Evaluation of a Solid-Phase Immunoassay for the Simultaneous Detection of Antibodies to Human Immunodeficiency Virus Type 1 and Human T-Cell Lymphotropic Virus Type I

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We describe the evaluation of a solid-phase immunoassay developed for the simultaneous detection of antibodies to human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotropic virus types I (HTLV-I) and II (HTLV-II) in human serum. The immunoassay employs a mixture of HIV-1 and HTLV-I whole viral lysates immobilized in the wells of microtiter plates. Evaluation of genetically well-pedigreed specimens along with normal blood donor samples indicated that the performance characteristics of the test were equivalent to the sensitivity and specificity of individual tests licensed by the Food and Drug Administration for antibodies to HIV-1 and HTLV-I. Furthermore, the test was also able to detect the presence of cross-reacting antibodies in HTLV-II-infected individuals. The use of such a test would greatly reduce the continually mounting costs associated with screening transfusable products for infectious agents.

Most blood products intended for transfusion or further manufacture into injectable products are screened for antibodies to two separate and distinct human retroviruses, human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotropic virus type I (HTLV-I). HIV-1 is the etiologic agent for AIDS (3, 9), and HTLV-I has been etiologically linked to adult T-cell leukemia and various neurologic disorders collectively referred to as HTLV-I-associated myelopathy (5, 10, 16).

Separate tests have been licensed by the U.S. Food and Drug Administration (FDA) for the detection of antibodies to these two viruses (2, 6). Although all donated blood in the United States is screened for antibodies to HIV-1, current federal recommendations require HTLV-I antibody screening to be performed only on blood that, when transfused, contains cellular components (i.e., plasma products are not screened for antibodies to HTLV-I) (2). Although the overall antibody prevalence rates for both HIV-1 and HTLV-I are similarly low in the normal blood donor population (1, 7, 17), blood screening is intended to further reduce the risk of transfusion-associated disease.

With the advent of additional required blood screening tests, such as hepatitis C testing, and the potential for still other required tests, the costs associated with blood screening tests continue to mount. It would therefore be desirable to develop methods to perform such testing in a more cost-effective and efficient manner. In addition, performance of additional tests also correspondingly increases the potential for experimental errors. Accordingly we have developed and characterized a solid phase enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection of antibodies to HIV-1 and HTLV-I. The resulting RETRO-TEK HIV-1/HTLV-I CombiELISA Test Kit (Cellular Products, Buffalo, N.Y.) employs antigen targets consisting of a mixture of viral lysates obtained from density gradient purified HIV-1 and HTLV-I virions. After detergent disruption and

inactivation, the viral lysate mixture is used to coat the wells of microtiter plates used in the ELISA format.

MATERIALS AND METHODS

Isolation of viral lysates. HIV-1 and HTLV-I viral lysates were individually prepared from their producer cell lines (Hut 78/HIV-1 and Hut 102.B2, respectively) by methods identical to those used to manufacture the previously FDA-licensed RETRO-TEK HIV-1 ELISA Test Kit and the RETRO-TEK HTLV ELISA Test Kit. Briefly, each cell line was grown in RPMI 1640 medium containing 10% fetal bovine serum to a final density of approximately 8×10^5 cells per ml. After the cells were removed by filtration with a 0.2- μ m-pore-size filter, the cell supernatant was concentrated 10 to 20-fold by ultrafiltration through a membrane with a molecular weight cutoff of 100,000. After the concentrate was centrifuged for 2 h at $80,000 \times g$, the crude viral pellet was suspended in TNE (0.01 M Tris-HCl [pH 8.0]-0.1 M NaCl-0.001 M EDTA) and layered over a linear sucrose gradient (22 to 65% sucrose in TNE) in a Sorval TZ-28 Zonal Rotor. After 12 h of centrifugation at $46,000 \times g$, the gradient was fractionated; all fractions between densities of 1.12 and 1.16 g/ml were pooled, diluted fourfold with TNE, and centrifuged for 2 h at $80,000 \times g$. The pelleted virions were suspended in 0.5% Triton X-100-0.6 M KCl, extracted for 1 h, clarified by centrifugation for 30 min at $100,000 \times g$, and further extracted twice with an equal volume of anhydrous ether. The individual titers of the resulting HIV-1 and HTLV-I viral lysates were immunologically determined; after checkerboard analysis, the optimum ratio of combined viral lysates was employed to coat the wells of microtiter plates.

