

EVIDENCE FOR HUMAN T CELL LYMPHOMA-LEUKEMIA
VIRUS INFECTION OF FAMILY MEMBERS OF HUMAN T CELL
LYMPHOMA-LEUKEMIA VIRUS POSITIVE T CELL
LEUKEMIA-LYMPHOMA PATIENTS

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The human T cell lymphoma-leukemia virus (HTLV)¹ is a novel, type-C retrovirus that was isolated from cells of some patients with lymphomas and leukemias of mature T cells (1, 2). To show an association of HTLV with particular human malignancies and elucidate a possible etiologic role of the virus, we have initiated sero-epidemiologic surveys of both patients and normal donors. Three main findings have emerged from these studies so far.² First, studies of Japanese with adult T cell leukemia (ATL) have shown a strong link with HTLV. The documented geographic clustering of this disease in southwestern Japan initially indicated an infectious vector, possibly viral, in the etiology of the disease (3, 4). Our studies have shown that 90% of ATL cases possess serum antibody to HTLV (5, 6). In addition, at least 10% of the normal population in the region where ATL is endemic possess HTLV-specific antibodies,² which suggests a widespread prevalence of the virus. Japanese investigators have also suggested by serological studies (7) that a virus was associated with ATL. Recently Yoshida et al. (8) isolated a virus from cells of an ATL patient. We have shown that HTLV is, in fact, the virus present in Japanese ATL cells by immunologic studies with purified HTLV p24 and with a monoclonal antibody to HTLV p19, and by nucleic acid hybridization studies (9). Second, we have recently shown that the Caribbean basin is another area where HTLV-associated disease apparently is en-

¹ Abbreviations used in this paper: ATL, adult T cell leukemia; CTCL, cutaneous T cell leukemias and lymphomas; HTLV, human T cell leukemia-lymphoma virus; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; T-LCL, T cell lymphosarcoma cell leukemia.

² Gallo, R. C., et al. The human type-C retrovirus: association with a subset of adult T cell malignancies. Manuscript submitted for publication.

demic. Antibodies to HTLV have been found in eight out of eight cases of West Indian patients (10) with an aggressive disease similar to ATL, called T cell lymphosarcoma cell leukemia (T-LCL) (11), and also in a small percentage of sera from normal healthy donors from the Caribbean (10).² Third, although HTLV was isolated from patients with cutaneous T cell leukemias and lymphomas (CTCL), a disease somewhat different from ATL and T-LCL, and although natural antibodies have been detected in sera from some CTCL patients (12, 13), most CTCL patients surveyed have lacked HTLV-specific antibodies.² We have postulated that a few of the HTLV-associated CTCL patients actually had a disease more akin to ATL, which is rarely diagnosed in the United States.² At least one of the antibody-positive CTCL patients, however, had a more typical case of CTCL. Therefore, we have not ruled out completely an association of HTLV with CTCL. Antibody-negative patients may still possess HTLV information as integrated proviral sequences. An HTLV association of this nature is currently being investigated by molecular hybridization studies. In addition, a few of the U.S. patients with an HTLV association have been diagnosed as having peripheral T cell lymphoma or malignant lymphoma of a diffuse, mixed cell type. A clear HTLV association may eventually aid in subclassification of the various malignancies of mature T cells.

The presence of antibody to HTLV proteins in sera of patients (or normal donors) indicates prior infection by the virus. The fact that the antibodies have shown specificity for internal structural proteins suggests the presence of replicating virus within the host cells. This evidence, together with previous results that showed the lack of HTLV sequences in DNA of numerous samples from normal uninfected people (14) and the presence of HTLV provirus in T cells but not B cells of the patient from whose cells HTLV was isolated (15), demonstrate that HTLV is not endogenous but rather is acquired by infection. In this paper we report on serologic studies of family members of patients with HTLV-associated disease. The results show that the family members are more likely to possess HTLV-specific antibody than the normal population, supporting the infectious nature of HTLV. The infectivity of HTLV is further illustrated by studies of one family in which four of five members possess either serum antibodies specific for HTLV or whose cells express HTLV proteins or release intact viral particles. A similar finding of type C viral particles in cultured T cells of relatives of a Japanese ATL patient was recently reported by Miyoshi et al. (16).

