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ARTICLE

An Effective, Direct Immunomagnetic Procedure for Purging Acute Lymphoblastic Leukemia Cells from Human Bone Marrow*

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The AB4 monoclonal antibody, which recognizes an HLA-DR epitope, was found to bind to a high percentage of malignant blast cells in samples obtained from 27 patients with ALL. These included 11 of 11 cases with c-ALL, 3 of 7 with pre-pre-B, and 8 of 9 cases with pre-B ALL. AB4 was used together with anti CD10 and anti CD19 antibodies and super-paramagnetic particles for developing a direct immunomagnetic procedure for purging human bone marrow of leukemic cells. In model experiments with KM3 cells admixed to mononuclear bone marrow cells, the individual antibodies each removed 2.8–3.1 logs and 3.6–4.1 logs of tumor cells with one and two purging cycles, respectively. In comparison, the efficacy of a mixture of the three antibodies was 4.4 logs with one treatment cycle, and > 5 logs with repeated treatments. Whereas the use of a commercially available anti-HLA-DR antibody resulted in a 90% reduction in the survival of CFU-GMs and normal blast colonies, AB4 had only a moderate effect on the progenitor cells (46% and 30% reduction). In conjunction with autologous transplantation, bone marrow from a patient was purged with the antibody mixture and 50% of the CFU-GMs and 47% of the CD34⁺ cells remained after treatment. The patient showed a normal engraftment, reaching a level of 0.5 x 10^9/l neutrophils by day 20 and 20 x 10^9/l platelets by day 30. It is concluded that the antibody cocktail may safely and effectively be used for rapid autograft purging in patients with c-ALL, and also in phenotypically selected cases with other subtypes of ALL. (Pathology Oncology Research Vol 1, No1, 32–37, 1995)

Key words: bone marrow, leukemia, transplantation, purging

Introduction

Autologous bone marrow transplantation (ABMT) is considered as an alternative therapy for patients with high risk acute lymphatic leukemia (ALL) who lack a histocompatible allogeneic donor.[1,2] In leukemia, the possible contribution of bone marrow (BM) purging to the efficacy of ABMT is unknown, as prospective clinical studies have not been performed.[1,2] However, gene-marking studies of autografted cells to trace the origin of relapse after ABMT have indicated that tumor cells remaining in the reinfused marrow contribute to recurrence of the disease.[3] The possible advantage of BM purging is further supported by results in patients with follicular lymphomas which indicate that efficient purging improves disease-free survival.[4]

In ALL patients the malignant event may occur in an early pluripotent stem cell. Hence, for purging purposes anti-HLA-DR antibodies that bind strongly to B-cell precursor ALL cells might be candidates for inclusion in the panel of antibodies used.[5,6,7] However, since HLA-DR gene products are also expressed on cells of the pluripotent stem cell compartment, it has been anticipated that purging with antibodies recognizing these proteins may also deplete early progenitor cells from the graft. We have, however, previously shown in a patient with non-Hodgkin lymphoma that a BM

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autograft purged with our AB4 anti-HLA-DR antibody[8] resulted in rapid and sustained engraftment.[9] Thus, it seems likely that the corresponding antigen, encoded by the B3 gene of the DR region, is not expressed in the most immature hematopoietic progenitor cells. To study whether the AB4 monoclonal antibody (MAb) binds to B-cell precursor ALL cells we first performed immunophenotyping of leukemic blasts in samples from patients with such disease, and found that the AB4 antigen was expressed on B-cell precursor ALL blasts in 22 of 27 patient samples.

When purging BM with immunobeads, the relevant antibodies can be used either in a direct or an indirect procedure.[10,11,12] With the direct method, the primary antibody is in advance attached to the beads, thereby eliminating both one incubation and a washing step.[10] This approach was tested in model purging experiments with a mixture of AB4, anti CD10 and anti CD19 antibodies, and highly promising results were obtained.

