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In Vitro Depletion of Clonogenic Cells in Adult Acute Lymphoblastic Leukemia with a CD10 (anti-cALLA) Monoclonal Antibody

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Abstract—*A semi-solid medium colony assay was used in common acute lymphoblastic leukemia (cALL) to test growth inhibition of leukemic progenitors (CFU-L) after exposure to monoclonal antibodies (MoAbs) directed against CD10 and CD9 antigens. Peripheral or bone marrow cells from 15 patients were plated after exposure to various concentrations of ALB2, a CD10 cytotoxic MoAb, followed by complement lysis. CFU-L inhibition was complete (no residual colony) in 5 cases (33%), marked (≥ 95%) in 4 cases (27%), but only moderate (64% ± 28) in 6 cases (40%). This inhibition was not related to the percentage of cALLA positive cells before exposure to MoAb. In addition, cells of 5 patients were exposed to BA1 (CD24) + complement. In these cases, the proportion of CFU-L inhibition was equal to or higher than with ALB2. In 3 cases, cells were exposed to an association of ALB2 and SB4 (CD19) MoAbs followed by complement lysis, with a marked inhibition (≥ 99%) in 2/3 cases.*

These observations give supplementary support to the use of several MoAbs directed against various antigens present at early stages of B differentiation.

INTRODUCTION

AUTOLOGOUS bone marrow transplantation (ABMT) following intensive therapy represents a new approach to the treatment of patients with acute leukemia [1–3]. Contamination of autologous marrow with tumor cells has been the main argument against ABMT in acute leukemia. Cytotoxic monoclonal antibodies directed against cell antigens present on leukemic cell surface could be used for *in vitro* depletion of residual leukemic cells [4, 5].

Several antigens are present on the cell surface of leukemic cells in common acute lymphoblastic leukemia (cALL). The common acute lymphoblastic antigen (cALLA), a 100,000 dalton glycoprotein (recognized by cluster of differentiation CD 10), was first identified [6–10] on cALL and was recognized by several MoAbs as J5 [11], BA3 [12] and one produced by one of us, ALB2 [13]. Other MoAbs, directed against cells committed to early B lineage differentiation, as BA1 (CD24) [14] and cytotoxic anti-B4 (CD19) [15], are candidates for *in vitro* purging in cALL.

To obtain a valid *in vitro* depletion, cytotoxic antibodies or immuno-toxins have also to be directed against clonogenic leukemic cells [16]. Such cells are probably representative of the leukemic progenitors responsible for leukemic regrowth after cytoreductive treatments administered before autograft. Previous studies [5, 17], using cell lines with a high *in vitro* cloning efficiency, have demonstrated the effectiveness of multiple MoAbs for *in vitro* purge of contaminated normal bone marrow. It is important to confirm this killing effect on fresh leukemic progenitor cells. In this study, we have used a culture assay for clonogenic leukemic cells (CFU-L) to appreciate the cytotoxicity of CD10, CD24 and CD19 MoAbs on these progenitors.

PATIENTS AND METHODS

Patients (Table 1)

Seventeen patients were studied: 13 at the time of diagnosis and 4 during relapse. The mean age was

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Table 1. Evolutive phase and biological data of the patients with in vitro *CFU-L culture*

Pt Nb	FAB sub-type	Phase of the disease	Nb of peripheral leukemic cells/mm³	Surface markers of fresh cells CD10 ALB2	CD9 ALB9	CD24 ALB6	CD19 B4	CD2 T11	CFU-L*
1	L1	P1	48,000	60%	80%	80%	nd	Neg	36
2	L2	P1	17,000	80%	90%	90%	nd	Neg	47
3	L2	P1	15,000	90%	90%	90%	nd	Neg	56
4	L2	P1	90,000	49%	88%	90%	94%	9%	66
5	L2	P1	75,000	95%	70%	95%	95%	2%	30
6	L1	P1	60 (BM: 97%)	80%	80%	80%	nd	Neg	36
7	L2	P1	325,000	90%	++	++	nd	Neg	34
8	L2	P2	5500	90%	60%	80%	nd	Neg	71
9	L2	P1	5000	38%	50%	70%	60%	30%	124
10	L2	P2	55,000	8%	23%	24%	nd	Neg	116
11	L1	P1	13,800	40%	60%	100%	85%	Neg	53
12	L2	P1	90,000	40%	80%	60%	nd	4%	50
13	L2	P1	400 (BM: 99%)	+	++	++	nd	Neg	53
14	L1	P1	156,000	35%	52%	82%	80%	12%	73
15	L1	P2	700 (BM: 86%)	95%	95%	95%	nd	Neg	37
16	L1	P2	200 (BM: 98%)	67%	6%	81%	96%	Neg	106
17	L2	P1	1500	42%	52%	45%	55%	10%	74

