HUMAN IMMUNE RESPONSE TO RAUSCHER LEUKEMIA VIRUS[1]

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Abstract

Twenty patients were immunized with formalin-killed Rauscher leukemia virus. No untoward side effects were observed. Approximately three-fourths of the patients developed cell-mediated immunity as assessed by *in vitro* lymphocyte blastogenic responses. Approximately two-thirds of the patients developed antibody responses as measured by radio-immunoprecipitation, and one-half of the patients developed delayed hypersensitivity to the immunizing antigen.

The responses illicited were specific for the immunizing viral antigen because little or no response was illicited *in vitro* among the immunized patients' lymphocytes to virus-free tissue culture vehicle. The immune response to the viral antigens was also evaluated by lymphocyte stimulation with solubilized from transformed cells.

These data suggest that human subjects (patients with metastatic cancer and acute leukemia) can mount immune responses to oncogenic viruses of both humoral and cell-mediated immunity.

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Introduction

The fundamental premise on which this study is based is that murine leukemia viruses and a hypothesized viral agent associated with human leukemia may share antigens or may induce common antigens in transformed cells. The observations which tend to support this hypothesis include the following. First, the bone marrow cells of certain patients with leukemia react with fluorescent antibody made in other species against the Rauscher leukemia virus (1). Second, serum of some patients with acute leukemia has neutralizing activity against Rauscher virus (2). Third, antigen of the virus envelope has been found in the serum of some patients with erythroleukemia (3). Finally, when human embryonic kidney cells are transformed by the Rauscher leukemia virus they develop a cell membrane antigen also present in human leukemia cells and lymphoblastoid cell lines (4).

These data suggested that a study of the human immune response to the Rauscher leukemia virus would be of interest. First, it would tell us if human subjects could respond immunologically to an oncogenic virus. Second, it would generate data regarding the possible interrelationship of human and murine leukemia. Third, it would generate useful reagents for the study of oncogenic viruses and their relationship to transformed cells.

Therefore, the immune response of 20 patients with advanced malignant disease to a killed Rauscher leukemia virus preparation was studied. The majority of patients mounted a cell-mediated and humoral immune response both to virion and to murine antigens present in the virus envelope. The data indicate that this may be useful in approaching the objectives mentioned above.

Materials and Methods

Twenty patients with advanced malignancy were selected. There were 13 patients with metastatic solid tumors and seven patients with acute leukemia. The objectives of the study and the possible risks were carefully explained to each patient and informed consent was obtained. Each potential candidate for study was skin tested with a battery of five antigens to which most individuals are sensitive, including dermatophytin, dermatophytin-0, candida, varidase and mumps. For entry into the study, at least two of these skin tests were required to be positive. Each patient also had a set of lymphocyte cultures set up which were stimulated with phytohemagglutinin and streptolysin-0. To enter the study, the responses to these mitogens were required to be at least 20,000 counts per minute and 3,000 counts per minute, respectively.

After this preliminary evaluation, each patient received Rauscher leukemia virus intradermally on day 1, 14, 28 and 42 of the study. The development of delayed hypersensitivity to the immunizing antigens was determined on the subsequent two days. *In vitro* lymphocyte responses and serum antibody responses to the immunizations were measured on day 1, 7, 14, 21, 28, 35, 42 and 49.

Virus for immunization was grown in JLSV9 cells, harvested from the supernatant fluid and purified by double banding by density gradient centrifugation. It was inactivated by exposure to 0.05 percent formalin for two weeks at 4 °C. The final virus concentration was 1.7 mg/ml. Virus for *in vitro* lymphocyte cultures was

prepared in the same fashion but not inactivated with formalin. The control for the Rauscher leukemia virus used *in vitro* was doubly banded culture fluid from JLSV9 cultures which were not infected with the Rauscher virus (virus vehicle).

Antigen from transformed cells was prepared from JLSV9 cells by the hypertonic KCl extraction method of Reisfeld (5). Antigen from JLSV9 non-infected cells for use as a control was prepared in the same fashion.