Materials and Methods

Sera. Samples of human sera or plasma were generally received as frozen or lyophilized aliquots. The latter were reconstituted to the original volume before assay. Occasionally, fresh samples were obtained for testing. No differences in antibody titers were noted in positive sera tested before or after freezing.

Serologic Assays. All sera were assayed by at least one of the techniques described below. Sera of family members were assayed by all three methods.

A solid-phase radioimmunoassay (RIA) using disrupted HTLV on the solid phase has been described (5). In this assay, antibody to any antigen in the HTLV preparation can be detected. Antibody specificity for HTLV was determined by competition assays. Sera were considered positive only if their reactivity was competed by extracts of HTLV-producing cells but not by extracts of nonproducing cells or fetal calf serum.

A competitive binding assay with a monoclonal antibody to HTLV p19 was used in conjunction with the solid-phase RIA for determining antibody to HTLV p19 (17). Briefly,

serial dilutions of human sera were mixed with a limiting dilution of monoclonal anti-p19 and incubated for 45 min at 37°C on a microtiter plate coated with disrupted HTLV. Those sera that possessed antibody to the same region of p19 as the monoclonal antibody competed for binding sites. After washing as in the solid-phase RIA, binding of the monoclonal anti-p19 remaining was detected using iodinated goat anti-mouse IgG. After 45 min incubation at 37°C and washing, microtiter wells were separated and counted. Competition was calculated based on binding of monoclonal antibody in the presence of a pooled normal human serum as 100% of maximum (0% competition).

A radioimmunoprecipitation (RIP) assay for purified HTLV p24 has been described previously (6). The specificity of this assay for the major core protein of HTLV and the lack of immunologic cross reaction with core proteins of other retroviruses or with cellular proteins has been documented (18).

Cell Culture. T cells of an ATL patient and his family members were cultured from viably frozen aliquots of peripheral blood lymphocytes. The use of partially purified T cell growth factor in culturing T lymphocytes has been described (19).

Detection of HTLV Antigens. Expression of HTLV p19 in cultured T cells was determined by indirect immune fluorescent assay on methanol:acetone-fixed cells using a monoclonal antibody to HTLV p19 as described (20). Expression of HTLV p24 was determined by competition RIP assay of the purified protein. The preparation of the cell lysates and their use as competitors has been described (18). The amount of p24 present in cell lysates was determined by comparison with a standard curve using purified HTLV p24 as competitor.

Results and Discussion

Relatives of T Cell Leukemia-Lymphoma Patients Possess HTLV-specific Antibodies. Sera of family members of patients known to be associated with HTLV by virtue of possession of HTLV-specific serum antibodies and/or expression of HTLV proteins or production of intact viral particles by their cultured T cells were assayed for HTLV-specific antibodies as described in Materials and Methods. Table I lists 19 patients known to be associated with HTLV and their family members who possess HTLV-specific antibodies. The families are grouped to demarcate those from the United States, the West Indies, and Japan. Although HTLV-associated disease is not known to be endemic in the United States, the involvement of HTLV in the U.S. patients listed is conclusive. HTLV was first and repeatedly isolated from cells of patient C.R. (1, 15) and a new isolate has recently been obtained from cultured T cells of patient M.J.³ Both patients were also the subjects of the initial reports on the presence of natural antibodies to HTLV in human sera (12, 13). The presence of antibody to p24 in the wife of patient C.R. was previously reported (13) and continues to be significant in supporting the infectivity of HTLV and also in suggesting a horizontal rather than a vertical mode of virus transmission. In addition to possessing HTLV-specific antibodies, patient W.A. is also the source of one of the new HTLV isolates.³

Sera were available from family members of four patients from the West Indies with aggressive HTLV-associated T cell malignancies (Table I). Cells of patient M.B. were the source of the second isolate of HTLV (2). The three other patients had T cell lymphosarcoma cell leukemia, an aggressive T cell malignancy similar to Japanese ATL (11) and recently shown to be HTLV associated (10). Although only a few cases have been identified to date, all T-LCL cases from the West Indies studied so far have possessed antibodies to HTLV (10), and of the four families studied here, three had members possessing serum antibodies to HTLV.

