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Influence of Cytokines and Autologous Lymphokine-Activated Killer Cells on Leukemic Bone Marrow Cells and Colonies in AML

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Key Words
Clonality · Cytokines · In vitro treatment · Lymphokine-activated killer cells

Abstract
We have already shown that cytokine cocktails (IL-1β, IL-3, IL-6, SCF, GM-CSF) and/or lymphokine-activated killer (LAK) cells can reduce the amounts of clonal, CD34-positive mononuclear bone marrow cells (BM-MNC) in acute myeloid leukemia (AML). In addition, the influence of those cocktails and/or LAK cells on the clonogenic potential of AML BM-MNC was investigated. BM colonies cultured in agar during different stages of the disease were immunophenotyped in situ: 17 patients at diagnosis, 14 patients in complete remission, 8 patients at relapse, 8 healthy donors. A significant reduction in leukemic cells and colonies positive for CD34 after in vitro culture of BM-MNC with cytokine cocktails was achieved with all samples obtained at diagnosis ($n = 8$, $p < 0.01$), in 6 of 8 cases in complete remission but only in 2 of 6 cases at relapse. Cytokine cocktails stimulated granulopoiesis as well as B and T lymphopoiesis. Colonies with leukemic phenotype could never be detected in healthy BM. A significant reduction in leukemic colonies was achieved by coculture of BM-MNC (uncultured or cytokine precultured) with autologous LAK cells in all 4 cases at diagnosis and in 1 case at relapse. An additive effect of in vitro cytokine preincubation of BM samples on the leukemia-reducing effect of LAK cells could be demonstrated in all samples studied ($p < 0.001$; diagnosis: $n = 10$, relapse: $n = 3$, complete remission: $n = 7$). Patients had a better prognosis if CD34-positive colonies in AML could be reduced by cytokine incubation ($p = 0.03$) or coculture with autologous LAK cells in vitro ($p = 0.04$). Our data show that cytokines as well as LAK cells alone and in combination can reduce, however not eliminate clonogenic AML cells. Such mechanisms might be responsible for maintaining stable remissions in AML.

Introduction

Acute myeloid leukemia (AML) results in an accumulation of leukemic blasts through clonal proliferation from one abnormal progenitor cell. Clonal cell populations can be identified by cytogenetics, polymerase chain reaction and Southern blot analyses [1, 2]. Leukemic cell populations can be identified by flow cytometry using a panel of

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different antibodies [3]. At colony level, leukemic colonies can be distinguished from nonleukemic colonies by culture of bone marrow mononuclear cells (BM-MNC) in a clonogenic assay with consecutive immunophenotyping in situ [4]. About 70% of patients with AML in complete remission relapse within 2 years. Therefore residual leukemic cells must have survived [5]. In patients who do not relapse, mechanisms must exist which inhibit or even eradicate those leukemic cells. Besides cellular mechanisms, several soluble factors and cytokines seem to play a role in the suppression of leukemic cells. Up to now, animals have been treated by several cytokines like IL-2 or interferon-α in order to find tumoricidal factors or to test the efficiency of different substances in restoring normal hematopoietic bone marrow cells. Moreover, IL-3, GM-CSF, or G-CSF are reported to regulate the proliferation of leukemic cells [6–8]. Preclinical data of others and ourselves show a positive synergistic effect of SCF, GM-CSF, IL-1β, IL-3, IL-6 and EPO on the proliferation of hematopoietic progenitor cells; moreover we could already demonstrate a reduction in CD34-positive, clonal cells in patients with AML [9, 10]. Cytotoxic mechanisms, mediated for example by natural killer (NK) or LAK cells, are known to suppress leukemic growth in vivo or in vitro, as already shown by others and ourselves [11, 12]. However, the influence of cytokines and/or LAK cells on the clonogenic potential of AML cells in vitro with respect to an influence on clonal CD34-positive cells has to be evaluated. We investigated the influence of LAK cells and of different combinations of growth factors on the survival of leukemic and normal cell colonies in vitro using untreated and cytokine-precultured BM-MNC of AML patients at diagnosis or in the course of the disease. Leukemic cell load in BM-MNC was evaluated by immunophenotyping of single cells (flow cytometry) and cell colonies after agar culture (enzyme immunoassay), and the proportion of clonal cells was evaluated by Southern blot analysis and densitometry. The clinical relevance of our in vitro studies was evaluated by survival analyses of patient groups formed according to response to in vitro cytokine treatment and/or LAK cell treatment.

Materials and Methods

Patients

Patients (mean age 49 years, male to female ratio 1.17:1) underwent cytological and cytochemical tests on diagnosis (17 patients), in complete remission (14) and at relapse (8 patients). Classification according to FAB criteria was as follows: 3 patients with AML-M0, 3 with AML-M1, 9 with AML-M2, 9 with AML-M4, 5 with AML-M5 and 10 with unknown FAB type. All the patients were included in the study at initial therapy and none had been previously treated. Patients were treated according to approved therapy standards. Complete remission was defined according to the criteria of the Cancer and Leukemia Groups (CALGB) when BM was normocellular, contained less than 5% blast, and at least 1.5×10^9/l neutrophil granulocytes in peripheral blood (PB) and at least 100×10^9/l platelets [6]. Relapse was diagnosed morphologically when the BM contained more than 5% leukemic blasts, or when leukemic cell infiltration occurred at any other site. BM cells obtained from 8 healthy donors served as controls.

Cell Preparation

BM cells were obtained by aspiration from the patients' posterior iliac crest after informed consent and collected in preservative-free heparin. MNC were obtained from BM cells by Ficoll (density 1.077; Seromed) density gradient centrifugation and washed in Hanks' balanced salt solution with $NaHCO_3$ (Seromed).

Culture of BM Cells with Cytokine Cocktails

BM-MNC of AML patients obtained at different times in the course of their disease and BM-MNC from healthy BM donors were cultured (1.0×10^6 cells/ml media) in cytokine-containing media for 14 days at 37 °C and 5% CO_2 in a humidified atmosphere. We used two different cytokine cocktails [7]:

(1) Cytok 1: IL-1β (10 ng/ml, Boehringer Ingelheim) + IL-3 (20 U/ml, Novartis) + IL-6 (100 U/ml, Novartis) + EPO (1 U/ml, Boehringer Mannheim) + SCF (100 ng/ml, Amgen) + GM-CSF (100 ng/ml, Novartis) in Iscove's modified Dulbecco's medium (ISC, GibcoBRL) and 20% fetal calf serum (FCS, HyClone).

(2) Cytok 2: had the same composition as Cytok 1 but without GM-CSF.

(3) ISC/FCS: control medium without added cytokines in ISC containing 20% FCS.

