

The Addition of Rituximab to Fludarabine Improves Clinical Outcome in Untreated Patients with ZAP-70-Negative Chronic Lymphocytic Leukemia

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BACKGROUND. Clinical trials of monoclonal antibodies in combination with chemotherapy have reported previously unattained response rates in patients with B-cell chronic lymphocytic leukemia (B-CLL); however, the analysis of ZAP-70 protein and/or CD38 may explain better the discordant outcomes independent of treatment.

METHODS. The authors conducted a Phase II study, in which rituximab was added to fludarabine for patients with symptomatic, untreated CLL, to evaluate clinical outcomes. Sixty patients with B-CLL received 6 monthly courses of fludarabine (25 mg/m² for 5 days) followed by 4 weekly doses of rituximab (375 mg/m²).

RESULTS. On the basis of National Cancer Institute criteria, 47 of 60 patients (78%) achieved a complete remission, 9 of 60 patients (15%) achieved a partial remission, and 4 of 60 patients (7%) had no response or progressive disease. It is noteworthy that the patients experienced a long progression-free survival (PFS) from treatment (68% at 3 yrs). A significantly shorter PFS was observed in ZAP-70-positive patients (25% vs. 100% at 3 yrs; $P = 0.00005$), in CD38-positive patients (18% vs. 91% at 3 yrs; $P = 0.0002$), and in patients who had more minimal residual disease (36% vs. 77% at 2.5 yrs; $P = 0.001$).

CONCLUSIONS. With the addition of rituximab to fludarabine, improved clinical outcomes were obtained, and the stratification of patients by using ZAP-70 and CD38 may help clinicians offer more aggressive and/or experimental approaches to the treatment of patients with high-risk B-CLL subtypes. *Cancer* 2005;104:2743–52. © 2005 American Cancer Society.

KEYWORDS: B-cell chronic lymphocytic leukemia, treatment, fludarabine, rituximab, ZAP-70, CD38, minimal residual disease, response, outcome.

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and is characterized by the relentless accumulation of functionally abnormal B cells. Despite the > 10-year life expectancy in early stages, patients who have progressive or advanced-stage CLL die rapidly, with a median survival between 18 months and 3 years.^{1,2} Promising results were observed when fludarabine was introduced for patients who were treated with alkylator agents and for patients who had symptomatic, untreated CLL.^{3,4} Although longer progression-free survival (PFS) was reported for the fludarabine arm in three randomized studies,^{5–7} significant improved survival with fludarabine therapy has not been observed to date in patients with CLL. There is an interest in developing new combination therapies for CLL with agents that can be added to fludarabine to increase the complete remission (CR) rate, because disease recurrence in patients with CLL most probably is the result of residual

mon Toxicity Criteria. Infusion toxicity was assessed according to the criteria previously described by Byrd et al.¹⁶ Patients who experienced Grade 3 or 4 neutropenia/thrombocytopenia or Grade 2 anemia were supported with growth factors (granulocyte-colony stimulating factor and erythropoietin α) and remained without treatment until these hematologic parameters recovered within 50% of baseline values. Thereafter, these patients received the same original dose. There were no dose reductions for rituximab therapy because of hematologic toxicity. Acute infusion toxicity after rituximab administration that was reversible did not require subsequent dose reduction of this agent. At the onset of fever, chills, rigors, or other infusional reactions, patients had their infusion discontinued and received an additional dose of diphenhydramine (50 mg) and acetaminophen (650 mg). After the resolution of symptoms, the rituximab infusion was restarted at a rate of 50 mg per hour and then was escalated, as tolerated, to 200 mg per hour. Patients who developed an infection were observed without further CLL treatment until the infection had resolved, but no dose reductions were implemented. Nonhematologic toxicities, including nausea, emesis, fatigue, diarrhea, and drug-related fever and chills, required no dose reductions.

