The first identified human retroviruses, the human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and -2), have the ability to transform lymphocytes in vitro (10, 11, 51, 62). T lymphocytes infected with HTLV demonstrate enhanced growth potential, marked by seemingly unlimited entry into the cell cycle. The two HTLV serotypes are approximately 65% homologous on the nucleotide level (56, 59) and demonstrate a correspondingly high serologic cross-reactivity (37). Nevertheless, they have distinct seroepidemiologic and clinical profiles. HTLV-1 is associated etiologically with adult T-cell leukemia (16, 26, 63) as well as with a peripheral neuropathy known as tropical spastic paraparesis or HTLV-associated myelopathy (1, 20, 31, 48). HTLV-1 is endemic to southern Japan, the Caribbean basin, central Africa, northeastern South America, and parts of the southeastern United States (3–6, 8, 25, 38, 43, 54). HTLV-2 is occasionally found in Native American Indians (12–15, 28, 34, 39, 41) as well as in a significant proportion of intravenous drug users (IVDUs) (2, 33, 35, 53). Recently, Hall et al. defined two subtypes of HTLV-2, HTLV-2a and HTLV-2b, isolated from peripheral blood lymphocytes (PBLs) of IVDUs (23). HTLV-2 has not been associated with any disease to date, though there have been isolated reports of HTLV-2-associated neuropathy mostly from south Florida and the Caribbean (24, 27, 30, 42, 58).

Persaud et al. (50) reported a limiting dilution infectivity assay for HTLV-1 which allowed early detection of infected cultures and interleukin 2 (IL-2)-driven expansion of clonal populations. The present study extends this method to HTLV-2 and characterizes the resultant transformants. Clonal HTLV-2 populations were routinely and reproducibly generated with the HTLV-2a laboratory strain LAMP/MO. Furthermore, Southern blot analysis was used to characterize both the number and size of proviral integrations in the transformants derived from the in vitro system. These results are compared with those previously noted for HTLV-1. Additionally, chromosomal in situ hybridization was used to localize both HTLV-1 and HTLV-2 proviral integrations.

**Infection-transformation efficiency and assay reproducibility.** The HTLV-2 transformation assay was performed as described by Persaud for HTLV-1 (50). Briefly, LAMP/MO cells (gift of Robert Gallo, National Cancer Institute, Bethesda, Md.) were exposed on ice to 11,700 rads (390 rads/min for 30 min) from a gamma source. Various numbers of irradiated cells (10, 100, and 1,000) were cocultivated with 10^4 activated peripheral blood mononuclear cells (PBMCs) in round-bottom 96-well plates in the presence of IL-2 (10 U/ml) (Boehringer-Mannheim Corp., Indianapolis, Ind.). HTLV-2-transformed cell populations were identified as cells that continued to proliferate beyond 6 weeks, the point at which most of the activated PBMCs not exposed to the HTLV-2-producing cells no longer proliferate in medium containing IL-2, and also by the continued production of HTLV p24 antigen (determined by enzyme-linked immunosorbent assay; Coulter Immunology, Hialeah, Fla.) in the culture supernatant. Control wells containing only activated PBMCs or only irradiated LAMP/MO (10^5 cells/well) were maintained in parallel. After 6 to 9 weeks of culture, cocultures which continued to grow were expanded in growth medium supplemented with 10 U of IL-2/ml. HTLV-2-transformed clones were defined as cells exposed to HTLV-2 that continued to proliferate in the presence of IL-2 and that continuously produced p24 antigen (>1,000 pg/ml) in the culture supernatant.
pg/ml) by 12 weeks after the initial coculture. Control wells, which contained 10⁴ phytohemagglutinin-activated PBMCs in the absence of HTLV-2-infected cells, proliferated for approximately 4 weeks and were never p24⁺; wells which contained only HTLV-2-irradiated cells never exhibited growth and remained positive for p24 at low levels for approximately 3 to 6 weeks after the assay set-up. Transformation efficiency was defined as the percentage of the total cultures exposed to irradiated LAMP/MO HTLV-2 producer cells that met the criteria for transformation. The lymphocyte transformation efficiency of HTLV-2 is demonstrated in Table 1, which summarizes the results of three different experiments. Different donor PBMCs were used for each experiment. With 1,000 HTLV-2-irradiated input cells, the overall transformation efficiency was 71%, with a range of 40 to 81%. With 100 and 10 input cells, the overall transformation efficiencies dropped to 58 and 10%, respectively, demonstrating that transformation efficiency is related to the number of input infected cells.

