

# Inhibition of Interleukin 10 by Rituximab Results in Down-Regulation of Bcl-2 and Sensitization of B-cell Non-Hodgkin's Lymphoma to Apoptosis<sup>1</sup>

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## ABSTRACT

Treatment of patients with non-Hodgkin's lymphoma (NHL) is frequently hampered by development of chemoresistance. Rituximab is a chimeric mouse antihuman CD20 antibody that offers an alternative; however, its mechanism of action is not clearly understood. Treatment of lymphoma cell lines with Rituximab sensitizes the cells to the cytotoxic and apoptotic effects of therapeutic drugs, *e.g.*, cisplatin, fludarabine, vinblastine, and Adriamycin. This study investigated the mechanism(s) involved in the reversal of drug resistance by Rituximab therapy. NHL cells synthesize and secrete antiapoptotic cytokines implicated in drug resistance, including interleukin (IL)-6, IL-10, and tumor necrosis factor  $\alpha$ . We hypothesized, therefore, that sensitization by Rituximab may be due in part to modification of cytokine production. In this study, examination of cytokine secretion by NHL 2F7 tumor cells revealed down-regulation of IL-10 by Rituximab treatment. Moreover, cytotoxicity assays using exogenous IL-10 and IL-10-neutralizing antibodies demonstrated that IL-10 serves as an antiapoptotic/protective factor in these tumor cells against cytotoxic drugs. Furthermore, expression in 2F7 cells of the protective factor, Bcl-2, was shown to be dependent on IL-10 levels and down-regulated by Rituximab. Other gene products such as Bax, Bcl-x, Bad, p53, *c-myc*, and latent membrane protein-1 (LMP) were not affected by Rituximab treatment. Drug sensitization, as well as down-regulation of both IL-10 and Bcl-2, was corroborated in experiments using the NHL cell line 10C9. The Ramos and Daudi NHL cell lines were not sensitizable, nor did their Bcl-2 or IL-10 levels change.

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These studies demonstrate that one mechanism by which Rituximab sensitizes NHL to chemotherapeutic drugs is mediated through down-regulation of antiapoptotic IL-10 autocrine/paracrine loops and Bcl-2. The clinical relevance of these findings is discussed.

## INTRODUCTION

Current therapies for NHL<sup>3</sup> elicit an initial response for most patients. However, whereas lymphomas are initially susceptible to conventional chemotherapeutic regimens, only a fraction of aggressive lymphomas are cured. Invariably, most NHL patients suffer a relapse after an average remission of only 1–3 years. Such patients are very difficult to treat by traditional means.

Monoclonal antibodies have provided a significant advantage in the treatment of hematological malignancies. The use of these antibodies provides higher tumor specificity than traditional systemic treatments. CD20, a  $M_r$  35,000 surface membrane phosphoprotein, has become a preferred target of monoclonal antibody immunotherapy. CD20 is expressed specifically within the B-cell lineage from the early pre-B-cell stage to the mature B-cell stage and disappears from the cell surface upon B-cell differentiation into immunoglobulin-secreting plasma cells (1). Although its function is still unclear, it is believed to play a role in B-cell proliferation and differentiation during lymphocyte development.

The chimeric mouse antihuman monoclonal antibody Rituximab (IDEC-C2B8) is specific for the CD20 antigen (2). Rituximab has been used in clinical trials to treat patients with NHL (3–7). These clinical trials included both indolent and aggressive NHL and have demonstrated a 30–50% overall response rate. The failure to garner higher levels of efficacy with Rituximab alone led to our studies investigating combination therapies using chemotherapeutic drugs and the mechanisms by which they may achieve synergistic activity.

The mechanisms by which Rituximab induces its antitumor effect are not fully understood. Tumor regression by Rituximab treatment *in vivo* is thought to involve complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, inhibition of cell proliferation, or anti-CD20-mediated programmed cell death. However, the mechanisms involved in

<sup>3</sup> The abbreviations used are: NHL, non-Hodgkin's lymphoma; ADR, Adriamycin; IL, interleukin; TNF, tumor necrosis factor; CDDP, cisplatin; LMP, latent membrane protein; XTT, sodium 3'-[1(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate; PI, propidium iodide; FIC, fractional index of cytotoxicity; RT-PCR, reverse transcription-PCR; IL-10R, IL-10 receptor; vIL-10, viral IL-10; PARP, poly(ADP-ribose) polymerase; ARL, AIDS-related lymphoma.

mediating the observed clinical efficacy may not necessarily be responsible for causing sensitization to chemotherapeutics. The means of sensitization is not to be confused with the ability of Rituximab ability to eradicate tumor cells *in vivo*. Although some overlap may exist, the major mechanisms of these phenomena are largely distinct.

Secretion of certain cytokines, particularly IL-6, IL-10, and TNF- $\alpha$  (8–10), by tumor cells has been shown to render them resistant to the cytotoxic effects of chemotherapeutic drugs. These cytokines work in autocrine/paracrine loops to induce their antiapoptotic effects on a variety of tumors, including prostatic carcinoma, B-cell NHL, and renal cell carcinoma (8–10). Although the regulation of apoptotic and antiapoptotic factors by protective cytokines has been well documented (11–15), the underlying mechanisms involved in maintaining drug resistance by these cytokines are not fully understood. Drug resistance factors, such as the apoptotic/antiapoptotic Bcl-2 family members, have also been implicated in the drug-resistant phenotype of many malignancies (16).

To circumvent drug resistance in NHL, we have investigated whether Rituximab can serve as a sensitizing agent against drug-resistant tumor cells. We theorize that Rituximab modulates the expression of protective factors involved in drug resistance of malignant B cells. The objective of this study was to delineate the mechanism(s) by which Rituximab modifies 2F7 B lymphoma cells, rendering them susceptible to certain chemotherapeutic drugs. Specifically, we investigated (a) the modulation of IL-10 secretion by Rituximab, (b) the role of exogenous and tumor-derived IL-10 on tumor cell susceptibility to cytotoxic drugs, and (c) the effect of Rituximab on the regulation of genes involved in apoptosis. Elucidating the mechanisms of chemosensitization may lead to rational development of therapeutic clinical trials.

## MATERIALS AND METHODS

**Tumor Cell Lines.** NHL B-cell lines 2F7 and 10C9 were derived from patients suffering from AIDS with Burkitt's lymphoma (17). The Ramos and Daudi cell lines were also of Burkitt's lymphoma origin (18, 19). The CD20-negative, CD4-positive T-lymphoblastoid leukemia line CEM was used as a CD20-negative control in Rituximab experiments. The 2F7 and 10C9 cell lines were kindly provided by Dr. Otoniel Martinez-Maza (Jonsson Comprehensive Cancer Center, Los Angeles, CA), whereas the CEM and Ramos cell lines were purchased from the American Type Culture Collection (Manassas, VA). The Daudi cell line was provided by Dr. Anahid Jewett (UCLA Dental Research Institute, Los Angeles, CA). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, N.Y.) with 10% heat-inactivated fetal bovine serum (Gemini, Calabasas, CA). Cultures were additionally supplemented with 1% bacteriostatic solution containing 10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25  $\mu$ g/ml fungizone (Irvine Scientific, Santa Ana, CA). Supplemented medium is hereafter referred to as complete medium. Cells were maintained at 37°C in 5% atmospheric CO<sub>2</sub>.

**Chimeric Mouse Antihuman CD20 Antibody, Rituximab (IDEC-C2B8).** Rituximab is a mouse antihuman monoclonal antibody specific for human CD20, an antigen found only

on normal and malignant B-cell lymphocytes (2). Rituximab was genetically engineered by fusing human IgG1 $\kappa$  constant regions to the murine antihuman CD20 antibody (IDEC-2B8) variable regions. The Rituximab antibody was expressed in Chinese hamster ovary cells, whereas the IDEC-2B8 antibody was produced from hybridoma. Both were kindly supplied by Dr. Nabil Hanna (IDEC Pharmaceutical Inc., San Diego, CA).

**Reagents.** ADR, CDDP, vinblastine, and emetine were purchased from Sigma (St. Louis, MO). Fludarabine was kindly provided by Berlex (Richmond, CA). PBS (pH 7.4; Sigma) was used to solubilize and store stocks of ADR (1 mg/ml), vinblastine (10  $\mu$ g/ml), and fludarabine (1 mM) at 4°C. CDDP was dissolved in DMSO (Sigma) at 5 mg/ml before use. [<sup>3</sup>H]Leucine was purchased from ICN (Costa Mesa, CA). The XTT cytotoxicity assay kit was purchased from Boehringer Mannheim (Indianapolis, IN).

Monoclonal capture antibodies for IL-6 and IL-10 ELISAs were purchased from Genzyme (Cambridge, MA) and Pharmingen (San Diego, CA), respectively. Antibodies against TNF- $\alpha$  were made in our laboratory from hybridomas. Polyclonal antibodies for all cytokines used for detection in ELISAs were produced from rabbits in our laboratory and partially purified by ammonium sulfate. Recombinant IL-10 was obtained from PeproTech (Rocky Hills, NJ).

Bcl-2 antibody was purchased from Dako (Carpinteria, CA), Bax and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Bcl-x and Bad antibodies were purchased from Transduction Laboratories (Lexington, KY).

**Cell Proliferation.** To characterize the effects of Rituximab on 2F7 and CEM, cells were cultured at 10<sup>4</sup> cells/ml (200  $\mu$ l) in 96-well plates (Costar, Cambridge, MA) in the presence of 0, 4, or 20  $\mu$ g/ml Rituximab for 5 days. Cells were cultured in the presence of normal mouse serum (1:1000) as a negative control. Cell number and viability were determined at 24-h intervals by trypan blue exclusion. Ten percent of the cell culture in each well (20  $\mu$ l) was added to 20  $\mu$ l of trypan blue (Life Technologies, Inc.) and counted by microscopy. The percentage of proliferation was calculated as shown below.

% proliferation

$$= \frac{\text{number of viable cells in treated samples}}{\text{number of viable cells in untreated samples}} \times 100\% \quad (1)$$

**[<sup>3</sup>H]Leucine Incorporation Assay.** 2F7 cells were grown in 96-well tissue culture plates (Costar) in 200  $\mu$ l of complete medium at a concentration of 10<sup>4</sup> cells/well. Cells were treated for 24 h at 37°C with Rituximab at 0.2, 2, 20, and 200  $\mu$ g/ml or with medium alone. Emetine (5  $\mu$ M), a protein synthesis inhibitor, was used as a positive control for inhibition of [<sup>3</sup>H]leucine incorporation/protein synthesis. After the incubation, 100  $\mu$ l of fresh medium containing 100  $\mu$ Ci/ml [<sup>3</sup>H]leucine were added. Cells were allowed to grow at 37°C for 6 h and then lysed on glass filters. Filters were solubilized in 1.0 ml of Bio-Safe II scintillation fluid (Research Products International Corp., Mount Prospect, IL) and analyzed on a 1211 Rackbeta liquid scintillation counter (Wallac Inc., Gaithersburg, MD). [<sup>3</sup>H]Leucine incorporation was defined to be the cpm of

each sample as a percentage of the cpm from cells grown in complete medium only.

% [<sup>3</sup>H]Leucine incorporation

$$= \frac{\text{cpm of treated sample}}{\text{cpm of cells grown in medium only}} \times 100\% \quad (2)$$

**PI Analysis for Apoptosis.** Cells were stained with PI and analyzed for DNA fragmentation (20) with an Epics-XL MCL flow cytometer (Coulter, Miami, FL). Briefly, 10<sup>6</sup> cells were treated with complete medium, 20 μg/ml Rituximab, 1 μg/ml CDDP, a combination of both Rituximab and CDDP, or varying concentrations of anti-IL-10 (1, 10, and 50 ng/ml) for 24 h. Cells were then washed three times in PBS/0.1% BSA. After washing, cells were permeabilized by resuspension in 500 μl of cold 75% ethanol and allowed to incubate at -20°C for 1 h. Cells were washed three times as described before and resuspended in 100 μl of PI solution (100 μg/ml PI and 50 μg/ml RNase). Incubation in PI solution was performed at room temperature for 30 min while samples were protected from light. After incubation with PI solution, 500 μl of PBS/0.1% BSA were added to each sample, and then the samples were analyzed by flow cytometry.