Delayed hypersensitivity skin test responses to immunization were read as previously described (6). *In vitro* lymphocyte cultures were set up, stimulated and harvested as previously described (6). Each set of cultures consisted of a control and cultures stimulated with phytohemagglutinin, streptolysin-0, a dose range of Rauscher leukemia virus, a dose range of Rauscher leukemia virus control, a dose range of JLSV9 Rauscher infected cell antigen and a dose range of JLSV9 non-infected cell antigen.

Antibody titers were performed by a radioimmunoprecipitation technique[7]. Briefly, a Rauscher preparation labeled during proliferation of the virus with ^{3}H-leucine was mixed with an aliquot of the test serum, all immunoglobulin was then precipitated with goat anti-human IgG and goat antihuman IgM, and the dilution of the serum at which either 30 or 50 percent of the virus was precipitated was taken as the titer.

Results

No untoward side effects of any kind were noted during this study. All patients tolerated the immunizations, skin testing and blood drawing procedures very well. There was transient local discomfort at the injection sites, lasting about 10 seconds during immunization. The immunization procedure did not appear to alter the natural history of the patient's disease in any way.

Table 1 shows the lymphocyte blastogenic responses before and after immunization to the various mitogens and antigens. It can be seen that there were no

Table 1. Lymphocyte Blastogenic Responses Pre- and Post-Immunization

Lymphocyte Stimulant	Response in CPM*		Response in SI**	
	Pre-Imm.	Post-Imm.	Pre-Imm.	Post-Imm.
Control	0.3	0.3	1.0	1.0
PHA	46.9	49.7	180.5	221.2
SLO	6.1	3.6	18.2	15.4
RLV***	0.8	5.5	4.3	21.6
RLV Vehicle Control	0.0	0.3	1.1	1.4
JLSV9R***	0.5	1.8	2.6	6.9
JLSV9C	0.0	0.2	1.1	1.7

* Net counts per minute per 10^{6} lymphocytes x 10^{3} (median)
** Stimulation Index (median)
*** Data obtained from RLV and JLSV9R doses giving highest response and appropriate dose-associated controls.

Table 2. Relative Specificity of Response to Various Rauscher Leukemia Virus Related Materials

Parameter	Virus/Vehicle Ratio		JLSV9R/JLSV9C Ratio	
	Pre	Post	Pre	Post
Number	16/19*	16/18	18/19	16/18
Median	3.5	12.1	3.1	4.0
Mean	6.4	20.4	11.6	20.0

* No. with ratio >1/no. in whom ratio studied

Table 3. Development of In Vitro Lymphocyte Blastogenesis after Immunization with Formalinized RLV

Antigen	Number of Patients	Response to Immunization				
		In CPM		In SI		Overall
		Pre	Post	Pre	Post	
RLV	Positive	2	15	3	16	
	Responding		14		15	14
JLSV9R	Positive	7	13	14	16	
	Responding		9		9	7

Table 4. Serial Study of Lymphocyte Response to RLV

Parameter	Week of Study							
	1	2	3	4	5	6	7	8
CMP*								
Median	0.5	1.8	1.9	1.8	1.2	1.3	2.8	0.6
Mean	1.0	2.2	3.5	1.5	2.0	3.2	5.0	2.3
SI								
Median	1.9	8.0	2.5	5.5	7.3	6.6	9.4	4.6
Mean	1.6	9.7	18.3	7.0	14.4	7.8	19.2	18.7

* CPM = Counts per minute per 10^6 lymphs x 10^3

significant changes either in terms of counts per minute or stimulation index in the control, PHA and SLO responses. There was a striking increase in response to the Rauscher leukemia virus and a modest increase in response to the JLSV9 Rauscher infected cell antigen. Since there was only a slight increase in the response to the RLV vehicle control and the JLSV9 noninfected control cell antigen, the responses were presumably virus or virus induced antigen specific.

This was further investigated by calculating a specificity ratio by dividing the virus response by the vehicle response, or the JLSV9 Rauscher infected cell antigen response by its appropriate control. It can be seen in Table 2 that both pre- and post-immunization these ratios were greater than one, indicating specificity. The median ratio rose from 3.5 to 12.1 for the virus and 3.1 to 4.0 for the antigen. The mean ratios rose from 6.4 to 20.4 for the virus and from 11.6 to 20.0 for the antigen.

