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Authors
Tobin, Evan
Denardo, Gerald
Zhang, Nan
et al.

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Combination Immunotherapy with Anti-CD20 and Anti-HLA-DR Monoclonal Antibodies Induces Synergistic Anti-lymphoma Effects in Human Lymphoma Cell Lines

Evan Tobin¹, Gerald DeNardo¹, Nan Zhang², Alan L. Epstein², Cathy Liu¹ & Sally DeNardo¹

¹ Department of Internal Medicine, University of California Davis, CA, USA
² Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA, USA

Running Title: Rituximab & ChLym-1 Combined Immunotherapy

Keywords: Lymphoma; immunotherapy; rituximab; Lym-1; CD20; HLA-DR

¹Address for correspondence:
Gerald L. DeNardo, M.D.
Division of Hematology and Oncology
1508 Alhambra Blvd., No. 3100
Sacramento, CA 95816
Telephone (916) 734-3787
Fax (916) 703-5014
E-mail: gldenardo@ucdavis.edu
ABSTRACT
Rituximab is effective in about one half of patients with indolent lymphoma. Even these patients relapse and develop rituximab resistance. To increase potency and circumvent resistance, the anti-lymphoma effects of rituximab, an anti-CD20 MAb\(^1\), combined with chLym-1\(^2\), an anti-HLA-DR MAb, were assessed in human lymphoma cell lines by examining growth inhibition and cell death, apoptosis induction, ADCC\(^3\) and CDC\(^4\). There were additive effects in all assays and synergism in cell lines, such as B35M, which displayed resistance to either MAb alone. In B35M cells, combined rituximab and chLym-1 induced a 27-fold direct reduction in viable cells, whereas equivalent concentrations of rituximab or chLym-1 alone induced only a 1-fold and 10-fold reduction in viable cells, respectively. Because these results occurred at MAb concentrations readily achievable in patients, they suggest that this combination immunotherapy regimen may increase the potency and range of effectiveness of these MAbs in lymphoma patients.
INTRODUCTION

Lymphoma treatment has traditionally relied on chemotherapeutic drugs used singly or in combination. While combination chemotherapy is generally more effective than monotherapy, it has been less successful in indolent lymphoma as most patients eventually relapse and succumb to the disease [1].

Monoclonal antibodies (MAbs) for immunotherapy provide high specificity for lymphoma antigens, alternate and potent mechanisms of cytotoxicity, and widespread tolerability in patients [2,3]. Rituximab has been incorporated into treatment regimens for B-cell lymphoma because it has efficacy as a single agent against indolent lymphomas, inducing a therapeutic response in approximately 50% of previously chemotherapy-treated patients with only mild toxicity [4]. Upon binding to CD20, an antigen present exclusively on mature normal and malignant B cells, rituximab activates several direct and indirect anti-lymphoma mechanisms, including growth inhibition, apoptosis, antibody dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) [5-8]. Like chemotherapy, however, patients eventually become resistant to rituximab immunotherapy [9,10].

When rituximab and cyclophosphamide, doxorubicin, vincristine, prednisone chemotherapy (CHOP) have been combined in clinical trials, more patients, of all lymphoma subtypes, experienced a therapeutic response of longer duration [11-13], presumably related to the advantage of different mechanisms of drug action [14-17]. However, at least 20% of patients with aggressive lymphoma [13] and 40% of those with indolent lymphoma [11] do not experience a complete response. This combined modality therapy is also limited, in part, to the dose-limiting toxicities of the chemotherapeutic drugs [11,13,18].
In comparison, clinical trials of MAbs alone have shown no dose-limiting toxicity up to 2,250 mg/m² [19] and 8 weekly doses [20]. Therefore, combination immunotherapy clinical trials have been conducted using rituximab with Hu1D10 (anti-HLA-DR) [21], epratuzumab (anti-CD22) [22], or Campath (anti-CD52) [23,24]. A recent multicenter phase II trial of epratuzumab combined with rituximab in patients with refractory or recurrent non-Hodgkin’s lymphoma showed promising anti-lymphoma activity and was well tolerated by patients [25].

In the study reported here, we selected the combination of rituximab and chimeric Lym-1 (chLym-1²), because of shared characteristics that offered remarkable potential. Although a single phase 1a trial of murine Lym-1 (mLym-1⁶) failed to demonstrate substantial responses in patients, the dosages used and the plasma MAb levels achieved were significantly below those currently known to be required for effective MAb therapy [26]. However, both mLym-1 and chLym-1 have since been shown to have significant anti-tumor effects in in vitro assays [27]. Both rituximab and chLym-1 have high affinity for the CD20 and HLA-DR antigens, respectively [28-32]. These antigens are attractive targets for immunotherapy, because they are expressed on a variety of B cell lymphomas at high surface densities, and are neither shed nor internalized following MAb binding [28,33]. There is also evidence of physical and functional linkage between these molecules [22,34,35], so binding of one MAb to its antigen may directly affect the other MAb’s binding, downstream molecular events, or antigen expression.