Materials and Methods

MAbs and immunobeads

An anti HLA-DR (IgG_{2A}) antibody was obtained from Becton Dickinson (Mountain View, CA). Our AB4 (IgM) antibody binds to an antigen encoded by the B3 locus of the DR gene. The F103.11 anti CD10 antibody (IgG_1) was a gift from T. Plesner (Amtssygehuset, Herlev, Copenhagen, Denmark), and the HD37 anti CD19 MAb (IgG_1) was kindly provided by B. Dörken (Robert Rossler Klinik, Free University, Berlin, Germany). The antibody against terminal deoxynucleotidyl transferase (TdT) was obtained from DAKO (Santa Barbara, CA). Dynabeads M-450 directly coated with the antibodies were delivered by Dynal (Oslo, Norway).

Immunophenotyping

Fresh BM or peripheral blood samples from 27 ALL patients, containing 80% blasts or more, were isolated by standard Ficoll-Hypaque density gradient centrifugation, and cell surface or intracytoplasmic antigens were examined by indirect immunofluorescence. MAbs to lymphoid-, myeloid-specific and non-lineage associated antigens, including the anti-HLA-DR and AB4 antibodies, were used as previously described.[13] Positive reaction is defined as $\geq$ 20% of the blasts expressing the surface markers or $\geq$ 10% blasts containing intranuclear/cytoplasmatic markers.[14] Immunophenotypic classification of B-cell precursor (i.e. pre-pre-B, common, Pre-B ALL) subtypes followed criteria described elsewhere.[5]

Tumor and BM cells

The KM3 non-T ALL cell line was cultured at 37°C in RPMI 1640 medium (RPMI) containing 10% fetal calf serum (FCS). Cultures were passaged frequently to assure that cells used in the experiments were in a proliferative log phase.

Normal BM cells were obtained from patients with nonhematological cancers aspirated for diagnostic purposes at the Norwegian Radium Hospital. None of the samples showed BM involvement of the disease as judged by BM biopsies and smears. Mononuclear cells were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and washed twice in phosphate-buffered saline (PBS) before use.

Assays of tumor cells and BM progenitor cells

The number of residual clonogenic KM3 tumor cells in control and purged samples was determined in a soft agar assay as previously described.[8] After 14 days of incubation at 37°C, colonies of more than 50 cells were counted using a Zeiss stereo microscope. The plating efficiency (PE) was defined as the number of colonies formed, expressed as the percentage of the number of viable cells plated. With the concentration of BM cells used, no colonies were formed in the absence of tumor cells.

The clonogenic capacity of the normal BM progenitor cells after purging was assessed in the CFU-GEMM and CFU-GM assays.[8] The blast colony formation assay on irradiated stromal feeder layers was performed as previously described.[15,16]

Incubation conditions and cell separation

10^6 KM3 cells mixed with $9x10^6$ mononuclear BM cells per ml in RPMI containing 10% FCS were incubated in 10 ml plastic tubes with the desired number of AB4, anti CD10 and anti CD19 immunobeads at 4°C for 30 min with gentle rotation. The concentration of beads was chosen on the basis of the total number of antibody-binding B-cells. The optimal ratio of beads to total nucleated cells was found to be 2 to 1 (data not shown), and this ratio was used in all experiments. The effect of the anti HLA-DR monoclonal antibody was tested by the indirect method. 10^7 mononuclear BM cells per ml of RPMI containing 10% FCS were incubated in 10 ml plastic tubes for 30 min at 4°C with 10 mg/ml of the anti HLA-DR antibody. The cell suspension was washed twice with cold PBS, resuspended to a final concentration of $1x10^7$ cells/ml in PBS and incubated with the desired number of Dynabeads SAM ST at 4°C for 30 min with gentle rotation.

In all cases the resulting cell/bead aggregates were removed by placing a Cobalt-Samarium magnet to the wall of tubes. After 1 min, the suspension was aspirated, and when desired a new batch of immunobeads was added to the cell suspension and the cycle was repeated.

A patient in first relapse of unclassified high grade non Hodgkin lymphoma, who had advanced disease with the BM heavily infiltrated with lymphoblasts expressing CD10,

CD19 and the AB-4 epitope, was brought into complete remission with second line chemotherapy. Bone marrow was harvested and the mononuclear cells were isolated using a CS-3000 Plus Blood Cell Separator (Baxter Healthcare Corporation, Deerfield, IL). The mononuclear cell fraction was purged with the AB4, anti CD10 and anti CD19 immunobeads under similar conditions to those described above, employing a MaxSep Magnetic Cell Separator (Baxter Healthcare Corporation). The number of CFU-GMs and $CD34^+$ cells before and after purging was estimated. Twenty days later, the patient was treated with total body irradiation in doses of 1.3 Gy twice daily for five days and with 60 mg/kg body weight of cyclophosphamide daily for two days. Two days after finishing the high dose treatment the purged BM was reinfused into the patient.

