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17. However, high extinction rates are generally balanced in offshore clades by high speciation rates that can also confer extinction-resistance [S. J. Gould and N. Eldredge, *Paleobiology* **3**, 115 (1977); T. A. Hansen, *ibid.* **6**, 193 (1980); S. M. Stanley, *Macroevolution* (Freeman, San Francisco, 1979)], so that probabilistic replacement of one clade by another due to differential species-extinction rates may not be sufficient to generate the pattern.
18. In addition to background extinctions, mass extinctions could also contribute to the pattern, especially if marine habitats are differentially affected.
19. The incessant local extinctions and recolonizations in frequently disturbed nearshore habitats may promote the origin of major new community types through repeated sorting and recombining of new and established species (*5*). Alternatively, the evolutionary novelties themselves may arise preferentially nearshore because new isolates in those habitats are commonly small and drawn from panmictic populations, and are thus more likely to undergo genetic revolutions or transiliences that could produce rapid shifts in morphology or physiology than the more frequent speciation events in offshore environments [see D. Jablonski and R. A. Lutz, *Biol. Rev. Phil. Soc.* **58**, 21 (1983); J. W. Valentine and D. Jablonski, in *Evolution, Time and Space: The Emergence of the Biosphere*, R. W. Sims, J. H. Price, P. E. S. Whalley, Eds. (Academic Press, New York, 1983); A. R. Templeton, *Evolution* **34**, 719 (1980); E. Mayr, *ibid.* **36**, 1119 (1982); but see B. Charlesworth, R. Lande, M. Slatkin, *ibid.* p. 474].
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Productive Infection and Cell-Free Transmission of Human T-Cell Leukemia Virus in a Nonlymphoid Cell Line

Abstract. *Human T-cell leukemia virus (HTLV), American PL isolate, was transmitted by cocultivation and by cell-free filtrates to a nonlymphoid human osteogenic sarcoma (HOS) cell line, designated HOS/PL, but not to nine other lines bearing receptors for HTLV. HOS and HOS/PL cells are not dependent on interleukin-2 and do not express interleukin-2 receptors that are recognized by anti-Tac monoclonal antibody. HTLV released by the Japanese MT2 cell line was also transmitted to HOS cells. The infected HOS cells release substantial titers of progeny HTLV which is antigenically indistinguishable from parental virus and is able to transform T cells.*

Human T-cell leukemia virus (HTLV) is a C-type RNA tumor virus associated with a mature form of adult T-cell leukemia-lymphoma (ATLL). HTLV was first isolated and characterized from patients in the United States (*1*) and later in Japan (*2*), and in patients of West Indian origin (*3*) and in Israel (*4*). Human umbilical cord lymphocytes and peripheral blood lymphocytes cocultivated with HTLV-releasing lymphoma cells become infected and transformed in vitro (*4, 5*). Transformation of simian and rabbit peripheral blood T cells by HTLV has also been reported (*6*). Several of the T-cell lines transformed in vitro produce larger quantities of HTLV particles than the original tumor lines.

We have recently demonstrated that cocultivation of HTLV-producing cells with a variety of human and animal nonlymphoid cell types induces cell fusion, leading to the formation of large, multinucleated syncytia as a result of HTLV expression (*7*). These observations indicate that HTLV interacts with the surface of a number of cell types. Further studies with vesicular stomatitis virus (VSV) pseudotypes bearing the envelope glycoproteins of HTLV showed that there is a broad range of cells susceptible to pseudotype infection (*8*). Thus the expression of HTLV receptors is not restricted to lymphoid cells, because many cell types derived from diverse mammalian species are permissive for HTLV adsorption and penetration.

In this report we describe the productive infection of a nonlymphoid human cell line by American and Japanese strains of HTLV. Furthermore, we show that cell-free transmission of HTLV is achieved in this line.

Permissivity of HOS cells to HTLV replication. Five human and five animal cell lines known to have receptors for HTLV (*8*) were cocultivated with HTLV-producing C91/PL T cells. The human cells were 7605L embryonic lung fibroblasts, HOS osteogenic sarcoma cells, RD rhabdomyosarcoma cells, HeLa cervical carcinoma cells, and EJ bladder carcinoma cells. Animal cells were Vero African green monkey kidney cells, Fcf2th canine thymus murine sarcoma virus (MSV)–transformed S^+L^- cells, feline CCC MSV-transformed S^+L^- cells, CCL64 mink lung cells, and XC Rous sarcoma virus (RSV)–induced rat sarcoma cells. In the first set of experiments HTLV-producing cells were not x-irradiated but during serial passage the lymphoma cells were soon lost from the adherent cultures. The cells were maintained in Dulbecco-modified Eagle's medium with 5 to 10 percent fetal calf serum and were passaged for 5 months.