Culture of LAK Cells and Coculture with Autologous BM Cells

We performed coculture experiments in order to test the antileukemic effect of LAK cells on autologous BM cells. LAK cells were obtained after 14 days of culture of $0.5–1.0 \times 10^6$ PB cells/ml in the presence of 1,000 U/ml IL-2 (Cetus Ltd., Ratingen, Germany) in LAK medium (69% ISC, GibcoBRL), 15% equine serum (HyClone), 5% FCS (HyClone), 1% penicillin-streptomycin (GibcoBRL) [12]. After 2 weeks those cells were analyzed for the expression of antigens like CD5, CD56, CD16, CD8 and CD25 typical for LAK cells [12, 13]. BM cells from AML patients were aspirated at different stages of the disease and from healthy BM donors. BM-MNC were (a) either frozen in liquid nitrogen by a computer-driven freezing procedure and thawed after 14 days for coculture with LAK cells or (b) were cultured for 14 days in Cytok 1, Cytok 2 or ISC/FCS as a control and afterwards added to autologous LAK cells [10]. LAK and BM cells were mixed in a proportion of 5:1. After 4 h, the cell mixtures were mixed with agar, plated on special slides (BioRad) and stimulated by GM-CSF (500 U/ml) for a 7-day colony assay [4]. In this colony assay LAK cells formed colonies under the influence of GM-CSF. Therefore the proportions of leukemic colony forming units (CFUs) under the influence of LAK cells in a 5:1 mixture with autologous BM cells had to be calculated according to the following formula:

$$\frac{(tc_p^{(1)}) - (5/6\ tLAK_p^{(2)})}{(tc_a^{(1)}) - (5/6\ tLAK_a^{(2)})},$$

where tc_p = total number of colonies positive for an antibody; tc_a = total number of all colonies counted; $tLAK_p$ = total number of LAK colonies positive for an antibody; $tLAK_a$ = total number of all LAK colonies counted; (1) = colony numbers obtained in the LAK/BM coculture, and (2) = colony number obtained in the LAK control culture.

Immunophenotyping of Agar Colonies in situ

To study the viability of BM-MNC as well as the influence of cytokines and autologous LAK cells on leukemic colonies, we cultured mononuclear cells for 7 days in a miniaturized culture assay directly on sterilized and specially prepared slides obtained from Bio-Rad as previously described [4]. 3×10^4 BM-MNC were suspended in 20 µl ISC/FCS and 0.25% agar agar (Fluka, Bio Chemika). After jelling at 37°C, 40 µl GM-CSF medium (500 U/ml (Novartis) in ISC/FCS) was layered on top of each plug. After 7 days of culture, colony counts were calculated. Moreover, immunologically stained colonies were counted after in situ incubation with a panel of different antibodies [4]. The total cell and colony counts before and after 14 days of cytokine incubation (Cytok 1, Cytok 2 or ISC/FCS as a control) or LAK cell treatment were evaluated to estimate the reductive effect on leukemic colonies as well as the proliferation-stimulating effect of those cytokine cocktails and LAK cells. The following unconjugated antibodies were used: CD34 (HPCA-I, Becton-Dickinson), CD20 (B 1, Coulter), CD13 (My 7, Coulter), CD15 (Leu M1, Becton-Dickinson), HLA-DR (Becton-Dickinson).

Surface Marker Analyses

Flow cytometric analyses were performed on uncultured and cultured BM-MNC in order to estimate the percentage of blast cells (positive for CD34), granulocytes (positive for CD15 and CDw65), T cells (positive for CD3) and B cells (positive for CD19 and CD20) [3, 4]. Antibodies conjugated with fluorescent dyes – phycoerythrin (PE) or fluorescein isothiocyanate (FITC) – were used: CD34 (class III clone 581, PE-labeled; Immunotech), CD15 (FITC or PE-labeled; Sigma), CDw65 (FITC-labeled; Ortho), CD19 (PE-labeled; Ortho), CD20 (PE-labeled, Dako) or CD3 (FITC-labeled; Ortho). Blast phenotypes at diagnosis were regularly evaluated using a panel of different leukocyte antibodies in combination according to consensus protocols [3]. In the further course of AML a patient's typical antibody combinations – including CD34 antibodies in combination with pan-myeloid (e.g. CD13, CD33) and pan-leukocyte antibodies (CD45) – were used to quantify CD34-positive cells. To avoid unspecific or epitope class-specific variations of antibody binding the same CD34 antibodies of the same company were always used. Analyses were performed on a flow cytometer (Cytoron Absolut, Ortho Diagnostic Systems). Data were evaluated using a special software (Immunocount 2) from Ortho Diagnostic Systems. The percentage increase or decrease of cells positive for the antibodies mentioned above before and after the cytokine treatment was calculated.

Statistics

Statistical tests (log-rank test, Mann-Whitney U test, repeated-measures Anova) were performed on a personal computer by a special software (Statistica 4.5, StatSoft Inc. 1993). All tests used were two-tailed. Differences were considered significant for $p < 0.05$. For survival analyses, patients were assessed at the time of BM transplantation and the data expressed by a Kaplan-Meier plot.

Results

Clonogenic Potential and Cellular Composition of Uncultured or Precultured BM-MNC from AML Patients at Different Stages of the Disease

Combinations of SCF with several cytokines are recommended to enrich hematopoietic stem cells [7]. The influence of those cytokine cocktails on clonogenic leukemic or healthy BM cells was studied. BM-MNC of AML patients at different stages of the disease were cultured in Cytok 1 (containing GM-CSF), Cytok 2 or ISC/FCS being used as controls. The clonogenic leukemic potential in BM samples (before and after cytokine culture) was evaluated after 7 days of culture in agar in the presence of GM-CSF. Moreover, cells were immunophenotyped before and after cytokine culture. In total, BM samples of 39 AML patients were studied: 17 at diagnosis, 8 at relapse and 14 in complete remission. BM-MNC from 8 healthy BM donors served as controls. The percentage of BM colonies of uncultured or precultured (with Cytok 1, Cytok 2 or ISC/FCS) BM cells positive for their respective antibodies are given.

Proportions of Leukemic and Nonleukemic Uncultured BM Cells or BM Colonies after 7 Days of GM-CSF Culture (table 1)

Proportions of undifferentiated (CD34-positive) or granulocytic (CD15-positive) cells or colonies after 7 days of culture in GM-CSF were evaluated in order to estimate the leukemic potential in BM samples obtained at diagnosis, at relapse, in complete remission or in control samples. On average, 54.4 ± 15.8, 13.3 ± 2.3, 7.0 ± 2.5 and 4.3 ± 0.9% CD34-positive cells could be detected in BM samples obtained at diagnosis, at relapse, in complete remission or in controls, respectively (table 1). At the same time, 35.3 ± 13.5, 33.2 ± 9.3, 56.5 ± 8.2 and 62.3 ± 6.4% CD15-positive cells could be detected at diagnosis, at relapse, in complete remission or in controls, respectively (table 1). After 7 days of culture of BM-MNC in the presence of GM-CSF, 30.2 ± 7.4, 7.6 ± 1.7, 2.5 ± 1.5 and 0% CD34-positive colonies could be detected in samples obtained at diagnosis, at relapse, in complete remission or in controls, respectively (table 1). At the same time, 26.3 ± 7.9, 22.6 ± 7.2, 39.8 ± 11.2 and 52.4 ± 6.3% CD15-positive colonies could be detected at diagnosis, at relapse, in complete remission or in controls (table 1). Proportions of CD15-positive cells or colonies were significantly higher in BM samples obtained from normal donors or in complete remission than in samples

Table 1. Proportions of CD34+ or CD15+ BM cells or colonies (in parentheses) after 7 day of culture with GM-CSF