³ Popovic, M., P. S. Sarin, M. Robert-Guroff, V. S. Kalyanaraman, D. Mann, J. Minowada, and R. C. Gallo. Isolation and transmission of human retrovirus (HTLV). Manuscript submitted for publication.

TABLE I
Presence of Natural Antibodies to HTLV in Family Members of Patients with HTLV-associated Leukemias and Lymphomas

Patient	Diagnosis*	Patient origin	Relative sera	Relationship of positive relative to patient	Antibody in relative serum to‡		
					Com- ponents of dis- rupted HTLV	HTLV p24	HTLV p19
					<i>titer</i>	<i>titer</i>	<i>titer</i>
C.R.	CTCL, M.F.	Alabama	1/4	Wife	200	200	—
M.J.	CTCL, Sezary	Massachusetts	0/4	—	—	—	—
W.A.	T cell lym- phoma	Georgia	1/4	Mother	95	3,160	20
M.B.	CTCL, Sezary	West Indies	1/9	Daughter	500	2,500	15
M.I.	T-LCL	West Indies	1/1	Husband	100	2,070	8
S.W.	T-LCL	Guyana	0/2	—	—	—	—
J.T.	T-LCL	West Indies	1/4	Mother	150	1,520	3
K.H.	ATL	Japan	1/4	Son	250	1,000	3
Y.Y.	ATL	Japan	0/2	—	—	—	—
K.N.	ATL	Japan	4/8	Wife	175	—	1
				Wife's mother	250	1,000	3
				Son	30	200	—
				Relative in en- demic area	200	1,500	2
Y.S.	ATL	Japan	1/1	Brother	150	6,400	24
S.K.	ATL	Japan	2/4	Mother	640	600	20
				Father	35	—	—
H.N.	ATL	Japan	4/4	Wife	100	1,710	28
				Daughter	20	35	—
				Daughter	90	45	—
				Son	55	20	—
H.S.	ATL	Japan	0/1	—	—	—	—
H.T.	ATL	Japan	2/3	Brother	35	540	8
				Aunt	30	90	—
S.H.	ATL	Japan	3/8	Daughter	40	630	1
				Daughter	65	540	—
				Daughter	30	—	—
K116	ATL	Japan	1/2	Daughter	40	1,710	5
K119	ATL	Japan	1/1	Wife	60	3,980	14
T.M.	ATL	Japan	0/2	—	—	—	—

* Diagnoses include: CTCL, M.F. (mycosis fungoides), T-LCL, and ATL.

‡ Serum antibodies were assayed as described in Materials and Methods. Titers are expressed as the reciprocal of the serum dilution giving 50% of maximal binding to components of disrupted HTLV in a 50- μ l assay; 20% precipitation of HTLV p24 in a 1-ml final volume; and 50% competition of monoclonal anti-p19 in a 50- μ l final volume.

The largest number of family member sera were obtained from Japan, where ATL is endemic and also HTLV associated. Of the 12 Japanese families investigated, 9 had antibody-positive family members. The specificity of the antibodies detected in

positive family member sera has been to HTLV internal components, which suggests not only mere exposure of the individuals to the virus but also actual replication of virus within these healthy donors. In only two cases was antibody to both HTLV p24 and p19 lacking in a family member: the father of patient S.K., and the daughter of patient S.H. (Table I). The antigenic component recognized in these cases has not been identified, but may be a viral envelope component.

HTLV Antibodies Are More Frequent in Healthy Relatives than in Random Healthy Donors. Among healthy donors studied, natural antibodies to HTLV occur with greater frequency in relatives of patients with HTLV-associated malignancies than in randomly selected normal donors (Table II). With regard to the United States, an endemic area of HTLV-associated disease has not been identified, although several HTLV-associated cases have originated from the southeastern United States. Sporadic cases of U.S. patients with adult T cell malignancies who possess HTLV-specific antibodies have been clearly demonstrated, but so far are rare.² Therefore, the two antibody-positive relatives of two U.S. patients (Table I) are all the more striking. The percentage of antibody-positive normal U.S. donors among relatives is much greater than the percentage among unrelated healthy donors (Table II), which strengthens the case for infection of individuals having close contact with infected patients or a very similar environment. Results from the two groups of healthy U.S. donors surveyed so far suggest that, as in Japan (see Fig. 1), the prevalence of HTLV will vary across the country. The apparent difference seen between the Washington, DC area and Georgia may well be explained by the proximity of Georgia to the Caribbean endemic area.

