

## Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma

(mycosis fungoides/T-cell growth factor/RNA tumor virus/reverse transcriptase)

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**ABSTRACT** Retrovirus particles with type C morphology were found in two T-cell lymphoblastoid cell lines, HUT 102 and CTCL-3, and in fresh peripheral blood lymphocytes obtained from a patient with a cutaneous T-cell lymphoma (mycosis fungoides). The cell lines continuously produce these viruses, which are collectively referred to as HTLV, strain CR (HTLV<sub>CR</sub>). Originally, the production of virus from HUT 102 cells required induction with 5-iodo-2'-deoxyuridine, but the cell line became a constitutive producer of virus at its 56th passage. Cell line CTCL-3 has been a constitutive producer of virus from its second passage in culture. Both mature and immature extracellular virus particles were seen in thin-section electron micrographs of fixed, pelleted cellular material; on occasion, typical type C budding virus particles were seen. No form of intracellular virus particle has been seen. Mature particles were 100-110 nm in diameter, consisted of an electron-dense core surrounded by an outer membrane separated by an electron-lucent region, banded at a density of 1.16 g/ml on a continuous 25-65% sucrose gradient, and contained 70S RNA and a DNA polymerase activity typical of viral reverse transcriptase (RT; RNA-dependent DNA nucleotidyltransferase). Under certain conditions of assay, HTLV<sub>CR</sub> RT showed cation preference for Mg<sup>2+</sup> over Mn<sup>2+</sup>, distinct from the characteristics of cellular DNA polymerases purified from human lymphocytes and the RT from most type C viruses. Antibodies to cellular DNA polymerase  $\gamma$  and antibodies against RT purified from several animal retroviruses failed to detectably interact with HTLV<sub>CR</sub> RT under conditions that were positive for the respective homologous DNA polymerase, demonstrating a lack of close relationship of HTLV<sub>CR</sub> RT to cellular DNA polymerases  $\gamma$  or RT of these viruses. Six major proteins, with sizes of approximately 10,000, 13,000, 19,000, 24,000, 42,000, and 52,000 daltons, were apparent when doubly banded, disrupted HTLV<sub>CR</sub> particles were chromatographed on a NaDodSO<sub>4</sub>/polyacrylamide gel. The number of these particle-associated proteins is consistent with the expected proteins of a retrovirus, but the sizes of some are distinct from those of most known retroviruses of the primate subgroups.

Retroviruses are involved in the cause of some leukemias, lymphomas, and sarcomas in various animal species (1, 2). Although some studies have suggested the presence of retrovirus information in some human tissues (3, 4), the detection and isolation of complete, well-defined particles from humans has proven exceedingly difficult. The recent development of continuously growing cell lines from patients with various neoplastic diseases, and in some cases from normal tissues, has led to reports of retroviruses in some of them (5-9). Most of these isolates are very closely related to previously isolated type C primate viruses (5-7), making interpretations of their origin difficult. The detection of retrovirus particles from some novel

cell lines from patients with diffuse histiocytic lymphoma has been recently described (8) and, although the DNA polymerase from these particles is also related to the reverse transcriptase (RT; RNA-dependent DNA nucleotidyltransferase) of type C primate viruses [simian sarcoma virus-gibbon ape leukemia virus (SSV-GALV) group], peptide maps of the purified DNA polymerases show significant differences between them (10, 11).

A new development in the culture of human hematopoietic cells is the growth of human T lymphoblasts made possible by the use of T-cell growth factor (TCGF) (12, 13). By use of TCGF, a number of normal T-cell lines and T-cell lines derived from patients with various T-cell neoplasms have been established (13-15). The data reported in this paper concern the separate detection of retroviruses from fresh blood cells and from two T-cell lines established a year apart from a patient with a cutaneous T-cell lymphoma (mycosis fungoides). The cell lines maintain the continuous release of these viruses.