Patient Monitoring and Response Evaluation

Baseline assessment included disease history, current disease stage, B symptoms, and measurement of lesions by physical examination and radiography (chest X-ray, computed tomography scan, ultrasound). The histologic examination of representative material (bone marrow biopsy and aspirate, immunophenotype of peripheral blood) had to be performed at least 6 months before enrollment. Laboratory testing involved CBC with differential, flow-cytometric phenotyping of lymphocytes of the peripheral blood, and clinical chemistry, including immunoglobulins (Ig), β_2 -microglobulin, and Coombs test.

Patients were assessed for response 4–6 weeks after the last rituximab infusion with a detailed clinical evaluation (physical examination with measurement of lymph nodes, liver, and spleen; CBC with differential; bone marrow biopsy and aspirate). Criteria for response used the revised 1996 NCI Working Group guidelines.²⁷ A CR or partial remission (PR) had to be maintained for ≥ 8 weeks. PFS was defined as the time from the end of treatment in the subgroup of patients who achieved a CR or PR until the last follow-up, the time at which progression occurred or further therapy for symptomatic CLL was required, or death that occurred from any cause. Survival was measured from

the date of initiation of treatment either until the last time the patient was seen alive or until death.

Sample Size Determination

A single-stage design according to the method of Fleming²⁹ was selected based on the following assumptions: 1) The treatment in this study would not be considered sufficiently effective if the global response rate (CR plus PR) was $< 60\%$. 2) The combination treatment with fludarabine and rituximab would be regarded as very promising for a Phase III comparative trial if the true response rate exceeded 80% . 3) The probability of erroneously declaring the drug active for further investigation, despite a true response rate $< 60\%$ (type I error), was set at 5% . 4) The probability of erroneously rejecting the therapy as not sufficiently active ($< 60\%$) if there was a promising true response rate ($> 90\%$) was 10% (type II error; power = 90%).

With regard to feasibility and tolerability, a failure rate of up to 10% was considered acceptable, and the sample size for the evaluation of treatment overall efficacy was set to 60 patients. Moreover, because a major objective of the study was to detect the role of classification of patients into groups of different sizes dichotomized according to specific biologic predictors, such as ZAP-70, CD38, and MRD, specific post-hoc power calculations were performed for PFS on any grouping, based on the number of events and on the hazard ratio between groups at the end of the observed time, according to the method described by Norman and Streiner.³⁰

Cellular Immunophenotypic Analysis

The following antibody conjugates were used: anti-CD23-phycoerythrin (PE), anti-CD5-fluorescein isothiocyanate (FITC), anti-CD38-PE, anti-CD19-allophycocyanin (APC), anti-CD45-FITC, anti-CD14-PE, anti-CD95-PE, and anti-CD10-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA). Peripheral blood mononuclear cells were analyzed for surface expression of CD19/CD5/CD38 and CD19/CD5/CD23 by triple-color immunofluorescence, as described previously.²⁰

ZAP-70 protein determination also was performed by flow cytometry, as described elsewhere.²³ Flow-cytometric analyses were performed on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems), and CellQuest software was used to acquire and analyze data. The threshold of positivity both for CD38 and for ZAP-70 was set at $> 20\%$ of CD19-positive/CD5-positive B-cells.

MRD Flow-Cytometric Study

Between 50,000 and 300,000 total cells were analyzed in each test that was performed in bone marrow approximately 1–2 months after the completion of combined therapy. The antibodies used were anti-CD5-APC, anti-CD20-PE, anti-CD19-peridin-chlorophyll protein, anti-CD38-PE, anti-CD20-FITC (Becton Dickinson Immunocytometry Systems) and anti-CD79b-FITC (Serotec, Oxford, United Kingdom). The 2 combinations CD19/CD5/CD20/CD79b and CD19/CD5/CD38/CD20 allowed to discriminate B-CLL cells from normal B cells in all samples, as described previously.²⁶ The threshold for positivity was set at > 5% CD19-positive/CD5-positive/CD79b-negative CLL cells.

