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Chemoimmunotherapy for Relapsed/Refractory and Progressive 17p13 Deleted Chronic Lymphocytic Leukemia (CLL) Combining Pentostatin, Alemtuzumab, and Low Dose Rituximab is Effective and Tolerable and Limits Loss of CD20 Expression by Circulating CLL Cells

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Abstract

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) patients with purine analogue refractory disease or TP53 dysfunction still have limited treatment options and poor survival.

Alemtuzumab containing chemoimmunotherapy regimens can be effective but frequently cause serious infections. We report a phase II trial testing the efficacy and tolerability of a short duration regimen combining pentostatin, alemtuzumab, and low dose high frequency rituximab (PAR) designed to decrease the risk of treatment associated infections and limit loss of CD20 expression by CLL cells. The study enrolled 39 patients with progressive CLL that was either relapsed/refractory (n=36) or previously untreated with 17p13 deletion (17p13-)(n=3). Thirteen (33%) patients had both 17p13- and TP53 mutations predicted to be dysfunctional and eight patients had purine analogue refractory CLL without TP53 dysfunction. Twenty-six (67%) patients completed therapy with only five (13%) patients having treatment limiting toxicity, and no treatment related deaths. Twenty-two (56%) patients responded to treatment with 11 (28%) complete responses (four with incomplete bone marrow recovery). Median progression free survival was 7.2 months, time to next treatment 9.1 months, and overall survival 34.1 months. The majority of deaths (82%) were caused by progressive disease including transformed diffuse large B cell lymphoma (n=6).

Correlative studies showed that low dose rituximab activates complement and NK cells without a profound and sustained decrease in expression of CD20 by circulating CLL cells. We conclude that PAR is a tolerable and effective therapy for CLL and that low dose rituximab therapy can activate innate immune cytotoxic mechanisms without substantially decreasing CD20 expression.

Keywords

Chronic lymphocytic leukemia/small lymphocytic lymphoma; CLL/SLL; relapsed/refractory; TP53/p53/17p13 deletion; alemtuzumab and low dose rituximab; trogocytosis

Introduction

Patients with purine analogue refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) and progressive disease with TP53 dysfunction have limited treatment options and poor survival [1]. Although alemtuzumab monotherapy is often effective at clearing CLL cells in the circulation and bone marrow, patients with bulky adenopathy and splenomegaly rarely achieve durable responses [2-4]. Alemtuzumab is also an effective therapy against CLL cells with TP53 dysfunction (deletion/mutation), although complete remissions are rare and progression free survival is usually short [5-7]. Alemtuzumab has thus been combined with other drugs in order to improve efficacy. Alemtuzumab and rituximab is effective and tolerable but also has limited efficacy against bulky disease [8-10]. Combination therapy with alemtuzumab and fludarabine has been reported to be more effective than fludarabine monotherapy [11, 12] but there are concerns about the safety of these chemoimmunotherapy regimens because of excess mortality for both patients undergoing their first therapy for progressive disease [13] and those treated for relapsed/refractory disease [14, 15]. A safer and more effective chemoimmunotherapy regimen could be of value for patients with very high risk progressive CLL.

We conducted a phase II clinical trial to test whether a combination therapy with pentostatin, alemtuzumab, and lower dose higher frequency rituximab (PAR) could be effective and tolerable for patients with relapsed/refractory or progressive CLL with 17p13 deletion (17p13-) and could limit loss of CD20 expression by circulating CLL cells. Pentostatin was included in the regimen with the aim of decreasing CLL tumor bulk by targeting the residual purine analogue sensitive clones of the CLL cell population with a drug that could have less myelotoxicity than fludarabine [16]. The rituximab administration schedule was based on data showing that lower dose and higher frequency therapy can decrease the loss of CD20 expression by circulating CLL cells that occurs with standard dose rituximab therapy [17, 18]. The duration of the therapy regimen was limited to three months to reduce the risk of prolonged immunosuppression. Our study shows that PAR is an effective and tolerable therapy for this patient population and that use of low dose rituximab decreases trogocytosis ("shaving") and thus loss of expression of CD20 by circulating CLL cells.

Methods

Patient Selection

This two-stage phase II clinical trial was conducted at the Mayo Clinic Rochester and University of Iowa with the approval of both Institutional Review Boards according to the principles of the Declaration of Helsinki and was registered with ClinicalTrials.gov (NCT00669318). Patients were eligible for accrual if they had a diagnosis of progressive CLL (including the small lymphocytic lymphoma variant) by standard criteria [19-21] and either had been previously treated for CLL (no more than three purine analogue containing regimens) or were previously untreated for their CLL and had 17p13- detected by interphase fluorescent in situ hybridization (FISH) analysis. Exclusion criteria were organ failure (creatinine >2x upper limit of normal (ULN), direct hyperbilirubinemia or AST >3x ULN, >class II New York Heart Association heart failure, or an ECOG performance status >3), infection with HIV, hepatitis B or C, active autoimmune cytopenia, and alemtuzumab therapy <2 months previously. Prognostic factor analysis was determined using FISH analysis of peripheral blood, *IGHV* mutation analysis, *TP53* mutation analysis, and expression of CD38 and ZAP-70 as previously described [22-26].