### MATERIALS AND METHODS

**Case History.** C.R. was a 28-yr-old black man referred to the National Cancer Institute-Veterans Administration Oncology Branch in May 1978 with a diagnosis of cutaneous T-cell lymphoma (mycosis fungoides) (16). He had no known unusual exposure to identifiable chemical carcinogens, no family history of leukemia or lymphoma, and no history suggestive of immune deficiency. Beginning in July 1977, he developed skin nodules over his body. Examination of cells from his peripheral blood, skin biopsy, lymph node biopsy, and metatarsal bone biopsy revealed malignant convoluted T cells. A T-lymphoblast cell line, HUT 102, was established from tumor cells derived from the lymph node biopsy. He was treated with concurrent whole-body electron-beam radiation therapy and combination chemotherapy (17), and had an apparent complete remission. In January 1979, his disease recurred with widespread systemic involvement. His therapy was switched to administration of cyclophosphamide, hydroxydaunomycin, vincristine, and the epipodophylotoxin, VP-16. He again attained a complete remission, but in May 1979, he was admitted for pneumonia. A blood cell sample was obtained at this time and used to establish a second T-cell line, CTCL-3, as described below. Soon, he

Abbreviations: TCGF, T-cell growth factors; HTLV<sub>CR</sub>, human cutaneous T-cell lymphoma virus (strain CR); IdUrd, 5-iodo-2'-deoxyuridine; RT, reverse transcriptase (RNA-dependent DNA nucleotidyltransferase); SSV(SSAV), simian sarcoma virus-simian sarcoma virus-associated virus; GALV<sub>H</sub>, gibbon ape leukemia virus (strain Hall's Island); MuLV<sub>R</sub>, murine leukemia virus (strain Rauscher); AMV, avian myeloblastosis virus; RD-114, feline endogenous virus.

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developed new pulmonary infiltrates and brain lesions, and died in August 1979.

**Establishment and Characterization of Cell Lines.** Cell line HUT 102 was derived from the patient's lymph node biopsy; CTCL-3 was established a year later from his peripheral blood cells. Both have been fully characterized (14, 15). HUT 102 initially required the presence of crude lymphocyte-conditioned medium containing TCGF (14), but after several passages it could be grown in RPMI-1640 medium containing 20% (vol/vol) heat-inactivated fetal calf serum. The growth of CTCL-3 (15) was initiated with partially purified TCGF (18) and remains dependent on added TCGF. Both cell lines are erythrocyte (E)-rosette positive and negative for surface immunoglobulin, Epstein-Barr virus nuclear antigen, terminal deoxyribonucleotidyltransferase, and mycoplasma contamination. Karyotypes of both cell lines have been routinely performed on selected passages by published procedures (19).

**Detection and Production of Type C Virus.** Cultured or fresh cells were grown at a concentration of  $10^6$  cells per ml in RPMI-1640 medium and 20% heat-inactivated fetal calf serum. Partially purified TCGF (18) was also added to the CTCL-3 cultures (15). Cell suspensions were centrifuged at  $1000 \times g$  for 10 min, the supernatant was removed for viral DNA polymerase assays, and the cell pellet was examined for virus by thin-section electron microscopy as described (15). The fetal calf serum was free of retrovirus particles, as determined by DNA polymerase assays and electron microscopic examination of material pelleted from 1 liter of fetal calf serum by polyethylene glycol precipitation.

Large volumes (20 liters) of cell suspensions were used for characterization of the virus particles and for analysis of the DNA polymerase and other viral proteins. These methods are given in the legend to Fig. 3.

**DNA Polymerase Assays.** Virus particles were precipitated from cell-free supernatant as follows: 0.3 ml of 4 M NaCl and 3.6 ml of 30% (wt/vol) polyethylene glycol (Carbowax 6000) were added to 7.5 ml of particle-containing medium and the suspension was placed on ice for 2 hr. The suspension was centrifuged in a Sorvall RC-3 centrifuge at 2100 rpm at  $4^\circ\text{C}$  for 45 min. The precipitate was resuspended in 200  $\mu\text{l}$  of 50% (vol/vol) glycerol/25 mM Tris-HCl, pH 7.5/5 mM dithiothreitol/50 mM KCl/0.025% Triton X-100. Virus particles