Performance of the CombiELISA. The RETRO-TEK HIV-1/HTLV-I CombiELISA was performed by diluting the test specimens in specimen diluent (at a 1/21 dilution) and incubating 200 μ l of the diluted specimen in the microtiter wells for 60 min at 37°C. After the microtiter plate wells were washed six times with 200 μ l of plate washing buffer, 200 μ l of goat anti-human immunoglobulin G conjugated to alkaline

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phosphatase was added, and plates were incubated for 60 min at 37°C. After the microtiter plate was washed six times with plate washing buffer as above, 200 µl of substrate (*p*-nitrophenylphosphate, 1 mg/ml) was added, and plates were incubated for 30 min at 37°C. The reaction was terminated by the addition of 50 µl of 3.0 N NaOH, and the A_{405} of the microtiter wells was read. A cutoff sample was simultaneously run in quadruplicate on all microtiter plates, and the resulting average A_{405} value of the cutoff sample was used as the cutoff value for that particular microtiter plate. To initially prepare the cutoff sample, HIV-1- and HTLV-I-positive serum specimens were mixed at various ratios and tested in the RETRO-TEK HIV-1/HTLV-I CombiELISA against an in-house panel of weakly HIV-1- and HTLV-I-seropositive and equivocal specimens. The cutoff sample was chosen based upon the combination of HIV-1 and HTLV-I seropositive serum specimens that consistently resulted in A_{405} values just below the expected equivocal zone, therefore providing assurance that true seropositive specimens would reproducibly yield A_{405} values greater than that resulting from the evaluation of the cutoff sample in the RETRO-TEK HIV-1/HTLV-I CombiELISA. During the routine performance of the RETRO-TEK HIV-1/HTLV-I CombiELISA, the mean A_{405} value of four replicates of the cutoff sample was defined as the cutoff value for the test. Any specimen with an A_{405} value equal to or greater than the cutoff value was considered reactive for antibodies to HIV-1 and/or HTLV-I.

Performance of HIV-1 and HTLV-I ELISA. To evaluate the various specimens for the presence of antibodies to HIV-1 and HTLV-I, the FDA-licensed RETRO-TEK HIV-1 ELISA Test Kit and RETRO-TEK HTLV-I ELISA Test Kit (Cellular Products), respectively, were utilized. Both ELISAs were performed exactly as recommended by the manufacturer.

PCR characterization of test samples. Where designated in the text, specimens were obtained from individuals who had been tested by using polymerase chain reaction (PCR) gene-amplification methods (11, 12, 14) with respect to the presence of genomic sequences corresponding to HIV-1, HTLV-I, and HTLV-II. In addition, all HIV-1 and HTLV-I PCR-positive samples were also found to be positive by HIV-1 or HTLV-I culture assay (see below). Various primer pairs specific for the three retroviruses were employed to distinguish the various integrated sequences. The primer pairs for HIV-1 *gag*, *pol*, and *env* were SK 38/39, SK 32/33, and SK 68/69, respectively. The HTLV-I and HTLV-II generic *pol* primer pair SK 110/111 was used along with the generic detector SK 115 and the HTLV-I- and HTLV-II-specific detectors SK 112 and SK 188, respectively. The generic HTLV-I and HTLV-II *tax* gene primer pair SK 43/44, the HTLV-I *pol* gene primer pair SK 54/55, and the HTLV-II *pol* gene primer pair SK 58/59 were also used.

HIV-1 and HTLV-I culture assay. Peripheral blood mononuclear cells were cultured for HIV-1 in a 30-day cocultivation assay. As previously described (15), cultures were analyzed for HIV-1 produced by analyzing aliquots of cell culture conditioned medium for HIV-1 p24 antigen with the RETRO-TEK HIV-1 p24 Antigen ELISA (Cellular Products). Similarly, peripheral blood mononuclear cells were cultured for HTLV-I or HTLV-II by culturing them in medium containing 10% delectinated T-cell growth factor (Cellular Products). Aliquots of conditioned media were periodically tested for HTLV-I antigens via competitive ELISA or antigen capture assay with the RETRO-TEK HTLV-I Antigen ELISA (Cellular Products). Furthermore,

TABLE 1. Comparative HIV-1 and HTLV-I sensitivity of the CombiELISA Test Kit

Patient status	No. reactive		
	HIV-1 ELISA	HTLV-I ELISA	CombiELISA
Frank AIDS, AIDS-related complex	40	0	40
Adult T-cell leukemia (U.S. patients)	0	11	11
Adult T-cell leukemia (Japanese patients)	0	10	10
Adult T-cell leukemia with immune deficiency	1	1	1

all cultured cells were also tested for HTLV-I or HTLV-II proviral DNA via Southern blot hybridization as previously described (4, 13).

