DETECTION OF THE HUMAN T CELL LYMPHOMA VIRUS p19 IN CELLS OF SOME PATIENTS WITH CUTANEOUS T CELL LYMPHOMA AND LEUKEMIA USING A MONOCLONAL ANTIBODY

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A novel human retrovirus, $HTLV_{CR}$, was isolated from a cutaneous T cell lymphoma-derived cell line (1). A highly related virus, $HTLV_{MB}$, was subsequently isolated from cells of a patient with cutaneous T cell leukemia (2). The uniqueness of these isolates is supported by the lack of relationship of their nucleic acids (3), proteins (4), and reverse transcriptase (5) to any other type-C RNA tumor virus. Because retroviruses are implicated in the cause of some animal leukemias and lymphomas (6), HTLV is of interest as a possible etiologic agent in similar human neoplasias. Our descriptions of natural antibodies to internal HTLV proteins in sera of patients with cutaneous T cell lymphomas and leukemias (CTCL)[1] strengthens this possibility (7, 8). To probe the patient population for indications of HTLV infection and to investigate the regulation of viral expression in HTLV-infected cells, we have developed monoclonal antibodies to HTLV proteins. We report on a monoclonal antibody for HTLV p19 and on the expression of HTLV p19 in cells of patients with leukemias and lymphomas.

Materials and Methods

Production of Monoclonal Antibody. Spleen cells of a BALB/c mouse hyperimmunized with disrupted HTLV were fused with NS-1 myeloma cells (9) using polyethylene glycol 1,000 (10). Cells were plated overnight in Dulbecco's modified Eagle medium containing 20% fetal calf serum, 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, 1% nonessential amino acids (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), 2 mM glutamine, 10% NCTC109 lymphocyte conditioned medium (Microbiological Associates), 0.5 mM sodium pyruvate, 0.2 U/ml insulin, and 1 mM oxalacetic acid. The cells were transferred to selective medium containing, in addition to the above, 4×10^{-7} M aminopterin and 50 μg/ml gentamicin (Schering Corporation, Kennilworth, N. J.), and were plated into 40 Costar 96-well plates. 5 d later, one-half of the medium was changed. Media from hybrid-positive wells were assayed by a solid-phase radioimmunoassay (RIA) for antibody to HTLV proteins (7). Positive hybridomas were cloned at one cell per 0.2-ml well until two successive clonings gave 100% positive cultures. Ascites fluids were generated by intraperitoneal inoculation of 10^6 cells into BALB/c mice primed 1 wk earlier with 1 ml pristane (2,6,10,14-tetramethylpentadecane).

Metabolic Labeling of Immunoglobulins. 10^6 cells were washed with Eagle's minimum essential

[1] *Abbreviations used in this paper:* CTCL, cutaneous T cell lymphomas and leukemias; IdU, iododeoxyuridine; PBS, phosphate-buffered saline; PMSF, phenyl methyl sulphonyl fluoride; RIA, radioimmunoassay; RIP, radioimmune precipitation; TCGF, T cell growth factor.

