A Colony Assay for In Vitro Transformation by Human T Cell Leukemia Viruses Type I and Type II

By Mordechai Aboud, David W. Golde, Noelle Bercsh, Joseph D. Rosenblatt, and Irvin S.Y. Chen

We report here the development of a rapid and quantitative method for measuring in vitro T cell transformation by human T cell leukemia viruses type I (HTLV-I) and type II (HTLV-II). This method is based on our finding that cocultivation of lethally irradiated HTLV-producing cells with peripheral blood lymphocytes (PBLs) preactivated for 24 hours with phytohemagglutinin and interleukin-2 (IL-2) induces colony formation in methylcellulose-containing medium. Colonies of about 200 cells can be clearly distinguished from background aggregates within four to six days after cocultivation. These colonies gradually increase in size and reach 300 to 1,000 cells within 14 days after cocultivation. Cells of these colonies were infected, as evidenced by expression of viral p19 antigen and the presence of HTLV proviral sequences. These cells proved to be transformed in terms of IL-2-independent continuous growth in liquid medium. Colony formation was found to depend in a linear fashion upon the percentage of the infected cells present in the irradiated cell population and is sufficiently sensitive to detect as few as 1% of virus-producing cells.

The human T cell leukemia viruses type I (HTLV-I) and type II (HTLV-II) have been etiologically implicated with specific human T cell malignancies. HTLV-I has been established by seroepidemiological and molecular studies as the causative agent of adult T cell leukemia (ATL), an aggressive disease endemic to southern Japan, the Caribbean basin, certain parts of the southeastern United States, and sporadically in other locations. HTLV-II has been associated with two cases of a variant of hairy cell leukemia, which, unlike ATL, is a rare and relatively indolent disease.

Both HTLV-I and HTLV-II are capable of immortalizing normal peripheral blood and umbilical vein T lymphocytes in vitro. Although these viruses can also infect a variety of other cells in culture, their in vitro transforming capacity seems to be restricted to human T cells. The mechanism of cell transformation by HTLV is still unknown and is likely to be different from that of other transforming retroviruses. Neither HTLV-I nor HTLV-II carries a classic retroviral oncoregion, and there is also no evidence that integration of the proviral DNA occurs in specific sites of the cellular genome in tumors. Clarification of this mechanism would be greatly facilitated if the HTLV-induced transformation of normal T lymphocytes could be measured by a rapid quantitative assay. In previous studies, transformation by HTLV-I and HTLV-II has been determined by cocultivating peripheral or cord blood lymphocytes in liquid cultures with lethally irradiated or mitomycin-treated HTLV-producing cell lines with or without interleukin-2 (IL-2). Transformation in this manner is defined by the appearance of IL-2-independent immortalized T cells. More recently, it has been found that the efficiency of HTLV-induced transformation can be enhanced by preactivation of the target lymphocytes with phytohemagglutinin (PHA) in the presence of IL-2 for 24 hours before cocultivation with the virus-producing cells. However, this procedure is inconvenient because of the length of time required to define IL-2-independent immortalization of the target lymphocytes. Moreover, this assay does not provide a quantitative analysis of the transformation potential.

We have developed a rapid colony assay that can quantitatively determine HTLV-induced transformation within four to six days. This assay relies on our finding that normal lymphocytes, preactivated with PHA in the presence of IL-2, produce colonies after cocultivation with lethally irradiated HTLV-producing cell lines in methylcellulose-containing medium.

MATERIALS AND METHODS

Cells and viruses. A variety of HTLV-I- and HTLV-II-producing T and B cell lines were tested for induction of colony formation by normal lymphocytes. The HTLV-I-producing cells were ME and ET T cell lines isolated from two different ATL patients: SLB-I, a T cell line generated from peripheral blood lymphocytes (PBLs) of a normal female blood donor by cocultivation with ME cells. The HTLV-II–producing cells were Mo-T and NRA T cell lines isolated from the first (Mo) and the second (NRA) variant hairy cell leukemia patients, respectively; JLB-I, a T cell line established from PBLs of the aforementioned blood donor by cocultivation with Mo-T cells; Mo-B, an Epstein-Barr virus (EBV)-transformed and HTLV-II–infected B cell line established from the patient Mo; J-WIL, an EBV-transformed B cell line (designated WIL) that was infected by cocultivation with Mo-T cells; and 729 pH6neo, an EBV-transformed B cell line (designated 729) that was stably transfected with an infectious cloned genomic DNA of HTLV-II. HUT-78 and MOLT-7 T cell lines and 729 and WIL B cell lines were used as HTLV-uninfected controls.