**Cytotoxicity Assay.** Cytotoxicity assays were performed using the XTT assay (Refs. 21 and 22; Boehringer Mannheim). Tumor cells were seeded in a final volume of 200 μl at a concentration of 10<sup>4</sup> cells/well in 96-well culture plates (Costar). Berinstein *et al.* (23) showed that 3 months after Rituximab treatment, Rituximab serum levels remain at 20.3 μg/ml. Thus, tumor cells were treated with Rituximab at 20 μg/ml, chemotherapeutic drugs, or a combination of both for 24 h. Drugs and concentrations included CDDP (1 μg/ml), fludarabine (20 μM), vinblastine (0.1 μg/ml), and ADR (1 μg/ml), all of which were used at levels detected in serum or tissue *in vivo* (24–27). CDDP, ADR, and vinblastine were concomitantly added to tumor cells along with Rituximab when treated in combination. Fludarabine was added 6 h after the addition of Rituximab. Control cells, *i.e.*, cells grown in complete medium alone, served as the baseline and negative control for cytotoxicity. Wells with 200 μl of complete medium only served as the plate blank. All samples were done in triplicate. After 24 h of incubation, the XTT assay was used to quantitate cell inhibition in each sample. The cytotoxic effect of each treatment was calculated as the percentage of viability as compared to the untreated cells.

% of control

$$= \frac{\text{absorbance of sample cells}}{\text{absorbance of untreated cells}} \times 100\% \quad (3)$$

**Statistical Analysis.** All values are presented as the mean ± SD of triplicate samples. ANOVA was used to test for significance. Pairwise analysis was performed by Bonferroni/Dunn *post hoc* test. *P* < 0.05 was considered to be significant.

**Isobolograms for Chemoimmunotherapy Using Rituximab and Therapeutic Drugs.** To establish whether the cytotoxic effect of the Rituximab/chemotherapy combination was more than additive, isobolograms (28) were constructed from a

battery of combinations combining Rituximab at various concentrations (0.1, 0.5, 1, 5, 20, and 50 μg/ml) with four therapeutic drugs: (a) CDDP (0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml); (b) vinblastine (5, 10, 50, 100, 500, and 1000 ng/ml); (c) ADR (0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml); and (d) fludarabine (1, 5, 20, 50, and 100 μM). Combinations yielding a cytotoxicity of 30 ± 5% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity.

$$\text{FIC} = \frac{A_{\text{sample}}}{A_{30\%}} + \frac{B_{\text{sample}}}{B_{30\%}} \quad (4)$$

**ELISAs for Cytokine Detection.** Briefly, supernatants were collected from tumor cells treated with Rituximab (20 μg/ml) for 24 and 72 h. Cytokine detection and quantification were accomplished as described previously (29). The absorbance values of each sample were determined with a Titertek plate reader (Titertek-Multiscan MCC/340; Titertek, Flow Laboratory, Huntsville, AL) at 405 nm.

**Determination of Transcriptional Regulation by Rituximab Using RT-PCR.** RT-PCR was used to detect transcriptional regulation of IL-10 and IL-10R messenger in tumor cells treated with or without Rituximab. Because 2F7 tumor cells are EBV positive, we also tested for vIL-10, which has been shown to have effects similar to those of human IL-10. Cells (10<sup>6</sup>) were treated in 12-well plates (Costar) with Rituximab (20 μg/ml) or medium alone for 4 h at 37°C. Total RNA was extracted from 2F7 cells treated or untreated with Rituximab using the single-step guanidinium thiocyanate-chloroform method with STAT 60 reagent (Tel-Test “B,” Inc., Friendswood, TX). Between 1 and 3 μg of total RNA were reverse transcribed to first-strand cDNA for 1 h at 42°C using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The RT-PCR reaction mixture contained 20 μM random hexamer primers, 10 μM DTT, 125 μM each deoxynucleotide triphosphate, and 4 μl of 5× first-strand buffer, all purchased from Life Technologies, Inc. PCR amplification to determine transcript expression was performed on 2F7 cDNA using the hot-start technique (30, 31). Amplification conditions for c-myc and LMP-1 involved denaturation at 95°C for 60 s and annealing at 60°C for 60 s, followed by extension at 72°C for 3 min. Reactions were amplified for 25 cycles and followed by an extension at 72°C for 10 min. All amplifications were performed with a DNA Thermo Cycler 480 (PerkinElmer, Norwalk, CT) and analyzed on 1% agarose (Sigma) gels in Tris-borate EDTA [89 mM Tris base, 89 mM boric acid, and 2 mM EDTA (pH 8.0)].

**Immunoblotting Analysis of Protein Expression.** Modulation of Bcl-2 by Rituximab treatment was tested by immunoblot analysis (32). Cells (1–5 × 10<sup>6</sup>) were treated in 6- or 12-well plates (Costar) at 10<sup>6</sup> cells/ml with 20 μg/ml Rituximab, complete medium alone, or varying concentrations of anti-IL-10 (1, 10, and 50 ng/ml). Analysis of PARP cleavage was done by treating 2 × 10<sup>6</sup> cells with 20 μg/ml Rituximab, 1 μg/ml CDDP, a combination of both, or medium alone for 24 h. After 24 h of treatment, cells were transferred to 1.5-ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and pelleted for 10 s at 3,000 × g in an Eppendorf microcentrifuge 5415 (Eppendorf Scientific Inc., Westbury, NY). Pellets were lysed on ice in cold radioimmunoprecipitation assay buffer (1% NP40,

0.1% SDS, 0.5% deoxycholic acid, and 1× PBS) supplemented with protease inhibitor mixture tablets (Boehringer Mannheim). Lysates were sheered using 1-ml insulin syringes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at  $14,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. Lysate supernatants were transferred to new microcentrifuge tubes and quantified for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Supernatants were then mixed with an equal volume of sample buffer [6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue], boiled for 10 min, and stored at  $-80^{\circ}\text{C}$  until immunoblotting was performed. Dilutions (1:500) of primary antibodies to Bcl-2, Bax, Bad, Bcl-x, or p53 were used. Densitometric analysis of the immunoblots was performed on a Power Mac computer using the public domain NIH Image program (developed at the NIH and available on the Internet<sup>4</sup>).

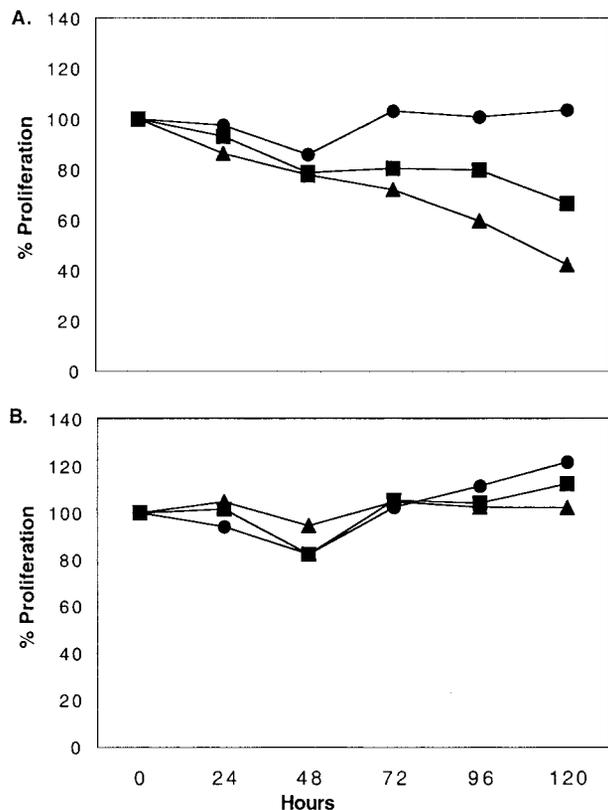
#### Gene-specific Primer Sequences for RT-PCR Analysis.

Sequence-specific primers used in this study included: (a) human IL-10 upstream (5'-CCAACAGAAGCTTCCATTCC-3') and downstream (5'-CACCGGTGCAACAATAAATATTG-3') primers; (b) IL-10R upstream (5'-CCATCTTGCTGACAAC-TCC-3') and downstream (5'-GTGTCTGATACTGTGTTGGC-3') primers; (c) vIL-10 upstream (5'-CTGAGAACCAAGAC-CCAGACATCAAGG-3') and downstream (5'-CAATAAGG-TTTTCTCAAGGGGCTGGGTC-3') primers; (d) c-myc upstream (5'-GTGGCACCTCTTGAGGACCA-3') and downstream (5'-TGGTGCTCCATAGGAGACA-3') primers; (e) LMP-1 upstream (5'-CGGAAGAGGTGAAAAACAAA-3') and downstream (5'-GTGGGGGTCGTCATCATCTC-3') primers; and (f) glyceraldehyde-3-phosphate dehydrogenase upstream (5'-GAACATCATCCCTGCCTCTACTG-3') and downstream (5'-CTTGCTGTAGCCAAATTCGTTG-3') primers.

## RESULTS

**Rituximab Inhibits Cellular Proliferation of the 2F7 B-cell Lymphoma but Does Not Induce Programmed Cell Death.** Treatment of 2F7 with Rituximab resulted in growth inhibition as determined by the percentage of cells grown in complete medium alone (Fig. 1A). Normal mouse immunoglobulin (serum) had no effect after 5 days. The fetal bovine serum was heat inactivated before usage to ensure that the Rituximab growth inhibition was complement independent. To verify that the Rituximab antibody was acting specifically through the CD20 antigen, the CD20-negative T-cell leukemia cell line CEM was used as a negative control. The Rituximab antibody had no effect on CEM growth (Fig. 1B).

