Cell-Mediated Immunity to Leukemia Associated Antigens in Experimental Models and in Man

Ronald B. Herberman

Laboratory of Immundiagnosis
National Cancer Institute
Bethesda, Maryland USA 20014

Cell-mediated immune reactions against leukemia associated antigens have been detected in experimental animal models and in patients with acute leukemia. The occurrence of specific cellular immune reactivity has important implications for the diagnosis and therapy of human acute leukemia. As summarized in Table 1,

Table I: Application of Assays of Cell-Mediated Immunity to Diagnosis and Therapy

1. Initial diagnosis
a. Specificity: Are antigens leukemia associated?
b. Reactivity: Is reactivity found only with leukemia patients?
2. Monitoring of patients during clinical course
a. Correlation with clinical status
b. Indication of prognosis or response to therapy
3. Therapy
a. Antigens: Can they induce transplantation protection?
b. Proximity of reactive cells to tumor cells
c. Augmentation of specific immune reactivity

assays of cellular immunity could be applied to the initial detection and diagnosis of leukemia, to the monitoring of patients during and after therapy, and to the planning of rational immunotherapeutic trials. For initial diagnosis, the specificity of the detected antigens and the distribution of reactivity among patients with leukemia and other individuals need to be carefully defined. For immunologic monitoring of leukemia patients, only assays which correlate with extent of disease or with responsiveness to therapy would be useful. Techniques which can measure leukemia associated transplantation antigens would be very useful for selection of materials for specific immunotherapy. In addition, such assays could be used to detect relevant responses to immunotherapeutic manipulations, and to subsequently design optimal schedules of immunotherapy. However, before such practical clinical applications can be realized, additional information in several areas needs to be accumulated. For all of these objectives, standardization of reagents will be very important. There appears to be considerable heterogeneity in the antigens on

leukemia cells and in the quantitative expression of each of these antigens (1). Some of the variability in results and some of the negative reactions could be ascribed to the use of poorly antigenic leukemic cells. Selection of large supplies of highly antigenic cells and extracts, with known specificities, and provision for long term preservation of antigenicity will help to ensure adequate sensitivity of the assays. Preservation of highly reactive lymphocytes, and reduction in day-to-day variability in the assays, will also be major steps in the standardization of the assays. Although progress in these areas is being made in clinical studies, there are a number of logistical problems. The use of leukemia models in syngeneic animal systems should be helpful to work out approaches might could then be applied to studies of leukemia patients. In this paper, I will review some of the information which has been obtained in animal systems, and try to relate this to the clinical objectives.

Specificity of Cell-Mediated Immunity in Experimental Mogels

Tumors induced by oncogenic viruses have been shown to have cell surface antigens specific for the particular virus and also antigens common to other types of tumors and to fetal cells (2, 3). We have shown, by quantitative absorption tests, that syngeneic mice and rats may respond to tumor cell inoculation by formation of antibodies with differing specificities. It is equally important to determine the nature of the antigens detected in assays of cell-mediated immunity. However, it is considerably more difficult to define the specificity of cellular reactions and few extensive studies have been done. Recently, an inhibition assay was developed which permits definition of the specificity of the ^{51}Cr release cytotoxicity assay for cell-mediated immunity (4). Unlabelled cells were added to the mixture of lymphocytes and labelled target cells. If the added cells had the same antigens as those on the target cells, competitive inhibition of lysis was seen. In studies of the Gross virus-induced leukemia (C58NT)D in W/Fu rats, the detected antigenic specificity was found to be quite distinct from any of those detected by humoral antibodies. The antigen was only found on rat cells and not on mouse cells transformed by Gross or other leukemia viruses (4). The antigen appears to be related to expression of rat endogenous type C virus (Table 2) and has been designated REV-SA-1 (5). We have also used this inhibition assay to study the specificity of the cell-mediated reactivity induced in mice by murine sarcoma virus (MSV). MSV induces local tumors in mice, which then usually

Table II: Specificity of Cell-Mediated Immunity in Experimental Models

1. Immunity to Gross virus-induced leukemia, (C58NT)D: antigens associated with rat endogenous type C viruses (REV-SA-1).
2. Murine sarcoma virus (MSV) induced immunity: antigens associated with mouse endogenous type C viruses (MEV-SA-1), present on leukemia cells but also on variety of other cells.
3. Immunity to Friend virus-induced leukemia, FBL-3:
   a. F and FMR antigens
   b. MEV-SA-1

regress. Many laboratories have studied the cell-mediated immune response in this system, and since positive reactions were seen with some leukemic cells, the detected antigens have been presumed to be related to FMR leukemia antigens (6–8). However, we have found that cytotoxic lymphocytes of immune C57BL/6 mice react against an antigenic specificity distinct from any of the expected specificities (9). This antigen has been designated MEV-SA-1 (Table 2) since it appears to be related to expression of mouse endogenous type C virus. The most direct evidence for the association of this antigen to endogenous virus has come from studies involving infection of a mouse cell line SC-1 and a rabbit cell line SIRC with viruses from antigen positive cells (10). Some of the infected cells rapidly became antigen positive (Table 3). Recent studies, with an ^{125}IUdR release cytotoxicity assay, of