Alternatively, the two MAbs in combination may activate distinctly different mechanisms of action thereby circumventing resistance to either MAb alone. Using assays to assess cell growth and viability, ADCC, CDC, and specific apoptosis related products, we compared the anti-lymphoma effects of rituximab and chLym-1 alone and in combination. We show at readily achievable MAb concentrations that this combination induced anti-lymphoma effects greater...
than either MAb alone in all assays and cell lines, and that the increased anti-lymphoma effect at times was synergistic.
MATERIALS AND METHODS

Antibodies

Rituximab (Rituxan®; Genentech, San Francisco, CA; Biogen Idec, San Diego, CA), a chimeric, IgG1 MAb, binds to the CD20 antigen on the surface membrane of mature and precursor B cells. chLym-1 was produced in the laboratory of Alan Epstein and is an IgG1 MAb, containing the variable regions of murine Lym-1 (mLym-1) and human γ1 and κ constant regions [30]. Although HLA-DR10 was the original immunogen used to generate the Lym-1 MAb, this MAb also targets many HLA-DR subtypes that share a conformationally determined epitope with HLA-DR 10 [36]. Chimeric L6 (chL6) (Oncogen, Seattle, WA), an IgG1 chimeric MAb, like rituximab and chLym-1 [37], binds an integral membrane glycoprotein that is highly expressed on lung, breast, colon, and ovarian tumor cells [37], but not on B cells. chL6 was used as an isotype-matched negative control for MAb binding, MAb direct and indirect anti-lymphoma effects.

Samples of MAbs were assayed for bacterial endotoxin, using a Limulus Amebocyte Lysate test (Bio-wittaker Pyrogent plus, Walkersville, MD). The test was performed as previously described [38]. The levels of endotoxin in all MAb samples were negligible in our experimental system.

Cells

The human Burkitt’s lymphoma cell lines, Raji and Ramos, were obtained from the American Type Culture Collection (Manassas, VA). The human Burkitt’s lymphoma cell line, B35M, and the human large cell lymphoma cell lines, SU-DHL-4 and SU-DHL-6, were provided by Alan Epstein. All cell lines were maintained in culture flasks with RMPI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine (200mM), 1% sodium pyruvate (100mM), 1%
nonessential amino acids and 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. The CD20 and HLA-DR density of these cell lines was determined by rituximab and chLym-1, respectively, binding followed by a goat anti-human IgG-flourescein-isothiocyanate (FITC⁷) and analyzed by fluorescence-activated cell sorting (FACS⁸). All cell lines were found to have high expression of both CD20 and HLA-DR antigens with the exception of Ramos cells, which had high CD20 expression, but very low HLA-DR expression (data not shown).

Study Design

The direct effects of rituximab and Lym-1 alone and in combination were assayed in parallel in five cell lines. Equal amounts of rituximab and chLym-1 were added simultaneously in all combination samples. Untreated samples, with cells and media only, and those treated with non-specific MAb, chL6, were included to provide baseline cell growth and death levels. Growth inhibition was indicated by a decrease in the number of total and viable cells, and cell death by an increase in non-viable cells. To determine the temporal effects of each MAb and to establish a threshold and saturating concentration, below and above which no significant difference in anti-lymphoma activity was observed, results were evaluated over 72 hours of incubation for individual MAb concentrations of 0.00025 – 20 μg/mL. Since the growth and viability of the untreated samples decreased at 96 hours (data not shown) due to exhaustion of the media, comparison between the untreated and treated samples was only made over 72 hours. Because no direct MAb anti-lymphoma effect was observed in Ramos, this cell line was not further studied.

To determine whether cell death was due to apoptosis, caspase-3 and poly (ADP-ribose) polymerase (PARP⁹) activation were determined over 24 hours using Western blot assay and 10 μg/mL of MAb, a concentration that provided maximum direct anti-lymphoma effects.
Camptothecin, a topoisomerase inhibitor, known to induce apoptosis in hemopoietic cell lines, including Raji [39], was used as a positive control and an untreated sample was used as a negative control. The relative ability of the MAbs alone and in combination to induce caspase-3 and PARP activation was compared to the camptothecin positive control, quantitated by densitometry, and expressed as the percent difference from the untreated control.

The indirect effects of rituximab and chLym-1 in combination and as single-agents, were assessed using ADCC and CDC assays. Since the ability of rituximab and chLym-1 to mediate ADCC has been shown to depend greatly on the types of effector cells used [10,29,40,41], each MAb was assessed in the presence of all effector cells, and a broad range of MAb concentrations and cell lines were utilized.

