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Primary Isolation of Human T-Cell Leukemia-Lymphoma Virus Types I and II: Use for Confirming Infection in Seroindeterminate Blood Donors

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We describe the use of an immunofluorescence assay and coculture to confirm human T-cell leukemia-lymphoma virus (HTLV) infection. Peripheral blood mononuclear cells from 32 of 32 seropositive donors were positive in the immunofluorescence assay, and 63% of their cocultures produced p24 antigen. Specific antibodies distinguished HTLV type I (HTLV-I) from HTLV-II. HTLV-I or HTLV-II was isolated from donors with indeterminate serologic test results.

Human T-cell leukemia-lymphoma virus type I (HTLV-I) causes leukemia and a neurologic syndrome, HTLV-associated myelopathy (3, 8, 24; reviewed in reference 9). Serologic testing for HTLV-I is routine in U.S. blood centers and is used diagnostically in patients with leukemia or HTLV-associated myelopathy (4, 28). Many seropositive donors are actually infected with a related virus, HTLV-II (11, 19), which has been associated with an HTLV-associated myelopathy-like illness as well (10). Positive enzyme-linked immunosorbent assays (ELISAs) are followed by confirmatory testing by HTLV-I Western immunoblotting (WB) and, in some cases, by radioimmunoprecipitation assay (1, 4). Definitive diagnosis by WB or radioimmunoprecipitation assay requires serologic recognition of at least two antigens ($p24^{gag}$ and an *env* gene product) of HTLV-I; any other seroreactivity is considered indeterminate (1, 4).

Isolation of HTLV-I and HTLV-II has been described previously (5, 13, 14, 22, 26). Peripheral blood mononuclear cells (PBMC) from infected patients are cocultivated with those from uninfected donors. The HTLVs produce low levels of reverse transcriptase, a useful marker for replication of other retroviruses. While culture has not been used extensively to diagnose HTLV, the polymerase chain reaction (PCR) is able to detect proviral DNA sequences present in PBMC in vivo and allows distinction of HTLV-I from HTLV-II (1, 2, 6, 7, 9–12, 15–19, 21, 26).

PCR studies have shown that some persons with HTLV-I or HTLV-II infection are seronegative, and other infected patients have indeterminate WB results (15, 17, 19, 27). One can rarely prove that a given PCR-positive sample represents true infection or contamination of the patient's PBMC DNA by previously amplified DNA. We sought to develop a method to verify positive PCR studies. To accomplish this, we determined the sensitivity of immunofluorescence assay (IFA) and coculture isolation of HTLV-I and HTLV-II in blood from seropositive and seroindeterminate blood donors, using an antigen capture ELISA (AC-ELISA) to detect virus. We found that coculture and IFA studies helped us verify that positive PCR assays represent genuine infection.

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Blood donors and serologic screening. All blood donors were tested for HTLV-I or HTLV-II antibody by ELISA after donation to the United Blood Services blood center in Albuquerque, N.M. Sera producing repeatedly positive results in the ELISA (Abbott Laboratories, North Chicago, Ill.) were used in WB analysis (Cambridge Biotech, Rockville, Md.) with lysates from HTLV-I-infected HUT-102 cells (1, 4, 20). Lysates for WB contained added $rgp21^e$, an HTLV-I envelope protein expressed in *Escherichia coli* (20). A serum specimen was considered positive if it reacted with $rgp21^e$ and $p24^{gag}$.

Seropositive donors to United Blood Services in Albuquerque are predominantly Native American (12). Most of these seropositive donors are infected with HTLV-II. Of those in this study, 27 of 32 were infected with HTLV-II; the rest had HTLV-I. All had been classified by virus type by PCR, but for 25 of the 32 it was also possible to differentiate the virus types by ELISA with virus-specific peptide antigens (11) (Coulter Immunodiagnostics, Hialeah, Fla.).

Seroindeterminate donors. PBMC from 19 sequential HTLV ELISA-positive, WB-indeterminate blood donors were obtained from donated blood slated to be discarded. PBMC from all 4 donors whose PBMC DNA produced a positive PCR signal were subjected to coculture assay, as were PBMC from 4 of the 15 with negative PCR results. Of the 19 serum samples, 3 had reactivity to *env* antigen ($rgp21^e$), and 16 reacted only to *gag* antigens (p19, p24, and/or p53, and in some cases intermediate forms). One (PCR-negative) donor's serum reacted with $rgp21^e$ and $p19^{gag}$.

