The immediate implication of the lower carbon storage in tropical forests is that less carbon would be released as CO_2 when tropical forests are burned or destroyed. Using the forest carbon densities of Whittaker and Likens and our earlier study and the FAO clearing rates (*10*), Detwiler *et al.* (*4*) estimated that the net flux of carbon to the atmosphere from tropical closed forests ranges from 0.68 to 0.74 $\times$ 10^9 ton/year for 1980. This flux would be reduced by approximately half if the lower volume-derived carbon densities were used (*25*). A lower carbon flux from the tropics would enable the global carbon budget to be almost balanced.

SANDRA BROWN
Department of Forestry, University of Illinois, Urbana 61801
ARIEL E. LUGO
Institute of Tropical Forestry, Southern Forest Experiment Station, Post Office Box AQ, Rio Piedras, Puerto Rico 00928

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12 September 1983; revised 4 January 1984

Transformation and Cytopathogenic Effect in an Immune Human T-Cell Clone Infected by HTLV-I

Abstract. *Human T-cell leukemia-lymphoma virus (HTLV) is a human C-type retrovirus that can transform T lymphocytes in vitro and is associated with certain T-cell neoplasms. Recent data suggest that, in the United States, patients with acquired immunodeficiency syndrome (AIDS), homosexual men with lymphadenopathy, and hemophiliacs have had significant exposure rates to HTLV, whereas matched and unmatched control American subjects have rarely been exposed to this agent. In the present experiments, T cells specifically reactive against HTLV were propagated from a patient whose HTLV-bearing lymphoma was in remission. The T cells were cloned in the presence of the virus and an HTLV-specific cytotoxic T-cell clone was isolated. This clone was infected and transformed by the virus, with one copy of an HTLV-I provirus being integrated into the genome. This T-cell clone did not exhibit the normal dependence on T-cell growth factor (interleukin-2) and proliferated spontaneously in vitro. Exposure of the clone to HTLV-bearing, autologous tumor cells specifically inhibited its proliferation and resulted in its death. These results may have implications for HTLV-associated inhibition of T-cell responses.*

The human type-C retrovirus known as human T-cell leukemia-lymphoma virus (HTLV) was first isolated from neoplastic cells derived from black patients in the United States with adult T-cell malignancies (*1, 2*). It has been suggested that HTLV and bovine leukemia virus (BLV) have a common ancestry (*3*).

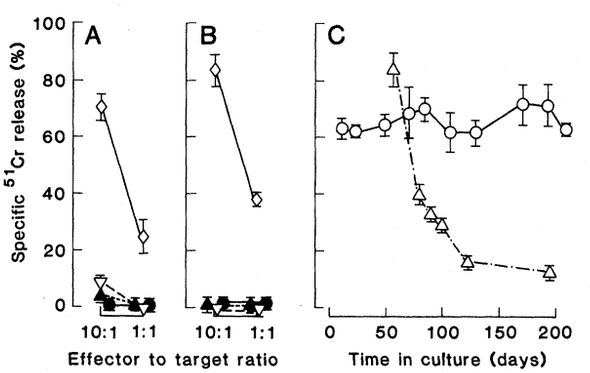

Fig. 1. Functional properties of clone K7. (A) Cytotoxic effector activity of the parent cultured T-cell line (MJ-CTL). (B) Initial cytotoxic effector activity of clone K7 cells derived from MJ-CTL. Standard 4-hour ^{51}Cr-release assays were used to assess the specific cytotoxic activity of cultured T cells as previously described (*15*). The release of radioactivity (R) into the surrounding medium by target cells labeled with ^{51}Cr is an index of cell destruction. The percentage specific release of ^{51}Cr was determined by the following formula: $\{[(R_{test}) - (R_s)]/[(R_{max}) - (R_s)]\} \times 100$, where R_{test} is the ^{51}Cr released in the assay, R_s is the spontaneous release, and R_{max} is the maximum release of radioactivity. MJ-CTL and clone K7 cells were tested on day 62 in culture. Target cells were HTLV-bearing autologous tumor cell line, MJ-tumor (◇); HTLV-bearing tumor cell line from an unrelated donor, HUT-102-B2 (▲); Epstein-Barr virus–transformed autologous B cells (●); and an erythroid line, K562 (▽). (C) Progressive loss of cytotoxic activity of clone K7. In each ^{51}Cr release assay, the cytotoxicity of the parent cultured T-cell line, MJ-CTL (○) and clone K7 (△) against autologous HTLV-bearing tumor cells (MJ-tumor cells), was determined. The ratio of effector to target cells for each determination was 10 to 1. Cloning took place on day 35 in culture.