Table 3 shows the numbers of subjects who showed positive responses *in vitro* to the virus and the antigen before and after immunization, in terms of counts per

Table 5. Development of Delayed Hypersensitivity after Immunization with Formalinized RLV

No.	Pre-Immune	Post-Immune	Response to Immunization
1	3*	3	No
2	0	0	No
3	3	16	Yes
4	0	0	No
5	0	4	Yes
6	0	10	Yes
7	0	0	No
8	0	0	No
9	6	10.5	No
10	0	4.5	Yes
11	0	3	Yes
12	8	12	No
13	4	18	Yes
14	0	12.5	Yes
15	0	5	Yes
16	0	0	No
17	0	0	No
18	0	5	Yes
19	0	0	No
20	0	7.5	Yes
Mean	1.2	5.55	10/20
± SD	2.4	5.78	
SE	.5	1.29	
Median	0	4.25	
Range	0-6	0-18	

* Diameter induration in mm

minute and stimulation index and the numbers of patients who showed a true response to immunization comparing pre- and post-immunization values. It can be seen that approximately three-fourths of the 20 subjects showed a response to the Rauscher leukemia virus and that somewhat more than one-third of the subjects showed a response to the solubilized cell antigen.

Table 6. Antibody Response after RLV Immunization by Radioimmunoprecipitation Assay

Patient Number	Pre-Immune		Post-Immune	
	30%	50%	30%	50%
1	0	0	1,024	379
2	0	0	512	48
3	0	0	0	0
4	0	0	256	96
5	0	0	256	128
6	0	0	64	0
7	0	0	1,024	512
8	0	0	198	64
9	0	0	1,500	512
10	0	0	0	0
11	0	0	512	256
12	0	0	2,000	1,024
13	0	0	0	0
14	0	0	0	0
15	0	0	16	0
16	0	0	16	0

Table 7. Immune Response after RLV Immunization Comparison of Various Absorption Methods

Serum Absorption	Reciprocal of Serum Dilution						
	16	32	64	128	256	512	1,024
None	84*	95	93	92	94	86	81
JLSV 6	87	86	84	82	75	64	48
3 T 3	95	95	95	94	87	84	66
In Vivo	95	92	90	87	82	72	42
None	87	87	85	82	71	63	48
In Vivo	20	17	16	16	16	14	12

* Percent precipitation at indicated dilution

Table 8. Correlation of Antibody Response to RLV and other Parameters of RLV Immunity

Immunoligical Parameter	Positive or Negative	Antibody Response	
		Positive or Negative	
Delayed	+	5	4
Hypersensitivity	–	4	3
Lymphocyte			
Response	+	8	1
to RLV (CPM)	–	5	2
Lymphocyte			
Response to	+	7	1*
JLSV9R (CPM)	–	2	6

* Significant Difference

Table 4 shows the kinetics of the response in terms of the median counts per minute and stimulation index. It can be seen that the vigorous responsiveness developed between one and two weeks after immunization or in the second and third week of the study that there was a decline in reactivity between the fourth and the sixth week with a return of vigorous activity subsequently and another decline at the end of the follow-up period.

The delayed hypersensitivity responses pre- and post-immunization are shown in Table 5. Only five subjects showed reactivity prior to immunization with a mean response of 1.2 mm in diameter of induration. After immunization, 13 subjects showed positive reactivity with a mean diameter of induration of 5.5 mm. Ten of the twenty gained activity after immunization or showing a greater than 100 percent increase in reactivity.

