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Detection of T-Cell Lymphoma-associated Antigens on Cord Blood Lymphocytes and Phytohemagglutinin-stimulated Blasts[1]

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Summary

Absorption studies demonstrate that T-cell lymphoma-associated antigens detected by rabbit antisera to human T-lymphoblast cell lines are present in suspensions of cord blood lymphocytes and phytohemagglutinin-stimulated adult blood lymphocytes in amounts similar to those found in T-cell lymphoma tumor cell suspensions. Smaller amounts of antigen activity are found in suspensions of tonsil cells, thymocytes, and unstimulated adult blood lymphocytes. Little or no antigen activity is found in suspensions of lymphoblasts from patients with other types of leukemia or from B-cell lines. T-cell depletion removes antigen activity from suspensions of normal lymphocytes. These findings suggest that T-cell lymphoma-associated antigens may be fetal antigens expressed by activated T-cells.

Introduction

We have previously reported that TLL[2] is a T-cell cancer (4) and have pointed out that several lymphoblastoid cell lines have been established in culture from patients with ALL 2° TLL. These lines were shown to differ from human B-lymphoblastoid lines in that they retain T-cell characteristics in culture and have not been transformed by Epstein-Barr virus (5). The evidence suggested that these lines are successfully established in culture because of malignant properties (2, 4, 5).

These observations led us to seek evidence for leukemia-lymphoma-associated antigens on T-cell lines. We found (6) that rabbit antisera prepared against T-cell line HSB-2, having been extensively absorbed with the autologous B-cell line CCRF-SB to remove reactivity against B-cell and histocompatibility antigens and further absorbed with normal thymocytes, reacted by complement-dependent cytotoxicity with malignant lymphoblasts obtained from patients with TLL or ALL 2° TLL. Furthermore, the absorbed antisera were cytotoxic for all T-cell lines. However, they did not react with leukemic cells from patients with ASCL or AML, nor did they kill B-cell lines, normal PBL, or thymocytes. These findings indicated that T-cell lines possess TLL-associated antigens. To determine whether such antigens are neoantigens unique to malignant T-cells or normal antigens expressed in larger amounts on malignant cells, extensive absorption studies have been carried out with tumor cells and normal cells from different tissues and from subjects of different ages. The results of these experiments now show that suspensions of cord blood lymphocytes and PHA-stimulated adult blood lymphocytes contain as much TLL-antigen activity as do TLL tumor cells, suggesting that TLL-associated antigens are fetal antigens reexpressed by activated T-cells.

Materials and Methods

T- and B-Cell Marker Assays. T- and B-cell markers were determined as previously described (4). T-cells were identified by E-rosettes. B-cells were identified by the presence of complement receptors (antibody- and complement-coated sheep erythrocyte-rosettes) and surface immunoglobulin.

Patients. The diagnostic criteria used in classifying tumor patients included histopathology, determination of T- or B-cell markers, and presence or absence of radiological evidence of a thymic mass. Patients with acute lymphoblastic leukemia were considered to have leukemia secondary to TLL if their tumor cells formed E-rosettes and/or if they presented with a thymic mass. All other patients with acute lymphoblastic leukemia were classified as having ASCL.

Tumor Cells. Tumor cells were obtained from pleural fluid of 1 patient with TLL and by Ficoll-Hypaque separation from peripheral blood of 4 patients with ALL 2° TLL, 4 patients with ASCL, 2 patients with AML, and 1 patient with AMML. All tumor cell suspensions contained more than 80% blasts as determined by examination of Wright-stained preparations. Tumor cells were used fresh or after frozen storage in 10% dimethyl sulfoxide at −130°.

Nonneoplastic Cells. Lymphocytes were obtained by Ficoll-Hypaque separation from peripheral blood of adults and neonates. Suspensions of thymocytes and tonsil cells were prepared by mincing surgically excised thymic and tonsil tissue in 0.15 M NaCl. PHA-stimulated lymphocytes were harvested from 72-hr cultures of adult PBL incubated

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[2] The abbreviations used are: TLL, thymic lymphoblastic lymphoma; ALL 2° TLL, acute lymphoblastic leukemia secondary to thymic lymphoblastic lymphoma; ASCL, acute stem cell leukemia; AML, acute myelogenous leukemia; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; E-rosettes, rosettes formed by T-cells and sheep erythrocytes; CI, cytotoxicity index.

at an initial cell concentration of 10^6/ml with 1:100 diluted PHA-M (Grand Island Biological Co., Grand Island, N.Y.). By morphological criteria, 80% of these cells were lymphoblasts. Control lymphocytes from 72-hr cultures without PHA were also obtained.