Although each of the ten cell types cocultivated with HTLV-producing cells was susceptible to HTLV penetration and eight were susceptible to HTLV-induced cell fusion, only one cell type, the HOS cell line (*9*), was permissive for HTLV replication. During the first 2 weeks of cocultivation, cell fusion occurred among the HOS cells, but with the loss of C91/PL cells on passage, the

Table 1. Virus production, syncytium induction, pseudotype formation, and antigen expression by HOS/PL cells.

Cell line	Reverse transciptase*	Syncytium induction†	VSV (HTLV) pseudotype titer‡	Percentage of cells immunofluorescent			
				HTLV antigens§		T-cell marker¶	IL-2 receptor ‖
				ATLL	p19		
C91/PL	16361	+++	3×10^3	87	89	72	85
HOS/PL	18890	++++	5×10^4	98	82	0	0
HOS	926	−	$< 10^1$	0	0	0	0

*Assay of viral RNA-directed DNA polymerase, expressed as the counts per minute of [^{3}H]TMP incorporated during incubation for 60 minutes at 37°C (*7*). †XC indicator cells were cocultivated with test cells for 18 hours and examined for syncytia (*7*). The results are expressed as the percentage of nuclei contained within syncytia: −, no syncytia; +++, 30 to 50 percent; ++++, > 50 percent. ‡Plaque-forming units per milliliter of vesicular stomatitis virus (VSV) with envelope antigens specific to HTLV (*8*). §Indirect immunofluorescence on fixed cells using serum from an antibody-positive ATLL patient (*7*) and monoclonal antibody to p19 (*10*). ¶Indirect immunofluorescence with UCHT1 monoclonal antibody (*12*) on live cells. ‖ Indirect immunofluorescence with anti-Tac monoclonal antibody (*14*) on live and fixed cells.

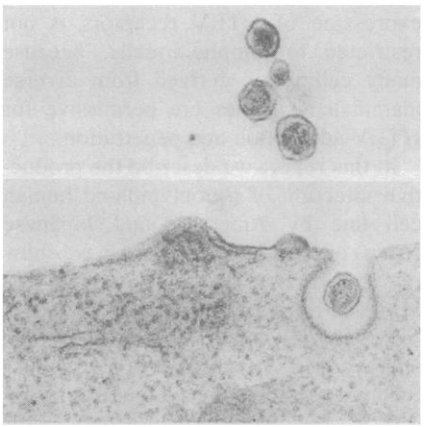

Fig. 1. Budding and mature virions resembling HTLV produced by HOS/PL cells. One particle is contained within a coated pit (×105,000).

syncytia disappeared. However, after 8 weeks' passage syncytia reappeared in the HOS cell monolayer indicating productive infection. By 10 weeks the cultures were full of syncytia, after which they gradually disappeared again. Virtually no syncytia could be seen by 12 weeks, although by this stage almost all cells expressed HTLV antigens. It appears that only the uninfected cells are sensitive to syncytium induction by HTLV. When all the cells express HTLV envelope antigens, the cell surface receptors become saturated and no further cell fusion takes place. The virus-infected subline was designated HOS/PL.

C-type particles with the morphology of HTLV were observed as budding and mature virions in electron micrographs of HOS/PL cultures (Fig. 1). No virus particles were evident in uninfected HOS cells. The relative amount of virus particles was measured by reverse transcriptase assay (Table 1) in which there was a preference for Mn^{2+} cations. HTLV production by HOS/PL cells was slightly higher than C91/PL cells, the T-cell line used for cocultivation.

HOS cells were also cocultivated with MT2 cells, producing a Japanese strain of HTLV known as ATLV (*2*). After 3 months syncytia appeared and by 4 months the HOS/MT2 subline produced substantial amounts of viral antigens.

Immunological properties of HTLV produced by HOS/PL cells. When the fully infected HOS/PL cells were mixed with uninfected HOS or XC "indicator" cells, syncytia were induced in the indicator cells (Table 1). Syncytium induction was specifically inhibited in the presence of ATLL patients' sera, as described previously for HTLV-producing T cells (*7*), demonstrating HTLV membrane antigen specificity.

VSV particles bearing HTLV envelope glycoproteins can be produced by infecting HTLV-producing T cells with VSV (*8*). VSV(HTLV) pseudotypes were detected following infection of HOS/PL cells with VSV (Table 1). The plaque-forming activity of these pseudotypes was completely neutralized by serum (at a 1:250 dilution) from a patient with ATLL.