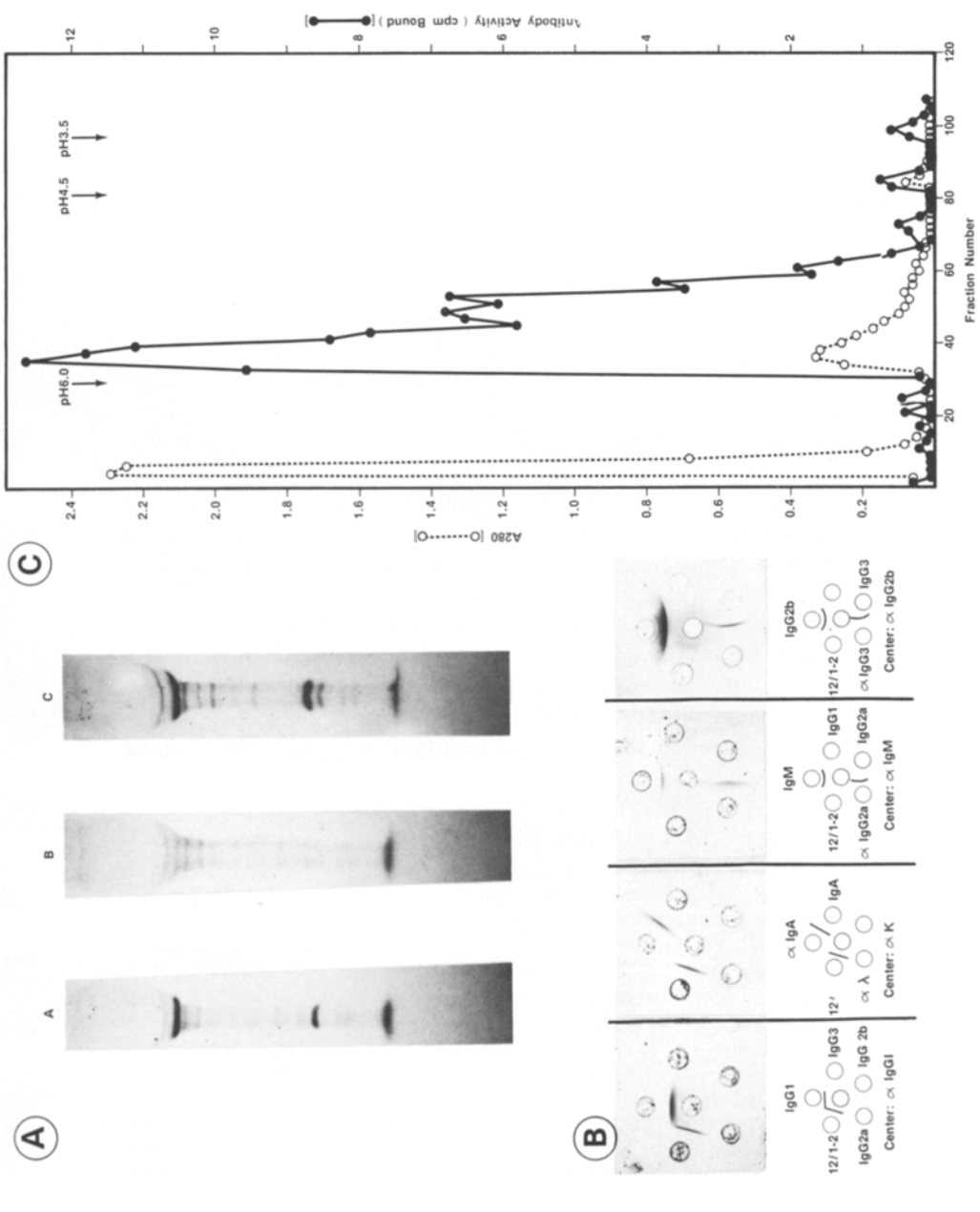

FIG. 1. Anti-p19 is an IgG_1. (A) Metabolic labeling of immunoglobulins. Media containing ~70,000 cpm [^{14}C]leucine were electrophoresed on a 10% polyacrylamide slab gel (12). Autoradiography was carried out at −70°C using Kodak X-Omat R film (Eastman Kodak Co., Rochester, N. Y.). Lane A, P3x63 myeloma line synthesizing IgG_1 and K light chain. Lane B, nonsecreting NS-1 parent line. Lane C, hybridoma 12/1-2 synthesizing IgG_1, K light chain. (B) Ouchterlony analysis of ascites fluid. 10 μl of mouse immunoglobulin (0.25 mg/ml) and specific antisera (Litton Bionetics, Inc.) were reacted, as diagrammed, with 10 μl of a 1:40 dilution of 12/1-2 ascites. Plates were dried and stained with Amido black. (C) Fractionation of 12/1-2 ascites fluid on protein A-Sepharose. Antibody activity, assayed by solid-phase RIA, is expressed as cpm ^{125}I-goat anti-mouse IgG bound.