Results

Immunophenotype of blast cells in BM and peripheral blood of ALL patients

BM or peripheral blood cells from 27 patients with diagnosed ALL were immunophenotyped with the different MAbs. Seven patients were found to have pre-pre-B-cell, 11 c-ALL and 9 pre-B-cell disease, according to criteria described by Does-van den Berg et al.[5] The expression in each of these subclasses of the antigens recognized by anti-TdT, anti-HLA-DR, and AB4 is listed in *Table 1*. It can be seen that all c-ALL patients, 3 of the 7 pre-pre-B ALL, 8 of 9 pre-B ALL expressed the AB4-associated antigen. The expression was heterogenous, ranging from 25% to 95% positive cells. When comparing the immunoreactivity of AB4 to that of anti-TdT, which is known to bind to malignant blasts but reacts with only about 1% of normal blast cells,[14] it can be concluded that AB4 binds a high fraction of leukemic c-ALL cells. Moreover, also in most of the AB4 positive pre- and pre-pre-B ALL cases the percentage of immunoreactive cells was high. The fraction of AB4 positive cells was consistently lower than that seen with the other HLA-DR antibody, which often bound to the surface of a higher percentage of cells than those stained intracytoplasmatically with the anti-TdT antibody.

Importantly, all c-ALL tumor cells expressed antigens recognized by at least one of the anti CD19, anti CD10, and AB4 MAbs (not shown), indicating that a mixture of these antibodies should be well suited for BM purging with immunobeads in this subgroup of ALL patients.

Recovery of progenitor cells after immunomagnetic purging with HLA-DR antibodies

In preclinical studies, HLA-DR antibodies used for BM purging have been reported to deplete hematopoietic stem cells. To further clarify the hematopoietic toxicity of immunomagnetic purging with AB4 beads, a comparison between the use of AB4 and the more broadly reactive HLA-DR antibody with immunobeads was performed. It is shown in *Table 2* that after 2 purification cycles with AB4, 54% of CFU-GM and 70% of blast colonies survived, whereas the use of the other HLA-DR antibody resulted in both assays in only 10% recovery of the normal progenitor cells.

Table 1. **Fraction of anti-TdT, AB4, and anti-HLA-DR reactive blast cells in bone marrow or peripheral blood from patients with B cell precursor ALL**

Immunophenotypic subclass	*Patient no*	*Marker expression on B-cell precursor precursor ALL blasts (%)**		
		TdT	*AB4*	*HLA-DR*
Pre-pre-B-ALL				
(TdT⁺, CD19⁺)	1	80	41	91
	2	85	49	82
	3	70	69	73
	4	65	11	82
	5	74	7	90
	6	50	0	44
	7	95	5	91
c-ALL				
(TdT⁺, CD19⁺,	1	62	52	67
CD10⁺, cyIgM⁻)	2	80	74	97
	3	90	52	94
	4	90	53	83
	5	31	61	78
	6	90	77	79
	7	75	70	91
	8	75	78	93
	9	90	50	91
	10	95	25	94
	11	95	47	96
Pre-B-ALL				
(TdT⁺, CD19⁺,	1	40	75	n.d.
CD10⁻/⁺, cyIgM⁺,	2	90	28	–
sIgM⁻)	3	60	0	–
	4	90	39	–
	5	90	95	–
	6	90	90	–
	7	60	58	–
	8	85	85	–
	9	68	63	–

* All results given as percent positive cells. Positive reaction was defined as 20% of the blast cells expressing the surface (AB4, HLA-DR) markers, or 10% of the blasts containing the cytoplasmic (TdT) marker.

