MONOCLONAL ANTIBODIES AGAINST HUMAN T CELL LEUKEMIA-LYMPHOMA VIRUS (HTLV) p24 INTERNAL CORE PROTEIN

Use as Diagnostic Probes and Cellular Localization of HTLV

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The human T cell leukemia-lymphoma virus (HTLV)[1] is a novel retrovirus associated with adult T cell leukemia (ATL) and lymphoma and shows a unique geographical distribution to southern Japan, the southeastern United States, the West Indies, and other areas (1). Studies using HTLV-associated patient serum (2–4) or a monoclonal antibody (12/1–2) against a 19,000 dalton component of HTLV (p19) (5, 6), as well as nucleic acid hybridization studies (7–9), have demonstrated that HTLV isolates from Japanese ATL and cutaneous T cell lymphoma from patients in the U. S. are highly related or identical. Serological studies demonstrated HTLV-associated patient sera immunoprecipitated HTLV proteins of 19,000 and 24,000 daltons from ^{125}I-labeled HTLV preparations (10), and that both p19 and p24 copurify with viral core structures, suggesting that they are structural core proteins of HTLV (11). Data from the complete nucleotide sequence of the proviral HTLV genome (12) as well as partial amino acid analysis of the p24 protein of HTLV (13, 14) indicate further that HTLV proteins p19 and p24 are encoded by the gag region of the viral genome.

Monoclonal antibody 12/1–2, which reacts with the p19 core protein of HTLV, has been a useful diagnostic reagent for the detection of HTLV-infected T cells (5, 6). However, the antigenic determinant recognized by monoclonal antibody 12/1–2 is also present on normal thymic epithelium and on other normal and neoplastic epithelial cells, and therefore is not strictly HTLV specific (15). In addition, no anti-HTLV p24 monoclonal antibodies have been made, and only limited quantities of polyclonal goat antiserum to HTLV p24 core

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[1] *Abbreviations used in this paper*: ATL, adult T cell leukemia; BSA, bovine serum albumin; F/P, fluorescein to protein ratio; HTLV, human T cell leukemia-lymphoma virus; PBL, peripheral blood leukocytes; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; SAC, fixed *Staphylococcus aureus* Cowan I strain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

protein are available as a probe for the detection of p24 in HTLV-infected cells. Moreover, use of the polyclonal goat anti-p24 in fluorescent assays has not been useful for HTLV cellular localization (10). Thus, in the present study, we raised monoclonal antibodies against HTLV p24 and used them as diagnostic reagents for detection of HTLV-infected cells, to study the cellular localization of p24, and to evaluate whether there is any shared antigenicity between p24 and normal human tissue, as has been found for HTLV p19 (15). We report here that four monoclonal antibodies (HTLV 6, 7, 8, and 9) raised against disrupted HTLV reacted with the p24 internal core protein of HTLV and with HTLV-infected T cells, but did not react with a wide range of normal, neoplastic, or non-HTLV-infected human cells and tissues. In addition, monoclonal antibodies HTLV 6, 7, 8, and 9 identified at least two antigenic sites on HTLV p24 that are both recognized by antibodies in HTLV$^+$ patient sera. Antibodies HTLV 6, 7, 8, and 9 also reacted with a T cell line from umbilical cord blood infected with HTLV-II_{MO}, a new type of HTLV. This virus was located from a patient (MO) with a T cell variant of hairy cell leukemia. Finally, HTLV p24 internal core protein was localized at the cell surface of HTLV-infected T cells and is likely associated with areas of budding virus particles.

Materials and Methods

Production of Monoclonal Antibodies. On days 0, 7, 14, and 21, BALB/c mice were immunized with subcutaneous and footpad injections of 50 μg disrupted HTLV (see below) in complete (day 0) and incomplete (days 7, 14, and 21) Freund's adjuvant. Before fusion, sera from immunized mice were screened for antibodies to HTLV by solid-phase radioimmunoassay (RIA) (6) and radioimmunoprecipitation (RIP) assay (16). Spleen cells from mice with circulating antibodies to HTLV were fused with one of the myeloma cell lines P3/63/Ag8, NS1-Ag4, or 653, using polyethylene glycol 1000 as previously described (17). After 10–14 d, colony supernatants were screened for anti-HTLV antibodies by solid-phase RIA. Positive colonies were passaged into 24-well plates and rescreened for reactivity to disrupted HTLV using the RIP assay or the Western blotting technique described below. Positive colonies were cloned by limiting dilution until successive clonings gave 100% positive cultures, and then passaged as ascites tumors.

Tissue and Cell Suspension Assays for Anti-HTLV Antibody Reactivity. Hybridoma supernatants and ascitic fluid were screened for reactivity with 4-μm frozen sections from a wide variety of types of tissues using indirect immunofluorescence as previously described (18). In some experiments, antibody HTLV 6 was directly fluoresceinated (fluorescein/protein ratio [F/P], 5.6) and used in direct fluorescence as described (18, 19). HUT 102, HUT 78, EB-3, SB, and HSB-2 cell lines were obtained and cultured as described (19). Cytocentrifuge preparations of cell lines, Hypaque-Ficoll-purified fresh normal peripheral blood leukocytes (PBL), and HTLV$^+$ patient PBL cultured in 25% T cell growth factor (Enzyme Center, Boston, MA) for 5 d were prepared, acetone fixed, and assayed for reactivity with anti-HTLV monoclonal antibodies or P3/63 control ascites fluid as previously described (10).

Viral Preparations and Antibody Reagents. HTLV was collected from HTLV$^+$ HUT 102 cell culture supernatants by continuous flow centrifugation and was purified by banding on sucrose density gradients as previously described (20). The virus was disrupted in Triton X-100 as described (16) and used in RIP or Western blot assays or extracted once with a threefold volume of diethyl ether and used in the solid-phase RIA. Goat polyclonal antisera against purified p24 and monoclonal antibody 12/1-2 that reacts with HTLV p19 core protein (5) were the generous gifts of Dr. V. Kalyanaraman and Dr. M. Robert-Guroff (NCI, NIH), respectively. HTLV$^+$ patient serum used in preclearing experiments was from patient SD with Japanese ATL (10).

