation of CD20 antigens by rituximab resulted in a significant sensitization of tumor cells towards standard chemotherapeutic agents, as demonstrated by the synergistic effects of rituximab and the chemotherapeutic drugs on the rate of apoptosis. Notably, the synergistic effects were achieved by simultaneous application of rituximab and the chemotherapeutic agents, suggesting that the sensitization caused by rituximab appears rapidly without requiring preincubation of cells with one component. These effects were also reproduced when only about half of the CD20 molecules per cell were saturated with rituximab, providing evidence that the absolute numbers of blocked CD20 molecules per cell is of lesser importance. We previously demonstrated that standard antineoplastic agents such as doxorubicin and mitoxantrone as well as newer agents used in lymphoma treatment such as cladribine and bendamustine exhibit considerable efficacy against *ex vivo* cells from patients with leukemic B-cell lymphomas and may, therefore, be promising options for clinical trials combining them with rituximab.[17,29] The data of the present study corroborate results of previous studies reporting synergistic interactions of fludarabine and cisplatin combined with rituximab on the cytotoxicity of various CD20-positive tumor cells.[28, 30] Of note, our data indicate that the drug sensitization induced by rituximab enhances the efficacy of all tested cytotoxic agents, independently of their mode of action (anthracyclines, purine analogs or alkylating agents). The intracellular mechanisms by which rituximab leads to apoptosis and sensitization of lymphoma cells towards treatment with other chemotherapeutic drugs still remain to be resolved. Upregulation of p53 – considered to be a central event in apoptosis by standard chemotherapeutic agents – was observed upon addition of chemotherapeutic agents and remained unaffected by addition of rituximab. Therefore, the intracellular events caused by rituximab seem to be independent of p53. A recent study described reduced levels of Bcl-2 in association with a down-regulation of IL-10 as the mechanisms responsible for induction of apoptosis after incubation with rituximab in an AIDS-related lymphoma cell line.[14] In that same work the authors demonstrated that neither rituximab nor the cytotoxic drug CDDP, used as single agents, was able to induce PARP-cleavage, whereas PARP-cleavage was observed upon incubation of lymphoma cells with both drugs. Since PARP-cleavage is mainly attributed to the activation of caspases, the data of Alas *et al.*[14] indicate that caspases are involved at least in sensitizing cells to drug-induced apoptosis. The central role of caspases in the sensitizing effect of rituximab is supported by our data, since the rate of apoptosis was markedly decreased in the presence of the general caspase inhibitor ZVAD-fmk. In contrast incubation of the cells with the specific inhibitor of caspase-3 Ac-DEVD-CHO did not influence the rate of apoptosis, PARP-cleavaging activity or activation of executioner caspase-7 when rituximab was combined with chemotherapeutic agents. The expression patterns of caspase-7 were also achieved for activated caspase-8 which is known to be an initiator caspase,[31] as well as an effector caspase in drug-induced apoptosis.[32] This minor role of caspase-3 corroborates findings by other groups which demonstrated that caspase-3 involvement was only found when rituximab was clustered with an second antibody.[12] In conclusion we demonstrate that a wide range of cytokines do not influence the expression of CD20 antigen of neoplastic lymphatic cells. Incubation with rituximab significantly decreases the IC_{30} and IC_{50} of standard chemotherapeutic drugs used in lymphoma treatment. This sensitization effect neither depends on the saturation of CD20 molecules by rituximab, nor on the presence of complement. Furthermore this study provides evidence that the sensitizing effect of rituximab used in combination with chemotherapeutic agents is independent of p53 and caspase-3, but may depend on activation of caspase-7 and –8.

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KUC co-ordinated the experimental work and wrote the paper. WDS performed cell cultures and analysis of apoptosis. SB carried out the Western blot analyses. BS was responsible for measurement of CD20-expression. GS carried out the cytokine experiments. MJR performed the statistical analysis. DH,PSM and EW supervised the study and critically reviewed the drafts of the manuscript for important intellectual content. All authors approved the final version of the manuscript and agreed to the order of the authors. The order of authorship strictly depended on the relevance of the contribution of each investigator to the complete study, as indicated above.

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Disclosures

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Peer Review Outcomes

What is already known on this topic

Rituximab is known to act via CDC and/or ADCC, although additional mechanisms are thought to include induction of apoptosis and an inhibition of proliferation.

What this study adds

New findings are that cytokines do not alter CD20 levels in fresh B-CLL and B-NHL and that incubation with Rituximab leads to chemosensitization of such cells to the pro-apoptostic activity of chemotherapeutic agents (bendamustine, doxorubicin, cladribine, mitoxantrone).

Manuscript processing

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Potential implications for clinical practice

Cytokine treatments are unlikely to increase the activity of Rituximab through an increase in CD20 levels. Rituximab may be particularly active in combination with chemotherapeutic agents because of its chemosensitizing activity.

Alessandro Rambaldi, Associate Editor