Therapy

Therapy started with rituximab 20 mg/m² intravenously (IV) Monday, Wednesday, and Friday (M-W-F). Subcutaneous (SQ) alemtuzumab therapy started on day 3 after administration of the second dose of rituximab with a daily dose escalation of 3-10-30 mg/d if tolerated and was then administered at 30 mg SQ M-W-F starting on day 8. Pentostatin 2 mg/m² IV was started on day 8 and repeated every 2 weeks. The first cycle of therapy included the week of alemtuzumab dose escalation and 4 subsequent weeks of full dose therapy for a total of 5 weeks. Subsequent cycles were 4 weeks of full dose therapy. Leukocyte growth factor (pegfilgrastim 6 mg SQ x 1 day or GM-CSF 500 µg/d SQ x 5 days) administration was started 48 hours after each dose of pentostatin and all patients had Pneumocystis and herpes virus prophylaxis during therapy and then for 6 months after the last dose of alemtuzumab. Blood cytomegalovirus (CMV) viral assays were done using a semi-quantitative polymerase chain reaction (PCR) based method weekly during treatment with alemtuzumab and all patients with detectable CMV viremia were treated with either valganciclovir or ganciclovir. Patients tolerating therapy and without disease progression received a minimum of 2 cycles of PAR therapy. After completing 2 cycles of therapy, those patients who had achieved a complete clinical response underwent a CT scan of their chest, abdomen and pelvis. Patients with no pathological radiological findings then underwent a bone marrow study to test for residual CLL. If the bone marrow biopsy showed no morphological evidence of residual CLL, immunohistochemical (IHC) staining (see below for details) was performed to evaluate for residual CLL cells. Those patients with an IHC negative (stringent) complete response (CR) had no further therapy. All other patients without progressive disease received a 3rd cycle of therapy.

Response Evaluation

Patients were evaluated by physical examination and blood testing one week after starting therapy, subsequently every 2 weeks during treatment, monthly for 6 months after

completing therapy, and then at 9 and 12 months after completing therapy. Adverse events were graded using the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 <http://ctep.cancer.gov/> with the exception of cytopenias which were graded using the Grading Scale for Hematologic Toxicity in CLL studies [19]. Response to treatment was measured 2 months after completion of therapy using standard National Cancer Institute-Working Group 1996 (NCI-WG96) criteria [21]. In patients who achieved a complete clinical remission by NCI-WG96 criteria, the bone marrow biopsy was evaluated for residual CLL cells by IHC studies (streptavidin-biotin peroxidase complex method) with antibodies directed against CD3, CD5, CD23 and PAX5 using standard techniques.

Correlative studies

The specific focus of the correlative studies was to examine the effects of the first week of therapy with low dose rituximab (20 mg/m² IV) on circulating CLL cells. Four peripheral blood samples were drawn from each patient: Sample 1 on day 1 of therapy prior to the first dose of rituximab, sample 2 on day 3 before therapy (48 hours after the first dose of rituximab), sample 3 on day 3 one hour after completion of therapy (rituximab infusion followed by SQ alemtuzumab injection), and sample 4 before treatment on day 8 of therapy.

Analysis of changes in CD20 expression and complement activation—

Immunophenotypic analyses on CLL cells were performed on the first 13 evaluable patients based on analyses starting with whole blood as previously described [27, 28]. These assays were performed within three days of the blood draw. Alternatively, in the next group of 18 patients, peripheral blood mononuclear cells (PBMC) were isolated within 2 hours of the blood draw and were then frozen and stored at -150°C as previously described [29]. The PBMC were subsequently thawed in AIM V media (Gibco) and analyzed as above except that the erythrocyte lysis step was omitted. Due to minor adjustments in the blood specimen collection protocols, the absolute lymphocyte counts were measured only in the first and fourth blood sample in the first group of patients, but absolute lymphocyte counts (ALC) were determined for all four freshly isolated blood samples for the second group of patients. Finally, in certain cases only three (of four) blood samples were obtained for some patients, and therefore the number of data points for specific times in the figures varied slightly.

All immunophenotypic assays were run in duplicate. CLL cells were identified as low side scatter, PerCP CD45+ (BD Pharmingen), and PE CD19+ (eBioscience). Levels of CD20 on CLL cells were measured as previously described by probing with ofatumumab or rituximab, followed by secondary development with Al488-labeled mAb HB43, specific for human Fc (American Type Tissue Collection)[30]. Deposition of complement C3d fragments was measured with Al488-labeled mAb 1H8 [27]. FITC-labeled mAbs specific for complement control proteins CD46 and CD59 were obtained from BD Pharmingen. Monoclonal antibody HD1A, specific for CD55, was the kind gift of Prof. Paul Morgan, University of Wales, Cardiff. Certain monoclonal antibodies, including HD1A, were labeled by reaction with Alexa 488 N-hydroxysuccinimide according to the manufacturers' directions (Molecular Probes). In order to test for a correlation between deposited levels of C3d and expression of CD20, a subset of Day 3 Post PBMC samples were first incubated

with ofatumumab, washed, and then probed with a cocktail of FITC mAb 1H8, PE CD19, PerCP CD45 and Al647 HB43.

