

## Evaluation of an Immunoblot Assay for Serological Confirmation and Differentiation of Human T-Cell Lymphotropic Virus Types I and II

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The confirmation of infection with human T-cell lymphotropic virus type I (HTLV-I) and type II (HTLV-II) currently involves multiple assays. These include Western blot (immunoblot) (WB) and/or radioimmunoprecipitation assay for detection of antibodies to HTLV-specific viral proteins and polymerase chain reaction and/or peptide-based enzyme immunoassays for differentiating between the two viruses. We undertook an evaluation of a modified WB assay that includes native HTLV-I viral proteins from MT-2 cells spiked with an HTLV-I transmembrane glycoprotein (recombinant p21e) and the HTLV-I- and HTLV-II-specific recombinant proteins MTA-1 and K55. The test panel consisted of well-characterized sera from U.S. blood donors, American Indians, intravenous drug users, and patients seen in sexually transmitted disease clinics. Of 158 HTLV-I/II-seropositive serum specimens tested, 156 (98.7%) were confirmed and typed as HTLV-I or HTLV-II. Of 82 HTLV-I/II-seroindeterminate or -seronegative serum specimens, only 1 was classified as HTLV-II positive: the sample had weak *gag* p19 and strong *gag* p24 and *env* p21e reactivity and was radioimmunoprecipitation assay negative for *env* gp61/68 but polymerase chain reaction positive for HTLV-II. The specificity of the modified WB for confirming and typing serum samples was therefore 100%. We conclude that this WB assay is useful for confirming and typing HTLV infection and can help simplify HTLV-I/II testing algorithms.

Human T-cell lymphotropic virus type I (HTLV-I), the etiological agent of adult T-cell leukemia/lymphoma and of HTLV-I-associated myelopathy or tropical spastic paraparesis, is endemic in populations of Japan, the Caribbean, and parts of Africa (1). HTLV-II is a virus closely related to HTLV-I, and antibodies to this virus cross-react with HTLV-I antigen. HTLV-II is prevalent in intravenous drug users (4, 10) and in some American Indian groups (7, 8). Little is known, however, regarding HTLV-II and disease associations.

In the United States, HTLV-I/II screening of volunteer blood donors was instituted in late 1988. Of approximately 2,000 (15 in every 100,000 donors) HTLV-I/II-seropositive individuals identified each year, approximately half are infected with HTLV-I and half are infected with HTLV-II (2). Counseling of these individuals is often limited by lack of knowledge as to whether they are infected with HTLV-I or HTLV-II.

Serological testing for antibodies to HTLV-I/II involves a number of different techniques. Most commonly, sera are screened by a licensed HTLV-I enzyme immunoassay (EIA). Repeatedly reactive specimens are tested further on the basis of the U.S. Public Health Service recommendations (17) to look for *gag* and *env* seroreactivity. Specific antibody reactivity to these HTLV proteins has traditionally necessitated Western blot (immunoblot) (WB) and, in in-

stances in which *env* antibodies are not detected by WB, also radioimmunoprecipitation assay (RIPA) (5). A recombinant envelope protein which is recognized by HTLV-I- and HTLV-II-infected serum, *env* p21e (transmembrane glycoprotein), has been developed and used to spike HTLV-I viral lysates for WB, resulting in markedly improved sensitivity for confirming HTLV-I/II infection (14). The specificity of envelope detection based on *env* p21e alone may, however, be suboptimal (6, 12).

Because HTLV-I and HTLV-II are closely related, neither the EIA nor the WB or RIPA reliably differentiate between them, though the pattern of *gag* reactivity obtained by WB can suggest HTLV-I or HTLV-II infection (18). To differentiate between the two viruses, polymerase chain reaction (PCR) has been used extensively, and more recently, specific HTLV-I and HTLV-II peptide-based EIAs have become available (13). Another approach to confirm and differentiate infections is the incorporation of specific recombinant proteins unique to HTLV-I and HTLV-II in a modified WB (15, 16). We report on the evaluation of a research WB with HTLV-I viral lysate, recombinant p21e, and additional recombinant proteins specific for detecting HTLV-I (MTA-1) (16) and HTLV-II (K55) (15) infection and describe its potential role in HTLV serological testing.