FAB type	AML at DIA (n = 17)		FAB type	AML at REL (n = 8)		FAB type	AML in CR (n = 14)		Healthy controls (n = 8)		
	CD34+ cells or colonies	CD15+ cells or colonies		CD34+ cells or colonies	CD15+ cells or colonies		CD34+ cells or colonies	CD15+ cells or colonies	n	CD34+ cells or colonies	CD15+ cells or colonies
M1	45 (36)	42 (21)	M4	12 (11)	27 (14)	M5a	10 (2)	62 (51)	1	5 (0)	75 (49)
M5	41 (27)	23 (28)	M2	15 (8)	32 (36)	M5b	10 (3)	42 (n.d.)	2	3 (0)	65 (54)
M2	55 (33)	15 (34)	M1	15 (6)	19 (16)	M2	3 (0)	70 (44)	3	5 (0)	65 (46)
M2	60 (21)	35 (36)	M0	16 (7)	43 (19)	M4	6 (3)	60 (38)	4	3 (0)	63 (64)
M2	70 (29)	20 (45)	M2	12 (9)	43 (23)	n.d.	6 (n.d.)	46 (59)	5	4 (0)	57 (51)
M0	87 (48)	40 (34)	M4	10 (7)	35 (21)	M2	7 (3)	65 (20)	6	4 (0)	57 (58)
M0	80 (33)	18 (29)	n.d.	n.d. (6)	n.d.(22)	M1	10 (5)	52 (26)	7	5 (0)	61 (45)
M2baso	54 (35)	48 (21)	n.d.	n.d. (7)	n.d. (30)	M4	4 (4)	60 (39)	8	5 (0)	55 (52)
M4	35 (24)	47 (20)				M5b	7 (3)	57 (32)			
M5b	50 (44)	37 (19)				M4	5 (2)	52 (28)			
n.d.	37 (26)	44 (24)				M4	9 (3)	55 (49)			
M2	60 (22)	35 (21)				n.d.	n.d. (1)	n.d. (46)			
M4	41 (29)	63 (24)				n.d.	n.d. (0)	n.d. (48)			
M4	47 (25)	27 (15)				n.d.	n.d. (3)	n.d. (37)			
n.d.	n.d. (31)	n.d. (34)									
n.d.	n.d. (28)	n.d. (21)									
n.d.	n.d. (23)	n.d. (21)									

DIA = Diagnosis; REL = relapse; CR = complete remission; n.d. = not determined.

Table 2. Influence of 14 days of incubation of BM-MNC with cytokine cocktails on the amounts of CD34-positive cells or colonies (in parentheses)

FAB type	AML at DIA (n = 10) CD34+ cells (%) or colonies after in vitro culture with ...			FAB type	AML at REL (n = 6) CD34+ cells (%) or colonies after in vitro culture with ...			FAB type	AML in CR (n = 10) CD34+ cells (%) or colonies after in vitro culture with ...			n	Healthy controls (n = 8) CD34+ cells (%) or colonies after in vitro culture with ...		
	Cytok 1	Cytok 2	ISC/FCS		Cytok 1	Cytok 2	ISC/FCS		Cytok 1	Cytok 2	ISC/FCS		Cytok 1	Cytok 2	ISC/FCS
M1	−62 (−54)	n.d. (n.d.)	−49 (n.d.)	M4	+25 (−20)	n.d. (n.d.)	+67 (−6)	M5a	+40 (−15)	+40 (n.d.)	+60 (+8)	1	+ 100 (± 0)	n.d. (n.d.)	+ 20 (± 0)
M5	−49 (−50)	n.d. (n.d.)	−49 (−21)	M2	+40 (−18)	n.d. (n.d.)	n.d. (+2)	M5b	n.d. (−4)	n.d. (n.d.)	n.d. (+14)	2	+ 100 (± 0)	n.d. (± 0)	+ 33 (± 0)
M2	n.d. (−50)	n.d. (n.d.)	n.d. (−18)	M1	+12 (+4)	+12 (n.d.)	+18 (+6)	M2	n.d. (−8)	n.d. (n.d.)	n.d. (n.d.)	3	± 0 (± 0)	n.d. (± 0)	± 0 (± 0)
M2	n.d. (−46)	n.d. (−36)	n.d. (−16)	M0	−57 (−8)	−24 (n.d.)	−11 (−6)	M4	−33 (−24)	n.d. (n.d.)	+17 (+4)	4	+ 233 (± 0)	+133 (± 0)	+ 67 (± 0)
M2	−74 (−43)	−64 (−37)	−57 (n.d.)	M2	−17 (−37)	+32 (−14)	+48 (−6)	M2	−29 (−38)	n.d. (−35)	−19 (−30)	5	+ 275 (± 0)	+200 (± 0)	+ 125 (± 0)
M0	−75 (−53)	−67 (−51)	−66 (−41)	M4	+45 (−16)	+55 (−10)	+73 (−9)	M1	−56 (−57)	−67 (n.d.)	−50 (−38)	6	+ 275 (± 0)	+170 (± 0)	+ 150 (± 0)
M0	−64 (−55)	−61 (−51)	−49 (−37)					M4	−75 (−42)	−50 (−34)	± 0 (−4)	7	+ 300 (± 0)	+200 (± 0)	+ 180 (± 0)
M2baso	−78 (−57)	−63 (−48)	−59 (−27)					M5b	+27 (−22)	± 0 (n.d.)	+67 (+17)	8	+ 300 (± 0)	+260 (± 0)	+ 220 (± 0)
M5b	−57 (−44)	−57 (n.d.)	−51 (−20)					M4	−80 (−29)	n.d. (−8)	n.d. (+13)				
M2	−37 (−43)	−28 (−28)	−27 (−17)					M4	−44 (−47)	n.d. (n.d.)	n.d. (n.d.)				

DIA = Diagnosis; REL = relapse; CR = complete remission; n.d. = not determined; + = increase of positive cells or colonies (%) compared to results with untreated BM-MNC; − = decrease of positive cells or colonies (%) compared to results with untreated BM-MNC.

obtained at diagnosis or at relapse ($p < 0.005$). Otherwise, proportions of CD34-positive cells and colonies were significantly higher in BM samples obtained at diagnosis than at relapse or in complete remission ($p < 0.05$). Proportions of B or T cells were not different in the groups compared (data not shown). Whereas low amounts of CD34-positive cells could be detected in control samples, no CD34-positive colonies could be grown from control samples (table 1). A high correlation between percentages of CD34-positive cells and colonies from BM-MNC (n =