Interphase Fluorescence in Situ Hybridization

Separate hybridizations were carried out for loci on chromosomes 11, 12, 13, and 17. For chromosomes 11 (q23), 13, and 17, commercial probes (ATM-2, *Rb-1*, and *p53*, respectively) were used (Vysis Inc., London, UK). An α satellite DNA probe, CEP12, which was labeled directly with SpectrumGreen, was used to detect aneuploidy of chromosome 12. LSIp53 labeled with SpectrumOrange (Vysis Inc.) was used to evaluate chromosome deletion at 17p13.1. The procedure used was described previously.³¹

IgV_H Mutation Analysis

Total RNA was extracted and reverse transcribed, as described previously.³² The resulting cyclic DNAs, which were checked for first-strand synthesis, were amplified using a mixture of sense primers and annealing either to the V_H1–V_H6 leader sequences or to the 5' end of V_H1–V_H6 FR1, as reported elsewhere.^{33,34} These primers were used in conjunction with a mixture of antisense primers complementary to the germline J_H regions. The purified, amplified products, which were inserted into the PCR2.1-TOPO vector (Invitrogen, Milan, Italy), were expanded in TOP10 One Shot™ competent cells (Invitrogen) and cloned. Plasmid DNAs were isolated from overnight cultures of randomly selected colonies and were sequenced by using an automatic DNA sequencer (Beckman CEQ2000XL; Beckman Coulter, Hialeah, FL). Comparisons between the obtained sequences and the sequences of the various germline IgV_H genes were performed with the IgBLAST directory (<http://www.ncbi.nlm.nih.gov/igblast>) using Mac Vector 7.1 sequence-analysis software (Accelrys; Symantec, San Diego, CA). Only when the same V_HDJ_H rearrangement was identified in at least 5–10 clones was a given IgV_H sequence analyzed further. Alignment of the IgV_H sequences available for each patient often revealed,

TABLE 1
Patient Characteristics (n = 60)

| Characteristic | No. of patients (%) |
|----------------------------------|---------------------|
| Median age in yrs (range) | 59 (37–74) |
| Male gender | 30 (50) |
| Rai modified stage | |
| Low | 5 (8) |
| Intermediate | 52 (87) |
| High | 3 (5) |
| ECOG performance status | |
| 0 | 37 (62) |
| 1 | 19 (32) |
| 2 | 4 (6) |
| B symptoms | 17 (28) |
| Time since first diagnosis | |
| ≤ 1 yr | 17 (29) |
| 2–5 yrs | 29 (48) |
| > 5 yrs | 14 (23) |
| Bone marrow infiltration pattern | |
| Nodular | 4 (7) |
| Mixed | 21 (35) |
| Diffuse | 35 (58) |

ECOG: Eastern Cooperative Oncology Group.

along with mutations shared by all the transcripts analyzed, several unique or partially shared mutations. For this reason, all mutational analyses were carried out in each IgV_H transcript separately, and the percentage mutation assigned to a given B-CLL was the mean value of the percentage of mutation found in each transcript. V_H gene sequences that deviated > 2% from the corresponding germline gene were defined as mutated.

Statistical Analysis

For the efficacy analysis, patients had to have received at least one cycle of therapy. All patients who had received at least one application of study therapy also were evaluable for toxicity. Rate comparisons between patients subgroups were performed by using the two-tailed Fisher exact test. Event-related data (survival and PFS) were estimated by using the Kaplan–Meier product-limit method. Prognostic subgroups were compared by using the log-rank test. Statistical analyses and Kaplan–Meier curves were performed using STATA 8.0 and Statistica® 6.0 for Windows.

RESULTS

Patient Characteristics

In total, 60 patients were enrolled in the current study between December 1997 and March 2004. The pretreatment features of these patients are summarized in Table 1. The median age was 59 years (range, 37–74 yrs). All patients met the guideline criteria for having

TABLE 2
Hematologic Toxicity During the Treatment Period

| Toxicity | No. of patients (%) | |
|------------------|---------------------|---------------|
| | NCI Grade 1-2 | NCI Grade 3-4 |
| Anemia | 3 (5) | 0 (0) |
| Neutropenia | 12 (20) | 29 (48) |
| Thrombocytopenia | 4 (7) | 3 (5) |

NCI: National Cancer Institute.