Efficacy of tumor cell depletion

We compared the efficacy of KM3 tumor cell removal by using beads directly coated with the primary MAbs. It is shown in *Fig.1* that with individual antibodies and one cycle of purification, a tumor cell depletion 2.8–3.1 logs was obtained. A second cycle of purification increased these

numbers to 3.6-4.1 log cell removal. When a mixture of all three antibodies was employed, 4.4 logs of tumor cells were removed with one cycle and no colony formation was observed after a second purging cycle. The latter result represents a tumor cell removal efficacy of at least 5 logs. Altogether, the data suggest that there is no complete overlap in tumor cell expression of the three antigens.

Table 2. **Recovery of blast colonies and progenitor cells after two cycles of immunomagnetic purging using either the anti-HLA-DR antibody and SAM-beads or the AB4-beads**

	*No of colonies formed**	
	CFU-GM/2x10^5 MNC	Blast colonies 2x10^5 MNC
Untreated control	160 ± 26 (100%)	189 ± 33 (100%)
HLA-DR/SAM-IB	15 ± 14 (10%)	19 ± 14 (10%)
AB4-IB	87 ± 32 (54%)	148 ± 38 (70%)

Fresh bone marrow cells (1x10^7/ml) were incubated in RPMI 1640 medium with the HLA-DR Mab followed by M-450 SAM beads, or with the direct AB4 beads. Conditions as in *Fig 1*.

* The results represent the mean SD of three independent experiments, each in triplicate, and the numbers in parentheses show the number of colonies relative to that in the untreated control.

Effect of immunomagnetic purging on hematopoietic stem cell recovery in a patient

The mixture of directly coated AB4, anti CD10, and anti CD19 immunobeads had, in an experimental setting, given a moderate effect on BM progenitor cells, with a 50% recovery of CFU-GMs (data not shown). When a full scale immunomagnetic purging procedure, employing the same immunobeads and with the same ratio of target cells and beads, was used on BM harvested from a patient with a relapsed high grade lymphoma, the recovery of CFU-GM cells and CD34$^+$ cells was 69% and 48%, respectively. After high dose treatment of the patient with total body irradiation and chemotherapy, the BM was reinfused. The patient showed a normal reconstitution with a level of 0.5 x 10^9/l neutrophils reached on day 20, and of 20 x 10^9 platelets on day 30.

Table 3. **Recovery of mononuclear BM cells, progenitor cells and CD34$^+$ cells after 2 cycles of immunomagnetic purging using a cocktail of AB4, anti-CD10 and anti-CD19 immunobeads**

Total no of	*Before purging*	*After purging*	*% Recovery*
MNC cells	4.5 x 10^9	2.3 x 10^9	51
CFU-GM cells	6.5 x 10^9	4.5 x 10^9	69
CD34$^+$ cells	1.7 x 10^8	0.8 x 10^8	47

Mononuclear bone marrow cells were isolated by using the CS3000 cell separator, and purging with immunobeads was performed with the MAX SEP cell separator as described in Materials and Methods.

Discussion

HLA-DR antigens are known to be strongly expressed on the surface of B-cell precursor ALL cells, also in the most immature types of the disease. Therefore, if the toxicity to normal BM progenitor cells was acceptable, the inclusion of an anti HLA-DR antibody in a panel of MAbs used for immunobead purging would be expected to be advantageous. Initial experiments showed that AB4 anti HLA-DR immunobeads were quite efficient in removing leukemic cells admixed to BM. Moreover, a good recovery of normal progenitor cells was seen after purging with these beads. Hence, it was of interest to examine the binding profile of AB4 in blast cells from ALL patients to examine the possibility of using this antibody for BM purging in such disease. Immunophenotyping of BM and peripheral blood cells from 27 patients showed that 11 of 11 c-ALL, 3 of 7 pre-pre-B ALL, and 8 of 9 pre-B ALL patients expressed the antigen recognized by AB4. The AB4 antigen was not uniformly expressed on the ALL blasts, but if used in a mixture with anti CD10 and anti CD19 antibodies the heterogeneity in antigen expression on B-cell precursor ALL cells should be well covered.