Unless otherwise noted, all results for these measurements are normalized to values obtained for the blood samples taken just before the first rituximab infusion. The results for the fresh and frozen samples are plotted in the same figures, but different symbols are used to identify CLL cells that were evaluated in either whole blood (open circles) or as PBMC (closed circles). In a few patients, levels of CD20 in the pre-treatment blood samples were very low (less than 5% of the median levels for the other patients, likely because of recent rituximab treatment), or ALC were less than $5 \times 10^9/L$, and these blood samples were not included in the analyses.

Analysis of changes in NK cells—NK cells were evaluated in blood specimens, taken before and 48 hours after the first dose of rituximab, for changes in numbers as well as expression of CD16 and CD54 as previously described [31]. Briefly, PBMCs were isolated from blood by Ficoll separation and red blood cells lysed with ACK buffer. 2×10^6 mononuclear cells were stained with CD54-PE, CD56-A647, CD16-FITC, CD3-APC-Cy7 and CD19-PE-Cy7, fixed and analyzed on a Becton Dickinson LSR II for changes in the percentage counts and expression of CD16 and CD54.

Statistical Analysis

This study used a two-stage Simon optimum design [32] with a primary endpoint of CR rate as defined by the NCI-WG96 criteria. The secondary end points were to determine the overall response rate, toxicity of treatment, time to subsequent therapy, and overall and progression free survival. Based on our experience with treatment of relapsed/refractory CLL and patients with progressive disease and 17p13-, we proposed that the largest CR rate where the proposed treatment regimen would be considered ineffective in this population was 10%, and the smallest success proportion that would warrant subsequent studies with the proposed regimen in this patient population would be 30%. A total of 38 evaluable patients provided 93% power, with a 7% Type I error rate. The progression-free survival (PFS) time is defined as the time from registration to progression or death due to any cause. Overall survival (OS) time is defined as the time from registration to death due to any cause. Time to subsequent therapy (TTT) is defined as the time from registration to the time of initiation of subsequent therapy for progressive CLL. Patients who underwent allogeneic transplant or received subsequent treatment before meeting criteria for disease progression were censored for TTT. The distribution of all time-to-event endpoints were estimated using the method of Kaplan-Meier [33] and differences between subgroups were evaluated using log-rank statistics. The results of rituximab infusions on cell phenotype were evaluated by the Wilcoxin signed-rank test and relationships between continuous variables were examined based on Spearman rank order correlation (Sigma stat).

Results

Patients

Forty-one patients were enrolled from July 2008 to February 2013 and 39 were evaluable. Two patients did not start treatment. One patient was found to have concomitant Hodgkin lymphoma and the other a serious systemic infection during pre-treatment evaluation. The characteristics of the 39 evaluable patients are summarized in Table I.

Complete prior therapy records were available for 34 of the 36 previously treated patients. Of these, 29 (85%) had previously received purine analogue based chemoimmunotherapy. Thirteen (45%) patients were considered to be purine analogue refractory because they had not responded to (n=12), or had a PFS of less than one year following their most recent purine analogue containing chemoimmunotherapy regimen (n=1). Thirty-four (94%) patients had previously received rituximab-based therapy and 8 (22%) had previously received alemtuzumab.

TP53 was evaluated by FISH analysis for deletion in all patients and for mutations predicted to be dysfunctional in exons 4-9 by sequencing in 34 patients including all 15 patients with 17p13-. *TP53* mutations were detected in 18 (53%) of evaluated patients. Of these patients 13 (72%) also had 17p13- and were thus presumed to have loss of *TP53* function (*TP53* dysfunction) in the affected CLL cells. Five patients had *TP53* mutations without 17p13- and two had 17p13- without detection of a *TP53* mutation in the remaining allele. Two patients had both 17p13- and 11q22- in the same CLL clone as previously described [34], and of these, one also had a *TP53* mutation. *IGHV* analysis showed somatic hypermutation in three (23%) of 13 patients with both 17p13- and *TP53* mutation, and two of five patients without 17p13- who had *TP53* mutation. Both patients with 17p13- and no detected *TP53* mutation had unmutated *IGHV*.

Twenty one (54%) patients on this study had very-high risk CLL based on predicted loss of *TP53* function (n=13) or purine analogue refractory disease in the absence of evidence of *TP53* dysfunction (n=8) [1]. Of the 13 patients with purine analogue refractory CLL, 5 (38%) had both a *TP53* mutation and 17p13-, one had a *TP53* mutation without 17p13-, three had 11q22-, and three were not tested for *TP53* mutations.

Treatment

Twenty-six (67%) patients completed all planned therapy including two patients who completed therapy per protocol after two cycles because they had achieved an IHC negative CR. Of the 13 patients who did not complete the planned therapy, four patients received only one cycle, five received two cycles, and four started but did not complete the third cycle of therapy. The reasons for not completing therapy were disease progression (n=4), adverse events (n=5), treating physician and patient choice (n=1), financial hardship (n=1), change to alternative therapy regimen (n=1), and diagnosis of metastatic carcinoma (n=1). There were 28 therapy delays in 23 patients because of adverse events (n=24) and for administrative reasons (n=4). There were no dose reductions for alemtuzumab or rituximab. Pentostatin doses were omitted 17 times in 11 patients and reduced five times in five patients because of adverse events.