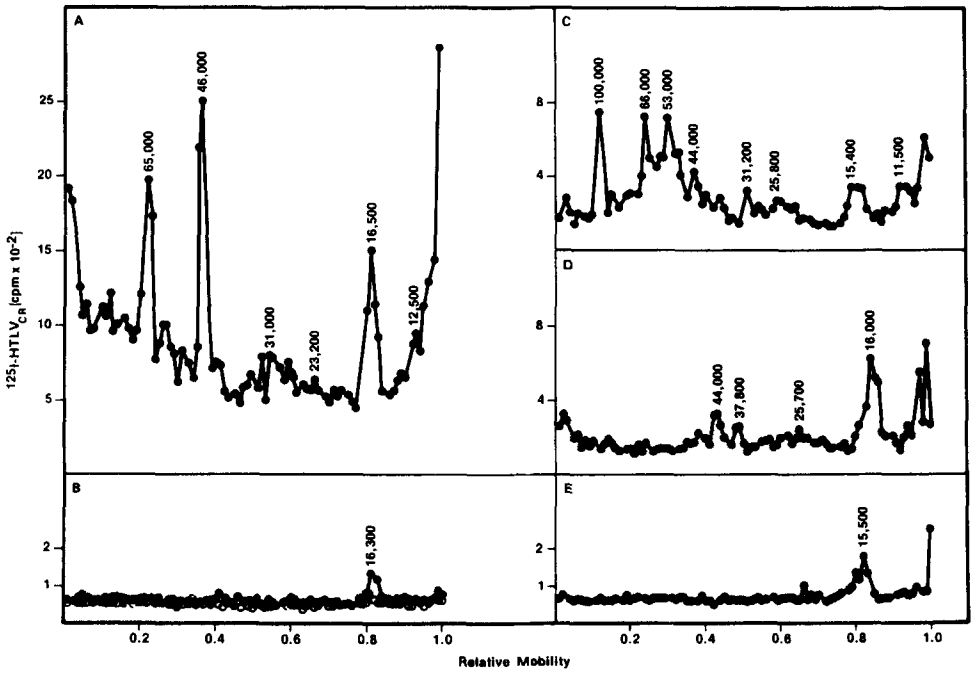

FIG. 2. Specificity of hybridoma 12/1-2 antibody. (A) Iodinated HTLV$_{CR}$ proteins, (~150,000 cpm) were electrophoresed on a 12% polyacrylamide gel (12). The gel was then sliced and counted. Molecular weights were calculated with reference to phosphorylase A, bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease standards. (B) Radioimmune precipitation of ^{125}I-HTLV$_{CR}$ proteins. ●——●, precipitation with 12/1-2 ascites; ○——○, precipitation with P3x63 ascites. (C, D, and E) Gel electrophoresis of ^{125}I-HTLV$_{CR}$ proteins after affinity chromatography on 12/1-2 IgG-Sepharose. (C) Unbound ^{125}I-HTLV proteins. (D) Bound ^{125}I-HTLV proteins eluted from the affinity column. (E) Bound ^{125}I-HTLV protein eluted from the affinity column after reapplication of the material in (D).

medium minus leucine, supplemented as the selective media, except that hypoxanthine, thymidine, and aminopterin were omitted. Labeling was accomplished by incubation overnight in 200 μl media containing 1 mCi [^{14}C]leucine.

Protein A-Sepharose Chromatography. 2 ml of ascites fluid was processed on a 7-ml protein A-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) column (11). Antibody binding to HTLV was assayed on 1:1,000 dilutions of eluted fractions by the solid-phase RIA (7).

Radioimmune Precipitation (RIP). Approximately 150,000 cpm of iodinated HTLV$_{CR}$ proteins (7) in 50 μl RIP buffer containing 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 10% glycerol, 0.2% Triton X-100, 0.1% gelatin, 0.02% NaN_3, and 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) were incubated 4–6 h at 4°C with 50 μl of a 1:5,000 dilution of ascites fluid. 250 μl of a 10% (vol/vol) suspension of protein A-Sepharose in RIP buffer was added. Samples were incubated overnight with mixing and were centrifuged. Pellets were washed with 1 ml RIP buffer. Immune precipitates were boiled in Laemmli sample buffer (12) and subsequently electrophoresed on 12% polyacrylamide gels.

Affinity Chromatography. Hybridoma 12/1-2 IgG_1, purified on protein A-Sepharose, was coupled to CNBr-Sepharose 4B (Pharmacia Fine Chemicals). ^{125}I-HTLV$_{CR}$ proteins in phosphate-buffered saline (PBS) were applied to the column and eluted with 0.2 M glycine-HCl, pH 2.8. The 2-ml fractions were neutralized with 100 μl 2 M Tris-HCl, pH 8.0. The unbound and bound proteins were pooled separately, dialyzed against 10 mM NH_4HCO_3, lyophilized, and dissolved in 10 mM sodium phosphate buffer, pH 8. 100-μl portions of each fraction were electrophoresed on 12% polyacrylamide gels (12) that were subsequently sliced and counted.