RESULTS

Before preclinical field studies were initiated, the performance characteristics of the RETRO-TEK HIV-1/HTLV-I CombiELISA were first compared with those of the previously FDA-licensed individual tests for the detection of antibodies to HIV-1 and HTLV-I, respectively. Forty serum samples obtained from individuals with documented HIV-1 infection (culture and PCR positive for HIV-1 genomic sequences) were analyzed with all three tests: all 40 samples were seropositive for antibodies to HIV-1 with both the HIV-1 ELISA and the CombiELISA. None of the samples was seropositive for antibodies to HTLV-I with the individual HTLV-I ELISA Test Kit (Table 1).

Twenty-two individuals with documented HTLV-I infection (culture and PCR positive for HTLV-I genomic sequences) were also analyzed with the same three tests (Table 1). All samples were found to be seropositive for antibodies to HTLV-I with both the individual HTLV-I ELISA Test Kit and the CombiELISA Test Kit. One specimen obtained from an adult T-cell leukemia patient with documented immunodeficiency was seropositive for antibodies to HIV-1 and HTLV-I with the individual tests. This specimen was also Western blot positive for both HIV-1 and HTLV-I as well as PCR and culture positive for both HTLV-I and HIV-1 (8).

Performance data of currently licensed HIV-1 and HTLV-I antibody tests indicate that antibodies present in specimens obtained from frank AIDS and adult T-cell leukemia or tropical spastic paraparesis patients are relatively easily detectable. Specimens obtained from individuals at high risk for infection for these retroviruses offer additional challenges to such testing. To determine whether the CombiELISA was able to detect seropositive reactions in samples obtained from high-risk individuals, nine such samples (one parent and two siblings of an adult T-cell leukemia patient, two intravenous drug abusers, three myelopathy patients, and one acute hepatitis patient) were evaluated with the separate FDA-licensed HIV-1 and HTLV-I ELISAs and the RETRO-TEK HIV-1/HTLV-I CombiELISA. In all cases, the samples were seropositive when the CombiELISA was utilized, consistent with the results obtained when the samples were evaluated on the individual HIV-1 and HTLV-I ELISAs (data not shown).

To determine whether the CombiELISA was hampered by potential false-positive determinations, a variety of patient specimens were analyzed on the CombiELISA as well as the

FIG. 1. The S/C value of each specimen initially analyzed in the CombiELISA was determined and plotted in histogram format. The S/C cutoff value of 1.0 is shown by the arrow. (The two specimens that were initially reactive were repeat tested and found to be nonreactive.)

individual FDA-licensed tests for HIV-1 and HTLV-I antibodies. The samples included in this portion of the study were obtained from patients infected with cytomegalovirus ($n = 8$), Epstein-Barr virus ($n = 6$), varicella-zoster virus ($n = 3$), hepatitis virus ($n = 7$), parainfluenza virus ($n = 1$), rubella virus ($n = 1$), and measles virus ($n = 1$). In addition, serum samples from individuals with diagnosed lung cancer ($n = 1$), rectal cancer ($n = 1$), and ovarian cancer ($n = 5$) were analyzed. All 34 samples were seronegative in all three tests (data not shown).

To estimate the specificity of the CombiELISA Test Kit, fresh serum samples from random blood donors were analyzed. Of 1,040 fresh serum samples, 2 were initially reactive, but none was repeatably reactive. Each of the two initially reactive samples was analyzed with the individual HIV-1 and HTLV-I ELISAs; both samples were nonreactive with both tests. The initial reactive rate was therefore estimated to be 0.19% with a repeat reactive rate estimated to be 0%.

Histogram analysis (Fig. 1) of the samples indicated that approximately 90% of the specimens yielded S/C (sample absorbance value/cutoff absorbance value) ratios of less than 0.3, with a tail of reactive samples spreading out toward the cutoff value. Such a profile is similar to those obtained when licensed tests for either HIV-1 or HTLV-1 are used to evaluate normal donor samples.

Field sensitivity was estimated by analyzing samples that had been well characterized with respect to the presence of HIV-1, HTLV-I, and HTLV-II DNA sequences. In all, 170 samples that were characterized with the PCR and culture assays were evaluated. All specimens obtained from individuals lacking PCR evidence of retroviral infection ($n = 40$) were scored as seronegative with the CombiELISA Test Kit, whereas all specimens obtained from HIV-1 PCR-positive individuals ($n = 50$) or HTLV-II PCR-positive individuals ($n = 30$) were scored as seropositive with the CombiELISA Test Kit (Fig. 2).