Adverse Events

There were 57 grade 3-4 adverse events at least possibly related to treatment in 27 patients. Of these 22 were neutropenia, 11 were thrombocytopenia, and 8 were infections/febrile neutropenia. Other grade 3-4 hematological events were anemia (n=2), autoimmune hemolytic anemia (AIHA)(n=2), hemorrhage (n=2), aplastic anemia (n=1), fatigue (n=3), fever (n=3), myalgia (n=1), hypotension (n=1) and hyperuricemia (n=1). There were no deaths attributable to treatment.

CMV reactivation occurred in 14 (36%) patients with a median viral load of 2,000 copies/ml (maximum 34,500), and was first detected in cycle 1 of therapy in 10 patients and cycle 2 of therapy in four patients. Eight patients had only one positive test for CMV viremia, five had positive tests for two sequential weeks, and one had positive tests for three sequential weeks. All patients were treated and there were no grade 3-4 events attributable to CMV reactivation, and no interruptions in treatment.

AIHA occurred for the first time in two patients during therapy with PAR. One patient developed AIHA in cycle 1 of therapy three days after his first dose of pentostatin and the other during cycle 3 of therapy. Both patients responded well to subsequent therapy with high dose methylprednisolone and rituximab.

Diffuse large B cell lymphoma (DLBCL, Richter's transformation) was diagnosed in 6 patients at a median time of 6.0 months (range 2.4–17.4) after initiation of PAR. Five of these patients had not responded to the PAR treatment regimen and one had achieved a CRi. Three of these patients had 17p13- and *TP53* mutations (*TP53* dysfunction) and one had a *TP53* mutation without 17p13- (one patient did not have *TP53* sequencing data). The DLBCL cells had the same immunoglobulin gene rearrangement as the patients CLL cells (clonal transformation) in all four patients tested. Only two patients had their DLBCL tissue tested for Epstein-Barr virus (EBV) by IHC and both were negative. Four patients developed DLBCL prior to any subsequent treatment for their CLL at a median time of 4.4 months (range 2.4–7.2 months). Two patients developed DLBCL after receiving subsequent treatment for progressive CLL. All six patients died from DLBCL with a median time from diagnosis of DLBCL to death of 9.3 months (5.2-19.3). No additional hematological malignancies were observed. One patient was diagnosed with metastatic carcinoma 3.2 months after initiation of the treatment regimen.

Of the five patients aged 70 years or older, two completed three cycles of therapy per protocol. Three patients received two cycles of therapy but did not receive the third cycle because of decisions by the patients and treating physicians based on the severity of cytopenias and social issues.

Response to Therapy

Twenty-two (56%, 95% CI 40-72) patients responded to therapy with 4 (10%) CR, 7 (18%) CR with incomplete bone marrow recovery (CRi), and 11 (28%) partial responses (PR). Seven (18%) patients had stable disease (SD), and 10 (26%) patients had progressive disease (PD) prior to response evaluation. Four patients (3 CR and 1 CRi) met the criteria for a stringent complete response (IHC negative bone marrow study). The median follow-up for

patients still alive was 35 months (range 3-58). Twenty-four patients have had progressive disease. The median progression free survival was 7.2 months (95% confidence interval (CI): 5.3,18.3) (**Figure 1A**). Twenty-one patients have received subsequent treatment for progressive CLL. In addition, four patients received additional treatment for their CLL at their physicians' discretion before meeting standard criteria for progression and were censored for time to re-treatment. Seven patients proceeded to planned reduced intensity allogeneic transplant after completing this treatment regimen. These patients had all responded to therapy (5 PR, 1 CRi, 1 stringent CR) and were censored for time to re-treatment. The median time to re-treatment was 9.1 months (95% CI: 5.7,27.0)(**Figure 1B**). Seventeen patients have died from progressive disease including DLBCL (n=14), infection (n=2, pneumonia, sepsis), and complications of RIC allotransplant (n=1). Median overall survival was 34.1 months (95% CI: 13.6, not reached)(**Figure 1C**).

Response in Patients with TP53 dysfunction—Seven (54%) of the 13 patients predicted to have loss of TP53 function (17p13- and dysfunctional *TP53* mutation) responded to therapy (3 CR/CRi, 4 PR). Of the 3 patients with a CR/CRi, one was censored at 6 months because of reduced intensity conditioning allogeneic hematopoietic stem cell transplant (RIC allotransplant). The other two patients, both of whom had mutated *IGHV* and one of whom had purine analogue refractory CLL, have not received subsequent treatment after being followed for 27.1 and 41.2 months. Of the 4 patients with a PR, two were previously untreated with 17p13- and were censored at 4.3 and 6.6 months because they underwent RIC allotransplant. The other two patients had subsequent treatment at 8.3 months and 27 months. The latter patient had mutated *IGHV*. One of the two patients with 17p13- without detectable *TP53* mutations had a PR and subsequently was censored at 5.4 months because of additional therapy including a RIC allotransplant. The other patient had PD and died from progressive CLL at 9.8 months. Two of the 5 patients with *TP53* mutations in the absence of 17p13- responded to therapy (CRi). Of these patients one with mutated *IGHV* had not yet been retreated at 46.8 months and the other had unmutated *IGHV* and required subsequent treatment at 7.5 months. Of the 3 patients who did not respond, one had mutated *IGHV*. Both patients with a CLL clone with 17p13- and 11q22- had PD before completing their PAR treatment.

