IMMUNOLOGIC ASPECTS OF LEUKEMIA VIRUS RESEARCH IN HUMANS[1]

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ABSTRACT

Recent theory concerning the mechanisms by which RNA-tumor viruses are transmitted in animals and their genetic information expressed during embryogenesis and adulthood is reviewed. An implication of such theory has been that adult animals are immunotolerant to the group-specific (gs) RNA-tumor-virus antigens, though there is evidence that refutes this implication.

On the basis that most mammals are not immunotolerant to the interspecies gs RNA-tumor-virus antigen, human sera from patients with various malignant diseases, from normal family members of these patients, and from other normal individuals were examined for antibodies to interspecies gs antigen. Using complement-fixation inhibition tests, a significant proportion of patients with rhabdomyosarcoma, osteogenic sarcoma, acute lymphocytic leukemia, or breast cancer gave positive reactions. A second test, inhibition of the paired radioiodine-labeled antibody technique, confirmed the results with sera from patients with rhabdomyosarcoma. The results are interpreted as serologic evidence of a human RNA-tumor virus. Other types of evidence for human RNA-tumor viruses also exist.

INTRODUCTION

The resurgence of interest in the role of immunologic factors in maligant disease has been accompanied by marked interest in antigenicity of malignant cells and, where appropriate, accompanying detailed antigenic analyses of the viruses responsible for their induction (Geering, *et al.*, 1968; Nowinski, *et al.*, 1972; Schafer, *et al.*, 1971). These studies have identified new (neo) antigens in or on tumor cells (Klein, 1966) and in some cases have differentiated between translation from viral genome or from host-cell genome derepressed by virus infection (Baluda, 1972; Gilden and Oroszlan, 1972; Temin, 1972a; Todaro and Huebner, 1972).

In the case of neoplasms induced by RNA tumor viruses, there is substantial evidence that a negative strand DNA copy of the RNA-virus genome is made in the infected cell (Baluda, 1972; Temin, 1972a). This copy serves as template for synthesis of positive RNA and DNA strands. The DNA copies appear to be integrated and function as host cell genes. In certain cases it has been demonstrated that the entire viral genetic code is integrated, but only portions of it are expressed (Gilden and Oroszlan, 1972). These expressions are identified by the presence of viral or viral-induced neoantigens in the cell and are often accompanied by morphologic changes in the cell (transformation).

Apparent partial expression, or conceivably total expression of incomplete viral genome without transformation—e.g. in chicken (Allen and Sarma, 1972) and in mouse and cat embryos (Huebner, *et al.*, 1971)—has contributed to the concept that RNA-tumor viruses are transmitted vertically in a genetic manner (Huebner and Todaro, 1969; Todaro and Huebner, 1972). The genes so transmitted have been called virogenes. The hypothesis also states that the genetic information responsible for oncogenesis, called the oncogene, is transmitted similarly in close association with the virogenes. (For an alternative and equally plausible theory see Temin, 1972a, b). The full expression of all virogenes and oncogenes results in a virus-producing malignant cell. In the adult mouse, the time (age) and degree of expression of each gene appears to be dependent on host genetic controls and the influence of other intrinsic and extrinsic factors (Huebner and Todaro, 1969).

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Although the oncogene-virogene hypothesis is feasible, and is supported by much evidence from studies in chickens and mice, its acceptance as the only mode of transmission of RNA tumor viruses is unwarranted. There has been no evidence presented that the viruses are present in germ cells following meiosis (Todaro and Huebner, 1972). Horizontal transmission of feline RNA tumor viruses, with both experimental and naturally occurring virus strains, has been documented (Essex, *et al.*, 1971a, b; Gardner, *et al.*, 1970; Hardy and Hirshaut, 1971; Rickard, *et al.*, 1969).

Spontaneous neoplasms have not been reported in germfree cats. Furthermore, in a colony of several hundred cats housed for eight years in a closed environment wherein natural feline pathogens have been excluded, we have yet to observe a single neoplasm. This may be highly significant since leukemia and lymphosarcoma are major diseases in cats (Hardy and Hirshaut, 1971; Schneider, 1971). A peak incidence of lymphoma occurs in conventional cats between the first and second year of life, and the potential lifetime incidence of neoplasms in cats has been estimated from registry data to be five to ten percent (Schneider, 1971). In germfree rats, spontaneous tumors arise but are rarely mailigant, and no RNA-tumor virus has been associated with them (Pollard, 1971).