Table III: Inhibition of MSV-Induced Cytotoxicity by MEV*-Infected Cells

Cell Line	Infected with virus from:	Inhibition of cytotoxicity
SIRC	none	–
	BALB/3T3 clones	
	S16 C1-2	+
	S2 C1-3	+
	S3	+
	EL-4 ascites	+
	E♂ G2	–
SC-1	MCDV-12 ascites	+
	none	–
	EL-4 ascites	+
	E♂ G2	–
	MCDV-12 ascites	–

* Mouse endogenous type C virus.

cell-mediated immunity against a Friend virus-induced leukemia FBL-3 have indicated reactivity against the FMR serologic specificity and also against MEV-SA-1 (11, 12).

Nature of Effector Cells in Experimental Models

A further complexity in the analysis of cell-mediated immunity is that different types of effector cells can be involved, even within the same tumor system (Table 4). These different subpopulations of cells can develop reactivity with disparate kinetics, and can be directed against different specificities. In each of the three animal model systems mentioned above, thymus derived lymphocytes (T cells) appear to be responsible for the reactivity in isotopic release cytotoxicity assays, of animals immunized by tumor cells or by virus (13–16). In addition, immune reactivity in a microcytotoxicity assay (16) and in a lymphocyte proliferation assay (17) in our MSV system, and the lymphoproliferative response of immune

Table IV: Nature of reactive cells in experimental models

1. T cells: responsible for specific immune reactivity (cytotoxicity and lymphocyte proliferation) induced by MSV, FBL-3, (C58NT)D.
2. Macrophages: responsible for nonspecific growth inhibiton induced by MSV and by other tumors.
3. N cells: responsible for specific natural immunity against mouse and rat leukemias.

cells against (C58NT)D (18) are dependent on T cells. Effector cells have also been detected in the MSV tumor system by a growth inhibition assay, in which cytostasis is measured by inhibition of uptake of ^{3}H-thymidine (19). Macrophages appear to be responsible for these effects, and in contrast to the specificity of the immune T cells, growth inhibitory activity appears to be nonspecific, with marked effects on a variety of leukemia cells and other tumor cells (19, 20). In addition to the T cells and macrophage effector cells in immune animals, the lymphocytes of many normal mice and rats have been shown to have specific cytotoxic reactivity against leukemia cells (21, 22). The natural cytotoxic reactivity of mice was not dependent on T cells, being unaffected by treatment with anti-⊖ serum plus complement, and with high levels being found in athymic, nude mice (Table 5; 23). Treatment of reactive mouse cells by procedures known to deplete or in-

Table V: Effects of various treatments on MSV immune and BALB/c nude spleen cell cytotoxicity against RBL-5 target cells

Treatment of spleen cells	MSV immune		BALB/c nude	
	Untreated	Treated	Untreated	Treated
Anti-⊖ + C	32*	4	38	25
Carbonyl iron/magnet	21	22	15	15
Nylon column	27	23	15	38
Carrageenan (200μg)	45	45	12	12
EA monolayer	24	31	28	21
EAC monolayer	24	28	28	34
Anti-γ globulin (1:20)	48	40	23	22

* % cytotoxicity.

activate macrophages, complement receptor bearing cells, or immunoglobulin receptor bearing cells also had no effect on cytotoxicity. Similarly, the natural rat effector cells had no identifiable surface marker (Table 6; 22). The subpopulation of lymphocytes responsible for the natural cytotoxicity has been tentatively designated N cells (23). The natural cytotoxicity appears to be directed against antigens associated with endogenous type C viruses of the particular species, but some differences have been noted between these specificities and those detected by immune T cells (22, 23). The finding of cytotoxic reactivity against some tumor

Table VI: Effects of various treatments on (C58NT)D immune and normal spleen cell cytotoxicity against (C58NT)D target cells

Treatment	Immune		Normal	
	Untreated	Treated	Untreated	Treated
Anti-T (NIH) + C	68*	3	13	12
Anti-T (Scripps) + C	32	3	12	11
Carbonyl Iron/magnet	11	24	6	8
Nylon column	24	41	8	10
EA monolayer	9	9	4	3
EAC monolayer	9	13	4	5
EAC column	46	53	4	5

* % cytotoxicity.

associated antigens in normal animals has important implications for the use of cell-mediated dytotoxicity assays for diagnosis of cancer. On the one hand, the wide distribution of reactivity, not only in tumor immune animals, but also in many normal individuals, would seem to eliminate the diagnostic usefulness of these assays. However, since different subpopulations of effector cells are involred, methods could be worked out to distinguish the nature of the reaction seen with a particular individual.