Similar observations were made with both the Caribbean and Japanese endemic populations, where the presence of antibodies to HTLV among patients is very high. Again, relatives of HTLV-associated patients from both groups possess HTLV antibodies with greater frequency than random normal donors. In the case of the

TABLE II
Prevalence of Natural Antibodies to HTLV in Sera of Patients with Malignancies of Mature T Cells, their Healthy Relatives, and Random Normal Donors

Serum donors	Antibodies to HTLV*	
	Positive/tested	Percent positive
Healthy relatives of U.S. patients with HTLV-associated malignancy	2/12	17
Unrelated healthy donors, Washington, DC	1/185	<1
Unrelated healthy donors, Georgia	3/158	2
Caribbean T-LCL patients	8/8	100
Healthy relatives of Caribbean patients	3/16	19
Random healthy donors, Caribbean	12/337	4
Japanese ATL patients	40/46	87
Healthy relatives of ATL patients	19/40	48
Random healthy donors, nonendemic area	9/600	2
Random healthy donors, endemic area	50/419	12

* Antibodies were detected by RIP of HTLV p24 or by the solid-phase RIA.

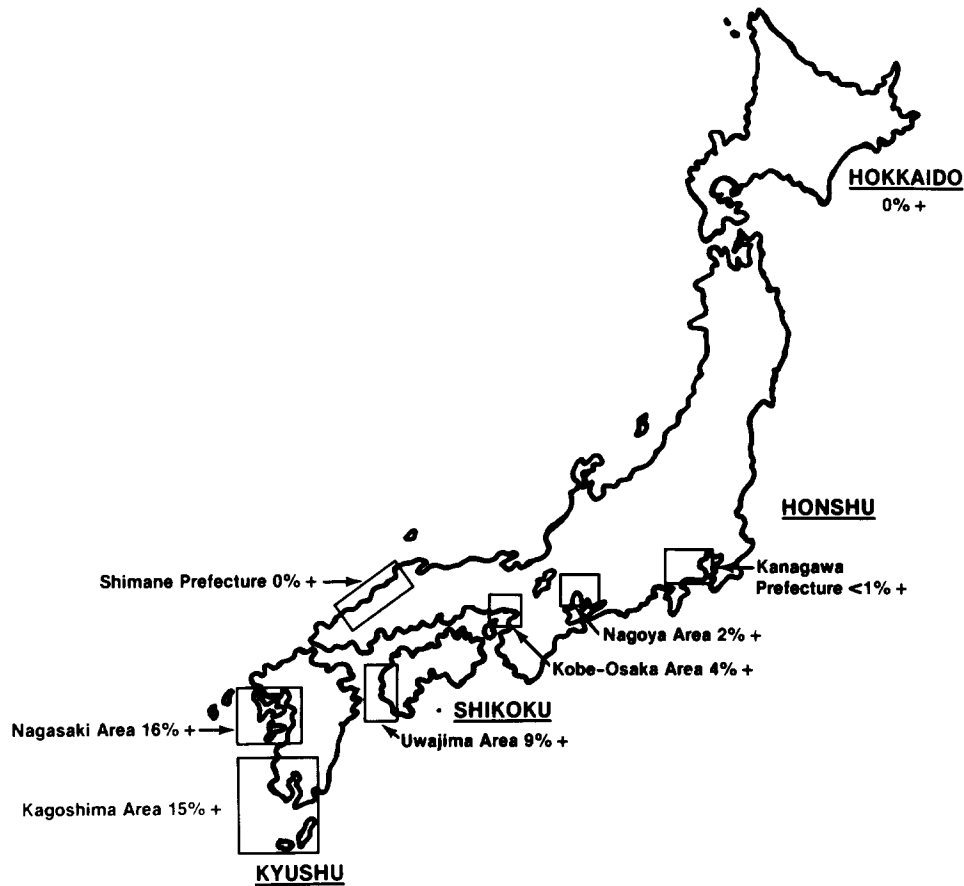


FIG. 1. Distribution of HTLV-antibody positive healthy Japanese donors from ATL endemic and non-endemic areas. The percentage of positive donors for several areas is indicated.