Tissue Culture Cells. The derivation and maintenance in culture of T-cell lines CCRF-CEM and HSB-2 and B-cell lines CCRF-SB, RAJI, and RPMI 7666 have been described previously (5). T-cell line MOLT-3 was kindly supplied by Dr. J. Minowada, and T-cell line JURKAT was kindly supplied by Dr. U. Schneider and maintained in the same manner.

Preparation and Absorption of Antisera. Rabbits received injections of 5×10^8 viable cells from T-cell line HSB-2. This dose was repeated 2 weeks later, and after another 9 days the animals were bled by cardiac puncture. Sera were heat inactivated at 56° for 30 min and stored at −20°. Antisera were then sequentially absorbed with B-cell line CCRF-SB and thymocytes by incubating a 1:10 dilution of serum with 10% v/v cells for 30 min at room temperature.

Cytotoxicity Assay. Complement-dependent cytotoxicity tests were performed by mixing 10^4 cells suspended in 10 μl diluent with 10 μl test serum (final dilution of 1:40) in V-bottom wells of microtiter plates (Cooke Engineering Co., Alexandria, Va.). Diluent in all instances consisted of Hanks' basal salt solution with 10% fetal calf serum. After 30 min at 37°, plates were centrifuged at 250 × *g* for 5 min at 37°, supernatant fluid was removed, and 10 μl of appropriately diluted rabbit complement and 10 μl diluent were added. After 30 min at 37°, 10 μl 1% trypan blue were then added, and the percentage of cells staining with dye was determined. Each test was done in quadruplicate and included controls for diluent and complement cytotoxicity. The CI was calculated by the formula:

$$CI = 100 \times [\%\ \text{dead (test)} - \%\ \text{dead (c' control)}]/[100 - \%\ \text{dead (c' control)}].$$

Absorption Tests. Absorption tests were performed by incubating 10^7 cells with 0.1 ml diluted antiserum for 30 min at 25°. Cells were then removed by centrifugation at 1500 × *g* for 10 min, and the supernatant was tested as above for cytotoxicity to T-cell line CCRF-CEM. Percentage of absorption was calculated by the formula:

$$\%\ A = 100 \times [CI\ (\text{unabsorbed}) - CI\ (\text{absorbed})]/CI\ (\text{unabsorbed}).$$

Quantitative absorption tests were performed using 10^6, 10^7, 5×10^7, and, when feasible, 10^8, and 10^9 cells. The number of cells required to give 50% absorption of a standard dilution of antiserum was then estimated from plots of percentage of absorption *versus* cell number. By assigning an absorptive capacity of 100 to the ability of 10^6 cells to result in 50% absorption, a relative absorptive capacity (RAC) for each cell type was then calculated as follows:

$$RAC = 100 \times \frac{1}{\text{cell no. needed for 50\% absorption} \times 10^6}$$

Purification of T- and B-lymphocytes. As described by Greaves and Brown (3), T-lymphocytes from tonsil cell suspensions were purified by a nylon wool column filtration method, and B-cells were purified by sedimentation of sheep erythrocyte-binding cells on a Ficoll-Hypaque gradient.

