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Tumor cell purging

A model of *in vivo* purging with Rituximab and high-dose AraC in follicular and mantle cell lymphoma

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Summary:

We studied a model of *in vivo* purging with Rituximab and high-dose (HD) cytarabine in 14 patients with relapsed/refractory follicular lymphoma and two with refractory mantle cell lymphoma enrolled in a program of HD chemotherapy and autotransplant. After two courses of debulking immunochemotherapy with Rituximab, Vincristine and Cyclophosphamide, we used a combination of Rituximab, HD cytarabine and granulocyte colony-stimulating factor for peripheral blood stem cells (PBSC) mobilization. The median number of CD34+ cells collected was 14.69×10^6/kg (range 5.74–73.2). Monitoring of peripheral CD19+ and CD20+ B cells prior to and throughout the purging period showed that a treatment with Rituximab, Vincristine and Cyclophosphamide results in a profound depletion of B cells in peripheral blood. B-cell depletion persists during mobilization with Rituximab and HD cytarabine allowing a collection of PBSC free of B cells (median CD19+ and CD20+ cells counts 0%). Of nine patients PCR positive for bcl-2 or bcl-1 in blood and marrow at the start of immunochemotherapy, all showed PCR-negative PBSC. In conclusion, in patients with indolent lymphoma, the concurrent administration of Rituximab and HD cytarabine is a safe and efficient method to obtain *in vivo* purged PBSC. Immunochemotherapy prior to mobilization produces B-cell depletion and seems to be a useful preparative step.

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Autologous stem cell transplantation has been shown to be effective in the long-term control of follicular lymphoma (FL).[1] Lymphoma, however, can progress after high-dose (HD) treatments. Lymphoma cells have been proved to contaminate bone marrow[2–4] and peripheral blood stem cell (PBSC)[5] collections and may contribute to relapse after autotransplant. The presence of PCR-detectable cells bearing the bcl-2 rearrangement in peripheral blood and marrow may be a surrogate marker of disease and the achievement of a bcl-2-negative status is associated with a lower risk of recurrence.[6,7] Several methods have been attempted to abolish graft contamination: *in vitro* treatment with cytotoxic agents,[8] *in vitro* treatment with anti-B-cell monoclonal antibodies and complement, immunomagnetic beads,[9,10] positive selection of CD34+ cells.[11] All these techniques usually produce loss of cells, are time-consuming and expensive, and neoplastic depletion is often partial with residual PCR-positive cells in the graft. At Dana Farber, only 42% of bone marrow grafts were purged with the use of three monoclonal antibodies.[6]

The chimeric anti-CD20 monoclonal antibody Rituximab has been shown to be an effective therapeutic option for low-grade lymphoma.[12] Owing to the different mechanism of action, the synergism with cytotoxic agents,[13] and non-overlapping toxicity, Rituximab is an ideal drug for combination with chemotherapy.[14] On this basis, Rituximab has been used during mobilization procedures for *in vivo* purging and collection of lymphoma-free progenitor cells. In addition, it has been demonstrated that the efficiency of harvested PBSCs is not adversely affected by Rituximab,[15,16] and that engraftment and hematopoietic recovery are not compromised.[15] The incorporation of Rituximab into sequential HD therapy programs[17–19] produced high rates of clinical and molecular remission in patients with indolent lymphoma, indicating that the antibody has an additive effect on chemotherapy.

We investigated the *in vivo* purging potential of Rituximab in 16 patients with advanced low-grade lymphoma (14 follicular, two mantle cell) enrolled in a program of immunochemotherapy and autotransplant. Immunophenotypic and molecular monitoring of peripheral B cells, in conjunction with analyses of the harvested PBSC, shows that the concurrent administration of Rituximab and HD cytarabine is a safe and efficient method for *in vivo* purging and mobilization of lymphoma-free PBSC.

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Patients and methods

Patients

We investigated the *in vivo* purging potential of Rituximab in 14 patients with relapsed/refractory FL and two patients with refractory mantle cell lymphoma (MCL) enrolled in a program of immunochemotherapy and autotransplant approved by the Institutional Ethic Committee. Patients were required to have histologic diagnosis of follicular or MCL, CD20 expression, age between 20 and 60 years, advanced Ann Arbor stage, normal cardiac, renal and hepatic function, negativity for HIV, HBV and HCV and informed consent.

