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Immunological Studies on Xenogeneic Antisera against Human O-ALL and T-ALL Leukemia Cells

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Abstract—*Xenogeneic antisera were produced against human O-ALL and T-ALL and tested against leukemia and normal cells by the complement-dependent cytotoxicity technique. After appropriate absorptions with normal cells, the two O-ALL antisera tested reacted with cells of about 50% of O-ALL patients. They did not react with cells of T-ALL, B-ALL, normal WBC, enriched B-lymphocytes or thymus lymphocytes. The antigen involved is present on cells of young human fetuses, since fetal liver cells are lysed in the cytotoxicity test and whole fetal tissue removes antibody activity against O-ALL cells. The three T-ALL antisera reacted with T-ALL cells and only with a minority of O-ALL cells. Among the other normal and malignant cells which have been tested, only thymus and fetal liver cells were positive. Thymus cells removed antibodies to thymus, but not to T-ALL and fetal liver. However, antibodies to T-ALL are completely removed by absorptions with fetal tissue. The experiments indicate that cells of O-ALL and T-ALL express fetal antigens and that the fetal antigens of the two types of ALL are immunologically distinct.*

INTRODUCTION

LEUKEMIAS and lymphomas may be classified by clinical, morphological, cytochemical and immunological methods. Immunological methods allow the detection of cell surface markers, the majority of which can be found on both malignant and normal cells. According to T- and B-lymphocyte markers, acute lymphoblastic leukemia (ALL) can be subtyped immunologically as T-cell ALL (T-ALL), B-cell ALL (B-ALL) or Null-cell ALL (O-ALL) [1–3].

However, serological tests and tests for cell-mediated immunity have shown that leukemia cells may also possess distinct leukemia-associated antigens (LAA) which are not present on blood cells of healthy donors and cells of leukemic patients in remission. Serologicallly, characteristic specificities of LAA are detectable by allogeneic [4] or xenogeneic [5–30] antisera against cells of acute, as well as chronic, forms of human leukemia. Subtype-specific antisera directed to O-ALL cells have been investigated by Greaves *et al.* [14, 15], Brown *et al.* [16, 17] and several other groups [18–23, 27]. The data indicate that the antisera may be used for diagnostic [15, 17] or therapeutic [21] purposes.

We have prepared antisera to both O-ALL and T-ALL in order to distinguish between these subtypes of ALL and to characterize the antigenic specificities by complement-dependent cytotoxicity (CDC).

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MATERIALS AND METHODS

Leukemia patients

Donors of leukemia cells for immunization and cytotoxicity tests were untreated patients of different ages and sex.

According to cytology and cytochemistry, smears from blood and bone marrow were diagnosed as ALL, acute myeloblastic leukemia (AML), chronic lymphatic leukemia (CLL) or chronic myeloid leukemia (CML).

Preparation of cells

Leukemia cells were derived from heparinized venous blood or bone marrow. According to cytological criteria the propor-

tion of blast cells among the mononuclear cells varied between 60 and 100%. The mean value was 84%. Mononuclear cells were separated by density gradient centrifugation [31]. Cells not used immediately were suspended in medium containing 15% dimethyl-sulfoxide and frozen in liquid nitrogen. After thawing in a water bath at 37°C, dead cells were removed by density gradient centrifugation [31]. For the tests, washed cells were suspended in MEM–Eagle's medium containing 10% calf serum.

Normal leukocytes were obtained from the blood of healthy donors or leukemic patients in remission. Spontaneous sedimentation of blood or density gradient centrifugation were used to obtain white blood cells (WBC) or mononuclear leukocytes, respectively. Cells spontaneously forming rosettes with sheep erythrocytes served as T-cell targets. T-lymphocyte (T-Ly) and B-lymphocyte (B-Ly) enriched fractions were produced by the separation of rosetting from non-rosetting cells. The non-rosetting fraction contained between 40 and 80% of immunoglobulin (Ig)-positive cells. Phytohemagglutinin (PHA)-induced blasts (PHA-B) were prepared by the incubation of mononuclear leukocytes with PHA-P (Wellcome Research Lab.) for 5 days.