Solid-Phase RIA. A solid-phase RIA (6) was used to detect hybridoma cell supernatants containing antibodies to HTLV proteins. An ether-extracted HTLV protein preparation was diluted in 15 mM $NaHCO_3$, 35 mM Na_2CO_3, pH 9.6 (coating buffer), and 100 ng in 50 μl coating buffer was added to detachable wells of Immulon plastic microtiter well strips (Dynatech Laboratories, Inc., Alexandria, VA). After overnight absorption at 4°C, wells were washed and incubated for 2 h at 4°C in coating buffer with 3% bovine serum albumin (BSA) and 0.02% NaN_3 to block nonspecific protein binding sites. Plates were stored at −20°C until used. Before assay, plate wells were washed three times with 100 μl of phosphate-buffered saline (PBS), pH 7.2, containing 0.5% Tween 20, 0.1% BSA, and 0.1% NaN_3 (PBS wash), and incubated for 1 h at 23°C with 50 μl of hybridoma supernatant diluted 1:2 in PBS, 0.5% Tween 20, 1% BSA, 0.1% NaN_3 (PBS diluent). After washing, 1:200 to 1:500 dilutions of rabbit anti-mouse IgG + M whole serum (Cappel Laboratories, Cochranville, PA) in PBS diluent were added and incubated for 30 min at 23°C. Plate wells were washed and incubated with 100,000 cpm of ^{125}I-protein A in 50 μl of PBS diluent for 30 min at 23°C. Wells were emptied, washed five times with PBS wash, separated, and counted for 1 min in a gamma counter.

Studies of Antigenic Sites on HTLV p24 Identified by HTLV 6, 7, 8, and 9 and by HTLV⁺ Patient Sera. Monoclonal antibodies HTLV 6, 7, 8, and 9 were isolated from mouse ascites fluid using protein A-Sepharose (21) and were iodinated using Iodobeads (Pierce Chemical Co., Rockford, IL). A competitive inhibition assay to compare the reactivities of HTLV 6, 7, and 8 and $HTLV^+$ patient sera to antigenic sites on HTLV p24 was performed as previously described (6) with some modifications. Briefly, duplicate microtiter wells precoated with HTLV proteins (1–2 μg/well) as described above were incubated with dilutions of unlabeled HTLV 6, 7, 8, and 9 or $HTLV^+$ patient sera in PBS diluent buffer for 45 min at 23°C. Wells were washed with PBS wash buffer and then further incubated with 10^5 cpm of ^{125}I-labeled HTLV 6, 7, 8, or 9 in PBS diluent buffer for 45 min at 23°C. Wells were washed as before and counted in a gamma counter. For controls, HTLV-coated wells were incubated with dilutions of P3X63 ascites fluid or pooled normal human serum and then used in the assay to estimate 100% binding of radiolabeled antibody. To estimate 0% binding of the radiolabeled antibody, HTLV-coated wells were incubated with a 1:100 dilution of the appropriate unlabeled anti-p24 antibody and the assay was completed as described.

Radioimmunoprecipitation of ^{125}I-HTLV. HTLV preparations were iodinated with Iodogen (Pierce Chemical Co.) in the following manner: Briefly, 10 μg of Iodogen in 100 μl chloroform was dried to the bottom of a 12 × 75-mm test tube under a stream of dry nitrogen and tubes were stored at 4°C in a dessicator until used. For labeling, 10 μg of HTLV protein in 10 μl PBS, pH 7.2, were incubated in Iodogen-coated tubes for 15 min at 23°C with 0.5 mCi ^{125}I (Amersham Corp., Arlington Heights, IL). The mixture was passed over a PD-10 column (Pharmacia Inc., Piscataway, NJ) equilibrated with RIP buffer (10 mM sodium phosphate, pH 7.5, 300 mM NaCl, 0.2% Triton X-100, 0.01% Tween 80, 0.1% gelatin, 0.02% NaN_3) and labeled HTLV proteins were eluted with RIP buffer and stored at −20°C. For screening of sera from mice immunized with HTLV, 5 μl of test or normal control serum was added to 500 μl of RIP buffer containing 5 × 10^5 cpm of ^{125}I-HTLV and incubated overnight at 4°C. Immune complexes were precipitated by adding 100 μl of 10% fixed *Staphylococcus aureus* Cowan I strain (SAC) (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) in RIP buffer and incubating for 1 h at 4°C. Pellets were washed three times in RIP buffer and immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) on 10% polyacrylamide gels.

Preclearing of ^{125}I-HTLV with anti-HTLV Monoclonal Antibodies or $HTLV^+$ ATL Patient Serum. To compare the reactivity of antibodies in sera of patients with HTLV with that of murine monoclonal antibodies raised to HTLV, preclearing experiments were undertaken in the following manner. To compare antibody A with antibody B, 5 × 10^5 cpm of ^{125}I-HTLV in 500 μl of RIP buffer were incubated overnight with the amount of antibody A that resulted in optimum precipitation of ^{125}I-HTLV, as determined by titration studies. To determine optimum preclearing conditions for precipitation of ^{125}I-HTLV with

