Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1653–1657, March 1982
Medical Sciences

Natural antibodies to the structural core protein (p24) of the human T-cell leukemia (lymphoma) retrovirus found in sera of leukemia patients in Japan

(RNA tumor virus/type C virus/radioimmunoassays/Japanese adult T-cell leukemia/T-cell malignant lymphoma)

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Communicated by Werner Henle, November 20, 1981

ABSTRACT **In Japan, adult T-cell leukemias and lymphomas are more common than in the United States and Europe, and in the southwest part of Japan these T-cell malignancy cases appear in clusters. Therefore, we investigated the involvement in these leukemias and lymphomas of the human T-cell leukemia virus (HTLV) that was previously isolated in one of our laboratories from cultured T cells of some patients in the United States with leukemias and lymphomas involving relatively mature T cells. High titers of antibodies capable of quantitative precipitation of ^{125}I-labeled p24, a well characterized core protein of HTLV, were detected in 12 of 12 patients with untreated adult T-cell leukemia (ATL). (One negative was a patient on chemotherapy.) Ten of the 12 positive samples were from an area where the disease is endemic. Strong precipitating antibodies were also detected in five of seven cases of T-cell malignant lymphoma (TML) which differs from ATL by having fewer leukemic cells in the peripheral blood. High antibody titers were also observed in one of five cases of acute monoblastic leukemia and one of eight cases of chronic myelogenous leukemia in the blast phase of the disease. Low to moderate titers of antibodies were detected in several categories of leukemia (two cases of blast-phase chronic myelogenous leukemia, two cases of acute lymphoblastic leukemia of the null-cell type, and one case of acute myelogenous leukemia). Among all categories of leukemias, except ATL and TML, more cases were negative than positive for anti-p24 activity. All of 79 sera from normal Japanese, including 39 collected from the endemic ATL area of southwest Japan, were negative for antibodies to HTLV p24. All the positive reactivities observed were highly specific to HTLV. The only competition observed in the precipitation of HTLV p24 was with HTLV or with cell lines expressing HTLV and not with various animal retroviruses or a large number of human and subhuman primate cell lines, not known to be producing HTLV. The data strongly indicate an association of HTLV with the increased incidence of ATL in parts of Japan, probably with other forms of leukemias in Japan, and, less commonly, with certain T-cell malignancies in the United States.**

Certain exogenous retroviruses (RNA tumor viruses) are now known to be involved in the natural cause of some leukemias of chickens, cats, cows, certain wild mice, and gibbon apes (see refs. 1–3 for reviews). Often the leukemias induced are of the T-cell type (3). These results stimulated a long series of studies by several groups designed to find related viruses in man and to determine whether an association with disease could be found. The bulk of these results were negative, suggesting that the majority of human leukemias do not contain detectable replicating type C virus of the same kind found in animal leukemias (see ref. 4 for review). However, after the finding of a method for long-term growth of mature human T lymphocytes in culture (5, 6) based on a protein termed T-cell growth factor (7), human neoplastic T cells could be routinely grown in suspension culture (8). Such experiments led to the isolation and characterization of a human type C retrovirus from cultures of cells from adults with certain leukemias or lymphomas involving relatively mature T lymphocytes (9, 10).

The human T-cell leukemia (lymphoma) retrovirus (HTLV) can be successfully propagated *in vitro*, and it has been extensively characterized (11–14). Two main features of HTLV have emerged from these analyses. (*i*) HTLV is distinct from previously identified animal retroviruses (11–14). (*ii*) HTLV is not transmitted in the germ line but must be acquired by postzygotic infection by some as yet unknown mode of transmission (11). These results stimulated a screening of human sera for the presence of antibodies against HTLV proteins and of populations for incidence of HTLV-associated leukemias. Among sera obtained from several hundred randomly selected normal donors in the United States and Europe, none contained detectable antibodies to HTLV. Similarly, sera from American cases of childhood leukemia [including childhood T-cell acute lymphocytic leukemia (ALL)], myeloid leukemias (adult or childhood), B-cell leukemias, or the typical relatively benign cutaneous T-cell lymphomas called mycosis fungoides, were negative. To date, all the American sera positive for anti-HTLV antibodies have come from patients with leukemia or lymphoma of mature T-lymphocytes (including C.R., the first patient from whose cells HTLV was isolated) (15, 16) and some of their close relatives.