Antibody titers have been run on the sera of 16 of the 20 patients. Before immunization, some of the patients showed antibody titers to the virus but these were reduced to the background level by *in vitro* absorption of the serum on JLSV6 cells or by *in vivo* absorption of the sera in BALB-C mice. After immunization, as shown in Table 6, 12 of the patients showed a 50 percent precipitation titer. This was after *in vivo* absorption of the sera. It might be noted that these titers remained positive after double absorption of the serum both *in vivo* and *in vitro*, that there were no positive pre-immune sera with true anti-viral activity, that a series of control subjects not immunized with virus but given BCG by scarification did not develop antibody and that these sera had no antibody activity using the same assay against the RD 114 virus. Table 7 shows the effects of absorption on the antibody titer in immune (upper four lines) and non-immune (lower two lines) serum. It can be seen that in the non-immune serum precipitating antibody was completely absorbed *in vivo*.

Table 8 shows an attempt to correlate the antibody response to the RLV to the other parameters of RLV immunity. The antibody responses did not correlate with delayed hypersensitivity responses or *in vitro* lymphocyte responses to the RLV. However, they did correlate with *in vitro* lymphocyte responses to the JLSV9 Rauscher infected cell antigen in terms of counts per minute.

Discussion

While the evidence is very strong that many animal malignant tumors are caused by viruses, the evidence is still circumstantial in man that any human malignancy is virus induced. For instance, attempts to isolate human cancer viruses by inoculation of cell-free extracts from human cancer materials into animals have failed (7a). Also, attempts to isolate oncogenic viruses from human cancer materials in tissue culture have failed (8). In general, attempts to unmask oncogenic viruses from human cancer materials in tissue culture have also failed. While initial enthusiasm greeted the observation that virus-like particles were observed in various malignant tissues, it is felt today that these were, for the most part, various cell fragments or artifacts (9). Human adenoviruses, which clearly cause malignancies in animal species, are apparently not oncogenic in man (10), and the Herpes virus group is probably only associated etiologically with one human malignancy, namely Burkitt's lymphoma (11), altough nasopharyangeal carcinoma and carcinoma of the cervix may also be caused by this virus group.

The strongest evidence for a viral etiology in cancer involves the RNA tumor viruses, namely type-B viruses in human breast cancer and type-C viruses in human leukemia and lymphoma. This includes discovery of antigenic relationships, observations of particles by electronmicroscopy, detection of viral genomes in cancer tissues by hybridization experiments and viral enzymes in human cancer tissue (12). Because of the observations relating the Rauscher leukemia virus with human leukemia cited in the introduction, we thought it would be worthwhile to study the human immune responses to such a virus. The data generated in the study of the first 20 patients indicates that human subjects can indeed mount an immune response to formalinized Rauscher leukemia virus. Approximately two-thirds of the patients studied mounted such an immune response and this involved both humoral and cell-mediated immunity. Both patients with solid tumors and with acute leukemia responded, so that apparently there is no immunological tolerance to viral antigens in leukemia patients.

The study generated some evidence of specificity. Thus, the response to the virus was greater than to the appropriate non-virus containing vehicle and the response to the antigens solubilized from virus infected cells was greater than the response to the antigen solubilized from the same cells which were not virus-infected.

In terms of the antibody response, while there was antibody to mouse antigens in both non-immune and immune subjects, in the immunized individuals there was also antibody to the virus capsule antigens themselves which could not be absorbed out by mouse tissue *in vivo* or *in vitro*. This was demonstrated by absorption studies. That antibody from immune subjects as well as non-immune subjects had antibody which reacted with virus but which could be partially or completely

absorbed out by normal mouse tissues, suggesting that mouse antigen is an integral part of the virus envelope structure. This is not surprising since the virus buds from the cell membrane. The nature of this antigen would be extremely interesting and it would be important to know whether it is normal cell surface antigen, such as H-2 antigen, or whether it is virus induced non-virion antigen or a cell associated antigen such as embryonic antigen.

These studies indicate that further work along these lines is warranted, first to clearly define the spectrum of reactivity of immunized subjects, second to determine the best preparation, dose and schedule of vaccine administration, third to prove specificity of anti-viral immunity induced by these immunization procedures, and finally to determine the relationship between the various viral and non-viral antigens to which the immune response is induced. Studies along these lines may well lead to knowledge on which the development of anti-viral vaccines for use in human cancer immunotherapy could be developed.

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