monoclonal antibodies or patient serum, titration curves were derived using 0.5–40 μl of monoclonal antibody ascites or patient serum, 5×10^5 cpm of ^{125}I-HTLV in 500 μl of RIP buffer, and 100 μl of 10% SAC. All antibody precipitations were done overnight at 4°C. To immunoprecipitate HTLV p24 optimally with HTLV 7, 8, or 9, immunoprecipitation was performed with SAC precoated with rabbit anti-mouse IgG + IgM (23). The amount of antibody that precipitated the maximum number of counts in titration studies was used in preclearing experiments described below. After precipitation of immune complexes with SAC, the precleared ^{125}I-HTLV samples were again precipitated overnight with a half volume of antibody A and SAC, followed by an additional 1 h incubation with SAC alone. When preclearing with HTLV$^+$ ATL patient serum, a third preclearing with a half volume of antibody plus SAC was required to preclear HTLV samples. For the final RIP, an optimum amount of antibody B plus SAC was added to the precleared ^{125}I-HTLV sample. For each preclearing experiment using antibodies A and B, the following controls were included: (*a*) preclearing with A and RIP with A, (*b*) preclearing with A and RIP with normal antibody control (normal human serum, normal mouse serum, or P3X63/Ag8 ascites) for A, (*c*) preclearing with A and RIP with B, (*d*) preclearing with A and RIP with normal antibody control for B (normal human serum, normal mouse serum, or P3X63/Ag8 ascites), (*e*) preclearing with normal control for A and RIP with A, (*f*) preclearing with normal control for A and RIP with normal antibody control for A, (*g*) preclearing with normal antibody control for B and RIP with B, (*h*) preclearing with normal antibody control for B and RIP with normal antibody control for B, and (*i*) RIP with A and B and normal antibody controls without preclearing. Immunoprecipitates were electrophoresed on 10% polyacrylamide gels and autoradiographed for 2 d at −70°C using Kodak X-omat film and Dupont Lightning Plus intensifying screens.

Western Blotting of HTLV. Western blotting of HTLV was performed according to the technique of Towbin et al. (24). Following SDS-PAGE of 0.5–2.5 μg of disrupted HTLV protein under reducing or nonreducing conditions, HTLV proteins were electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH) using an electrode buffer of 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, pH 8.3, for 45 min at 0.7–0.9 A. Following the transfer, the nitrocellulose sheet was treated with 3% BSA in Tris-buffered saline (TBS) (0.9% NaCl, 10 mM Tris, pH 7.4) for 2 h at 23°C to block additional nonspecific protein binding sites. Individual lanes were then incubated with monoclonal antibody supernatants diluted 1:5 for 2 h at 23°C. Strips were then washed in TBS five times for 5 min each in 16×150 test tubes, incubated for 30 min at 23°C in 2 ml of 3% BSA-TBS containing 10^6 cpm/ml of ^{125}I-labeled sheep anti-mouse IgG + M $F(ab')_2$ fragment (Amersham Corp.), washed five to eight times in TBS, blotted dry, and autoradiographed for 2 h with an intensifying screen. ^{14}C-labeled molecular weight markers that were transferred from the gel to the nitrocellulose served as internal molecular weight standards.

Electron Microscopy. HUT 102 cells were fixed by the addition of a volume of 2% glutaraldehyde in 0.1 M phosphate buffer with 1.8% sucrose equal to the volume of media in the flask. After 30 min, cells were pelleted at low speed, and fresh 1% glutaraldehyde in phosphate/sucrose buffer was added for an additional 60 min. Postfixation was in 1% osmium tetroxide in phosphate buffer for 30 min followed by staining with 1% uranyl acetate in 0.11 M veronal acetate buffer for 30 min. Cells were embedded in EmBed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were poststained with uranyl acetate and lead citrate and viewed in a Philips EM 300 electron microscope.

Results

Production of Anti-HTLV p24 Monoclonal Antibodies. After the fusion of mouse spleen cells with P3X63/Ag8, NS1-Ag4, or 653 myeloma cell lines, cells were plated into five 96-well microtiter plates per myeloma cell line. A total of 542 colony supernatants were screened in the solid-phase RIA for reactivity to HTLV proteins, 107 of which were positive. Four of the colony supernatants immuno-

precipitated a 24,000 dalton protein (p24) from ^{125}I-labeled disrupted HTLV, and these colonies were cloned, passaged as mouse ascites tumors, and designated as antibodies HTLV 6, 7, 8, and 9. Table I shows the fusion parents and isotypes of the anti-HTLV p24 monoclonal antibodies produced.

Monoclonal antibodies HTLV 6, 7, 8, and 9 all reacted with a 24,000 dalton (p24) protein of HTLV when tested in the Western blotting procedure against reduced and blotted HTLV proteins (Fig. 1). HTLV 6, 7, 8, and 9 also reacted with a 24,000 dalton protein of HTLV under nonreducing conditions (data not shown). A control lane that contained blotted HTLV proteins was negative when similarly incubated with P3X63 ascites (Fig. 1*D*).

To further characterize monoclonal antibodies HTLV 6, 7, 8, and 9, the following criteria were established for reactivity with HTLV gag-encoded p24 internal core proteins: (*a*) Monoclonal anti-p24 antibodies should immunoprecipitate the same p24 HTLV protein that both HTLV$^+$ ATL patient (SD) serum and known polyclonal goat anti-HTLV p24 precipitate; and (*b*) monoclonal anti-p24 antibodies should react with HTLV-infected but not noninfected T cells grown in culture. Thus, preclearing studies were undertaken to compare the reactivities of HTLV 6, 7, 8, and 9 with those of HTLV$^+$ ATL patient (SD) serum and goat antiserum raised against HTLV p24. To preclear HTLV p24

TABLE I

Anti-HTLV P24 Monoclonal Antibodies

	HTLV 6	HTLV 7	HTLV 8	HTLV 9
Clone	6G9	15C8	11G6	6F10
Fusion parent	NS-1	P3X63/Ag8	P3X63/Ag8	NS-1
Immunoglobulin isotype	IgG_1	IgG_1	IgG_2	IgG_1

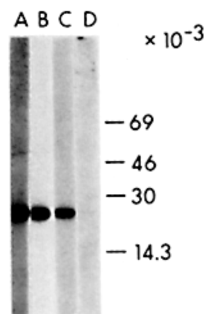

FIGURE 1. Reactivity of monoclonal antibodies HTLV 6, 7, 8, and 9 with p24 of HTLV. SDS-PAGE of disrupted HTLV under reducing conditions and electrophoretic transfer to nitrocellulose were performed as described in Materials and Methods. After electrophoretic transfer of HTLV proteins, nitrocellulose strips were blocked with 3% BSA and incubated with hybridoma supernatants containing monoclonal antibody HTLV 6 (*A*), HTLV 7 (*B*), HTLV 9 (*C*), or supernatant from mouse myeloma cell line P3X63 as negative control (*D*). Binding of monoclonal antibodies to blotted HTLV proteins was evaluated by incubating nitrocellulose strips with ^{125}I-labeled $F(ab')_2$ fragment of sheep anti-mouse IgG + M, followed by washing and autoradiography for 2 h. HTLV 6, 7, and 9 recognized a 24,000 D protein of HTLV. HTLV 8 (not shown) also reacted with a p24 protein of HTLV in the Western blotting technique. ^{14}C molecular weight markers were transferred to the nitrocellulose and used to derive molecular weight estimates.