Outcomes in patients with TP53 dysfunction (17p13- and *TP53* mutation, n = 13) were compared to the other patients on this clinical trial. There were no significant differences in the overall response rate (54% vs. 58%, p=1), CR/CRi (23% vs. 31%), TTT (median 9.1 vs. 8.3 months, p=0.92) or OS (median 16.8 months vs. not reached, p=0.20) (**Supplementary Figure 1A**). The comparison of patients with 17p13- to those without 17p13- showed no significant differences in these parameters (data not shown).

Response in Patients with Purine Analogue Refractory CLL—Seven (54%, 95% CI:25,81) of the 13 patients with purine analogue refractory CLL responded to therapy with 4 CR/CRi and 3 PR. This was not significantly different from the 56% response rate (95%CI: 30,80) among the 16 patients responsive to their most recent purine analogue containing treatment regimen. Of the seven (54%) purine analogue refractory patients who had no detectable *TP53* defects, all had unmutated *IGHV* and 3 had 11q22-. Five of these

patients responded to treatment (2 CRi, 3 PR). Three patients were censored because of subsequent treatment at 4.2, 6.8, and 8.5 months. The TTT in the other four patients ranged from 1.3–11.4 months and three patients have died. There was no difference between purine analogue refractory and sensitive patients for TTT (median 9.1 vs. 25.9 months, $p=0.97$) or OS (18.3 months vs. not reached, $p=0.27$)(**Supplementary Figure 1B**).

Response in Patients with Very High Risk CLL—CLL patients with TP53 dysfunction or purine analogue refractory disease in the absence of detectable TP53 dysfunction comprise a cohort with a very high risk of disease progression and poor prognosis. Patients with very high risk CLL ($n=21$) did not have a significantly different TTT (median 9.1 months vs. 8.3 months, $p=0.90$) or OS (median 18.3 months vs. not reached, $p=0.18$) (**Supplementary Figure 1C**) compared to the 18 patients who did not have very high risk CLL.

Response in Patients with Previous Alemtuzumab Therapy—Three of the 8 patients previously treated with alemtuzumab responded to treatment (all PR).

Response in Patients 70 years Old—Of the 5 patients 70 years or older ($n=5$), three responded to treatment (1 CR, 2 PR). The median TTT in this older population was 8.3 months (range 2.7–33.9), three patients died at 7.1, 10.8, and 23.4 months after initiation of PAR and two were alive at 50.8 and 58.0 months.

Correlative studies

Effect of low dose rituximab on CD20 expression and complement activation

—We studied all 26 patients with a baseline ALC $>5 \times 10^9/L$ who had not received rituximab therapy for at least one month prior to starting the PAR regimen and had the appropriate specimens collected per protocol. The results from evaluation of the effects of IV infusion of low doses (20 mg/m²) of rituximab on normalized levels of ALC ($n=14$) and of CD20 on circulating CLL cells ($n=26$) extends and generalizes our previous report in which only six patients received low dose rituximab therapy [17]. The results of infusion of rituximab on day 1 were still evident on day 3, as manifested by decreases in ALC, based on measurements in blood samples (Day 3 Pre) taken just before the second rituximab infusion ($p<0.001$)(**Figure 2A**). Moreover, the *second rituximab infusion* (on day 3) led to rapid and substantial decreases in ALC; on average ALC were reduced to 30% of the respective values before initiation of therapy ($p<0.001$)(**Figure 2A**). The first 3 mg dose of alemtuzumab was injected SQ on day 3 after the rituximab infusion, but it is unlikely that this low SQ dose had an immediate effect on ALC. Moreover, although rituximab was also infused on day 5, and alemtuzumab was administered SQ on days 4 and 5 (10 mg and 30 mg, respectively), by day 8 there was a modest, but significant *increase* in ALC ($p<0.05$)(**Figure 2A**), very likely reflecting re-equilibration, between day 5 and day 8, of additional CLL cells into the bloodstream from other compartments [17, 35]. Although cells were indeed cleared from the bloodstream on day 3 (**Figure 2A**, Day 3 Post), we observed simultaneous *loss of CD20* from surviving circulating cells (**Figure 2B**), and the % of CD20 lost was *independent* of the starting CD20 levels for a given patient CLL cell population ($p=0.733$ for fresh samples and $p=0.467$ for PBMC, Spearman Rank Order correlation). Thus, as we reported previously,