Cross-linking of surface Fc receptors on B cells has been shown to induce inhibitory signals as well as apoptosis (33–35). To illustrate that the antiproliferative effect of Rituximab was not due to triggering of Fc receptors, the mouse antihuman CD20 antibody IDEC-2B8, the Fc portion of which is mouse and does not bind to human Fc receptor, was used in parallel with Rituximab. Growth inhibition produced by both antibodies was identical (data not shown), indicating that possible binding



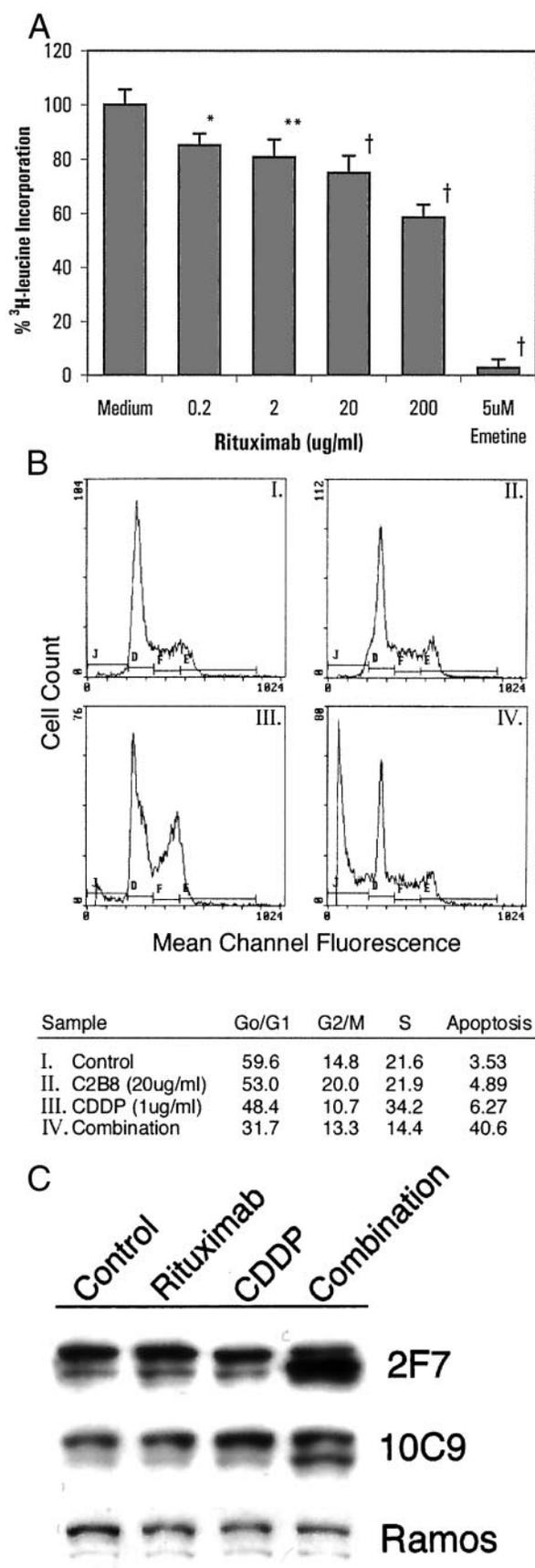
**Fig. 1** Rituximab (IDEC-C2B8) inhibits the growth of B-cell lymphoma. **A**, 2F7 cells ( $10^4$ ) were grown in 96-well plates in the presence of normal mouse serum (●) or Rituximab at 4 (■) and 20  $\mu\text{g}/\text{ml}$  (▲). Treatment with Rituximab resulted in a decrease in cell number as determined by cell viability over a 5-day period. Normal mouse serum had no effect on cell growth. Tumor cells grown in complete medium alone were established as 100% at each time interval. Cell number was determined by trypan blue exclusion. **B**, the CD20-negative T-cell leukemia cell line CEM showed no change in proliferation in the presence of Rituximab, demonstrating that the antiproliferative effect was anti-CD20 specific. Results for cell viability number by trypan exclusion assays are representative of three independent experiments.

to Fc receptors on the surface of tumor B cells by Rituximab did not contribute to inhibition.

Rituximab-mediated growth inhibition was also shown to be dependent on the concentration of the antibody, as demonstrated by [<sup>3</sup>H]leucine incorporation (Fig. 2A). After 24 h, cells treated with varying concentrations of Rituximab (0.2, 2, 20, and 200  $\mu\text{g}/\text{ml}$ ) showed a decrease in protein synthesis in a concentration-dependent manner. Emetine (5  $\mu\text{M}$ ) was used as a positive control for inhibition of [<sup>3</sup>H]leucine incorporation.

Previous studies, both in our laboratory and others, have revealed that signaling through CD20 by Rituximab can induce certain lymphoma cell lines to undergo apoptosis (36–38). 2F7 cells treated with 20  $\mu\text{g}/\text{ml}$  Rituximab for 24 h, however, did not undergo programmed cell death, as determined by PI staining (Fig. 2B) or PARP cleavage (Fig. 2C). Combination treatment with both Rituximab and CDDP elicited an apoptotic response in 2F7 cells (Fig. 2, B and C). 2F7 cells were resistant to Rituximab-induced apoptosis at concentrations up to 200  $\mu\text{g}/\text{ml}$ .

<sup>4</sup> <http://rsb.info.nih.gov/nih-image>.

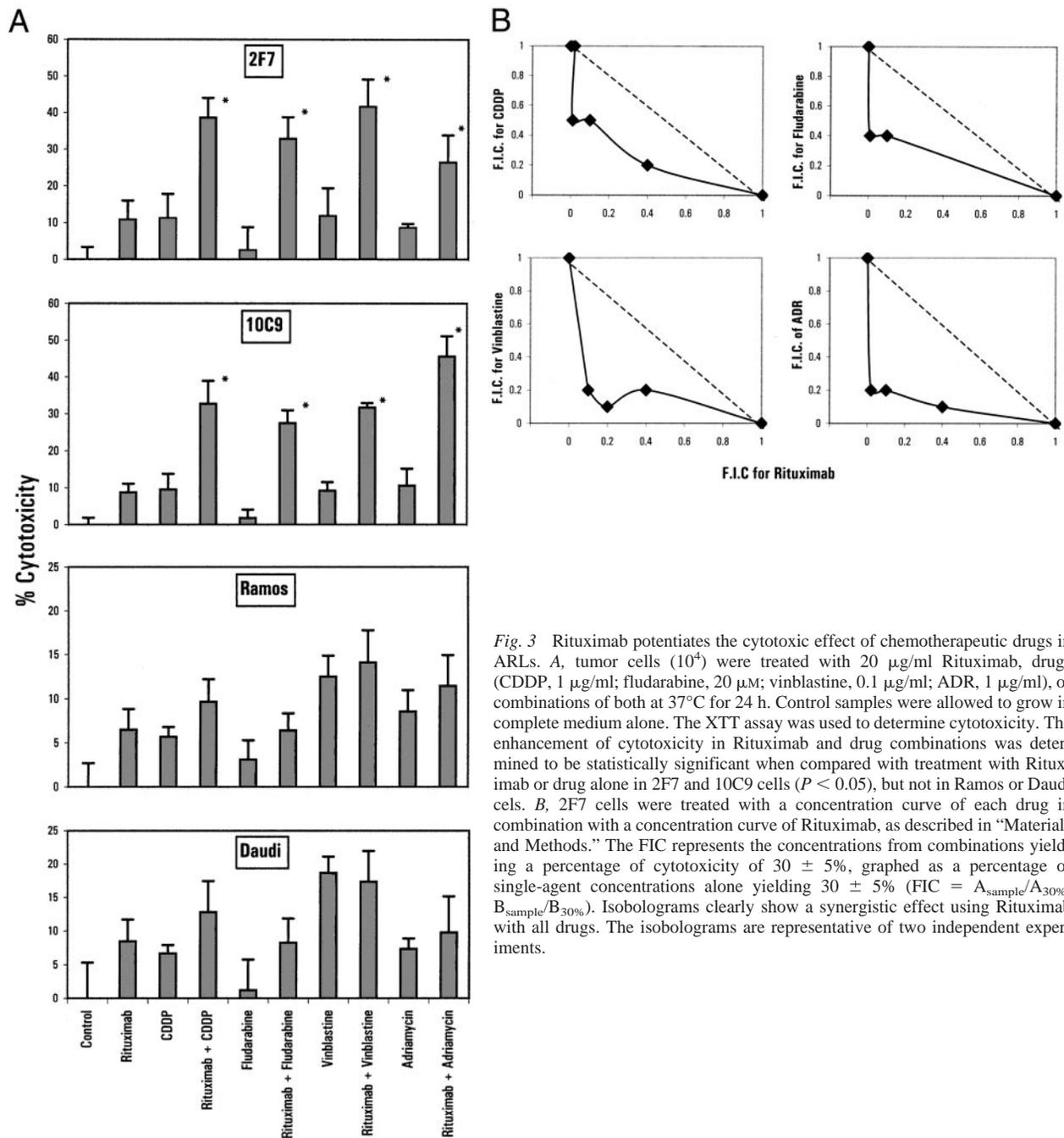


Cells were also shown not to apoptose after 72 h of treatment (data not shown). These results were corroborated by acridine orange staining, which demonstrated an equal percentage of apoptotic cells in Rituximab-treated cells as compared with untreated 2F7 cells (data not shown) and a sharp increase in apoptotic cells in samples treated with drug-Rituximab combinations. Additional experiments confirmed that Rituximab, by itself, was not apoptotic to the NHL cell lines 10C9, Ramos, and Daudi by PARP cleavage (Fig. 2C). The ARL cell lines 2F7 and 10C9 were induced to apoptose with the combination of Rituximab and CDDP, but non-ARLs Ramos and Daudi were not.

**Rituximab Sensitizes Drug-resistant NHL to Conventional Chemotherapy.** To determine the feasibility of Rituximab-drug combinations for NHL treatment, we sought to establish whether Rituximab reverses the resistant phenotype of the NHL tumor cells to chemotherapeutic drugs. Tumor cells were concomitantly treated with Rituximab (20  $\mu$ g/ml), chemotherapeutic drugs, or a combination of both for 24 h. The cytotoxicity results demonstrate that Rituximab potentiates the effects of various chemotherapeutic agents in the drug-resistant 2F7 and 10C9 cell lines but has little or no effect on the non-ARL cell lines (Fig. 3A). The extent to which Rituximab potentiates the cytotoxic effect of drugs varies with each drug. Furthermore, the combinations of Rituximab with the four chemotherapeutic drugs presented here on 2F7 work in a synergistic manner, as demonstrated by isobologram analysis (Fig. 3B).

**Regulation of IL-10 by Rituximab and Involvement of IL-10 in Drug Resistance.** IL-6, IL-10, and TNF- $\alpha$  have been previously shown to act as drug resistance factors in tumor cells (8–10). To analyze whether the drug resistance in 2F7 cells reversed by Rituximab involved these cytokines, we looked for their presence and possible regulation by the Rituximab antibody. 2F7 cells were treated with Rituximab (20  $\mu$ g/ml) for 24 and 72 h. ELISAs were performed on supernatants to determine human IL-10 levels. Treatment with Rituximab dramatically inhibited the secretion of IL-10 by 2F7 cells (Fig. 4A). After 24 h, IL-10 decreased by 58.8% in the Rituximab-treated culture, whereas a 69.3% decrease was observed after 72 h. IL-6 was not detected in the culture supernatant, and TNF- $\alpha$  showed no change (data not shown).

**Fig. 2** Rituximab-mediated growth inhibition was concentration dependent, but not due to induction of apoptosis. **A**, tumor cells were treated for 24 h with increasing concentrations of Rituximab (0.0, 0.2, 2, 20, and 200  $\mu$ g/ml). Emetine (5  $\mu$ M) was used as a positive control for protein synthesis inhibition. After treatment, cells were further grown in the presence of [<sup>3</sup>H]leucine for 6 h. [<sup>3</sup>H]Leucine incorporation was analyzed by scintillation counter and revealed a proliferative decrease in 2F7 cells treated with Rituximab. All treatments were compared to medium control and revealed statistical significance: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and †,  $P < 0.001$ . **B**, 2F7 tumor cells were treated with complete medium, Rituximab (20  $\mu$ g/ml), CDDP (1  $\mu$ g/ml), or a combination of Rituximab and CDDP for 24 h. Cells were stained with PI and analyzed by flow cytometry. Cells treated with Rituximab or CDDP alone showed almost no change in the number of apoptotic cells above that of control cells. The combination of Rituximab and CDDP, however, resulted in synergistic apoptosis. **C**, the PI results were confirmed by immunoblotting for PARP, which also showed induction of apoptosis in 2F7 cells. The 10C9 cell line also responded apoptotically to combination treatment, whereas the Ramos and Daudi lines remained resistant to cell death.