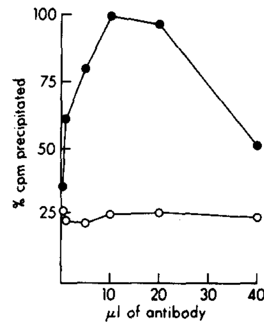

FIGURE 2. Precipitation of ^{125}I-labeled HTLV p24 by monoclonal antibody HTLV 6. To determine the amount of HTLV 6 antibody needed to precipitate the maximum number of counts from ^{125}I-labeled HTLV proteins, varying amounts of HTLV 6 were incubated overnight with 5×10^5 cpm of ^{125}I-labeled HTLV proteins, immune complexes were precipitated with fixed *Staphylococcus aureus*, and pellets were washed and counted on a gamma counter. The titration curve shows that 10 μl of HTLV 6 (●) was needed to precipitate the maximum amount of radiolabeled HTLV proteins. Ascites fluid from mouse myeloma cell line P3X63 (○) was used as a negative control.

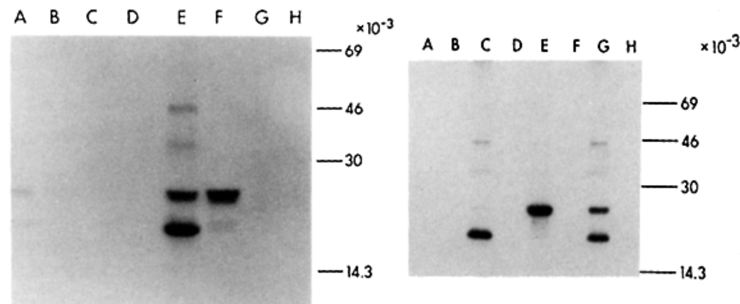

FIGURE 3. Monoclonal antibody HTLV 6 and antibodies in HTLV-associated ATL patient serum reacted with the same p24 protein of HTLV. Preclearing experiments were performed as described in Materials and Methods. Samples containing 5×10^5 cpm of ^{125}I-labeled HTLV proteins were first precleared with HTLV ATL patient SD serum and then radioimmunoprecipitated (RIP) with HTLV 6 (*left*) or precleared first with HTLV 6 and then precipitated with patient serum (*right*). Preclearing with patient serum removed the p24 protein recognized by HTLV 6 (*left, B*), while preclearing with normal human serum (NHS) did not affect precipitation of p24 by HTLV 6 (*F*). (*Left*) Other controls were: (*A*) preclearing with patient serum, RIP with same; (*C*) preclearing with patient serum, RIP with NHS; (*D*) preclearing with patient serum, RIP with P3X63 ascites fluid; (*E*) preclearing with NHS, RIP with patient serum; (*G*) preclearing with NHS, RIP with NHS; (*H*) preclearing with NHS, RIP with P3X63 ascites fluid. (*Right*) Preclearing with HTLV 6 removed the HTLV p24 recognized by patient serum (*C*) while preclearing with P3X63 ascites fluid did not affect precipitation of p24 by HTLV ATL patient serum (*G*). Other controls are: (*A*) preclearing with HTLV 6, RIP with same; (*B*) preclearing with HTLV 6, RIP with P3X63; (*D*) preclearing with P3X63, RIP with NHS; (*F*) preclearing with P3X63 and RIP with same; (*H*) preclearing with NHS and RIP with same.

protein optimally from ^{125}I-labeled disrupted HTLV with all polyclonal goat, patient, or murine monoclonal antibodies assayed, titration curves were first derived by precipitating radiolabeled HTLV proteins with varying amounts of antibody plus SAC. Fig. 2 shows a representative precipitation curve derived with monoclonal antibody HTLV-6. The amount of HTLV 6 ascites (10 μl) that precipitated the maximum amount of ^{125}I-labeled p24 was used in subsequent

preclearing studies. Similar titration curves were generated using SD serum, goat anti-p24, and HTLV 7, 8, and 9.

To determine whether SD serum and HTLV 6 precipitated the same 24,000 dalton protein, ^{125}I-labeled HTLV samples (5 × 10^5 cpm/sample) were extensively precleared by precipitation with SD serum and SAC. Precleared HTLV samples were then precipitated with HTLV 6 plus SAC, and immunoprecipitates were compared with those obtained with control antibodies on a 10% polyacrylamide gel (Fig. 3, *left*). Preclearing of HTLV samples with SD serum removed most of the p19 and p24 proteins (lane *A*) and also removed the p24 protein recognized by HTLV 6 (lane *B*). In contrast, after HTLV samples were precleared with normal human serum, SD serum precipitated HTLV p19 and p24 and other proteins (lane *E*), while HTLV 6 precipitated p24 (lane *F*). Control precipitation experiments are shown in lanes *C*, *D*, *G*, and *H* (see figure legend). When ^{125}I-HTLV samples were first precleared with HTLV 6 and then immu-

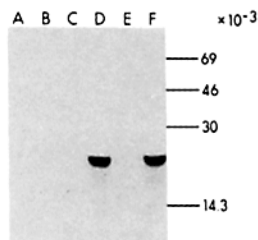

FIGURE 4. Monoclonal antibodies HTLV 6 and 7 each reacted with the same p24 of HTLV. Preclearing experiments were performed as described in Materials and Methods. Samples containing 5 × 10^5 cpm of ^{125}I-labeled HTLV proteins were first precleared with HTLV 6 and then radioimmunoprecipitated with HTLV 7. Preclearing with HTLV 6 removed the p24 protein of HTLV recognized by HTLV 7 (*C*), while preclearing with P3X63 did not inhibit precipitation of p24 by HTLV 7 (*F*). Other controls are: (*A*) preclearing with HTLV 6, RIP with same; (*B*) preclearing with HTLV 6, RIP with P3X63; (*D*) preclearing with P3X63, RIP with HTLV 6; (*E*) preclear with P3X63, RIP with same. HTLV 6 also precleared the p24 protein precipitated by monoclonal antibodies HTLV-8 and HTLV 9 (data not shown).