TABLE I

Specificity of Hybridoma 12/1-2 for HTLV-producing Cells by Immune Fluorescence Assay

Cell type	Immune fluorescence	Cell type	Immune fluorescence
	% positive		*% positive*
HTLV-producing CTCL cells		*Transformed CTCL cells not producing HTLV*	
HUT102	+(60–70)	HUT78	—
HUT102, Clones A9, B2, A15	+(60–70)	*Immature or pre-T cell lines*	
CTCL-3	+(12)	Molt-4	—
CR-CTC	+(38)	8402	—
CTCL-2	+(5)	CCRF-CEM	—
Animal retrovirus infected cells		CCRF-HSB	—
LBv20B/SSAV (N.P.)	—	TALL-1	—
LBv20A/GaLV (N.P.)	—	HPB-MLT	—
NC37/SSV	—	HPB-ALL	—
88/M28	—	JM	—
FEA/FeLVA, FeLVB, or FeLVC	—	PEER	—
		DND-41	—
JLSV9/RLV	—	*Normal peripheral blood lymphocytes*	
A7573/SSAV	—	NPBO-19	—
KHOS (N.P.)	—	NPBO-20	—
88/GaLV$_{Thai}$	—	NPBO-24	—
B cell lines		0-49	—
Daudi	—	0-50	—
CCRF-SB	—	0-51	—
LBv20E	—	0-52	—
NC37	—	T.O.M.	—
CR-B cells	—	NPB1-9	—
80-81 (EBV)	—		
80-84 (EBV)	—		

Indirect Immune Fluorescence Assay. Cells (3×10^6) were washed with PBS and suspended in 200 μl of a 1:15 dilution of PBS. Aliquots (1–2 μl) were spotted on slides, air dried, and fixed for 10 min at room temperature in 50% methanol:50% acetone. Slides were stored at −20°C until use. Ascites of hybridoma 12/1-2 or control ascites of P3x63 cells, diluted 1:400 in PBS, was applied to cells and incubated 30 min. The fluorescein-conjugated F(ab')$_2$ fragment of sheep anti-mouse IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was purified through a human IgG-Sepharose column, diluted, and applied to cells. After 30 min incubation at room temperature, slides were washed for 1–2 h in PBS before microscopic examination.

Cells. The isolation of HTLV$_{CR}$ from HUT102 cells derived from a patient (C.R.) with CTCL has been described (1), as have CTCL-3 cells derived from a second blood sample of patient C.R. (1). CR-CTC cells were derived independently from a third blood sample of patient C.R. by Dr. T. Waldmann. Dr. M. Maeda cloned the HUT102 cells. CTCL-2 cells derived from cells of a patient with cutaneous T cell leukemia were the source of the second HTLV isolate, strain MB (2). Other patient lymphocytes were examined either as fresh or fresh-frozen cells, or else were examined after culture in the presence of T cell growth factor (TCGF) (13). Normal peripheral blood lymphocytes were cultured using phytohemagglutinin and TCGF (13). Virus-infected cells included human B cells infected with but not producing woolly monkey (simian sarcoma associated) virus (LBv20B/SSAV); gibbon ape leukemia virus (LBv20A/GaLV) (14); human lymphoblasts infected with simian sarcoma virus (NC37/SSV); bat lung cells infected with baboon endogenous virus (88/M28); feline embryo fibroblasts infected with strain A, B, or C of feline leukemia virus (FEA/FeLVA, FEA/FeLVB, FEA/FeLVC); bat lung cells infected with GaLV, Thai strain (88/GaLV$_{Thai}$); mouse fibroblasts