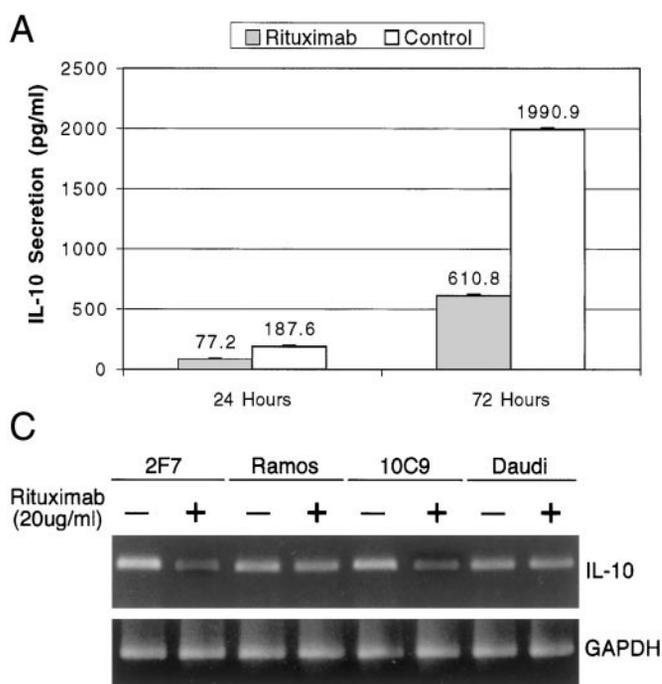


**Fig. 3** Rituximab potentiates the cytotoxic effect of chemotherapeutic drugs in ARLs. **A**, tumor cells ( $10^4$ ) were treated with 20  $\mu\text{g}/\text{ml}$  Rituximab, drugs (CDDP, 1  $\mu\text{g}/\text{ml}$ ; fludarabine, 20  $\mu\text{M}$ ; vinblastine, 0.1  $\mu\text{g}/\text{ml}$ ; ADR, 1  $\mu\text{g}/\text{ml}$ ), or combinations of both at 37°C for 24 h. Control samples were allowed to grow in complete medium alone. The XTT assay was used to determine cytotoxicity. The enhancement of cytotoxicity in Rituximab and drug combinations was determined to be statistically significant when compared with treatment with Rituximab or drug alone in 2F7 and 10C9 cells ( $P < 0.05$ ), but not in Ramos or Daudi cells. **B**, 2F7 cells were treated with a concentration curve of each drug in combination with a concentration curve of Rituximab, as described in “Materials and Methods.” The FIC represents the concentrations from combinations yielding a percentage of cytotoxicity of  $30 \pm 5\%$ , graphed as a percentage of single-agent concentrations alone yielding  $30 \pm 5\%$  ( $\text{FIC} = A_{\text{sample}}/A_{30\%} + B_{\text{sample}}/B_{30\%}$ ). Isobolograms clearly show a synergistic effect using Rituximab with all drugs. The isobolograms are representative of two independent experiments.

To verify that the decrease in IL-10 was due to regulation by Rituximab and not merely due to the decrease of cell number in culture, RNA extracts were isolated from 2F7 cells treated with Rituximab (20  $\mu\text{g}/\text{ml}$ ) or complete medium alone for 4 h. PCR amplification of IL-10 transcripts revealed a down-regulatory effect by Rituximab (Fig. 4B). Down-regulation was specific for IL-10 because IL-6 and TNF- $\alpha$  transcripts were visible by RT-PCR but remained unchanged (data not shown). Additional RT-PCR experiments found that IL-10 transcripts were also decreased in 10C9 cells but remained unchanged in Ramos and Daudi cells (Fig. 4C).

vIL-10 has been shown to mimic the activity of IL-10 (39), as well as induce its secretion in Burkitt’s lymphoma (40, 41). Cells treated with Rituximab (20  $\mu\text{g}/\text{ml}$ ) for 4 h were analyzed for vIL-10 gene expression, whereas cells treated for 24 h were tested for cytokine secretion. RT-PCR revealed no change in vIL-10 transcription (Fig. 5A). After 24 h, the vIL-10 protein was not detectable in the 2F7 cell culture, with or without treatment. (data not shown).

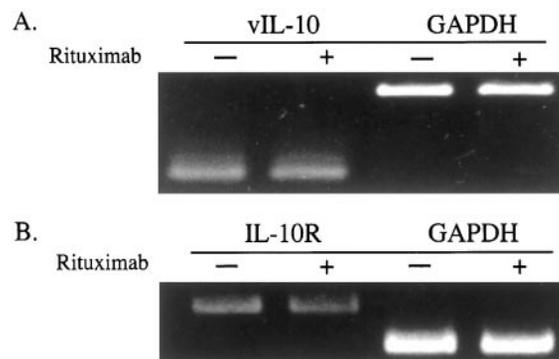
To determine whether a decrease in human IL-10 was responsible, at least in part, for the reversal of resistance, exogenous IL-10 was used in synergistic experiments with 2F7. Cells



**Fig. 4** Rituximab down-regulates IL-10 secretion and transcription by tumor B cells. **A**, 2F7 cells were treated with or without Rituximab for 24 and 72 h. Supernatants were then isolated and assayed using ELISA to quantitate IL-10 secretion. In the presence of Rituximab, IL-10 decreased by 58.8% after 24 h and 69.3% after 72 h. The difference in IL-10 secretion at both time points was statistically significant ( $P < 0.001$ ). The IL-10 ELISA data are representative of four independent experiments. **B**, 2F7 cells were incubated with or without Rituximab (20  $\mu$ g/ml) for 4 h. RNA was isolated and reverse transcribed to cDNA. PCR was performed using primer-specific sequences for human IL-10 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Transcripts for IL-10 were down-regulated in the presence of Rituximab, corroborating the regulation of IL-10 secretion in ELISA studies and demonstrating IL-10 control by Rituximab at the single-cell level. RT-PCR results are representative of four independent experiments. **C**, the RT-PCR experiment was repeated with the additional cell lines Ramos, 10C9, and Daudi. Down-regulation of IL-10 was again seen in both ARLs (2F7 and 10C9) but did not appear to change in Ramos or Daudi.

were treated with Rituximab (20  $\mu$ g/ml) and drugs, as described before, in the presence or absence of exogenous IL-10 (1 ng/ml). Cells were cultured for 24 h and analyzed using the XTT assay. As described before, Rituximab was shown to sensitize the tumor cells to the drugs. Results also revealed that the ability of Rituximab to sensitize tumor cells was abrogated in the presence of IL-10 (Fig. 6A). IL-10 proved not only to be a growth factor in these cells but also served as a protective factor because viability remained high in the presence of IL-10 treatment in all four combinations of Rituximab and drugs. IL-10 is therefore shown to be a contributor to drug resistance in this NHL cell line.

To further test the involvement of tumor-derived IL-10 in bestowing drug resistance, antibody against IL-10 was used to neutralize its activity. Alone, anti-IL-10 antibody decreased tumor cell viability, whereas isotype control had no effect. Using combinations of drugs and anti-IL-10 or isotype, XTT assays confirmed the involvement of IL-10 in drug resistance. Tumor cells were sensitized to chemotherapeutic drugs in the presence of IL-10-neutralizing antibody after 24 h (Fig. 6B). Combinations of drugs and isotype were no more cytotoxic than drugs alone. The antitumor effect of the combination of Rituximab and neutralizing IL-10 antibody was not significantly different from that of either agent alone (Fig. 6C). This would imply that down-regulation of IL-10 plays a large part in the antitumor effect of Rituximab against 2F7 *in vitro*. If it is supposed that Rituximab was acting primarily through another mechanism, neutralizing IL-10 in addition to Rituximab's additional mechanism(s) would most likely result in a greater effect. Other mechanisms cannot be entirely excluded, however, because CDDP in triple combination with Rituximab and anti-IL-10 showed a slight increase in loss of viability over CDDP in double combinations (Fig. 6C). Furthermore, a concentration



**Fig. 5** Impediment of IL-10 autocrine/paracrine loops did not involve the down-regulation of vIL-10 or IL-10R in 2F7. Unlike IL-10, treatment of cells by Rituximab (20  $\mu$ g/ml) for 4 h did not induce a transcriptional regulation in (A) vIL-10 or (B) IL-10R expression, as determined by RT-PCR. Samples were amplified for 25 and 30 cycles, respectively, and run on a 1% agarose gel.

range of anti-IL-10 antibody showed that, like Rituximab, it did not induce apoptosis (Fig. 6D).

Because the disruption of IL-10 autocrine/paracrine loops may potentiate the cytotoxicity of chemotherapeutic drugs, we sought to test whether IL-10Rs underwent down-regulation during Rituximab exposure. After a 4-h treatment with or without Rituximab (20  $\mu$ g/ml), RNA was extracted from tumor cells and used in RT-PCR of the IL-10R. Unlike the IL-10 cytokine regulation seen after 4 h of Rituximab treatment, IL-10R demonstrated no change in mRNA levels (Fig. 5B). Therefore, the impediment of IL-10 to sustain resistance to drugs was achieved at the level of cytokine secretion and not at the level of the receptor.