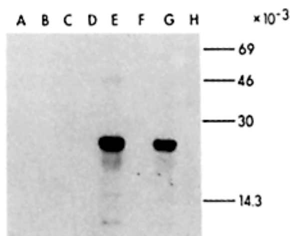

FIGURE 5. Goat antiserum to purified HTLV p24 and HTLV 6 reacted with the same p24 protein of HTLV. Preclearing experiments were performed as described in Materials and Methods. Samples containing 5 × 10^5 cpm of ^{125}I-labeled HTLV proteins were precleared first with goat antiserum to purified p24 (goat anti-p24) and then precipitated (RIP) with HTLV 6. Preclearing with goat anti-p24 removed the p24 band recognized by HTLV 6 (*C*) while preclearing with normal goat serum (NGS) did not inhibit precipitation of p24 by HTLV 6 (*G*). Other controls are: (*A*) preclearing with goat anti-p24, RIP with same; (*B*) preclearing with goat anti-p24, RIP with NGS; (*D*) preclearing with goat anti-p24, RIP with P3X63; (*E*) preclearing with NGS, RIP with goat anti-p24; (*F*) preclearing with NGS, RIP with same; (*H*) preclearing with NGS, RIP with P3X63.

noprecipitated with SD serum (Fig. 3, *right*), the p24 band was selectively removed (lane *C*) from those proteins immunoprecipitated by SD serum (lane *G*), confirming that antibody HTLV 6 precipitated the HTLV p24 recognized by patient serum antibodies. Additional controls are shown in lanes *A*, *B*, *D*, *F*, and *H* (see figure legend).

The reactivity of HTLV 6 was then compared with reactivities of HTLV 7 in preclearing experiments shown in Fig. 4. Preclearing of radiolabeled HTLV proteins with HTLV 6 removed the p24 protein recognized by HTLV 7 (Fig. 4*C*), while preclearing with control P3X63/Ag8 ascites did not remove the p24 protein recognized by HTLV 7 (Fig. 4*F*). In similar experiments, HTLV 6 also precleared the p24 protein recognized by HTLV 8 and HTLV 9, indicating that monoclonal antibodies HTLV 6, 7, 8, and 9 all react with the same p24 protein.

To further demonstrate that HTLV 6 reacts with the p24 internal core protein of HTLV, preclearing experiments were undertaken using goat antiserum raised to purified HTLV p24. As shown in Fig. 5, the goat antiserum to p24 precleared the p24 protein recognized by HTLV 6 (Fig. 5*C*), whereas preclearing with the normal goat serum did not affect the subsequent precipitation of p24 by HTLV 6 (Fig. 5*G*). In contrast, preclearing with monoclonal antibody 12/1-2 directed against the p19 protein of HTLV did not affect the sequential precipitation of p24 by HTLV 6, and preclearing with HTLV 6 did not inhibit precipitation of the p19 protein by monoclonal antibody 12/1-2 (data not shown).

To summarize the results of the preclearing experiments, monoclonal antibodies HTLV 6, 7, 8, and 9, goat anti-p24 antiserum and HTLV$^+$ ATL patient serum all reacted with the same p24 protein of HTLV.

Antigenic Sites on HTLV p24 Recognized by HTLV 6, 7, 8, and 9 and by HTLV$^+$ Patient Sera. To determine whether or not HTLV 6, 7, 8, and 9 recognize the same antigenic site or proximal sites on the p24 molecule, HTLV 6, 7, 8, and 9 were purified over protein A-Sepharose 4B columns, radiolabeled, and allowed to compete with unlabeled HTLV 6, 7, 8, and 9 for binding to HTLV p24 in a competitive inhibition plate assay (Fig. 6*A–D*). As controls, competition experiments performed with dilutions of unlabeled P3X63 ascites fluid and ^{125}I-labeled HTLV 6, 7, 8, and 9 were used to estimate 100% binding in the plate assay. First, monoclonal antibodies HTLV 6 and 7, and to a lesser extent HTLV 8, inhibited the binding of ^{125}I-labeled HTLV 6 to HTLV p24 in a dose-dependent manner, while HTLV 9 did not inhibit binding (Fig. 6*A*). We concluded that HTLV 6, 7, and possibly HTLV 8 recognized either the same or closely proximal antigenic sites on HTLV p24. Next, in the same assay, unlabeled HTLV 6, 7, and 8 each inhibited the binding of ^{125}I-HTLV 7 (Fig. 6*B*) and ^{125}I-HTLV 8 (Fig. 6*C*) to HTLV p24, thus confirming that HTLV 6, 7, and 8 recognize either the same or closely proximal antigenic sites on p24. Neither HTLV 6 nor 7 inhibited the binding of ^{125}I-HTLV 9 to p24, although HTLV 8 partially inhibited ^{125}I-HTLV 9 binding (Fig. 6*D*). However, since unlabeled HTLV 9 did not inhibit the binding of ^{125}I-HTLV 6, 7, or 8 to HTLV p24 (Fig. 6*A–C*), we concluded that HTLV 9 recognizes a different site than the site or sites recognized by HTLV 6, 7, and 8. Thus, at least two antigenic sites on HTLV p24 were identified by HTLV 6, 7, 8, and 9: site A, which appears to react only