Japanese, random normal donors have been categorized as from either the known endemic ATL areas of Kyushu and Shikoku islands (3, 4) or from other areas of Japan not known to be endemic. The distribution of the healthy normal Japanese we have surveyed is illustrated in Fig. 1. Not all the individuals studied have been mapped according to their birthplace. However, certain areas of Kyushu and Shikoku clearly have an increased incidence of HTLV exposure. Even taking this geographic distribution into account, relatives of ATL patients still have a greater frequency of antibodies to HTLV (42%), which again suggests greater exposure to the virus.

HTLV Infection of Relatives of One ATL Patient. The availability of peripheral blood cells from one Japanese ATL patient and his family members has allowed an in-depth study of HTLV infection of this family. A detailed report on this family, including preliminary results on the HTLV association, has been submitted.⁴ In addition to serological studies showing antibodies to HTLV in the sera of the mother and father

⁴ Aoki, T., A. Shibata, Y. Ohnishi, Y. Aoyagi, H. Miyakoshi, I. Emura, V. S. Kalyanaraman, M. Robert-Guroff, M. G. Sarngadharan, M. Popovic, P. S. Sarin, P. C. Nowell, and R. C. Gallo. High incidence of the human type-C retrovirus HTLV in members of a family of an HTLV-positive Japanese T-cell leukemia patient and suggestions of a preleukemic state in one member. Manuscript submitted for publication.

of the patient (S.K., Table I), T cells of each family member were cultured using TCGF and subsequently examined for expression of HTLV proteins. HTLV p19 was detected in cells of four of the family members, including the patient (Table III), by an indirect immune fluorescent assay using a monoclonal antibody to HTLV p19.⁴ Representative fluorescent staining of p19-positive T cells is illustrated in Fig. 2 with cells of the mother and father of patient S.K.

The cultured T cells of this family were also examined for expression of HTLV p24 by means of a competition RIP assay for the major core protein using cell extracts as competitors. Again, the same four individuals, the ATL patient and his mother, father, and brother, expressed clearly detectable levels of HTLV p24 (Fig. 3, Table III). It is of interest that although three healthy family members express HTLV

TABLE III
Expression of HTLV Proteins in Cultured Cells of a Japanese ATL Patient and His Family Members

Cell donor	Cell passage	Expression of HTLV proteins	
		p19	p24
		<i>Percent positive cells</i>	<i>ng p24/mg cell protein</i>
ATL patient (S.K.)	2	39	174
	7	61	ND*
Healthy mother (T.K.)	2	54	800
Healthy father (H.K.)	3	38	400
Healthy brother (B.K.)	2	2	20
	3	13	ND
Healthy sister (M.K.)	2	0	ND
	3	0	<1

Expression of HTLV proteins was determined as described in Materials and Methods.

* Not done.