Correlation of Cell-Mediated Immunity in Experimental Models with Tumor Growth

In order to evaluate the usefulness of assays of cell-mediated immunity for monitoring of individuals with leukemia, it is important to determine the relationship of reactivity to course of disease. In each of our three animal model systems, it has been possible to induce progressive tumor growth (such animals are designated progressors) or transient tumor growth followed by complete regression (such animals are designated regressors). In both regressors and progressors, the T cell cytotoxic responses were transient, with peak levels soon after inoculation (8, 12, 24). However, the responses of progressors in each of these systems were considerably lower than those seen in regressors (Table 7). In the growth inhibition assay, more reactivity has been seen in progressors than in regressors. The activity appeared to be correlated with presence of large tumors, and it persisted in the progressors (19, 20). In the lymphocyte proliferation response to (C58NT)D, the difference between regressors and progressors was particularly striking. Reactivity has been detected in regressors at 15 days or more after tumor cell inoculation. In contrast, no reactivity was seen with lymphocytes from progressors (25). This lack of detectable direct reactivity was found not to be due to an absence of lymphocytes capable of response, but rather to the presence of suppressor cells. After removal of these suppressor cells, positive proliferative responses could then be observed.

In addition to these *in vitro* studies of correlation with *in vivo* host resistance

Table VII: Correlation of cell-mediated immunity with tumor growth

	Regressors	Progressors
Cytolysis		
MSV	peak at 14 days; then rapid decline to low levels	similar time course, but low levels
FBL-3	biphasic response	similar time course, but much lower levels
(C58NT) D	peak at 10 days; then rapid decline to low levels	similar time course, but low levels
Growth inhibition	transient activity at peak of tumor growth	persistent activity
Lymphocyte proliferation		
(C58NT) D	detectable after 15–20 days	not detectable, except after removal of suppressor cells
Adoptive transfer of immunity		
MSV	good protection against	weak protection against cell
FBL-3	tumor cell challenge	challenge

to tumor growth, we have also performed some systemic adoptive transfer experiments in the MSV and FBL-3 systems, with lymphocytes from regressors and progressors (15). Cells from regressors conferred considerably better protection against challenge with leukemia cells than did cells from progressors.

Secondary Cytotoxic Responses in Experimental Model Systems

One of the major apparent discrepancies between the results of *in vitro* cytotoxicity assays and *in vivo* events in our model systems was the short duration of strong cytotoxic reactivity after tumor or virus inoculation. This was in contrast to the very long-lasting resistance of previously inoculated animals to rechallenge with leukemia cells. This raised serious questions about the *in vivo* relevance of the cytotoxic reactions. Recently, a series of experiments has been performed which resolves this problem. When immune animals were rechallenged with tumor cells,

Table VIII: In vivo generation of secondary cytotoxic response to tumor cells

Tumor system	% Cytotoxicity* no challenge	challenge with tumor cells
MSV	–0.2	34.1
FBL-3	3.5	33.0
(C58NT) D	2.3	47.2

* Immune animals were challenged intraperitoneally with tumor cells and 3–5 days later, peritoneal exudate cells were tested for cytotoxicity against labelled target cells. In the MSV system, RBL-5 ascites tumor cells were used for challenge and as target cells.

strong cytotoxic reactivity rapidly developed but tended to be restricted to the region of challenge (Table 8; 26–28). Similar results have also been obtained *in vitro*, by incubation of immune lymphocytes with tumor cells for 5–9 days (Table 9; 29–31). These data clearly indicate that immune animals have memory cells for cytotoxicity, but re-exposure to antigen is needed for generation of reactivity. Since the *in vivo* transplantation protection experiments involve such re-exposure to tumor cells, the data from the secondary response experiments are quite concordant with the persistence of *in vivo* resistance to challenge.