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based on clinical studies [27, 28] and in vitro models [30, 36], we find that both trogocytosis (“shaving”) of CD20 and clearance (both processes likely mediated by fixed tissue macrophages [30, 36–38]) occurs simultaneously for populations of circulating CLL cells that are opsonized with anti-CD20 monoclonal antibodies such as rituximab. In order to test for additional validation of this idea, we also examined surviving CLL cells for deposition of C3d fragments. It is well established that cells that are opsonized with rituximab should activate complement, thus allowing in vivo covalent tagging with C3b fragments which will rapidly decay to C3d [27, 28, 39]. In the present low-dose rituximab paradigm, if these C3d-tagged cells are not cleared, but CD20 is *partially removed*, then evidence that the cells *must have been previously opsonized with rituximab* would be the “residual” covalently bound C3d. Indeed, we find that among the surviving CLL cells, significant amounts of C3d fragments are indeed demonstrable (**Figure 2C**). We examined CLL cells collected on day 3 after therapy (Day 3 Post) for different patients, and we also find that there is a linear correlation between the amount of deposited C3d on the cells and the residual levels of CD20 (correlation coefficient=0.833, $p=0.005$) (**Figures 2D,E**). Moreover, dot plots for CLL cells analyzed in these Day 3 Post samples from two representative patients also reveal an approximately linear correlation between the amount of deposited C3d and residual levels of CD20 (**Figure 2F**). This is reasonable because within a given CLL cell population, the cells with the most CD20 *before* rituximab infusion will have the most CD20 after, because the % of CD20 loss is not dependent on starting CD20 levels, and higher starting levels of CD20 should of course presage more complement activation and more C3d deposition. Thus, even though CD20 is reduced, the “signature” on cells that had been opsonized with rituximab will be residual CD20 along with deposited C3d. Finally, it is important to note that by day 8, expression of CD20 on circulating cells had increased compared to the day 3 post (**Figure 2B**), indicating that the lower rituximab doses had less profound long-term effects on CD20 levels than has been found for the standard 375 mg/m² dose of rituximab (>90% loss of CD20 expression) [17, 27]. In addition, because the CD20 target is “restored” on circulating cells, the next rituximab dose would, as previously reported, be expected to continue to target and clear the CD20 positive cells [17].

We also probed the CLL cells from the Day 3 post treatment samples with the monoclonal anti-human Fc antibody HB43 in order to determine if any rituximab was still bound to the cells soon after completion of the second rituximab infusion. Of 23 patient samples that could be evaluated, 15 had no measureable bound rituximab, but 8 had modest level of rituximab bound, corresponding to $16\pm7\%$ ($p<0.001$) of the maximum binding that was possible, based on reacting naïve CLL cells (sample 1) with excess rituximab (or ofatumumab) followed by monoclonal antibody HB43 [28].

Several lines of evidence suggest that complement control proteins (CCP) on cancer cells are likely to promote resistance of these cells to monoclonal antibody-based therapies that depend upon complement activation to promote cell elimination [40–43]. Indeed, under certain conditions CCP might be up-regulated in response to complement-dependent therapies; that is, cells under attack by rituximab and alemtuzumab that express the highest levels of CCP are more likely to survive and this could lead to evolution of more CDC-resistant phenotypes. Therefore, we examined levels of the three key CCP on CLL cells:

CD46, CD55 and CD59, before, and after the rituximab and alemtuzumab infusions. Our results (**Supplementary Figures 2A-C**) indicate that soon after infusion of rituximab there appear to be small but significant decreases in expression of these proteins on surviving CLL cells. These results may be most readily explained based on “innocent bystander” decreases in the levels of one or more of these proteins, which can accompany trogocytosis of CD20 mediated by rituximab [44-46] However, by day 8, after the three infusions of rituximab and alemtuzumab, expression of the three CCP had increased modestly and had returned to close to baseline levels, following the general pattern seen for CD20.

Effect of low dose rituximab on NK cells—We have previously shown in vitro that rituximab-coated target cells can induce changes in NK cell phenotype consistent with NK cell activation including a rapid down-modulation of CD16 and up regulation of a number of markers including CD54 [47]. In vivo studies confirmed these changes take place within four hours of therapy with standard dose rituximab, and also are associated with trafficking of NK cells out of the circulation [48]. In this study 8 patients had suitable blood samples taken before and 48 hours after the initiation of RTX therapy with an NK count of at least 0.5% of mononuclear cells in the pre-therapy sample. For logistical reasons, the post rituximab sample was obtained 48 hours after the initiation of the rituximab infusion. As illustrated in **Figure 3**, low dose rituximab therapy resulted in a significant decrease in NK cell CD16 expression ($p=0.001$) (**Figure 3A**), up regulation of surface CD54 ($p=0.04$) (**Figure 3B**), and a reduction in NK cell numbers ($p<0.0001$)(**Figure 3C**), which indicates NK cell activation by treatment with low dose rituximab.

Discussion

We report the results of treatment of patients with high risk and relapsed/refractory CLL using a novel chemoimmunotherapy regimen combining a purine analogue with alemtuzumab and low dose rituximab. This regimen was relatively well tolerated with 67% patients receiving the planned duration of therapy. Only 5 (12.8%) patients did not complete therapy because of treatment related adverse events and there were no treatment related deaths. The overall response rate was 56% of patients with half of the responders achieving a CR/CRi. Despite the short median progression free survival, the median overall survival was 34 months. In addition, patients with very-high risk CLL as defined by TP53 dysfunction or purine analogue refractory disease ($n=21$) had similar responses to treatment compared to those patients without these adverse characteristics. The correlative laboratory studies showed that the low dose rituximab regimen activated both complement and NK cells and resulted in more limited loss as well as more rapid recovery of CD20 expression by CLL cells compared to that previously demonstrated for treatment with standard dose rituximab.