In all assays, the combination MAb effect was considered to be synergistic if there was evidence that the combined MAb potency was greater than that of either MAb and one of the two MAbs was not potent when used alone [42].

**Assessment of Cell Number and Viability**

Microscopy and trypan blue dye (960 kD) were used to determine cell number and viability at study time zero and after 24, 48, and 72 hours of incubation. B35M, SU-DHL-4, Raji, and Ramos cells were at least 95% viable at study time zero; SU-DHL-6 cells were at least 90% viable at study time zero. To eliminate the influence of complement, cells in log phase of growth were centrifuged for 5 minutes at 1100 rpm to pellet the cells. The supernatant was removed and the cells resuspended in RPMI-1640 media supplemented with 10% heat inactivated fetal calf serum (HIFCS\textsuperscript{10}) and supplemental nutrients to twice their final concentration (5 x 10\textsuperscript{5} cells/mL). Similarly, rituximab + chLym-1, rituximab, chLym-1, and chL6 MAbs were diluted in the same media to twice their final concentration (single MAb concentration: 0.0005 μg/mL – 50 μg/mL).
Finally, 100 μl of cells were seeded into each well of a 96-well plate followed by addition of 100 μl of MAb or 100 μl of media for the untreated control and then incubated at 37°C in a humidified 5% CO₂ environment. At each time point, 90 μl of cells were removed from each well after gentle pipetting to break up cell aggregations. The aliquot of cells was then combined with 10 μl of filtered trypan blue. 10 μl of this mixture was then placed in a hemocytometer and counted using bright field microscopy, under which non-viable cells appeared blue and viable cells appeared bright and translucent. The numbers of viable and non-viable cells in each sample were summed to quantify the total population.

Caspase-3 and PARP Activation

The levels of caspase-3 and PARP activation products were determined by Western blot assay. B35M, SU-DHL-4, Raji, and SU-DHL-6 cell lines were treated with a saturating concentration of rituximab and chLym-1 in combination or alone (single MAb concentration: 10 μg/mL) in parallel with a camptothecin (1μM) positive control and an untreated negative control. After 6 and 24 hours incubation, the cells were sedimented at 300 x g for 10 min, washed once with ice-cold phosphate buffered saline (PBS¹¹), and solubilized at a concentration of 5 x 10⁷ cells/mL in lysis buffer (50mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM EDTA¹², protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)). The cells were incubated on ice for one hour and centrifuged at 4°C and 13,000g for 30 minutes. The supernatant was recovered and the protein concentration assayed by bicinchoninic acid (BCA¹³) (Pierce, Rockford, IL). 100 μg of total protein from each sample, recovered from approximately 5 x 10⁵ cells, was mixed with lithium dodecyl sulfate (LDS¹⁴) sample buffer (Invitrogen, Carlsbad, CA), heated at 95°C for 5 min, and migrated in a 4-12% (w/v) gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE¹⁵) gel at 175V for 1 hour. The
total cell protein was transferred to a polyvinylidene difluoride (PVDF) membrane at 30V for 1 hour. The PVDF membrane was blocked with 1% nonfat milk for 1 hour followed by an overnight incubation with a 1:200 dilution of an anti-caspase-3 MAb (Oncogene, San Diego, CA) that binds to the activated enzyme. The PVDF membrane was thoroughly washed 5 times for 5 minutes each followed by a 1 hour incubation with a 1:40,000 dilution of an anti-mouse-HRP conjugate (KPL, Gaithersburg, MA). After thorough washing as indicated above, the PVDF membrane was incubated with Supersignal substrate (Pierce, Rockford, IL) for 1 minute immediately prior to exposure to a chemiluminescent-sensitive film. Subsequently, these PVDF membranes were assayed for PARP and activated PARP with a 1:1000 dilution of MAb (Pharmingen, San Diego, CA) otherwise using the same method as for the anti-caspase-3 MAb. Each PVDF membrane was also assayed for equal protein between lanes with a 1:10,000 dilution of anti-β-actin MAb (Sigma, St. Louis, MO).