Fetal liver cells (FLC) were from fetuses of the 9th up to the 15th week of gestation. After mechanical dispersion of the tissues in medium the single cell suspension contained more than 90% viable cells. Other target cells were from cord blood or neonatal thymus (THYC).

Immunological typing of cells

ALL-cells were subtyped by testing them for T- and B-cell markers. Criteria for T-cells were spontaneous rosetting with sheep red-blood cells and sensitivity to an anti-human-thymus serum in the presence of rabbit complement [32, 33]. Immunofluorescence with FITC-labeled anti-human-Ig sera (Behringwerke AG., Marburg) was used as an indicator of B-cells.

ALLs having more than 40% of T marker-positive cells were considered T-ALL. One leukemia identified as B-ALL had 40% Ig-positive cells. The majority of ALL's, however, had less than 10% T- or B-cell markers. They were classified as O-ALL.

Preparation of anti-leukemia-sera

Altogether 10 antisera were produced by immunization of rabbits and by immunization of a goat using living leukemia cells. Each animal received the cells from one patient only. The animals were given 4–5 intravenous injections each of 10^8 cells at weekly intervals. One week after the last injection the animals were bled and the sera were stored frozen at −20°C. In this paper we present the results obtained with 5 antisera.

For absorption, complement-inactivated antisera were diluted 1:5 with physiological saline and absorbed for 1 hr each with human AB red cells 3 times and human WBC from healthy donors or cells from cadaver spleens (SPLC) 2–3 times.

CDC tests

All tests were set up in Takatsy-Microtitrator plates. Twenty microliters of antiserum dilution were incubated at 37°C for 1 hr with 20 µl target cells (1×10^7 cells per ml) and as a source of complement with 20 µl of undiluted rabbit serum which had been absorbed with human spleen cells. After cooling, one drop of 0.2% trypan blue was added to each well. The cells were counted in a hemocytometer. Samples with more than 30% dead cells were considered positive; a weak reaction is suggested if the range is between 30 and 60% of killed cells while more than 60% of killed cells indicates a strong reaction. Control samples (cells in the presence of complement or antiserum, respectively) showed less than 15% of dead cells.

RESULTS

Xenogeneic rabbit and goat antisera were produced against cells from two patients with O-ALL and three patients with T-ALL. Some basic data on the patients and leukemia cells are listed in Table 1. Results of CDC tests with five of the 1:5 diluted antisera which had been absorbed with AB red cells and normal WBC or SPLC are shown in Tables 2 and 3. According to the results the antisera detected LAA on the surface of acute lymphoblastic leukemia cells but not on blood cells of hematologically normal donors.

Specificities of two O-ALL antisera

The two O-ALL antisera reacted with about 50% of O-ALL but not with the 4 T-ALLs tested till now. Cells of B-ALL, CLL and CML were like T-ALL, negative with O-ALL antisera. The reactivity with 2 out of 10

Table 1. *Basic data on ALL cells, the corresponding antisera and patients who spent the cells for immunization*

ALL-subtype	Age (in years) and sex of donors	WBC per mm^3	Source of leukemia cells* (% blasts)	Per cent T-marker-positive cells	Per cent B-marker-positive cells	Prepared antiserum-No.	Origin of antiserum
O-ALL	6, f.	31,000	BM (100)	3	8	O-ALL antiserum-1	rabbit
O-ALL	15, f.	10,900	PBL (67)	13	16	O-ALL antiserum-10	rabbit
T-ALL	13, f.	193,000	PBL (93)	40	6	T-ALL antiserum-2	rabbit
T-ALL	8, f.	89,000	PBL (100)	45	0	T-ALL antiserum-5	rabbit
T-ALL	5, f.	5,500	BM (80)	40	n.t.†	T-ALL antiserum-7	goat

*BM—bone marrow, PBL—peripheral blood.
†n.t.—not tested.