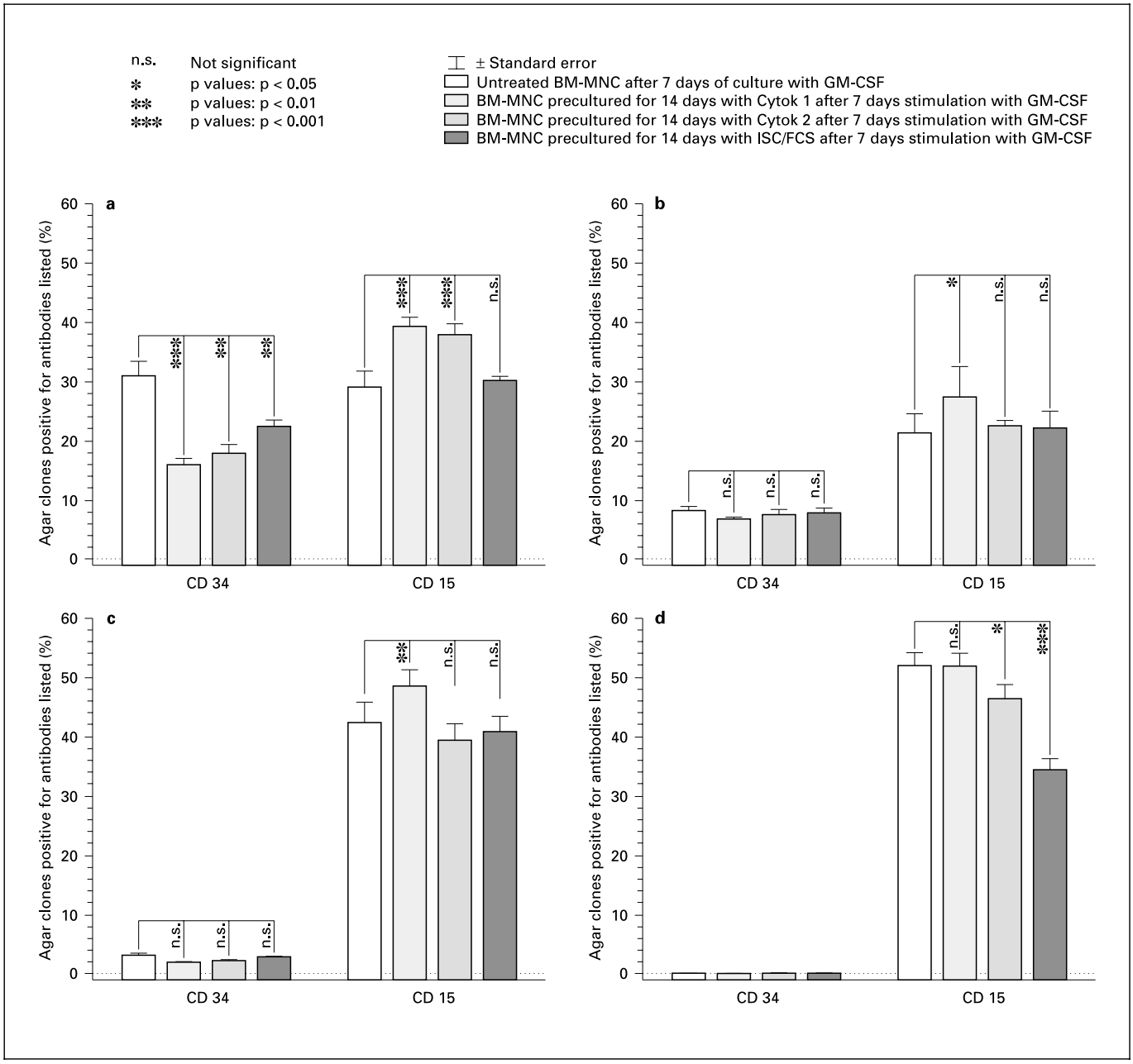

Fig. 1. Influence of cytokines on BM-MNC. Percentages of CD34- or CD15-positive colonies before and after 14 days of culture in Cytok 1, Cytok 2 or ISC/FCS. **a** AML at diagnosis (n = 10). **b** AML at relapse (n = 6). **c** AML in complete remission (n = 10). **d** BM from healthy donors (n = 8).

30, r = 0.89, $p < 0.0001$, data not shown) as well as between CD15-positive cells and colonies could be demonstrated (n = 38, r = 0.50, $p < 0.005$, data not shown), proving the clonogenic results.

Influence of Cytokine Cocktails on Proportions of Leukemic and Nonleukemic BM Cells and BM Colonies (table 2, fig. 1)

Results of Diagnosis of AML (table 2, fig. 1a). At diagnosis (n = 10), a significant reduction in CD34-positive cells ($p < 0.001$) and CD34-positive agar colonies (p <

0.01) was achieved by 14 days of incubation of BM-MNC in Cytok 1 or Cytok 2 or ISC/FCS as compared to BM-MNC not pretreated by cytokines (table 2, fig. 1a). In all BM samples a reduction of at least 27–78% CD34-positive cells as well as of 16–57% CD34-positive colonies was achieved at diagnosis (table 2). Cell culture in cytokine-containing medium resulted in significantly elevated granulocyte counts ($p < 0.001$, data not shown) or granulocyte colonies ($p < 0.001$, fig. 1a). Moreover, cytokine cocktails stimulated lymphopoietic B and T cells ($p < 0.005$, data not shown). In summary, our data show that compared to control cultures (ISC/FCS), cytokines in the culture cocktails (Cytok 1, Cytok 2) significantly increased the described effects ($p < 0.05$, table 2, fig. 1a).

Results at Relapse of AML (table 2, fig. 1b). In contrast to the results at diagnosis, at relapse (n = 6) no significant reduction of CD34-positive cells or colonies could be achieved by culture of BM-MNC with cytokine cocktails (table 2, fig. 1b): a reduction in CD34-positive cells in cytokine-containing medium of at least 57% was seen in only 2 of 6 cases (table 2). In the other cases, CD34-positive cells expanded by 55% under the influence of cytokines (table 2). The amounts of CD34-positive colonies could be reduced by cytokine cocktails in 5 of 6 cases by Cytok 1, in 2 of 2 cases by Cytok 2, but only in 4 of 6 cases by ISC/FCS. Cytokine cocktails stimulated granulopoiesis ($p < 0.05$, fig. 1b) as well as B and T lymphopoiesis compared to control cultures without added cytokines ($p < 0.01$, data not shown). Again the culture cocktails increased the effects demonstrated compared to control cultures with ISC/FCS (table 2, fig. 1b).

Results in Complete Remission of AML (table 2, fig. 1c). In complete remission (n = 10), CD34-positive cells on average remained more or less the same after incubation with cytokine cocktails (table 2, fig. 1c). A reduction in CD34-positive cells was achieved in 6 of 8 cases studied by Cytok 1, in 2 of 4 cases by Cytok 2 and in 2 of 6 cases by ISC/FCS (table 2). At the colony level, amounts of CD34-positive agar colonies were reduced by Cytok 1 and Cytok 2 by at least 4–57% in 10 of 10 cases (table 2). However, in only 3 of 8 cases cultured with ISC/FCS could a reduction of CD34-positive colonies be demonstrated (table 2). A stimulating effect of cytokine cocktails on granulopoiesis ($p < 0.01$, fig. 1c) as well as on B and T lymphopoiesis could be seen ($p < 0.05$, data not shown). Again cytokines in the culture medium increased the described effects compared to ISC/FCS (table 2, fig. 1c).

Results in Healthy BM Samples (table 2, fig. 1d). In 7 of 8 healthy BM samples a significant increase in CD34-positive cells ($p < 0.05$) (table 2), of granulocytes as well as of B and T cells (data not shown) could be demonstrated following incubation of BM-MNC with cytokine cocktails. CD34-positive colonies in agar could not be detected in any culture condition (table 2). Without cytokines added to the culture medium, a significant decrease of CD15-positive colonies was found ($p < 0.001$, fig. 1d). An increase in granulopoiesis as well as in B and T lymphopoiesis was observed at single-cell level (data not shown) following incubation with cytokine cocktails.