### MATERIALS AND METHODS

**Serum specimens.** A total of 240 serum specimens, repeatedly reactive by an HTLV-I EIA and additionally tested by

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TABLE 1. Serum specimens included in study

Serum source	No. of samples with HTLV serologic classification <sup>a</sup>		
	Positive	Indeterminate	Negative
Blood donors <sup>b</sup>	37	52	23
IVDU/STD <sup>c</sup>	45	0	1
Pregnant women <sup>d</sup>	20	2	0
ATL or HAM/TSP <sup>e</sup>	19	0	0
Panama Indians <sup>f</sup>	37	3	1
Total	158	57	25

<sup>a</sup> U.S. Public Health Service criteria define HTLV positivity as seroreactivity to *gag* and *env* viral proteins. HTLV-indeterminate serum demonstrates reactivity to some viral bands, usually *gag* p19 or p24, but does not meet positivity criteria. Negative samples are repeatedly reactive by HTLV-I EIA and WB negative and have no viral bands.

<sup>b</sup> U.S. blood donors from American Red Cross, Atlanta, Ga.

<sup>c</sup> Intravenous drug users and patients seen in sexually transmitted disease clinics.

<sup>d</sup> HTLV-I/II-infected pregnant women, New Orleans, La.

<sup>e</sup> Adult T-cell leukemia/lymphoma or HTLV-I-associated myelopathy or tropical spastic paraparesis patients from Japan and the United States.

<sup>f</sup> HTLV-II-infected Guaymi Indians, Panama (11).

WB and RIPA as previously described (17), were included in this evaluation. Of these specimens, 158 were classified as HTLV-I/II seropositive (reactivity to *gag* p24 and *env* gp46 or gp61/68), 57 were classified as seroindeterminate (reactivity to *gag* p19 and/or p24 but no reactivity to *env* gp46 or gp61/68), and 25 were classified as seronegative (no viral bands) (Table 1). Of the 158 seropositive specimens, 39 required RIPA to demonstrate HTLV-I/II *env* reactivity.

Peripheral blood mononuclear cells from 50 of the 158 HTLV-I/II-seropositive subjects were available for study by PCR as described elsewhere (9). Of these, 14 subjects were found to be infected with HTLV-I and 36 were found to be infected with HTLV-II.

Of the remaining 108 HTLV-I/II-seropositive specimens, 89 were tested by synthetic peptide-based EIA to differentiate HTLV-I from HTLV-II infection (SynthEIA HTLV-I and SynthEIA HTLV-II [Olympus Corp., Lake Success, N.Y.], and/or Select-HTLV [Coulter/IAF Biochem International Inc., Montreal, Quebec, Canada]). Test results were interpreted according to the manufacturers' recommendations. Of these samples tested, 26 were HTLV-I positive, 52 were HTLV-II positive, and 11 were untypeable by synthetic peptide-based EIA. These tests have been shown to be 100% specific for differentiating HTLV-I and HTLV-II infections (18).

**HTLV-I/II modified WB.** The study WB strips were prepared by using HTLV-I viral lysate spiked with recombinant *env* p21e protein and specific recombinant proteins to HTLV-I (MTA-1) and HTLV-II (K55) as described previously (16). After WB strips were incubated overnight with human test sera (diluted in BLOTTO 1:100), they were rinsed briefly with wash buffer and then washed twice for 10 min in wash buffer. Bound human immunoglobulin was detected by incubating WB strips for 1 h with goat anti-human immunoglobulin G ( $\gamma$  chain specific) conjugated to alkaline phosphatase (diluted in BLOTTO 1:1,000) (Sigma, St. Louis, Mo.). Color was developed after another series of washes with substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in 100 mM Tris (pH 9.5) prepared as recommended by the manufacturer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg,

