0041-1337/78/2603-0157$02.00/0
TRANSPLANTATION

Vol. 26, No. 3
Printed in U.S.A.

TRANSPLANTATION OF SYNGENEIC BONE MARROW INCUBATED WITH LEUKOCYTE ANTIBODIES

II. CYTOTOXIC ACTIVITY OF ANTI-cALL GLOBULIN ON LEUKEMIC CELLS AND NORMAL HEMOPOIETIC PRECURSOR CELLS IN MAN[1]

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SUMMARY

Bone marrow removed from leukemic patients during remission, for retransplantation during relapse, may contain residual leukemic cells. Antisera against surface antigens of these cells should not react with hemopoietic stem cells. In order to produce an appropriate antiserum, rabbits were given injections of cells from the common form of acute lymphoblastic leukemia (cALL) of childhood. The resultant antiserum was absorbed with liver-kidney homogenate, chronic lymphoid leukemia cells (CLL), normal peripheral lymphocytes, and lymphoblastoid cells from B cell lines. The purified globulin fraction showed high complement-dependent cytotoxicity against the cells of 39 of 56 patients with ALL. It did not react with the cells of 11 T-ALL, 1 B-ALL, and 5 undifferentiated (without known markers) ALL. Furthermore, the antiserum did not react with the cells from acute myeloid leukemias, chronic lymphoid leukemias, B-type lymphoblastoid cell lines, normal bone marrow cells, and peripheral blood lymphocytes. Unabsorbed anti-cALL globulin was found to be highly cytotoxic against hemopoietic colony-forming cells (CFU-c) and completely inhibited the growth of marrow cells and CFU-c in diffusion chambers. Absorption of anti-cALL with liver-kidney homogenate, CLL, and peripheral blood lymphocytes removed only part of the cytotoxic antibodies cross-reacting with antigens present on CFU-c. An additional absorption with lymphoblastoid cell lines removed the cytotoxic effect of anti-cALL against CFU-c completely and did not inhibit proliferation of marrow cells and CFU-c in diffusion chambers, while high cytotoxicity against cALL blasts was preserved.

It follows that cALL antiserum lacks an inhibitory effect on normal hemopoietic stem cells when measured in CFU-c and diffusion chamber assays.

The nonreactivity of cALL antiserum against hemopoietic stem cells would be a prerequisite for a therapeutic use of the antiserum in "antileukemic autotransplantation". This is an approach to eliminate by antibodies against cell surface antigens residual leukemic cells in the bone marrow taken during remission and stored for retransplantation in relapse. Its principle was tested in a previous study on mice, where anti-T cell globulin was shown to eradicate leukemic cells without inhibiting stem cells required for bone marrow transplantation (*25*).

In this study the question was raised whether anti-cALL made specific against common ALL (*21*), affects normal hemopoietic stem or precursor cells. The inhibition of colony-forming cells was used to test for a possible cytotoxic or inhibitory effect of the antiserum (*18*). This system was applied along with the diffusion chamber technique as an indicator for stem cell function.

[1] Supported by a grant from Deutsche Forschungsgemeinschaft, SFB 37, B 8, and Euratom Grant 217-76-1 BIOD.

MATERIALS AND METHODS

Preparation of antisera. Anti-cALL sera were raised in rabbits using human ALL cells that were negative for surface Ig, T antigen, and E rosette formation. Cells (10^8) incubated with crude rabbit antithymocyte globulin at a final dilution of 1/30 (*10*) were injected s.c. to cover nonleukemic determinants. The rabbits were boosted i.v. on days 14, 15, 16, and 23, and were bled 7 days later. Serum complement was inactivated by heating at 56 C for 1 hr. Except where otherwise stated, the antisera were absorbed once with thoroughly washed liver-kidney homogenate, three times with normal peripheral blood lymphocytes obtained by leukapheresis using the Haemonetics Model 30 blood cell processor, three times with cells from patients with chronic lymphoid leukemia (CLL), and three times with lymphoblastoid cells from selected continuous B cell lines in a weight ratio of sediment to serum of 1:4.