By linear regression analysis, the log input number of LAMP/MO cells correlated with the efficiency of transformation with an r value of 0.88 and a P value of <0.0001. There was no evidence of a significant difference in donor lymphocytes relative to lymphocyte transformation. All continuous lymphocyte growth was HTLV related: there were no cultures which remained positive for growth and negative for p24. By week 10, 70% of the wells with 10³ LAMP/MO cells/well were p24 positive. By week 12, all cultures which demonstrated growth had detectable p24, and no additional positive cultures were noted. Supernatant from cells in control wells containing only irradiated LAMP/MO cells were p24 negative at the earliest time point tested. Most of the wells that continued to proliferate could be readily expanded and continued to produce p24. These cultures have been maintained in culture for over 2 years with the addition of IL-2. Abrupt removal of IL-2 results in cessation of cell growth, but it is possible to generate IL-2 independence of HTLV-2-transformed cells (data not shown). We conclude that these HTLV-2 lymphocyte lines are IL-2 dependent in the initial phases of transformation.

**Cell surface phenotypes of HTLV-2 transformants.** The cell surface phenotypes of 41 cultures from experiment C (Table 1) were determined at the earliest time possible after coculture (approximately 12 weeks). As shown in Table 2, all HTLV-2-transformed cultures were positive for the T-lymphocyte markers CD2 and/or CD3 and negative for the B1 cell surface antigen. Forty-four percent of the HTLV-2 transformants generated consisted of pure (>95%) populations of CD4⁺ or CD8⁺ T lymphocytes, and the remainder consisted of mixtures of the two subsets. A greater percentage of the transformants (39 versus 5%) were CD4⁺ than CD8⁺ in this system. In contrast, analyses of lymphocytes in vivo indicate that HTLV-1 is detected primarily in the CD4⁺ subset (52) and HTLV-2 is found in the CD8⁺ population (29). The findings reported here indicate that in vitro, the CD4⁺ cell is at least as susceptible to infection and transformation with HTLV-2 as the CD8⁺ T cell. One possible explanation for the difference between the in vitro and in vivo observations is that HTLV-2-infected CD4⁺ T lymphocytes may be eliminated in vivo (45) or suppressed by the host immune system.

**T-cell receptor rearrangement of HTLV-2 transformants.** The cell surface analyses indicated that several of the HTLV-2 transformants were homogenous T-lymphocyte populations. Southern blot analysis for T-cell receptor beta chain (TCRβ) gene rearrangement was performed to confirm lineage and to establish clonality (44, 60). A representative blot is shown in Fig. 1. All 15 cultures analyzed were confirmed to be T cells by virtue of rearrangement. Ten were determined to be clonal populations, and 5 were oligoclonal. Notably, the TCRβ gene pattern demonstrated by LAMP/MO (Fig. 1, lane MO) was distinct from that of the other samples analyzed, indicating that the cultures generated in the infectivity assay were in fact new transformants and not the result of outgrowth of insufficiently irradiated LAMP/MO.

**Proviral copy number in HTLV-2-transformed cell lines.** LAMP/MO and the 15 HTLV-2 transformants studied above were next analyzed for viral copy number by Southern blot analysis. Digestion of DNA from the HTLV-2 transformants with the enzyme HindIII, which does not cut in the proviral sequence, was utilized to determine the number of integrated proviruses. Each band generated by HindIII digest on Southern blot analysis represents a distinct proviral integration site. A representative blot is shown in Fig. 2. LAMP/MO contained a single major band and several (at least three) other, much fainter appearing bands. Of the 10 cultures determined to be clonal by TCRβ rearrangement, 5 had single integration sites, 3 had a single strong band and several faint bands, and 2 had multiple bands. The five samples which were oligoclonal by TCRβ rearrangement had all multiple integration sites. No

**T-lymphocyte lines are IL-2 dependent in the initial phases of transformation.** Any possible explanation for the difference between the in vitro and in vivo observations is that HTLV-2-infected CD4⁺ T lymphocytes may be eliminated in vivo (45) or suppressed by the host immune system.