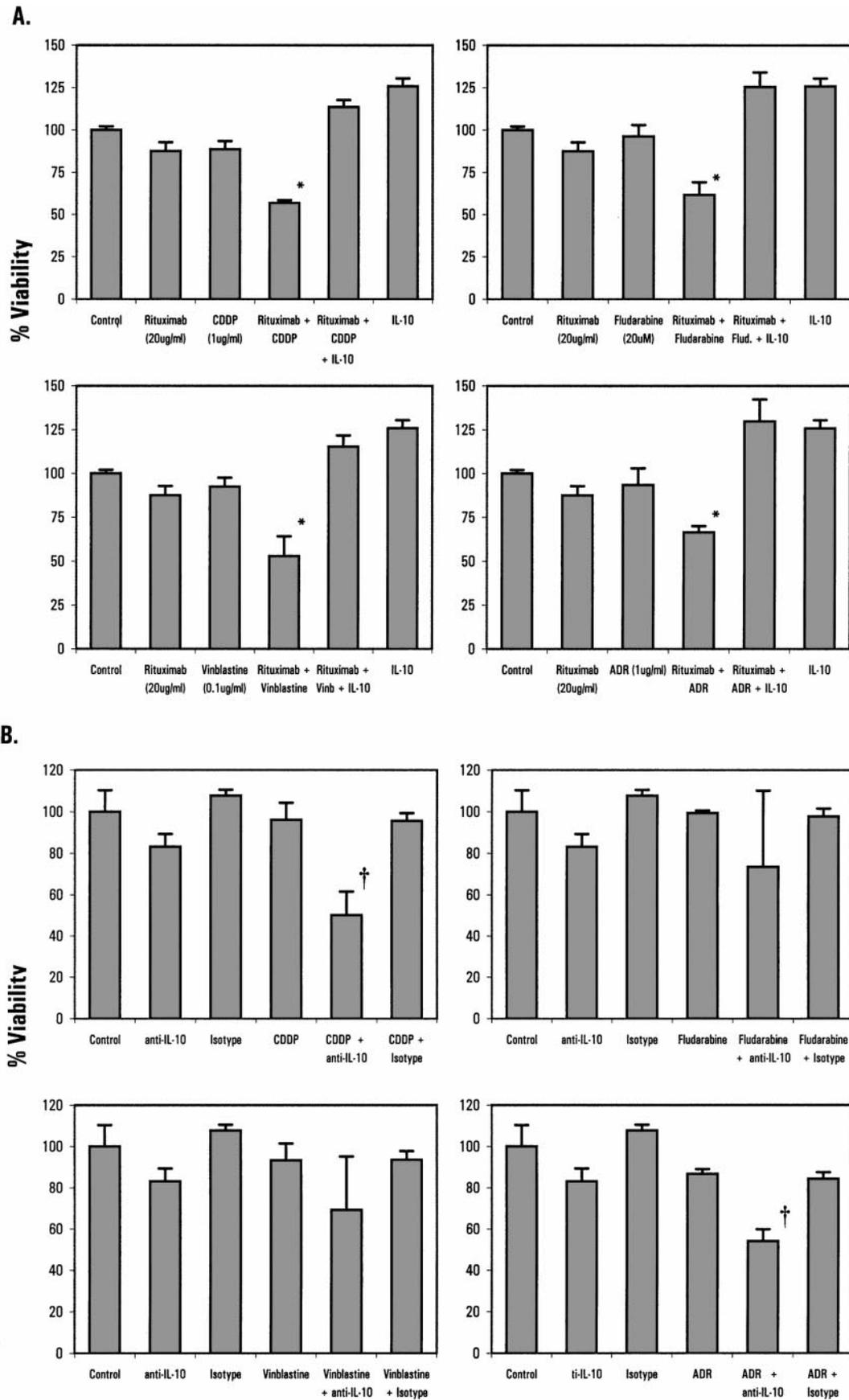


Fig. 6

**Fig. 6** IL-10 confers resistance to Rituximab-mediated drug sensitization. **A**, a total of 200  $\mu$ l of 2F7 tumor cells were seeded in 96-well plates at  $10^4$  cells/ml and subsequently treated using synergistic combinations of Rituximab (20  $\mu$ g/ml) and drugs in the presence or absence of exogenous human IL-10 (1 ng/ml). Drug concentrations remained as follows: CDDP, 1  $\mu$ g/ml; fludarabine, 20  $\mu$ M; vinblastine, 0.1  $\mu$ g/ml; and ADR, 1  $\mu$ g/ml. Cells were cultured for 24 h at 37°C, followed by analysis using the XTT assay. Drug treatment in combination with Rituximab results in synergistic antitumor activity after only 24 h, as shown before (Fig. 3);  $P < 0.05$ . XTT assays also show that in the presence of IL-10, the ability of Rituximab and drugs to synergize is abrogated. IL-10 acts as a protective factor against all four chemotherapeutic drug combinations. The differential in cytotoxicity between Rituximab and drugs in the presence or absence of IL-10 was highly significant (\*,  $P < 0.001$ ). **B**, cytotoxicity assays were done to examine whether the effects of chemotherapeutic drugs could be potentiated in the presence of IL-10-neutralizing antibody or its isotype control (PharMingen). 2F7 cells were treated with combinations of drugs and antibodies for 24 h at 37°C, as described above. Both antibodies were used at concentrations of 5 ng/ml. Blocking the activity of endogenous IL-10 rendered the tumor cells more susceptible to chemotherapeutic drugs, although only CDDP and ADR were deemed statistically significant ( $\dagger$ ,  $P < 0.05$ ). Results for exogenous IL-10 XTT assays are representative of five independent experiments, whereas results for neutralizing IL-10 XTT assays are representative of three independent experiments. **C**, 2F7 cells were treated in the presence of Rituximab, IL-10-neutralizing antibody, and CDDP. The combination of Rituximab and IL-10-neutralizing antibody did not result in a significant decrease in cell viability when compared to both agents alone. Both agents were able to sensitize 2F7 cells to CDDP (\*,  $P < 0.05$ ). **D**, 2F7 tumor cells were treated with complete medium or a concentration range of IL-10-neutralizing antibody for 24 h. Cells were stained with PI and analyzed by flow cytometry. Cells treated with anti-IL-10 antibody showed little change in the number of apoptotic cells above that of control cells.

Confirmatory experiments on IL-10 gene regulation demonstrate that, to a lesser extent, 10C9 is induced to express less IL-10 by Rituximab treatment (Fig. 4C). The Ramos and Daudi cell lines show no change in IL-10 gene expression. The differential regulatory effects of IL-10 in 2F7 and 10C9 *versus* Ramos and Daudi are not clear.

**Regulation of Bcl-2 by Rituximab and Involvement of Bcl-2 in Drug Resistance.** The Bcl-2 family of pro- and antiapoptotic proteins remains a key component in the ability of B-cells to respond to apoptotic stimuli. Because IL-10 has been shown to maintain high Bcl-2 levels in hematopoietic cells (42–44), and, as shown above, IL-10 down-regulation was found to occur with Rituximab treatment, 2F7 cells were examined for Rituximab-mediated regulation of Bcl-2. The 2F7 cells were incubated with Rituximab (20  $\mu$ g/ml) or complete medium alone for 24 h. Lysates run on SDS-PAGE and immunoblotted for Bcl-2 demonstrated a decrease in Bcl-2 protein expression in a concentration-dependent manner (Fig. 7A). Similar blots were performed for Bcl-x, Bax, Bad, and p53. No changes were observed in these proteins (Fig. 7B). Thus, regulation of Bcl-2 may be a viable mechanism whereby tumor cell sensitivity to drugs is modulated. Helping to confirm a regulatory mechanism for Rituximab in AIDS-related NHL, 10C9 cells also displayed a decrease in Bcl-2. Ramos and Daudi levels remained unchanged (Fig. 7C).

The mechanisms involved in controlling Bcl-2 expression, however, are not fully understood. To delineate whether down-regulation of Bcl-2 was due to depleted levels of IL-10 or

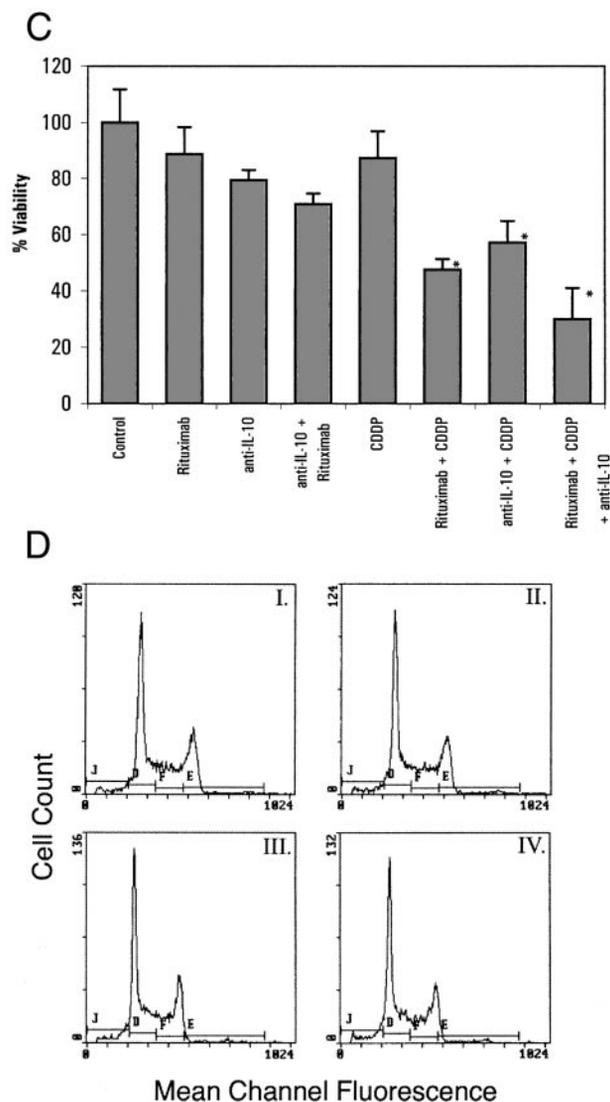
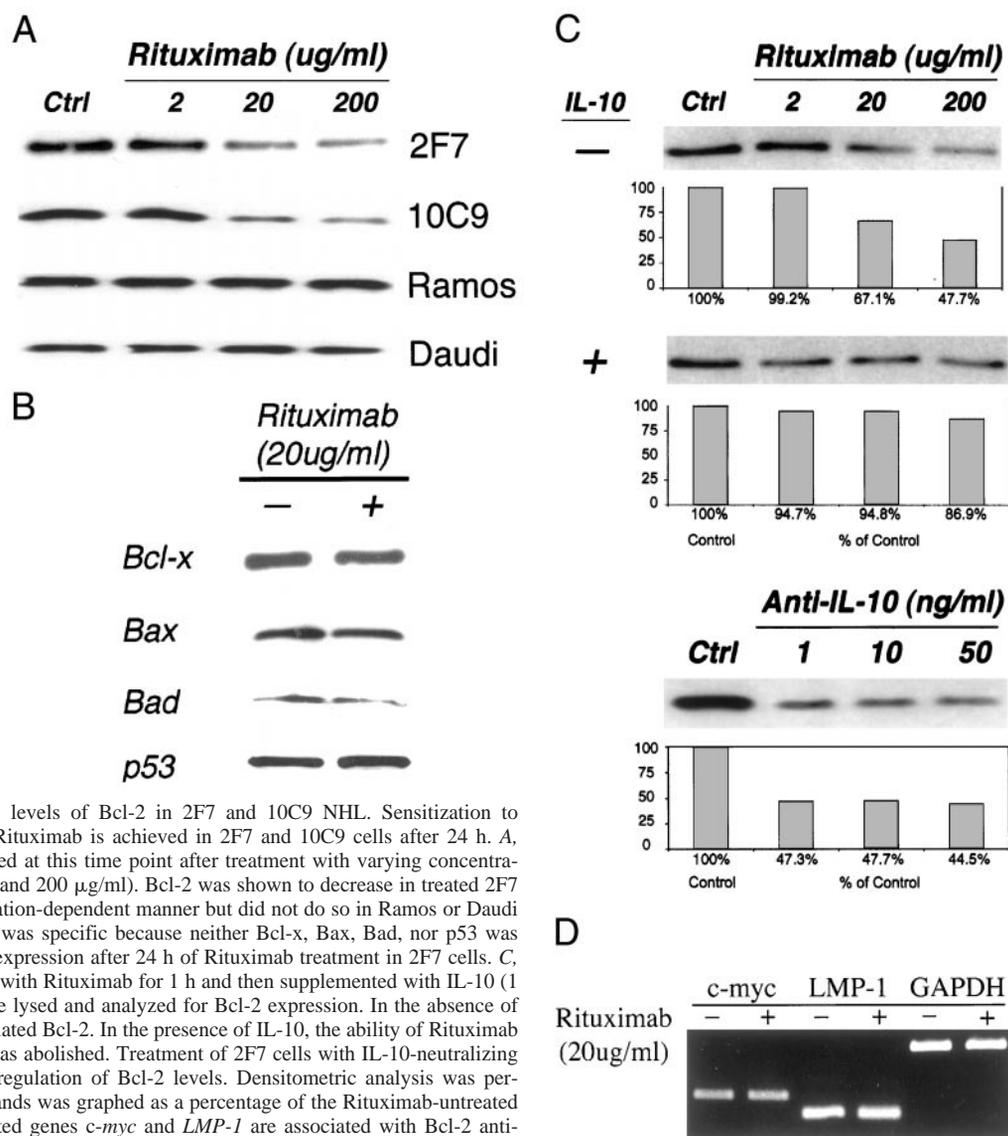


Fig. 6 Continued.

induced directly by CD20 signaling, exogenous IL-10 (1 ng/ml) was added to 2F7 cultures in the presence of Rituximab, and Bcl-2 expression was analyzed. The addition of IL-10 one h after Rituximab treatment recovered levels of Bcl-2 in the tumor cells when analyzed after 24 h of treatment (Fig. 7D). The addition of IL-10 was performed 1 h after treatment with Rituximab to allow uninhibited CD20 signaling to take place before IL-10 signaling began. Although the expression of Bcl-2 was recovered, the addition of exogenous IL-10 did not induce higher levels of Bcl-2 than in the control population. This may be due to the saturation of IL-10Rs with IL-10 after 24 h in cells



**Fig. 7** Rituximab decreases levels of Bcl-2 in 2F7 and 10C9 NHL. Sensitization to chemotherapeutic drugs by Rituximab is achieved in 2F7 and 10C9 cells after 24 h. **A**, Bcl-2 expression was analyzed at this time point after treatment with varying concentrations of Rituximab (0, 2, 20, and 200 µg/ml). Bcl-2 was shown to decrease in treated 2F7 and 10C9 cells in a concentration-dependent manner but did not do so in Ramos or Daudi cells. **B**, regulation of Bcl-2 was specific because neither Bcl-x, Bax, Bad, nor p53 was seen to have altered protein expression after 24 h of Rituximab treatment in 2F7 cells. **C**, 2F7 tumor cells were treated with Rituximab for 1 h and then supplemented with IL-10 (1 ng/ml). After 24 h, cells were lysed and analyzed for Bcl-2 expression. In the absence of IL-10, Rituximab down-regulated Bcl-2. In the presence of IL-10, the ability of Rituximab to impair Bcl-2 expression was abolished. Treatment of 2F7 cells with IL-10-neutralizing antibody also caused down-regulation of Bcl-2 levels. Densitometric analysis was performed, and the density of bands was graphed as a percentage of the Rituximab-untreated controls. **D**, the EBV-regulated genes *c-myc* and *LMP-1* are associated with Bcl-2 anti-apoptotic activity and expression, respectively. Neither gene was shown to be modulated after 4 h of Rituximab (20 µg/ml) treatment by RT-PCR.

exogenously treated with IL-10 and in untreated cells that constitutively secrete IL-10. Furthermore, treating cells with anti-IL-10 antibody decreased Bcl-2 protein levels after 24 h (Fig. 7D). These findings demonstrate that IL-10 is a regulator of Bcl-2 expression in lymphocytes and that Bcl-2 levels are largely dependent on IL-10 autocrine/paracrine loops in these tumor B cells.