The target normal PBLs were prepared by Ficoll-Hypaque (P.L. Biochemicals, Milwaukee) density centrifugation from fresh blood samples of random male or female blood donors. The banded mononuclear cells were washed twice with Iscove’s medium (Irvine Scientific, Santa Ana, CA) containing 20% fetal calf serum (FCS) and antibiotics (Irvine Scientific) and incubated in flasks with this
medium for two hours at 37°C to remove adherent cells, and the remaining nonadherent cells were used for the colony assay.

**Colony formation assay.** The virus-producing cells and the nonproducing control cells received fresh Iscove's medium containing 20% FCS two days before the assay. Immediately before plating, their density was adjusted to 10⁶ viable cells (determined by trypan blue exclusion) per milliliter and irradiated with 12,000 rad. The target lymphocytes were activated for 24 hours before the assay by 1% PHA in the presence of IL-2. As a source for IL-2, we used a 1:1,000 dilution of conditioned medium collected from cultures of COS cells 48 and 72 hours after transfection with a recombinant eukaryotic expression plasmid carrying the IL-2 gene that was provided by S.C. Clark (Genetics Institute, Boston). The conditioned medium contained about 1 ng IL-2/mL. Both the irradiated and target cells were centrifuged and resuspended in Iscove's medium containing 20% FCS by extensive dispersion of cell aggregates through a 25-gauge syringe needle. Aliquots of 10⁴ irradiated cells were mixed with an equal number of target lymphocytes in a final volume of 1 mL of Iscove's medium containing 20% FCS and 3.14% methylcellulose (Dow Chemical Co, Midland, MI), and after repeated dispersion, the mixture was plated in 35-mm-diameter tissue culture dishes with 2-mm grids (Falcon Labware, Oxnard, CA). In some preliminary experiments, IL-2 was also added to this mixture. After one to two hours of incubation at 37°C, the cultures were inspected for background cell aggregates.

**Analysis of HTLV antigen expression.** Large colonies were picked 14 days after plating, propagated in liquid to about 10⁹ cells, and tested for expression of HTLV p19 antigen on their surface as previously described.

## RESULTS

**Colony formation by HTLV-infected PBLs.** When PBLs, preactivated for 24 hours with PHA in the presence of IL-2, were cocultivated with lethally irradiated HTLV-I- or HTLV-II-producing cell lines in methylcellulose, colony formation was consistently observed. In some experiments, IL-2 was included in the methylcellulose plating mixture. Although these conditions allowed the formation of large colonies of transformed cells, they were found to be inconvenient for scoring these colonies because of a heavy background apparently resulting from IL-2–supported proliferation of the nontransformed PBLs. Omission of IL-2 from the plating mixture was subsequently found to permit similar colony formation without significantly affecting their number. More important, elimination of IL-2 arrested the growth of untransformed PBLs and thus reduced the density of the background. Under such conditions, colonies of transformed cells could be easily detected microscopically as early as four to six days after plating. At this early stage, these colonies were relatively small (about 200 cells) but clearly distinguishable from background aggregates of less than 70 cells. Moreover, upon further incubation, the colonies of transformed cells gradually grew in size, whereas the background aggregates remained the same or gradually degenerated. Figure 1A illustrates an example of large colonies, with some small aggregates of apparently dead cells observed 14 days after cocultivating PBLs with HTLV-II–producing cell lines. Such colonies may grow to more than 1,000 cells as shown in Fig 1B. Table 1 presents repeated assays with PBLs from random different normal male and female blood donors that used the same HTLV-producing cell line (Mo-T). Colony formation was consistently detected in all of these experiments, thereby indicating that the assay can be carried out with PBLs of any available healthy blood donor.

**Cell morphology in colonies.** Large colonies were randomly picked, fixed by cytocentrifugation, and stained with repeated dispersion, the mixture was plated in 35-mm-diameter tissue culture dishes with 2-mm grids (Falcon Labware, Oxnard, CA). In some preliminary experiments, IL-2 was also added to this mixture. After one to two hours of incubation at 37°C, the cultures were inspected for background cell aggregates.

**Analysis of HTLV antigen expression.** Large colonies were picked 14 days after plating, propagated in liquid to about 10⁹ cells, and tested for expression of HTLV p19 antigen on their surface as previously described.