Results

A panel of test cells to be used for cytotoxicity and absorption reactions was first developed and examined for T- and B-cell markers as delineated in Table 1. The T-cell marker of spontaneous rosette formation with sheep erythrocytes was found on tumor cells from 1 patient (Case 1) with TLL and 2 patients with ALL 2° TLL (Cases 3 and 4). Tumor cells from 2 other patients with ALL 2° TLL (Cases 2 and 5), all 3 patients with ASCL, 2 patients with AML, and 1 patient with AMML had neither T- nor B-cell markers. Cell lines HSB-2, CCRF-CEM, MOLT-3, and JURKAT, all derived from patients with ALL 2° TLL, had T-cell properties. Cell lines CCRF-SB, RAJI, and RPMI 7666 had the B-cell properties of antibody- and complement-coated sheep erythrocyte-rosette formation and/or surface immunoglobulin. Almost all of the thymocytes and PHA-stimulated adult lymphoblasts had T-cell markers. Mixtures of T- and B-cells were found in suspensions of PBL from normal adults and neonates as well as in tonsil cell suspensions.

This cell panel was then used to test the specificity of the anti-T-cell line antisera. As shown in Table 2, the pattern of cytotoxic reactivity of a representative antiserum, A12, was identical to that previously reported for other similarly prepared antisera (6). Of the tumor cells tested, only lymphoblasts from subjects with TLL or ALL 2° TLL reacted strongly with the antiserum. By comparison, little or no cytotoxicity was seen with other types of leukemic cell. The 4 T-cell lines, all derived from patients with ALL 2° TLL, reacted strongly. In contrast, all 3B-cell lines examined, including CCRF-SB, derived from the same leukemic patient as HSB-2, failed to react in the cytotoxicity test. The antiserum showed little or no cytotoxicity for normal lymphocytes including adult and cord blood lymphocytes, adult PHA blasts, tonsil cells, and thymocytes.

Because of the possibility that absorption tests might reveal antigens not detected by complement-dependent cytotoxicity, experiments were carried out in which 10^7 of each test cell type in the panel was used to further absorb antiserum A12. The percentage of absorption of cytotoxicity of the antiserum for T-cell line CCRF-CEM was then determined. As also shown in Table 2, in most instances the results of absorption tests paralleled the results of the cytotoxicity assays. However, in contrast to their cytotoxicity response, cord blood lymphocytes and PHA-stimulated adult lymphocytes strongly absorbed antiserum reactivity. Weak but definite absorption reactivity was consistently obtained with tonsil cell suspensions, thymocytes, and adult PBL. These data confirmed our previously reported finding that appropriately absorbed rabbit antisera to T-cell lines detect non-HLA antigens which are shared by T-cell lines and lymphoblasts from patients with TLL and ALL 2° TLL and that these antigens are absent from B-cell lines and tumor cells from subjects with other types of leukemia. The absorption studies revealed, however, that these antigens are also present on some normal lymphocytes and that they

are particularly prevalent on cord blood lymphocytes and PHA blasts.

To assess further the relative amounts of TLL-associated antigens on different cell populations, quantitative absorption tests were performed using graded increases in numbers of absorbing cells. As shown in Chart 1, antiserum A12 was progressively absorbed by increasing numbers of cells from suspensions of T-cell line CCRF-CEM, TLL tumor cells, cord blood lymphocytes, adult blood lymphocytes, tonsil cells, and thymocytes, confirming that TLL-associated antigens are present in each of these suspensions. In contrast, the failure of even as many as 5×10^8 cells from B-cell lines CCRF-SB and RAJI or from a patient with AMML to absorb antiserum A12 reactivity shows that such cells lack TLL-associated antigens.

The similarities in the slopes of the absorption curves between 20 and 80% absorption suggested that a reasonable numerical estimate of the relative amounts of antigens in different cell suspensions might be obtained by comparing the number of cells in each type of suspension resulting in 50% absorption. Thus, by assigning a relative absorptive capacity of 100 to the ability of 10^6 cells to absorb 50% of the cytotoxicity of A12 for T-cell line CCRF-CEM, a relative absorptive capacity for each cell type was calculated (Table 3). By this method of estimation, T-cell line CCRF-CEM had the highest TLL antigen activity of all the cell types tested. Amounts of TLL antigens on cord blood lymphocytes and TLL tumor cells were comparable to each other and were approximately 5 to 10 times greater than those found on tonsil cells, thymocytes, and blood lymphocytes from older children and adults. The absorptive capacities of the latter cell types were roughly similar to each other.