In this small study the CR/CRi rate of 28% was lower than the 30% considered the smallest success proportion that would warrant subsequent studies with the regimen. However, the response to treatment compared favorably with the results of recently reported previous studies in similar patients using other alemtuzumab based combination therapies [15, 49]. The toxicity of the PAR regimen appears to have been less severe than that previously reported for CLL treatment regimens including fludarabine and alemtuzumab used either

concomitantly [13-15, 50] or sequentially [51, 52]. Factors contributing to the lower toxicity of the PAR regimen could have included required prophylactic antimicrobial therapy and CMV monitoring with treatment of CMV reactivation, and the limited duration (maximum of 3 months) of alemtuzumab therapy.

Diffuse large B cell lymphoma was subsequently diagnosed in 6 (15%) patients treated with PAR and was an important cause of treatment failure and mortality. The study population had a high frequency of advanced stage disease (59%), TP53 dysfunction (33%), 11q22- (15%), unmutated *IGHV* (69%), and ZAP70 expression (72%), all of which are associated with an increased risk of DLBCL in patients with CLL [53, 54]. Although treatment of CLL with alemtuzumab [55] and purine analogues [56] has also been reported to be associated with development of DLBCL, the role of these drugs in the development of DLBCL remains unclear. In this study, the median interval between starting PAR therapy and the diagnosis of DLBCL was only 4.4 months from start of PAR in patients who did not receive subsequent therapy for their CLL. This short interval suggests that DLBCL developed prior to the start of PAR therapy and the treatment of the CLL could have accelerated the diagnosis of the DLBCL rather than being a causative factor in these patients. Of note, a similar high rate of DLBCL was recently reported in patients with relapsed/refractory CLL treated with the Bruton's tyrosine kinase inhibitor ibrutinib [57]. The high rate of DLBCL in our study population is clearly a serious problem, but we cannot determine whether or not PAR therapy contributed to this complication.

There is now substantial evidence that use of conventional doses of rituximab (375 – 500 mg/m²) for the treatment of CLL results in a considerable decrease in CD20 expression by the circulating CLL cells, which results in a loss of efficacy of this drug [17, 18]. In this study, we tested the effect of higher frequency, low dose IV rituximab on expression of CD20 by circulating CLL cells and on the activation of NK cells. Our correlative measurements confirm that at rituximab doses as low as 20mg/m², cells undergo three separate and interrelated fates. These doses provide sufficient rituximab to opsonize and clear the majority of circulating cells, because at the end of the infusion on day 3, ALC levels are reduced considerably (**Figure 2A**). However, the surviving cells do suffer some loss of CD20 and are also covalently tagged with C3d, indicating that they were also opsonized with rituximab, but were not cleared (**Figures 2B,C**). Indeed, as demonstrated previously, both clearance and loss of expression of CD20 appear to occur simultaneously, and both processes are mediated in part by the same effector cells, fixed tissue macrophages which express Fcγ receptors [30, 36-38]. We also can confirm that loss of CD20 is less pronounced under these low-dose rituximab conditions, and that considerable restoration of CD20 on circulating cells is readily seen based on analyses of blood samples on day 8 (**Figure 2B**). Moreover, we found that complement was activated based on the appearance of C3d-tagged cells, and the present studies, although limited in time, suggest that CCP are not up-regulated in response to rituximab or alemtuzumab therapy (**Supplementary Figure 2**). We also found that cells with the most residual CD20 had the greatest amount of deposited C3d. This analysis cannot be performed after infusion of large quantities (> 300 mg) of rituximab or ofatumumab, because under these conditions loss of CD20 reaches values in excess of 90% [27, 28, 35]. Our correlative studies also demonstrated that a single,

low dose (20 mg/m² IV) of rituximab therapy can induce activation of NK cells within two days of therapy as indicated by down-modulation of CD16, upregulation of CD54 and a decrease in the number of circulating NK cells (**Figure 3**). These findings are similar to those found in our prior studies exploring the effect of full dose rituximab on NK cell activation despite a number of differences between the current and prior studies including dose of rituximab (20mg/m² vs. 375mg/m²), patients (CLL versus lymphoma without significant numbers of circulating B cells) and timing of the post-rituximab sampling (48 hours versus 4 hours)[48]. Together, this data suggests the short-term effect of low dose rituximab on NK cell activation is similar to that seen with standard dose therapy.

The strengths of our study are detailed characterization of the biology of the patients' malignant B cells, and the comprehensive follow up of all patients after completion of therapy. The study design allowed for collection of serial blood samples that could address important questions about the optimal dosing of anti-CD20 monoclonal antibodies. The limitations of this study include the relatively small number of patients and the limited data on TTT because of early initiation of subsequent treatment, especially in patients proceeding to planned RIC allogeneic hematopoietic stem cell transplant. This limitation is especially important with regard to determining the benefit of PAR therapy in CLL patients with very high risk disease, where the lack of differences in outcome could reflect the small sample size rather than a true equivalence of treatment efficacy.