were disrupted by addition of 100  $\mu\text{l}$  of 0.9% Triton X-100/1.5 M KCl (solution 2). DNA polymerase assays were performed at  $37^\circ\text{C}$  for 1 hr with a 10- $\mu\text{l}$  aliquot of the disrupted virus solution in a final volume of 100  $\mu\text{l}$  containing 40 mM Tris-HCl (pH 7.8), 4 mM dithiothreitol, 45 mM KCl, and 50  $\mu\text{g}$  of template-primer poly(A)-dT<sub>12-18</sub> and poly(C)-dG<sub>12-18</sub> per ml (with 10 mM Mg<sup>2+</sup>) or 50  $\mu\text{g}$  of poly(dA)-dT<sub>12-18</sub> per ml (with 0.25 mM Mn<sup>2+</sup>). The mixture also contained 15  $\mu\text{M}$  of the appropriate labeled deoxyribonucleoside triphosphates, [<sup>3</sup>H]dTTP (16 Ci/mmol; 1 Ci =  $3.7 \times 10^{10}$  becquerels) or [<sup>3</sup>H]dGTP (12 Ci/mmol). The peak activities were also assayed with various Mg<sup>2+</sup> and Mn<sup>2+</sup> concentrations with each template-primer, and this activity was compared with that of SSV(simian sarcoma virus-associated virus) [SSV(SSAV)] RT and human DNA polymerases  $\alpha$  and  $\gamma$ .

**Cellular and Viral DNA Polymerases and Antisera.** Human DNA polymerase  $\alpha$  and  $\gamma$  were purified from NC37 B-lymphoblast cells (20). RT was purified by described methods with final chromatography on a poly(U)-Sepharose column (21) from the following viruses: SSV(SSAV) grown in 71AP1 marmoset fibroblasts; feline endogenous virus (RD-114) grown in RD human cells; Rauscher murine leukemia virus (MuLV<sub>R</sub>) grown in JLSV-9 mouse fibroblasts; GALV (strain Hall's Island) (GALV<sub>H</sub>) grown in the original gibbon leukemic cell line (6G-1) (22); and avian myeloblastosis virus (AMV) from plasma virus

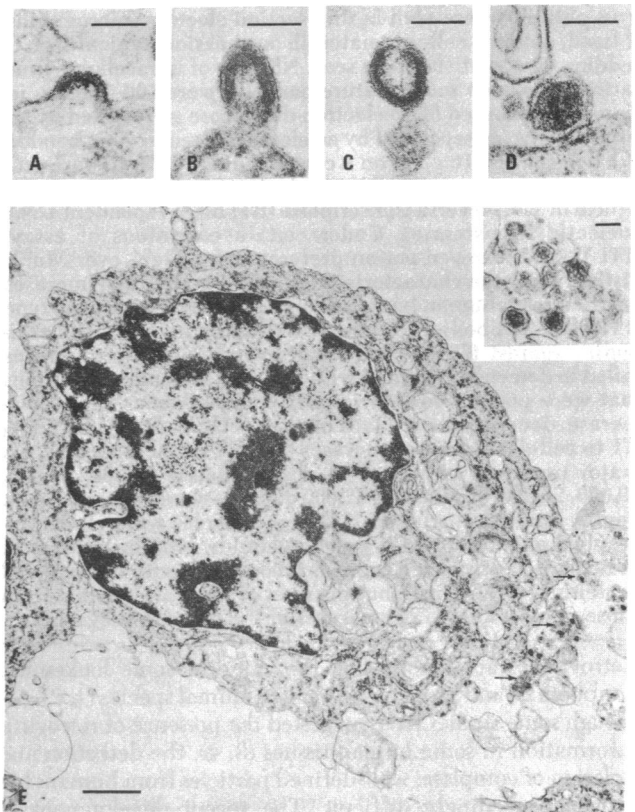


FIG. 2. Thin-section electron micrographs of budding HTLV<sub>CR</sub> particles seen in pelleted HUT 102 cells. The methods have been described (15). (A) Early viral bud; (B) late viral bud with nearly completed nucleoid (specimen was treated with tannic acid prior to chrome osmium); (C) "immature" extracellular virus particle (top) with incomplete condensation of the nucleoid; (D) "mature" extracellular virus particle with condensed, centrally located nucleoid surrounded by an outer membrane separated by an electron-lucent area; (E) HUT 102 cell 72 hr after induction by IdUrd. Many mature virus particles are found in the extracellular space in clumps associated with cellular debris (Inset). (Bars in A-D and Inset = 100 nm; bar in E = 1000 nm.)