Most of the HTLV isolates studied are very similar to one another (*4, 5*) and belong to the subgroup HTLV-I, a family of acquired viruses with T-cell tropism (*6*). In coculture, cells producing HTLV can infect other cells and transform them (*7, 8*). This transformation reduces or eliminates the normal requirement for T-cell growth factor (TCGF) or interleukin-2.

Despite recent advances, much remains to be learned about the relation between HTLV infection and immune function. Populations in which HTLV is endemic can be found in the West Indies and southern Japan, where the virus is associated with the development of a T-cell leukemia or lymphoma, often made up of neoplastic cells with suppressor immunoregulatory function (*9, 10*). Other regions of the world where HTLV is endemic include the southeastern United States, parts of South America, and portions of Africa (*11*).

In the endemic areas of Japan and the Caribbean, 6 percent (and in selected districts up to 37 percent) of adults are asymptomatic carriers of the virus (*12*). In the United States, HTLV infection is linked to fulminant T-cell lymphoproliferative disorders in adults, complicated by hypercalcemia and opportunistic infections (*11*).

The full spectrum of diseases associated with HTLV is not known. There are data supporting the hypotheses that certain patients with the recently defined acquired immunodeficiency syndrome (AIDS) either have an increased risk of infection with viruses in the HTLV family or developed the disease as a result of infection with a strain of HTLV (*13*). Studies with outbred cats indicate that there is a clear precedent for the latter possibility in that feline leukemia virus (FeLV) can mediate an infectious form of immunodeficiency (*14*). Indeed, such an immunodeficiency involves an increased risk of bacterial, viral, and parasitic infections and it is commonly encountered in veterinary practice. However, the factors that govern whether the retrovirus will cause a neoplasm, an immunodeficiency, or both, are not defined.

We have investigated populations of HTLV-specific, cytotoxic T cells that were derived from a patient (M.J.) whose HTLV-bearing lymphoma was in remission (*15*). We now report a human T-cell clone that has been infected and transformed by the HTLV that itself had served as its target antigen. The clone initially had potent specific cytotoxic activity that waned with time in culture, and it spontaneously proliferated in the absence of TCGF. The spontaneous proliferation of the clone was profoundly and specifically inhibited by exposure to autologous HTLV-bearing tumor cells. The genome of the clone contained one copy of an HTLV provirus.

We generated cultures of immune T cells from the peripheral blood of patient M.J. after his neoplasm was in remission (*15*). These uncloned, starting T-cell populations required TCGF for propagation in vitro. Such T cells mediated specific cellular immune reactions in vitro against HTLV-I, including proliferation in response to cultured HTLV-I–bearing tumor cells and a form of HTLV-specific cytotoxicity restricted by products of the major histocompatibility complex (MHC). In the current studies, immune T cells were exposed to infectious HTLV and cloned. Cloning of the immune T cells was undertaken by limiting dilution (0.5 cells per well) in round-bottom microtiter wells containing lectin-free TCGF and a feeder-layer of irradiated mononuclear cells obtained from the patient in remission. The wells also contained a lethally irradiated population of HTLV-producing autologous tumor cells. Within 21 days, one microtiter well was noted to contain a very rapidly growing population of cells (designated clone K7). These cells expressed the same surface markers of mature cytotoxic T cells (OKT-3$^+$, -4$^-$, -8$^+$, DR$^+$, and Tac$^+$) that characterized the uncloned, starting T cells. They also expressed the patient's HLA phenotype: A1, B8, B27, Cw6, Cw7, DR3, and DR7. (These phenotypic properties remained stable throughout the course of these studies.) The K7 cells proliferated in the absence of TCGF (although the rate of replication could be increased by exogenous TCGF). The cells were propagated in the absence of MJ-tumor cells for further study.