Another argument originally submitted in support of the virogene-oncogene hypothesis is that chickens, mice, and cats appeared to be immunologically unresponsive to viral antigens expressed during fetal life (Huebner, *et al.*, 1971), particularly those viral internal antigens known as group-specific (gs) antigens. However, evidence has been reported from four different laboratories (Armstrong, 1969; Rabotti and Blackham, 1970; Roth, *et al.*, 1971; Weber and Yohn, 1972) that chickens are not immunotolerant to avian gs antigens, and two laboratories, including ours, have described antibodies in cats to feline gs antigens (Noronha, *et al.*, 1972; Yohn and Olsen, 1972).

At this point it would seem appropriate to define briefly the antigens of RNA tumor viruses. Simply stated, these viruses consist of an outer envelope derived from modified host cell-membranes, usually the cytoplasmic membrane, and an inner nucleoid of proteins and RNA. The outer membrane contains at least two specific viral glycoproteins of molecular weights of approximately 100,000 and 70,000 (Nowinski, *et al.*, 1972; Oroszlan, *et al.*, 1971). These glycoproteins contain type-specific antigens, but also may contain species-specific and possibly interspecies antigenic determinates (Schafer, 1972). The core proteins, usually three or four in mammalian RNA tumor viruses, contain mainly species-group-specific (gs) antigens. One of these, gs-3 is shared by all C-type mammalian RNA tumor viruses (Geering, *et al.*, 1968) and is known as an "interspec" antigen (Schafer, *et al.*, 1971). This latter antigen is also present in two human candidate RNA viruses, ESP–1 (Priori, *et al.*, 1971) and RD–114 (McAllister, *et al.*, 1972). It should be mentioned that the major gs protein of RD–114, gs-1, is not antigenically related to murine and feline gs-1 antigens (McAllister, *et al.*, 1972; Nelson-Rees, *et al.*, 1972). It has been suggested that the reported relationship between ESP–1 virus and murine viruses (Gilden, *et al.*, 1971) may be associated with a viral protein other than gs-3 that may have interspecies antigenic determinants (Schafer, 1972).

If ESP–1 and/or RD–114 are truly human RNA tumor viruses, one question of considerable importance is whether specific antibodies to these viruses are present in human sera. To answer this question unambiguously would require highly specific antibodies to the human RNA tumor virus gs and membrane antigens. Lacking such reagents, a more preliminary question may be asked, namely, do human sera contain antibodies reactive with the mammalian RNA tumor virus "interspec" antigen. This question is answerable because mammals are not immunotolerant to the antigen (Yohn and Olsen, 1972) and produce antibodies that can be used as reference reagents (Geering, *et al.*, 1968).

To answer this important question we selected three serologic procedures, direct micro-CF tests and two tests which do not depend on direct binding of complement, namely, complement-fixation inhibition (CFI) and inhibition of the paired radioiodine-labeled antibody technique (PRILAT inhibition). The latter two procedures have been used to demonstrate the presence of gs antibodies in chickens (Weber and Yohn, 1972; Yohn, *et al.*, 1971) and in cats (Olsen and Yohn, 1972; Yohn and Olsen, 1972). Briefly, the CFI test (Rice, 1948) involves incubation of dilutions of a human serum with two units of ether-disrupted murine leukemia virus (MuLV) gs antigen, followed by the addition of two units of reference antibody and three units of guinea-pig complement (C'50). In the presence of binding of interspec antigen by the human serum, free complement is available to lyse sensitized indicator sheep red-blood-cells. In the absence of competitive binding, the reference antibody reacts with the test antigen and fixes all the complement. The reference antibody employed in this system was rabbit anti-FeLV (Richard strain) and is known to contain antibodies to "interspec" antigens. The antigen was ether-disrupted Rauscher MuLV.

The PRILAT inhibition test has been described in detail by us (Evans and Yohn, 1970). Briefly, fixed cells known to contain murine gs antigens were incubated with human sera. Appropriate control cells, known to be free of gs antigens, were similarly incubated. Control cells were human embryonic-kidney (HEK) while Rauscher-MuLV-infected HEK (HEK–HRV) cells served as test cells. After appropriate rinsing of the cells, the direct PRILAT test was performed. The antibody in the test was ^{125}I-labeled goat Igg to FeLV mixed with an equal concentration of ^{131}I-labeled normal goat Igg. The antiserum contained antibodies to "interspec" antigen and had been absorbed with fetal calf serum proteins and feline tissue antigens. Inhibition of the PRILAT was accepted as significant when the proportional uptake of the ^{125}I-labeled antibody on antigen-containing cells was two standard errors less than the uptake on cells which had not been incubated with the human serum.