After the last absorption the serum was ultracentrifuged (Beckman Spinco) for 30 min at 30,000 rpm, and the pure IgG fraction was isolated by ammonium sulfate precipitation and DEAE-cellulose ion exchange chromatography (*8*). It was dialyzed against phosphate-buffered saline, reconcentrated to a protein content of 10 mg/ml, and passed through a 0.22-μ Millipore sterile filter and stored at −20 C. Four different antisera preparations prepared against ALL cells from four different donors were used during these studies and revealed identical specificity. Absorption of the antisera with cALL cells removed all of the activity noted against cALL cells.

T and B cell surface markers. The presence of T antigen on blast cells was assayed by the indirect immunofluorescence test and complement fixation test using specific anti-T cell globulin as described by Rodt et al. (*22*). Assays for blasts forming spontaneous rosettes with sheep erythrocytes (E rosettes) were performed using a method according to Jondal et al. (*15*). The presence of SmIg on blast cells was assayed by indirect immunofluorescence microscopy as described previously (*22*).

Cell preparations. Bone marrow and blood samples were collected using preservative-free heparin as an anticoagulant and separated by Ficoll-Isopaque density sedimentation (*4*). For the immunisation procedure and for most of the investigations (CFU-c and diffusion chamber tests), leukemic cells and normal bone marrow cells were cryopreserved at a controlled rate of freezing in 10% dimethyl sulfoxide with or without 10% AB serum and stored in liquid nitrogen. Viability was more than 90% as assessed by trypan blue dye exclusion. Lymphoblastoid cell lines were selected for absorption procedures from

a stock of 32 B lymphoblastoid cell lines established at our institute (*17*).

Cytotoxicity test. The microcytotoxicity test was used with rabbit complement and eosin for dye exclusion (*16*). The cells were suspended in RPMI 1640 and 10% heat-inactivated fetal calf serum and finally adjusted to a concentration of 2×10^6 cells/ml. Selected normal rabbit serum diluted to a ratio of 1:4 served as a source of complement. Four hundred cells were counted in an inverted phase contrast microscope.

Immunofluorescence. Binding of anti-cALL was studied by indirect immunofluorescence using anti-cALL (protein concentration 10 mg/ml) at a final dilution of 1/40, and fluorescein-conjugated goat anti-rabbit IgG (Behring AG) at a dilution of 1/20. Staining was performed at 4 C and finally sodium azide was added in a concentration of 2×10^{-2} M. The cells were examined with a Leitz fluorescence microscope, and 400 cells were investigated for each sample.

Colony inhibition assay. Nucleated marrow cells (4×10^6) were incubated with the antibody preparation in a final volume of 0.5 ml of TC Medium 199 for 30 min at 4 C. Selected normal rabbit serum was added as the source of complement (12% final dilution), and the suspension was further incubated for 60 min at 37 C. Thereafter, the cells were washed once and counted for the respective culture assays. Normal rabbit globulin instead of the antibody preparations was added as a control.

The number of granulocyte-monocyte precursor cells surviving after antiserum treatment was evaluated by a double-layer agar technique, as described by Pike and Robinson (*20*), with minor modifications. After gelation at room temperature, the agar dishes were incubated for 12 to 14 days at 37 C in a fully humidified atmosphere continuously flushed with 5% CO_2. Colonies (defined as groups of 50 or more cells) were counted using a Leitz Diavert microscope.