In Japan the incidence of adult T-cell leukemias (ATL) is greater than in most countries studied, and a significant cluster of so-called ATL was reported around the Kyushu district (17–19). In addition, several features of Japanese ATL are similar to those in some of the American cases positive for HTLV (20). Therefore, for an understanding of the epidemiological significance of HTLV in T-cell malignancies it was important to screen sera of Japanese patients with T-cell leukemias for natural antibodies toward HTLV antigens.

In the present communication, we report that sera from a significant proportion of Japanese leukemia patients contain natural antibodies that precipitate the purified internal struc-

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Abbreviations: HTLV, human T-cell leukemia (lymphoma) retrovirus; ATL, adult T-cell leukemia; TML, T-cell malignant lymphoma; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; AMoL, acute monoblastic leukemia.

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tural protein (p24) of HTLV. These results are in concordance with data from solid-phase immunoassays and immunofluorescence studies (unpublished data). This included 12 of 13 patients with ATL and 5 of 7 with T-cell malignant lymphoma (TML) but also included other forms of leukemia. None of 79 random normals were positive. However, the number of normal specimens collected is still small and it is possible that "healthy carriers" might be found during the ongoing survey (see *Note Added in Proof*). Thus, HTLV is an unambiguous human retrovirus, and it is strongly associated with some unusual forms of human T-cell leukemia and lymphoma and, at least in Japan, with some other leukemias as well.

MATERIALS AND METHODS

Viruses. HTLV was obtained as described (9). Virus was harvested from culture supernatants and concentrated and purified by continuous-flow centrifugation and sucrose density gradient banding (9). Sources of other viruses and cells used have been described (12).

Sera. Sera from normal donors and leukemic patients were collected in Japan and shipped to the United States as lyophilized samples. All leukemic sera were from sporadic cases with no specific family history of neoplasia. Normal sera and the leukemic sera included those collected from the area endemic for ATL in southwest Japan and random cases from elsewhere.

Purification of HTLV p24. The major core protein of HTLV was purified to homogeneity as described (12). Briefly, density-banded virus was lysed with nonionic detergent at high ionic strength and the extract was passed through a DEAE-cellulose column to remove nucleic acids. The nucleic acid-free extract was chromatographed on a phosphocellulose column at pH 6.5, and elution of bound proteins was performed by using a linear NaCl gradient. Fractions containing the bulk of p24, as judged by polyacrylamide gel electrophoretic analysis in the presence of $NaDodSO_4$, were pooled, concentrated by ammonium sulfate precipitation (80% saturation), and further purified by gel filtration through a Bio-Gel p60 (Bio-Rad) column equilibrated with 10 mM sodium phosphate/1 M NaCl/0.1 mM phenylmethylsulfonyl fluoride. Purified p24 was labeled, as necessary, with ^{125}I to specific activities of 5–20 μCi/μg (1 Ci = 3.7×10^{10} becquerels) by the chloramine-T method (21).

Immunoprecipitation and Radioimmunoassay. ^{125}I-Labeled HTLV p24 (8000–10,000 cpm) was incubated with serial 1:2 dilutions of the human sera in a final volume of 200 μl of 20 mM Tris·HCl, pH 7.5/200 mM NaCl/1 mM EDTA/0.3% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride containing 2 mg of bovine serum albumin per ml. After 2 hr at 37°C, the incubation was continued overnight at 4°C. A 20-fold excess of goat anti-human IgG or a predetermined amount of inactivated *Staphylococcus aureus* cells (Pansorbin, Calbiochem) was then added and the reaction mixture was diluted to 1 ml with the above buffer. After further incubation for 1 hr at 37°C followed by 2 hr at 4°C, the precipitates were collected by centrifugation at 2500 rpm for 15 min in a Beckman centrifuge and the radioactivity in the precipitates was determined. For competition radioimmunoassays, limiting dilutions of the appropriate serum resulting in 20–30% precipitation of the labeled antigen in the absence of competition, were preincubated with serial dilutions of the unlabeled competing antigens (viral and cellular extracts) for 1 hr at 37°C. ^{125}I-Labeled p24 (8000–10,000 cpm) was then added and the reaction mixture was incubated and processed as described above.