P1: first evolutive phase, P2: first relapse; nd: not done.
In 4 instances with low WBC counts (cases 1, 6, 15 and 16), CFU-L were obtained from bone marrow (see text). In these cases, % and phenotype of bone marrow blasts (BM) are given.
ALB6 and ALB9 are not cytotoxic.
T markers (CD2): Neg ≤ 1% cells.
*CFU-L first plating efficiency: number of colonies/2 × 10^4 cells plated.

32 ± 20 (range: 15–69). The cytological subtype according to FAB classification is L1 in 6 cases and L2 in 11 cases. Blood was used for *in vitro* culture of clonogenic cells in 13 patients with a mean circulating leukemic cell count 69,000/mm³ (range 1500–325,000). However 4 patients had low circulating blast cell count; in these patients, the bone marrow (95% ± 5 blast cells) was used for culture.

The presence of surface antigens on leukemic cells was investigated with MoAbs [13]: ALB2 for CD10, ALB9 for CD9, ALB6 for CD24 (these 3 MoAbs were produced by one of us and commercialized by Immunotech, Marseilles, France), B4 (provided by Dr. Nadler for Boston International Workshop) for CD19 and OKT11 (Ortho Lab.) for pan-T antigen.

Methods

Cytotoxicity. Leukemic cells were used to test the cytotoxic activity of ALB2 and rabbit complement. Fresh leukemic cells (10^6 cells) from 6 patients with cALLA⁺ leukemias (case 6 and 5 additional cases, Nos 18–22) and cells from KM3 continuous cell line (cALLA⁺) were incubated for 30 min at 4°C with 0.5, 1 and 2 μg (and 0.05 μg for KM3) of ALB2 and exposed to rabbit complement (Centre de Transfusion Sanguine, Besançon, France) (final dilution 1/2) for 1 h at room temperature. After washing and Trypan Blue incubation, the percentage of surviving cells and that of cells positive for ALB2 among them were counted.

Colony assay for cALL progenitors. The method used for colony assay in ALL was that described by Izaguirre *et al.* [18, 19]. Mononuclear cell populations were obtained by centrifuging heparinized blood or bone marrow through a 1077 density gradient (M.S.L. Lab.). Cells from the interface were incubated with sheep erythrocytes during 1 h (or overnight) and the T lymphocyte rosettes removed using a second density cut. Light cells contained less than 2% T cells as assayed by SRBC rosette formation. The T cell depleted population (E^- cells) was washed in growth medium (alpha medium with 10% fetal calf serum) and resuspended.

The T-cells (E^+ cells) were recovered from the pellet after the second density cut by lysing the SRBC and incubated with mitomycin C (Sigma Lab.), for 30 min at 100 μg/ml in growth medium, to avoid proliferation. Conditioned medium (PHA-TCM) was prepared by culturing normal T cells with 1% PHA in growth medium for 3 days. The supernatant was collected and filter-sterilized.

2×10^4 E^- cells were mixed with 3×10^4 E^+ treated cells (feeder cells), and plated in alpha-MEM (Eurobio Lab.), with 10% fetal calf serum, 20% PHA-TCM and 0.8% methyl cellulose (Fluca Lab.), into microwells (Titerteck, Limbro Lab.). Cells were cultured at 37°C in incubator chamber (Billup, Rothenberg) containing 5% O_2 concentration, 5% CO_2 and balanced N. Control cultures with feeder cells alone (mitomycin-treated T cells)

+ 20% PHA-TCM were also plated. After 5–7 days colonies containing more than 20 cells were counted. Cells from colonies were collected, washed with warm PBS and resuspended in PBS for May–Grunwald–Giemsa staining and immunological analysis (ALB2, ALB9, anti-B4 and OKT11) using a fluorescence microscope.

In vitro *depletion.* E^- negative cells were incubated before plating with various concentrations (0.5, 1, 5 μg/10^7 cells) of ALB2 (CD10, Immunotech, Marseilles, France), BA1 (CD24, Hybritech) (in 5 cases) or SB4 (CD19, Immunotech) (in 2 cases) for 30 min at 4° C followed by exposure (1 h at room temperature) to new-born rabbit complement (dilution 1/2). In 6 cases, a simultaneous exposure to ALB2 and BA1 or ALB2 and SB4 (5 μg/10^7 cells of each) was performed according to the same schedule. After washing, cells were resuspended in 1 ml and plated as described before. Controls with exposure to MoAb alone, complement alone and without *in vitro* exposure were plated. For each MoAb concentration, at least 4 microwells were counted.