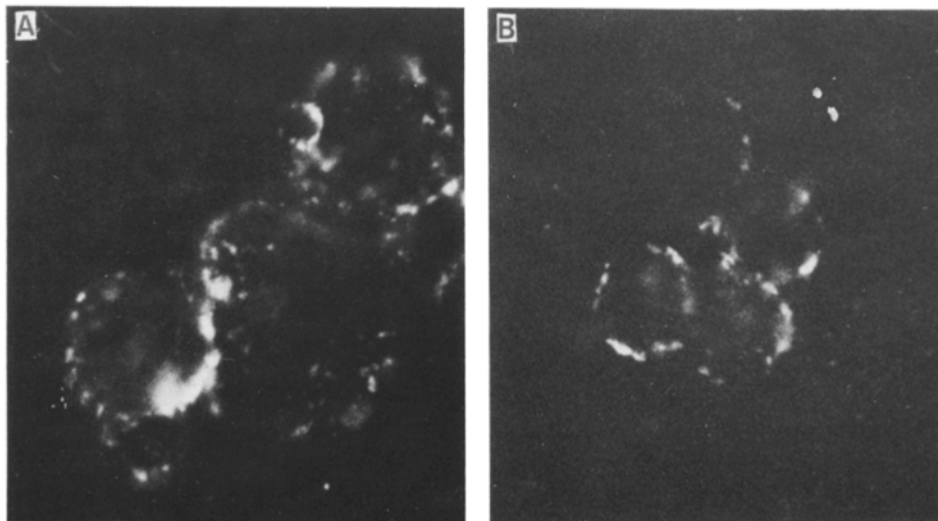


FIG. 2. Fluorescent staining of cultured T cells for HTLV p19: The indirect immune fluorescent assay using a monoclonal antibody to p19 was described in Materials and Methods. Representative staining of p19-positive cells include those from the mother of Japanese ATL patient S.K. (A), and those of his father (B). Original magnification $\times 540$.

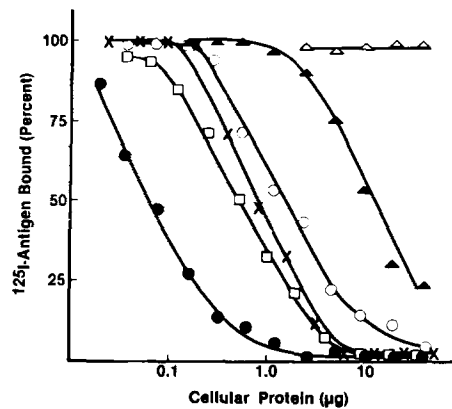


FIG. 3. Competition RIP assay of HTLV p24 by lysates of cultured T cells of an ATL patient and his relatives. The ability of a limiting dilution of goat antiserum to purified p24 to precipitate iodinated HTLV p24 was competed by the indicated amounts of cellular protein. Competition curves are indicated for HTLV producer cells (x) and for cultured T cells of ATL patient S.K. (o), and the patient's mother (●), father (□), brother (▲), and sister (△).

proteins, the healthy brother expressed low amounts of both p19 and p24 and in fact also lacked detectable serum antibody to HTLV antigens. It will be of interest to follow this individual with time, as the data suggest he is possibly newly infected. The healthy mother of patient S.K. is also of particular interest. Other data have suggested that she is in a preleukemic state. In particular, 7% of her peripheral blood cells resemble neoplastic T cells histologically.⁴ A determination of whether her fresh uncultured malignant cells also express HTLV proteins is of obvious importance. Her cultured T cells do in fact release HTLV, as shown by electron microscopy and biochemical studies (data not shown).

The pedigrees of all but two of the families illustrate the relationships of the family members surveyed and effectively summarize both the serologic and cultured cell data (Fig. 4). In only a few cases have cells been available for culturing, even among the patients represented, so the lack of data on expression of HTLV in cultured cells does not mean that HTLV proteins are not expressed in particular patients or family members, or that HTLV particles are not released. Cells have been cultured from U.S. patients C.R. and M.J., West Indian patients M.I. and M.B., and the Japanese ATL patient S.K. In each case, after culture with TCGF we have been able to obtain cells either expressing HTLV proteins or releasing intact viral particles (1, 2).^{2,4} One might expect, therefore, that if cells were cultured from other antibody-positive patients, HTLV expression would also be detected.