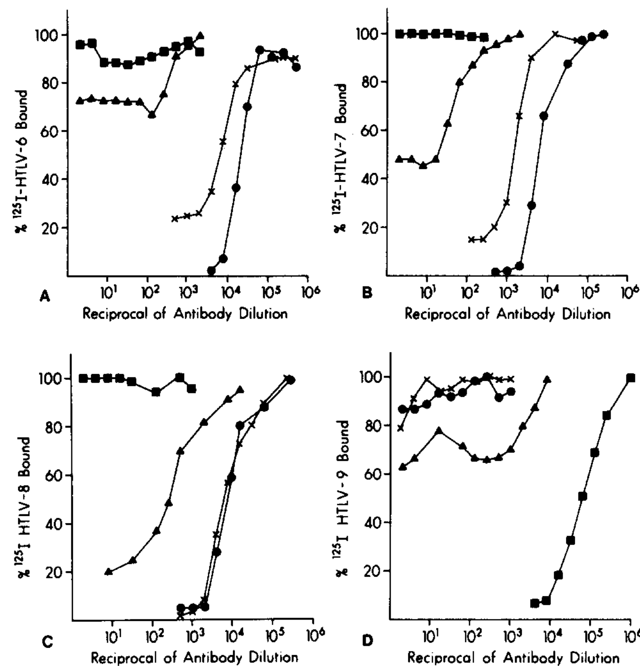

FIGURE 6. Monoclonal antibodies HTLV 6, 7, 8, and 9 defined at least two distinct antigenic sites on HTLV p24. Competitive inhibition studies were performed as described in Materials and Methods. Briefly, duplicate microtiter wells precoated with HTLV proteins were incubated with dilutions of unlabeled HTLV 6, 7, 8, and 9, washed, and then further incubated with 10^5 cpm of iodinated HTLV 6, 7, 8, and 9. Wells were washed and the percent binding of radiolabeled antibody was evaluated. Control wells preincubated with dilutions of P3X63 or unlabeled HTLV 6, 7, 8, and 9 at 1:100 dilution were used as controls to estimate 100 and 0% binding, respectively (HTLV 6 [●], HTLV 7 [x], HTLV 8 [▲], HTLV 9 [■]). (*A*) Binding of ^{125}I-HTLV 6 to HTLV p24 is inhibited by unlabeled HTLV 6 and 7 and by HTLV 8 at low dilutions, but not by HTLV 9. (*B*) Binding of ^{125}I-HTLV 7 to HTLV p24 is inhibited by unlabeled HTLV 6, 7, and 8, but not by HTLV 9. (*C*) Binding of ^{125}I-HTLV 8 to p24 is inhibited by unlabeled HTLV 6, 7, and 8 but not by HTLV 9. (*D*) Binding of ^{125}I-HTLV 9 to HTLV p24 is inhibited by unlabeled HTLV 9 and by HTLV 8 at low dilutions, but not by HTLV 6 or 7. Since unlabeled HTLV 9 does not block the binding of ^{125}I-HTLV 6, 7, or 8 to HTLV p24, it appears that HTLV 9 recognizes an antigenic site different from the site or sites recognized by HTLV 6, 7, and 8. Also, HTLV 6, 7, and 8 each inhibit the binding of ^{125}I-HTLV 6, 7, and 8 to p24 and thus appear to recognize either the same antigenic site or proximal antigenic sites on the p24 molecule.

with antibody HTLV 9, and an additional antigenic site (site[s] B) identified by antibodies HTLV 6, 7, and 8.

To determine whether or not HTLV$^+$ patient sera or other human sera positive for anti-HTLV antibodies recognize the same sites on HTLV p24 identified by monoclonal antibodies HTLV 6, 7, 8, and 9, human sera were used to inhibit the binding of ^{125}I-HTLV 6, 7, 8, and 9 to HTLV p24 in a competitive binding assay. As shown in Table II, HTLV$^+$ ATL or HTLV$^+$ cutaneous T cell lymphoma patient serum partially inhibited the binding of ^{125}I-labeled HTLV 6, 7, 8, and 9 to HTLV p24. In addition, two sera obtained from clinically normal HTLV-infected family members of HTLV$^+$ patients contained antibodies to HTLV p24 that also inhibited the binding of ^{125}I-HTLV 6, 7, 8, and 9 to p24.

TABLE II

Inhibition of Binding of ^{125}I-labeled HTLV 6, 7, 8, and 9 to HTLV P24 by HTLV$^+$ Human Sera

HTLV$^+$ human sera	Percent inhibition of ^{125}I-labeled monoclonal antibody binding to HTLV p24*			
	HTLV 6	HTLV 7	HTLV 8	HTLV 9
SD[‡]	27	23	ND	30
WA[§]	40	27	28	27
No. 1[‖]	60	44	34	28
No. 2[‖]	69	57	56	47
Pooled normal serum	0	0	0	0

* 100% inhibition obtained with 1:100 dilutions of unlabeled HTLV 6, 7, 8, or 9 and 0% inhibition obtained with pooled normal human serum from four subjects. All HTLV$^-$ control and HTLV$^+$ human sera were used at 1:2 dilution. ND, not done.
‡ HTLV$^+$ Japanese ATL patient serum.
§ HTLV$^+$ American cutaneous T cell lymphoma patient serum.
‖ Sera from clinically normal HTLV-infected family members of HTLV$^+$ patients.

TABLE III

*Reactivity of Anti-p24 Monoclonal Antibodies with Human Cells**

Cell line or source[‡]	Percent positive			
	HTLV 6	HTLV 7	HTLV 8	HTLV 9
HUT 102 HTLV$^+$ T cell	85	80	80	90
SD Japanese ATL PBL after culture[§]	100	100	100	100
C3-44/MO HTLV$_{II}^+$ cells[‖]	21	98	100	99
Fresh normal PBL	0	0	0	0
Fresh normal PBL after 4 d in culture[§]	0	0	0	0
Normal PBL after Con A (50 μg/ml) activation[¶]	0	0	0	0
HSB-2 HTLV$^-$ T cell	0	0	0	0
EB-3 B cell	0	0	0	0
HUT 78 HTLV$^-$ T cell**	0	0	0	0

* Reactivity of monoclonal antibodies determined by indirect immunofluorescence on acetone-fixed cytocentrifuge preparations of cells.
§ Japanese ATL cells cultured 5 d in 25% crude T cell growth factor. Fresh PBL cultured 4 d in media.
¶ Normal PBL cultured 4 d in Con A (50 μg/ml).
** HUT 78 cells are p19 (15) and p24 (11) negative.
‡ Other tissues tested in indirect immunofluorescence assays with HTLV 6, 7, 8, and 9 and found to be negative: thymus, lymph node, tonsil, spleen, kidney, liver, pancreas, adrenal, brain cortex and medulla oblongata, adenocarcinoma of lung and prostate, melanoma, breast carcinoma (3), and infiltrating T cells in skin from HTLV$^+$ ATL patient SD.
‖ C3-44/MO is a T cell cord blood line infected with HTLV$_{IIMO}$, a new type of HTLV (25).