**ADCC Assay**

ADCC was assessed in the presence of all peripheral effector cells to assess the overall anti-lymphoma effect in vivo. The effect of 0.01-10 μg/mL (single MAb concentration) rituximab and chLym-1 alone or in combination was assessed in a 14 hour 51Cr release assay. B35M, Su-DHL-4, Raji, and SU-DHL-6 (2 x 10^6) were labeled for 2 hours at 37°C with 200 μCi 51Cr (New England Nuclear, Boston, MA) in 200 μl normal saline. The labeled cells were washed three times with RPMI-1640 media and resuspended in the same. Cell viability was assessed by trypan blue exclusion and then 10,000 cells were seeded in each 12 x 75 mm tube. All samples were assayed in triplicate. Peripheral blood leukocytes (PBLs) collected from a volunteer donor were purified by lysing the red blood cells (RBCs) with lysis buffer (0.17M NH₄Cl, 0.01M KHCO₃, 0.08 mM tetrasodium ethylenediaminetetraacetic acid (EDTA), pH 7.3) for no
more than 5 min, and washing the PBLs in cold PBS twice. 100 ul of a 1.5 x 10^7 cells/mL suspension of effector cells was added to each tube, consisting of 1 x 10^6 polymorphonuclear cells (effector:target ratio of 100:1) and 0.5 x 10^6 mononuclear cells (effector:target ratio of 50:1), for a total effector:target ratio of 150:1. The effector:target ratio of 150:1 was chosen on the basis of preliminary experiments using an effector:target ratio of 50:1, which showed little ADCC activity with all MAbs. The final volume of each well was brought to 300 μL by adding the RPMI-1640 media alone or RPMI-1640 media containing MAb. After 14 hours incubation at 37°C in a humidified 5% CO2 environment, the tubes were centrifuged at 300g for 10 minutes. One half of the supernatant was aliquoted, and the radioactivity was measured in a gamma counter. The percent lysis was determined by the following equation: \[\frac{(E-T)}{(H-T)}\times 100\], where E is the mean cpm released in the test samples with effector cells, H is the mean cpm released in the presence of 1N HCl, and T is the mean cpm released by target cells incubated with medium alone.

**CDC Assay**

The ability of 0.5 - 12 μg/mL (single MAb concentration) rituximab and chLym-1, alone or in combination, to mediate CDC was assayed in duplicate and in parallel with the chL6 negative control. B35M, SU-DHL-4, Raji, and SU-DHL-6 cells were counted for viability and cell number using trypan blue exclusion and placed into RPMI-1640 medium containing no additives. The cells were immediately dispensed into round bottom, 96-well cell plates, with 1 million cells per well in 100 μl volume. 50 ul of MAb, diluted in RPMI-1640, was added, followed by the addition of 50 μl of human serum obtained from a volunteer donor. After the plates were incubated for 1 hour at 37°C in a humidified 5% CO2 environment, cell viability was determined by microscopy and trypan blue exclusion.
**Statistical Analysis**

To assess the significance of the cell counts, a two sample, one-tailed, student T-test was performed on the duplicate counts within an experiment. Results were deemed significant if the p-value was equal to or less than 0.05. The Kendall Tau test was used to determine whether a significant concentration-response relationship existed. To assess whether this relationship was linear, Pearson’s correlation was employed. A Pearson’s product-moment correlation coefficient or a rank-order correlation coefficient for the Kendall Tau test of less than 0.05 was considered to be significant. To assess the difference between the treated and untreated samples, the percent difference observed in the numbers of replicate experiments (n = 2-3) were averaged. The difference was considered to be significant if the p-value was less than 0.05.
RESULTS

Growth Inhibition and Cell Death

Using microscopy and trypan blue exclusion, the ability of rituximab and chLym-1 alone or in combination to induce growth inhibition and cell death was evaluated in the B35M, SU-DHL-4, Raji, SU-DHL-6, and Ramos cell lines over 72 hours incubation with MAb. The cell populations, when treated with the control MAb, chL6, was never significantly different than the untreated cells (data not shown, $p \geq 0.96$). When incubated with $10 \mu g/mL$ of rituximab and chLym-1 in combination ($20 \mu g/mL$ total MAb), significant growth inhibition and cell death were observed over 72 hours in all cell lines except Ramos cells; Ramos consistently showed little effect with either MAb or the MAb combination (Figure 1). The MAb combination induced a synergistic direct anti-lymphoma effect in B35M and SU-DHL-4 cells (Figure 2). In the B35M cell line, rituximab and chLym-1 in combination induced a 27-fold reduction in viable cells, whereas rituximab or chLym-1 alone induced a 1-fold reduction and a 10-fold reduction, respectively. In a separate experiment, synergistic cell death was apparent even after adjustment for the MAb concentration to make the total MAb concentration in the combination equivalent to the single MAb concentration (data not shown). Under the same conditions in SU-DHL-4 cells, the combination induced a 2-fold reduction in viable cells, whereas rituximab and chLym-1 alone had little effect. At all MAb concentrations and incubation times, the direct anti-lymphoma effects of the MAb combination were at least as great as either MAb alone in all cell lines.