CLL.²⁷ According to Rai staging criteria,¹ 5 patients (8%) had low-risk (Stage 0) CLL, 52 patients (87%) had intermediate-risk (Stage I or II) CLL, and 3 patients (5%) had high-risk (Stage III or IV) CLL. In this modified classification scheme, patients with intermediate-risk CLL have lymphadenopathy or hepatosplenomegaly without significant cytopenias, whereas patients with high-risk CLL have anemia or thrombocytopenia. Much greater than one-half of patients (62%) had a good ECOG performance status of 0. The time from diagnosis ranged from 1 year to 10 years. Only 28% of patients exhibited B symptoms. Finally, the histologic patterns of bone marrow infiltration were mainly diffuse (58%) or mixed (35%).

Toxicity

All patients were evaluable for toxicity. The three most frequent side effects were infusion-related toxicity, myelosuppression, and infections. Five of 58 patients who were treated with rituximab experienced a mild, infusion-related symptom complex, which consisted of fever, chills, and rigors, during the first infusion. There were 13 opportunistic infections, including 3 dermatomal herpes zoster infections, 7 localized herpes simplex infections, 2 episodes of Grade 3 pulmonary toxicity (isolated interstitial pneumonitis and Candida pneumonia), and 1 incident of fulminant B hepatitis. The reported infectious toxicity was confined to the first 12 months of therapy, but no significant opportunistic infections were noted after this period in patients observed in CR or PR without further treatment. Hematologic toxicity (Table 2) included neutropenia (Grade 1 and/or 2 in 12 patients, Grade 3 and/or 4 in 29 patients), thrombocytopenia (Grade 1 and/or 2 in 4 patients, Grade 3 and/or 4 in 3 patients), and anemia (Grade 2 in 3 patients). In particular, Grade 3 and 4 neutropenia was observed in 25 of 60 patients (41%) during fludarabine infusions and in 9 of 58 patients (16%) during rituximab treatment. Thrombocytopenia and anemia were noted only during fludarabine cycles.

TABLE 3
Comprehensive Response Rate According to Baseline Patient Features (n = 60)

| Variable | No. of patients (%) | | | | |
|-----------------------------|---------------------|--------|-------|-------|-------|
| | OR | CR | PR | SD | PD |
| Total group (n = 60) | 56(93) | 47(78) | 9(15) | 1(2) | 3(5) |
| Low stage (n = 5) | 5(100) | 5(100) | — | — | — |
| Intermediate stage (n = 52) | 49(94) | 41(79) | 8(15) | — | 3(6) |
| High stage (n = 3) | 2(66) | 1(33) | 1(33) | 1(33) | — |
| No B-symptoms (n = 43) | 43(100) | 42(98) | 1(2) | — | — |
| B-symptoms (n = 17) | 13(76) | 5(29) | 8(47) | 1(8) | 3(16) |

OR: overall response; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

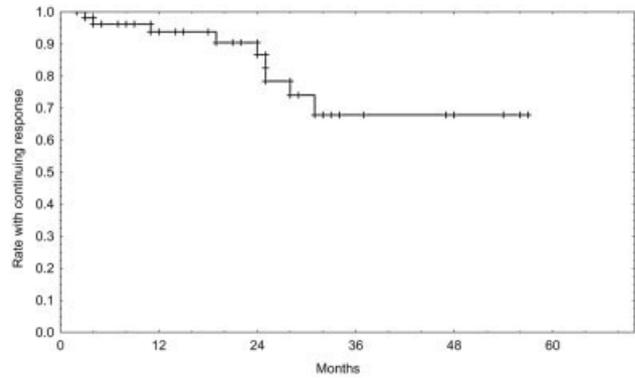


FIGURE 2. Kaplan–Meier analysis of progression-free survival showed the proportion of 56 patients who had a response to treatment and remained in complete or partial remission. The estimated 3-year progression-free survival rate was 68%, and 9 of 56 patients (16%) experienced a recurrence.