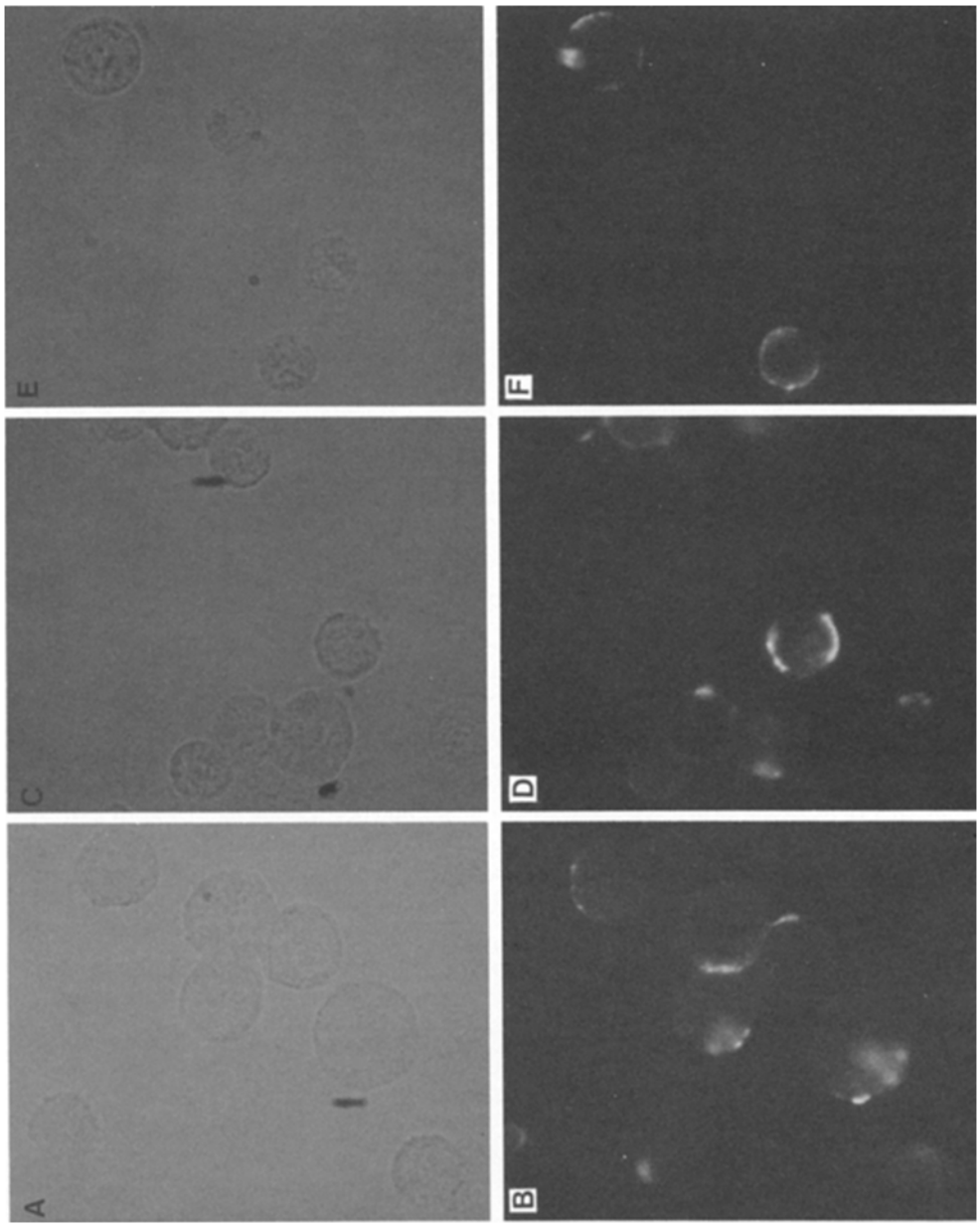

FIG. 3. Immune fluorescence of HTLV-producing cells using anti-p19. (A–D) HUT102, clone B2 cells; (E–F) CTCL-3 cells. Upper panels, bright field view; lower panels, fluorescent cells in same field.

TABLE II

Prevalence of HTLV p19 Expression in Cells of Patients with Leukemias and Lymphomas

Cell type	Number tested	Number positive	Cell type	Number tested	Number positive
Cutaneous T cell lymphomas			*Acute lymphocytic leukemia*		
(Mycosis fungoides)			Fresh	7	0
Fresh	6	0	Fresh frozen	1	0
Cultured	12	3*	Cultured	4	0
Cultured + IdU	2	0	Cultured + IdU	1	0
Cutaneous T cell leukemias			*Acute myelocytic leukemia*		
(Sezary syndrome)			Cultured	2	0
Fresh	8	0	*Chronic lymphocytic leukemia*		
Fresh frozen	2	0	Fresh	3	0
Cultured	2	1‡	Cultured	1	0
Cultured + IdU	1	0	fresh, T cell type	2	0
T cell lymphoma			*Hairy cell leukemia*		
Fresh	5	0	Fresh	2	0
Cultured + IdU	1	0	Cultured	1	0
Promyelocytic leukemia			*Unclassified leukemias*		
Cultured	3	0	Fresh	1	0
Hodgkins			Cultured	3	0
Cultured	1	0	Cultured + IdU	1	0

* The positive cells include HUT102 cells, CTCL-3 cells,and CR-CTC cells.

