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Immunology

Stable growth transformation of human T lymphocytes by Herpesvirus saimiri

(CD4 and/or CD8 cell lines/oncogenic herpesvirus)

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ABSTRACT **Herpesvirus saimiri induces T-cell lymphomas in various species of New World monkeys and in rabbits, and it is able to immortalize monkey T lymphocytes *in vitro*. Sequences responsible for these effects have been localized to a region of the genome that varies significantly among the virus subgroups A, B, and C. We now report that infection of human blood lymphocytes and thymocytes with strains of subgroup C, in contrast to viruses of the other subgroups, yields continuously proliferating T-cell lines with the phenotype of mature CD4- or CD8-positive cells. Infection with strains of Herpesvirus saimiri subgroup C can thus be used to generate human T-cell lines for a variety of immunological and developmental studies.**

Studies of normal human lymphocyte function are largely dependent on clones of stimulated cells since tumor lines usually express aberrant phenotypes. Expansion of human B-lymphocyte cultures by infection with the Epstein–Barr virus, a γ_1-herpesvirus, has provided continuously growing human B cells for the study of B-cell differentiation and immunoglobulin synthesis. The distantly related γ_2 prototype, Herpesvirus saimiri, is indigenous to and apathogenic in squirrel monkeys (1) but causes fulminant T-cell lymphomas and acute lymphocytic leukemias in other New World primates (1–3) and rabbits (4). It also immortalizes cultured peripheral blood lymphocytes of cotton top marmosets (*Saguinus oedipus*) (5) and common marmosets (*Callithrix jacchus*) (6). Genes required for lymphocyte growth transformation and oncogenicity were localized to the left-terminal region of the 112-kilobase-pair (kbp) L-DNA in the Herpesvirus saimiri genome (7–12). Based on extreme sequence variations at the same left terminus of L-DNA, the known Herpesvirus saimiri strains have been divided into three subgroups (A, B, and C) (13, 14) that differ in their oncogenic capacity (10). We now report that strains of Herpesvirus saimiri subgroup C efficiently transform human lymphocytes from the thymus or peripheral blood to continuously proliferating T-cell lines. The transformed cells grow independently of antigen stimulation and have a phenotype of activated mature T lymphocytes, expressing CD4 or CD8 at their surface. This approach will likely be useful in generating human T-cell lines for a variety of immunological and developmental studies.

MATERIAL AND METHODS

Cell Culture and Infection. Peripheral blood leukocytes from healthy donors, cord blood leukocytes, or thymocytes from children undergoing cardiac surgery were used in two protocols. One procedure used centrifugation through Histopaque density gradients (1.1 g/ml; Pharmacia) to isolate mononuclear cells, which were subsequently stimulated with phytohemagglutinin P (PHA; Sigma) for 1 day. Alternatively, erythrocytes were depleted from whole blood by dextran sedimentation, and leukocytes were sedimented from the supernatant. Thymocytes were extracted from minced thymus tissue. Stimulated and unstimulated cells were seeded into appropriate tissue culture plastic ware at a density of 1×10^6 cells per ml and inoculated with 10^4–10^6 tissue culture infectious doses of virus. PHA-stimulated cells were supplemented with recombinant interleukin 2 (50 units/ml; Eurocetus, Amsterdam). Production of infectious virus was monitored by cocultivation of lymphocytes with permissive owl monkey kidney cells.

Detection of Viral DNA. Total cellular DNA was isolated and analyzed by Southern blot hybridization to the *Acc* I fragment specific for strains of group C (Fig. 1) according to standard protocols (20). To determine whether the viral genome was integrated into cellular DNA or persisted as an episome, 1×10^6 cells were lysed on top of a 1% agarose gel by the procedure of Gardella *et al.* (21), and fractionated DNA was transferred to nitrocellulose filters. Hybridization was performed with a *Kpn* I fragment conserved in all virus strains (Fig. 1).

Analysis of Surface Markers. Cells were incubated at $1–5 \times 10^5$ cells per well in 96-well microtiter plates. Monoclonal antibodies OKT4 (CD4), OKT6 (CD1), OKT8 (CD8), OKT11 (CD2), OKT26a (CD25, interleukin 2 receptor β chain), and OKB-PanB (CD19) (Ortho Diagnostics); Leu1 (CD5), Leu4 (CD3), Leu9 (CD7), and Leu19 (CD56, NKH-1) (Becton Dickinson); TCR1 [α/β chains of T-cell receptor (TCR)], and TCR2 (γ/δ chains of TCR) (T-Cell Sciences, Cambridge, MA); ATCC TIB200 (CD57, HNK-1), ATCC HB96 (HLA-class II molecules), and Ki-1 (CD30) (Dianova, Hamburg, F.R.G.) were used for single-color flow microfluorometry (FMF). For double-color FMF fluorescein isothiocyanate-conjugated anti-CD4 and phycoerythrin-labeled anti-CD8 antibodies (Immunotech, Marseille, France) were applied. After incubation for 30 min at 4°C, cells were washed and used for FMF double fluorescence or stained with fluorescein isothiocyanate-conjugated mouse anti-human IgG plus IgM $F(ab')_2$ (Becton Dickinson) for single-color FMF. FMF analysis was performed using an electronically programmable individual cell sorter (Epics Elite or Epics Profile, Coulter) (22). Nonviable cells and cellular debris were gated out by propidium iodide staining and forward light scatter.