Response to Treatment and Treatment Outcome

All patients enrolled on this study were evaluable for response. Response evaluation occurred at least 1 month after the completion of fludarabine therapy (induction response) and then again 2 months after the completion of all therapy (fludarabine plus rituximab). The response rate included an induction CR rate of 70% and an overall response rate of 92%. Of 13 patients who had a PR after induction who received rituximab, 4 patients (31%) had a conversion to a CR. The comprehensive response rate included a 78% CR rate and an overall response rate of 93% (95% confidence interval [95% CI], 84–98%) (Table 3). Only 1 patient with stable disease after fludarabine therapy had a conversion to a CR after receiving rituximab therapy. Outcome data relative to PFS and OS are shown in Figures 2 and 3. After a median of 27 months of follow-up, 9 of 56 patients (16%) experienced a recurrence. The estimated 3-year PFS rate was 68% (95% CI, 46–83%) (Fig. 2). Among the 60 patients

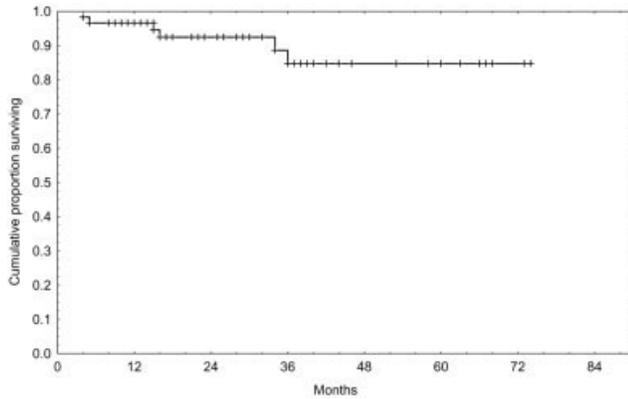


FIGURE 3. Kaplan–Meier analysis of overall survival included all 60 patients with chronic lymphocytic leukemia who were treated with a sequential fludarabine and rituximab regimen. Six patients died either from progressive disease or from opportunistic infections.

enrolled in this study, 6 patients died. One patient died in CR of fulminant B hepatitis, two other patients died of progressive CLL that was resistant to fludarabine induction, and the other three patients died of progressive CLL after they received the prescribed protocol therapy.

Clinical and Biologic Features Predicting Response and Outcome

We examined whether age, gender, or β_2 -microglobulin level predicted a CR during fludarabine plus rituximab therapy. A higher CR rate was not correlated with gender, age, or β_2 -microglobulin level ($P > 0.30$ for all variables). Conversely, there was a trend toward a correlation between the CR rate and the modified Rai stage, because all patients with low Rai stage CLL ($n = 5$ patients) achieved a CR ($P = 0.05$).

Twenty-eight patients (46.7%) had ZAP-70 expression levels $>$ higher than 20% (range, 4–65%), and 19 of 60 patients (31.7%) presented CD38 levels $>$ 20% (range, 1–66%). Tests for MRD, which were performed on bone marrow by flow cytometry after the protocol therapy, were positive ($>$ 5% CD19-positive/CD5-positive/CD79b-negative CLL cells) in 15 of 60 patients (25%). Forty patients were analyzed by interphase fluorescence in situ hybridization (FISH): Twenty patients (50%) had a normal karyotype, 14 patients (35%) had a 13q– abnormality, 4 patients (10%) had trisomy 12, 1 patient (2.5%) had 11q–, and 1 patient (2.5%) had 17p–. No significant correlation was found between these cytogenetic abnormalities and ZAP-70 or CD38 expression levels. Thirty-two patients were analyzed for IgV_H mutations: Fifteen of 16 patients with IgV_H mutations $<$ 2% presented with ZAP-70 protein levels $>$ 20% ($P = 0.0006$). Moreover, the strict corre-