As very few BM samples were studied in parallel after Cytok 1 and Cytok 2 culture, a statistical comparison of cases divided in different stage groups was not possible. When all AML cases (cases at diagnosis, at relapse and in complete remission pooled), that were studied in parallel with Cytok 1 and Cytok 2 were compared, a significantly higher reduction in CD34-positive cells could be demonstrated with (Cytok 1) than without GM-CSF (Cytok 2) ($p < 0.05$; n = 14). At colony level, a better, however not significant, reduction in CD34-positive colonies with Cytok 1 than with Cytok 2 could be demonstrated.

In summary, our data show that cytokine cocktails (Cytok 1) led to a reduction in CD34-positive cells and agar colonies in all 8 AML cases studied at diagnosis, in 2 of 6 cases studied at relapse and in 6 of 8 cases studied in complete remission. In 7 of 8 healthy BM samples, CD34-positive cell proportions were increased; however, CD34-positive agar colonies could not be detected. These data prove that CD34-positive cells in AML are clonal cells, whereas CD34-positive cells from healthy donors grown in cytokine cocktails (Cytok 1, 2) are healthy, nonclonal stem cells.

Control Experiments

Whenever possible, cell counts and cloning efficiency were measured before and after cytokine treatment. The absolute cell counts were increased by Cytok 1 and Cytok 2, but not by ISC/FCS treatment: in normal BM samples, the absolute cell counts increased by 79.3 ± 29.1% (Cytok 1) or 84.8 ± 28.6% (Cytok 2), whereas in BM-MNC obtained at diagnosis of AML cell counts increased by 47.0 ± 25.3% or 49.9 ± 24.6%, by 95.6 ± 36.9% or 130.3 ± 56.9% at relapse and by 56.1 ± 24.0% or 82.8 ± 30.3% in complete remission. However, ISC/FCS led to a decrease in cell counts by 39.4 ± 12.6% in healthy BM samples, by 20.8 ± 14.4% at diagnosis and by 39.2 ± 9.5% in complete remission. At relapse, cell counts increased by 8.0 ± 20.0%. The viability of BM-MNC was not influenced by Cytok 1, Cytok 2 or ISC/FCS treatment: the absolute colony counts in a colony assay before the

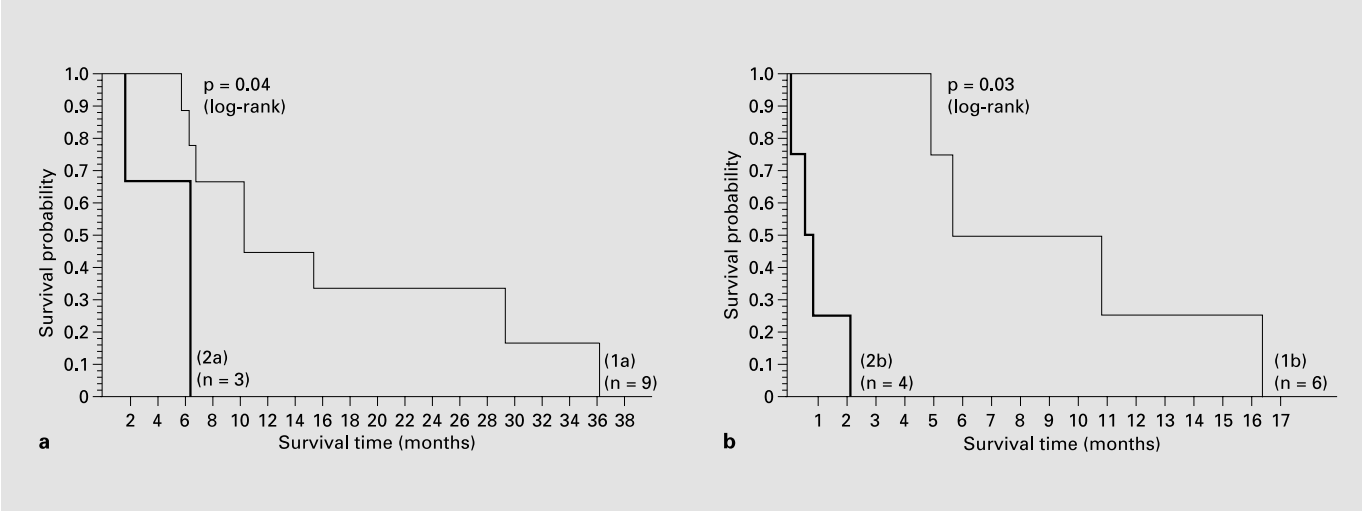

Fig. 2. Survival probability of AML patients at diagnosis; the proportions of CD34-positive BM cells (**a**; n = 12) or BM colonies (**b**; n = 10) could be reduced by more (1) or less (2) than 50% by Cytok 1 in vitro.

treatment were more or less the same as compared to counts after treatment (data not shown).

In conclusion, cytokine treatments as described here have no negative influence on cell growth or viability of BM-MNC. Addition of cytokines (Cytok 1, Cytok 2) to the control medium (ISC/FCS) resulted in an increase in the absolute single cell and colony counts.

Prognostic Value of Cytokine Treatments in vitro (fig. 2)

Statistical evaluation of our data revealed that AML patients studied at diagnosis survived longer, if CD34-positive cells (p = 0.04, fig. 2a) or CD34-positive colonies (p = 0.03, fig. 2b) grown from BM-MNC in cytokine-containing medium (Cytok 1) could be reduced by more than 50% in vitro. No significant differences could be found when other cut-off points were chosen (data not shown). Relapse-free survival times were significantly longer in those patients in whom a reduction of CD34-positive cells or CD34-positive colonies could be demonstrated in vitro (p = 0.04, data not shown). At the clonal level, the probability to achieve or stay in complete remission for a longer time was significantly higher in those patients studied at diagnosis or at relapse in whose BM samples a reduction in the amount of rearranged DNA could be achieved by culture of BM-MNC in Cytok 1 in vitro (p = 0.02, data not shown). Hematological parameters such as white blood cells, hemoglobin values or proportions of granulocytes, B or T cells or CD34-positive cells were not different in the groups compared before cytokine treatment (data not shown).

In summary, our results have clinical significance: patients had a better prognosis if CD34-positive cells or colonies or amounts of clonal DNA could be reduced by Cytok 1 in vitro.

Cytotoxic Effect of Autologous LAK Cells on Uncultured or Precultured Leukemic or Nonleukemic BM Cells and BM Colonies (table 3, fig. 3)

The potential cytotoxic effect of LAK cells on untreated or cytokine-precultured leukemic BM cells was evaluated after coculture. Proportions of CD34-positive BM agar colonies before and after coculture with LAK cells were evaluated and compared.

Influence of LAK Cells on Uncultured (Thawed) AML-BM Cells (table 3)

Coculture of LAK cells with untreated BM-MNC from AML patients at diagnosis resulted in a reduction in CD34-positive colonies (by 16–40%, table 3) as well as in CD15-positive colonies (by 3–5%) in all 4 cases examined (data not shown). In the case studied at relapse, a reduction in CD34-positive colonies by 14% could be demonstrated (table 3). No experiments were performed with AML patients in complete remission or with healthy BM samples.