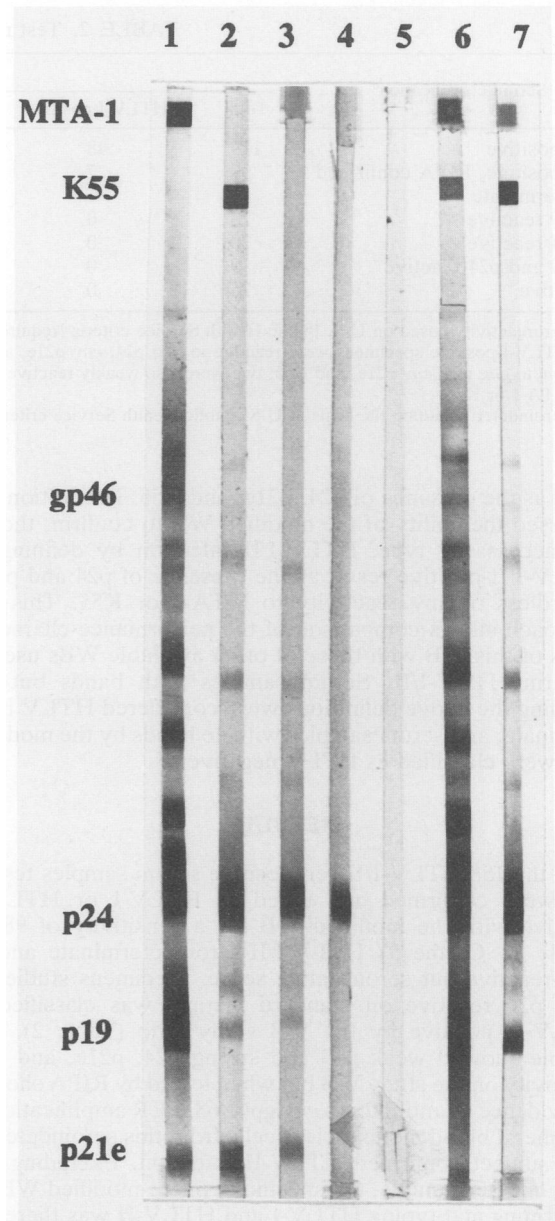


FIG. 1. MTA-1 and K55 WB strips. Lane 1, HTLV-I-positive sample demonstrating reactivity to MTA-1, *gag* p24, and *env* p21e but not K55; lane 2, HTLV-II-positive sample reactive to K55, *gag* p24, and *env* p21e but not MTA-1; lane 3, HTLV-I/II-positive sample reactive to *gag* p24 and *gag* p21e but not MTA-1 or K55; lane 4, HTLV-I/II-indeterminate sample reactive to HTLV-I/II *gag* proteins but no reactive to HTLV-I/II type-specific recombinant proteins or envelope proteins; lane 5, HTLV-I/II-negative sample with no reactivity to HTLV-I/II proteins; lane 6, HTLV-I-positive sample cross-reactive with K55; lane 7, HTLV-II-positive sample cross-reactive with MTA-1.

Md.). Results were tabulated on the basis of the presence or absence of specific HTLV bands on the WB (p19, p24, p21e, MTA-1, and K55; Fig. 1).

**Definitions.** To evaluate the ability of the modified WB to both confirm and type HTLV-I/II infection, an HTLV-I-positive result was defined as the presence of p24, p21e, and MTA-1 reactivity and an HTLV-II-positive result was de-

TABLE 2. Test results obtained by modified WB

Serum classification <sup>a</sup>	No. of specimens <sup>b</sup>					
	Total	HTLV-I positive	HTLV-II positive	HTLV-I/II positive	Indeterminate	Negative
Seropositive	119	48	70	1	0	0
Seropositive, RIPA confirmed	39	7	31	1	0	0
Indeterminate						
p19 reactive	28	0	0	0	28	0
p24 reactive	20	0	1 <sup>c</sup>	6	12	1
p19 and p24 reactive	9	0	0	1	8	0
Negative	25	0	0	0	12	13

<sup>a</sup> Seroreactivity based on U.S. Public Health Service criteria (require *gag* p24 and *env* gp46 and/or gp61/68 reactivity).

<sup>b</sup> HTLV-I-positive specimens were reactive to *gag* p24, *env* p21e, and MTA-1; four were also weakly reactive to K55. HTLV-II-positive specimens were reactive to *gag* p24, *env* p21e, and K55; two were also weakly reactive to MTA-1. HTLV-I/II-positive specimens were reactive with *gag* p24 and *env* p21e but not MTA-1 or K55.

