POLYMERASE CHAIN REACTION MONITORING SHOWS A HIGH EFFICACY OF CLINICAL IMMUNOMAGNETIC PURGING IN PATIENTS WITH CENTROBLASTIC-CENTROCYTIC NON-HODGKIN’S LYMPHOMA

To the Editor:

Bone marrow (BM) involvement almost invariably compromises autologous BM transplantation (ABMT) in patients with follicular non-Hodgkin’s lymphoma who are considered for this therapy. Because the reinfusion of clonogenic tumor cells into the patient may give rise to relapse, several ex vivo purging methods have been applied to remove lymphoma cells from the autograft. Recently, it has been reported that the use of anti-B-cell monoclonal antibodies (MoAbs) plus complement in patients with B-cell lymphoma seems to be efficient in only 50% of the cases, resulting in an impaired disease-free survival in the cases in which purging was not successful. Although this finding suggests that reinfusion of clonogenic lymphoma cells contributes to relapse, alternative reasons, like a higher tumor burden in these patients or a more resistant disease, which is reflected in a reduced susceptibility to complement-mediated cytolysis of lymphoma cells, must also be taken into account.

We have used immunomagnetic purging for autografts of lymphoma patients and monitored the efficacy of this procedure in four patients with a t(14;18) translocation-positive centroblastic-centrocytic lymphoma in clinical remission who all had a history of BM involvement. We usually use a cocktail of five different MoAbs against B-cell antigens: anti-CD19 (HD37), anti-CD20 (1F5), anti-CD22 (HD39), anti-CD23 (HD50), and anti-CD37 (HD28). Ten micrograms of each MoAb were used per $1 \times 10^6$ B cells. The

Fig 1. PCR amplification of t(14;18) targets. One microgram of genomic DNA was subjected to in vitro enzymatic amplification with 2.5 U Taq DNA polymerase (Amersham, UK) in a volume of 100 $\mu$L. The PCR buffer consisted of 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L MgCl$_2$. The concentration of dNTPs was 0.2 mmol/L. A JH universal primer 5' ACCTGAGGAGACGGTGACC 3' as well as two primers for reaction control $\beta$-globin sequences, 5' GGTTGGCCAATCTACTCCAGG 3' and 5' TGGTCTCTTTAACCTGCTT 3', which induced amplification of a 268-bp DNA fragment, were present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany). In DNA dilution experiments, 1 $\mu$g of t(14;18)-negative human control DNA was present in the reaction mixture at a concentration of 1 $\mu$mol/L each. Forty PCR cycles (94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes) were performed in a thermocycler (Bio-med, Reutlingen, Germany).
sheep antimoise (SAM) beads (M-450 Dynabeads; Dynal, Oslo, Norway) were added to give a bead/total B-cell ratio of 50 to 1, and the bead/cell rosettes and excess beads were removed using our own purging device as previously reported.3 A second cycle of purging was performed by adding the same amount of SAM beads.

The various cell and bead/cell fractions separated by immunomagnetic purging were analyzed by t(14;18) polymerase chain reaction (PCR),4 which is feasible in approximately 80% of patients with a follicular lymphoma.3 One microgram of genomic DNA, which is the equivalent of 150,000 diploid cells, was present in each reaction and a single round of amplification consisting of 40 cycles was performed. After electrophoretic separation of reaction products, the agarose gels were blotted and the respective blots hybridized with a 32P-labeled bcl-2 major breakpoint region (MBR) probe.6 This analysis showed a strong enrichment of lymphoma cells in the bead/cell fractions, which were removed from the autografts, in comparison to the PCR signal of the autograft before purging (Fig 1A). In three of four cases, immunomagnetic purging resulted in a PCR-negative transplant.