TABLE 4
Comprehensive Response Rate According to ZAP-70 and CD38 Expression

| Expression status | No. of patients (%) | | | | |
|------------------------------------|---------------------|---------|-------|------|-------|
| | OR | CR | PR | SD | PD |
| ZAP-70 | | | | | |
| Positive ($n = 28$) ^a | 24(86) | 16(57) | 8(29) | 1(4) | 3(10) |
| Negative ($n = 32$) | 32(100%) | 31(97%) | 1(3) | — | — |
| CD38 | | | | | |
| Positive ($n = 19$) ^b | 16(84) | 11(58) | 5(26) | — | 3(16) |
| Negative ($n = 41$) | 40(98) | 36(88) | 4(10) | 1(2) | — |

OR: overall response; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

^a $P = 0.001$ (CR vs. PR vs. SD vs. PD; two-tailed Fisher exact test).

^b $P = 0.027$ (CR vs. PR vs. SD vs. PD; two-tailed Fisher exact test).

lation between CD38 and IgV_H mutational status was confirmed (15 of 16 patients with IgV_H mutations $>$ 2% showed CD38 levels $<$ 20%, $P = 0.0006$).

With regard to clinical outcomes, all 32 patients who had ZAP-70 expression levels $<$ 20% achieved a CR or a PR ($P = 0.001$), and a significant higher overall response rate was found among CD38-negative patients (98% vs. 84%; $P = 0.027$) (Table 4). No significant correlation was found between cytogenetic abnormalities and overall response. Moreover, 42 of 47 patients (93%) who achieved a CR based on NCI criteria were negative for MRD according to flow cytometry ($<$ 5% CD19-positive/CD5-positive/CD79b-negative CLL cells in bone marrow; $P < 0.0001$). Finally, all but 1 patient (15 of 16 patients) who presented with IgV_H mutations $>$ 2% achieved a CR ($P < 0.01$).

A significantly shorter PFS was observed in ZAP-70-positive patients (25% [95% CI, 5–54%] vs. 100% at 3 yrs; $P = 0.00005$) (Fig. 4A), in CD38-positive patients (18% [95% CI, 1–52%] vs. 91% [95% CI, 66–98%] at 3 yrs; $P = 0.0002$) (Fig. 4B), and in patients with higher MRD after treatment (36% [95% CI, 6–69%] vs. 77% [95% CI, 48–91%] at 2.5 yrs; $P = 0.001$) (Fig. 4C). Moreover, unmutated patients also showed a significantly shorter PFS rate (0% vs. 50% at 2 yrs; $P = 0.01$).

Similarly, OS was significantly shorter in ZAP-70-positive patients (68% [95% CI, 40–85%] vs. 100% at 3 yrs; $P = 0.006$) and in MRD-positive patients (72% [95% CI, 42–88%] vs. 89% [95% CI, 62–97%] at 3 yrs; $P = 0.018$) and showed a significant trend in CD38-positive patients (70% [95% CI, 36–89%] vs. 92% [95% CI, 68–98%] at 3 yrs; $P = 0.059$). Finally, cytogenetic abnormalities showed no significant prognostic impact with regard either PFS or OS.

With regard to PFS, post-hoc power was not calculable for ZAP-70 group comparisons, because we