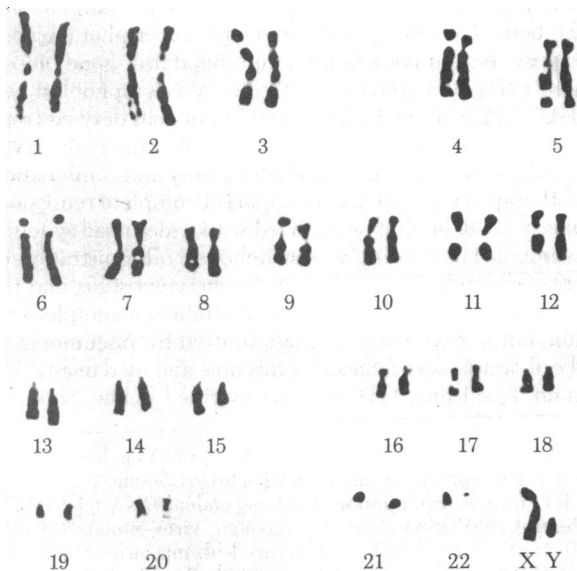


FIG. 1. Karyotype analysis of HUT 102 cells, passage 60 (46 chromosomes, XY, - chromosome 22, + minute chromosome).

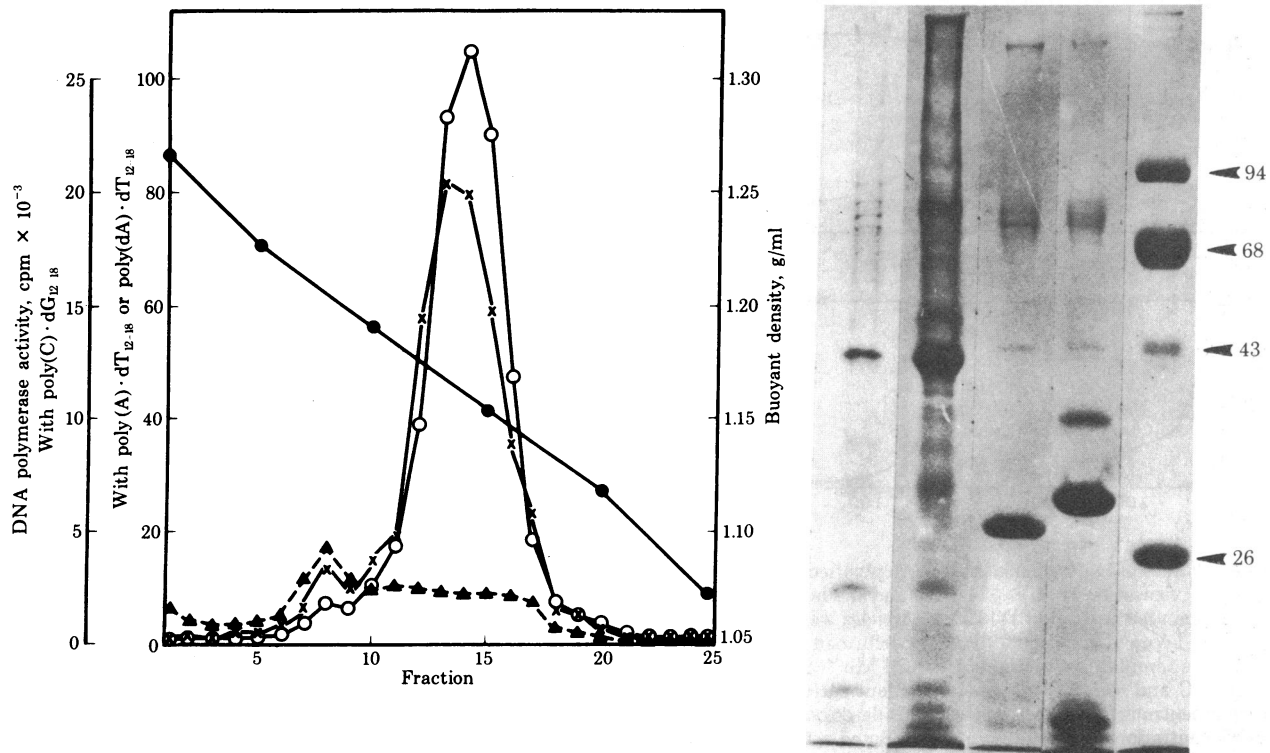


FIG. 3. DNA polymerase activities (Left) and protein profiles (Right) of concentrated, doubly banded, disrupted HTLV<sub>CR</sub> particles. For these experiments, large volumes (20 liters) of cell suspensions were centrifuged at 1000 × g for 10 min. The supernatant was kept on ice and the virus particles were subsequently concentrated on a RNase-free sucrose gradient (22–65%) by zonal centrifugation on a model K ultracentrifuge (25). Fractions (100 ml) were collected; those with buoyant densities of 1.19–1.12 g/ml were pooled, diluted 1:1 with 10 mM Tris-HCl, pH 7.4/0.1 M NaCl/1 mM EDTA (TNE buffer), and centrifuged in a type 19 rotor at 18,000 rpm for 2 hr. The pelleted material was resuspended in TNE buffer, assayed for DNA polymerase activity, and examined by negative-stain electron microscopy. The resuspended particles were then pelleted through 30% glycerol in TNE buffer onto a 100% glycerol cushion by centrifugation in a SW 41 rotor at 30,000 rpm for 2 hr. This pelleted material was again resuspended in TNE buffer and applied to a 10-ml continuous sucrose gradient (22–65%). The gradient was collected, 10-μl samples from each fraction were assayed for DNA polymerase activity, and electron microscopy was performed on negatively stained samples as described [ref. 22 and Kramarsky, B. (1979) *Annu. Meeting Am. Soc. Microbiol.