The extent and pattern of precipitation of labeled p24 by human sera were identical whether we used bovine serum albumin or ovalbumin as the protein diluent. Furthermore, there was no nonspecific competition in the immune precipitation by irrelevant cellular or viral components as was encountered in some earlier investigations on natural human antibodies reactive generally against glycoproteins and polysaccharides (22, 23).

RESULTS

Antibodies to HTLV p24 in Sera of Japanese Patients with ATL or TML. Sera of ATL patients in Japan along with those of patients with other types of leukemias were screened for antibodies to HTLV p24. Serial dilutions of sera were incubated with ^{125}I-labeled, homogeneous p24 of HTLV, and the extent of immune precipitation obtained was measured. Fig. 1*A* shows

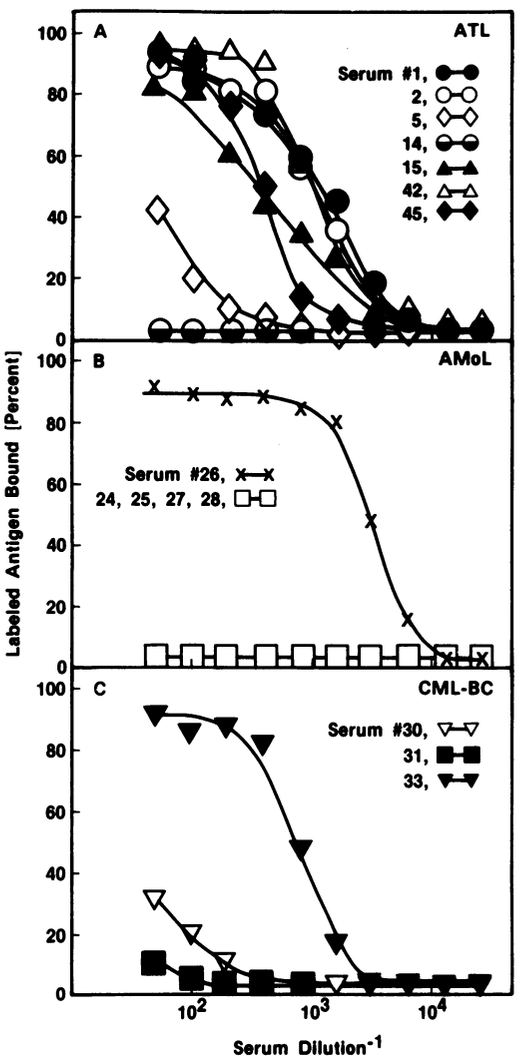

FIG. 1. Immunoprecipitation of ^{125}I-labeled HTLV p24 by different human sera. The labeled p24 (8000–10,000 cpm) was mixed with 1:2 serial dilutions of human sera in 200 μl of buffer 1 (20 mM Tris·HCl, pH 7.5/200 mM NaCl/1 mM EDTA/0.3% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride containing 2 mg of bovine serum albumin per ml). The reaction mixture was incubated for 2 hr at 37°C and overnight at 4°C. A 20-fold excess of goat anti-human IgG was then added and the volume was made up to 1 ml with buffer 1. The samples were incubated for 1 hr at 37°C and an additional 2 hr at 4°C and then centrifuged at 2500 rpm for 15 min in a Beckman centrifuge. The supernatants were aspirated and the radioactivity in the pellets was measured in an LKB Ultrogamma counter. (*A*) ATL sera; (*B*) acute monoblastic leukemia (AMoL) sera; (*C*) chronic myelogenous leukemia (CML) sera from patients in blast stage.