An indirect immunofluorescence assay for HTLV structural antigens was carried out with serum from an antibody-positive ATLL patient. Nearly 100 percent of HOS/PL cells expressed HTLV antigens (Fig. 2). Bright fluorescent regions in the cytoplasm and spots on the cell surface were observed as well as diffuse fluorescence around the nucleus. In addition, immunofluorescence revealed by monoclonal antibody to the HTLV core antigen p19 (*10*) was positive in HOS/PL cells (Table 1). No significant fluorescence was observed in uninfected HOS cells.

HTLV-specific proteins synthesized by HOS/PL cells were also detected by radioimmunoprecipitation and gel electrophoresis (Fig. 3). Immunoprecipitation by monospecific antiserum to p24 (*11*) revealed the synthesis in HOS/PL cells of proteins with apparent molecular weights of 27,000, 37,000, and 57,000.

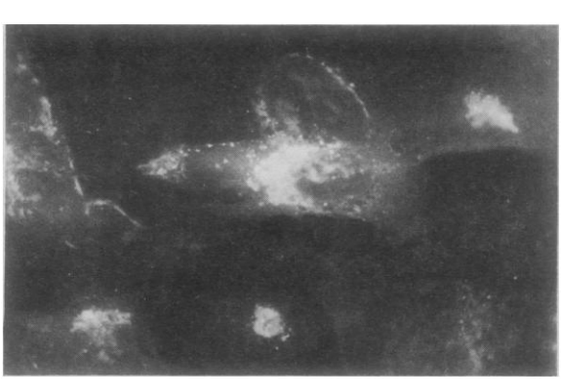

Fig. 2. Immunofluorescence of fixed HOS/PL cells treated with serum from a patient with ATLL and goat antiserum to human immunoglobulin G conjugated with fluorescein isothiocyanate.

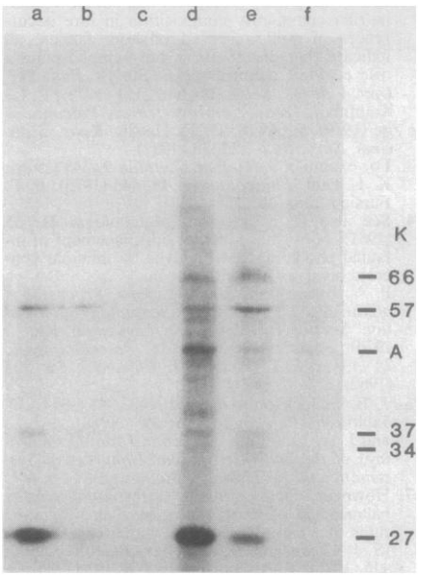

Fig. 3. Radiofluorograph of immunoprecipitation followed by polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins synthesized in HTLV-infected and uninfected cell lines. Lanes a, b, and c: precipitation of C91/PL, HOS/PL, and HOS lysates, respectively, by rabbit antiserum to p24 serum (*10*). Lanes d, e, and f: precipitation of C91/PL, HOS/PL, and HOS lysates, respectively, by human ATLL serum. HTLV-specific polypeptides with molecular weights of 27,000 (27K), 34K, 37K, 57K, and 66K are indicated. Defined proteins ranging from 21K to 85K (not shown) were used as molecular weight markers in the same gel. *A*, actin.

With serum from an ATLL patient, further labeled proteins were precipitated, the major one being a broad band of 66,000 apparent molecular weight. All the major proteins present in C91/PL cells were also present in HOS/PL cells but with the exception of actin were not seen in uninfected HOS cells.

The results of the antibody-specific immunofluorescence, immunoprecipitation, syncytium inhibition, and pseudotype neutralization experiments show conclusively that the virus produced by HOS/PL cells is HTLV.

HOS cells lack T-cell markers and IL-2 receptors. HOS and HOS/PL cells were tested for expression of a surface antigen common to all T cells, recognized by UCHT1 monoclonal antibody (*12*). Neither cell type was positive for this antigen, providing additional evidence besides their morphology and reported origin (*9*) that HOS cells are not T cells.

T-cell growth factor, also known as interleukin-2 (IL-2), is a glycoprotein synthesized and secreted by certain T lymphocytes following activation with antigen or mitogen (*13*). IL-2 is required for proliferation and maintenance of ma-

ture T cells in long-term culture. Monoclonal antibody specific for the IL-2 membrane receptor (anti-Tac) suppresses IL-2–induced proliferation of T cells, and blocks the binding of IL-2 to cells (*14*). Several of the HTLV-producing T-lymphoma cells do not require exogenous IL-2 for proliferation in culture. All HTLV-transformed T-cell lines, however, express the IL-2 receptor which becomes preferentially associated with the virions of HTLV (*15*). It has been suggested that the IL-2 receptor may play a role in HTLV infection and transformation (*15*). We therefore investigated whether the expression of IL-2 receptors was involved in the infection of HOS cells by HTLV.