In contrast, pooled normal human serum did not inhibit binding of ^{125}I-HTLV 6, 7, 8, and 9 to p24. Thus, anti-p24 antibodies in HTLV$^+$ human sera reacted with antigenic sites on p24 that are also recognized by HTLV 6, 7, 8, and 9.

Tissue and Cellular Specificity of Anti-HTLV p24 Monoclonal Antibodies. Table III demonstrates that all four anti-p24 monoclonal reagents reacted only with the HTLV$^+$ malignant T cells tested and did not react with HTLV$^-$ T cells, fresh normal PBL, B cell lines, mitogen-activated normal T cells, or any of a large panel of normal human tissues. Also, HTLV 6, 7, 8, and 9 reacted with acetone-

fixed cord blood T cells (C3-44/Mo) that were infected with $HTLV_{II}$, a new type of HTLV (25). Thus, the antigenic sites of HTLV p24 identified by these antibodies are conserved in a cell line infected by $HTLV_{II}$.

When treated with HTLV 6, 7, 8, and 9 in an indirect immunofluorescence assay, acetone-fixed HUT-102 HTLV-infected T cells demonstrated positive fluorescence localized in discrete patches at or near the cell surface or at the interface of cell to cell contact (arrows, Fig. 7). Also, 5% of HUT 102 cells appeared to contain intracytoplasmic fluorescence (arrowhead, Fig. 7). Type C virus particles were frequently seen between adjacent HUT 102 cells by electron microscopy (10) (arrows, Fig. 8) and probably account for the intense anti-p24 fluorescent staining localized between adjacent HUT-102 cells (Fig. 7). In contrast, none of the anti-p24 monoclonal antibodies reacted with cell surface antigens on viable non–acetone-fixed HUT-102 cells in suspension, as shown by the fluorescence-activated cell sorter histogram in Fig. 9. These data are compatible with the notion that p24 is an internal viral core protein and suggest that p24 is sequestered in budding virus particles at or near the surface of most HUT-102 cells.

Discussion

In this study we report the production and diagnostic use of four monoclonal antibodies to disrupted HTLV, HTLV 6, 7, 8, and 9, all of which reacted with the p24 internal core protein of HTLV. All four monoclonal antibodies reacted with the same p24 HTLV protein. Moreover, these antibodies all reacted with the same p24 HTLV protein recognized by antibodies in $HTLV^+$ ATL patient (SD) serum and by polyclonal goat antisera to purified HTLV p24 internal core

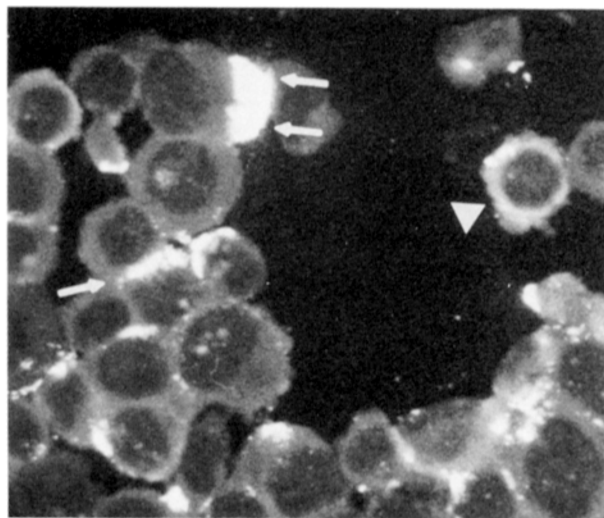

FIGURE 7. Immune fluorescence of $HTLV^+$ acetone-fixed HUT-102 cells using monoclonal antibody HTLV 6. HTLV p24 was localized in discrete polar patches at or near the cell surface and between adjacent cells (arrows). Also, 5% of cells had intracytoplasmic anti-p24 fluorescence (arrowheads). Similar fluorescent staining of HUT 102 cells was also seen with HTLV 7, 8, and 9 (not shown). × 400.