Coculture assay. PBMC from ELISA-positive donors were obtained and purified as described elsewhere (11). Aliquots were cryopreserved for later culture. Cocultures were prepared by mixing 5×10^6 to 1×10^7 PBMC from seropositive donors in equal amounts with phytohemagglutinin-stimulated (3 days) PBMC from uninfected donors in Iscove's modified Eagle's medium containing 15% fetal calf serum and 5 μg of phytohemagglutinin per ml. Uninfected donor cells were prepared from pooled buffy-coat PBMC from three donations (typically 5×10^8 cells per donation) and were frozen in aliquots of 10^8 cells. This helped to maintain consistency in our target cell preparations. Cells were cul-

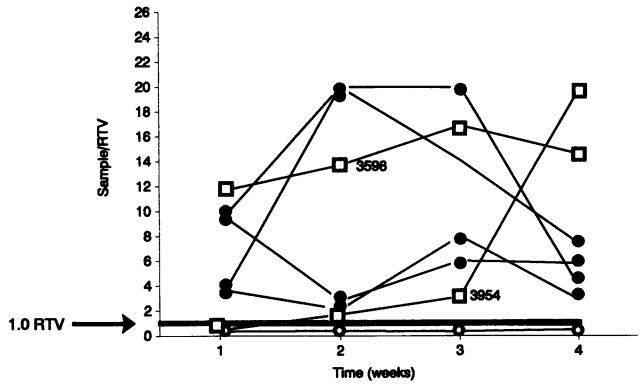

FIG. 1. Production of p24 by PBMC cocultures from a representative sample of 32 blood donors infected with HTLV-I or HTLV-II, as determined by AC-ELISA. p24 expression is expressed as sample absorbance divided by reactive threshold value (Sample/RTV). Closed circles represent cultures from donors with WB-confirmed serologic test results, whereas open squares represent cultures from donors with WB-indeterminate serologic test results. AC-ELISA ratios from a typical negative culture are charted with open circles.

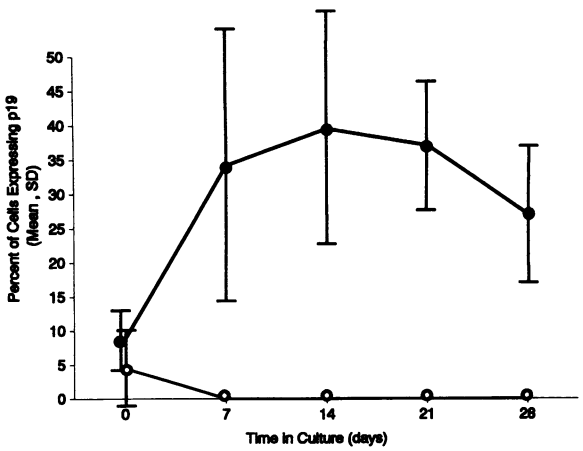

FIG. 2. Correlation of IFA reactivity of cocultured PBMC at various time points of culture with production of extracellular p24 antigen, determined by AC-ELISA, in PBMC cocultures from 18 seropositive donors. IFA reactivity is expressed as the percent of cells reacting to a monoclonal antibody to HTLV-I p19. Closed circles represent cocultures that produced p24 (n = 9); open circles represent those that did not (n = 9). SD, standard deviation (indicated by error bars).

tured in 12-well plates in medium containing phytohemagglutinin for 7 to 10 days and then switched to medium containing 5% interleukin-2 (Advanced Biotechnologies, Columbia, Md.) but lacking phytohemagglutinin. On day 0 and at 7-day intervals thereafter for 4 weeks, aliquots of cells were removed for IFA, and the supernatant was removed for AC-ELISA.

