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Characterization of Antibody Reactivity to Human T-Cell Lymphotropic Virus Types I and II Using Immunoblot and Radioimmunoprecipitation Assays

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We have characterized the immunoreactivity to human T-cell lymphotropic virus type I (HTLV-I) among 26,983 persons of various seroprevalence groups by using enzyme immunoassay, immunoblot (IB), and radioimmunoprecipitation assays (RIPA) in accordance with Public Health Service recommended guidelines for the interpretation of serologic test results for HTLV-I infection. IB-indeterminate serum specimens (*n* = 178) were reactive to HTLV-I *gag* proteins, and no serum contained only *env* reactivity. Overall, RIPA resolved 40% of IB-indeterminate serum samples; however, the probability that RIPA would confirm IB-indeterminate samples depended on the seroprevalence of the population tested. HTLV-I *gag* p19-only reactivity on IB was not a reliable marker of HTLV-I infection, while *gag* p24 reactivity on IB was clearly associated with positive seroreactive specimens. IB and RIPA tests did not clearly distinguish between HTLV-I and HTLV-II seroreactivities. These data emphasize that patterns of immunoreactivity to HTLV-I antigens are dependent upon the seroprevalence of the risk groups tested. In addition, RIPA detected antibodies to *env* proteins present in low titer in a substantial number of IB *gag*-only reactive sera and resolved the HTLV-I antibody status of these sera.

Human T-cell lymphotropic virus type I (HTLV-I), the first reported human retrovirus (15), has been etiologically associated with adult T-cell leukemia/lymphoma (ATLL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (2). HTLV-I infection is endemic in southwestern Japan (6), the Caribbean (20), some regions of Africa (4), and certain risk groups in the United States (18). The virus is transmitted through infected lymphocytes, and those at increased risk of acquiring HTLV-I infection include intravenous drug users (IVDU) (18), recipients of cellular blood products (11), and infants of infected mothers (7). HTLV-I appears to be present in low prevalence in the U.S. blood donor population (21) and in higher prevalence among IVDU (18) and prostitutes (R. Khabbaz, W. Darrow, T. Hartley, J. Witte, J. Cohen, J. French, P. Gill, J. Potterat, K. Seikes, R. Reich, J. Kaplan, and M. Lairmore, J. Am. Med. Assoc., in press). In addition, HTLV-I-associated diseases have been documented in the United States (17), although the incidence of these diseases has not been determined. Because of the risk of transmitting HTLV-I through infected blood, the U.S. Food and Drug Administration has recommended the use of licensed serologic tests to screen donated blood for the presence of HTLV-I. Virtually all donated blood in the United States is now being tested for this virus (16).

HTLV-II is genomically closely related to HTLV-I, and screening tests for HTLV-I antibodies are cross-reactive with serum specimens from HTLV-II-infected persons. There is a limited amount of information known about the epidemiology, disease associations, and modes of transmission of HTLV-II. Recent evidence suggests that certain IVDU populations may have elevated infection rates for this virus (9).

The algorithm used by most laboratories in testing for HTLV-I antibodies involves initial screening with an enzyme immunoassay (EIA) or particle agglutination assay followed by more specific supplementary testing of repeatedly reactive specimens by immunoblotting (IB), radioimmunoprecipitation assay (RIPA), or immunofluorescence. In this report, we describe HTLV-I immunoreactivity of serum specimens from patients with ATLL and HAM/TSP and indeterminate IB patterns (reactivity to HTLV-I but not meeting Public Health Service criteria for seropositive) and their frequency in low, intermediate, and high HTLV-I seroprevalence groups. We also report our experience with RIPA in resolving indeterminate IB results and in attempting to serologically distinguish between HTLV-I and HTLV-II infections.

MATERIALS AND METHODS

Sera. We examined a total of 26,983 serum specimens from various previously studied populations (Table 1). These specimens were stored frozen at −20°C and initially tested by EIA, and dually reactive samples were further tested by IB (see below); samples with indeterminate IB results (*n* = 178) were also tested by RIPA (see below). A serum specimen was considered positive for HTLV-I antibody if immunoreactivity to both *gag* p24 (matrix) and *env* gp46 (external envelope) or *env* gp61/68 (envelope precursor) could be demonstrated by IB alone or after IB and RIPA according to previously published Public Health Service guidelines (16). A serum specimen with immunoreactivity to at least one HTLV-I gene product (tested by IB and RIPA) but not satisfying the above criteria was called indeterminate. All other serum specimens, including those not repeatably reactive in EIA and therefore not tested by IB, were considered negative for HTLV-I.

On the basis of these criteria, study populations were classified as having low (<0.1%, *n* = 20,210), intermediate (>0.1 but <2.0%, *n* = 5,960), and high (>2.0%, *n* = 813)

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TABLE 1. HTLV-I indeterminate immunoblot reactivity versus seroprevalence

Group studied	Seroprevalence	No. of serum specimens tested	No. (%) indeterminate in IB	No. (%) of indeterminate specimens		
				p19+p24+	p19−p24+	p19+p24−
U.S. blood donors, gay men, and hemophiliacs	≤0.1	20,210	24 (0.1)	3 (12.5)	0 (0.0)	21 (87.5)
West Africans, STD[a] clinic patients, and Puerto Rican blood donors	>0.1, <2.0	5,960	94 (1.6)	25 (26.6)	36 (38.3)	33 (35.1)
IVDU and female prostitutes	≥2.0	813	60 (7.4)	35 (58.3)	22 (36.7)	3 (5.0)
Total		26,983	178 (0.7)	63 (35.4)	58 (32.6)	57 (32.0)

[a] STD, Sexually transmitted disease.