Table 3. Influence of autologous LAK cells on uncultured or cytokine precultured CD34-positive BM colonies

FAB type	AML at DIA (n = 10)				FAB type	AML at REL (n = 4)				FAB type	AML in CR (n = 7)			
	BM (thawed, uncult.)	Cytok 1	Cytok 2	ISC/FCS		BM (thawed, uncult.)	Cytok 1	Cytok 2	ISC/FCS		BM (thawed, uncult.)	Cytok 1	Cytok 2	ISC/FCS
M1	n.d.	−22	n.d.	−21	M4	n.d.	n.d.	n.d.	−21	M5a	n.d.	−25	n.d.	± 0
M5	n.d.	−30	n.d.	−24	M2	n.d.	−17	n.d.	−13	M5b	n.d.	−20	n.d.	+ 1
M2	n.d.	−26	−12	−9	M1	n.d.	−25	−20	−8	M4	n.d.	−51	n.d.	−28
M2	n.d.	−9	n.d.	−4	M2	−14	−25	−18	−16	M2	n.d.	−2	n.d.	+ 11
M2	−24	−37	−33	n.d.						M4	n.d.	−28	n.d.	± 0
M0	−16	−31	−26	−25						M5b	n.d.	−16	n.d.	+ 24
M0	−17	−10	−10	−5						M4	n.d.	−23	−14	−13
M2baso	−40	−31	−26	−16										
M5b	n.d.	−28	n.d.	−6										
M2	n.d.	−29	n.d.	−13										

n.d. = Not determined; uncult. = uncultured; + = increase of positive colonies (%) compared to results before LAK/BM coculture; – = decrease of positive colonies (%) compared to results before LAK/BM coculture.

Influence of LAK Cells on Cytokine-Precultured AML-BM Cells (table 3, fig. 3)

The influence of LAK cells on cytokine-pretreated BM cells was studied. After coculture of LAK cells with BM-MNC, the amounts of CD34-positive colonies were evaluated after an additional 7 days of culture in agar under GM-CSF stimulation to estimate the leukemic potential of the cocultured cell mixture.

Results at Diagnosis of AML (table 3, fig. 3a). At diagnosis, a significant reduction of CD34-positive colonies ($p < 0.001$) and an increase of CD15-positive granulocytic colonies ($p < 0.001$) could be achieved by coculture of LAK cells with cytokine-pretreated BM-MNC (table 3, fig. 3a). In all BM samples (n = 10) studied at diagnosis, a reduction in CD34-positive colonies by at least 4–37% could be achieved (table 3). The highest reduction of leukemic CD34-positive colonies could be achieved if BM-MNC were precultured with Cytok 1 (table 3, fig. 3a).

Results at Relapse of AML (table 3, fig. 3b). LAK cell coculture of cytokine-pretreated BM-MNC from AML patients at relapse resulted in a minimal, not significant, reduction by 8–25% of CD34-positive colonies (table 3). If BM-MNC were precultured with Cytok 1 and then cocultured with LAK cells, an increase in CD15-positive granulocyte colonies could be seen ($p < 0.05$, fig. 3b). Without cytokines in the culture medium (ISC/FCS), the percentage of granulocytic colonies decreased ($p < 0.05$, fig. 3b).

Results in Complete Remission of AML (table 3, fig. 3c). LAK cell coculture of cytokine-pretreated BM-MNC (Cytok 1) from AML patients in complete remission resulted in a reduction by 2–51% of CD34-positive colonies (table 3). Without added cytokines (ISC/FCS) the amounts of CD34-positive colonies could be decreased by LAK cells in only 2 of 7 patients (table 3). Coculture of LAK cells and BM cells after Cytok 1 preincubation of BM-MNC resulted in elevated CD15-positive granulocyte colonies ($p < 0.05$, fig. 3c), whereas without cytokine preincubation the amounts of CD15-positive colonies decreased (ISC/FCS, $p < 0.05$, fig. 3c).

Results in Healthy BM Samples (fig. 3d). No CD34-positive agar colonies could be grown from healthy BM samples, either before or after LAK cell coculture. LAK cell coculture resulted in a significant reduction of CD15-positive granulocyte colony counts ($p < 0.05$, fig. 3d). With cytokine preculture this reduction was less pronounced (Cytok 1: $p < 0.05$, Cytok 2: $p < 0.01$, ISC/FCS: $p < 0.001$, fig. 3d).

Prognostic Value of LAK Cell Treatment in vitro (fig. 4)

Statistical evaluations revealed that patients studied at diagnosis, whose BM-MNC were pretreated with Cytok 1 and whose CD34-positive colonies were reduced by more than 20% by autologous LAK cells in vitro (n = 8) had a significantly better survival probability than cases without such a reduction (n = 2) (fig. 4, $p = 0.04$). No significant differences were found (data not shown) with other cut-off percentages. Moreover, we could show that patients studied in complete remission, whose clonal gene rearranged BM-DNA could be reduced by LAK cells in vitro had a significantly better survival probability as