EBV controls the levels of *c-myc* expression, which is known to work cooperatively with Bcl-2 to protect cells from apoptotic stimuli (45, 46). EBV encodes LMP-1, which is known to up-regulate IL-10 and Bcl-2 expression. We examined whether these Bcl-2-associated genes controlled by EBV were also subject to Rituximab regulation. After 4 h of treatment with Rituximab (20 µg/ml) or complete medium alone, RT-PCR was used to detect gene expression in both *c-myc* and LMP-1. No obvious difference in expression occurred with Rituximab treatment (Fig. 7E).

These findings demonstrate that the sensitization of 2F7 by Rituximab to cytotoxic drugs is the result of down-regulation of IL-10 and, consequently, the down-regulation of Bcl-2.

## DISCUSSION

The intent of this study was to delineate the mechanisms involved in the drug sensitization of NHL tumor cells by the chimeric mouse antihuman CD20 antibody Rituximab. We hypothesized that protective or trophic factors in NHL tumor cells conferred a drug-resistant phenotype and that signaling through the CD20 antigen via Rituximab modified expression of these protective factors, inducing their drug susceptibility.

We used the B-cell lymphoma 2F7 as a model to study the mechanisms of Rituximab-mediated sensitization, with corroborative work performed on 10C9, Ramos, and Daudi cell lines. The evidence we present demonstrates that Rituximab can in-

hibit ARL cell growth and sensitizes the tumor cells to cytotoxicity by various clinically relevant chemotherapeutic drugs, at levels shown to be clinically practiced by *in vivo* studies (24–26), by one mechanism involving the selective disruption of IL-10 autocrine/paracrine loops. This mechanism of down-regulation was not seen in the non-ARL tumor cells, and, correspondingly, no sensitization was observed in these cell lines to any of the drugs studied by Rituximab treatment. Disruption of IL-10 loops by Rituximab is achieved through the down-regulation of tumor-derived IL-10 transcription and subsequent down-regulation of the expression of the antiapoptotic Bcl-2 gene product. The combination treatment of Rituximab and drugs results in synergistic cytotoxicity and apoptosis.

The events responsible for changes in B-cell behavior and phenotype on CD20 signaling remain enigmatic. Different anti-CD20 antibodies have been shown to induce distinct proliferative responses in B cells. Depending on the epitope specificity of the antibody, anti-CD20 can signal for G<sub>0</sub>-arrested B cells to enter the cell cycle at the G<sub>1</sub> phase (47) or to inhibit the proliferation of stimulated B cells (48, 49). Rituximab, specifically, is shown here and elsewhere to inhibit tumor cell progression. Unlike other reports that show cell death induced by Rituximab treatment alone (36–38), we did not detect Rituximab-mediated apoptosis in 2F7, 10C9, Ramos, or Daudi. The resistance of these cells to Rituximab-induced apoptosis is not known. The apoptotic cascade induced by Rituximab in other studies has yet to be defined. Although CD20 signal transduction remains largely uncharacterized, three principal aspects have been reported. On stimulation with anti-CD20, the CD20 antigen (*a*) recruits src family members (specifically, Lyn, Fyn, and Lck) and an undetermined adaptor molecule to its cytoplasmic regions (50, 51), (*b*) migrates to lipid raft microdomains (52, 53), and (*c*) induces transmembrane Ca<sup>2+</sup> conductance (54–56). The relevance of these signaling events to the proliferative and apoptotic variation seen in B lymphocytes by anti-CD20 antibody treatment *in vitro* remains unclear at present.

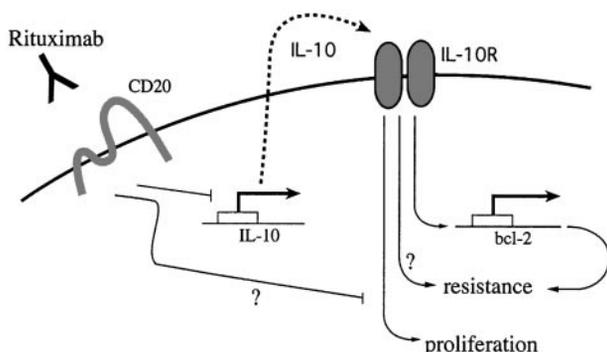
Several studies have revealed that IL-10 acts as a protective factor, enhances growth progression, and aids in the pathogenesis of NHL through autocrine/paracrine loops (9, 57, 58). NHL tumor cells taken from patients maintain higher viability and increase their proliferation when cultured in the presence of IL-10 or IL-6 (57). It has also been shown that IL-10 production by constitutively activated CD4<sup>+</sup> T cells in mice drives the proliferation of chronically activated B cells (59–61). More importantly, it is known that serum levels of IL-10 are elevated in many NHL patients and correlate to a poor rate of survival (62, 63). Thus, our laboratory examined whether IL-10 was involved in NHL drug resistance and demonstrated that IL-10 serves as a protective factor in AIDS-related NHLs against such cytotoxic drugs as CDDP, etoposide, ADR, and diphtheria toxin (9). We expand our findings here to show that IL-10 not only protects 2F7 cells from drug cytotoxicity but also abrogates the ability of Rituximab to sensitize tumor cells to drugs. Moreover, we illustrate that the sensitization of 2F7 tumor B cells by Rituximab is mediated through the down-regulation of IL-10 secretion. The ability of Rituximab to sensitize NHL cells to chemotherapeutic drugs with various cytotoxic mechanisms by down-regulating IL-10 correlates directly with the ability of IL-10 to protect cells against a range of apoptotic stimuli.

Additionally, our experiments using antibody to neutralize IL-10 and sensitize cells to drugs revealed that IL-10 is a key component in the resistance of NHLs to drugs (Fig. 6C). When anti-IL-10 and Rituximab are used in combination, the decrease in viability is not significant. We infer this to be due to the overlapping effects of the antibodies. Additional effects by Rituximab in tumor cells must exist, however, because a triple combination of CDDP, Rituximab, and anti-IL-10 is slightly more cytotoxic than double combinations of CDDP-Rituximab or CDDP-anti-IL-10.

The regulation of human IL-10 gene expression is not well understood. Three transcriptional elements have been identified as IL-10 inducers in human cells. Recent work has implicated transcription factor complexes consisting of cAMP-responsive element binding protein 1 and activating transcription factor 1 in the cAMP-dependent activation of IL-10 in monocytes (64). IL-10 transcription was driven by the binding of cAMP-responsive element binding protein 1/activating transcription factor 1 to the cAMP-responsive elements located in the enhancer of the IL-10 promoter. Activation of the transcription factor nuclear factor  $\kappa$ B is associated with higher rates of survival in tumor cells and has also been shown to activate IL-10 expression in the Hut78 T-cell lymphoma cell line (65). Factors responsible for down-regulation of IL-10 have not been reported. Characterization of the IL-10 promoter revealed that DNA segments upstream of the gene possess both positive and negative regulatory elements (66). Putative IL-10 repressors may serve as a means by which IL-12 arrests IL-10 expression in Th1/Th2 paracrine loops. Likewise, they could also play a role in the down-regulation of IL-10 by Rituximab-mediated CD20 signaling.

The means by which IL-10 induces its protective effects are currently not known, although it appears to involve a common pathway diverse drugs use to induce cell death. However, IL-10 is a known promoter of Bcl-2 expression in hematopoietic cells (42–44). Bcl-2 plays an important role in a tumor cell's ability to survive cytotoxic stimuli. It is specifically known to confer resistance to various apoptotic stimuli including UV radiation, serum starvation, and drug treatment (16, 67, 68). Due to the ability of Rituximab to regulate endogenous IL-10 levels, we analyzed Bcl-2 expression on Rituximab treatment. We demonstrate that expression of Bcl-2 in these NHL tumor cells is dependent on IL-10 levels and that both are down-regulated by the Rituximab antibody. Bcl-2 down-regulation could serve as a possible mechanism for the sensitization of B lymphoma cells to a variety of chemotherapeutic drugs.

A number of Bcl-2 mechanisms to suppress apoptosis have been reported. Among them are the ability to act as an antioxidant (69, 70), block caspase activity (71, 72), and regulate Ca<sup>2+</sup> flux (73, 74). Taken together, these commonalities between Bcl-2 function, chemotherapeutic drugs, and CD20 signaling allude to possible mechanisms involved in the reversal of drug resistance. For instance, although Bcl-2 does not reduce the amount of DNA damage incurred by a cell when treated with cytotoxic drugs, it prevents them from undergoing apoptosis by such mechanism. Through the Rituximab-mediated depletion of Bcl-2, the block of apoptosis by DNA-damaging drugs, such as CDDP and fludarabine, may be circumvented. Likewise, prevention of reactive oxygen species accumulation by Bcl-2 can potentially suppress the effects of ADR, which includes the



**Fig. 8** Schematic diagram representing the mechanism of Rituximab-mediated drug sensitization. We illustrate a mechanism whereby signaling through the CD20 pathway after Rituximab treatment down-regulates the transcription of IL-10 and most likely factors involved in B-cell proliferation. The disruption of this IL-10 autocrine/paracrine loop leads to lower levels of expression of IL-10-responsive genes. Reduced levels of IL-10 in the tumor microenvironment result in diminished Bcl-2 expression in 2F7 lymphoma tumor cells. A decrease in Bcl-2 is thought to lower the threshold of drug tolerance and reverse the drug-resistant phenotype in these cells.

generation of free radicals. Bcl-2 has been shown to prevent  $\text{Ca}^{2+}$  fluxes within the mitochondria and endoplasmic reticulum (73). This may inhibit any  $\text{Ca}^{2+}$ -dependent apoptotic pathway induced by CD20 signaling.

Our proposed model of sensitization in the 2F7 tumor cells involves a sequential order of events necessary to turn off protective factors (Fig. 8). Signaling through the CD20 antigen via Rituximab down-regulates IL-10 transcription and therefore secretion. IL-10 is responsible for the overexpression of Bcl-2 protein in these NHL cells. Thus, depletion of IL-10 by Rituximab in the tumor microenvironment leads to the down-regulation of Bcl-2 protein expression and subsequent sensitivity to conventional chemotherapeutic drugs. Changes in cellular phenotype involve a complex network of factors and invariably depend on modulation of a multitude of determinants. Therefore, the IL-10/Bcl-2 loop does not exclude the possibility that other elements involved in drug resistance are also modulated, through either cytokines or CD20 signal transduction.