## RESULTS

**Colony formation by HTLV-infected PBLs.** When PBLs, preactivated for 24 hours with PHA in the presence of IL-2, were cocultivated with lethally irradiated HTLV-I- or HTLV-II-producing cell lines in methylcellulose, colony formation was consistently observed. In some experiments, IL-2 was included in the methylcellulose plating mixture. Although these conditions allowed the formation of large colonies of transformed cells, they were found to be inconvenient for scoring these colonies because of a heavy background apparently resulting from IL-2–supported proliferation of the nontransformed PBLs. Omission of IL-2 from the plating mixture was subsequently found to permit similar colony formation without significantly affecting their number. More important, elimination of IL-2 arrested the growth of untransformed PBLs and thus reduced the density of the background. Under such conditions, colonies of transformed cells could be easily detected microscopically as early as four to six days after plating. At this early stage, these colonies were relatively small (about 200 cells) but clearly distinguishable from background aggregates of less than 70 cells. Moreover, upon further incubation, the colonies of transformed cells gradually grew in size, whereas the background aggregates remained the same or gradually degenerated. Figure 1A illustrates an example of large colonies, with some small aggregates of apparently dead cells observed 14 days after cocultivating PBLs with HTLV-II–producing cell lines. Such colonies may grow to more than 1,000 cells as shown in Fig 1B. Table 1 presents repeated assays with PBLs from random different normal male and female blood donors that used the same HTLV-producing cell line (Mo-T). Colony formation was consistently detected in all of these experiments, thereby indicating that the assay can be carried out with PBLs of any available healthy blood donor.

**Cell morphology in colonies.** Large colonies were randomly picked, fixed by cytocentrifugation, and stained with

**Table 1.** Colony Induction by Mo-T Cell Line in PBLs From Different Blood Donors

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gender of Blood Donor</th>
<th>No. of Colonies per Dish*</th>
<th>PBLs Alone</th>
<th>Mo-T Alone</th>
<th>PBLs + Mo-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>536</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>424</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>560</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>648</td>
<td></td>
</tr>
</tbody>
</table>

*Large colonies of more than 300 cells were counted 14 days after plating. Data are presented as averages of duplicate cultures.
Fig 2. Giemsa staining of colonies. A large colony induced by Mo-T cells (A) (original magnification × 25; current magnification × 16) and a small clump picked from a control culture of irradiated Mo-T cells alone (B) (original magnification × 25; current magnification × 25) or from a culture of PBLs alone (C) were spun down onto a glass slide and photographed (original magnification × 25; current magnification × 23) after Giemsa staining.

Giemsa for morphological analysis. Figure 2A illustrates a representative colony induced by Mo-T cells. It can be seen that the colony consists of mononuclear cells growing around an apparent, degenerating, irradiated virus-producing cell. Such degenerating cells, obtained from clumps picked from dishes containing irradiated Mo-T cells alone, are shown in Fig 2B. Figure 2C, on the other hand, shows mononuclear cells derived from nonspecific aggregates picked from control dishes containing PBLs alone.

Specificity of colony induction by HTLV-producing cells. To verify that this assay was a specific measure for HTLV infection, a variety of HTLV-I- and HTLV-II-producing T or B cell lines was tested in comparison to HTLV-uninfected cells. The data summarized in Table 2 clearly indicate that colony formation was induced, under our experimental conditions, only by the HTLV-producing cell lines, not by any of the tested nonproducing cell lines.

Cell morphology of representative large colonies, randomly picked from each of these experiments for Giemsa staining, was the same as shown in Fig 2A, whereas aggregates picked from mixed cultures of PBLs and uninfected irradiated cells were similar to those shown in Fig 2C for PBLs alone (not shown).

To confirm that the transformed cells were indeed infected by HTLV, we examined the expression of HTLV antigens on the surface of cells from various colonies. Large colonies were randomly picked from various experiments 14 days after plating, grown in liquid medium to about 10⁶ cells, and subjected to direct immunofluorescence analysis using monoclonal antibodies against HTLV p19 antigen. A representative experiment is depicted in Table 3. In this particular experiment, colony formation was induced by Mo-T and 729 pH6neo, and as can be seen, all of the tested colonies contained p19-expressing cells, although in variable proportion, ranging from 5% to 95% of their cell population. Similar data were obtained with colonies induced by other HTLV-producing cells (not shown). Such a variation is frequently observed between different HTLV-infected cell clones, apparently reflecting different efficiencies of virus expression in different cells. Final confirmation of HTLV infection