During the course of these studies, two specimens were also identified that were infected with both HIV-1 and HTLV-I. Both of these samples were seropositive when evaluated on the individual FDA-licensed tests for HIV-1 and HTLV-I antibodies. Furthermore, both samples were

FIG. 2. Sera obtained from patients characterized with the PCR as containing genomic sequences for HIV-1 ($n = 50$), HTLV-I ($n = 50$), or HTLV-II ($n = 30$) as being uninfected ($n = 40$) were analyzed in the CombiELISA; the resulting A_{405} values ($\times 1,000$) are shown for each individual sample. The A_{405} cutoff value of 0.352 is shown.

also seropositive when evaluated by the criteria of the CombiELISA Test Kit (data not shown).

DISCUSSION

The use of a combination ELISA has been demonstrated to be as sensitive and specific as individual, previously FDA-licensed tests for the detection of antibodies to HIV-1 and HTLV-I. Furthermore, presumably cross-reacting antibodies in genetically documented HTLV-II-infected individuals also strongly reacted in the CombiELISA Test Kit. As concern continues to mount over the presence of additional infectious agents in donated blood, it will become more imperative that the overall cost of performing the requisite testing be reduced. The total cost associated with blood screening comprises the purchase cost of the tests, the actual performance of the test and analysis of the resulting data, and counseling. In addition, an indirect cost of unusable blood is also associated with a screening test of poor specificity. The magnitude of the financial investment associated with the purchase, performance, and analysis of the tests would be reduced if combination ELISAs of excellent sensitivity and specificity were utilized. The data presented here indicate that the RETRO-TEK HIV-1/HTLV-I CombiELISA is able to detect antibodies to either HIV-1 or HTLV-I with the same level of sensitivity as the individual FDA-licensed tests for HIV-1 and HTLV-I. Furthermore, evaluation of specimens well pedigreed by PCR analysis indicates that the CombiELISA is able to detect the presence of antibodies to HTLV in individuals infected with

either HTLV-I or HTLV-II. Presumably, cross-reacting antibodies found in HTLV-II-infected individuals are responsible for the detected reactions. Since many of the seropositive reactions observed in FDA-licensed tests for HTLV-I antibody detection are, in fact, due to HTLV-II reactions, the data presented herein indicate that such reactions will not go undetected in the combination format. The estimated sensitivity of 100% indicate that the CombiELISA can readily meet the strict requirements of the blood banking community.

Present testing algorithms for both HIV-1 and HTLV-I antibody testing require that initially reactive specimens be retested in duplicate with the same test and sample. If one or both of the duplicate repeat tests is reactive, the sample is considered repeatably reactive for that particular antibody and the donor is deferred pending additional, more specific tests such as Western blotting. Since the CombiELISA Test Kit was not designed to distinguish between seropositivity for HIV-1 and seropositivity for HTLV-I or HTLV-II, initially reactive tests results indicate that the sample is potentially seropositive for antibodies to HIV-1 and/or antibodies to HTLV-I or HTLV-II. With a testing algorithm similar to that employed for the individual tests, repeat testing in duplicate with the CombiELISA would further indicate whether a sample was repeatably reactive for antibodies to one or both of these human retroviruses. If a testing establishment wishes to determine the actual identity of the seroreactivity, it would obviously be necessary to perform individual tests for HIV-1 and for HTLV-I and HTLV-II. Inasmuch as more specific tests such as a Western blot would be required to confirm repeatably reactive specimens, it would be more appropriate to perform the repeat ELISA testing using individual HIV-1 and HTLV-I ELISAs, thus permitting the appropriate Western blot to be performed without the unnecessary expense of performing two separate Western blots. The CombiELISA, therefore, could be used as an efficient initial screen, thus eliminating the vast majority of ELISA testing required for antibody detection to these separate viruses.

The overall cost savings resulting from the use of such a CombiELISA is quite significant, since the amount of repeat testing would be directly proportional to the false-positive rate, which, as indicated by our data, is acceptably quite low. In addition, assuming an actual experienced false-positive rate of 0.1%, for every 1,000 samples the use of such a CombiELISA would result in the savings of nearly 1,000 tests. The costs associated with the purchase, performance, and evaluation of the required tests would be dramatically reduced.

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