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Research Article

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Specificity of Heteroantisera to Human Acute Leukemia-Associated Antigens

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ABSTRACT Antisera have been raised to human leukemic blast cells from individual patients in mice rendered tolerant with cyclophosphamide to remission leukocytes from the same individual. 10 antisera were raised against acute myelogenous leukemia (AML) cells and 5 antisera were raised against acute lymphoblastic leukemia (ALL) cells. Antisera to AML cells were absorbed with ALL cells, and antisera to ALL cells were absorbed with AML cells. Unabsorbed and absorbed antisera as well as antisera raised in nontolerant mice were tested for cytotoxicity against various cells of a panel containing myeloblasts from 35 patients with AML, lymphoblasts from 7 patients with ALL, myeloblasts from 7 patients with chronic myelogenous leukemia (CML) in blast crisis, peripheral blood leukocytes from 12 patients with acute leukemia in remission and 30 nonleukemic patients, and nucleated bone marrow cells from 10 nonleukemic patients. Unabsorbed antisera to AML or ALL cells raised in tolerant mice were highly cytotoxic to leukemic blast cells but significantly less cytotoxic to remission and control cells. Antisera to AML cells absorbed with ALL cells retained measurable cytotoxicity against AML cells but were not cytotoxic to ALL cells or control cells. Similarly, antisera to ALL cells absorbed with AML cells retained significant cytotoxicity only to ALL cells. Control antisera raised in nontolerant mice were cytotoxic to all cells tested. Although species specific, histocompatibility, differentiation, maturation, and cell cycle-associated antigens may be responsible in part for the cytotoxic activity of the unabsorbed antisera, the absorbed antisera are probably detecting antigens specific for their leukemic cell type.

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INTRODUCTION

The serologic detection and characterization of antigens associated with experimental animal tumors has been central to our understanding of how tumors develop and how they might be treated by immunological means (1-4). Analogous studies in man have progressed more slowly because of the ethical and logistic difficulties of human tumor allografting, and the lack of syngeneic subjects (5-13). If a species other than man is used for raising antisera against human tumor tissue, the immune reaction is ordinarily directed against numerous xenospecific determinants, most of which are also constituents of normal tissues (4, 8, 14). Attempts to circumvent these difficulties have included induction of neonatal tolerance to human nonleukemic tissue (5, 7) or appropriate absorptions of antisera with nonleukemic tissue (8, 10, 13). Although some heteroantisera have detected antigenic differences between leukemic and nonleukemic cells, antigenic differences between leukemic cells of different morphology have been less frequently reported, and the results have been variable (4, 9, 11, 14, 15).

Previously, we reported a method for raising antisera to leukemia cells of an individual patient in mice rendered tolerant with cyclophosphamide to remission leukocytes of the same individual (16). The use of cells from a single patient avoids many of the problems of using pooled or third party cells, such as alloantigenic differences or antigens acquired in tissue culture (17-19). In the current study, we have further defined the nature of human leukemia-associated antigens using a panel of heteroantisera raised in this fashion. Each antiserum was then tested against a panel of human leukemia cells to determine whether the leukemia-associated antigens identified by this technique were individual or group specific.

METHODS

Leukocytes were separated from whole blood by sedimentation and frozen to -79° or -196°C at a controlled rate in 10% dimethyl sulfoxide. When required, cells were thawed rapidly in a 37°C water bath. Leukemic blast cells were obtained from the peripheral blood of patients with acute leukemia having a differential white count of greater than 90% blast cells. Remission leukocytes were collected from the peripheral blood of patients with no circulating blast cells and less than 5% blast cells in the bone marrow. The diagnosis of acute leukemia and the definition of the cell type were made by the standard hematological criteria (20).

3-mo-old male mice of the CBA/J strain (Jackson Laboratories, Bar Harbor, Me.) were injected intraperitoneally with cyclophosphamide 200 mg/kg and 4 h later with 10^8 frozen stored remission leukocytes intravenously. 7, 14, and 21 days later, the mice were injected subcutaneously and intraperitoneally with 10^8 frozen stored leukemic blast cells from the same patient in relapse. Cells for injection on days 14 and 21 were suspended at a concentration of 10^8 cells in 0.2 ml of Eagle's minimal essential medium (MEM, [Grand Island Biological Co., Grand Island, N. Y.]¹ and mixed with 0.2 ml of complete Freund's adjuvant. The resulting emulsion was divided and equal parts injected subcutaneously and intraperitoneally.