The immune cytotoxic effector function of the noncloned, starting population and clone K7 is illustrated in Fig. 1, A and B. Shortly after cloning, K7 cells mediated substantial cytotoxic activity against autologous HTLV-bearing tumor cells and other histocompatible cells infected with HTLV. Clone K7 was cytotoxic only for cells that were infected

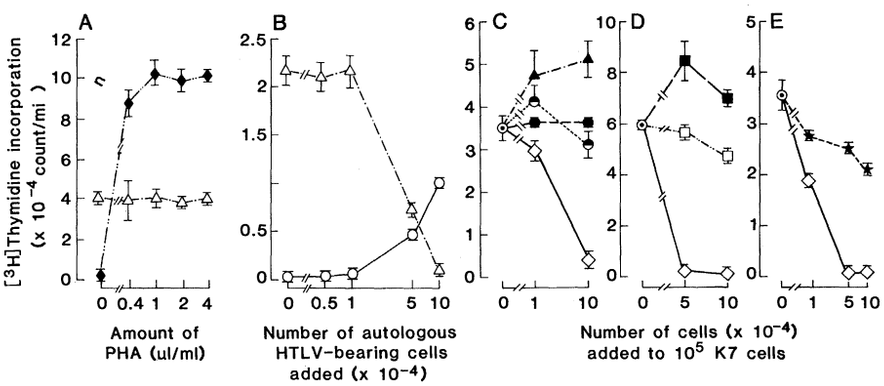

Fig. 2 (A and B). Altered properties of clone K7. (A) Loss of proliferative response to the nonspecific mitogen phytohemagglutinin-M (PHA-M). Clone K7 cells (△) (10^5) and peripheral blood mononuclear cells from a normal individual (◆) were cultured for 3 days with various amounts of PHA-M (Gibco) in the absence of exogenous TCGF in 200 μl of RPMI 1640 medium supplemented with 10 percent heat-inactivated fetal calf serum, 4 m*M* L-glutamine, and penicillin (50 unit/ml) and streptomycin (50 μg/ml) at 37°C in humidified air containing 5 percent CO_2. In the final 5 hours of culture the cells were exposed to 0.5 μCi of [^{3}H]thymidine. They were then harvested onto glass fibers and assessed for the incorporation of isotope as an indicator of proliferation. The results are expressed as the mean counts per minute ± one standard deviation for triplicate determinations. Clone K7 proliferates spontaneously and the proliferative rate is not affected by PHA; this provides a control showing that nonspecific stimuli at the surface membrane do not inhibit proliferation of the clone. (B) Inhibition of proliferation upon exposure of clone K7 to autologous HTVL-bearing cells. Clone K7 cells (10^5) (△) and the parent line, MJ-CTL (○), were cocultured with various numbers of irradiated autologous HTLV-bearing cells (MJ-tumor cells) for 3 days, exposed to [^{3}H]thymidine, and harvested as described above. The spontaneous proliferation of clone K7 was inhibited by the addition of irradiated MJ-tumor cells. By contrast the parent line was stimulated to proliferate under the same conditions. (C to E) Specificity of clone K7 inhibition. Clone K7 cells (10^5) were cultured in the presence or absence (⊙) of a variety of irradiated (12,000 rad) cells for 3 days. Assays of [^{3}H]thymidine incorporation were the same as in (A) and (B). Cells cocultured with clone K7 cells were MJ-tumor (◇); HUT-102-B2 (▲), Epstein-Barr virus–transformed B cells from patient M.J. (●), and a normal individual M.M. (◓) who shared A1, B8, Cw6, DR3, and DR7 with patient M.J. [shown in (C)]; freshly harvested peripheral blood mononuclear cells from patient M.J. (□) and a normal individual M.M. (■) [shown in panel (D)]; and PHA-induced TCGF-dependent T-cell blasts from patient M.J. (★) that were generated after his tumor was in remission [shown in (E)]. None of these irradiated cells incorporated a significant amount of [^{3}H]thymidine when cultured alone.

with HTLV, and then only when histocompatible target cells were used. However, with time in culture, clone K7 progressively lost cytotoxic activity (Fig. 1C). It was not possible to restore the cytotoxic activity by recloning the cells. Clone K7 spontaneously proliferated in the absence of TCGF, and phytohemagglutinin (PHA), a polyclonal T-cell mitogen, did not affect the spontaneous proliferation of clone K7 (Fig. 2A).