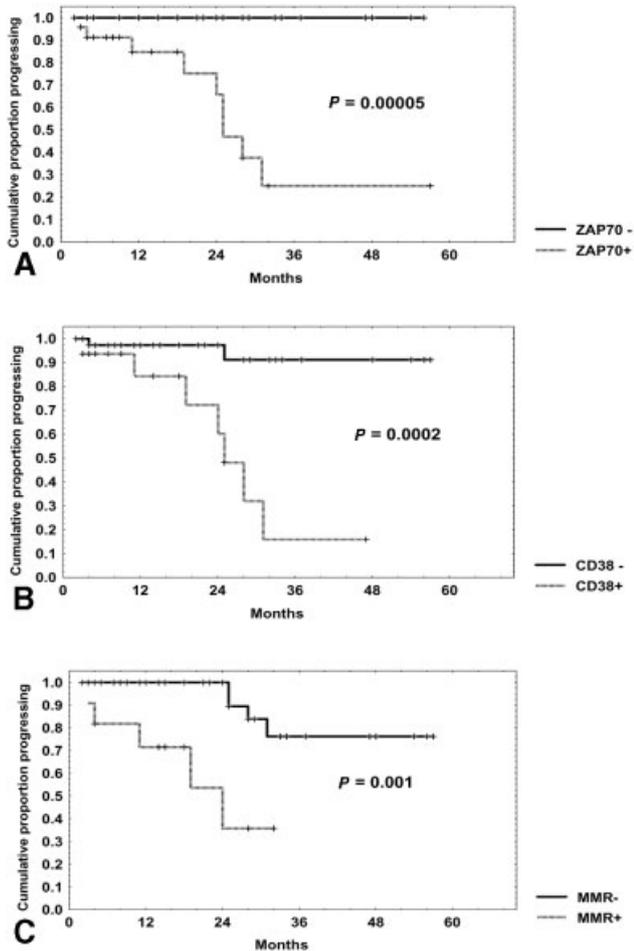


FIGURE 4. Progression-free survival curves were based on ZAP-70, CD38, and minimal residual disease (MMR) expression. Progression-free survival was significantly shorter in patients with ZAP-70 levels > 20% (ZAP-70 positive [+]) (A), CD38 levels > 20% (CD38+) (B), and (C) MRD > 5% (MMR+).

found that no event (progression) took place in the group of ZAP-70-positive patients. For CD38, post-hoc power was 0.79; whereas for MRD, post-hoc power was 0.22.

DISCUSSION

In this Phase II study, we demonstrated clearly that combined immunochemotherapy with six cycles of fludarabine followed by four cycles of rituximab was safe and very effective in patients with previously untreated B-CLL. The overall response rate was 93%, with a CR rate of 78% and a PR rate of 15%. This very high CR rate probably was achieved because almost all patients (57 of 60 patients) had CLL with low or intermediate Rai stage. In any event, all treated patients had progressive disease and presented with at least 1 of the following parameters: a lymphocyte doubling

time < 1 year, disease progression to a more advanced Rai modified stage, or the development of systemic symptoms. Schulz et al.¹⁵ also demonstrated that there were more CRs among patients who had Binet B CLL (43%) compared with patients who had Binet C CLL (11%) in a similar Phase II trial that was based on a regimen combining rituximab and fludarabine. A CR rate of 28% (PR rate, 49%) was obtained in patients with B-CLL who had both Rai intermediate-risk CLL (58%) and Rai high-risk CLL (42%) and were treated with a sequential scheme based on fludarabine plus rituximab.¹⁶ Our sequential regimen was very effective in the patients with intermediate Rai stage disease, as shown above (see Results), and the treatment of this progressive subset before the appearance of anemia or thrombocytopenia allowed us to obtain very good results in terms of both response and duration of response. The long-term benefit of this therapy as it relates to PFS and OS is not yet clear, and even if it is superior to fludarabine alone remains undetermined. It is noteworthy that our patients with B-CLL who were treated sequentially with fludarabine and rituximab experienced a long PFS after treatment (68% were alive at 3 yrs). Recently, Cancer and Leukemia Group B (CALGB) tested this combined regimen against monotherapy with fludarabine in a retrospective, Phase III clinical trial and demonstrated that, with fludarabine plus rituximab, significantly longer PFS ($P < 0.0001$) and OS ($P = 0.0006$) were achieved.³⁵

The fludarabine plus rituximab treatment regimen is feasible and can be administered safely on an outpatient basis. Rituximab causes important and prolonged depletion of B lymphocytes. Conversely, fludarabine leads to profound depletion of T cells.^{36,37} The risk of opportunistic infections may increase greatly with the combination of fludarabine and rituximab. In the current study, toxicity was moderate, and there was a slightly increased risk of severe infections. The majority of these opportunistic infections were viral and often localized. There were only 3 patients with Grade 3 or 4 infections, 1 of which was fatal (B hepatitis). Grade 3 and/or 4 hematologic toxicity was observed almost exclusively for neutropenia (48%), compared with thrombocytopenia (5%). These percentages of serious neutropenia were inferior to those published by Byrd et al.,¹⁶ who reported that 76% of their patients had Grade 3 or 4 neutropenia, probably because of the greater numbers of rituximab infusions. Nonetheless, this excess neutropenia did not predispose to an excess number of neutropenic fever episodes or life-threatening infections either in the study by Byrd et al.¹⁶ or in our current study.