Results of MoAbs and complement exposure were expressed as % of CFU-L inhibition.

RESULTS

ALB2 + rabbit complement was strongly cytotoxic for total leukemic cells (Table 2), with, at minimum, a percentage of killed cells equivalent to the proportion of $ALB2^+$ cells; all surviving cells were negative for this marker. A dose–response curve was obtained for KM3 cells, with only 25% dead cells after exposure to 0.05 μg/.10^6.

The mean plating efficiency of untreated leukemic samples was 62 ± 22 colonies for 2×10^4 E^- cells plated (range: 30–124), with no relationship between the number of circulating blast cells and plating efficiency ($r = 0.23$).

The phenotype of the cells from pooled colonies was determined in 8 cases. Morphology and surface markers of pelleted cells (Table 3) confirmed the leukemic origin of the colonies. The percentage of cALLA positive cells in cultures varied from patient to patient, but the presence of CD9 and CD19 antigens, when tested, and the absence of T cell antigens argue for the pre-B origin of the colonies. The only case (patient 10) with cALLA negative colonies ($CD9^+$, $T11^-$) had few cALLA-positive circulating cells (8%). A good correlation was found ($r = 0.92$, $P = 0.01$) between the percentages of cALLA positive blast cells assessed by immunofluorescence before plating and after culture.

We did not observe a dose-dependent CFU-L inhibition curve after exposure to various concentrations of ALB2 varying between 5 and 0.5 μg/10^7 cells (Table 4). The association of ALB2 (2 or 5 μg/10^7 cells) and complement (Table 5) resulted in a good cytotoxic effect in 9 cases: a complete inhibition was obtained in 5 cases, and in the 4 other cases only 1 (± 0.5) residual colony (inhibition ≥ 95%) was observed. However, in the 6 other cases tested (40%), the inhibitory effect was only moderate, with 34–84%.

No relationship was found between the proportion of cALLA-positive cells before plating or in colonies and the inhibition of cell growth after exposure to MoAb + C′ ($r = 0.27$ and $r = 0.29$, respectively). Exposure to BA1 (5 μg/10^7 cells) and complement gave better (or at minimum equivalent) inhibition of CFU-L growth than ALB2, but was not sufficient in 3 cases to eradicate colony growth. In the 3 cases tested with an association of ALB2 and BA1, results were (at minimum) equivalent to those obtained with each MoAb alone in the 2 cases

Table 2. Cytotoxicity of $ALB2^+$ complement on fresh leukemic cells and KM3 continuous cell line

Pts	% of $CD10^+$ cells	% cytotoxicity ALB2 2 μg	C′ 1/2	ALB2+C′ 2 μg	ALB2+C′ 1 μg	ALB2+C′ 0.5 μg	% of $ALB2^+$ residual cells
6	80%	4%	25%	nd	80%	nd	<5%
18	56%	6%	30%	95%	88%	87%	nd
19	62%	nd	12%	60%	nd	63%	nd
20	92%	nd	15%	91%	94%	94%	0%
21	56%	0%	15%	67%	74%	70%	0%
22	16%	0%	18%	24%	16%	22%	0%
KM3	95%	nd	nd	nd	97%	95%	0%

Cytotoxicity activity of ALB2 (30 min at 4°C with batch used for all *in vitro* depletion) and new-born rabbit complement, final dilution 1/2, 1 h at room temperature.
Cytotoxicity was assayed by Trypan Blue staining.
Patient 18–22 were not tested for CFU-L depletion.
ALB2 alone was used at 2 μg/10^6 cells.
% of $ALB2^+$ surviving cells were tested for the minimal MoAb concentration exposure (0.5 μg).

Table 3. Surface markers in colonies

Pt	Surface markers of cells from colonies			
	CD10	CD24	CD19	CD2
Nb	ALB2	ALB9	B4	T11
4	44%	95%	95%	Neg
5	95%	nd	nd	5%
9	58%	90%	70%	Neg
10	Neg	+	nd	Neg
12	62%	80%	nd	Neg
14	60%	95%	95%	4%
15	95%	95%	nd	5%
16	86%	94%	36%	4%

Cells from colonies were pooled, washed and tested for surface markers. Percentages of viable labelled cells are scored after indirect fluoresence test.