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell Lineage</th>
<th>HTLV Type</th>
<th>Colony No. per Dish *</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>T</td>
<td>I</td>
<td>960</td>
</tr>
<tr>
<td>ET</td>
<td>T</td>
<td>I</td>
<td>536</td>
</tr>
<tr>
<td>SLB-I</td>
<td>T</td>
<td>I</td>
<td>1,040</td>
</tr>
<tr>
<td>Mo-T</td>
<td>T</td>
<td>II</td>
<td>648</td>
</tr>
<tr>
<td>NRA</td>
<td>T</td>
<td>II</td>
<td>192</td>
</tr>
<tr>
<td>JLB-I</td>
<td>T</td>
<td>II</td>
<td>728</td>
</tr>
<tr>
<td>Mo-B</td>
<td>B</td>
<td>II</td>
<td>268</td>
</tr>
<tr>
<td>729 pH6neo</td>
<td>B</td>
<td>II</td>
<td>360</td>
</tr>
<tr>
<td>J-WIL</td>
<td>B</td>
<td>II</td>
<td>176</td>
</tr>
<tr>
<td>HUT-78</td>
<td>T</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>MOLT4</td>
<td>T</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>729</td>
<td>B</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>WIL</td>
<td>B</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are presented as averages of duplicate cultures.
was made by Southern hybridization (data not shown). We define HTLV cellular transformation as growth in the absence of exogenous IL-2. The transformed state of the cell in individual colonies in our experiments was thus confirmed by their growth in liquid medium without IL-2.

Sensitivity and quantitative application of the colony assay. Finally, we analyzed the sensitivity of the assay by determining the lowest proportion of HTLV-infected cells that it can detect in tested cell populations and whether it is adequate for quantitation of these infected cells. For this purpose, variable numbers of HTLV-infected cells (such as Mo-T and 729 pH6neo) were mixed with uninfected cells (729 cell line) before irradiation, the total cell number of cells in the mixture kept constant. This mixture was cocultivated with activated PBLs. From the results illustrated in Fig 3, it is evident that this assay can detect HTLV-infected cells even if they constitute as few as 1% of the irradiated cell population. Furthermore, the assay is fairly linear within the range of its sensitivity.

DISCUSSION

Both HTLV-I and HTLV-II can infect a variety of cells in culture. They also exhibit an in vitro transforming activity, but this activity seems to be restricted to T cells with a mature activated phenotype. Our current investigations on the mechanism of the HTLV-induced transformation have motivated us to search for a rapid assay to measure this transforming capacity. Preactivation of target PBLs with PHA and IL-2 before cocultivation with lethally irradiated HTLV-producing cells enhances the formation of IL-2-independent transformed T cells; whereas malignant T cells produce colonies in methylcellulose. We therefore elucidated whether preactivated normal PBLs would produce such colonies upon cocultivation with lethally irradiated HTLV-producing cell lines. We examined a variety of T and B cell lines producing either HTLV-I or HTLV-II and showed an efficient and rapid colony formation that could conveniently be used for assaying the HTLV transforming capacity. Such colonies could be visualized and counted as early as four to six days after plating. Cells from such colonies were found to be capable of IL-2-independent continuous proliferation in liquid medium, thus confirming their transformed phenotype. Furthermore, these cells expressed HTLV p19 antigen, thereby indicating that they were productively infected by HTLV. It is worthwhile to emphasize, in this context, that in contrast to virus expression in such in vitro transformed cells, no viral antigen or RNA can be detected in primary leukemic T cells from patients with HTLV-associated T cell malignancies, although these primary cells harbor HTLV DNA in their genomes. Virus expression can be demonstrated in such cells only if they are grown in culture. This contrast may suggest that in vitro T cell transformation is probably analogous to an early event that initiates the in vivo T cell malignancy by altering the growth regulation of the infected cells. However, in the in vivo leukemogenic process, this event is apparently followed by additional events leading to the eventual malignancy that is no longer dependent on viral functions.

Finally, our data demonstrate that the colony assay is sufficiently sensitive to detect HTLV-infected cells, even if they constitute as few as 1% of the examined cell population. Under our experimental conditions, this means that roughly one out of $10^3$ virus-producing cells can be detected as an infectious center. Such an infectious center may be derived from more than one PBL that proliferates into a colony; therefore, these colonies are not necessarily monoclonal. However, since the colony-inducing capacity of such populations is linearly proportional to the percentage of the virus-producing cells, this assay can nevertheless be used for quantitative analysis of the HTLV-induced in vitro transformation. This quantitative potential of the assay may be useful for examining the early events associated with T cell immortalization by these viruses. It may also provide a tool for correlation between structural and functional properties of various HTLV isolates and mutants.

NOTE ADDED IN PROOF

Since acceptance of this report for publication, another report was published describing a different HTLV colony assay (Graziano et al: Cancer Res 47:2468, 1987).

ACKNOWLEDGMENT

We thank Shirley Quan for doing the indirect immunofluorescence analysis and Wendy Afi for preparation of the manuscript.
REFERENCES


A colony assay for in vitro transformation by human T cell leukemia viruses type I and type II

M Aboud, DW Golde, N Bersch, JD Rosenblatt and IS Chen