*, p. 297, Abstr. S(H)18]. (Left) Activities of DNA polymerase were assayed on 25 fractions as described in *Materials and Methods* except that in these and other assays using large volumes of medium, polyethylene glycol precipitation was not needed and, to each 10-μl sample, 5 μl of solution 2 was added to disrupt viral particles. O, Poly(A)-dT<sub>12-18</sub> and 10 mM Mg<sup>2+</sup>; X, poly(C)-dG<sub>12-18</sub> and 10 mM Mg<sup>2+</sup>; ▲, poly(dA)-dT<sub>12-18</sub> and 0.5 mM Mn<sup>2+</sup>. Fractions with peak activity with poly(A)-dT<sub>12-18</sub> were also assayed for terminal deoxyribonucleotidyltransferase activity as described (26) and were always negative. ●, Density determined by refractometry. (Right) Coomassie blue-stained NaDodSO<sub>4</sub>/polyacrylamide slab gel of doubly banded, disrupted HTLV<sub>CR</sub>, SSV(SSAV), and MuLV<sub>R</sub>. Lane 1, IdUrd-induced HTLV<sub>CR</sub>; lane 2, constitutively produced HTLV<sub>CR</sub>; lane 3, SSV(SSAV); lane 4, MuLV<sub>R</sub>; lane 5, molecular weight markers (shown ×10<sup>-3</sup>): phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen (26,000).

supplied by Joseph Beard. Detailed descriptions of the antibodies to DNA polymerase λ (23) and antibodies to RT of each of the above viruses (21–23) have been described.