<sup>c</sup> Seroindeterminate on the basis of U.S. Public Health Service criteria; PCR positive for HTLV-II.

defined as the presence of p24, p21e, and K55. In addition, we assessed the ability of the modified WB to confirm, though not necessarily type, HTLV-I/II infection by defining an HTLV-I/II-positive result as the presence of p24 and p21e, regardless of any reactivity to MTA-1 or K55. This last approach allows comparison of the performance characteristics of this WB with those of other available WBs used to confirm HTLV-I/II. Serum samples with bands but not fulfilling the above definitions were considered HTLV indeterminate, and serum samples with no bands by the modified WB were classified as HTLV negative.

## RESULTS

Of the 158 HTLV-I/II-seropositive serum samples tested, 156 were confirmed and typed as HTLV-I or HTLV-II positive with the modified WB for a sensitivity of 98.7% (Table 2). Of the 57 HTLV-I/II-seroindeterminate and 25 EIA-reactive but seronegative serum specimens studied, 1 (*gag* p24 reactive on standard testing) was classified as HTLV-II positive by the WB study strip (Table 2). The sample showed weak p19 and strong p24, p21e, and K55 reactivity on the study WB but when tested by RIPA showed no evidence of antibody to *env* gp61/68. PCR amplification of peripheral blood mononuclear cells from this seroindeterminate subject confirmed HTLV-II infection. Excluding this truly infected sample, the specificity of the modified WB for confirming and typing HTLV-I and HTLV-II was therefore 100% (81 of 81). An additional seven serum samples (six *gag* p24 reactive and one *gag* p19 and p24 reactive) reacted with *gag* p24 and *env* p21e on the WB study strip but not with MTA-1 or K55 (Table 2). Of these seven specimens, five were PCR negative (the other two were not tested by PCR). An additional five HTLV-seronegative and six *gag* p19-reactive samples (five of which were tested and found to be negative by PCR) also reacted with *env* p21e but not with *gag* p24, and seven other HTLV-seronegative samples showed *gag* reactivity with the modified WB (Table 2).

Two HTLV-I/II-seropositive samples showed reactivity to *gag* p19, p24, and *env* p21e but not to MTA-1 or K55 and were therefore classified as HTLV-I/II positive (Table 2). The sensitivity of the modified WB for serological confirmation of HTLV-I/II infection was therefore 100% (158 of 158). However, seven HTLV-I/II-indeterminate serum specimens also showed *gag* p24 and *env* p21e reactivity (Table 2), resulting in a specificity of 91.4% (74 of 81) for confirming HTLV-I/II infection.

We next assessed the accuracy of the modified WB for

typing HTLV-I and HTLV-II infection by examining MTA-1 and K55 reactivity in PCR-typed or peptide-based EIA-typed serum specimens. Of the 14 HTLV-I-positive specimens typed by PCR, all seroreacted with the HTLV-I-specific recombinant protein MTA-1; 1 of the 14 also reacted weakly with K55. The dually reactive sample reacted with much greater intensity to MTA-1 than to K55 (Fig. 1). All 36 PCR-classified HTLV-II-positive specimens reacted to K55 recombinant protein; 1 of these samples cross-reacted with the HTLV-I recombinant protein MTA-1, but with weaker intensity than to K55 (Fig. 1). Of 26 additional peptide-based EIA-typed HTLV-I-seropositive serum samples, 25 were reactive to MTA-1 and not to K55 and 1 was reactive to neither. Of 52 peptide-based EIA-typed HTLV-II-positive serum specimens, 51 reacted to K55; 1 of these cross-reacted with MTA-1 but at a lower intensity than with K55. One peptide-based EIA typed HTLV-II-positive serum sample did not react to K55 or to MTA-1. The study strip thus correctly distinguished between HTLV-I and HTLV-II infection in 50 of 50 PCR-typed samples and 76 of 78 peptide-based EIA-typed samples. Of 11 HTLV-I/II-seropositive samples that were nontypeable by peptide-based EIA, 1 reacted with MTA-1 and not K55, 9 reacted with K55 and not MTA-1, and 1 reacted with both MTA-1 and K55, but with a much stronger reactivity to MTA-1 than to K55. Immunoreactivity with type-specific recombinant proteins on the study WB thus possibly allowed typing an additional 11 HTLV-I/II-seropositive specimens.