To estimate lymphoma cell concentrations, DNA dilution experiments were performed with the t(14;18)-positive cell line Karpas 422(7) and the various purging fractions. In the case of the Karpas 422 cell line, the last detectable PCR signal was found at the dilution step 10^-4, which is consistent with the ability of PCR to detect single DNA target molecules in the reaction (Fig 1B). In the patient samples, the highest concentration of lymphoma cells was found in the first cell/bead fraction, for which three positive 1 log dilution steps could be performed. Within the last positive dilution sample, DNA corresponding to 150 cells of the first cell/bead fraction was present, at least one t(14;18) target. This means that the lymphoma cell concentration in the first cell/bead fraction was equal or higher than 0.7%. On the other hand, DNA corresponding to 15 cells of the original sample was present at the dilution step 10^-4, which was PCR negative. This showed that the lymphoma cell concentration was lower than 0.7%. Therefore, we could assume a lymphoma cell concentration of approximately 1% to 5% in the first cell/bead fraction. This estimation was confirmed by the presence of rearranged JH bands on Southern blots in two investigated cases in our series (cases no. 3 and 4). Also, the specific t(14;18) junction fragments could be visualized on the agarose gels after ethidium bromide staining when DNA from the first cell/bead fraction was used as a template for one round of amplification. The different sizes of the distinct DNA bands (ranging between 150 and 230 bp) identified the individual tumors (data not shown). We calculated that 0.01% to 0.05% or 0.1% to 0.5% of total nucleated BM cells before purging were lymphoma cells. In the second cell/bead fraction, lymphoma cells varied between 0.001% to 0.005% and 0.1% to 0.5% in the different cases. Lymphoma cells in the one PCR-positive autograft after purging made up 0.001% to 0.005%. The DNA dilution experiment in case no. 1 is shown in Fig 1C.

Our preliminary results (Table 1) confirm the high efficacy of immunomagnetic purging in a clinical setting, which was originally demonstrated for artificial settings in which mixtures of lymphoma cell lines with normal BM were used.55 Two rounds of immunomagnetic purging are required to efficiently purge the marrow of lymphoma cells.3 PCR failed to detect lymphoma cells in three autografts after purging in our series. Even in case no. 2, in which a PCR signal still could be detected after purging, purging succeeded in reducing the concentration of lymphoma cells approximately 100-fold. However, negative PCR after purging does not necessarily indicate a complete lack of lymphoma cells, as lymphoma cells were still present in the second cell/bead fraction. Therefore, such negative PCR results of the autografts may merely reflect a lymphoma cell contamination less than 0.001%. Whether a third round of immunomagnetic purging can lead to a "lymphoma cell-free" autograft is very doubtful. In corresponding experiments using our method, such a third treatment cycle did not appear to result in further benefit, because no bead-cell rosettes were seen and clonogenic assays did not show a further lymphoma cell removal (G. Kvalheim, personal communication).

Recently, data were presented12 that confirmed our previous finding6 that immunomagnetic purging is superior to complement-mediated cytolysis. The results were obtained in small-scale experiments with patient samples, rather than the large-scale, clinical purging in our report here. Direct comparisons between these studies are further complicated by differences in the purging protocols (5 MoAbs plus Dynabeads reported here compared with 3 to 4 MoAbs plus Advanced Magnetics beads in Gribben et al12), the antibodies themselves, and the bead/target cell ratios.

The PCR analysis is highly compatible with immunomagnetic purging in the assessment of its efficacy because immunomagnetic purging does not lead to the production of nonvalid t(14;18) targets, as is the case with immunologic purging involving the lysis of target cells. The performance of one round of amplification followed by detection of tumor-specific DNA fragments with radiolabeled probes on Southern blots is sufficient to provide the desired sensitivity of the PCR reaction.

The question of whether BM purging with immunomagnetic beads actually is of benefit for lymphoma patients who receive ABMT is being addressed in a multicenter randomized trial.

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REFERENCES

Table 1. Evaluation of Immunomagnetic BM Purging. Cell Counts and PCR t(14;18) Signals

<table>
<thead>
<tr>
<th>Case No.</th>
<th>TNC (x10^9)</th>
<th>T (x10^9)</th>
<th>TNC</th>
<th>1.Cell/Bead</th>
<th>2.Cell/Bead</th>
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Abbreviations: TNC, total nucleated BM cells before purging; 1.Cell/Bead, first cell/bead fraction; 2.Cell/Bead, second cell/bead fraction; T, total nucleated BM cells after purging that were used for transplantation; +, detection of a t(14;18) signal (every additional + indicates the 1 log dilution steps that could be performed still leaving a PCR signal); (+), very weak PCR signal at the last positive dilution step.

*This patient so far has not received ABMT.


Polymerase chain reaction monitoring shows a high efficacy of clinical immunomagnetic purging in patients with centroblastic-centrocytic non-Hodgkin's lymphoma [letter]

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