Concentration Effects of Rituximab and chLym-1 in Combination

To find the threshold and saturating concentrations, below and above which no significant differences in effect were observed, the concentrations of rituximab and chLym-1 alone and in
combination were reduced stepwise from 20 μg/mL to a minimum of 0.0025 μg/mL and examined at 24, 48, and 72 hours in the four cell lines (Figure 2). The threshold concentration of the rituximab and chLym-1 combination in all cell lines was ≥ 0.05 μg/ml, p ≤ 0.04, which was lower than the threshold concentration of either MAb alone, 0.25 to 0.5 μg/mL, p ≤ 0.05. Whereas the concentration-dependence of chLym-1 or rituximab alone varied considerably between cell lines, the MAb combination at concentrations greater than 0.05 μg/mL showed a consistent concentration-dependent induction of growth inhibition and cell death in all cell lines when compared to the untreated cells (p ≤ 0.05). These data suggest that the combination of rituximab and chLym-1 has a lower dose threshold than either MAb alone, as well as a concentration-dependent effect in all cell lines. The saturating concentration of MAb beyond which no further anti-lymphoma effect was seen was ≤ 10 μg/ml for all 4 cell lines. Even when MAb concentrations were increased up to 200 μg/ml, no additional antilymphoma effect was seen (data not shown). The maximum achievable cell death effect of the MAb combination required a higher concentration than that of either MAb alone in both B35M and Raji cells (data not shown for Raji) (p ≤ 0.03) (Figure 2).

Temporal Effects of Rituximab and chLym-1 in Combination

To determine the temporal effects of a saturating concentration of the rituximab and chLym-1 combination, 10 μg/mL of each MAb was incubated over 72 hours with B35M, SU-DHL-4, Raji, or SU-DHL-6 cells. At all time points and in all cell lines, greater anti-lymphoma effects were seen using 10 μg/mL of each MAb in the combination (20 μg/mL of total MAb) than with either MAb alone. The difference was greatest in the B35M cell line, in which rituximab alone had little effect over 72 hours (Figure 3). The magnitude of MAb effect, manifested by a decrease in
viable and increase in non-viable cells from the untreated population, was greatest after 72 hours incubation in all cell lines.

**Activation of Caspase-3 and PARP**

Using Western blot assay, an increase in caspase-3 and PARP activation was observed in all cell lines treated with 10 μg/mL (single MAb concentration) rituximab or chLym-1 alone or in combination when compared to untreated cells (Figure 4). At both 6 hours and 24 hours, camptothecin induced apoptosis to a greater extent than that seen in the untreated samples and showed maximum apoptosis induction by 6 hours. The combination of MAbs induced a synergistic increase in apoptosis in B35M cells by 24 hours. At this time point, the percent difference of activated caspase-3 by densitometry due to the MAb combination was 6X greater than that of the untreated B35M cells, and 4X greater than either rituximab or chLym-1 alone (Figure 4). Rituximab and chLym-1 in combination also induced greater caspase-3 activation than either MAb alone in Raji cells at both 6 and 24 hours incubation. In SU-DHL-4 and SU-DHL-6 cells, however, the MAb combination did not induce substantial apoptosis after 24 hours incubation.

**Antibody-Dependent Cellular Cytotoxicity (ADCC)**

The relative abilities of rituximab and chLym-1 to mediate ADCC in Raji, B35M, and SU-DHL-6 cells were evaluated in comparison with an untreated control sample (Figure 5). Using a total effector:target cell ratio of 150:1 (polymorphonuclear cells of 100:1 and mononuclear cells of 50:1), purified PBLs from a volunteer exhibited 22-41% cytotoxicity against the three cell lines in the absence of MAb. In all cell lines and at all concentrations of MAb, chLym-1 alone mediated ADCC much more efficiently than rituximab alone. Combination MAb treatment mediated synergistic ADCC effects in SU-DHL-6 cells at concentrations of 1 μg/mL and above.
and additive effects at concentrations of 0.1 μg/mL and below. For example, while chLym-1 induced specific lysis of 30% of SU-DHL-6 cells and rituximab only 1% at 1 μg/mL, the combination effect (46%) was substantially greater than the effect of either MAb alone. In Raji and B35M cells, the combination of rituximab and chLym-1 was as effective as either MAb alone, but did not induce synergistic or additive effects. The combination of rituximab with chLym-1 showed a significant and consistent relationship between the single MAb concentration and the response, reaching threshold at 0.1 μg/mL and maximum response at 1 μg/mL in all cell lines (p ≤ 0.04). The MAb combination was at least as effective as either MAb alone in mediating ADCC in all lymphoma cell lines, and in some instances, their effect was much greater used in combination.