Table 4. Clonogenic leukemic cell inhibition after exposure (30 min, 4°C *to different ALB2 concentrations* (5, 1, 0.5 μg/10^7 *cells) followed by C'* (1 h *room temperature*)

Pt	Nb of CFU-L/2 × 10^4 cells			
		ALB2 + C' (1/2 dilution)		
Nb	Control	5 μg	1 μg	0.5 μg
4	66	0 (100%)	0 (100%)	0 (100%)
5	30	0 (100%)	0 (100%)	0 (100%)
7	53	13 (80%)	28 (58%)	11 (83%)
8	71	1 (99%)	1 (99%)	1 (99%)
14	73	42 (43%)	36 (51%)	39 (47%)
15	37	26 (30%)	23 (38%)	17 (54%)

Percentages of inhibition are noted in brackets.

of complete inhibition, but without additive effect in the third case. The effect of SB4 alone on CFU-L was documented in 2 cases, with a total inhibition in one case and 24% surviving CFU-L in the other case. The cells issued from these surviving CFU-L expressed B4 antigen (74% of the cells). The simultaneous exposure to SB4 + ALB2 in 3 patients gave a good inhibition in 2/3 cases (≥ 99%).

DISCUSSION

We have tested the cytotoxic effect of CD10 — and in some cases of CD24 and CD19 — MoAb on clonogenic leukemic cells in ALL using an adequate method of culture on semi-solid medium [18]. The leukemic origin of colonies was assessed by determining the phenotype of cells obtained from colonies, with a significant correlation between the percentage of $cALLA^+$ cells before plating and in the colonies. However, there was no correlation between this percentage and the rate of inhibition of CFU-L by anti-cALLA MoAb + C'; these data suggest that the antigen profile of the precursors of colonies can differ from the one of more mature leukemic progeny.

The absence of T cell proliferation was demonstrated by the low percentage of cells bearing T markers in the colonies. Feeder T cells permitted the growth of pre-B cells but were previously treated with mitomycin C and were unable to proliferate after plating. A complete or marked inhibition of CFU-L after exposure to ALB2 was observed only in 9 out of the 15 cases tested. Several hypotheses can be raised to explain the failure to get a satisfactory inhibition of leukemic growth in near half of the patients: (a) A growth of residual T cell progenitors due to incomplete T depletion before plating; this is an unlikely explanation since in the 8 cases tested, the percentage of T cells in the colonies was lower than 5% and, in the only case tested, the residual colonies were $B4^+$. (b) An incomplete lysis by complement of ALB2− coated cells; the complete lysis of all leukemic cells by ALB2 + C' at the dose used was demonstrated by Trypan blue staining and argues against this supposition, but the more sensitive colony forming assay is more likely to detect these residual incompletely lysed cells. Since other authors [5] have emphasized the importance of multiple MoAb and complement exposures, perhaps an extra round of treatment may have improved the data. (c) Antigenic modulation: the internalization of the immuno-complexes renders the cells resistant to complement-dependent lysis [20], and could occur within a short time after exposure to MoAb. Modulation did not appear in our experience as a prominent phenomenon, since all cALLA-positive leukemic cells, as shown by immunofluorescence labelling, were destroyed during the same time exposure — 30 min for MoAb and 60 min for C' — than the one utilized for inhibition of CFU-L. Except in the case of a rather improbable restriction of antigenic modulation to the leukemic clonogenic cells, modulation cannot explain the incomplete inhibition of CFU-L in an appreciable number of $cALLA^+$ ALL. (d) The existence of cALLA negative — more primitive? — clonogenic cells in cALL. Such a hypothesis, first suggested by Izaguirre *et al.* [21], was supported more recently by Touw *et al.* [22]: these authors, using a cell sorter, have separated the cALLA-positive and cALLA-negative cells in 4 cases of cALL and observed that colonies (of $cALLA^+$ cells) are obtained in both $cALLA^+$ and $cALLA^-$ fractions after plating. Consolini *et al.* [23] confirmed these data in 11 patients with cALL during CR: E^+ and residual $cALLA^+$ depleted blood mononuclear cells gave colonies with variable proportion of $cALLA^+$ cells. Morever, one could hypothesize that the cALLA-negative precursors are the more primitive, with the cALL antigen

Table 5. In vitro *inhibition of CFU-L after exposure to cytotoxic MoAb: incubation with MoAbs alone did not inhibit growth* (≤ 5%), *data not shown*