**Neutralization and Binding Assays.** Antisera against RT from each of the viruses mentioned above are active in neu-

Table 1. Comparison of various DNA polymerase activities with different template-primers and divalent cations

Source of DNA polymerase	Activity ( <sup>3</sup> H cpm incorporated × 10 <sup>-3</sup> ) with:					
	Poly(A)- (dT) <sub>12-18</sub>		Poly(dA)- (dT) <sub>12-18</sub>		Poly(C)- (dG) <sub>12-18</sub>	
	Mg <sup>2+</sup>	Mn <sup>2+</sup>	Mg <sup>2+</sup>	Mn <sup>2+</sup>	Mg <sup>2+</sup>	Mn <sup>2+</sup>
HTLV <sub>CR</sub>	104	76	2	10	22	10
SSV	2	537	1.5	1	2	60
NC37-α	1	4	12	93	0.5	0.5
NC37-γ	144	411	413	325	0.5	2

Assays were performed with a 10-μl aliquot of the indicated DNA polymerase sample with either 10 mM Mg<sup>2+</sup> or 0.25 mM Mn<sup>2+</sup> as the divalent cation. The Mg<sup>2+</sup> and Mn<sup>2+</sup> concentrations are optimized for HTLV<sub>CR</sub> RT with poly(A)-(dT)<sub>12-18</sub> and are not necessarily the optimal divalent cation concentrations for the other DNA polymerases tested.

tralization assays. The methods used have been reported (23) and are summarized in the legend to Fig. 4. Goat antibodies (IgG) directed against human DNA polymerase γ and against feline leukemia virus RT, which do not neutralize polymerase activity, are active in binding assays as reported (21, 23). We used these assays to compare antigenic relatedness of human cutaneous T-cell lymphoma virus (HTLV) RT to these polymerases.

**Polyacrylamide Gel Electrophoresis.** Doubly banded virus particles (50 μl) were disrupted, electrophoresed on a 12% polyacrylamide/NaDodSO<sub>4</sub> vertical slab gel, and stained with Coomassie blue (24). For comparison, similarly treated, doubly banded SSV and MuLV<sub>R</sub> virus particles were run in the same gel.

## RESULTS

**Detection of Type C Virus (HTLV<sub>CR</sub>) in Cell Line HUT 102.** HUT 102 T lymphoblasts have been grown independent of TCGF since passage 5. Their karyotypes in early passages were hypodiploid, pseudodiploid, and diploid, but the predominant karyotype in late passage has been that of a pseudodiploid human male with a missing chromosome 22 plus a