Diffusion chamber technique. The diffusion chamber technique used was that developed by Benestad (*2*) and Boyum and Borgstrøm (*6*). MF Millipore filters with a porosity of 0.22 μ were glued to perspex rings to form chambers. After the incubation procedure with anti-cALL globulin, 5×10^5 nucleated bone marrow cells from normal volunteers were filled into each chamber. Two chambers at a time were implanted i.p. into male CBA/J mice (25 to 30 g) irradiated with 650 R. Reimplantation into freshly irradiated mice was carried out on day 7. On days 6 and 12, 10 chambers were harvested for each antibody preparation and shaken for 1 hr in 0.5% of pronase-buffered medium to liquefy the clot. The resulting cell suspensions were counted for total number of nucleated cells and smears were made for differential counts. The remaining cells were washed and transferred to soft agar cultures for the CFU-c test. Colonies were counted as described above.

RESULTS

Cytotoxic activity of anti-cALL on various leukemias. Fifty-six patients with ALL (43 at diagnosis, 13 in relapse) were investigated for cell surface markers. The anti-cALL reacted with blast cells from 39 patients (70%) in the cytotoxicity test (titer 1:128 to 1:2048) and the indirect immunofluorescence test (Table 1). The blasts of these cases did not carry surface Ig and did not form E rosettes (so-called O-ALL). The leukemic cells from 11 patients with T antigen-positive ALL, 1 patient with surface Ig (B-ALL), and 5 patients with undifferentiated (without known markers) ALL were negative with anti-cALL in both test systems. No positive reaction was found with leukemic cells from 4 patients with acute myeloid leukemia, 7 patients with chronic myeloid leukemia in blast crisis, 10 patients with chronic lymphoid leukemia, 10 B lymphoblastoid cell lines, bone marrow cells from 16 normal donors, and peripheral blood lymphocytes from 12 normal donors.

The cytotoxic activity of anti-cALL in three representative cases of cALL at diagnosis (>99% blasts in bone marrow) and one case in relapse (48% blasts) is shown in Figure 1. All blast cells were lysed up to a dilution of 1/256. In contrast, no cytotoxic effect was observed against leukemic cells of T-ALL (>99% blasts in bone marrow), normal bone marrow cells, and normal peripheral blood lymphocytes.

Indirect immunofluorescence confirmed the specificity of the antiserum preparations. Table 2 summarizes the results obtained with anti-cALL from 15 patients with cALL at diagnosis and 4 patients in relapse. There is a clear correlation between the number of blast cells identified by morphological criteria and the number of positive blasts in the immunofluorescence test.

Cytotoxic activity of anti-cALL on normal hemopoietic precursor cells. Incubation of marrow cells with crude anti-cALL and complement completely inhibited the clonal growth of precursor cells committed to granulocyte-monocyte differentiation (CFU-c). Absorption of the antiserum with liver-kidney homogenate, peripheral blood lymphocytes, and CLL decreased

TABLE 1. Reactivity of anti-cALL globulin with leukemic blasts and normal cells by cytotoxicity and immunofluorescence

	No. of cases	Cytotoxicity titer (1/x)	Immunofluorescence
Common ALL	39	128–2048[a]	++-+++
T-ALL	11	Negative	Negative
B-ALL	1	Negative	Negative
Undifferentiated ALL	5	Negative	Negative
Common ALL in complete Remission	9	Negative	Negative
Acute myeloid leukemia	4	Negative	Negative
Chronic myeloid leukemia	3	Negative	Negative
CLL	12	Negative	Negative
B-type LCL	10	Negative	Negative
Normal bone marrow	16	Negative	Negative
Peripheral blood lymphocytes	12	Negative	Negative

[a] Titer that shows >95% lysis of blast cells.