Mice were bled for antisera on the 28th and 35th days. Control antisera were produced in mice injected at first with cyclophosphamide alone and then blast cells on days 7, 14, and 21. A second control group received three immunizing doses of blast cells but no cyclophosphamide or remission cells. To test whether blast cells were intrinsically more susceptible to *in vitro* injury, horse antihuman thymocyte serum (ATS) (Upjohn Co., Kalamazoo, Mich) was used as an additional control.

Antiserum was titered in MEM in microtiter plates and tested for cytotoxicity against remission leukocytes and leukemic cells using a modification of Amos' two-stage method (21). Fluorescein diacetate was used as an indicator of cell viability (22), and simultaneously, ethidium bromide 0.01% was used to indicate cell death (23). Rabbit serum diluted 1:10 was the source of complement. Results were expressed as that titer which killed 50% of cells viable in the presence of normal mouse serum.

Antisera raised against myeloblasts were absorbed with lymphoblasts and lymphoblast antisera were absorbed with myeloblasts. Two antisera raised against acute lymphoblastic leukemia (ALL) cells were absorbed with the patients' autologous remission bone marrow. Cells used for absorption were stored at -79°C or in liquid nitrogen and thawed as above. 5×10^7 cells were suspended in MEM in a 0.4 ml Beckman microcentrifuge tube (Beckman Instruments, Inc., Fullerton, Calif.) and centrifuged at 1,000 *g* for 10 min at 4°C . The supernate was discarded, 10 μl of unabsorbed antisera was added, and the suspension was incubated at 4°C for 40 min. The incubated suspension was centrifuged at 3,000 *g* for 3 min in a Fisher model 59 centrifuge (Fisher Scientific Company, Toronto) by placing the microcentrifuge tube inside a 1-ml polystyrene tube. 3 μl of supernate was then removed for testing, and the

¹ Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATS, antithymocyte serum; CML, chronic myelogenous leukemia; HL-A, human leukocyte antigen; MEM, Eagle's minimal essential medium.

remaining supernate was transferred to a second microcentrifuge tube containing 5×10^7 cells for absorption. The absorption process was repeated until three absorptions were completed, with antisera set aside for testing after each absorption. If the absorption curves did not show relative flattening after the third absorption, the cell number was increased to as high as 5×10^8 per absorption step.

Antisera obtained before and after each absorption were tested in triplicate for residual activity against myeloblasts, lymphoblasts, remission leukocytes, normal allogeneic leukocytes, and normal allogeneic bone marrow cells.

RESULTS

10 unabsorbed antisera raised against acute myelogenous leukemia (AML) cells and 5 unabsorbed antisera to ALL cells were tested against myeloblasts from 35 patients with AML, lymphoblasts from 7 patients with ALL, myeloblasts from 7 patients with chronic myelogenous leukemia (CML) in blast crisis, and peripheral blood leukocytes from 12 acute leukemia patients in remission (Table I). Control antisera raised in nontolerant mice, normal mouse serum, and ATS were similarly tested. Both the mean and the range of cytotoxic titers were calculated. Antisera made against AML cells (unabsorbed) yielded a high cytotoxic titer against myeloblasts, lymphoblasts, and CML blasts, whereas almost no cytotoxicity was seen against remission cells. Antisera raised against ALL blasts gave high titers against myeloblasts and lymphoblasts and somewhat lower titers against CML blasts. Very low titers occurred with remission cells. Antisera against AML or ALL blasts raised in nontolerant mice gave uniformly high titers against myeloblasts, lymphoblasts, CML blasts, remission cells, and other control cells. Normal mouse sera or rabbit complement alone were not significantly cytotoxic and blasts cells and remission leukocytes were equally vulnerable to ATS.

Four antisera to myeloblasts absorbed with lymphoblasts (Table II) still yielded measurably stronger cytotoxicity against myeloblasts than against lymphoblasts. Absorption of ALL antisera with myeloblasts yielded corresponding results, with significant cytotoxicity being retained against lymphoblasts compared with myeloblasts. Antisera against the lymphoblasts of two patients with ALL, absorbed with their own remission bone marrow cells, retained somewhat reduced cytotoxic activity against both myeloblasts and lymphoblasts. Absorbed antisera gave titers of less than 1:2 with remission leukocytes. Four examples of cytotoxicity tests with absorbed antisera are given in Table III. In each case the absorbed antiserum retained significant activity against the parent cell type when compared with the absorbing cell. Absorptions done in microliter quantities showed a slight downward trend of the cytotoxic titer after the third absorption, due to the difficulty of completely removing the supernate.