**Complement-Dependent Cytotoxicity (CDC)**

The ability of rituximab and chLym-1 alone and in combination to mediate CDC against B35M, SU-DHL-4, Raji, and SU-DHL-6 cells was tested by comparing them with the untreated and chL6 negative controls (Figure 6). Complement-mediated lysis due to the serum alone or the chL6 negative control was never greater than 10%. Although rituximab did not mediate CDC in B35M cells, its effect in combination with chLym-1 substantially increased the CDC effect over either MAb alone. At low concentrations of MAb, the effect due to combination treatment appeared to be synergistic. In SU-DHL-4, Raji, and SU-DHL-6 cells, rituximab alone mediated CDC more efficiently than chLym-1 alone, but no greater than the MAb combination (Table I).
DISCUSSION

In multiple human lymphoma cell lines expressing CD20 and HLA-DR antigens, we have demonstrated enhanced direct and indirect anti-lymphoma effects when rituximab and chLym-1 are used in combination. Using microscopy and trypan blue exclusion to distinguish viable from non-viable cells, greater direct anti-lymphoma effects were consistently observed in all cell lines given rituximab and chLym-1 in combination as opposed to equivalent doses of either MAb alone (Table I). The difference between the mono- and combination therapy seemed to be greatest in cell lines such as B35M and SU-DHL-4 that exhibited some resistance to either rituximab or chLym-1 alone. In these two cell lines, the effect of the MAb combination was synergistic in nature [42]. Stein et al. [43] similarly showed an enhanced anti-proliferative effect in SU-DHL-4 cells when a humanized anti-CD20 MAb and anti-CD22 MAb were used in combination. Our studies further showed substantially increased non-viable B35M and Raji cells due to the MAb combination when compared to either MAb alone, indicating that there was at least an additive cell death effect in these cells at all incubation times. Cell death was particularly dramatic in B35M cells at 48 and 72 hours and at all concentrations of MAb combination, even when adjusting for the total concentration of MAb present, indicating that the effect was synergistic in these cells (Figure 2).

Interestingly, significant cell death was not observed in the SU-DHL-4 or SU-DHL-6 cell lines at any concentration of either MAb alone, or in combination, perhaps due to a t(14:18) chromosomal translocation in these cells leading to bcl-2 overexpression and inhibition of apoptosis that provided these cells with a survival advantage [44]. Since this mutation occurs in approximately 50% of NHLs (80% low grade; 30% intermediate grade), it seems likely that this gene plays a major role in MAb resistance.
In our study, Western blot assay showed increased caspase-3 and PARP activation in all rituximab and chLym-1 treated cell lines (B35M, SU-DHL-4, Raji, SU-DHL-6), indicating that ligation of HLA-DR or CD20 induced apoptosis, perhaps not exclusively, through a caspase-3 mediated pathway. Combination treatment induced greater apoptosis than rituximab or chLym-1 alone in all cell lines, but the effect was greatest in B35M cells in which caspase-3 activation by the combination was at least 4x greater than that of rituximab or chLym-1 alone and 6x greater than that of the untreated cells. These results suggest that the substantial cell death observed in B35M cells after combination treatment was due to synergistic induction of apoptosis.

In addition to direct anti-lymphoma effects, MAbs can activate indirect effector mechanisms, such as ADCC and CDC. In the presence of all peripheral effector cells, chLym-1 was more efficient than rituximab in mediating ADCC in all cell lines, consistent with the observation that anti-HLA-DR MAbs are potent activators of polymorphonuclear cells that represent the majority of effector cells normally in the blood [40,41]. At all concentrations of MAb and in all cell lines (B35M, SU-DHL-4, Raji, SU-DHL-6), the combination of rituximab and chLym-1 mediated at least as much ADCC as either MAb alone. Rituximab primarily induces ADCC effects in the presence of purified mononuclear cells [10]. Hence, the combination of rituximab and chLym-1 will take advantage of the cytotoxic capability of both mononuclear and polymorphonuclear effector cells. In addition, CDC assays, performed in the presence of human serum, showed a greater effect for the MAb combination compared to monotherapy in all cell lines. In B35M cells, the combination induced synergistic CDC effects whereas rituximab alone had no effect at any MAb concentration.

In this study of an anti-CD20 and an anti-HLA-DR MAb, neither MAb showed strong potency uniformly across all cell lines and assays (Table I). However, the combination of
rituximab and chLym-1 produced greater anti-lymphoma effects that were synergistic at times in cell lines that exhibited resistance to either MAb alone (Table I). Past studies have shown a direct relationship between resistance to rituximab and low CD20 antigen density in cell lines [45] and in patients with B-chronic lymphocytic leukemia [46,47]. In addition, rituximab treatment has also been associated with the loss or down-modulation of CD20 antigen expression [48-50]. To overcome resistance in patients with low CD20 levels, higher and more doses of rituximab have been used to increase response rates, although these responses have tended to be short-lived [51].