With regard to the serum antibody-positive healthy relatives, we have had the opportunity to culture cells of six individuals: the wife of C.R., the daughter of M.B., and the four family members of patient S.K. The cultured T cells of the first two individuals have been negative for expression of HTLV proteins, whereas three of the latter were positive for HTLV antigen expression. As we mentioned above, the healthy brother of S.K. lacked serum antibody to HTLV, but expression of HTLV proteins was observed in his cultured T cells. The factors controlling expression or lack of expression of HTLV in these healthy individuals exposed to HTLV are under investigation. Items of major importance include length of time post-HTLV infection,

PEDIGREES OF PATIENT FAMILIES INFECTED WITH HTLV

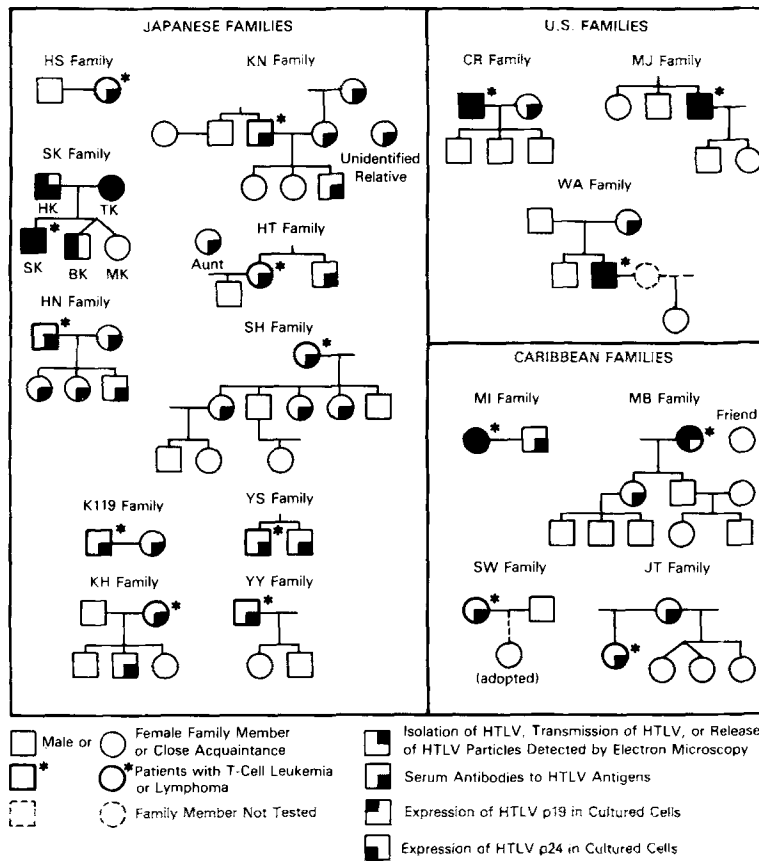


FIG. 4. Pedigrees of families infected with HTLV. Both the presence of natural antibodies to HTLV and the expression of HTLV proteins in cultured cells of patients and family members are summarized graphically. Cells for culture were available in only a few cases (see text). The lack of information on HTLV antigen expression or particle release therefore does not necessarily indicate the absence of HTLV information in particular family members.

age at time of infection, and immunological competence of infected individuals. This healthy brother of S.K. further serves to emphasize the point that antibody-negative cases are not necessarily uninfected by HTLV.

With regard to only the serologic studies, the data summarized in Fig. 4 would not rule out any particular mode of HTLV transmission. Although strict vertical genetic transmission in the sense of endogenous retroviruses has been ruled out by nucleic acid hybridization studies (14, 15), one cannot rule out other possibilities, including congenital infections, milk-borne transmissions, and other types of horizontal transmission.

Of the 19 families studied here, 14 had at least 1 healthy member who possessed HTLV antibodies. Of the five families with no antibody-positive healthy members, four were represented by a very small number of donors who were perhaps not representative of the entire family. The fact that the overall prevalence of HTLV

infection of family members, reflected by their possession of HTLV antibodies, is greater than that of randomly selected normal donors (Table II) supports the infectious nature of HTLV and further suggests its involvement with the particular diseases represented here.

Summary

Sera of family members of patients from the United States, the Caribbean, and Japan, with human T cell lymphoma-leukemia virus (HTLV) associated T cell malignancies, possess HTLV-specific antibodies directed against internal structural components of HTLV, p24 and p19. The prevalence of antibodies to HTLV is greater in family members than in random healthy donors, which supports the infectious nature of HTLV and its association with particular aggressive T cell malignancies. Expression of HTLV p24 and p19 has also been observed in cultured T cells of some healthy relatives, and intact virus particles have been released from cells of one possibly pre-leukemic family member.

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