Table 2. *Complement-dependent cytotoxic (CDC) activities of ALL antisera* (1:5 *diluted*) *against leukemia cells*

	No. of patients with positive tests over total no. of leukemia patients tested Target cells					
Antiserum	O-ALL	T-ALL	B-ALL	AML	CML	CLL
O-ALL antiserum-1	10/17	0/4	0/1	2/10	0/1	0/3
O-ALL antiserum-10	4/9	0/4	0/1	0/5	n.t.	n.t.
T-ALL antiserum-2	6/14	5/5	1/1	0/9	0/2	0/6
T-ALL antiserum-5	2/12	4/4	0/1	0/9	0/2	0/6
T-ALL antiserum-7	2/6	3/3	1/1	0/9	0/1	0/6

Table 3. *CDC activities of ALL antisera* (1:5 *diluted*) *against cells derived from hematologically normal donors*

	No. of positive specimens over total no. of specimens tested Target cells						
Antiserum	WBC	T-Ly	B-Ly	PHA-Bl	Cord-Ly	THYC	FLC
O-ALL antiserum-1	0/12	n.t.	0/2	n.t.	n.t.	0/2	4/4
O-ALL antiserum-10	0/8	n.t.	0/2	n.t.	n.t.	0/2	n.t.
T-ALL antiserum-2	0/38	0/2	0/4	0/4	0/7	3/3	6/6
T-ALL antiserum-5	0/24	0/2	0/4	0/4	0/7	3/3	6/6
T-ALL antiserum-7	0/15	0/2	0/3	0/2	0/7	3/3	4/4

cases of AML could be removed successfully by absorption with AML cells (Table 4). This did not eliminate the activity against O-ALL cells. The direct cytotoxicity tests show that O-ALL antiserum-1 reacted with O-ALL cells of 10 out of 17 patients and O-ALL antiserum-10 with 4 out of 9 cases. In the case of parallel tests, both antisera displayed the same pattern of reactivity, i.e., antiserum-10 lysed cells from the same leukemia as antiserum-1 and it was also negative against cells from the same patients.

About 50% of patients with O-ALL were negative when the cells were tested against O-ALL antiserum. The absence of this LAA in at least one of the negative O-ALL tested is indicated by the findings that the cells did not remove antibodies to O-ALL. Absorption was effective, however, with antigen-positive O-ALL cells. Both O-ALL antisera did not react in the CDC test with WBC, B-Ly and THYC. O-ALL antiserum-1 was also tested against FLC, it was found strongly cytotoxic in 4 out of 4 cases (Table 3). Absorption of O-ALL

Table 4. CDC activity of O-ALL antiserum-1 after absorption with leukemia cells or fetal tissue

Cells used for absorption		No. of positive specimens over total no. of specimens tested Target cells			
		O-ALL		AML	FLC
WBC	(3 ×)	5/5	(s or w)*	2/2 (w)	1/1 (w)
SPLC	(1 ×)	5/5	(s or w)	0/2	1/1 (w)
AML	(2 ×)				
SPLC	(2 ×)	0/1		n.t.	n.t.
O-ALL†	(2 ×)				
SPLC	(2 ×)	1/1	(s)	n.t.	n.t.
O-ALL‡	(2 ×)				
WBC	(2 ×)	0/4		n.t.	0/1
fetal tissue	(2 ×)				

*Strength of the cytotoxic reaction: s—strong, w—weak (see Methods).
†O-ALL sensitive to O-ALL antiserum.
‡O-ALL resistant to O-ALL antiserum.

antiserum-1 with homogenates of whole fetuses removed total antiserum activity against both FLC and O-ALL cells.