To determine whether those normal lymphocytes that reacted with the antiserum were T-cells or B-cells, suspensions were prepared of tonsil cells depleted of B-cells by nylon column filtration or depleted of T-cells by Ficoll-Hypaque gradient separation of sheep erythrocyte-binding cells. These suspensions were then compared with unpurified tonsil cells for their ability to absorb reactivity of antiserum A12. As shown in Table 4, compared to unseparated tonsil cells, nylon column-filtered tonsil cells contained a high proportion of T-cells, whereas Ficoll-Hypaque-separated cells were mostly B-cells. Absorption tests with 10^8 cells from each of these 3 tonsil suspensions showed that E-rosette depletion but not nylon column filtration abrogated the ability of tonsil cells to absorb antiserum A12, indicating that the antiserum reacts exclusively with T-cells.

Discussion

The absorption studies reported here demonstrate: (*a*) that T-cell lymphoma-associated antigens detected by complement-dependent cytotoxicity tests exclusively on TLL tumor cells and T-cell line lymphoblasts are also components of some normal lymphocytes, and (*b*) that significantly larger amounts of these antigens are present in suspensions of cord blood lymphocytes and adult PHA blasts than in suspensions of normal thymocytes, tonsil cells, and adult PBL. Together with the observation that cell suspensions

Table 1
T- and B-cell marker determinations

Cell type	Case	Thymic mass	Diagnosis	Cell source	E-rosettes (%)	EAC[a] rosettes (%)	SIg (%)
Tumor cells	1	+	TLL	Pleural fluid	31	10	1 3
	2	+	ALL 2° TLL	Blood	8	4	1
	3	+	ALL 2° TLL	Blood	91	2	8
	4	+	ALL 2° TLL	Blood	28	15	1
	5	+	ALL 2° TLL	Blood	6	1	13
	6	−	ASCL	Blood	3	13	4
	7	−	ASCL	Blood	1	2	2
	8	−	ASCL	Blood	3	7	5
	9	−	ASCL	Blood	2	8	27
	10	−	AML	Blood	17	11	0
	11	−	AML	Blood	17	0	0
	12	−	AMML	Blood	0	13	
Cell lines	HSB-2		ALL 2° TLL		51	0	0
	CCRF-CEM		ALL 2° TLL		72	0	0
	MOLT-3		ALL 2° TLL		39	0	0
	JURKAT		ALL 2° TLL		43	0	0
	CCRF-SB		ALL 2° TLL		0	2	90
	RAJI		Burkitt's		0	63	96
	RPMI 7666		Normal		0	3	100
Normal lymphocytes	Adults (16)[b]			Blood	60 ± 8[c]	17 ± 4	27 ± 5
	Newborns (3)			Blood	46	17	
	Tonsil (4)				43 ± 5	61 ± 6	20 ± 4
	Thymus (2)				98	1	0
	PHA-stimulated adult blood				96		

[a] EAC, antibody- and complement-coated sheep erythrocytes; SIg, surface immunoglobulin.
[b] Numbers in parentheses are number of different samples tested.
[c] Mean ± S.E.

Table 2

Reactivity of antiserum A12[a] for tumor cells, cell lines, and normal cells

Cell types	Case	Diagnosis	CI	Absorption by 10^7 cells (%)
Tumor cells	1	TLL	48	86
	2	ALL 2° TLL	39	80
	3	ALL 2° TLL	73	85
	4	ALL 2° TLL	12	81
	5	ALL 2° TLL	43	92
	6	ASCL	0	18
	7	ASCL	0	13
	8	ASCL	3	1
	9	ASCL	0	22
	10	AML	2	0
	11	AML	2	10
	12	AMML	2	0
T cell lines	HSB-2		19	95
	CCRF-CEM		63	95
	MOLT-3		16	78
	JURKAT		28	80
B cell lines	CCRF-SB		0	4
	RAJI		2	8
	RPMI 7666		0	13
Normal	Adult PBL (20)[b]		2	22 ± 2[c]
	Neonatal PBL		2	84
			4[d]	72[d]
	Adult PHA blasts		5	85
	72-hr diluent control			0
	Tonsil (7)[b]		1	23 ± 7[e]
	Thymus		6	18

[a] A12 diluted 1:40.
[b] Numbers in parentheses, number of different samples tested.
[c] Mean ± S.E. of 20 normal adults.
[d] A pool of 9 cord blood specimens.
[e] Mean ± S.E. of 7 children's tonsils tested separately.