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**Table 1. HTLV-2 transformation efficiency and reproducibility.**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Total no. (%) of antigen-positive cultures with LAMP/MO input cell number of:</th>
<th>10</th>
<th>100</th>
<th>1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6/58 (10) 28/48 (58) 32/45 (71)</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2/8 (25) 4/8 (50) 6/8 (75)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3/40 (8) 22/30 (73) 22/27 (81)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as the number (%) of antigen-positive (p24 concentration >1,000 pg/ml) cultures over the total number of wells seeded that could be established as cell lines after cocultivation of 10⁴ PHA-stimulated PBMCs with different cell concentrations (10, 100, and 1,000) of irradiated LAMP/MO.

**Table 2. Cell surface phenotypes of HTLV-2 transformants.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of cultures with indicated phenotype</th>
<th>Total no. (%) of cultures with indicated phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2⁺ or CD3⁺</td>
<td>2⁵ 19⁶ 20⁷</td>
<td>41</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>1 13 2</td>
<td>16 (39)</td>
</tr>
<tr>
<td>CD6⁺</td>
<td>0 0 2</td>
<td>2 (5)</td>
</tr>
<tr>
<td>CD4⁺ and CD8⁺</td>
<td>1 6 16</td>
<td>23 (56)</td>
</tr>
</tbody>
</table>

* Cells lines were designated as CD4⁺ or CD8⁺ if >95% of the cells were positive for the subtype by flow cytometric analysis as described in the text. All HTLV-2-positive transformants in these experiments were B1 negative. The cell lines were phenotyped by one- or two-color fluorescence by using a FACSscan (Becton-Dickinson and Co., Mountain View, Calif.) and were analyzed using the Lysys 2.0 software package. The following antibodies were used: CD2, CD3, CD4, CD6, B1, KC56 (T200 Ag), and MsIgG (mouse isotypic control containing immunoglobulin subtype G1, G2a, G2b, G3) (Coulter Immunology).

* From three HTLV-2-transformed cultures.

* From 22 HTLV-2-transformed cultures.

* From 24 HTLV-2-transformed cultures.
difference in growth properties in cells with single or multiple integration sites was noted (with regard to IL-2 requirements and generation time).

Defective proviral forms associated with HTLV-2. Clonal populations of HTLV-1-infected lymphocytes have been shown to contain both full-length and subgenomic, or defective, proviruses. The presence of three proviral forms (full-length, 8.8 kb; defective, 6.5 and 3.5 kb) in LAMP/MO has been described previously (9). However, the transmissibility of these forms has not been investigated qualitatively in vitro. The HTLV-2 transformants derived from this transformation assay provided the opportunity to determine whether defective proviral forms are associated with \( T \) lymphocytes transformed in vitro with HTLV-2. For these analyses, a combination of the enzymes \( \text{AseI} \) and \( \text{EcoRV} \), which each cut once in the 5' (bp 1032) and 3' (bp 8035) long terminal repeat, respectively, were used. A full-length provirus digested with the combination \( \text{EcoRV/AseI} \) would be visualized at 7.0 kb; a defective with internal deletions would be detected as a smaller band.

LAMP/MO and a total of 18 HTLV-2 transformants were analyzed for proviral structure. As demonstrated in Fig. 3, all transformants contained a full-length proviral copy and no transformant contained only a subgenomic fragment. LAMP/MO (Fig. 3, lane 2) contains, in addition to the full-length provirus (visualized here as a band of 7.0 kb) one major defective form of approximately 6.0 kb. One sample (Fig. 3, lane 5) contained, in addition to the full-length form, a band slightly larger than the full-length form. Two other HTLV-2 transformants harbored a smaller HTLV-2 proviral form in addition to the full-length provirus (Fig. 3, lane 3, and data not shown). With these three exceptions, all of the other 15 samples examined contained exclusively a full-length provirus.

Production of subgenomic, or defective, provirus by HTLV-1 has been demonstrated in clinical samples (47). Similarly, analyses of HTLV-1-transformed \( T \) lymphocytes demonstrated that most transformants contained defective proviral forms in addition to the full-length provirus (data not shown). In contrast to the data for HTLV-1 transformants, our studies present little evidence for the existence of defective HTLV-2 provirus. The findings reported herein suggest either that defectives are not efficiently generated in HTLV-2 replication, and therefore play little role in HTLV-2-mediated viral transformation, or that HTLV-2 defectives that are generated in vitro are “lethal-dominants” and cannot support replication.