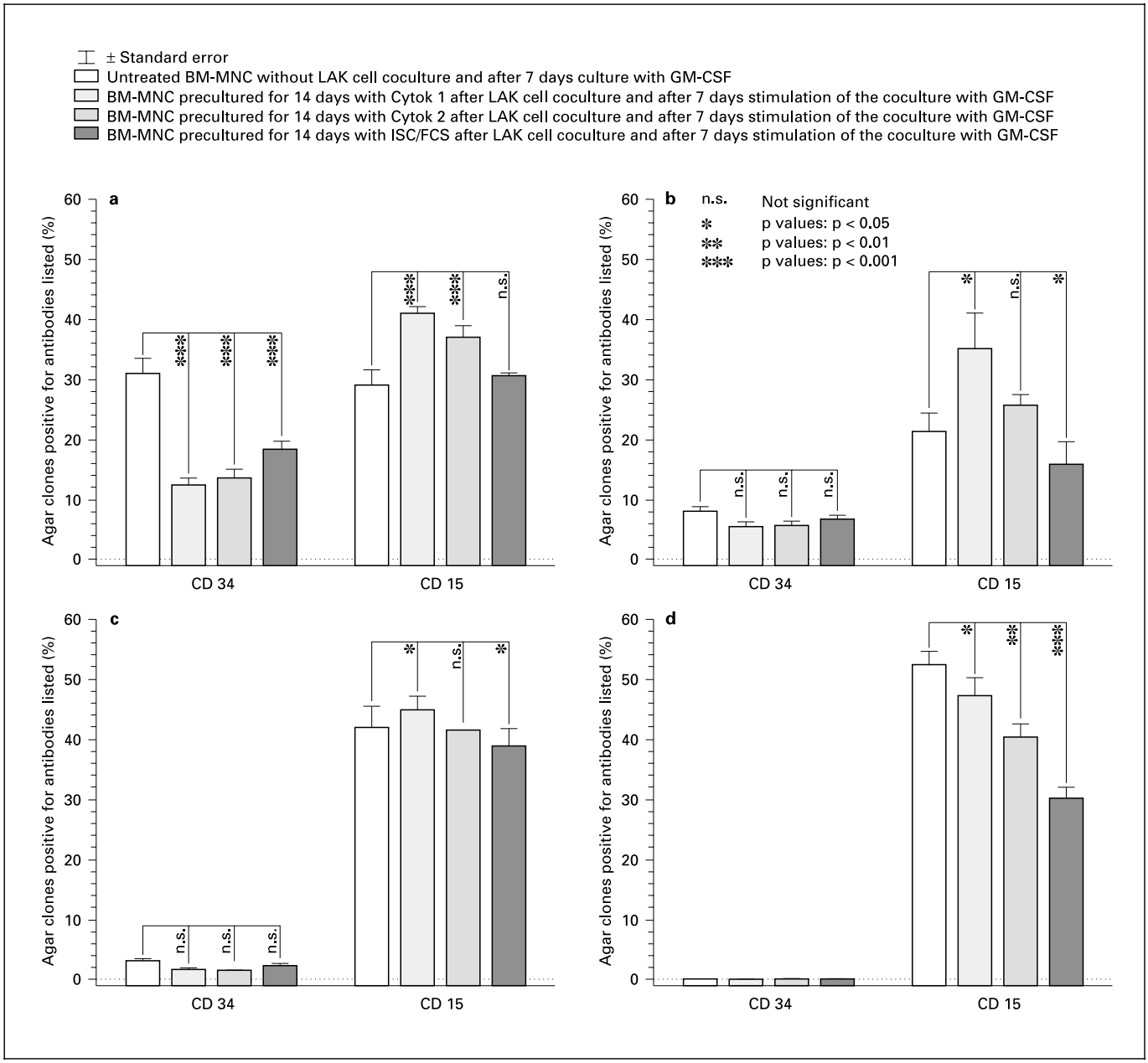

Fig. 3. Influence of LAK cells on autologous untreated (thawed) BM-MNC or BM-MNC precultured with cytokines for 14 days (Cytok 1, Cytok 2, ISC/FCS). Percentages of CD34- or CD15-positive colonies before and after the LAK/BM coculture. **a** AML at diagnosis (n = 10). **b** AML at relapse (n = 4). **c** AML in complete remission (n = 7). **d** BM from healthy donors (n = 7).

compared to cases without such a reduction (p = 0.05, data not shown).

Our data show that clonal and CD34-positive BM cells or colonies can be reduced by coculturing with autologous LAK cells. The cytotoxic effect of LAK cells on leukemic cells can be improved if BM cells are precultured in Cytok 1 or Cytok 2. Moreover, our results have clinical relevance: patients had a better prognosis if clonal cells or CD34-positive cells or colonies could be reduced by LAK cells in vitro.

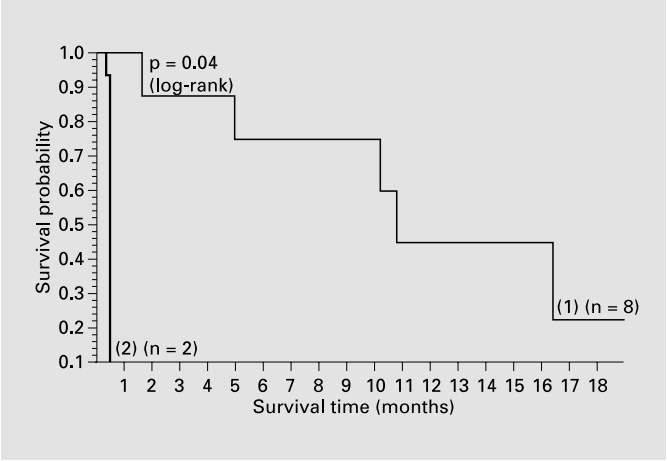

Fig. 4. Survival probability of AML patients at diagnosis (n = 10); the proportions of CD34-positive colonies obtained after cytokine treatment in vitro and coculture with autologous LAK cells could be reduced by more (1) or less (2) than 20% in vitro.

Discussion

Clonogenic Assays Are an Appropriate Method to Determine and Quantify Leukemic Cells

AML is a stem cell disease developing clonally but heterogeneously with respect to cell differentiation. Publications investigating surface marker expression in AML have so far focused on uncultured cells [3] or on clonogenic cells studied by cytotoxic tests [14], but to a lesser extent on the analysis of in vitro colonies. In previous work, our group has shown that leukemic colonies can be identified both at diagnosis and during remission by the expression of 'early' markers like CD34, CD20 and CD10 whereas late antigens (like CD15) are expressed both on normal and leukemic colonies [4, 15]. This differentiation between normal and leukemic cells was carried out at a clonogenic level by immunophenotyping of agar colonies in situ. With this method, the leukemic tumor load of untreated BM samples as well as of BM cells after cytokine treatment and/or LAK cell treatment was evaluated. As in previous studies [4, 16, 17], we could again demonstrate the presence of large amounts of CD34-positive cells and agar colonies after 7 days of culture in the presence of GM-CSF in active stages of AML and low amounts in remission. In previous experiments we could also demonstrate that the amounts of leukemic agar colonies are predictive of relapse [16]. CD34-positive agar colonies could also be detected in samples obtained from patients in remission of AML, but not in samples from healthy BM donors. As already shown, the persistence of clonogenic leukemic cells can also be proved at the clonal level by Southern blot analyses: clonal, gene-rearranged cells can be detected regularly in remission of AML [5, 17, 18]. Moreover, the differentiation capacity of cells can be estimated by evaluating the amounts of CD15-positive granulocyte agar colonies: proportions of CD15-positive cells or colonies were significantly higher in BM samples obtained from healthy BM donors or in remission of AML than in samples obtained at diagnosis or at relapse. It can be concluded that the colony assay used here is an appropriate method to detect and quantify clonogenic leukemic cells in the course of AML.

Cytokines Can Reduce but Not Eliminate Clonogenic Leukemic Cells in AML in vitro

Recombinant human cytokines may provide a potentially interesting new approach to the treatment of AML [8, 19]. GM-CSF, IL-3 and G-CSF are known to be regulators of proliferation of leukemic cells, although these and other cytokines (IL-1, IL-4, IL-6) have been demonstrated to be capable of stimulating leukemic cell growth too [8, 20]. Recombinant human SCF – alone or in combination with other cytokines – can be used for the ex vivo expansion of stem cells [7]. We could already demonstrate that cytokine combinations like Cytok 1 or Cytok 2 can increase the number of healthy, CD34-positive cells and reduce that of clonal, leukemic cells in cases with AML in vitro [10]. These data prove that CD34-positive cells in AML are clonal cells, whereas CD34-positive cells from healthy donors grown in cytokine cocktails are healthy, nonclonal stem cells. Those cells could be retransfused to patients after high-dose chemotherapy for an early reconstitution of the hematopoietic system [7]. The aim of this study was to investigate the influence of SCF-containing cytokine cocktails on the proliferation and differentiation capability of leukemic and nonleukemic BM-MNC in vitro. Moreover, the clinical significance had to be evaluated. At diagnosis of AML reduction in leukemic cells and colonies as well as induction of granulopoietic and lymphopoietic cell differentiation could be shown in all examined cases. At relapse of the disease, the effect of cytokine treatment could only be demonstrated in 50% of the cases which means that leukemic cells are more sensitive to cytokine treatment at diagnosis than at relapse. This might be explained by a different autocrine production of cytokines by BM cells at relapse. IL-1β and IL-6 are known to be able to inhibit or stimulate AML blast proliferation depending on the 'history' of the leukemic cells [21]. As already shown by others and ourselves, leukemic

cells can differentiate – without losing their leukemic signs. That could mean that a 'preleukemic state', but no complete remission might be achieved by cytokine treatment in vitro [10, 17, 22]. In complete remission, a reduction in CD34-positive colonies was achieved in all examined cases. In healthy BM, leukemic CD34-positive colonies could never be detected. GM-CSF has been shown to favor AML cell proliferation and healthy BM cell differentiation [18]. The GM-CSF-containing medium (Cytok 1) was more effective in reducing the leukemic burden in AML. This might be due to the additional tumor cytotoxic effect of GM-CSF [23]. Eradication of leukemic cells could not be achieved by incubation of AML cells with those cytokines. A reduction in clonogenic cells could also be achieved (to a lower degree) by culturing cells in ISC/FCS medium, which is probably due to unknown cytokines in the FCS. Cytokine treatments as described here have no negative influence on cell growth or viability of BM-MNC. Addition of cytokines (Cytok 1, Cytok 2) to the control medium (ISC/FCS) resulted in an increase in single cell and colony absolute counts. Our results have clinical significance. AML patients studied at diagnosis survived longer, if clonal, CD34-positive cells or leukemic colonies grown from BM-MNC in cytokine-containing medium could be reduced by more than 50% in vitro [10]. Relapse-free survival times were significantly longer in those patients in whom a reduction of CD34-positive cells in vitro could be demonstrated.