‡ The positive cells were CTCL-2 cells.

infected with Rauscher murine leukemia virus (JLSV9/RLV); dog thymus cells infected with SSAV (A7573/SSAV); and human osteogenic sarcoma cells infected with but not producing the Kirsten rat leukemia virus (KHOS [N.P.]). Among the standard B and T cell lines used were several T cell lines kindly supplied by Dr. J. Minowada (CCRF-CEM, CCRF-HSB, JM, TALL-1, HPB-MLT, HPB-ALL, PEER, and DND-41). CR-B cells refers to B cells of patient C.R. The normal B cell lines 80–81 and 80–84 were established in culture after transformation with the Epstein-Barr virus.

Results and Discussion

Of several monoclonal antibodies to HTLV, one produced by hybridoma 12/1–2 has been especially useful. Ouchterlony analysis and metabolic labeling indicated that this hybridoma synthesizes IgG_1 with a kappa light chain (Fig. 1 a and b). A confirmation of this subtype and evidence that binding of 12/1–2 ascites fluid to HTLV was antibody mediated was obtained by coincident elution of antibody and binding activity from a protein A-Sepharose column at pH 6 (11; Fig. 1 c). An RIP assay for HTLV proteins illustrated the specificity of the antibody. Among the iodinated HTLV proteins (Fig. 2 a) only p19 could be specifically precipitated (Fig. 2 b). (The molecular weight of p19 varies with measurement from 16,000 to 19,000.) Hybridoma 12/1–2 IgG_1-Sepharose also specifically bound the p19 protein (Fig. 2 c, d, and e). The column used was too small to quantitatively bind all the p19 (Fig. 2 c and d). However, reapplication of the first eluate to the column resulted in specific binding of HTLV p19 (Fig. 1 e).

In indirect immune fluorescent assays this anti-p19 specifically labels only HTLV-producing cells (Table I). Transformed HUT78 cells, also derived from a patient with CTCL, do not produce HTLV and are negative for p19. The percentage of fluorescent cells in a positive sample varies. Both HUT102 parental cells and clones are 60–70% positive, suggesting that viral expression might be related to cell cycling. CTCL-2 and CTCL-3 cells are 5 and 12% positive, respectively. Presumably, not all the cells are producing virus. Alternatively, some cells might express virus at levels too low to

detect by this assay. In fact, CTCL-2 cells require iododeoxyuridine (IdU) induction for significant virus production (2). Normal peripheral blood lymphocytes and human T and B cell lines examined were all negative for the presence of HTLV p19 (Table I), emphasizing the viral specificity of the antibody. Anti-p19 does not recognize cells producing other mammalian RNA tumor viruses, which further substantiates the lack of relationship of HTLV to these viruses.

The fluorescent labeling of virus-positive cells is restricted to the cell membrane (Fig. 3). Only fixed cells can be labeled, supporting the notion that p19 is an internal HTLV protein (4) and not an envelope component. Whereas conventional antisera to an internal viral protein might stain the cytoplasm of virus-producing cells, the monoclonal antibody only recognizes p19 when the virus is assembled at the cell membrane.

TCGF has enabled growth of malignant mature T cells, resulting in isolation of HTLV (1, 2). Two of the HTLV-producing cell lines, HUT102 and CTCL-2 cells, now grow independently of exogenous factor. Presumably, TCGF caused stimulation of virus production by expansion of the appropriate cell population. We have looked for expression of HTLV p19 in cells of other patients with CTCL as well as of patients with other leukemias. Both fresh and cultured cells have been examined. So far, all attempts to detect HTLV in this fashion have been negative, including those in which we tried to induce HTLV production using IdU (Table II). It is clear that HTLV p19 is not expressed in most malignant T cells, indicating that replicating HTLV is not present. Whether HTLV is present more commonly but is not completely expressed, is present only in the early stages of disease, or is present in only a subset of T cell leukemias is not known. The monoclonal antibody to HTLV p19 provides an assay for HTLV expression for determining the frequency of HTLV replication in human T cells and for studying the regulation of viral gene expression.

Summary

A monoclonal antibody specific for the internal p19 protein of a type-C retrovirus (HTLV) isolated from human neoplastic T cells has been developed. Its specificity has been shown by radioimmune precipitation and by affinity chromatography of iodinated HTLV proteins. By indirect immune fluorescence this antibody recognizes only HTLV-producing cells. Examination of cells from patients with cutaneous T cell lymphomas and leukemias and with other types of lymphomas and leukemias indicated that HTLV p19 expression is rare. The monoclonal antibody will be useful in determining the natural reservoir of HTLV, possibly in a subset of mature T cell neoplasias.

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