Specificities of three T-ALL antisera

The three T-ALL antisera reacted strongly in CDC with cells from the T-ALL patients tested (Table 2). They reacted with cells from less than 50% of the patients with O-ALL. In the positive cases the reactions with O-ALL cells were weaker as compared with T-ALL cells. It should be mentioned here (not shown in the Table) that the three T-ALL antisera exhibited a non-regular pattern of reactivity with O-ALL cells. As an example T-ALL antiserum-2 lysed O-ALL cells which T-ALL antiserum-5 did not harm. Two out of the three antisera also reacted with the B-ALL cells from one patient tested.

As shown in Table 3, the T-ALL antisera did not lyse blood leukocytes from healthy donors. The tests included WBC, T-Ly, B-Ly, PHA-Bl and cord blood lymphocytes. Furthermore, the tests were negative with WBC from leukemia patients in remission (not shown in the Table).

Like O-ALL antiserum, the T-ALL antisera reacted strongly with FLC. In contrast to O-ALL antisera, the T-ALL antisera also reacted with THYC.

Absorption tests with T-ALL antisera (Table 5) show that repeated treatments with SPLC or adult liver tissue did not remove antibodies against T-ALL cells, THYC and FLC. Treatment of two T-ALL antisera with THYC removed antibodies to THYC but not to T-ALL cells and FLC. However, after absorption with homogenate from fetuses the three T-ALL antisera had lost their activities against FLC as well as T-ALL cells. The positive reaction of fetal tissue-absorbed T-ALL antiserum-7 with THYC observed in one experiment needs further confirmation.

DISCUSSION

After absorption with erythrocytes and normal leukocytes, rabbit and goat antisera against O-ALL and T-ALL detect leukemia-associated antigens on O-ALL and T-ALL cells. In our tests the pattern of reactivity against leukemia and normal white blood cells as detected by O-ALL antisera corresponds well with that of Greaves *et al.* [14, 15] and Rodt *et al.* [19, 20], i.e., O-ALL antisera reacted exclusively with O-ALL cells but not with cells of T-ALL, B-ALL, CLL, normal WBC and THYC. The cross-reactivity occasionally observed with AML could be removed by absorption with AML cells. This did not decrease the activity against O-ALL cells. In addition to our experiments, Greaves *et al.* [15] and Janossy *et al.* [18] have also found reactivity of O-ALL antisera against CML cells of certain patients in a blastic crisis and with some cases suffering from undifferentiated acute leukemia.

Absorptions of O-ALL-antisera with fetal tissue and direct cytotoxic tests with FLC

Table 5. CDC activities of T-ALL antisera after absorption with normal cells and fetal tissue

T-ALL antisera no.	Cells used for absorption		No. of positive specimens over total no. of specimens tested Target cells: T-ALL	THYC	FLC
2	SPLC	(4 ×)	4/4 (s or w)*	1/1 (w)	1/1 (s)
5	SPLC	(4 ×)	4/4 (s)	1/1 (w)	1/1 (s)
7	SPLC	(5 ×)	4/4 (s)	1/1 (w)	1/1 (s)
2	SPLC and adult liver	(2 ×)	2/2 (s or w)	n.t.	1/1 (s)
5		(2 ×)	1/1	n.t.	1/1 (s)
2	SPLC and THYC	(2 ×)	2/2 (s or w)	0/1	1/1 (s)
5		(2 ×)	2/2 (s)	0/1	1/1 (w)
2	SPLC and fetal tissue	(2 ×)	0/3	n.t.	0/1
5		(2 ×)	0/4	n.t.	0/1
7			0/4	1/1 (w)	0/1

*Strength of the cytotoxic reaction: s—strong, w—weak (see Methods).