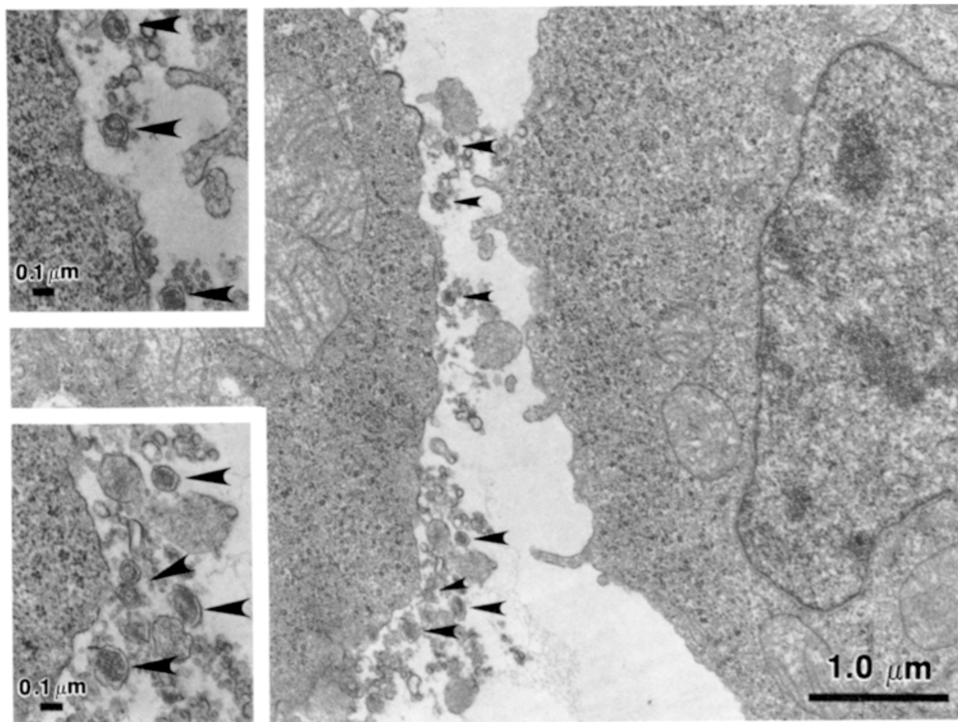

FIGURE 8. Electron micrographs of HTLV from HUT-102 cells. Electron micrograph of adjacent HUT 102 cells showing extracellular HTLV particles (arrows) between cells. Inserts: High magnification of extracellular particles (arrows) between adjacent HUT-102 cells.

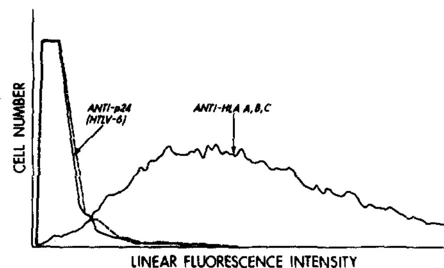

FIGURE 9. Fluorescence-activated cell sorter histogram of nonreactivity of monoclonal antibody HTLV 6 on viable HTLV$^+$ HUT 102 cells, showing negative cell surface fluorescence. HUT 102 T cells were positive when stained for the presence of nonpolymorphic HLA with monoclonal antibody 3F10. P3X63 ascites fluid (– – –) was used as a negative control.

protein. Screening of HTLV 6, 7, 8, and 9 against a comprehensive panel of normal, neoplastic, and virus-infected human tissues and cells indicated that these monoclonal antibodies selectively identify only those cells that are infected with HTLV and express mature virions. A previous study (15) with monoclonal antibody 12/1–2 directed against the p19 protein of HTLV demonstrated that antibody 12/1–2 react with an antigenic determinant on normal non–HTLV-infected human endocrine thymic epithelium and on the basal layer of keratinocytes in squamous epithelium (15). In contrast, the reactivity of monoclonal

anti-HPLV p24 antibodies HTLV 6, 7, 8, and 9 is restricted to T cells infected with HTLV.

In competitive inhibition studies, HTLV 6, 7, and 8 recognized either the same antigenic site or proximal antigenic sites on HTLV p24, while HTLV 9 reacted with a unique site distinct from the site or sites recognized by HTLV 6, 7, and 8. Also, HTLV 6, 7, 8, and 9 reacted with fixed cord blood T (C3-44/Mo) cells that were infected with $HTLV_{II}$, a human type C retrovirus reported to be a new type of HTLV (25). Thus, the HTLV p24 antigenic sites identified by these antibodies are conserved in a cell line infected by $HTLV_{II}$ and are likely to be associated with the p24 of $HTLV_{II}$. In addition, the antigenic determinants of p24 detected by our monoclonal antibodies are immunogenic in man, since $HTLV^+$ patient sera blocked the binding of ^{125}I-labeled HTLV 6, 7, 8, and 9 to HTLV p24 in a competitive RIA.

Using anti-HTLV p24 antibodies, we have shown that HTLV p24 is localized at or near the cell surface of 95% of acetone-fixed $HTLV^+$ HUT 102 cells. The p24 antigen was not detectable on the cell surface of viable $HTLV^+$ cells, as demonstrated by data obtained with indirect fluorescence techniques and cell sorter analysis (Fig. 9). Polar areas of $HTLV^+$ T cells and areas between adjacent HUT 102 cells were strongly p24 positive by indirect immunofluorescence (Fig. 7); these are areas where HTLV virions frequently are demonstrated with electron microscopy (10) (Fig. 8). These data suggest that HTLV p24 is sequestered in the virus particles budding from the surface of the HTLV-infected cells. In addition, monoclonal antibodies HTLV 6, 7, 8, and 9 and 12/1-2 (anti-HTLV p19) infrequently (5) react with diffuse cytoplasmic components of $HTLV^+$ T cells. This may indicate that the precursor molecule encoded by the gag region of HTLV undergoes posttranslational modification at or near the cell membrane, thereby giving rise to the p19 and p24 epitopes defined by these monoclonal antibodies (26). Finally, the use of directly fluoresceinated anti-HTLV p24 monoclonal antibodies in a one-step fluorescent assay on 5-d cultured PBL appears to be a simple and highly specific test for HTLV-infected cells.

It is hoped that these monoclonal antibodies will be useful diagnostic probes to detect HTLV-infected cells and to discriminate between the subtypes of HTLV associated with adult T cell leukemia, the T cell variant of hairy cell leukemia, and other diseases potentially associated with HTLV or variants of HTLV, such as the acquired immunodeficiency syndrome.

Summary

Four monoclonal antibodies, human T cell leukemia-lymphoma virus (HTLV) 6, 7, 8, and 9, which react with the 24,000 dalton internal core protein of $HTLV_I$, have been developed. These monoclonal antibodies reacted with only HTLV-infected cells and not with a broad spectrum of normal, neoplastic, mitogen-stimulated, or virus-infected cells and tissues. HTLV 6, 7, 8, and 9 identified at least two different antigenic determinants on HTLV p24 that were also recognized by antibodies present in $HTLV^+$ patient sera. Monoclonal antibodies HTLV 6, 7, 8, and 9 reacted in indirect immunofluorescence assays with HTLV p24 localized at the cell surface of 5-d cultures of HTLV-infected T cells and, as well, reacted with T cells infected with $HTLV_{II}$, a new type of

HTLV isolated from a patient (MO) with a T cell variant of hairy cell leukemia. Thus, HTLV 6, 7, 8, and 9 should prove to be useful diagnostic reagents in the identification of HTLV- and $HTLV_{II}$-infected T cells.

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