IFA and AC-ELISA. The IFA was performed by treating cold-acetone-fixed cells (3×10^4 per slide) with a murine monoclonal antibody (1:500 dilution) to HTLV-I p19 antigen (Pan-Data Systems, Rockville, Md.) (25) for 30 min at 37°C. After the slides were washed three times in phosphate-buffered saline (PBS) containing 0.5% fetal calf serum, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (1:300 dilution) was added. After three more washes in PBS containing 0.5% fetal calf serum, the slides were rinsed with water and counterstained with 0.01% Evans blue in PBS. We could distinguish uninfected PBMC from a mixture composed of 10^6 uninfected PBMC and 10^2 Mo-T cells (chronically HTLV-II-infected T cells).

AC-ELISA (Coulter) was performed as recommended by the manufacturer. After 24 h of incubation, we were able to distinguish a culture containing 10^2 MT-2 or MO-T cells admixed with 10^6 uninfected PBMC from a culture containing only uninfected PBMC.

An AC-ELISA procedure that employs an antibody specific for HTLV-I (Cellular Products, Inc. [CPI], Buffalo, N.Y.) was used to distinguish cultures producing HTLV-I from those producing HTLV-II.

Electron microscopy. Cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and stained with uranyl acetate and lead citrate as described elsewhere (23).

PCR. All individuals studied had been previously determined to have either HTLV-I or HTLV-II by PCR performed with *tax* and *pol* gene primers and probes as described elsewhere (11, 12, 18).

Coculture assay for HTLV infection. PBMC from 32 seropositive donors were cultured. Figure 1 shows typical antigen production data for the cultures. Twenty cultures (63%) produced antigen after 1 to 4 weeks of culture. Absorbance values near the reactive threshold value were rare, and positive cultures remained positive throughout the 4-week period. The results of the quantitative IFA for cells producing p19 antigen (performed on 18 cocultures) correlated well with the AC-ELISA results for antigen production (Fig. 2). All cocultures from HTLV seropositive donors had at least a few p19-positive cells (Fig. 3). Such cells represented 0.7 to 13% of all input cells. Those with high initial fraction of p19-positive cells (>8%) were much more likely to subsequently produce p24 antigen in coculture (Fig. 2). The fraction of p19-positive cells increased in cultures for which the AC-ELISA was positive, while cultures that did not produce p24 antigen were uniformly nearly devoid of p19-positive cells by day 7. The fraction of p19-positive cells in AC-ELISA-positive cultures ranged from 12 to 57%.

That cultures producing AC-ELISA readings well above the background were productively infected with HTLV was

TABLE 1. Comparison of AC-ELISA results obtained with Coulter p24 antibody and those obtained with CPI antibody

Donor	Cell background	Result obtained by:			
		WB	PCR	AC-ELISA with antibody from:	
				Coulter	CPI
NA[a]	PBMC coculture	−	−	−	−
56539	PBMC coculture	+	HTLV-I	+	+
3529	Jurkat cell line		HTLV-I	+	+
3958	PBMC coculture	I[b]	HTLV-I	+	+
55376	PBMC coculture	+	HTLV-I	+	+
3887	PBMC coculture	+	HTLV-I	+	+
3594	PBMC coculture	I	HTLV-II	+	−
3564	PBMC coculture	+	HTLV-II	+	−
3971	PBMC coculture	+	HTLV-II	+	−
NA	MT2 cell line		HTLV-I	+	+
NA	Mo-T cell line		HTLV-II	+	−

[a] NA, not available.
[b] I, indeterminate. See text.