The PRILAT inhibition and CF inhibition tests with murine RNA-tumor-virus antigen and FeLV reference antibody were designed to test for antibodies in human sera to "interspec" antigen, since, as far as could be determined, this antigen-antibody reaction was the only one occurring between these reagents.

Tests of approximately 250 human sera, representing point bleeds from patients with various malignancies or leukemia and from normal individuals and family members of patients, were performed. Direct CF tests (using guinea-pig complement) between the human sera and the MuLV gs antigens were performed in conjunction with the CFI tests as controls for the latter test. In general, any sera reactive in direct CF did not react in CFI; only 6 exceptions were noted. The direct CF results (Table 1) indicated a significant ($P<.05$) number of positive reactions among patients with acute lymphocytic leukemia, breast carcinoma, and metastatic breast carcinoma, and also among normal family members of cancer patients when compared with the results of tests made on other normal individuals. However, because of the several different antigens in the virus material, the reactions can not be interpreted as indicative of antibody to the same antigen in all sera.

The CFI results (Table 2), however can be interpreted as indicative of antibody to "interspec" antigen. In these tests, 40% or more of sera from the majority of disease categories examined were reactive. The only disease categories with a non-significant number of reactive sera were Hodgkin's disease, 8/32, and acute leukemia (non-lymphocytic), 1/11. Among normal individuals (non-family), 9/70 sera (13%) were reactive, while 12/63 (19%) sera from normal family members of cancer patients were reactive.

One hundred and five sera were tested by PRILAT inhibition (Table 3); of these, 83 were also tested by CF and CFI (Table 4). The correspondence was

TABLE 1

Direct CF tests between human sera and Rauscher MuLV (HEK–HRV) gs antigens using guinea pig complement

Disease Category	CF Titers			Positive/total	P by (x^2)
None (Normal)	62	10	1	11/73	——
None (Family members)	37	22	1	23/60	<.05
Sarcoma					
Rhabdomyo	8	2	0	2/10	ns
Osteogenic	8	0	0	0/8	ns
Reticulum cell	10	1	0	1/11	ns
Hodgkin's	25	6	1	7/32	ns
Lympho	16	2	1	3/19	ns
Lipo	5	1	0	1/6	ns
Leukemia					
Acute lymphocytic	11	6	0	6/17	<.05
Acute (other)	8	2	0	2/10	ns
Chronic lymphocytic	3	0	0	0/3	ns
Breast					
Carcinoma	7	6	0	6/13	<.02
Adenocarcinoma	4	0	0	0/4	ns
Metastatic	2	3	1	4/6	<.02

essentially 70%, that is, 70% of the sera positive by PRILAT inhibition were also positive by CF or CFI, and 68% of the sera negative by CF and CFI were negative by PRILAT inhibition. The criterion that the specific uptake quotient (S.U.Q.) in the PRILAT inhibition test must be reduced by 2 standard errors may have resulted in the designation of many sera as falsely negative. By reducing the inhibition level to 1 standard error, essentially 60% of the PRILAT inhibition

TABLE 2

Complement-fixation inhibition tests for antibody in human sera to "interspec" antigen using rabbit anti-FeLV and Rauscher MuLV (HEK–HRV) gs antigens as reference reagents

Disease Category	CFI Titers			Positive/ Total	P by (x^2)
	<1:2	1:2 to 1:8	≧1:16		
None (Normal)	61	7	2	9/70	——
None (family members)	51	5	7	12/63	ns
Sarcoma					
Rhabdomyo	5	1	4	5/10	<.05
Osteogenic	0	1	6	7/7	<.01
Reticulum cell	3	1	6	7/10	<.05
Hodgkin's	24	2	6	8/32	ns
Lympho	5	3	7	10/15	<.05
Lipo	1	0	5	5/6	<.01
Leukemia					
Acute Lymphocytic	11	3	4	7/18	<.05
Acute (other)	10	1	0	1/11	ns
Chronic Lymphocytic	1	0	2	2/3	<.10
Breast					
Carcinoma	9	3	4	7/16	<.05
Adenocarcinoma	0	0	4	4/4	<.05
Metastatic	3	1	3	4/7	<.05

TABLE 3

PRILAT-inhibition tests for antibody in human sera to "interspec" antigen using goat anti-FeLV and Rauscher MuLV infected HEK cells (HEK–HRV)