Moreover, there were no episodes of autoimmune hemolytic anemia, probably because the transient B-

cell depletion caused by rituximab may avoid the development of fludarabine-associated hemolytic anemia. This hypothesis is corroborated by the findings of some authors who observed effective treatment of autoimmune hemolytic anemia with rituximab.³⁸ One concern about the use of rituximab in patients with CLL is infusion-related toxicity. This problem arises from studies demonstrating that rituximab can cause severe infusion-related toxicity in a minority of patients and that a high number of circulating B-CLL cells may predispose patients to this toxicity.^{39,40} In our study, only 5 patients presented with mild infusion-related symptom complex, consisting of fever, chills, and rigors, during the first infusion. Probably, prior treatment with fludarabine greatly diminished the infusion toxicity observed with subsequent rituximab treatment, mainly because of lower leukocyte counts.

The low CR rate obtained with previous therapies in patients with CLL relates to several different mechanisms of disrupted apoptosis.⁴¹ Both fludarabine and rituximab enhance different proapoptotic mechanisms, based both on *bcl-2* down-modulation and caspases activation, so that this combined regimen allowed us to achieve better responses compared with single-agent fludarabine or rituximab.⁴²

Various clinical features, including age, Rai stage, and serum β_2 -microglobulin levels, have been associated with lower response rates and poor long-term treatment outcomes after alkylator-based and purine analog-based therapy for patients with CLL.^{43,44} Several standard prognostic factors, including age, gender, Rai stage, and β_2 -microglobulin levels, did not reach a level of significance for predicting treatment outcomes either in the current study or in the randomized Phase II study by CALGB 9712.¹⁶

Recent studies examining molecular aberrations (i.e., *p53* mutations), unfavorable cytogenetics, CD38 expression, somatic VH gene mutational status, and ZAP-70 expression, have demonstrated that all of these factors are important determinants for treatment outcomes in patients with CLL.⁴⁵ Therefore, the analysis of unmutated V_H genes, CD38 expression, and/or ZAP-70 protein expression may be better for explaining the discordant outcomes independent of treatment that were observed in these studies. It is noteworthy that, in the current study, ZAP-70 and CD38 overexpression identified a subset of patients with a poor prognosis in terms of overall response, PFS, and OS. Thus, these two markers prospectively may identify subsets of patients who have a low likelihood of responding to combination therapy with fludarabine and rituximab. In our experience, FISH analysis did not allow us to obtain significant different

risk subsets with regard to response and outcome, perhaps because of the small number of patients analyzed. Conversely, MRD assays by flow cytometry allowed us to identify patients who were at significantly greater risk of recurrence. This is important, because, based on NCI criteria, patients in CR who are MRD-positive (5 of 47 patients in the current study) may be eligible for some consolidation therapy to avoid disease recurrence. These observations may encourage the use of cyclophosphamide combined with fludarabine and rituximab in these patient subsets with "poor-risk" B-CLL. In fact, investigators at The University of Texas M. D. Anderson Cancer Center used this triple combination and reported a CR rate of 66% in patients with previously untreated CLL.⁴⁶ The ultimate objective in CLL therapy is not only to achieve high CR rates but also mainly to avoid recurrence, thus prolonging remissions and, finally, survival. The introduction for stratification purposes of some biologic markers, such as ZAP-70 and CD38, into clinical practice may allow us prospectively to isolate patients who respond poorly to fludarabine plus rituximab and who may be potential candidates for more aggressive and/or experimental approaches.⁴⁷

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