What also remains unclear is why the ARL cell lines 2F7 and 10C9 responded to Rituximab by down-regulating IL-10 and Bcl-2, whereas the non-ARLs, Ramos and Daudi, showed no change. None of the cell lines have a Bcl-2 translocation, all are of Burkitt's type, and only 2F7 is EBV positive. The primary difference that distinguishes the ARL lines from the non-ARL lines is that the non-ARLs have translocated *c-myc* genes (75, 76), whereas the ARL lines do not (17). However, treatment in the 2F7 cell line with Rituximab showed no change in *c-myc* expression (Fig. 7D). The presence of *c-myc*, therefore, does not appear to be involved in the regulation of drug resistance or the regulation of IL-10 and Bcl-2.

In previously reported studies (36, 77) and unpublished work,<sup>5</sup> we showed that Rituximab can sensitize the B-cell NHL

<sup>5</sup> A. Jazirehi, C. Emmanouilides, S. Alas, and B. Bonavida, unpublished observations.

lines DHL-4, 2F7, and Ramos, as well as fresh patient tumor samples, to the lethal effects of a variety of therapeutic drugs. The Rituximab-mediated drug sensitization model can be applied to patients and has clinical relevance. Information on the correlation between IL-10 secretion in patients' NHL tumor cells and relapse is poorly understood (78–80). IL-10 levels reported in patients are generally assessed using serum levels. Drug resistance mediated through IL-10 in the tumor microenvironment of these semisolid tumors may not result in enough oversecretion of IL-10 to elevate serum levels. Of more relevant interest would be the characterization of IL-10 mRNA levels and IL-10R expression in refractory NHL. The observation made here with IL-10, however, does not preclude the fact that other mechanisms are involved in both resistance and sensitization. For instance, when cells were treated with the combination of both Rituximab and anti-IL-10 antibodies in the presence of CDDP, the cytotoxic effect was higher than that seen in cells treated with anti-IL-10 antibody alone in the presence of CDDP (Fig. 6C). This suggests that the sensitization effect of Rituximab includes mechanisms independent of IL-10 involvement. Recent studies using Bcl-2 antisense therapy demonstrate that the decrease in Bcl-2 renders tumor cells more sensitive to the human immune system and chemotherapeutic drugs (81–83). The decrease, although not necessarily the disappearance, of Bcl-2 appears to be an important factor in treating B-cell NHL (84).

The results described in this study delineate a novel mechanism involved in the reversal of drug resistance by the anti-CD20 antibody Rituximab. The observations indicate that IL-10 autocrine/paracrine loops play an important role in NHL drug resistance to therapeutic drugs. The new findings lend reason for other antitumor modalities combining Rituximab with chemotherapeutic drugs, cytokine therapy, or antisense therapy.

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## REFERENCES

1. Stashenko, P., Nadler, L. M., Hardy, R., and Schlossman, S. F. Characterization of a human B lymphocyte-specific antigen. *J. Immunol.*, 125: 1678–1685, 1980.
2. Reff, M. E., Carner, K., Chambers, K. S., Chinn, P. C., Leonard, J. E., Raab, R., Newman, R. A., Hanna, N., and Anderson, D. R. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood*, 83: 435–445, 1994.
3. Maloney, D. G., Liles, T. M., Czerwinski, D. K., Waldichuk, C., Rosenberg, J., Grillo-Lopez, A., and Levy, R. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. *Blood*, 84: 2457–2466, 1994.
4. Maloney, D. G., Grillo-Lopez, A. J., Bodkin, D. J., White, C. A., Liles, T. M., Royston, I., Varns, C., Rosenberg, J., and Levy, R. IDEC-C2B8: results of a Phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. *J. Clin. Oncol.*, 15: 3266–3274, 1997.
5. Maloney, D. G., Grillo-Lopez, A. J., White, C. A., Bodkin, D., Schilder, R. J., Neidhart, J. A., Janakiraman, N., Foon, K. A., Liles, T. M., Dallaire, B. K., Wey, K., Royston, I., Davis, T., and Levy, R.

- IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood*, 90: 2188–2195, 1997.
6. McLaughlin, P., White, C. A., Grillo-Lopez, A. J., and Maloney, D. G. Clinical status and optimal use of Rituximab for B-cell lymphomas. *Oncology (Huntingt.)*, 12: 1763–1769, discussion 1769–1770, 1775–1767, 1998.
  7. Coiffier, B., Haioun, C., Ketterer, N., Engert, A., Tilly, H., Ma, D., Johnson, P., Lister, A., Feuring-Buske, M., Radford, J. A., Capdeville, R., Diehl, V., and Reyes, F. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter Phase II study. *Blood*, 92: 1927–1932, 1998.
  8. Borsellino, N., Belldegrun, A., and Bonavida, B. Endogenous interleukin 6 is a resistance factor for *cis*-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res.*, 55: 4633–4639, 1995.
  9. Demidem, A., Dantam, L., Gan, X. H., Levin, A., Salahuddin, Z., and Bonavida, B. Endogenous IL-10 is a protective factor in drug-resistant AIDS-related lymphoma (ARL). *Proc. Am. Assoc. Cancer Res.*, 36: 323, 1995.
  10. Mizutani, Y., Bonavida, B., Nio, Y., and Yoshida, O. Overcoming TNF- $\alpha$  and drug resistance of human renal cell carcinoma cells by treatment with pentoxifylline in combination with TNF- $\alpha$  or drugs: the role of TNF- $\alpha$  mRNA downregulation in tumor cell sensitization. *J. Urol.*, 151: 1697–1702, 1994.
  11. Lotem, J., and Sachs, L. Regulation of bcl-2, bcl-X<sub>L</sub>, and bax in the control of apoptosis by hematopoietic cytokines and dexamethasone. *Cell Growth Differ.*, 6: 647–653, 1995.
  12. Li, L., Krajewski, S., Reed, J. C., and Choi, Y. S. The apoptosis and proliferation of SAC-activated B cells by IL-10 are associated with changes in Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 expression. *Cell. Immunol.*, 178: 33–41, 1997.
  13. Puthier, D., Derenne, S., Barille, S., Moreau, P., Harousseau, J. L., Bataille, R., and Amiot, M. Mcl-1 and Bcl-x<sub>L</sub> are co-regulated by IL-6 in human myeloma cells. *Br. J. Haematol.*, 107: 392–395, 1999.
  14. Schwarze, M. M., and Hawley, R. G. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-x<sub>L</sub>. *Cancer Res.*, 55: 2262–2265, 1995.
  15. Kumakura, S., Ishikura, H., Tsumura, H., Iwata, Y., Endo, J., and Kobayashi, S. C-Myc and Bcl-2 protein expression during the induction of apoptosis and differentiation in TNF- $\alpha$ -treated HL-60 cells. *Leuk. Lymphoma*, 23: 383–394, 1996.
  16. Reed, J. C. Bcl-2 family proteins: regulators of chemoresistance in cancer. *Toxicol. Lett.*, 82–83: 155–158, 1995.
  17. Ng, V. L., Hurt, M. H., Fein, C. L., Khayam-Bashi, F., Marsh, J., Nunes, W. M., McPhaul, L. W., Feigal, E., Nelson, P., Herndier, B. G., *et al.* IgMs produced by two acquired immune deficiency syndrome lymphoma cell lines: Ig binding specificity and VH-gene putative somatic mutation analysis [published erratum appears in *Blood*, 84: 995, 1994]. *Blood*, 83: 1067–1078, 1994.
  18. Klein, G., Giovannella, B., Westman, A., Stehlin, J. S., and Mumford, D. An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by *in vitro* infection. *Intervirology*, 5: 319–334, 1975.
  19. Klein, E., Klein, G., Nadkarni, J. S., Nadkarni, J. J., Wigzell, H., and Clifford, P. Surface IgM- $\kappa$  specificity on a Burkitt lymphoma cell *in vivo* and in derived culture lines. *Cancer Res.*, 28: 1300–1310, 1968.
  20. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, 139: 271–279, 1991.
  21. Jost, L. M., Kirkwood, J. M., and Whiteside, T. L. Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. *J. Immunol. Methods*, 147: 153–165, 1992.
  22. Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.*, 48: 4827–4833, 1988.
  23. Berinstein, N. L., Grillo-Lopez, A. J., White, C. A., Bence-Bruckler, I., Maloney, D., Czuczman, M., Green, D., Rosenberg, J., McLaughlin, P., and Shen, D. Association of serum Rituximab (IDEC-C2B8) concentration and anti-tumor response in the treatment of recurrent low-grade or follicular non-Hodgkin's lymphoma. *Ann. Oncol.*, 9: 995–1001, 1998.
  24. Andersson, A., Fagerberg, J., Lewensohn, R., and Ehrsson, H. Pharmacokinetics of cisplatin and its monohydrated complex in humans. *J. Pharm. Sci.*, 85: 824–827, 1996.
  25. Gandhi, V., Kemena, A., Keating, M. J., and Plunkett, W. Cellular pharmacology of fludarabine triphosphate in chronic lymphocytic leukemia cells during fludarabine therapy. *Leuk. Lymphoma*, 10: 49–56, 1993.
  26. Rahmani, R., and Zhou, X. J. Pharmacokinetics and metabolism of vinca alkaloids. *Cancer Surv.*, 17: 269–281, 1993.
  27. Stewart, D. J., Grewaal, D., Green, R. M., Mikhael, N., Goel, R., Montpetit, V. A., and Redmond, M. D. Concentrations of doxorubicin and its metabolites in human autopsy heart and other tissues. *Anticancer Res.*, 13: 1945–1952, 1993.
  28. Berenbaum, M. C. Criteria for analyzing interactions between biologically active agents. *Adv. Cancer Res.*, 35: 269–335, 1981.
  29. Gan, X. H., and Bonavida, B. Preferential induction of TNF- $\alpha$  and IL-1 $\beta$  and inhibition of IL-10 secretion by human peripheral blood monocytes by synthetic aza-alkyl lysophospholipids. *Cell. Immunol.*, 193: 125–133, 1999.
  30. Birch, D. E. Simplified hot start PCR. *Nature (Lond.)*, 381: 445–446, 1996.
  31. Chou, Q., Russell, M., Birch, D. E., Raymond, J., and Bloch, W. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.*, 20: 1717–1723, 1992.
  32. Itakura, M., Mori, S., Park, N. H., and Bonavida, B. Both HPV and carcinogen contribute to the development of resistance to apoptosis during oral carcinogenesis. *Int. J. Oncol.*, 16: 591–597, 2000.
  33. Pearse, R. N., Kawabe, T., Bolland, S., Guinamard, R., Kurosaki, T., and Ravetch, J. V. SHIP recruitment attenuates Fc  $\gamma$  RIIB-induced B cell apoptosis. *Immunity*, 10: 753–760, 1999.
  34. Ono, M., Okada, H., Bolland, S., Yanagi, S., Kurosaki, T., and Ravetch, J. V. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell*, 90: 293–301, 1997.
  35. Coggeshall, K. M. Inhibitory signaling by B cell Fc  $\gamma$  RIIB. *Curr. Opin. Immunol.*, 10: 306–312, 1998.
  36. Demidem, A., Lam, T., Alas, S., Hariharan, K., Hanna, N., and Bonavida, B. Chimeric anti-CD20 (IDEC-C2B8) monoclonal antibody sensitizes a B cell lymphoma cell line to cell killing by cytotoxic drugs. *Cancer Biother. Radiopharm.*, 12: 177–186, 1997.
  37. Shan, D., Ledbetter, J. A., and Press, O. W. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood*, 91: 1644–1652, 1998.
  38. Taji, H., Kagami, Y., Okada, Y., Andou, M., Nishi, Y., Saito, H., Seto, M., and Morishima, Y. Growth inhibition of CD20-positive B lymphoma cell lines by IDEC-C2B8 anti-CD20 monoclonal antibody. *Jpn. J. Cancer Res.*, 89: 748–756, 1998.
  39. Hsu, D. H., de Waal Malefyt, R., Fiorentino, D. F., Dang, M. N., Vieira, P., de Vries, J., Spits, H., Mosmann, T. R., and Moore, K. W. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *Science (Washington DC)*, 250: 830–832, 1990.
  40. Burdin, N., Peronne, C., Banchereau, J., and Rousset, F. Epstein-Barr virus transformation induces B lymphocytes to produce human interleukin 10. *J. Exp. Med.*, 177: 295–304, 1993.
  41. Nakagomi, H., Dolcetti, R., Bejarano, M. T., Pisa, P., Kiessling, R., and Masucci, M. G. The Epstein-Barr virus latent membrane protein-1