show that O-ALL cells and fetal cells share a common antigen. Human fetuses were at an age of less than 15 weeks of gestation. Our conclusion thah O-ALL-antisera may detect a fetal antigen is also supported by earlier findings of Greaves *et al.* [14], who reported on positive reactions between O-ALL-antisera and cells from 9-week old fetal liver. However, 22-week old fetal liver tissue was antigen-negative in their experiments. Thus, it seems possible that antigen presence in fetal tissue is age-dependent and the antigen appears only early in differentiation. Fetal liver as a main site of antigen localization is suggested indirectly by the inability of O-ALL-specific antisera to react with fetal spleen, thymus and bone marrow cells [14, 19, 20]. As compared with other fetal organs, the proportion of hemopoietic cells in young fetal liver is very high [34], so that the antigen shared by the O-ALL and fetal cells could be an early differentiation antigen of hemopoietic cells.

The data of cytotoxicity after absorption of the O-ALL antiserum with fetal tissue indicate that the O-ALL antigen is a fetal one. However, more sensitive techniques such as immunofluorescene need to be included in future experiments in order to test whether our absorbed sera did contain antibodies to O-ALL-associated antigens. The existence of such antigens has been described by several groups [14–19].

As Greaves *et al.* [35] and Janossy *et al.* [36] have shown, an O-ALL-associated antigen also occurs on 1–20% of cells from juvenile bone marrow and fetal liver.

T-ALL antisera absorbed with erythrocytes and WBC or SPLC reacted with all cases of T-ALL tested and with about 30% of O-ALL. They did not react with cells of AML, CML, CLL, remission leukocytes, WBC, T-Ly, B-Ly, PHA-BL and cord blood lymphocytes.

A certain degree of T-ALL specificity was also obtained by Borella *et al.* [24, 25] using xenogeneic antiserum against E-rosette-forming ALL cells. It is assumed that the phenotype of Thy-ALL is identical to that of normal THYC [15, 18, 24]. The reaction of T-ALL antiserum against THYC was also found in our experiments. Moreover, like O-ALL antisera the 3 T-ALL-antisera reacted against FLC. Reactivity of T-ALL antisera against fetal liver cells was not removed by THYC, although this absorption eliminated antibodies to THYC. Antibodies against T-ALL cells, however, could be completely absorbed with fetal tissue. Absorption with fetal tissue was not an unspecific effect. As shown in tests with antisera against AML cells (Thränhardt *et al.*, in preparation), fetal tissue removed antibodies which are reactive against ALL-cells and FLC but did not remove antibodies to AML cells. We may conclude from these experiments that T-ALL cells carry a

THYC-specific antigen and in addition to that a T-ALL-associated fetal antigen.

Both O-ALL and T-ALL antisera show the presence of fetal antigens on O-ALL and T-ALL cells. Apparently, these fetal antigens are immunologically distinct since the O-ALL antisera reeact only with O-ALL and not with T-ALL cells, and vice versa the T-ALL antisera are mainly reactive against T-ALL cells. It would be very useful to know whether antisera against fetal liver may be used for the detection of leukemia-associated antigens. Preliminary results [37] show that a rabbit antiserum against human FLC reacted with some ALL but not with AML and CML cells. Obviously another antigen common to fetal tissue, leukemia cells, B-lymphocytes and *in vitro* cultured skin was described by Jerry *et al.* [38] when testing a rabbit antiserum against fetal tissue. A direct comparison would be necessary, however, to analyze the antibody specificities of the different antisera. This is also true for the antisera which have been tested by different groups [5, 6, 39, 41] and which detected certain antigenic relationships between leukemia and fetal tissue.

If we assume that the O-ALL and T-ALL antigen-carrying cell in the fetal tissue is a stem cell, there might be two possibilities: a stem cell carrying both antigens or 2 stem cells each carrying only one of the antigens. The present stage of investigation does not allow a distinction between the alternatives. It should be mentioned, however, that Rodt *et al.* [20] and Netzel *et al.* [21] were unable to find any inhibitory effects of their O-ALL antisera to human bone marrow stem cells *in vitro*.

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