Cytotoxicity Assay. Natural killer (NK) activity was determined with a chromium release assay using K562 cells (23).

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Abbreviations: PHA, phytohemagglutinin; TCR, T-cell receptor; FMF, flow microfluorometry; ORF, open reading frame; NK, natural killer.

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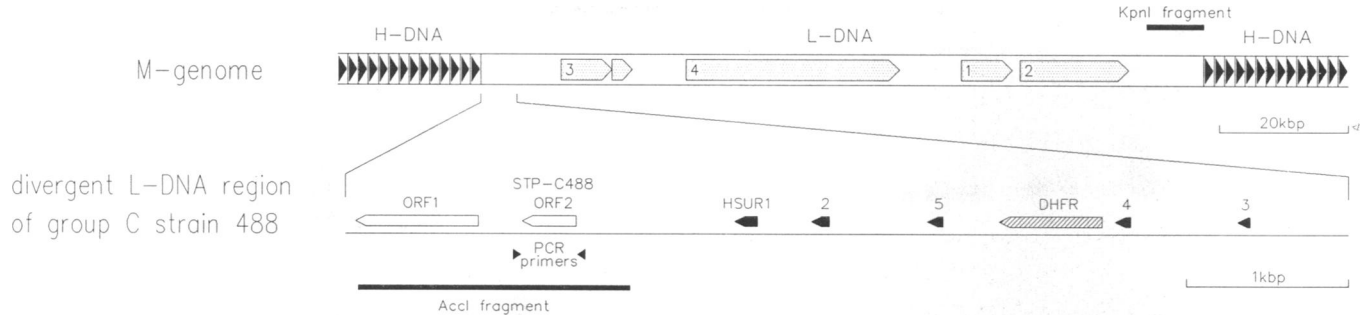

FIG. 1. Structural organization of the Herpesvirus saimiri M-genome (15) and its left-terminal L-DNA. The 112-kbp L-DNA region encompasses four blocks of genes (arrows 1–4) that are partially conserved among all known herpesviruses (16); it is flanked by repetitive H-DNA (17). The left-terminal 6 kbp of L-DNA in strain 488-77 of group C are widely divergent from L-regions of virus strains of groups A and B (11). The region contains five U-RNA genes (solid arrows) (11, 18), a transcription unit for dihydrofolate reductase (DHFR) (19), and two open reading frames (ORF; ORF-1 and ORF-2/STP-C488) related to the oncogenic phenotype (11, 12). The hybridization probe (*Kpn* I fragment) detecting nonintegrated viral DNA (as shown in Fig. 2), the *Acc* I fragment used for Southern and Northern blot analyses, and oligonucleotides for PCR amplification of viral DNA and mRNA are indicated.

RESULTS

Cultures of human mononuclear cells from peripheral blood, cord blood, or thymus infected with strains 484-77 and 488-77 of Herpesvirus saimiri subgroup C (13, 14) yielded continuously growing lymphoblastoid cell lines, whereas all cells infected with strains 11 and OMI (group A) or SMHI (group B) and uninfected cells ceased to proliferate within ≈4 weeks. Numerous lymphoid cell lines were consistently established by infection with the subgroup C strain 488, of which six lines were selected for further characterization (Table 1). All lines have been observed for 6–10 months. When total cellular DNA on Southern blots was hybridized with a 1.7-kbp *Acc* I fragment from the left end of L-DNA, covering the transformation-associated gene (Fig. 1), genomic Herpesvirus saimiri DNA sequences were found in all cell lines, except line PB-W, at 30–60 genome equivalents per diploid cellular genome. The lymphoid cell lines contained the multicopy viral genomes as episomes (Fig. 2), as has been observed in lymphoma-derived monkey cells (24) and in persistently infected human tumor cell lines (25, 26). Estimates based on results of a polymerase chain reaction (PCR) indicated that the peripheral blood-derived line PB-W probably carries 1 genome copy per cell. The immortalized human lymphoblastoid lines did not secrete detectable amounts of infectious virus into the culture supernatant. Transcription of the putative oncogene STP-C488/ORF-2 (12) was demonstrated in all cell lines by hybridization of RNA on Northern blots with an ORF-2 probe (Fig. 1) and by PCR amplification of cDNA with ORF-2-specific primers (Fig. 1 and data not shown).