Of 47 HTLV I/II-indeterminate serum specimens from subjects who were also tested by PCR and found to be negative, 4 reacted with K55 and 43 did not demonstrate reactivity to either of the type-specific recombinant proteins. None of the four specimens that reacted with K55 demonstrated reactivity with other viral proteins (none were reactive to *gag* p24 and *env* p21e). These samples were retested in duplicate in separate laboratories, and reactivity to K55 could not be confirmed.

## DISCUSSION

HTLV serologic testing has been limited by the need for multiple assays to confirm seroreactivity and by the inability of standard supplementary serologic assays (WB and RIPA) to differentiate between HTLV-I and HTLV-II. Recent developments in recombinant-protein and synthetic-peptide technologies have simplified the diagnosis of HTLV-I and HTLV-II infections. The addition of the recombinant transmembrane glycoprotein *env* p21e to testing assays has de-

creased the number of RIPAs needed to confirm envelope reactivity (6). However, concerns regarding the specificity of recombinant p21e make it prudent not to rely on the presence of *env* p21e without additional proof of *env* reactivity in low-prevalence populations. This specifically applies to blood donor screening and other serodiagnostic usage in which individuals are notified regarding their infection status. The availability of synthetic peptide-based EIAs that differentiate between HTLV-I and HTLV-II, with reported sensitivities of over 80% (13), has also been important. Although they require additional testing of a serum specimen, they are a great improvement over PCR, which has been the "gold standard" for typing infection but is not practical for large-scale studies or rapid diagnosis.

The modified WB assay, which combines *env* p21e and HTLV-I- and HTLV-II-specific recombinant proteins with an HTLV-I whole-virus lysate in a WB format, offers the possibility of confirming and differentiating HTLV-I and HTLV-II infection with a single test. In evaluating this assay, we examined both its ability to confirm and type HTLV infection and its ability to only confirm HTLV infection. The modified WB had a sensitivity of 98.8% and a specificity of 100% for confirming and classifying HTLV-I/II infection. Thus, samples that are confirmed and typed with this assay do not need additional testing. In our hands, the limited number of samples with reactivity to both the HTLV-I- and HTLV-II-specific recombinant proteins demonstrated a marked difference in the intensity of reaction to these two proteins, allowing us to properly classify them. It is possible that additional experience with this assay will reveal instances of dual reactivity that may not be that easy to classify and may need additional testing to differentiate HTLV-I from HTLV-II. Such results, when they occur, may represent infections with both HTLV-I and HTLV-II.

As expected on the basis of the reported sensitivity of p21e (6, 14), the sensitivity of this modified WB was excellent (100%) for confirming HTLV-I/II infection. Therefore, serum samples not showing p21e reactivity with the modified WB can be resolved as HTLV-I/II indeterminate without a need for additional testing. However, as is the case with other assays using *env* p21e, relying on this protein alone for *env* reactivity resulted in false positivity. While this may be acceptable for anonymous testing and in the context of seroepidemiologic studies, it is desirable that individuals with *env* p21e and *gag* p24 but without additional evidence of *env* reactivity be further tested by RIPA or PCR prior to notification and counseling.

In conclusion, the modified WB strip appears to be useful. On the basis of its performance characteristics, we suggest that it be further evaluated with samples from other populations and geographic areas. Its use could benefit serosurveillance of HTLV; analysis of clinical specimens to aid in diagnosing HTLV-I; differentiation between HTLV-I and HTLV-II infection, which will be important to identify new disease associations; and confirmation of HTLV infection in conjunction with blood donor screening. Our evaluation of the strip addressed its role as a supplementary serologic test in conjunction with licensed HTLV-I whole-virus EIAs. Other issues have been raised regarding the sensitivity of the licensed screening assays for detection of HTLV-II (3). We studied serum samples screened with these assays and therefore are unable to address how the modified WB strip might perform with such HTLV-II-positive EIA-nonreactive samples.

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