In conclusion, PAR is effective and tolerable therapy that could be of value in management of patients with very-high risk CLL who fail targeted tyrosine kinase inhibitors, and in patients requiring disease control before therapy with allogeneic transplant or chimeric antigen receptor therapy. This study showed that low dose rituximab activates components of the innate immune system known to be important in its anti-CLL effect without substantially decreasing CD20 expression by circulating CLL cells after treatment. This finding strongly supports the conduct of clinical trials comparing higher frequency low dose and standard dose anti-CD20 monoclonal antibody therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Response to Therapy

The median progression free survival was 7.2 months (95% confidence interval (CI): 5.3;18.3) (A) and 21 patients received subsequent treatment for progressive CLL with a median time to next treatment of 9.1 months (95% CI: 5.7; 27.0)(B). The 11 patients who received subsequent treatment before disease progression are censored and 7 patients did not receive subsequent treatment. Median overall survival was 34.1 months (95% CI: 13.6; not reached)(C).

Figure 2. Effects of rituximab infusion on absolute lymphocyte count, CD20 levels, and C3d deposition

A. Absolute lymphocyte counts (ALC) are reduced as a result of rituximab therapy (20 mg/m²/dose) on day 1, day 3 and day 5 with additional therapy with alemtuzumab on days 3, 4 and 5. All values are normalized to the pre-infusion levels on day 1 for each patient. There was a decrease in ALC 48 hours after the first rituximab infusion, and a further decrease was observed over the course of day 3 treatment with a second rituximab infusion, followed by a small increase on day 8, despite the additional monoclonal therapy on days 4 and 5. Initial ALC varied between 9.8 and $240.0 \times 10^9/L$ (mean $63 \times 10^9/L$). All post treatment normalized ALC values were significantly lower than pre-treatment values ($p < 0.001$).

B and C. Infusion of rituximab leads to transient reduction of CD20 levels (B), and to deposition of C3d fragments on surviving, circulating cells (C). Normalized CD20 and C3d levels are based on analyses of fresh washed whole blood (open circles) or of PBMC which were isolated and then frozen (closed circles) for later analyses. The reduction in CD20 is most evident in the day 3 post-treatment samples. By day 8, CD20 levels had increased, even though rituximab was also infused on day 5. C3d levels are reported as the normalized increase relative to the values for day 1 pre-treatment samples.

D and E. The amount of C3d deposited on the cells of patients in the Day 3 post samples is positively correlated with the levels of CD20 on the cells at that time point. In this case MESF values are reported, and the signals are not normalized. D. filled circles: PBMC samples; E. open circles: Fresh samples, Analyses based on Spearman rank correlation gave a correlation coefficient of 0.833 and $p = 0.00526$ for both plots.

F. Within the CLL cell populations of individual patients, in the day 3 post-treatment samples there is a correlation between the amount of C3d deposited and the levels of CD20. Panel i is a plot for cells in a pre-treatment sample. Panels ii and iii are plots for Day 3 Post samples for two different patients. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Figure 3. Effect of Low Dose Rituximab on NK Cells

Treatment of patients with low dose rituximab resulted in significant NK cell activation, as evidenced by significant down modulation of surface CD16 expression (A) ($p=0.001$) and up regulation of surface CD54 expression (B) ($p=0.04$). All values are expressed as median fluorescence intensity (MFI). Circulating NK cell percentages showed a significant drop following therapy with low dose rituximab (C) ($p<0.0001$). NK cell percentages of post-therapy samples were normalized to that of respective pre-therapy samples.

Table I

Age	
Median (Min, Max)	61 (47, 78)
Sex	
Male	30 (77%)
PS	
0	32 (82%)
1	7 (18%)
Disease group	
Relapsed or Refractory CLL	36 (92%)
Untreated CLL with 17p13del	3 (8%)
Number of previous regimens	
Median (Min, Max)	2 (0, 10)
Rai Stage	
1	7 (18%)
2	9 (23%)
3	6 (15%)
4	17 (44%)
CD38	
Positive (30%)	10 (26%)
Negative (<30%)	27 (69%)
Unknown	2 (5%)
Mutation	
Mutated (2%)	11 (28%)
Unmutated (<2%)	27 (69%)
Unknown	1 (3%)
ZAP-70	
Positive (20%)	28 (72%)
Negative (<20%)	9 (23%)
Unknown	2 (5%)
CD49d	
Positive (45%)	14 (36%)
Negative (<45%)	19 (49%)
Unknown	6 (15%)
FISH [*]	
17p	15 (38%)
11q	6 (15%)

trisomy 12	5 (13%)
13q	8 (21%)
Other	2 (5%)
None	3 (8%)
<hr/>	
TP53 mutation (exons 4-9)	
Mutation predicted to be dysfunctional	18 (46%)
No mutation	16 (41%)
Unknown	5 (13%)

* Hierarchical classification[58]