- (LMP1) induces interleukin-10 production in Burkitt lymphoma lines. *Int. J. Cancer*, 57: 240–244, 1994.
42. Levy, Y., and Brouet, J. C. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J. Clin. Investig.*, 93: 424–428, 1994.
  43. Cohen, S. B., Crawley, J. B., Kahan, M. C., Feldmann, M., and Foxwell, B. M. Interleukin-10 rescues T cells from apoptotic cell death: association with an upregulation of Bcl-2. *Immunology*, 92: 1–5, 1997.
  44. Weber-Nordt, R. M., Henschler, R., Schott, E., Wehinger, J., Behringer, D., Mertelsmann, R., and Finke, J. Interleukin-10 increases Bcl-2 expression and survival in primary human CD34+ hematopoietic progenitor cells. *Blood*, 88: 2549–2558, 1996.
  45. Lombardi, L., Newcomb, E. W., and Dalla-Favera, R. Pathogenesis of Burkitt lymphoma: expression of an activated c-myc oncogene causes the tumorigenic conversion of EBV-infected human B lymphoblasts. *Cell*, 49: 161–170, 1987.
  46. Marin, M. C., Hsu, B., Stephens, L. C., Brisbay, S., and McDonnell, T. J. The functional basis of c-myc and bcl-2 complementation during multistep lymphomagenesis *in vivo*. *Exp. Cell Res.*, 217: 240–247, 1995.
  47. Golay, J. T., Clark, E. A., and Beverley, P. C. The CD20 (Bp35) antigen is involved in activation of B cells from the G<sub>0</sub> to the G<sub>1</sub> phase of the cell cycle. *J. Immunol.*, 135: 3795–3801, 1985.
  48. Tedder, T. F., Forsgren, A., Boyd, A. W., Nadler, L. M., and Schlossman, S. F. Antibodies reactive with the B1 molecule inhibit cell cycle progression but not activation of human B lymphocytes. *Eur. J. Immunol.*, 16: 881–887, 1986.
  49. Tedder, T. F., Boyd, A. W., Freedman, A. S., Nadler, L. M., and Schlossman, S. F. The B cell surface molecule B1 is functionally linked with B cell activation and differentiation. *J. Immunol.*, 135: 973–979, 1985.
  50. Deans, J. P., Kalt, L., Ledbetter, J. A., Schieven, G. L., Bolen, J. B., and Johnson, P. Association of 75/80-kDa phosphoproteins and the tyrosine kinases Lyn, Fyn, and Lck with the B cell molecule CD20. Evidence against involvement of the cytoplasmic regions of CD20. *J. Biol. Chem.*, 270: 22632–22638, 1995.
  51. Popoff, I. J., Savage, J. A., Blake, J., Johnson, P., and Deans, J. P. The association between CD20 and Src-family tyrosine kinases requires an additional factor. *Mol. Immunol.*, 35: 207–214, 1998.
  52. Polyak, M. J., Taylor, S. H., and Deans, J. P. Identification of a cytoplasmic region of CD20 required for its redistribution to a detergent-insoluble membrane compartment. *J. Immunol.*, 161: 3242–3248, 1998.
  53. Deans, J. P., Robbins, S. M., Polyak, M. J., and Savage, J. A. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. *J. Biol. Chem.*, 273: 344–348, 1998.
  54. Bubien, J. K., Zhou, L. J., Bell, P. D., Frizzell, R. A., and Tedder, T. F. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca<sup>2+</sup> conductance found constitutively in B lymphocytes. *J. Cell Biol.*, 121: 1121–1132, 1993.
  55. Kanzaki, M., Lindorfer, M. A., Garrison, J. C., and Kojima, I. Activation of the calcium-permeable cation channel CD20 by  $\alpha$  subunits of the Gi protein. *J. Biol. Chem.*, 272: 14733–14739, 1997.
  56. Kanzaki, M., Nie, L., Shibata, H., and Kojima, I. Activation of a calcium-permeable cation channel CD20 expressed in Balb/c 3T3 cells by insulin-like growth factor-I. *J. Biol. Chem.*, 272: 4964–4969, 1997.
  57. Voorzanger, N., Toutou, R., Garcia, E., Delecluse, H. J., Rousset, F., Joab, I., Favrot, M. C., and Blay, J. Y. Interleukin (IL)-10 and IL-6 are produced *in vivo* by non-Hodgkin's lymphoma cells and act as cooperative growth factors. *Cancer Res.*, 56: 5499–5505, 1996.
  58. Benjamin, D., Park, C. D., and Sharma, V. Human B cell interleukin 10. *Leuk. Lymphoma*, 12: 205–210, 1994.
  59. Cerny, A., Merino, R., Makino, M., Waldvogel, F. A., Morse, H. C. d., and Izui, S. Protective effect of cyclosporin A on immune abnormalities observed in the murine acquired immunodeficiency syndrome. *Eur. J. Immunol.*, 21: 1747–1750, 1991.
  60. Yetter, R. A., Buller, R. M., Lee, J. S., Elkins, K. L., Mosier, D. E., Fredrickson, T. N., and Morse, H. C. d. CD4+ T cells are required for development of a murine retrovirus-induced immunodeficiency syndrome (MAIDS). *J. Exp. Med.*, 168: 623–635, 1988.
  61. Gazzinelli, R. T., Makino, M., Chattopadhyay, S. K., Snapper, C. M., Sher, A., Hugin, A. W., and Morse, H. C. d. CD4+ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. *J. Immunol.*, 148: 182–188, 1992.
  62. Sarris, A. H., Kliche, K. O., Pethambaram, P., Preti, A., Tucker, S., Jackow, C., Messina, O., Pugh, W., Hagemester, F. B., McLaughlin, P., Rodriguez, M. A., Romaguera, J., Fritsche, H., Witzig, T., Duvic, M., Andreeff, M., and Cabanillas, F. Interleukin-10 levels are often elevated in serum of adults with Hodgkin's disease and are associated with inferior failure-free survival. *Ann. Oncol.*, 10: 433–440, 1999.
  63. Blay, J. Y., Burdin, N., Rousset, F., Lenoir, G., Biron, P., Philip, T., Bancheau, J., and Favrot, M. C. Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. *Blood*, 82: 2169–2174, 1993.
  64. Platzer, C., Fritsch, E., Elsner, T., Lehmann, M. H., Volk, H. D., and Prosch, S. Cyclic adenosine monophosphate-responsive elements are involved in the transcriptional activation of the human IL-10 gene in monocytic cells. *Eur. J. Immunol.*, 29: 3098–3104, 1999.
  65. Mori, N., and Prager, D. Activation of the interleukin-10 gene in the human T lymphoma line HuT 78: identification and characterization of NF- $\kappa$ B binding sites in the regulatory region of the interleukin-10 gene. *Eur. J. Haematol.*, 59: 162–170, 1997.
  66. Eskdale, J., Kube, D., Tesch, H., and Gallagher, G. Mapping of the human IL10 gene and further characterization of the 5' flanking sequence. *Immunogenetics*, 46: 120–128, 1997.
  67. Tsujimoto, Y. Stress-resistance conferred by high level of bcl-2  $\alpha$  protein in human B lymphoblastoid cell. *Oncogene*, 4: 1331–1336, 1989.
  68. Domen, J., Gandy, K. L., and Weissman, I. L. Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood*, 91: 2272–2282, 1998.
  69. Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Millman, C. L., and Korsmeyer, S. J. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, 75: 241–251, 1993.
  70. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Washington DC)*, 262: 1274–1277, 1993.
  71. Miyashita, T., and Reed, J. C. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.*, 52: 5407–5411, 1992.
  72. Strasser, A., Harris, A. W., Jacks, T., and Cory, S. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell*, 79: 329–339, 1994.
  73. Baffy, G., Miyashita, T., Williamson, J. R., and Reed, J. C. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J. Biol. Chem.*, 268: 6511–6519, 1993.
  74. Genestier, L., Bonnefoy-Berard, N., Rouault, J. P., Flacher, M., and Revillard, J. P. Tumor necrosis factor- $\alpha$  up-regulates Bcl-2 expression and decreases calcium-dependent apoptosis in human B cell lines. *Int. Immunol.*, 7: 533–540, 1995.
  75. Croce, C. M., Erikson, J., Huebner, K., and Nishikura, K. Co-expression of translocated and normal c-myc oncogenes in hybrids between Daudi and lymphoblastoid cells. *Science (Washington DC)*, 227: 1235–1238, 1985.
  76. Wiman, K. G., Clarkson, B., Hayday, A. C., Saito, H., Tonegawa, S., and Hayward, W. S. Activation of a translocated c-myc gene: role of

- structural alterations in the upstream region. *Proc. Natl. Acad. Sci. USA*, 81: 6798–6802, 1984.
77. Alas, S., Emmanouilides, C., and Bonavida, B. Potentiation of fludarabine cytotoxicity on non-Hodgkin's lymphoma by pentoxifylline and Rituximab. *Anticancer Res.*, 20: 2961–2966, 2000.
78. Stasi, R., Zinzani, P. L., Galieni, P., Lauta, V. M., Damasio, E., Dispensa, E., Dammacco, F., Papa, G., and Tura, S. Prognostic value of serum IL-10 and soluble IL-2 receptor levels in aggressive non-Hodgkin's lymphoma. *Br. J. Haematol.*, 88: 770–777, 1994.
79. Cortes, J., and Kurzrock, R. Interleukin-10 in non-Hodgkin's lymphoma. *Leuk. Lymphoma*, 26: 251–259, 1997.
80. Edelman, L., Deveau, C., Raphael, M., Monchatre, E., Gabarre, J., Deville-Chabrol, A., Pialoux, G., Emilie, D., Joab, I., and Galanaud, P. Serum interleukin-10 in acquired immunodeficiency syndrome lymphoma patients. Seroco-Hemoco Study Group. *Eur. Cytokine Netw.*, 7: 785–791, 1996.
81. Zangemeister-Wittke, U., Schenker, T., Luedke, G. H., and Stahel, R. A. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. *Br. J. Cancer*, 78: 1035–1042, 1998.
82. LeVan, T. D., Dow, S. B., Chase, P. B., Bloom, J. W., Regan, J. W., Cunningham, E., and Halonen, M. Evidence for platelet-activating factor receptor subtypes on human polymorphonuclear leukocyte membranes. *Biochem. Pharmacol.*, 54: 1007–1012, 1997.
83. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat. Med.*, 4: 232–234, 1998.
84. Webb, A., Cunningham, D., Cotter, F., Clarke, P. A., di Stefano, F., Ross, P., Corbo, M., and Dziewanowska, Z. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet*, 349: 1137–1141, 1997.

# Clinical Cancer Research

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