Chromosomal localization of HTLV integration. Several studies have addressed the site specificity of HTLV-1 proviral chromosomal integration (40, 55, 65). No obvious clustering of proviral integration has been observed, indicating that HTLV-1 probably integrates randomly into chromosomal DNA. The localization of HTLV-2 proviral integration has not been previously addressed. In this series of experiments, chromosomal in situ hybridization of several HTLV-1 and HTLV-2 transformants was used to determine the proviral chromosomal localization. If there were a preferential site(s) of proviral integration, the transformants derived from this assay could facilitate its localization because multiple transformants from a single donor can be analyzed. Figure 4 shows an example of in situ

**FIG. 1.** TCR\( \beta \) rearrangement of HTLV-2 transformants. DNAs from selected HTLV-2 lines were digested with \( \text{HindIII} \), subjected to Southern blotting as previously described (50), and hybridized with a 0.42-kb probe to the constant regions, CT\( \beta \)1 and CT\( \beta \)2, of the \( \beta \) chain of the T-cell receptor (Oncor, Gaithersburg, Md.). CT\( \beta \)1 DNA was labeled with \( ^{32} \text{P} \) by using a random-priming kit (GIBCO/BRL) and was purified on a Sephacyl G-50 column (Pharmacia, Piscataway, N.J.). The germline control (lane PBL) consisted of DNA from normal PBLs and gave the predicted bands of 3.7 and 7.7 kb. LAMP/MO (lane MO) demonstrated a clonal pattern distinct from the HTLV-2 transformants. Lanes 3, 4, and 5 are clonal populations by this analysis, and lanes 1 and 2 are oligoclonal.

**FIG. 2.** Detection of HTLV-2 proviral integration sites in transformed populations of \( T \) lymphocytes. Southern blot analyses of DNA from HTLV-2 transformants determined to be clonal by TCR\( \beta \) rearrangement analysis were digested with \( \text{HindIII} \) and hybridized with HTLV-2 probe pMO-4, which consisted of inserts of the entire HTLV-2 genome (57) (kindly provided by George Shaw, University of Alabama, Birmingham, Ala.). Lane MO, LAMP/MO; lanes 1 to 9, HTLV-2 transformants; lane 10, negative control (PBLs from HTLV-negative donor).

**FIG. 3.** Detection of HTLV-2 proviral integration forms in clonal populations of \( T \) lymphocytes. Southern blot analyses of HTLV-2 transformants digested with a combination of \( \text{AseI/EcoRV} \) as described in the text and hybridized with probe pMO-4. Lane 1, negative control; lane 2, LAMP/MO; lanes 3 to 10, HTLV-2 transformants. Low-molecular-weight bands (<1 kb) seen in lanes 2, 4, 5, 7, and 10 most likely represent cellular-probe degradation products.
The present studies are the first qualitative analyses of the HTLV-2 proviral structure in transformed T lymphocytes. Clonal T-cell populations were shown to contain either single or multiple integration sites. HTLV-1 proviral integration has been characterized by numerous studies: adult T-cell leukemia cells contain oligo- or monoclonal viral integration (64), while in cells from patients with tropical spastic paraparesis/HTLV-infected patients and differ qualitatively from HTLV-1 in disease potential.

The present studies do not illuminate one of the more intriguing biologic questions about HTLV-1 and HTLV-2, namely, the viral properties that account for the markedly different ecologic niches. In recent years, the HTLV seroprevalence among IVDUs has been attributed predominantly to HTLV-2, indicating a possible quantitative or qualitative difference in infectivity between the two viruses. Since in vitro transformation with HTLV-1 and HTLV-2 are apparently equivalent, perhaps other factors are important determinants of transmission and explain the seeming differences in the niches occupied by these closely related retroviruses. Particularly puzzling from a seroepidemiologic perspective is the relative absence of HTLV-2 in the general population and, in spite of a low level of HTLV-1 in the general population, the relative absence of HTLV-1 in IVDUs (2, 33, 35, 53). It is clear that HTLV-1 is etiologically associated with clinical disease; it seems possible that HTLV-2 may be as virulent as HTLV-1 but the high mortality of infected patients from other causes (49) may obscure HTLV-2-specific disease. Alternatively, HTLV-2 may produce only subclinical infections in the vast majority of HTLV-2-infected patients and differ qualitatively from HTLV-1 in disease potential.