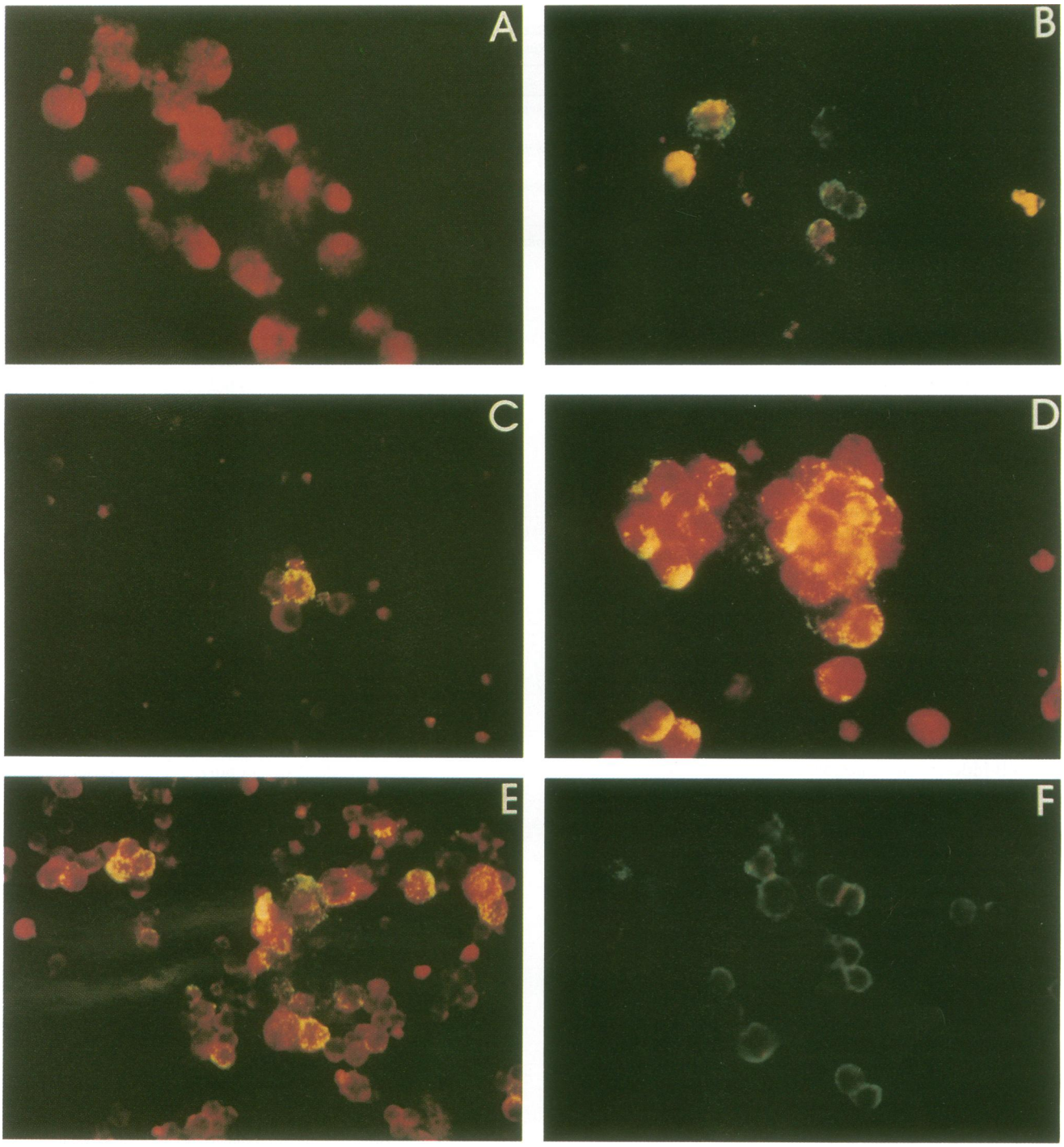

FIG. 3. Examples of p19 IFAs of PBMC from a seronegative donor (A), an HTLV-I-positive donor (donor 3387) whose p24 assay was positive (B), an HTLV-II positive donor (3930) whose coculture did not produce p24 (C), seroindeterminate donor 3594 (determined by PCR to be infected with HTLV-II) (D), and seroindeterminate donor 3958 (determined by PCR to be infected with HTLV-I) (E), and Mo-T cells chronically infected with prototype HTLV-II (F).

verified by electron microscopic examination of two of two such cultures (data not shown), from donors 3887 (HTLV-I positive) and 3564 (HTLV-II positive). PBMC from four of five (80%) HTLV-I-infected donors produced p24 antigen in culture, compared with 16 of 27 (59%) of HTLV-II-infected cultures.

HTLV-I-specific monoclonal antibody distinguishes HTLV-I from HTLV-II. Using the anti-p19 antibody from CPI, we could distinguish HTLV-I-producing cultures from those producing HTLV-II (Table 1). Even HTLV-II cultures that had very high absorbance values with the Coulter antibody were negative with the CPI antibody (18).