The capacity to recognize an antigen and respond by proliferating in the absence of exogenous TCGF is one of the hallmarks of immune T cells. The uncloned, starting population of immune T cells from patient M.J. proliferated specifically in response to irradiated, autologous HTLV-bearing tumor cells (MJ-tumor cells) (Fig. 2B). However, under the same conditions, exposure of K7 to autologous HTLV-bearing tumor cells caused an inhibition of proliferation and cell death in a dose-dependent fashion. The addition of TCGF to the K7 cells at the time of initial exposure to irradiated MJ-tumor cells did not prevent the inhibitory effects (data not shown). Neither HTLV-infected nor -uninfected cells from unrelated individuals could bring about these inhibitory effects. Similarly, a variety of autologous cells obtained from the patient after his neoplasm was in remission (including fresh peripheral blood mononuclear cells, Epstein-Barr virus–transformed B cells, and PHA-induced T-cell blasts) did not cause these inhibitory effects (Fig. 2, C to E). Comparably treated MJ-tumor cells had no inhibitory effects on the proliferation of MOLT-4 (an HTLV-negative T-cell line), HUT-102-B2 (an HTLV-positive T-cell line), or PHA-stimulated T cells from an unrelated normal individual in control coculture experiments. A monoclonal antibody to the p19 group-specific antigen (*gag*) protein of HTLV-I did not appreciably affect the responses of K7 cells discussed above. However, in preliminary experiments we observed that the inhibitory effect exerted by MJ-tumor cells on clone K7 could be partially abrogated by the addition of irradiated syngeneic (but not allogeneic) peripheral blood mononuclear cells taken from patient M.J. in remission.

We then determined whether K7 cells were infected with HTLV. K7 cell DNA was digested with the restriction endonuclease Eco RI, which does not cleave within the proviral DNA. The resultant Southern blot contains a single proviral fragment (Fig. 3), indicating that there is only one copy of HTLV and that the K7 cell population is monoclonal with respect to the HTLV integration site. This provides an independent indicator of a monoclonal origin for the K7 cell population. This contrasts with the multiple integration sites observed in MJ-tumor cells (the long-term line of autologous HTLV-bearing tumor cells), a pattern commonly observed in cultured HTLV-producing tumor cells (*16*). Digestion with the restriction endonuclease Sst I, which cleaves the HTLV-I_{MJ} provirus once internally, results in two fragments labeled by an HTLV probe for a long terminal repeat (LTR) (Fig. 3). This implies that there are LTR sequences at both the 5′ end and the 3′ end of the provirus. The restriction endonuclease Pvu II cuts several times within the HTLV provirus. Pvu II digests of K7-cell DNA give two fragments that label with the LTR probe and are, therefore, proviral-host DNA junction fragments. This confirms that the proviral population is integrated in the same site in all the K7 cells and that the provirus indeed has LTR sequences at both the 5′ and 3′ ends. In addition, in each of the Pvu II and Sst I digests, the size of at least one of the junction fragments differs from any of the junction fragments in MJ-tumor cell DNA (Fig. 3), indicating that the cell lineage of clone K7 is different from that of the neoplastic cells from the same patient. Digestion with Sma I gives a characteristic 4.3-kb fragment labeled with a probe consisting of the Cla I–Hind III fragment of HTLV clone λCR-1 (*17*), indicating that the 3′ half of the virus is present (data not shown). Southern blots with other enzymes and a complete HTLV proviral probe, λ23-3 (*18*), suggest that there are no gross deletions or rearrangements of the HTLV provirus. In spite of the apparently complete HTLV provirus, virus expression was restricted during the course of these studies. There was no detectable expression of the *gag* proteins p19 or p24 during the first 275 days after cloning [about 30 passages] (not shown). During this time, viral RNA was not detectably expressed in the majority of the K7 cells as determined by in situ hybridization to fixed K7 cells. A few cells (10 to 20 percent) appeared to express low levels of viral RNA. Since the same cells were negative for viral *gag* proteins, it appears that either the RNA did not include functional *gag* messenger RNA or it was not translated at detectable levels. However, we do not yet know the reasons for the observed restriction of HTLV expression in K7 cells (*19*).