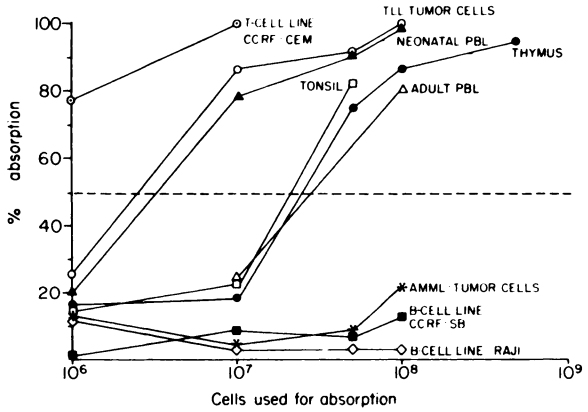

Chart 1. The percentage of absorption of cytotoxicity of antiserum A12 for T-cell line CCRF-CEM by varying numbers of different cell types. - - - -, 50% absorption level.

Table 3

Relative absorptive capacities[a] of cells for antiserum A12

T-cell line CCRF-CEM	>100
T-cell lymphoma (Case 1)	40
Cord blood lymphocytes	29
Tonsil	5
Thymus	4
Adult blood lymphocytes	3
B-cell line CCRF-SB	<1
B-cell line RAJI	<1

[a] Relative absorptive capacity =

$$100 \times \frac{1}{\text{cell no.} \times 10^6 \text{ causing 50\% absorption of A12}}$$

depleted of T-cells contain less TLL antigen activity than do untreated cell suspensions, the findings suggest that TLL-associated antigens are fetal antigens reexpressed by mature T-cells during the cell replicative cycle.

Other fetal antigens have been found not only on neoplastic cells, but also on adult cells when they replicate. For example, α-fetoprotein has been detected in large amounts not only in subjects with hepatomas and teratoblastomas, but also during the recovery phase of viral hepatitis and in some subjects with cirrhosis (8). Similarly, α-fetoglobulin, a murine fetal protein analogous to human α-fetoprotein, has been shown to be produced not only by experimentally induced hepatomas but also by dividing adult liver cells following partial hepatectomy (1).

Observations such as these have led to the concept that

Table 4
Absorption of cytotoxicity of antiserum A12 for T-cell line CCRF-CEM by purified tonsil T- and B-cells

Tonsil cell treatment	E-rosettes (%)	EAC[a]-rosettes	Sig (%)	Absorption by 10^6 cells (%)
Unseparated	46	33	32	76
Nylon column filtration	77	4	5	78
E-rosette depletion	1	56	54	18

[a] EAC, antibody- and complement-coated sheep erythrocyte; Sig, surface immunoglobulin.

derepression of fetal antigen production occurs in association with normal cell division as well as during malignant transformation. Since the fetal phase of development is characterized by widespread cell replication, an alternative interpretation is that fetal antigens are actually cell division differentiation antigens. Thus, cells may express large amounts of a certain differentiation antigen during a specific phase of development such as fetal life, not because such an antigen is a fetal antigen, *i.e.*, unique to the fetal phase of development, but rather because more cells that express that particular antigen are in the process of dividing. Adult cells may express only small amounts of this differentiation antigen because relatively few of them are in the process of dividing. In the experiments described here, cord blood lymphocytes may express larger amounts of TLL-associated antigens than adult blood lymphocytes, not because they are representative of fetal cells, but because, when compared to adult blood lymphocytes, more of them are dividing. This is reflected by their higher spontaneous uptake of [^{3}H]thymidine (7).

Regardless of whether TLL-associated antigens are described as fetal antigens, differentiation antigens, or cell cycle antigens, it will be important to determine whether host immune responsiveness to these antigens exists. Since attempts to answer this question would be facilitated by availability of isolated, soluble TLL antigens, we are currently attempting to prepare and characterize TLL antigens using T-cell lines as an abundant and convenient source of antigenic material.

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