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hybridization of metaphase spreads of two HTLV-2-transformed cell lines with probe pMO4: proviral integration at 2p24 was noted for cell line 7MO3-27, and proviral integration at 1q21-22 was noted for cell line 7MO2-7. A minimum of 20 cells were analyzed for each cell line. Signals detected more than three times at the same chromosome locations were scored as specific proviral integration sites. Eight HTLV-2 transformants were examined, and a total of 17 integration locations were detected; 10 HTLV-1 transformants were also examined, and a total of 25 integration locations were noted (data not shown). No specific chromosomal integration site or pattern was identified with transformants from either virus.

The chromosomal localization studies of the transformants derived from this in vitro transformation system confirm and extend the conclusions of previous studies for HTLV-1. Analysis of multiple transformants, including several from a single donor, have failed to reveal a conserved integration locus. However, the possibility of HTLV integration adjacent to conserved, genome-wide repetitive sequences has not been eliminated. Recent work indicating HTLV proviral integration in the GC-rich fraction of the genome (65) supports the concept that HTLV-1 integration may be governed by yet undefined rules. Further studies are necessary to elucidate completely the relationship between HTLV integration and T-lymphocyte transformation.

The data presented in this report show that clonal populations of lymphocytes transformed with HTLV-2 can be reproducibly generated by using a modified limiting dilution cocultivation assay. Clonal transformants are typically CD3+, B1−, CD4+, or CD8+ T lymphocytes demonstrating IL-2-dependent long-term growth and continuous p24 production. The time course of transformation and transformation efficiency, as well as the phenotypic profile of the resultant HTLV-2 clones, are comparable to the results obtained when HTLV-1 is used to transform PBMCs (50). One notable difference is the frequent detection of defective proviral formation with HTLV-1 transformants and the rarity of defective proviruses noted with HTLV-2 transformants.

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FIG. 4. In situ hybridization of HTLV-2-transformed cell lines. Metaphase preparations of two HTLV-2 transformants (7MO2-7 and 7MO3-27) were hybridized with probe pMO4. Cell cultures were harvested for metaphase preparation with colcemid by standard cytogenetic laboratory procedures. Probes were labelled by nick translation with either biotin-dUTP or digoxigenin-dUTP (Boehringer-Mannheim Corp.) and detected by nonfluorescent methods which use peroxidase-tagged avidin (Vector Lab Inc., Burlington, Calif.) or peroxidase-tagged anti-digoxigenin (Boehringer-Mannheim, followed by diaminobenzidine and silver amplification (Amersham Corp., Arlington Heights, Ill.). This method was originally described by Burns et al. (7), and hybridization and signal detection were performed as described by Lee et al. (36) with modifications (61). The probe concentration used was 10 μg/ml, and hybridization was performed overnight by standard in situ hybridization protocols (Oncor). For each probe and transformant cell line, several amplification times were tested: the best signal localization was achieved with the minimal amplification time (ranging from 20 to 40 min) sufficient to observe specific signal while minimizing the background. Cells were chosen for further analysis when the silver signals were confined to a single small dot on each chromatid. Images were recorded and analyzed with Cytovision software (Applied Imaging, Pittsburgh, Pa.). These results are representative of the results obtained with the other HTLV-2-transformed cell lines analyzed. Similar results were also achieved when HTLV-1-transformed cell lines were hybridized with the HTLV-1 probe pMT2, a full-length (8.25 kb) HTLV-1 probe as previously described (57).
event occurs; alternatively, the transformed phenotype may represent the cumulative effect of multiple integrations. In vitro data presented above provide support for the former hypothesis.

HTLV transformation confers enhanced growth potential on the infected lymphocyte. This transforming capacity has been exploited in the laboratory as a method of immortalizing lymphocytes for further study, in a manner similar to Epstein-Barr virus transformation of B lymphocytes. PBLS from patients with adenosine deaminase deficiency (32) and paroxysmal nocturnal hemoglobinuria (46) have been immortalized with HTLV-1, and a series of papers have examined hormone responsiveness in Pygmy and normal HTLV-2-transformed T-cell lines (17–19). The transforming ability of HTLV-2, the relative frequency of monoclonal integrants, and the absence of discoid association render HTLV-2 a suitable candidate for the immortalization of T lymphocytes from patients for multiple purposes.

REFERENCES