To our knowledge, clone K7 is the first example of a functional cytotoxic T cell transformed by a human retrovirus. Perhaps the current results can be viewed in the context of the receptor-mediated leukemogenesis theory, which to date has been predicated on data from studies of retrovirus-associated lymphomas of rodents (*20*). The theory proposes that antigen-specific lymphocytes can be a target for infection by an oncogenic retrovirus, a point that has not been fully resolved in animals (*21*). According to this theory, retrovirus-induced lympho-

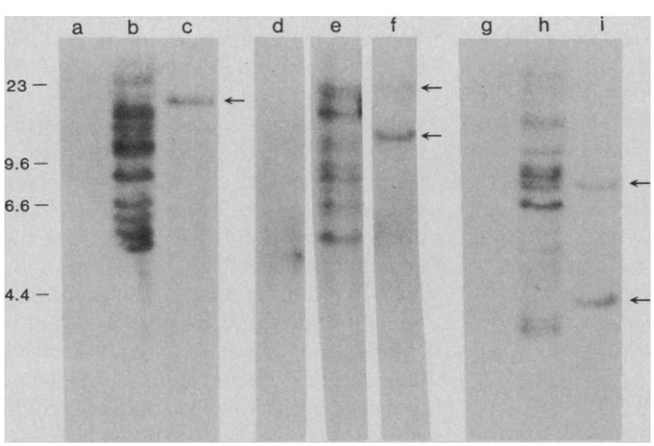

Fig. 3. HTLV Proviral sequences in clone K7 DNA. The DNA was digested with Eco RI (lanes a to c), Sst I (lanes d to f), or Pvu II (lanes g to i) and analyzed by the Southern blotting technique with an HTLV LTR (R-U5) probe. The DNA was from an uninfected cord blood B-cell line (lanes a, d, and g), HTLV-bearing tumor cells from patient M.J. (lanes b, e, and h), or clone K7 (lanes c, f, and i). Arrows show the provirus-containing K7 DNA fragment (or fragments), and the lines on the left-hand side show the position of the Hind III fragments of λ phage DNA as a marker. High molecular weight DNA was digested with Pronase–sodium dodecyl sulfate and extracted with organic solvents essentially as described (*16*). For Southern blots, 25 to 30 μg of DNA was digested for 16 hours at 37°C with 60 units of the indicated enzyme and a buffer as specified by the manufacturer. Restriction endonuclease digests were subjected to electrophoresis overnight at 40 V in 0.5 percent agarose, transferred to nitrocellulose, and analyzed by hybridization to a nick-translated ^{32}P-labeled excised insert containing the R-U5 region of the LTR sequences of HTLV-I_{CR} cloned in plasmid PBR-322 (*17*). Filters were washed extensively in 0.075*M* NaCl, 7.5 m*M* sodium citrate (*p*H 7) at 65°C. Sst I was from Bethesda Research Laboratories, Gaithersburg, Maryland, and Eco RI and Pvu II were from Boehringer Mannheim, Indianapolis, Indiana.

mas can be the progeny of normal cells that bear antigen-specific T-cell receptors for engaging viral envelope antigens. Binding of a retrovirus to these cells is postulated to provide both a portal of infection and a continuous mitogenic signal. Such events could have played a role in the emergence of the human T-cell clone K7 in the current studies, although the receptor-mediated leukemogenesis theory per se would not explain the inhibitory effects of exogenous HTLV after the clone emerged.