Pt Nb	C′ (1/2)	ALB2 + C′	BA1 + C′	ALB2 + BA1 + C′	SB4 + C′	ALB2 + SB4 + C′
1	17%	100%	nd	nd	nd	nd
2	0%	100%	nd	nd	nd	nd
3	nd	100%	nd	nd	nd	nd
4	11%	100%	100%	100%	nd	nd
5	0%	100%	100%	100%	nd	nd
6	34%	97%	nd	nd	nd	nd
7	64%	95%	nd	nd	nd	nd
8	76%	98%	nd	nd	nd	nd
9	nd	98%	nd	nd	76%	99%
10	33%	84%	nd	nd	nd	nd
11	nd	80%	91%	nd	nd	nd
12	18%	76%	nd	nd	nd	nd
13	19%	66%	nd	nd	nd	nd
14	0%	43%	73%	nd	nd	nd
15	24%	34%	73%	68%	nd	nd
16	0%	nd	nd	nd	100%	100%
17	nd	nd	nd	nd	nd	80%

Incubation with new-born rabbit complement alone (1/2 dilution, 1 h, room temperature), with ALB2 (CD10, Immunotech, Marseilles) (5 μg/10^7 cells) followed by complement (1/2 dilution).
Incubation with BA1 (CD24, Hybritech) 5 μg/10^7 + complement (1/2 dilution), and association of (ALB2 + BA1) + C′ (1/2 dilution, 1 h at room temperature).
Incubation with SB4 cytotoxic CD19 MoAb (Immunotech), 5 μg/10^7 cells, 30 min, 4° C + C′.
Simultaneous exposure to ALB2 + BA1 or ALB2 + SB4 (5 μg/10^7 cells of each) followed by C′.
Inhibition is expressed in percentage of CFU-L inhibition compared to control, and is the mean of a minimum of 4 microwells counting.

appearing at a later stage of differentiation. A recent observation from Keating *et al.* [24] on human marrow stromal cells is in favor of such a hypothesis: long term cultures could be generated after destruction of most of the stroma cells by anti-cALLA MoAb + C′, suggesting that the cALLA$^+$ stromal cells derive from cALLA-negative precursors.

The fact that, in our experience, CFU-L in cALL may not bear the cALL antigen, consequently favors the use of a cocktail of MoAbs and/or of MoAb directed against antigens present at a more primitive level of differentiation.

In children with ALL in second or subsequent CR, Takvorian *et al.* [25], using a purged ABMT treated with one (J5 anti-cAllA) or two (J5 + J2, anti-gp26) MoAbs, observed 27% early relapses, a proportion lower than the percentage of patients with a poor *in vitro* and probably *in vivo* CFU-L depletion by ALB2$^+$ C′ in our experience.

The higher inhibition of CFU-L with BA1 was related to the higher percentage of leukemic cells positive for this marker, and confirms the usefulness of such MoAb belonging to CD24 in a program of bone marrow purging in ALL. The ALB2 + SB4 association seems to be interesting, despite one incomplete inhibition out of 3 patients tested.

With our assay, we could detect a maximum of two log depletions corresponding to complete absence of CFU-L growth, but we could not determine whether the association of two MoAbs is more potent than one. The superiority of a cocktail of 3 MoAbs + C′ was demonstrated, however, *in vitro* by Le Bien *et al.* [17], using a clonogenic assay to detect residual cells after MoAb depletion of contaminated normal bone marrow with cALLA$^+$ continuous cell lines. This method of purging by 3 different MoAbs has been tried by Ramsay *et al.* [3] in 23 patients, treated by autologous BMT during second or subsequent remission, with 7 patients alive and without relapse for 6–32 months. However, these authors noticed that the number of antibodies with which the bone marrow leukemic cells reacted *in vitro* did not influence the rate of relapse.

The use by Favrot *et al.* [26] of a liquid culture system to monitor the efficacy of the *in vitro* depletion method in Burkitt leukemia–lymphoma was not possible in cALL because of the very low plating efficiency of cALL CFU-L.

In conclusion, whenever a purging of bone marrow is considered for an autologous transplantation, it is advisable to select cytotoxic MoAbs directed against several antigens probably present on the

leukemic progenitor cell surface: CD24 and CD19 in addition to CD10. Moreover an *in vitro* testing of the effectiveness of these combined MoAbs on CFU-L could be helpful to choose the cocktail that could be employed in each individual patient.

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