In model experiments, the mixture of the 3 immunobeads removed more than 4 log KM3 tumor cells with one, and at least 5 logs with two purging cycles. Importantly, in these experiments a procedure with the specific antibodies attached directly onto magnetic beads was used, thereby eliminating one incubation and one washing step compared to the more commonly used indirect immunobead method. With the purging efficacy obtained, the direct method offers clear advantages with respect to speed and simplicity, and two cycles of purification can be performed within one hour.

The present, and also previous results,[8,9] showed a good recovery of hematopoietic progenitor cells after immunomagnetic purging, both with AB4 beads alone and when used in the mixture. Therefore, it can be concluded that AB4 recognizes an epitope that is not expressed, or has a low level of expression, in hematopoietic stem cells. In contrast, purging with the other anti-HLA-DR antibody resulted in depletion of 90% of CFU-GMs and blast colonies (*Fig. 1*).

Philadelphia chromosome positive (Ph$^+$) ALL is associated with poor prognosis and such patients may be considered for high dose treatment at first complete remission.[18] It has been reported that 55% of adult c-ALL patients have the typical BCR/ABL rearrangement and that its presence correlates with poor overall survival and remission duration.[7] In our study, all the 11 phenotyped c-ALL patients had Ph$^+$ blast cells, which also expressed the antigens recognized by the anti CD10, CD19 and AB4 antibodies. Based on these findings a phase-I study in Ph$^+$ ALL patients treated with ABMT and BM purged with the directly coated beads has been initiated. In such patients

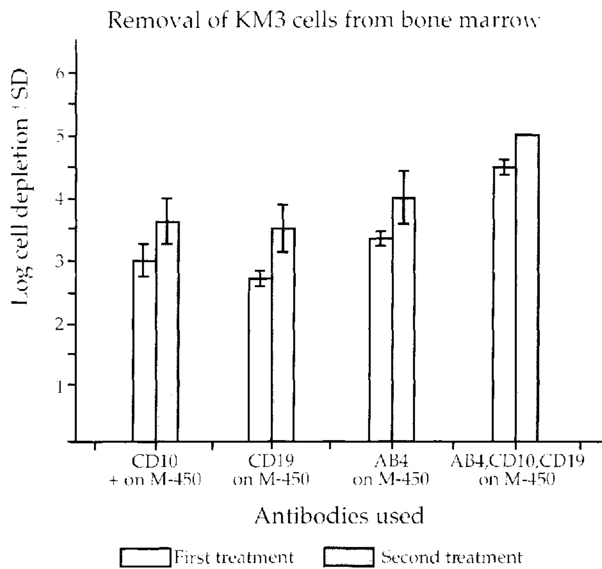

Figure 1. Efficacy of immunobeads involving three different antibodies in removing KM3 leukaemia cells. One x10[5] KM3 cells admixed with 9x10^{6} MNC bone marrow cells were incubated with M-450 beads coated individually with different primary antibodies. The bead/total nucleated cell ratio was 2 to 1. After magnetic separation the number of remaining tumor cells was determined as described in "Material and Methods". The results represent the mean ± SD of 3 independent experiments in triplicate.

the purging efficacy may be assessed by a polymerase chain reaction (PCR) method that detects the presence of possible BCR-ABL chimeric transcripts remaining in the autograft. The procedure is in principle similar to that recently reported for use in patients with follicular non-Hodgkin's lymphoma.[19] It should be noted that the immunomagnetic method facilitates the use of PCR procedures for analyzing the purging efficacy. Thus, since the target cells are removed from the autograft, problems with false positive results are avoided. In contrast, when methods involving complement or immunotoxins are used positive PCR amplification may result from contaminating target DNA carrying breakpoint sequences liberated from lysed tumor cells. Moreover, with immunobeads the risk of false negative results is also greatly diminished, as PCR amplification of specific sequences can be performed on the cell suspension containing the bead-removed target cells.

The insignificant effect of AB4 immunobeads on normal stem cells has been shown in a clinical study in which 4 non-Hodgkin lymphoma patients autotransplanted with BM purged with CD19 and AB4 beads showed normal and sustained engraftment.[20] These results were confirmed here in a patient autotransplanted with BM purged with AB4, anti-CD10 and anti-CD19 immunobeads. It is concluded that the direct immunobead technique with these antibodies can safely and efficiently be used to eradicate ALL cells in BM autotransplants.

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