We conclude that Cytok 1 and Cytok 2 are appropriate cocktails to (1) expand healthy nonclonal CD34-positive cells and (2) reduce clonogenic CD34-positive cells or colonies in AML, especially in remission of the disease. (3) Our data have clinical significance: patients in whom a reduction of the leukemic tumor load could be achieved by cytokine treatment in vitro had a better survival probability.

LAK Cells Can Reduce, but Not Eliminate Clonogenic Cells in AML

Cytotoxic mechanisms against tumor cells can be mediated by NK or LAK cells and can result in suppression of leukemic cells [12]. However, the mechanisms of action of those NK or LAK cells are not fully understood [24]. Cytotoxic cytokines released by NK, LAK or other cells or adhesion molecules or histocompatibility antigens might sensitize effector cells for the NK/LAK cell attack. Our study was aimed at finding out whether leukemic progenitors from AML patients can be reduced by autologous LAK cells and whether the cytotoxic effect of LAK cells can be improved by preincubation of BM-MNC with different cytokine cocktails. The potential cytotoxic effect of LAK cells on untreated or precultured leukemic BM-MNC was evaluated after coculture. Proportions of CD34-positive BM agar colonies before and after LAK cell coculture were evaluated and compared. Our data showed that autologous LAK cells can reduce, but not eliminate CD34-positive BM colonies obtained at active stages of AML as well as in remission. This means that the cytotoxic effect of LAK cells might be efficient in reducing residual leukemia, but that LAK cells are more or less ineffective as the only cytotoxic agent at active stages of AML, as already shown by others and ourselves [12, 25, 26]. This might be due to different expression of adhesion molecules like CD54 or CD58 in active disease as compared to remission [24, 27]. In complete remission, the percentage of NK cells in BM decreases when relapse occurs [28]. Preincubation of BM-MNC with cytokine cocktails containing SCF, IL-1β, IL-3, IL-6, EPO with/without GM-CSF resulted in a more efficient reduction in CD34-positive leukemic colonies by LAK cells compared to results with not prestimulated BM cells. If the cytokine cocktail (Cytok 1) contains GM-CSF, the antileukemic effect of LAK cells seems to be better than that of cocktails without GM-CSF (Cytok 2) as already described [12, 22, 27]. Preculturing cells in ISC/FCS as a control showed that a reduction in clonogenic cells could also be achieved (to a lower degree) by this medium, which is probably due to cytokines in the FCS. Our data are of prognostic significance: patients studied at diagnosis, whose BM cells were pretreated with Cytok 1 and whose CD34-positive colonies were reduced by more than 20% by autologous LAK cells in vitro had a significantly better survival probability as compared to cases without such a reduction. In preliminary experiments we could already show that the prognosis of AML patients was better if clonal, gene-rearranged DNA could be reduced by autologous LAK cells in vitro [12]. The advantage of measuring the antileukemic effect of LAK cells by clonogenic assays and flow cytometric estimation of CD34-positive cells as compared to ^{51}Cr-release assays [29] is that specific cells responsible for leukemic proliferation, which survive also in remission, are also picked up.

We conclude hat autologous LAK cells can reduce but not eliminate leukemic colonies and this LAK cell effect can be increased by coculture of BM effector cells with cytokine cocktails like Cytok 1 or Cytok 2. This means that AML cells can be sensitized to LAK cell treatment by cytokines.

Conclusion and Perspectives

Patients have 10^{12} malignant cells at diagnosis. In complete remission there may still be as many as 10^{10} neoplastic cells [30]. Therefore different techniques to study 'minimal residual disease' with the goal of identifying early relapsing patients are under investigation. This includes cytogenetic techniques such as in situ hybridization, polymerase chain reaction or immunophenotyping [30–32]. Our results show that clonogenic assays combined with immunophenotyping can be used to study 'minimal residual disease' in AML [4] and the persistence or reemergence of leukemic progenitors by quantifying the leukemic tumor load in the course of the disease. In summary, our data show that (1) leukemic progenitors are detectable in BM samples obtained at diagnosis and at relapse as well as in complete remission of AML; (2) incubation of BM-MNC with cytokine cocktails and/or autologous LAK cells leads to a reduction of the leukemic tumor load; elimination of leukemic progenitors, however, could not be achieved; (3) BM-MNC are less susceptible to cytokines or LAK cell treatment at relapse than at diagnosis of AML. Clinical trials have been conducted to investigate the ability of IL-2 to generate killer cells in AML patients in vivo [33]. Because of the high incidence of side effects, this therapy on its own is not safe. Additional malignant hematolymphoid disorders can be induced by NK cells [34]. However, AML patients might benefit from a LAK cell treatment in vivo, especially if this treatment is combined with cytokines, although adverse clinical effects such as stimulation of leukemic proliferation might be induced [35]. Moreover, new cytokine combinations (SCF, TPO, Flt-3-ligand) have to be tested in order to reduce clonogenic leukemic cells in AML, for example by in vitro purging of BM samples or for use in vivo. Moreover, 'priming' effects of cytokines on the proliferation of leukemic cells have to be evaluated [9]. In addition, the generation of cytotoxic, NK-like effector cells such as 'cytokine-induced killer' (CIK) cells or 'adherent LAK cells' (A-LAKs) have to be stimulated and studied in depth to learn about cell-cell contacts of AML blasts with accessory cells and with cytotoxic effector cells in the BM. First results point to a regulating and tumor-suppressing role of NK cells in this direct or indirect cytokine-mediated cytotoxicity [27]. Moreover, first clinical and in vitro results demonstrate that histamine and IL-2 synergize to kill AML blasts in vitro and that prolonged remission times could be achieved in 5 patients treated in vivo [36]. Other data show that apoptotic death of AML cells can be induced through fas/Apo-1 (CD95) mediated cytotoxicity with the CD95 ligand expressed on IL-2-activated T cells [37].

In conclusion, these data demonstrate the important role of LAK cells in the cytotoxic cascade against tumor cells. Many problems have to be solved to find the most cytotoxic 'killer cells', the best way of producing them in vitro, the best activity confined to certain leukemia types or to certain patients, the optimal clinical settings and the optimal clinical outcomes. Nevertheless, we think that our results can contribute important data for the development of innovative strategies in the treatment of AML.

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