Disease Category	PRILAT-Inhibition Number Positive/ Number Tested	P by (x^2)
None (Family Members)	2/15	——
Sarcoma		
Rhabdomyo	3/3	<.05
Osteogenic	2/3	<.10
Reticulum cell	1/4	ns
Hodgkin's	4/13	<.10
Lympho	1/7	ns
Leiomyo	1/2	ns
Leukemia		
Acute Lymphocytic	1/5	ns
Acute (other)	1/7	ns
Chronic Lymphocytic	0/2	ns
Breast		
Carcinoma	1/11	ns
Adenocarcinoma	0/1	ns
Metastatic	3/7	<.05
All other tumors	6/25	ns

ns = not significant.

negative sera would have been called positive. Future studies will include analyses of the validity of employing reduction of the S.U.Q. by 1 standard error rather than by 2. The truly discordant sera included 10/83 (12%), which were PRILAT positive, but negative by CF and CFI; these differences are not understood.

In spite of the probable high level of false negatives in the PRILAT inhibition tests, four disease categories were identified that contained a noteworthy proportion of reactive sera. This included patients with rhabdomyosarcoma, (3/3), osteogenic sarcoma, (2/3), Hodgkin's disease, (4/13), and metastic breast carcinoma (3/7). These four diseases and acute lymphocytic leukemia (Table 5) are considered worthy of further study for antibodies to interspec antigen.

TABLE 4

Correlations between PRILAT inhibition, CF and CFI tests

	Ratio	Percent
Negative by all 3 tests	21/83	25
Positive by PRILAT and by CF or CFI	24/83	29
Negative by PRILAT and positive by CF or CFI	28/83	34
Positive by PRILAT and negative by CF and CFI	10/83	12
Percent of PRILAT positive sera that were CF or CFI positive	——	70
Percent of CF and CFI negative sera that wer PRILAT negative	——	68

TABLE 5

Composite results of CF, CFI and PRILAT tests in selected diseases for antibodies to "interspec" antigen

Disease Category	Number Positive/Number Tested		
	CF	CFI	PRILAT
None (Family Members)	23/60	12/63	2/15
Rhabdomyosarcoma	2/10	5/10*	3/3*
Osteogenic Sarcoma	0/8	7/7*	2/3
Hodgkin's Disease	7/32	8/32	4/13
Acute Lymphocytic Leukemia	6/17*	7/18*	1/5
Breast Cancer	10/23*	15/27*	4/19

*P = <.05 by X^2.

The evidence presented is indicative that many patients with the diseases listed in Table 5 have responded immunologically to the mammalian interspec RNA-tumor virus antigen. Does this mean that their disease has been induced by an RNA-tumor virus? At this point one can not answer the question. However, if one evaluates the data in Table 5 in the light of other already-existing evidence that human RNA-tumor viruses exist, one can not overlook the possibility that RNA-tumor viruses are involved in the etiology of human cancer and leukemia.

The evidence to date includes:

1) observations, by electron microscopy, of RNA-tumor-virus particles in human clinical material (Dmochowski, 1971; Hall, *et al.*, 1970; Seman, *et al.*, 1969; Seman, *et al.*, 1971; Shigematsu, *et al.*, 1971; Schlom, *et al.*, 1971);
2) isolation of infectious RNA-tumor viruses from cell cultures of human origin (Priori, *et al.*, 1971; McAllister, *et al.*, 1972; Stewart, *et al.*, 1972);
3) presence of RNA in human tumors which hybridizes with appropriate copies of nucleic acid from RNA-tumor virus (Kufe, *et al.*, 1972; Schlom, *et al.*, 1972);
4) presence of viral-like RNA-dependent DNA polymerase in human clinical material (Gallo, *et al.*, 1970; Gallo, *et al.*, 1971, and Gallo, *et al.*, 1972; Kiessling, *et al.*, 1971; Schlom, *et al.*, 1971);
5) antigens in human cancer cells which appear to be disease specific (Eilber and Morton, 1970; Giraldo, *et al.*, 1971; morton and Malgren, 1968);
6) antibodies in human sera which appear to be disease specific (Eilber and Morton, 1970; Morton and Malmgren, 1968); and finally,
7) the evidence given herein that in human sera antibodies are present that react with mammalian RNA-tumor virus interspec antigen.

Perhaps the most hopeful conclusion one can make from the present studies is that humans appear to be immunoresponsive to RNA-tumor-virus antigens. This may mean that early immunodiagnostic tests and control by immunologic procedures are feasible.

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