TABLE 2. Background and laboratory data for WB-indeterminate donors found positive for HTLV-I or HTLV-II by PCR

Donor	Age (yr)	Sex	Ethnic background	Risk	Result of[a]:		Seroconversion	Result of[b]:		Culture
					WB	RIPA		PCR	peptide ELISA	
3302	37	Female	Native American	None	$p19^{gag}$, $p28^{gag}$	–	No	HTLV-II	–	–
3958	37	Male	Black	None	$rgp21^{e}$	Indet $p63^{env}$	Yes	HTLV-I	HTLV-I	HTLV-I
3594	65	Male	Non-Hispanic white	TR[c]	$p19^{gag}$, $p28^{gag}$, $p32^{gag}$	–	No	HTLV-II	HTLV-II	HTLV-II
53814	37	Male	Native American	None	$p19^{gag}$, $p28^{gag}$, $p32^{gag}$	–	NT[d]	HTLV-II	HTLV-II	–

[a] Antigens with which samples reacted are listed. RIPA, radioimmunoprecipitation assay; Indet, indeterminate. A minus sign indicates a negative result.
[b] A minus sign indicates a negative result.
[c] TR, transfusion recipient.
[d] NT, not tested.

HTLV AC-ELISA was positive with two of four PCR-positive, WB-indeterminate PBMC samples. Four of 19 donors with WB-indeterminate sera had HTLV DNA by PCR when both *tax* and *pol* primers were used (one donor [3958] had HTLV-I DNA, and three others [3302, 3594, and 53814] had HTLV-II DNA). Three of the four donors' sera reacted in the Coulter peptide epitope ELISA (Table 2). PBMC from 4 of the 15 seroindeterminate donors with negative PCR were also tested by coculture, and all failed to produce p24.

In HTLV coculture assays, PBMC from donors 3594 and 3958 produced p24 antigen (Fig. 1). In the AC-ELISA for HTLV-I p19 antigen (CPI), the coculture from donor 3958 was positive while that from 3594 was not (Table 1), and HTLV particles were observed in cocultures from both (reference 22 and data not shown). IFAs for p19 antigen were initially positive with PBMC from all four donors, but the p24 AC-ELISA was negative for cocultures from donors 3302 and 53814.

Repeat phlebotomies were performed on the three of the four WB-indeterminate donors at 17 months (donor 3302), 14 months (donor 3594), and 9 months (donor 3958). In the second sample, donor 3958 had small amounts of antibody to p19 and p24 and was thus considered to have seroconverted. In retrospective examination, extremely faint p24 and p19 reactivity was present originally but could be detected only with certain lots of WB strips. WBs of specimens from donors 3302 and 3594 remained essentially unchanged. None of the four had any risk for recent exposure to HTLV.

A method for HTLV culture suitable as an adjunct in diagnosis. Serologic assays are believed capable of detecting most infections with HTLV-I and HTLV-II, and some believe that infection is rare in blood donors who are seroindeterminate for HTLV-I and HTLV-II (17, 21). However, previously reported experience with PCR includes several examples of apparent infection among individuals with indeterminate WB patterns or even a negative screening test (15, 17, 19, 27). PCR can detect minute amounts of contaminating amplified DNA, and new viruses or endogenous viruses only slightly related to HTLV-I or HTLV-II could potentially be detected. We wished to determine whether an HTLV coculture assay using other methods of detection would improve the confidence with which we could diagnose infection in seronegative or seroindeterminate donors.

While the relatively low sensitivity (63%) of AC-ELISA combined with coculture may impose some limits on its ability to detect infection in serologically ambiguous donors, it still proved helpful in those situations in which it is positive.

Four of 19 donors with indeterminate serologic results whom we examined had PCR evidence of HTLV infection. Use of the coculture assay, including AC-ELISA and p19 immunofluorescence, demonstrated that (i) p19-positive cells were present in PBMC from all four, (ii) two of the four produced abundant p24 antigen, (iii) viral particles resembling HTLV were observed in the two cultures that were producing p24, and (iv) reactivity to the HTLV-I-specific anti-p19 antibody in p24-positive supernatants was consistent with PCR viral typing. Thus, some WB-indeterminate blood donors are infected with an HTLV, and our results suggest that HTLV can be propagated from the cells of PCR-positive, WB-indeterminate donors at a frequency similar to those from serologically confirmed donors.

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