Table IX: In vitro generation of secondary cytotoxic response to tumor cells

Tumor system	% Cytotoxicity * immune cells alone	% Cytotoxicity * immune cells + tumor cells**
MSV	9.1	33.5
FBL-3	3.0	69.1
(C58NT) D	6.1	66.7

* At end of 5–9 days in culture, effector cells tested against ^{51}Cr labelled target cells, at ratio of 50:1, or in FBL-3 system, against $^{125}IUdR$ labelled cells, at 15:1 ratio. In MSV system, RBL-5 ascites tumor cells were used as target.
** Immune spleen cells were obtained 30–40 day after virus or tumor cell inoculation. Incubation performed with mitomycin C treated tumor cells.

Standardization of Assays of Cell-Mediated Immunity

For reliable practical applications of the assays of cell-mediated immunity, standardization of reagents and reduction in day-to-day variability of the assays is quite important. We have focused on the ^{51}Cr release cytotoxicity, and have been able to cryopreserve functional effector cells and tumor target cells (32, 33). Cryopreserved tumor cells have also been quite useful in the cytotoxicity inhibition assays. These cyropreserved cells have yielded very reproducible results over a long period of time, and have reduced the number of variables in the assays. In addition, they have allowed us to include internal standards in assays, so that we can accurately compare experimental results among different experiments. Cryopreserved tumor cells have also been effective for stimulation of lymphocyte proliferation and for *in vitro* generation of secondary cytotoxic responses.

Cell-Mediated Immunity to Human Leukemia Associated Antigens

Cell-mediated immune reactions have also been detected against human leukemia associated antigens. My laboratory has primarily used skin tests for delayed hypersensitivity and ^{51}Cr release cytotoxicity assays for these studies. Some of the characteristics of the observed reactions are summarized in Table 10. The initial delayed hypersensitivity studies were performed with membrane extracts of autologous leukemic and remission leukocytes. Positive reactions were obtained with leukemic extracts and not with the extracts of remission cells (34). Although these data indicate an association of the antigens with leukemia, it was not possible

Table X: Characteristics of cell-mediated immunity in human leukemias

Assay	Specificity	Clinical Correlation
Skin tests	leukemia associated	higher incidence of reactivity in remission than in relapse
autologous cells		
allogeneic cells	leukemia (same type) associated	higher incidence of reactivity in remission than in relapse
Raji	broader specificity: mainly ALL* and Burkitt's lymphoma	higher incidence of reactivity in remission than in relapse
HKLY 28	broad specificity: mainly NPC** and ALL	higher incidence of reactivity in remission and with localized tumor
^{51}CR release cytotoxicity		
autologous cells	leukemia associated	?
allogeneic cells	antigens on blast cells and remission cells of leukemia patients; considerable normal reactivity	?
tissue culture lines	high incidence of normal reactivity	depressed during chemotherapy

* Acute hymphocytic leukemias.
** Nasopharygeal carcinom.

to evaluate the distribution of a particular antigen. Therefore, a series of experiments were performed with extracts of allogeneic cells (35). Extracts of allogeneic acute leukemia cells produced positive reactions in about one third of patients with the same type of leukemia, but were unreactive in patients with the dissimilar type of leukemia. Extracts of allogeneic remission cells or of normal leukocytes gave negative results. Therefore, the skin tests with the blast extracts detected reactions to antigens common to acute leukemia (ALL) and to distinct common acute myelogeous leukemia (AML) antigens. However, these antigens did not appear to be equally represented in all extracts. Some preparations gave positive reactions in more than half of the appropriate leukemia patients, whereas others were poorly or non-reactive. Partly as an attempt to obtain large amounts of standard test materials, extracts have been prepared from human lymphoid tissue culture cell lines, derived from leukemia, other tumors, or from normal keukocytes (36). Extracts of some of these lines have given positive skin reactions in patients. A summary of data with some of these extracts is given in Table 11. The Raji line, derived from a patient with Burkitt's lymphoma, has given reactions in 40 % of American patients with ALL, several patients with AML, and over 60 % of patients with Burkitt's lymphoma (37). Only a small proportion of French ALL patients (38, 39) and none of the Canadian ALL patients reacted to Raji (40). This could

Table XI: Delayed skin reactions to extracts of lymphoid cell lines

Patients	Tests positive/total tests (% +) Tumor derived cell lines			
	RAJI	MOLT	HKLY 28	F-265
ALL* (U.S.)	26/65 (40)	6/17 (35)		1/54 (2)
ALL (French-Oldham)	4/49 (8)			0/49 (0)
ALL (French-Levine)	1/12 (8)		1/10 (10)	0/10 (0)
ALL (Canada)				
before therapy	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)
remission	0/22 (0)	0/22 (0)	7/22 (32)	0/22 (0)
Burkitt's lymphoma	29/47 (62)			2/47 (4)
NPC**	10/34 (29)		24/36 (67)	6/35 (17)
Other carcinomas	10/72 (14)		12/59 (20)	1/69 (2)