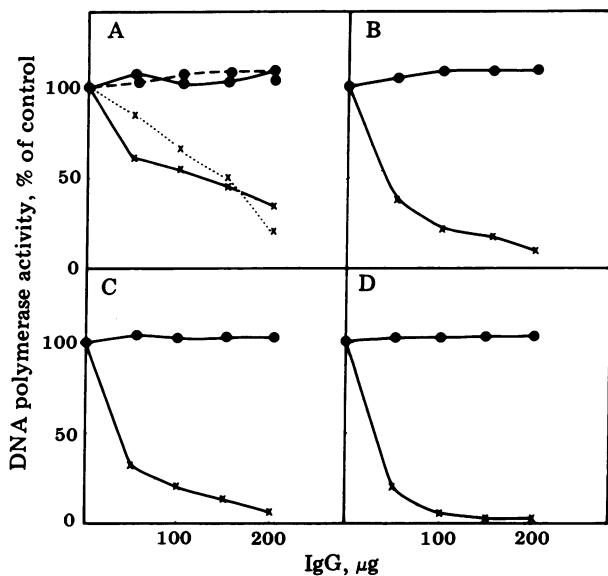


FIG. 4. Effect of neutralizing antisera to purified RT from different type C viruses on HTLV<sub>CR</sub> RT (●) and the homologous RT (X). Double-banded disrupted HTLV<sub>CR</sub> particles were used as the source of HTLV<sub>CR</sub> RT. RT assays were performed with poly(A)-dT<sub>12-18</sub> as the template-primer and 0.25 mM Mn<sup>2+</sup> as the divalent cation in A-C and 5 mM Mg<sup>2+</sup> as the divalent cation in D. Antipolymerase neutralization assays were done as described (23). Briefly, various concentrations of hyperimmune IgG diluted with pre-immune IgG were incubated for 4 hr at 0°C with either RT from HTLV<sub>CR</sub> or RT from the virus used to prepare the antibody (homologous system). Fractions were then removed and assayed for RT activity under optimal conditions. (A) Effect of anti-SSV(SSAV) RT (solid lines) or anti-GALV RT antibodies (broken lines); (B) effect of anti-RD-114 RT antibody; (C) effect of anti-MuLV<sub>R</sub> RT antibody; (D) effect of anti-AMV RT antibody. One hundred percent activity is approximately 50,000 cpm of <sup>3</sup>H. The concentration of immunoglobulin used in A is one-tenth that indicated for the antisera in B-D.

minute (Fig. 1). From passage 4 to passage 50, the production of virus particles required induction with 5-iodo-2'-deoxyuridine (IdUrd). However, constitutive production occurred by passage 56 and has persisted up to and including the current passage 200. Although infrequent, typical type C viruses were observed budding from cell membranes in several samples (Fig. 2 A and B). No forms of intracellular particles have been found. Most particles were present as immature (Fig. 2C) and mature (Fig. 2D) extracellular virions, which sediment in clumps adjacent to the lymphoblasts (Fig. 2E). No particles with eccentric or cylindrically shaped nucleoids were found. The virion shape, morphology on budding, and appearance of centrally located nucleoids in the mature particles suggest that the virus is type C with no evident type D or type B forms. Peak virus budding and subsequent release into the extracellular fluid, as measured by electron microscopy and DNA polymerase activity, occurred 24 hr and 72 hr, respectively, after induction by IdUrd.

**Detection of HTLV<sub>CR</sub> from Fresh Lymphocytes and Cell Line CTCL-3.** Fresh mononuclear cells were obtained from the peripheral blood of patient C.R. 1 yr after establishment of the HUT 102 cell line. When these cells were put into culture with RPMI medium containing fetal calf serum and induced with IdUrd, virus particles were again detected 3 days later. Cell line CTCL-3, established from this peripheral blood sample, is also now a constitutive producer of retrovirus particles. Preparations of HTLV<sub>CR</sub> from both cell lines (HUT 102 and CTCL-3), whether induced by IdUrd or not, have given similar results in subsequent studies. Most of the data presented below are from HUT 102 after it had become a constitutive producer of HTLV<sub>CR</sub>.

**Production and Purification of HTLV<sub>CR</sub>.** DNA polymerase assays of resuspended polyethylene glycol precipitates and singly banded virus particles from clarified HUT 102 cell culture medium had considerable activity with poly(dA)-dT<sub>12-18</sub>. Because this template-primer is not used by retrovirus RT, this finding indicated persistent cellular contamination (20). However, sedimentation of these resuspended, singly banded particles through 30% glycerol onto a 100% glycerol cushion and subsequent application to a second continuous sucrose gradient resulted in samples with little or no DNA polymerase activity with poly(dA)-dT<sub>12-18</sub>. DNA polymerase activity using poly(A)-dT<sub>12-18</sub> and poly(C)-dG<sub>12-18</sub>, the appropriate template-primers for viral RT, peaked at a density of 1.16 g/ml (Fig. 3 *Left*). Electron micrographs of negatively stained material banding at 1.16 g/ml revealed typical retrovirus particles. All subsequent characterization was performed on this peak fraction or similarly prepared material.

**Protein Profiles of Doubly Banded HTLV<sub>CR</sub>.** Protein profiles from IdUrd-induced and constitutively produced, doubly banded, and disrupted HTLV<sub>CR</sub> particles were compared to profiles from similarly prepared MuLV<sub>R</sub> and SSV(SSAV) particles on Coomassie blue-stained slab gels (Fig. 3 *Right*). The protein bands of both HTLV<sub>CR</sub> samples appeared virtually identical, but some differed from those in either MuLV<sub>R</sub> or SSV. The dominant bands appeared at molecular weights corresponding to approximately 10,000, 12,000, 19,000, 24,000, 42,000, and 52,000. Two areas of diffuse staining are seen between molecular weights 30,000 and 36,000 and 70,000 and 80,000. Subsequent gels have demonstrated that these areas consist of multiple protein bands. Purified HTLV<sub>CR</sub> particles also have a poly(A)-containing 70S RNA, which will be described elsewhere.