Immunochemotherapy, in vivo *purging and PBSC mobilization*

After a debulking phase with VACOP-B for 6 weeks, treatment program calls for a phase of immunochemotherapy consisting of two cycles with Rituximab 375 mg/m^2 on day 1, Vincristine 1.4 mg/m^2 on day 2 and Cyclophosphamide 400 mg/m^2 on days 2–6. This is followed by a mobilization phase with Rituximab 375 mg/m^2 on day 1, and cytarabine 2 g/m^2 every 12 h on days 2 and 3. A second infusion of Rituximab 375 mg/m^2 is given on day 9. Granulocyte colony-stimulating factor (G-CSF) 5 μg/kg/day is started subcutaneously on day 6 (Figure 1). Leukaphereses were started at values of CD34+ cells in peripheral blood $>30/\mu$l. PBSC were collected with a continuous-flow blood cell separator Spectra (COBE BCT), processing a total volume per leukapheresis of two to three blood mass volumes.

Evaluation of in vivo *purging*

Flow cytometry. CD34 surface marker analysis was performed using anti-CD34 phycoerythrin (PE)-conjugated antibody (Becton Dickinson), anti-CD45 fluorescein isothiocyanate (FITC)-conjugated antibody (Becton Dickinson) and LDS-751, a nuclear stain. Aliquots of peripheral blood and leukapheresis products were incubated with monoclonal antibodies for 15 min at 20°C and then lysed with a lyse-no-wash standard assay. Data acquisition was performed with Cellquest software (Becton Dickinson). Analysis was performed according to the guidelines of ISHAGE (International Society for Hematotherapy and Graft Engineering) protocol. The analysis of B cells in peripheral blood and in the harvest was performed using the monoclonal antibodies CD20-FITC, CD19-PE, CD45-peridin chlorophyll protein (PerCP) (Becton Dickinson), while for surface immunoglobulins the monoclonal antibodies κ-FITC, λ-PE and CD19-PerCP (Becton Dickinson) were used.

Molecular evaluation. DNA samples of peripheral blood, bone marrow and leukapheresis product were assessed using nested PCR amplification of either the bcl-2/IgH or the bcl-1/IgH rearrangements as described previously.[18] The limit of detection of minimal residual disease was reproducible at the level of 10^{-6} for the bcl-2/IgH rearrangement and at 10^{-5} for the bcl-1/IgH rearrangement.

Rituximab pharmacokinetics

A pharmacokinetic study of plasma concentrations of Rituximab during the PBSC mobilization was carried out in eight cases. Serum samples were collected immediately before and at the end of each infusion of Rituximab. Rituximab concentrations were determined by a previously validated ELISA method, with a limit of detection of 5 μg/ml.[20]

Statistical analysis

Continuous variables are summarized as the median and range. Categorical variables are reported as the count and relative frequency. All computations were carried out using STATISTICA for Windows 5.5, StatSoft, Inc. (2000).

Results

Patients' characteristics

The clinical characteristics of the 16 patients, at the start of salvage treatment, are summarized in Table 1. Of these, 15 had FL (nine grade 1, three grade 2, one grade 3, one transformed to high grade) and two MCL. Nine patients had bone marrow involvement. All patients were evaluated for the presence of a molecular marker of disease: seven

Days		1	2	3	4	5	6	7	8	9
Drug	mg/m^2									
Rituximab	375	•								•
Cytarabine *	2000/12 h		•	•						

* 2-hour infusion

Figure 1 Mobilization scheme with Rituximab and HD cytarabine. G-CSF 5 μg/kg/day subcutaneously is given from day 6 until the end of peripheral stem cells' collection.

Table 1 Patients' characteristics at study entry

Characteristic	*No.*
Total number	16
Sex M/F	11/5
Median age (years) (range)	51 (44–59)
Histology	
Follicular grade 1	9
Follicular grade 2	3
Follicular grade 3	1
Follicular, transformed	1
Mantle cell	2
Ann arbor stage	
II bulky	3
III–IV	13
Bone marrow involvement	9
Relapsed	12
Refractory	4
Median no. of previous treatments (range)	1 (1–3)
Previously treated with anthracyclines	16
Previously treated with radiotherapy	2
Previously treated with Rituximab alone	4

patients with FL were positive for bcl-2 rearrangement on bone marrow and peripheral blood; the two patients with MCL were positive for bcl-1 rearrangement.