the results obtained with 7 of 13 Japanese ATL sera tested. Six sera had strong antibody titers; the seventh was essentially nonreactive. Strong antibody reactivities were obtained with the six additional ATL sera not shown in the figure. The negative ATL serum happens to be from a patient who had received chemical treatment for the leukemia; the other ATL sera were from untreated cases. Eleven of the 13 ATL patients were born in the endemic areas of southwest Japan (Kyushu and Shikoku Islands). The one negative was from Shikoku Island. Similarly, high-titer antibodies to HTLV p24 were observed in the sera of five of seven patients with TML, a closely related T-cell disease. This disease is distinguished from ATL by the presence of fewer leukemic cells in the peripheral blood. In contrast to the ATL and TML sera, none of the 79 sera from normal donors contained detectable antibodies to HTLV p24. Among the normal sera, 40 were collected from random donors around Kyoto and 39 were from the endemic area for ATL. The number of normal sera tested in this study is low and we cannot exclude the possibility of finding antibody-positive normal sera in a more extensive survey (see *Note Added in Proof*).

Antibodies to HTLV p24 in Other Types of Leukemia. Strong anti-p24 titer was detected in the serum sample of a 16-year-old man with a diagnosis of acute monoblastic leukemia (AMoL) in the first relapse phase (Fig. 1*B*); the leukocyte count was 188,000/mm^3 with 93.5% leukemic leukocytes. Because no meaningful cytochemical study was done on the cells and the diagnosis was based on cell morphology, this diagnosis may be considered tentative. Four other AMoL patients had no serum antibody against HTLV p24. Three cases of chronic myelogenous leukemia (CML) in the acute blast stage of the disease were seropositive, one of them showing a fairly strong reactivity (Fig. 1*C*). Five other sera from CML patients in blastic phase were negative (data not shown). It is known that in a small proportion of cases of CML the cells that undergo proliferative response during blastic conversion are of T-cell lineage (24), but we cannot correlate the positive cases in the present study with T-cell proliferation. Detectable but low antibody titers were also present in two of six cases of the null-cell type of ALL and one of four cases of acute myelogenous leukemia (AML).

Table 1 summarizes the data obtained with all the sera we analyzed for anti-p24 activity. With the exception of the single case of AMoL, the diagnosis of which is somewhat tentative,

Table 1. Natural antibodies to HTLV p24 in sera of leukemic patients

Diagnosis	Tested	Positive	Max. precipitation*	Antibody titer†
ATL	13	12	76–95	100–3200
TML	7	5	62–95	178–1845
Null-cell ALL	6	2	25;78	30;100
CML (blast stage)	9	3	30–91	25–1450
AMoL	5	1‡	91	5800
AML	4	1	73	200
ALL	6	0	—	—
B-cell CLL	4	0	—	—
Normal§	79	0	—	—

* Maximal precipitation was the amount of p24 precipitated at 1:25 dilution of the sera.

† Titer is expressed as the reciprocal of serum dilution for 20% precipitation of p24.

‡ The diagnosis of AMoL for this patient was based on cell morphology and pathology and was not aided by distinguishing cell cytochemistry.

§ The normal donors included 40 who were randomly selected around Kyoto and 39 others who were from southwest Japan, an area where ATL is endemic.

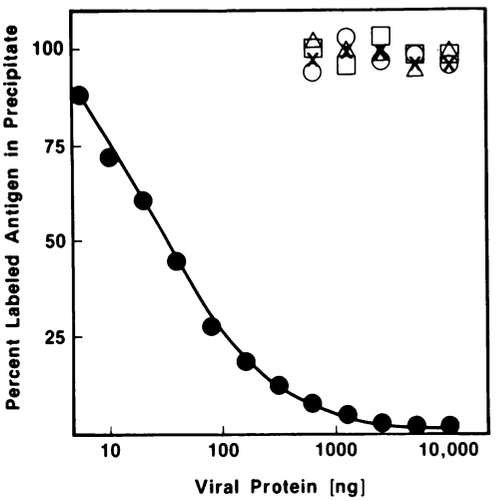

FIG. 2. Viral competition in the precipitation of HTLV p24 by human ATL serum. Competition radioimmunoassays were performed with ^{125}I-labeled HTLV p24 and limiting dilution (1:1500 in the final assay) of the human ATL serum 2. Serial dilutions of the unlabeled antigens were preincubated with the serum for 1 hr at 37°C. Labeled p24 (8000–10,000 cpm) was then added and the reaction mixture was incubated and processed as described in Fig. 1. ●, HTLV; ○, simian sarcoma virus; x, baboon endogenous virus; △, squirrel monkey retrovirus; □, bovine leukemia virus.