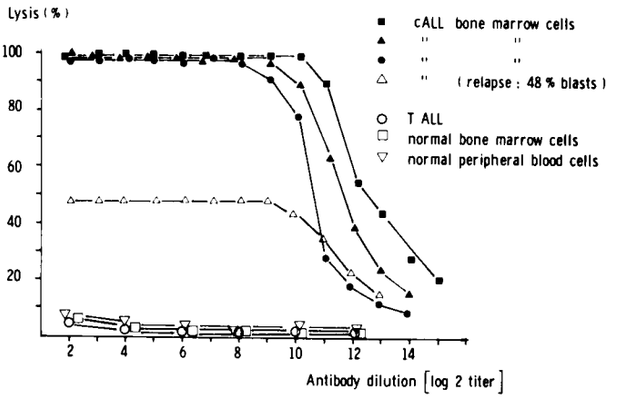

FIGURE 1. Cytotoxic activity of anti-cALL globulin against three cases of cALL at diagnosis (>99% blasts in bone marrow), one case in relapse (48% blasts), one case of T-ALL (>99% blasts in bone marrow) and against normal bone marrow and peripheral blood cells.

TABLE 2. Percentage of blast cells identified by anti-cALL globulin in the immunofluorescence test and by morphological criteria from 15 patients with common ALL at diagnosis and 4 patients in relapse

Patient	Sex/Age	Initial WBC	Cytotoxicity titer (1/x)	Immunofluorescence (%)	Morphological blasts (%)	
1	Female/7	3,400	>1024	ND	98	BM[a]
2	Female/4	10,200	1024	99	97	BM
3	Male/12	112,000	512	95	91	PB
4	Male/5	13,700	1024	ND	95	BM
5	Female/14	87,200	>1024	ND	98	BM
6	Male/4	60,000	>1024	87	98	BM
7	Male/5	7,800	1024	71	82	BM
8	Male/5	6,300	512	97	99	BM
9	Female/5	16,000	512	99	99	BM
10	Female/9	32,000	>1024	100	99	BM
11	Male/10	2,400	512	89	90	BM
12	Male/3	10,600	1024	100	100	BM
13	Male/8	3,500	>1024	98	95	BM
14	Male/6	7,900	1024	87	92	BM
15	Male/2	60,000	128	77	75	PB
16[b]	Male/10	17,000	>1024	81	75	BM
17[b]	Male/3	155,200	>1024	65	62	BM
18[b]	Male/10	4,100	128	85	87	BM
19[b]	Male/7	8,700	>1024	96	98	BM

[a] BM, bone marrow; PB, peripheral blood.
[b] Relapse.

TABLE 3. Toxicity of crude and differently absorbed anti-cALL globulin on CFU-c[a]

	Antibody dilution	CFU-c/2 × 10^5 nucleated marrow cells
Anti-cALL globulin[a]		
Not absorbed	1/4 + C	0
Absorbed with liver-kidney,	1/4 + C	27.3 + 1.5[c]
PBL,[b] and CLL	1/64 + C	73.6 ± 2.0
Absorbed with liver-kidney,	1/4 + C	69.5 ± 1.7
PBL, CLL, and LCL	1/32 + C	79.3 ± 1.5
	1/64 + C	79.0 ± 3.4
	1/128 + C	73.3 ± 2.4
Normal rabbit globulin	1/4 + C	72.1 ± 2.7

[a] All preparations were adjusted to a protein content of 10 mg/ml.
[b] PBL, peripheral blood lymphocytes.
[c] CFU-c ± SEM from triplicates. Control value without rabbit globulin 73.1 ± 2.1 CFU-c/2 × 10^5 nucleated marrow cells.

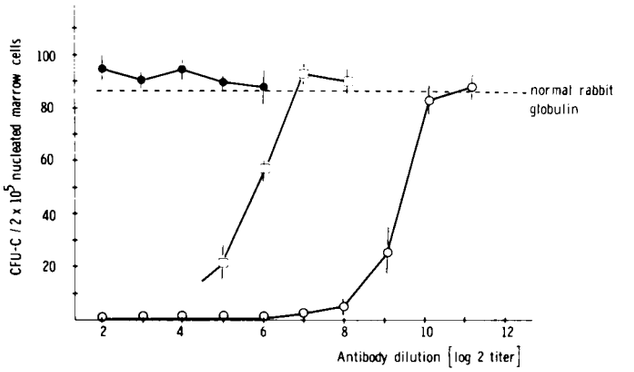

FIGURE 2. Recovery of CFU-c after incubation of normal marrow cells with increasing dilutions of differently absorbed anti-cALL and complement. ○, anti-cALL not absorbed; □, absorbed with red blood cells and CLL; ●, absorbed with red blood cells, CLL, and LCL.