* Acute lymphocytic leukemia.
** Nasopharyngeal carcinoma.

be partially ascribed to differences between lots of antigen, even from the same cell line; however, American ALL patients reacted well to the same lot as that used in the study in France. The extract from HKLY28, a lymphoid cell line derived from a patient with nasopharyngeal carcinoma (NPC), gave a different pattern of reactivity. Patients with NPC had a high incidence of reactivity (38), Canadian ALL patients also reacted (40), but only 1 of 10 French ALL patients reacted (39). It is clear from these data that the antigens detected on the cultured cells have considerably broader specificity than the blast cell extracts. However, the reactions showed some clear specificity, since patients with carcinomas other than NPC had a low incidence of reactivity. The HKLY28 cell line produces Epstein-Barr virus (EBV) and Raji contains the EBV genome. The skin reactive antigens on these cells could be related to EBV. However, the F-265 cell line has a similar expression of EBV genome as Raji, yet elicited very few reactions. Furthermore, the MOLT cell line, derived from a patient with ALL and lacking the EBV genome, produced almost as high an incidence of reactions in American ALL patients as did Raji.

In the skin tests with membrane extracts of either blast cells or of cell lines, reactivity has correlated with clinical status (Tabele 10). Patients with ALL or Burkitt's lymphoma in remission had a significantly higher incidence of reactivity than did patients in relapse (35, 37, 38). Patients with positive skin tests have also tended to have a better prognosis. Similarly, NPC patients with localized disease have had a higher incidence of reactions to HKLYL8 than those with more advanced disease (41).

The main limitation in the skin test studies has been the inability, for ethical reasons, to test the extracts in control individuals, with benign or no disease. *In vitro* studies of cytotoxicity, using the ^{51}Cr release assay against cryopreserved leukemic target cells, have allowed a more extensive study of reactivity in controls as well as in patients (Table 10). Testing of autologous reactivity of leukemia

patients showed reactions against leukemia associated antigens, with no reactions against remission target cells (42). In allogeneic testing, a broader pattern of reactivity was seen. Positive results were obtained against remission lymphocytes of leukemia patients, as well as against blast cells (43, 44). A considerable number of normal individuals reacted against the target cells from leukemia patients, but hey did not react against their own lymphocytes or against allogeneic normal lymphocytes. Reactivity of normal individuals, higher than that of leukemia patients, was also seen against lymphoid tissue culture target cells, including the F-265 line (45). As noted in Table 10, cytotoxic reactivity of the leukemia patients has not correlated well with clinical status. Patients with bone marrow relapse, as well as those in complete remission, gave positive reactions (42).

Major Areas for Further Study of Human Leukemia

Considerably more work will be needed before the assays of cell-mediated immunity can be used for practical clinical problems. Table 12 outlines some of the major problems which need to be addressed. From the tests performed thus far, it

Table XII: Major areas in human leukemia to be investigated

1. Preparation of large, standardized batches of antigenic materials.
2. Better characterization of antigens.
3. Nature of effector cells in patients and in controls.
4. Conditions for augmentation of cell-mediated immunity to leukemia associated antigens.

appears that the antigens on human leukemia cells, similar to those on experimental leukemias, are heterogeneous and quite complex. In order to sort these antigens out, it will be important to work with large, standardized batches of cells or extracts. Cells obtained by the cell separator machines or from tissue culture lines should be quite useful in this regard. Further characterization of the antigens, both by fractionation studies and also by further specificity testing (e.g. by the cytotoxicity inhibition assay), is needed. It will be of interest to determine whether any of the antigenic specificities detected by the cellular immunity assays are the same as those described by others at this Workshop, using serologic techniques.

It will also be of interest to determine the nature of the effector cells in the cytotoxicity reactions. It has recently been suggested that cytotoxicity by effector cells from patients is mediated by T cells, whereas the natural cytotoxicity is mediated by complement receptor bearing cells (46, 47). This would be analogous to the differences seen in the experimental tumor models. However, our preliminary studies have indicated that both T cells and non-T cells of normal individuals can be cytotoxic against the lymphoid cell lines (48). Further studies will be needed to resolve this issue, and determine whether the cytotoxicity assay can be used for diagnosis of leukemia or for monitoring of patients.

The assays of cell-mediated immunity should be helpful to determine conditions for immunotherapy. If the antigens detected are relevant to host resistance, as some appear to be in the experimental model systems, then procedures which

augment reactivity would be expected to have a beneficial *in vivo* effect. Better schedules of therapy, and selection of antigenic materials for inoculation, could be determined by appropriate immunologic testing.

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