and the one case of CML in blast phase, the only sera capable of quantitative precipitation of HTLV p24 were from the ATL and TML cases. In addition, only ATL and TML sera had high antibody titers except for the same two cases. Although some other sera (one AML, one of two ALL of the null-cell type, and one CML in blast phase) showed >70% precipitation of HTLV p24, they did so only at the highest serum concentration tested and their antibody titers ranged between 100 and 200. The only totally nonreactive leukemic sera were from patients with B-cell chronic lymphocytic leukemia (CLL) and ALL. In addition, all the normal sera including those from southwest Japan and those collected from random population were nonreactive against HTLV p24.

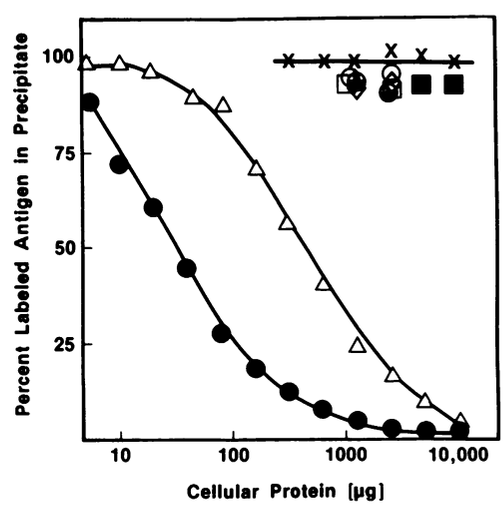

FIG. 3. Cellular competition in the precipitation of HTLV p24 by human serum. The competition radioimmunoassays were done as in Fig. 2, except cell extracts were used as competing unlabeled antigen. ●, HUT102 cells; △, CTCL-2 cells; x, HUT78 cells; ○, normal human T cells cultured with T-cell growth factor; ◒, P3HR-1 cells; ◇, Daudi cells, B-95 cells, and YT-4E cells; □, cultured human ALL cells; ■, cultured human melanoma cells.

Table 2. Viral and cellular specificities in the precipitation of HTLV p24 by human leukemia sera

		Percent competition by viruses and cells, %									
		Viruses								Cells	
Serum	Diagnosis	HTLV	R-MuLV	FeLV	SSV	BaEV	MPMV	SMRV	BLV	HUT102	HUT178
1	ATL	100	10	5	8	7	2	1	0	89	7
22	AML	95	2	5	11	12	9	11	9	89	11
26	AMoL	99	6	12	7	5	11	2	12	94	0
33	CML-BC	100	10	7	6	5	10	8	11	95	0
56	TML	93	0	0	0	0	0	0	0	91	0

Competition was measured with 10 μg of viral protein or 50 μg of cellular protein. R-MuLV, Rauscher strain of murine leukemia virus; FeLV, feline leukemia virus; SSV, simian sarcoma (wooly monkey) virus; BaEV, baboon endogenous virus; MPMV, Mason–Pfizer monkey virus; SMRV, squirrel monkey retrovirus; BLV, bovine leukemia virus.

In the above immunoprecipitation, we used a goat anti-human IgG serum as the second antibody in a double-antibody precipitation system. When inactivated *S. aureus* cells were used instead of goat anti-human IgG serum, slightly higher titers were obtained with certain sera. This may indicate that other classes of immunoglobulins in addition to IgG may be involved in the immune reaction.