All cell lines tested expressed mature T-cell phenotypes ($CD2^+$, $CD3^+$, $CD5^+$, $CD7^+$, and $TCR1^+$) but did not have the markers CD1 and TCR2. All lines, except one (Lucas), were either $CD4^+CD8^-$ or $CD4^-CD8^+$ (Table 1 and Fig. 3). The thymus-derived cell line Lucas had a mixed phenotype. Double-fluorescence analysis indicated that the Lucas cell line contains $CD4^+$ and $CD8^+$ single-positive cells and smaller subpopulations of CD4/8 double-positive and -negative cells (Fig. 3*D*). All cell lines were negative for the nonspecific NK cell marker CD57 (HNK-1). A second marker for lymphocytes with NK activity, CD56 (NKH-1), was found on all lines with the exception of PB-W. Strong cytotoxic activity on K562 cells was observed for the $CD8^+$ lines (Table 1). The $CD4^+CD8^-$ lines showed no (PB-W) or low (CB-15) NK activity. All cell lines appeared to represent activated T cells expressing HLA-class II molecules and were positive for the T-cell activation marker CD30. They all expressed the interleukin 2 receptor β chain. No reactivity was observed with the pan-B-cell marker CD19.

DISCUSSION

Convenient protocols for the immortalization of human T cells by viruses have been lacking up to now. Expansion of primary T lymphocytes by repeated antigen or PHA stimulation and cloning of these cells is laborious and usually yields limited numbers of cells growing <1 year. Immortalization with human T-cell leukemia virus type I (HTLV-I) is limited in terms of frequency and by its CD4 specificity. Herpesvirus saimiri has long been known to transform monkey T cells. Unlike Epstein–Barr virus or HTLV-I, penetration of Herpesvirus saimiri into cells is not restricted by a membrane receptor limited to few cell types (27). However, strains from Herpesvirus saimiri groups A and B did not transform the growth of human T cells in numerous attempts. Grassmann *et al.* (28) constructed recombinants of an attenuated noncogenic Herpesvirus saimiri with X-region genes of HTLV-I. These recombinants, expressing the X-region proteins $p42^{tax}$ and $p27^{tax}$, were capable of generating $CD4^+$ lymphoblastoid cell lines from human cord blood cells and thymocyte pri-

Table 1. Origin and surface properties of herpesvirus-transformed human cell lines

Cell line	Origin	Donor sex/age	Stimulation of primary culture by PHA	Presence of surface marker			Cytotoxicity against K562 cells
				CD4	CD8	CD56	
CB-15	Cord blood	Newborn	−	+	−	+	+
P-1080	Cord blood	Newborn	+	−	+	+	+
PB-W	Adult blood	Female/23 years	−	+	−	−	−
P-1083	Adult blood	Female/26 years	+	−	+	+	+
Lucas	Thymus	Male/17 months	−	+	+	+	+
P-1084	Thymus	Female/4.5 years	+	−	+	+	+

All cells expressed CD2, CD3, CD5, CD7, CD25, CD30, TCR1 (α/β chains), and HLA class II molecules and were negative for CD1, CD19, CD57, and TCR2 (γ/δ).

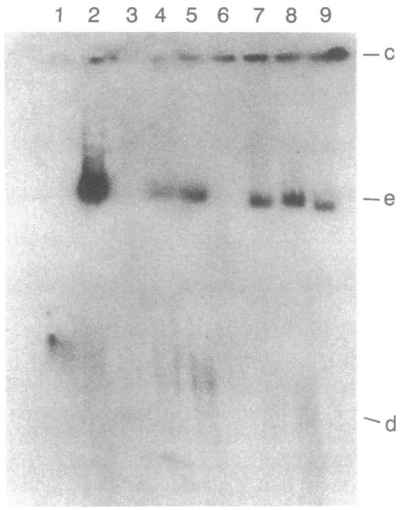

FIG. 2. Detection of nonintegrated viral DNA in human T-lymphocyte lines transformed by strain 488 of Herpesvirus saimiri group C. Positions of chromosomal DNA (bands c), high molecular weight episomes (bands e), and degraded linear DNA (bands d) are indicated. Virion DNA was used as a marker for the linear form of DNA. The marmoset tumor cell line 1670 had been shown (24) to contain ≈210 genome equivalents of strain 11 (Herpesvirus saimiri group A) as episomes per cell. The cell line Hut78 served as a negative control. Episomal DNA from the human cell lines CB-15, P-1080, P-1083, Lucas, and P-1084 comigrated with the circular viral DNA. The estimated single copy of PB-W was below the sensitivity of this assay. Lanes: 1, virions; 2, 1670; 3, Hut78; 4, CB-15; 5, P-1080; 6, PB-W; 7, P-1083; 8, Lucas; 9, P-1084.