the toxicity on CFU-c, but considerable inhibition was still observed at higher antibody concentrations (1/4 dilution) (Table 3). In order to remove completely cross-reacting antibodies against CFU-c, additional absorption with B lymphoblastoid cell lines was required. Figure 2 shows the recovery of CFU-c after the incubation of normal marrow cells with increasing dilutions of crude or differently absorbed anti-cALL and complement. The antiserum that had been absorbed with red blood cells and CLL still inhibited more than 50% of CFU-c at a dilution of 1/32. In contrast, no inhibition at all was observed after the antiserum had been absorbed additionally with lymphoblastoid cell lines (LCL). Similar results were obtained with three other antiserum batches from different cell donors.

Diffusion chamber studies. The effect of anti-cALL on pluripotent stem cells was investigated in diffusion chamber cultures. This culture system appears to provide a suitable milieu for the growth of early stem cells. Kinetic studies were performed using two different antiserum preparations in four different experiments. Figure 3 summarizes the results of one representative experiment. Incubation of marrow cells with crude anti-cALL and complement suppressed the total number of nucleated cells and led to a loss of differentiation.

Treatment of marrow cells with absorbed anti-cALL and complement decreased the total number of nucleated cells up to day 6, followed by an increase up to day 12 exceeding the implanted cell number. The morphological distribution of nucleated cells revealed an increase of immature granulocyte cells (myeloblasts, promyelocytes, and myelocytes) on day 12. Despite the decrease in cell number during the first 6 days, the concentration of CFU-c per chamber increased during this period and exceeded the initial CFU-c number, with a further increase up to day 12. The rate of increase of bone marrow CFU-c in the presence of anti-cALL was similar to that of the normal rabbit globulin as a control, whereas no colony formation was observed throughout the study when marrow cells were treated with unabsorbed anti-cALL.

DISCUSSION

The results demonstrate that rabbit antisera against blasts from patients with cALL reveal specific cytotoxic activity,

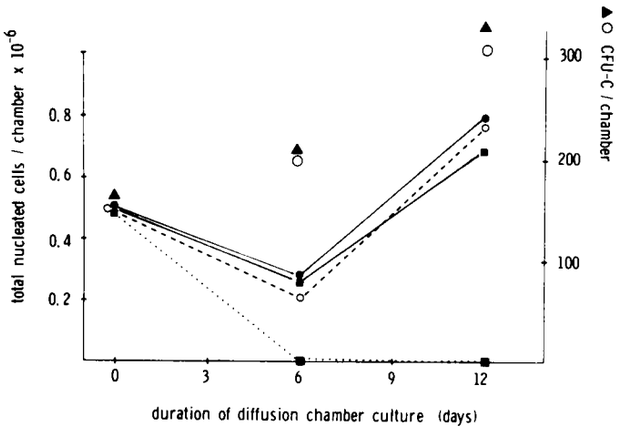

FIGURE 3. Effect of crude (■---■ = 1/64 dilution + C), absorbed (● = 1/32 + C, ■——■ = 1/64 + C) anti-cALL or normal rabbit globulin (○---○ = 1/64 + C) on the number of nucleated cells and of absorbed (▲ = 1/32 + C) anti-cALL or normal rabbit globulin (○ = 1/64 + C) on the number of CFU-c, following antibody incubation and cultivation of normal bone marrow cells in diffusion chambers.

provided the crude antisera had been appropriately absorbed. After an absorption of the antisera with liver-kidney homogenate, peripheral blood lymphocytes, CLL, and LCL, specific activity was found against 70% of ALL (common ALL), but not against T-ALL, B-ALL, and undifferentiated ALL without known markers. Anti-cALL was negative with all other leukemias as was shown in both the cytotoxic and immunofluorescence tests. Cells labeled with fluorescein isothyocyanate-conjugated anti-cALL antibodies correlated with the number of blast cells identified by routine morphological criteria.