In a previous study comparing another humanized anti-HLA-DR MAb with rituximab, Stein et al. [32] also showed that the combination of MAbs was more effective than rituximab alone. Synergy between anti-CD20 and anti-HLA-DR MAbs could be related to the physical and functional linkage of CD20 and HLA-DR antigens on B cells [22,34,35]. It is possible that the binding of one MAb to its antigen may directly affect the other MAb’s binding, downstream molecular events, or antigen expression. It has been demonstrated in several studies that CD20 ligation with MAb redistributes the antigen into lipid rafts [52,53]. Binding of rituximab to CD20 may then co-aggregate HLA-DR molecules as well as CD20 molecules on B cells. Associating these molecules in close proximity increases the likelihood of antigen crosslinking by MAb, which has been shown to be necessary for significant rituximab-induced apoptosis [14,54,55]. The formation of lipid rafts is also required for the activation of certain HLA-DR signal transduction pathways [56,57]. The mechanism of synergy between rituximab and chLym-1, therefore, may involve a dependent interaction of CD20 and HLA-DR molecules following combination immunotherapy. Studies of this nature are in progress.
Monoimmunotherapy is limited by variable and heterogenous antigen expression and the inevitability of drug resistance in an evolving biological system, whereas combination immunotherapy exploits the coexpression of distinct antigens and the possibility for synergy given the physical and functional linkages of those antigens, such as between CD20 and HLA-DR. Supporting combination immunotherapy, results for clinical trials of rituximab in combination with other MAbs (Hu1D10, epratuzumab, Campath) [21-24], have shown that it is safe, effective, and possibly synergistic in certain lymphoma histologies. Bispecific antibodies targeting two separate antigens are in development and promise to take advantage of combination immunotherapy, while minimizing cost and infusion times [58,59].

We have shown that combining rituximab and chLym-1 induces synergistic anti-lymphoma effects, such as growth inhibition, cell death, and apoptosis, while mediating greater indirect effects, such as ADCC and CDC, than monotherapy in all cell lines tested. Importantly, these effects were seen at MAb levels readily achievable in patients using the currently approved four, once weekly rituximab dosing [60]. The ability of the rituximab and chLym-1 combination to induce greater effects than either MAb alone in cell lines, even those with low antigen expression and resistance to the individual MAbs, suggests that combination immunotherapy may be more effective in the clinic over a broader range of lymphoma types. In particular, the potency of rituximab and chLym-1 alone and in combination over a range of B-cell lymphomas at clinically achievable MAb levels suggests that this combination may have clinical utility and that a combination immunotherapy trial of rituximab and chLym-1 is warranted. Trials of this type are under exploration.
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FIGURE LEGENDS

Figure 1. Effect of rituximab and chLym-1 on cell viability. Non-viable (□-) and viable (■-) cell populations were quantified using microscopy and trypan blue. Results reflect the percent increase in nonviable cells or the percent decrease in the viable cells as compared to the untreated cells. The combination of rituximab and chLym-1 caused significant growth inhibition and cell death in all cell lines except for Ramos. The antilymphoma effect of the MAb combination in B35M and SU-DHL-4 cells was synergistic in nature. B35M cells (upper left; A), SU-DHL-4 cells (upper right; B), Raji cells (middle left; C), SU-DHL-6 cells (middle right; D), and Ramos cells (lower; E). $5 \times 10^4$ cells were incubated for 48 h with 10 g/ml (single MAb concentration) rituximab or chLym-1 alone or in combination, diluted in RPMI media supplemented with 10% HIFCS. These data are representative of replicate assays that showed reproducible results.

Figure 2. Concentration effects of rituximab and chLym-1 on B35M and SU-DHL-4 cells. B35M (left; A) and SU-DHL-4 (right; B) data illustrate the concentration relationship at 48-h incubation. The x-axis reflects single MAb concentrations. Cells treated with rituximab and chLym-1 in combination (●-) showed greater growth inhibition (upper; A,B) and cell death (lower; A,B) than rituximab (□-) or chLym-1 (△-) alone at all concentrations of MAb from 0.025 to 20 g/ml. A lower concentration threshold was also observed for the combination compared with either MAb alone. Although both the combination and chLym-1 treatments induced substantial cell death in B35M cells, no MAb treatment induced significant cell death in SU-DHL-4 cells. Population numbers represent an average of duplicate samples, and error bars provide the range between the two samples. These data are representative of replicate trials that showed reproducible results.
Figure 3. Temporal effects of rituximab and chLym-1. The effect of a saturating concentration (10 g/ml single MAb concentration) of rituximab and chLym-1 in combination (-●-), rituximab alone (-□-), or chLym-1 alone (-Δ-) on B35M cells was observed over 72-h incubation. Treated B35M cells demonstrated growth inhibition (upper; A) and increased cell death (lower; B), when compared to the untreated samples (-○-) and chL6 treated samples (data not shown, same as untreated). The magnitude of the decrease in viable cells (upper; A) and increase in nonviable cells (lower; B) was greater after longer incubation. Population numbers represent an average of duplicate samples, and error bars provide the range between the two samples. These data are representative of four replicate trials that showed reproducible results.