mary cultures. They did not, however, induce continuous lymphoproliferation from adult peripheral blood cells. We were thus surprised to find that the wild-type strains of Herpesvirus saimiri group C employed in this study are less restricted in their host range, transforming both $CD4^+$ and $CD8^+$ human T lymphocytes. The repertoire of human T lymphocytes amenable to immortalization by this herpesvirus has not yet been fully determined. Like three of six human cell lines described in this report, marmoset lymphocytes transformed by strains of Herpesvirus saimiri subgroup A were $CD8^+$, had NK activity upon sensitive cell targets, and expressed the NK-associated surface marker NKH-1 (29). CD56/NKH-1 and NK activity are also present in most, but not all, of the transformed human T cells described here. Efficient clonal expansion of differentiated human T lymphocytes, including $CD8^+$ cells, may become one important application of the transformation protocol described in this report. To this end, we have recently obtained evidence that human $CD4^+$ or $CD8^+$ lines that are preselected by antigen stimulation can be permanently expanded with Herpesvirus saimiri. Study of activation pathways may be another useful application of this technique. Future experiments will need to be directed toward identification of the range of differentiated T lymphocytes, T-lymphocyte precursors, and leukemia/lymphoma cells susceptible to indefinite expansion with this herpesvirus. The mechanism by which the virus exerts its effects on lymphocyte growth regulation also needs to be investigated.

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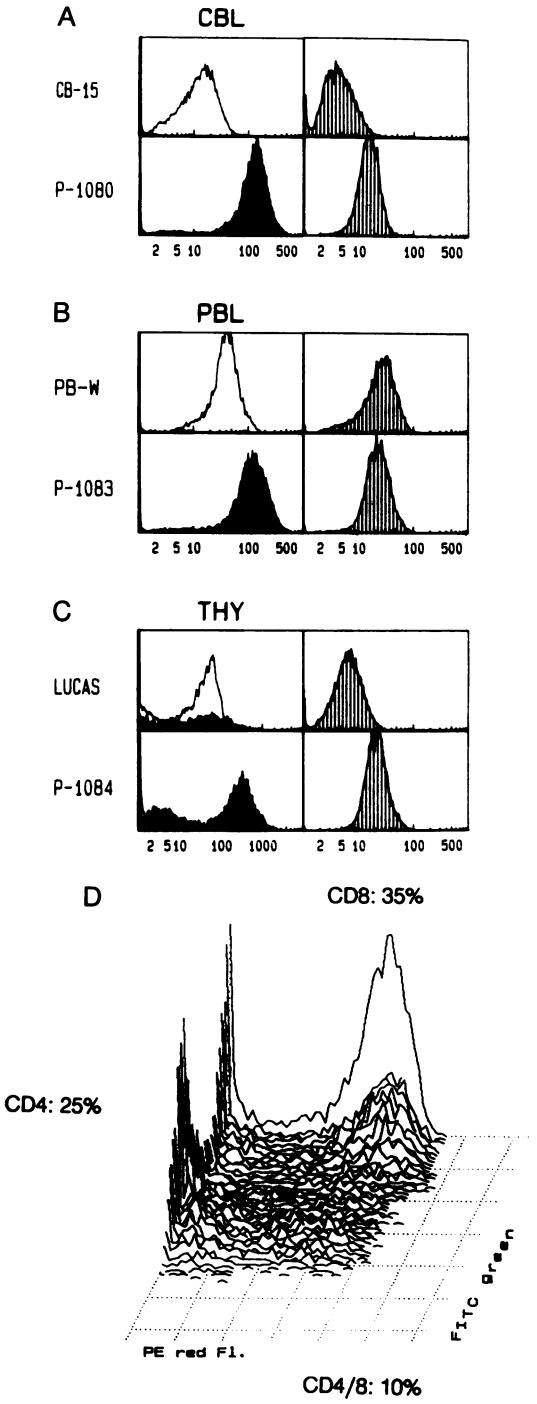

FIG. 3. (*A–C*) Expression of surface markers CD4, CD8, and CD3 in human T-cell lines infected with Herpesvirus saimiri strain 488. Cell lines CB-15 and P-1080 were derived from cord blood leukocytes (*A*), lines PB-W and P-1083 were from peripheral blood leukocytes (*B*), and lines Lucas and P-1084 were from thymocytes (*C*). Expression of CD4 (open peaks), CD8 (solid peaks), and CD3 (shaded peaks) is shown. (*D*) Two-color immunofluorescence of CD4 and CD8 expression on the thymus-derived cell line Lucas infected with Herpesvirus saimiri strain 488. CD8 expression (red fluorescence) is shown on the horizontal axis; CD4 (green fluorescence) is on the oblique axis. Approximately 10% of cells express high levels of both CD4 and CD8 >5 months after infection.

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