In vivo *purging and pharmacokinetic analysis*

At the start of the immunochemotherapy with Rituximab, Vincristine and Cyclophosphamide, 15 patients had CD19+ and CD20+ B cell counts in the normal range, while one patient who had received Rituximab as a single agent 5 months prior to study entry showed a marked peripheral B-cell depletion. After two cycles of immunochemotherapy, peripheral CD19+ and CD20+ B cells were substantially absent in all patients: median CD19+ cells count/μl was 0 (0–13), with a median percent value of 0 (0–3.9); median CD20+ B cells count/μl was 0 (0–45), with a median percent value of 0 (0–13.7). Monitoring of B cells in peripheral blood at days 1 and 9 of the mobilization phase showed no immunologically detectable B cells.

Before mobilization all patients were in clinical and molecular remission. The mobilizing regimen consisting of two standard doses of Rituximab coupled with four infusions of HD cytarabine was well tolerated. No adverse events occurred during Rituximab administration. No grade 3–4 nonhematologic toxicities were observed after HD-AraC and no patient experienced neutropenic fever. Patients needed a median of 1 platelet transfusion (range 0–2), while no erythrocyte support was given. Patients were hospitalized for 3 days (for the first Rituximab infusion and 2 days of HD-AraC). All the other procedures, including leukaphereses, were performed on an outpatient basis. Leukaphereses were started after a median of 12 days (range 11–14) after the first dose of HD-AraC. The median number of CD34+ cells harvested was 14.69×10^6/kg (range 5.74–73.24), with a median of two procedures (range 1–2). The median cellularity in the leukapheresis product was 6.58×10^8/kg (range 2.43–12.88). The engraftment of harvested PBSC was regular without significant delay in hematopoietic recovery. During follow-up post-transplant, which ranged from 2 to 4 years, patients did not show increased tendency to infections.

The median Rituximab concentrations quantified before and after the antibody infusion preceding HD-AraC were 20.6 μg/ml (range 5.0–44.7 μg/ml) and 238.9 (range 170.0–297.4 μg/ml), respectively. After 24 h, they were 168.3 μg/ml (range 12.5–270.9 μg/ml). Since the very long distribution and elimination of Rituximab half-lives, drug concentrations declined slowly during the post-treatment period, so that just before the following infusion median Rituximab levels were 65.95 μg/ml (range 42.4–157.2 μg/ml). Figure 2 illustrates the behavior of the median CD34+ cells counts in peripheral blood and of the median Rituximab serum levels during the mobilization phase. It shows that the harvest of PBSC occurs in the presence of high serum levels of Rituximab. The low tumor burden and the low circulating B-cell levels of patients enrolled in this study may have contributed to the sustained Rituximab concentrations during the course of therapy.

Immunophenotypic and molecular evaluation of the mobilization product

The median CD19+ cells count in the grafts was 0/μl (range 0–3.28), with a median percentage of 0 (range 0–0.3). The median CD20+ B cells count was 0/μl (range 0–14.06), with a median percentage of 0 (range 0–1.4). In none of the grafts was the κ/λ ratio unbalanced. In all the nine patients who were PCR positive in blood and marrow at the start of immunochemotherapy, PBSC collection proved PCR negative.

Discussion

HD therapy with autologous stem cell support may determine long-lasting remissions in FL.[1,7] Relapses, however, can occur caused either by incomplete eradication of neoplastic cells or reinfusion of lymphoma cells contaminating the harvest. Techniques for purging stem cell collection *in vitro* are expensive, time-consuming and cause stem cell loss. Rituximab, a human/mouse chimeric anti-CD20 monoclonal antibody, induces host effector