**Analysis of DNA Polymerase Activity of Purified HTLV<sub>CR</sub> Particles.** The optimal DNA polymerase activity of disrupted HTLV<sub>CR</sub> virions with poly(A)-dT<sub>12-18</sub> occurs at about 10 mM Mg<sup>2+</sup> and 0.25 mM Mn<sup>2+</sup> (not shown). The activity of HTLV<sub>CR</sub> RT was compared to purified human DNA polymerase  $\alpha$  and  $\gamma$  and to RT purified from SSV(SSAV) by using different template-primers with Mg<sup>2+</sup> or with Mn<sup>2+</sup> (Table 1). As anticipated from the known properties of viral and cellular DNA polymerases (20), the relative lack of utilization of poly(dA)-dT<sub>12-18</sub> and preference for poly(A)-dT<sub>12-18</sub> and poly(C)-dG<sub>12-18</sub> distinguished the viral enzymes from the two cellular DNA polymerases. The SSV(SSAV) RT prefers Mn<sup>2+</sup> over Mg<sup>2+</sup> with both poly(A)-dT<sub>12-18</sub> and poly(C)-dG<sub>12-18</sub> as template-primers, whereas RT from HTLV<sub>CR</sub> slightly favors Mg<sup>2+</sup> at the concentrations used.

None of the antibodies to cellular or to viral polymerases tested showed detectable crossreactions with HTLV<sub>CR</sub> RT. Antibody against DNA polymerase  $\gamma$  had no effect on HTLV<sub>CR</sub> RT, but it bound approximately 75% of DNA polymerase  $\gamma$ . Antisera to feline leukemia virus RT also failed to bind to HTLV<sub>CR</sub> RT (data not shown). Antisera directed against the purified RTs from SSV(SSAV), GALV<sub>H</sub>, RD-114, MuLV<sub>R</sub>, and AMV failed to neutralize HTLV<sub>CR</sub> RT despite the use of concentrations that achieved substantial inhibition of their homologous DNA polymerases (Fig. 4). More extensive studies using antisera made against RT from several other retroviruses also did not detect interaction with RT from HTLV (unpublished results).

## DISCUSSION

Retrovirus particles with type C morphology and budding were isolated from fresh peripheral blood lymphocytes and from two human T-lymphoblast cell lines derived a year apart from a man with mycosis fungoides, a variant of a group of rare dis-

eases known collectively as cutaneous T-cell lymphomas. The amount of HTLV<sub>CR</sub> produced from both the HUT 102 and CTCL-3 cell lines is much less than that seen with most lines producing retrovirus, and attempts at obtaining a more efficient producer by transmission of the virus to other cell lines have so far been unsuccessful. For these reasons, concentration of the virus particles from large amounts of cells is necessary for analytical studies. Because T-lymphoblastoid cells are relatively fragile, a certain percentage (10–20%) of the cells are disrupted during centrifugation, releasing cellular components into the medium. This requires substantial rebanding of the particles in order to achieve reasonably purified viral preparations.

The protein profile and characteristics of the DNA polymerase of doubly banded HTLV<sub>CR</sub> particles are consistent with the expected properties of the structural proteins and RT of a retrovirus (27), but further experimentation is required to determine whether all of the protein bands seen on NaDodSO<sub>4</sub> gel chromatography of these particles are of viral origin. HTLV<sub>CR</sub> differs in some respects from the common animal type C viruses (avian, murine, rat, feline, and subhuman primates). Some of its characteristics, most notably the difficulty in visualizing viral buds, the slight preference of its RT for Mg<sup>2+</sup>, and the size of some of its proteins, suggest a possible relationship to certain retroviruses that are difficult to classify (28, 29).

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