The mechanisms for the specific inhibition of this clone by its antigen HTLV are not known. Normal human T-cell clones that are specific for discrete peptides of influenza A virus can be rendered unresponsive to antigen by incubation with high concentrations of the appropriate peptide (*22*). This state of specific unresponsiveness is defined by a failure to mount a proliferative response to antigen in vitro and is perhaps akin to certain forms of immunologic tolerance. Perhaps clone K7 represents HTLV-induced transformation and expansion of a T cell that had already been programmed for such unresponsiveness. In this context, K7 cells might serve as an in vitro model for immunologic tolerance based on a mechanism of clonal deletion. Alternatively, the insertion of HTLV (or a mutant strain of the virus) into a critical domain within the genome could induce or predispose reactive T cells to somatic errors that cause them to recognize exogenous viral antigens as a negative signal. These are topics for future research that could have implications in understanding the relation between retroviruses in T-cell neoplasms and acquired immunodeficiency disease states.

HIROAKI MITSUYA
HONG-GUANG GUO
MARY MEGSON
CECELIA TRAINOR
MARVIN S. REITZ, JR.
SAMUEL BRODER*

Clinical Oncology Program and *Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20205*

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19. After approximately 1 year in culture (50 passages), some K7 cells were found to express p19 and p24 proteins as well as virus particles, confirming the presence of a complete provirus and indicating that the restriction of HTLV expression was a reversible phenomenon.
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* Address reprint requests to S.B.

7 December 1983; accepted 21 February 1984

Heparin Affinity: Purification of a Tumor-Derived Capillary Endothelial Cell Growth Factor

Abstract. *A tumor-derived growth factor that stimulates the proliferation of capillary endothelial cells has a very strong affinity for heparin. This heparin affinity makes it possible to purify the growth factor to a single-band preparation in a rapid two-step procedure. The purified growth factor is a cationic polypeptide, has a molecular weight of about 18,000, and stimulates capillary endothelial cell proliferation at a concentration of about 1 nanogram per milliliter.*

The proliferation of capillary endothelial cells is a key component of angiogenesis (*1*). It has been postulated that in tumor-induced angiogenesis tumor cells produce growth factors that stimulate the proliferation of capillary endothelial cells (*2*). A class of compounds of very low molecular weights (200 to 1000) that can stimulate endothelial cell proliferation in vitro and angiogenesis in vivo have been isolated from tumor cells (*3*, *4*) but are as yet uncharacterized. Two recent developments have helped considerably in efforts to purify and characterize tumor-derived endothelial cell mitogens of larger molecular weights. The first is the availability of cultured capillary endothelial cells (*5*). Capillary endothelial cells differ structurally and functionally from aortic endothelial cells (*6*) and respond differently to tumor-derived factors. For example, tumor-derived factors have been shown to stimulate proliferation (*3*), motility (*7*), and collagenase production (*8*) in capillary but not in aortic endothelial cells. Thus capillary endothelial cells appear to be the target cell of choice in the analysis of growth factors that may be involved in capillary growth.

The second development, described in this report, is the finding that capillary endothelial cell growth factors derived from tumors have a strong affinity for heparin. This affinity enabled us to develop a rapid, two-step procedure for purifying a capillary endothelial cell growth factor found in chondrosarcoma. The chondrosarcoma-derived growth factor is a cationic polypeptide of about 18 kilodaltons (kD) that stimulates capillary endothelial cell proliferation at a concentration of about 1 ng/ml.

Growth factor isolated from extracts of chondrosarcoma extracellular matrix (ECM) bound tightly to columns of heparin-Sepharose. The growth factor activity, as measured by the ability to stimulate DNA synthesis in BALB/c mouse 3T3 cells (*10*), eluted at about 1.5*M* NaCl (Fig. 1A). The strong affinity of chondrosarcoma-derived growth factor for heparin was not shared by other growth factors that stimulate 3T3 cell DNA synthesis, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). The EGF did not bind at all; PDGF bound but eluted at about 0.5*M* NaCl (Fig. 1A). The much tighter binding of chondrosarcoma-derived growth factor to heparin than PDGF is surprising. Both growth factors are cationic polypeptides of similar charge (isoelectric points between 9.5 and 10), and both adhered to the cationic exchange resin Bio-Rex 70 and eluted at about 0.5*M* NaCl (Fig. 1B). From the similarities in positive charge it might be expected that