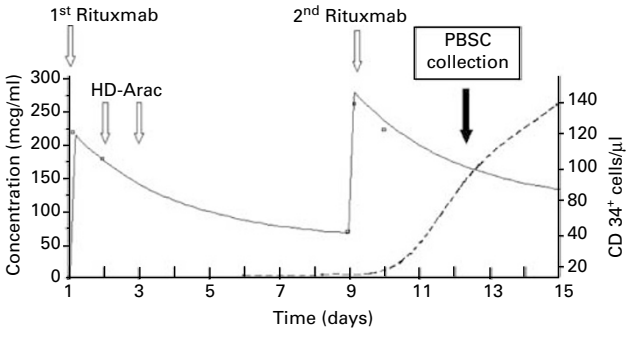

Figure 2 Median Rituximab levels and median CD34+ counts during the mobilization phase of peripheral stem cells with Rituximab and HD AraC.

mechanisms to kill lymphoma cells, determines apoptosis of CD20-positive cells and produces a rapid and long-lasting depletion of B-cell in peripheral blood.[21] The antilymphoma activity of Rituximab has been demonstrated in indolent[12] and aggressive[22] lymphoma either as a single agent or in combination with chemotherapy.[14,23,24] Rituximab shows peculiar pharmacokinetic properties: in a study with Rituximab administered once weekly for 8 weeks, the mean peak serum concentration increased with each successive infusion, ranging from 242.6 μg/ml after the first infusion to 460 μg/ml after the fourth infusion.[25] On the basis of its biological characteristics and clinical activity, several investigators have employed Rituximab as *in vivo* purging to obtain lymphoma-free PBSC collections. For this task, however, the optimal dose and timing of Rituximab administration was not defined. Buckstein *et al*[15] used a single dose of Rituximab before G-CSF without chemotherapy, while Salles *et al*[26] and Belhadj *et al*[27] employed the traditional scheme of four weekly infusions before mobilizing chemotherapy. Flinn *et al*[16] administered Rituximab on day 1 followed by HD cyclophosphamide on day 4. Using an HD sequential (HDS) chemotherapy approach,[28] the addition of Rituximab (R-HDS) ameliorated purging *in vivo* in FL and MCL allowing lymphoma-free harvests.[18]

In a program of immunochemotherapy and autotransplant for patients with refractory/relapsed follicular and MCL,[19] we employed a scheme of *in vivo* purging that uses Rituximab in two distinct phases: a preparative immunochemotherapy phase based on the combination of Rituximab with cyclophosphamide and a mobilization phase that combines Rituximab with HD-AraC. Rituximab was employed during the mobilizing phase as *in vivo* purging also in patients already PCR negative, because a negative molecular status in blood/marrow is not predictive of a molecularly negative harvest.[18] The effect of the preparative phase was a profound peripheral B-cell depletion that was maintained throughout the mobilization phase. In fact, a low level of CD19+ B cells in the peripheral blood at the time of mobilization has been shown to predict a low tumor load in the apheresis product.[29] The subsequent mobilizing phase with Rituximab (days 1 and 9) and HD-AraC (days 2 and 3) was aimed at collecting PBSC in the presence of high serum levels of monoclonal antibody, therefore enhancing lymphoma cells' clearing. The number of stem cells harvested was not affected by Rituximab administration. In fact, all 16 patients in this study collected a number of CD34+ cells sufficient to support HD therapy. A pharmacokinetic analysis of Rituximab during mobilization lends further support to the rationale of this strategy (Figure 2). In fact, sustained serum levels of Rituximab were maintained during the entire mobilization period, with particularly high levels corresponding to the peaks of CD34+ cells (start of harvest). The favorable pharmacokinetic profile of Rituximab translated into an evident *in vivo* purging effect: B cells were virtually absent at cytofluorimetric evaluation in all aphereses, and in no case κ/λ ratio was unbalanced. In nine molecularly informative patients (seven bcl-2 positive and two bcl-1 positive), the graft proved negative at PCR evaluation. This mobilizing schedule was devoid of unexpected hematological and extrahematological toxicity. However, given the intensive nature of this program, it should be better employed in the early phases of resistance, before deterioration with multiple lines of chemotherapy occurs.

In conclusion, the integration of Rituximab with chemotherapy on the basis of the pharmacokinetic properties of the antibody seems a rational way to exploit the antilymphoma potential of the combination. A preparative immunochemotherapy phase prior to mobilization produces complete B-cell depletion that is maintained throughout the harvesting phase. The combination of Rituximab with HD cytarabine permits collection of PBSC in the presence of high serum levels of the antibody, resulting in the clearing of lymphoma cells from the graft confirmed at the immunophenotypic and molecular level.

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