TABLE I
Cytotoxicity of Unabsorbed Heteroantisera to Leukemic Blast Cells

Unabsorbed antisera made against	Mean cytotoxic titer vs.:			
	AML blasts (35)	ALL blasts (7)	CML blasts (7)	Remission cells (12)
AML blasts (10)	1:512* 1:64-1:1,024† 8.9±0.9§	1:512 1:128-1:512 8.5±0.7	1:256 1:32-1:1,024 7.6±2.0	1:16 1:8-1:32 4.1±0.9
ALL blasts (5)	1:256 1:64-1:1,024 7.8±2.0	1:256 1:64-1:1,024 7.9±1.5	1:64 1:16-1:256 6.1±2.1	1:16 1:8-1:32 4.0±0.8
Normal mouse sera	<1:2	<1:2	<1:2	<1:2
ATS	1:32 1:16-1:64 5.3±0.8	1:64 1:16-1:256 5.8±1.5	1:32 1:16-1:64 5.0±0.8	1:32 1:16-1:64 5.0±0.8

* Mean titer.

† Range of titers.

§ Mean±SD of log₂ titer.

All antisera, both absorbed and unabsorbed, were further tested against a panel of 30 normal allogeneic leukocytes and 10 normal allogeneic nucleated bone marrow cells (Table IV). The 15 unabsorbed antisera yielded very low titers and the 8 absorbed antisera were not cytotoxic.

DISCUSSION

Heteroantisera to human leukemic cells have been prepared by a variety of techniques (4-10), but the significance and specificity of the antigens detected have remained in doubt (14). In the present study, the unabsorbed antisera from tolerant mice were selectively cytotoxic to leukemic blast cells as compared to control cells. Since antisera from nontolerant mice were equally cytotoxic to both blast cells and remission cells, the differential cytotoxicity of the antisera from tolerant mice was likely due to recognition of antigenic differences in the target cells. These differences might be related to species or histocompatibility antigens, differentiation or maturation antigens, cell cycle-specific antigens, or antigens associated specifically with acute leukemia.

Antisera raised against myeloblasts and absorbed with lymphoblasts retained a measurable level of cytotoxicity against myeloblasts while losing all cytotoxicity to lymphoblasts. Absorptions done in the reverse direction gave the corresponding results, indicating that myeloblasts have associated antigens not found on lymphoblasts and vice versa. Differentiation of myeloblasts and lymphoblasts by means of serological determinations have also been reported elsewhere (10, 11, 15, 24).

Walford, Finkelstein, Neerhout, Konrad, and Shanbrom (25) and other groups (26) have postulated that human leukocyte antigens (HL-A) HL-A2 and -A12 are seen in higher than expected frequency in children with ALL. Pegrum, Balfour, Evans, and Middleton (27) have examined a more heterogeneous group including patients with AML and found an increase in HL-A1 and -A3. Several authors have found no increase in expected frequencies of HLA types in the

TABLE II
Cytotoxicity of Absorbed Heteroantisera to Leukemic Blast Cells

Antisera made against	Absorbed with	Cytotoxicity against	
		AML blasts (10)	ALL blasts (7)
AML (4)	ALL	1:32* 1:8-1:64† 4.9±1.4§	<1:2*
ALL (4)	AML	<1:2*	1:32* 1:8-1:64† 4.5±1.2§
ALL (2)	Remission bone marrow	1:16* 1:8-1:32† 3.8±0.9§	1:16* 1:8-1:32† 3.8±0.9§

* Mean titer.

† Range of titers.

§ Mean±SD of log₂ titer.