Viral and Cellular Specificity in the Immune Precipitation of HTLV p24 by Human Sera. The specificity of the p24 precipitation was examined by measuring the competition exerted by extracts from a large number of animal retroviruses and from cells. Of the viruses tested, only extracts of HTLV competed in the precipitation of the ATL sera. Two primate type C retroviruses [simian sarcoma (wooly monkey) virus and baboon endogenous virus], a primate type D retrovirus (squirrel monkey retrovirus), and bovine leukemia virus showed no effect even at 1000-fold higher concentrations than HTLV (Fig. 2). The specificity was further examined by using extracts of cells that produce HTLV and cell lines that do not. Competition was obtained only with extracts of HUT102 cells and CTCL-2 cells (Fig. 3). These are T-cell lines that produce HTLV strains that have been derived from two different patients with T-cell malignancies (9, 10). Extracts from HUT78 cells, another T-cell line derived from a T-cell malignancy similar to that of HUT102 cells but not known to produce HTLV, were totally devoid of competition as were extracts from several other cells. Extensive competition analyses were also performed on the immune reactivity found in each group of sera. The precipitation of HTLV p24 by antibodies in all classes of leukemic sera was specifically inhibited only by HTLV and a cell line (HUT102) that produces HTLV (Table 2). None of a large number of viruses or a virus nonproducer T-cell line (HUT78) was competitively inhibiting in these reactions.

DISCUSSION

The experimental system used in the present study involved measurement of specific immune precipitation of a well-characterized (12, 14) core protein (p24) of HTLV, an exogenously acquired human virus (11) with no immunological crossreactivity (12) or nucleic acid sequence homology (11) with other animal retroviruses. The extent of precipitation obtained in most cases was quantitative, and the reaction was blocked only by HTLV extracts or extracts of cells known to be producing HTLV (Figs. 2 and 3; Table 2).

Leukemias and lymphomas of T cells are relatively less common than B-cell lymphomas in the United States and Europe, especially in adults (25, 26), and as yet no clusters of such diseases have been identified in these areas. Therefore, the rare findings of HTLV-positive cases in the United States were chance observations and may not be repeated with any degree of predictability. The low-frequency finding of antibodies to HTLV in our initial screen of sera of leukemic patients and normal donors from the United States and Europe is consistent with this. On the other hand, the incidence of adult T-cell leukemias and lymphomas is relatively high in Japan, and they cluster in southwest Japan (17–19). The results reported here clearly indicate the presence of antibodies to HTLV p24 among a high percentage of the leukemic sera obtained from Japan (Fig. 1; Table 1). The results with ATL and TML are striking not only in the percentage positive but also in the high antibody titers. Positives were found in a few other categories of leukemias, but the T-cell malignancies have a much higher proportion of antibody positive sera. Sera from random normal donors including those collected from the area endemic for ATL have been without significant anti-p24 activity. Some recent data from our laboratories show the presence of antibodies to HTLV in the sera of some relatives of patients with T-cell malignancies (unpublished observations). This might indicate a more widespread distribution of antiviral antibodies among even healthy individuals, especially in the areas endemic for ATL.

Among the antibody-positive ATL and TML patients we could not find any consistent age or sex pattern. The ages ranged between 42 and 85, and there were 8 men and 9 women. No major correlation was noted between antibodies in the sera and the patient's clinical features, such as peripheral blood leukocyte count, fraction of cells that were leukemic, and extent of bone marrow involvement.

Because HTLV is not endogenous to humans (11) it will be of considerable interest to determine its origin, mode of transmission, and natural reservoir. It will also be important to search for other clusters of HTLV-associated leukemias. In addition, more extensive studies of leukemias and lymphomas of T-cell origin in and around southwest Japan should be pursued.

Note Added in Proof. Subsequent to the submission of this manuscript we have screened 120 additional sera of random normal donors from the so-called endemic areas of Japan for antibodies to HTLV p24 and approximately 10% of them have been positive. Samples from nonendemic areas have not yet been screened in large numbers.

We appreciate the excellent technical assistance by M. Jarvis and the cooperation of Drs. T. Masaoka, S. Mitsui and M. Sakurai in making available the leukemia serum samples for the present study.

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