Four antiserum preparations from four cell donors were investigated and revealed identical specifities. No cytotoxic activity against the donors' remission bone marrow or peripheral blood lymphocytes was observed. In addition, no cross-reactivity against bone marrow cells and peripheral blood lymphocytes from normal donors was observed either. So far, no antibodies against HLA and HLA-linked B cell alloantigens have been noted in the specifically absorbed antibody preparations. Crude rabbit anti-cALL antisera contain antibodies against a variety of antigeneic determinants. Apart from antibodies against leukemia-associated determinants, cross-reactions with species-specific and organ-specific structures present on both normal and leukemic cells can be found. Some of these antibodies were responsible for the inactivation of hemopoietic stem cells. As a consequence of it, the incubation of normal marrow cells with crude anti-cALL globulin and complement completely inhibited the clonal growth of precursor cells in soft agar, and led to decreasing growth curves and lack of differentiation of normal bone marrow cells in diffusion chambers. Previous studies showed the sensitivity of the colony inhibition assay (*18*). This was also evident from the wide range of toxicity of the crude anti-cALL on granulocyte precursor cells. A dilution of 1/256 still revealed more than 95% inhibition of CFU-c.

Absorption studies with liver-kidney homogenate, peripheral blood lymphocytes, CLL, and LCL showed significant differences in their ability to remove cross-reacting antibodies against hemopoietic precursor cells. It has already been reported that anti-cALL antisera, without absorption with LCL, cross-react with immature granulocytic and erythrocytic cells of normal bone marrow (*21*). The present studies underline the importance of an absorption of the antiserum with LCL, in order to remove completely cytotoxic antibodies cross-reacting with antigens on bone marrow stem cells. The detected cross-reaction of anti-cALL not absorbed with LCL may be explained by B cell alloantigens on early precursor cells. These antigens are also present on normal, early myeloid cells (*7*) and were found on a variety of leukemic cells (*3, 9, 23*).

The diffusion chamber system was used because it appears to provide a suitable milieu for the growth of pluripotent stem cells (*1, 5, 24*). The growth kinetics of CFU-c, investigated after various periods of diffusion chamber cultures, indicate an influx from the pluripotent stem cell pool in addition to the limited self-replication of the committed precursor cells. Our data show that the proliferative activity of colony-forming and diffusion chamber stem cells is not affected by an incubation of anti-cALL. They do not support the postulate of Janossy et al. (*13, 14*) that cALL antigen is a stem cell antigen. Our experimental design does, on the other hand, not preclude the possibility of the cALL antigen being expressed at a concentration too low for antibody-derived cell toxicity. The CFU-c sparing, antileukemic effect was already verified in vivo after administration of anti-cALL to a patient with a relapse of cALL. Although the circulating leukemic cells disappeared from the blood within hours after injection of anti-cALL globulin, the pool of circulating CFU-c remained unaffected (*12*). Further investigations should clarify the possibility of eliminating in vivo residual ALL cells resistant to conventional therapy.

This study aims for an in vitro elimination by antibodies of residual leukemic cells in the marrow taken from patients in remission and stored for retransplantation in relapse. The lack of activity against hemopoietic precursor cells and the high specific toxicity against cALL cells makes anti-cALL a candidate antiserum for such an approach, which would have the advantage of avoiding fatal graft-versus-host reactions and the lack of compatible donors so frequent in allogeneic bone marrow transplantation.

Acknowledgements. The authors wish to thank Cornelia Dalibor, Maximiliane Greifenegger, Monika Renner, Ursula Scherello, and Gerda Weber for their excellent technical assistance.

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Received 5 January 1978.
Accepted 20 March 1978.