TABLE III
Cytotoxicity of Absorbed Heteroantisera; Four Representative Examples

Antisera made against	Absorbed with	Tested against	Cytotoxic titer after absorption			
			0	1	2	3*
1. AML blasts	ALL blasts	AML blasts	1:64	1:16	1:32	1:16
		ALL blasts	1:64	1:4	<1:2	<1:2
2. AML blasts	ALL blasts	AML blasts	1:1,024	1:128	1:64	1:32
		ALL blasts	1:512	1:2	1:4	<1:2
3. ALL blasts	AML blasts	ALL blasts	1:1,024	1:128	1:32	1:32
		AML blasts	1:1,024	1:2	<1:2	<1:2
4. ALL blasts	AML blasts	ALL blasts	1:1,024	1:128	1:8	1:8
		AML blasts	1:512	1:2	1:2	<1:2

* 5×10^7 cells used per absorption step in examples 1, 2, and 4. 10^8 cells per absorption step were required in example 3.

acute leukemias (28-31). In our study, the remission leukocytes used to induce tolerance were from the same individual as the leukemic blast cells to which the antiserum was raised, so that the effect of any increase in frequency of HL-A phenotypes would be canceled out during the preparation of the antisera.

In HL-A typing of leukemic blast cells, extra reactions may be seen that are not present on autologous remission cells and may not conform to a consistent HL-A genotype (14, 32). HL-A antisera may actually be detecting leukemia-associated antigens by virtue of weak antibodies present in the HL-A typing sera, thus accounting for the extra reactions (14, 32). Such atypical reactions occur irregularly in acute leukemia so that antisera such as ours which are cytotoxic to a large panel of leukemic cells could not only be directed towards extra HL-A antigens.

TABLE IV
Cytotoxicity of Heteroantisera to Control Cells

Antisera made against	Remission leukocytes (12)	Allogeneic leukocytes (30)	Normal bone marrow (10)
AML blasts (10)	1:16*	1:32	1:16
	1:8-1:32†	1:2-1:64	1:2-1:64
	4.1±0.9§	4.9±1.5	3.8±1.8
ALL blasts (5)	1:16	1:16	1:8
	1:8-1:32	1:2-1:64	1:4-1:64
	4.0±0.8	3.7±1.9	3.3±1.5
Absorbed antisera (8)	<1:2	<1:2	<1:2

* Mean titer.

† Range of titers.

§ Mean ± SD of log₂ titer.

Antigens characteristic of specific differentiated tissues have been well documented in the mouse (33) and may occur in the human white cell series (34). It is possible that myeloblasts and lymphoblasts carry such differentiation antigens. Antigens related to cell maturation have also been documented in both animals (35-37) and man (4, 38, 39), but only the red cell series has been studied. It is possible however that leukemic blast cells carry antigens not expressed on remission or normal leukocytes by virtue of blast cell immaturity. The reappearance of antigens characteristic of the fetus is known to occur in some human malignancies, and there is evidence that this may occur in leukemia (40). Cell membrane characteristics reflecting the metabolic changes of the phases of the replicative cell cycle may include changes in cell-surface antigenicity (18, 19, 41-43). Leukemic blast cells by virtue of dominance of certain cell-cycle phases may then manifest antigens not seen on remission and normal leukocytes. Species-specific antigens would be represented on both blast and remission leukocytes but might influence the activity of the unabsorbed antisera since the distribution of these antigens amongst blast cells and remission cells is unknown. In the present study, neoantigens appearing by virtue of cell culture techniques (17-19) are not a factor as they may be in other heteroantisera studies (9).

Although species specific, histocompatibility, differentiation, maturation, and cell cycle-associated antigens may be responsible in part for the cytotoxic activity of the unabsorbed antisera, it is unlikely that any of these antigens would be represented on one blast cell type to the exclusion of the other. Since the absorbed antisera retained activity against the parent cell type and had no residual activity against other blast cells, remission cells, or normal cells, these antisera are therefore probably detecting antigens specific for their leukemic cell type.

The antisera raised in this study are detecting one or several antigens associated with lymphoblasts and myeloblasts that are specific for each cell type and that may comprise true leukemia-associated antigens. This observation may be of both diagnostic and therapeutic significance. Diagnostically, the absorbed heteroantisera might be useful in distinguishing AML from ALL in patients where morphological criteria are inconclusive. Furthermore, an antiserum capable of detecting leukemic blasts cells could be used to screen the bone marrow of patients with preleukemic states or patients with acute leukemia in remission, for early appearance of blast cells. Therapeutically, absorbed heteroantisera with defined specificity might be used for passive immunotherapy of human leukemia with more success than past trials of less specific antisera. Studies pursuing these goals are currently in progress.

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