Figure 4. Detection of activated caspase-3 and PARP by Western blot. B35M, SU-DHL-4, Raji, and SU-DHL-6 cells were treated for 6 h (A) or 24 (B) h with 10 g/ml (single MAb concentration) rituximab or chLym-1 alone or in combination, lysed, and prepared immediately for Western blot assay. Untreated samples were used as a negative control and 1 M camptothecin treated samples were used as a positive control for apoptosis. All MAb treatments induced apoptosis compared with the untreated control. The MAb combination induced caspase-3 activation as great or greater than either MAb alone at both 6 and 24 h. The Western blots were subsequently probed for the pro- and activated forms of PARP. A similar relationship between the amount of activated PARP and activated caspase-3 in the treated and untreated samples was observed in all four cell lines. Finally, the Western blots were probed for β-actin to document equal protein loading between lanes (data not shown). The percent difference (C) from the untreated control of activated caspase-3 in B35M cells treated with rituximab and chLym-1 in combination, rituximab alone, chLym-1 alone, or the camptothecin control for 6 h (-○-) and 24 h (-●-) was determined by densitometry.
Figure 5. Antibody-dependent cellular cytotoxicity (ADCC) assay. ADCC against lymphoma cells mediated by rituximab and chLym-1 in combination (-●-), rituximab (-□-), chLym-1 (-Δ-), or mLym-1 (-Δ-) was adjusted for spontaneous lysis of the untreated control in a 14 h $^{51}$Cr release assay. Fresh PBLs from a volunteer donor were added to $^{51}$Cr labeled Raji, B35M, or SU-DHL-6 cells using a 150:1 effector:target ratio in the presence or absence of MAb. B35M (upper left; A), SU-DHL-4 (upper right; B), Raji (lower left; C), and SU-DHL-6 cells (lower right; D). The x-axis reflects single MAb concentrations. The MAb combination was generally more potent than either MAb alone. ADCC data is representative of three replicates performed in parallel and plotted as the percent specific lysis (see text).

Figure 6. Complement-dependent cytotoxicity (CDC) assay. CDC mediated by rituximab and chLym-1 in combination (-●-), rituximab (-□-), or chLym-1 (-Δ-) on B35M cells was adjusted for nonspecific lysis in the untreated samples. Fresh serum (25% by volume) from a volunteer donor was incubated with $1 \times 10^6$ B35M cells for 1 h and cytotoxicity was measured immediately by microscopy and trypan blue dye exclusion. The x-axis reflects single MAb concentrations. Combined rituximab and chLym-1 showed greater CDC activity at lower MAb concentrations in B35M cells. CDC data is representative of duplicate trials performed in parallel.
Figure 1
Figure 2
Figure 3

(A) Viable Cells/mL x 10^5 over time (h) from 0 to 72.

(B) Non-Viable Cells/mL x 10^5 over time (h) from 0 to 72.
Figure 4
Figure 5
Figure 6
Table I. Relative antilymphoma effect of rituximab and chLym-1 (10 μg/ml) using direct (trypan blue and caspase-3) and indirect (ADCC and CDC) assays*.

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*The effects in each assay were graded (0 to ++++) wherein an approximate linear estimation was made. Each (+) was assigned a particular percentage value for each effect indicated below.

†(+) ≥ 300% decrease in viable cell population from the untreated after 72 h incubation.

‡(+) ≥ 150% increase in the non-viable cell population from the untreated after 48 h incubation.

§(+) ≥ 40% increase in activated caspase-3 from the untreated after 6 h incubation.

**(+ ≥ 15% specific lysis after 14 h incubation.

††(+) ≥ 15% specific lysis after 1 h incubation.
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ABBREVIATIONS

1. MAb, monoclonal antibody
2. chLym-1, chimeric Lym-1
3. ADCC, antibody-dependent cellular cytotoxicity
4. CDC, complement dependent cytotoxicity
5. CHOP, cyclophosphamide, doxycyclin, vincristine, prednisone
6. mLym-1, murine Lym-1
7. FITC, fluorescein-isothiocyanate
8. FACS, fluorescence-activated cell sorting
9. PARP, poly (ADP-ribose) polymerase
10. HIFCS, heat-inactivated fetal calf serum
11. PBS, phosphate buffered saline
12. EDTA, ethylenediaminetetraacetic acid
13. BCA, bicinchoninic acid
14. LDS, lithium dodecyl sulfate
15. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
16. PVDF, polyvinylidene difluoride
17. PBLs, peripheral blood leukocytes
18. RBCs, red blood cells