To detect the presence of IL-2 receptors on the membranes of HOS and HOS/PL cells, we performed indirect immunofluorescence with anti-Tac monoclonal antibody (Table 1). With this assay the presence of IL-2 receptors was detected in C91/PL T cells but neither in virus-producing HOS/PL nor in uninfected HOS cells, indicating that the IL-2 receptor is not necessary for HTLV replication and is not equivalent to the HTLV receptor.

HOS HTLV transforms T cells. To determine whether HTLV produced by HOS/PL cells is able to transform T lymphocytes, x-irradiated HOS/PL cells were cocultivated with phytohemagglutinin (PHA)-stimulated mixed human tonsil lymphocytes initially in the presence of IL-2. A transformed, HTLV-producing line of IL-2–independent T cells became established within 6 weeks. Thus the T-cell transforming property of HTLV was not lost upon passage through HOS cells.

Cell-free transmission of HOS HTLV. The infection of HOS cells was repeated and confirmed with x-irradiated (5000 R) HTLV-producing C91/PL and HOS/PL cells. When fresh HOS cells were cocultivated with x-irradiated HOS/PL cells, the HOS cells became infected and produced HTLV within 3 weeks, rather than 10 weeks that elapsed after cocultivation with C91/PL cells. Since the amount of virus released from HOS/PL and C91/PL cells was not markedly different as measured by reverse transcriptase (Table 1), this result suggests that HTLV produced by HOS/PL cells has become adapted for more efficient replication in this cell type.

It may be argued that cocultivation of HOS cells with HTLV-infected T cells allowed the transfer of chromosomes bearing HTLV proviruses into the HOS cells by cell fusion, even though the HOS cells did not bear T-cell markers. We therefore attempted to perform cell-free transmission of the HTLV. Clarified and filtered (0.2 μm) medium harvested from HOS/PL cell cultures was added to subconfluent cultures of HOS cells in the presence of DEAE-Dextran (25 μl/ml). After incubation for 1 hour at 37°C, growth medium was added and the cells were passaged twice a week. After 3 weeks the infected cells produced syncytia, and complete infection was established within 7 weeks.

On repeating cell-free infection of HOS cells, the appearance of HTLV antigens and syncytia was followed by immunofluorescent and cytological assays performed at 1, 3, 7, 14, 21, 28, and 35 days after infection. Syncytia first became evident 14 days after infection and by 21 days numerous syncytia were seen. HTLV antigens were first detectable by immunofluorescence 21 days after infection, increasing in cell number and intensity thereafter. It is likely that a very small proportion of cells are initially infected and that the virus progressively spreads through the culture during subsequent passage. However, we cannot rule out the possibility that the majority of cells become infected by the original inoculum but do not express viral antigens until several weeks later.

Discussion. HTLV has been successfully transmitted by cocultivation to other human, simian, and rabbit cells of lymphoid origin (*4–6*). Cell-free transmission of HTLV to human bone marrow cells has recently been observed (*16*). Here we show that HTLV is transmissible to nonlymphoid cells, by cocultivation with infected cells as well as by culture with filtered medium from such cells, and that virus-producing cell lines can be established. The uninfected HOS line and the HOS/PL and HOS/MT2 sublines proliferate in culture medium supplemented with fetal calf serum, without a requirement for specific growth factors. Bovine leukosis virus, which is distantly related to HTLV, can also be transmitted to nonlymphoid cells (*17*).

Because HTLV-transformed T cells represent cells derived from an unknown, minority T-cell subset in fresh lymphocyte cultures, there exist no control, uninfected cell lines directly comparable to the HTLV-infected cells. HOS, HOS/PL, and HOS/MT2 cells provide such a system, which is now being used in serological studies of ATLL and AIDS patients. This system enables us to distinguish in immunofluorescence studies between virus-specific and cell-specific antibodies in these patients' sera. HOS/PL cells are also useful as a source reagent for serological assays because they consistently produce high titers of HTLV antigens.

Experiments on the infection of HOS cells with HTLV-2 (*18*) and with HTLV-like viruses isolated from AIDS patients (*19*) and from nonhuman primates (*20*) should make possible detailed comparisons of the various HTLV strains.

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