HTLV-I seroprevalence (Table 1). Also studied were 14 serum samples from patients with HTLV-I-associated disease (ATLL or HAM/TSP).

EIA. Serum specimens were screened with a commercially manufactured HTLV-I EIA approved for research purposes (Dupont, NEN Research Products, Boston, Mass.). Sera whose absorbances were repeatedly above the cutoff defined by the manufacturer, as well as sera whose absorbances were within 20% below the cutoff (our gray zone), were tested further for antibody to HTLV-I by IB. We have previously reported the sensitivity (93.8%) and specificity (98.8%) of this EIA (using the manufacturer's cutoff) when used to test a HTLV-I risk group (R. Khabbaz, T. Hartley, M. Lairmore, and J. Kaplan, Am. J. Public Health, in press). This EIA uses detergent-disrupted HTLV-I-infected HUT-102 cells as antigen, and serum is tested at a 1:21 dilution.

IB. IB was performed as described previously (8), with modifications. Both purified HTLV-I antigen (MT-2 cell line) (10) and HTLV-II antigen (Mo-T cell line) (5) were obtained from a commercial source (Hillcrest Biologicals, Cypress, Calif.). Both HTLV-I and HTLV-II antigens were used at the same concentration (10 μg/cm of gel well). Each antigen was suspended in 0.1 M Tris buffer, pH 6.8, containing 0.5% sodium dodecyl sulfate, 0.10 μg of bromophenol blue per ml, 20% (vol/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol. The antigen suspension was then heated at 95°C for 4 min and electrophoresed on a 10% polyacrylamide gel with a 3% stacking gel at a constant 40 V for 16 h at room temperature. Proteins resolved by polyacrylamide gel electrophoresis were electrophoretically transferred to nitrocellulose sheets (constant 0.10 mA for 16 h then 0.20 mA for 2 h at 4°C). The nitrocellulose sheet was blocked for 5 h in phosphate-buffered saline (pH 7.4) containing 0.5% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and 5 g of nonfat dry milk per dl and then cut into 3-mm strips. Individual strips were incubated overnight at room temperature with a 1:100 dilution of serum, washed, and incubated for 1 h with 5 μg of biotinylated goat anti-human (heavy- and light-chain) immunoglobulin G (Vector Laboratories, Burlingame, Calif.) per ml. Following reaction with an avidin-biotin-horseradish peroxidase conjugate and further washing, immune reactions were visualized with diaminobenzidine-nickel chloride-hydrogen peroxide as a substrate.

We determined the locations of viral bands on our immunoblots with mouse monoclonal antibodies to p19 (12), p24 (13), and gp46/68 (14) of HTLV-I. The specificity of our IB strips was previously determined by testing the strips against serum specimens with antibodies to potentially interfering viral infections (i.e., human immunodeficiency virus types 1 and 2, Epstein-Barr virus, cytomegalovirus, and hepatitis viruses) and against serum specimens from patients with a variety of immunological disorders (i.e., rheumatoid factor and anti-HLA-Dr) (1). Serum reactivity with other presumed viral bands (p15, p21E, and p40X) is not reported in this study. Studies to determine the sensitivity and specificity of these reactivities in our IB system are in progress.

RIPA. HTLV-I-infected MT-2 cells and HTLV-II-infected Mo-T cells were metabolically labeled (200 μCi of each amino acid per ml per 10^7 cells) with [^{35}S]cysteine and [^{35}S]methionine (40 TBq/mmol; New England Nuclear Corp., Boston, Mass.), disrupted with RIPA lysing buffer (0.2 M NaH_2PO_4, 0.15 M NaCl, 1% phenylmethylsulfonyl fluoride, 100 U of aprotinin), and centrifuged for 45 min at 38,000 rpm (T 75 rotor). The lysate supernatants were reacted with 20 μl of test serum for 16 h at 4°C. Immune complexes were precipitated with protein A-Sepharose CL-4B (Sigma) for 1.5 h at 4°C. Bound immune complexes were washed (three times) with RIPA lysing buffer and then eluted with sample buffer (see above) by boiling for 4 min. Samples were electrophoretically analyzed in 10% polyacrylamide gels, and then the dried gel was analyzed by autoradiography.

RESULTS

A total of 14 serum specimens from patients with HTLV-I-associated diseases (ATLL and HAM/TSP) were repeatedly EIA reactive and further tested by HTLV-I IB. All of these sera met our criteria for seropositivity, reacting with both *gag* p24 (matrix) and *env* gp46 (external envelope) or gp61/68 (envelope precursor) on IB. Eight of these samples were also tested on HTLV-II IB strips; seven of eight were also reactive with HTLV-II *gag* p24 and *env* gp46 proteins (Fig. 1). Similar to previous reports, our HAM/TSP patients had higher mean IB antibody titers to HTLV-I proteins than the ATLL patients, and both groups had mean IB antibody titers to *gag* proteins that were higher than mean antibody titers to *env* glycoproteins (data not shown).

Testing of 26,983 serum specimens from groups without suspected HTLV-I-associated disease revealed more specimens repeatedly reactive in EIA with indeterminate IB seroreactivity than with positive IB reactivity (Table 1); this pattern of increased percentage of indeterminate samples was present in all prevalence groups tested. A total of 291 serum specimens repeatedly reactive in EIA were tested by IB. Of these, 21 were positive by IB alone, 92 were negative, and 178 were indeterminate by IB alone and required further testing by RIPA. The IB patterns of serum specimens of patients with indeterminate IB seroreactivity were characterized for all seroprevalence groups (Table 1). All of these 178 IB-indeterminate serum specimens reacted with only

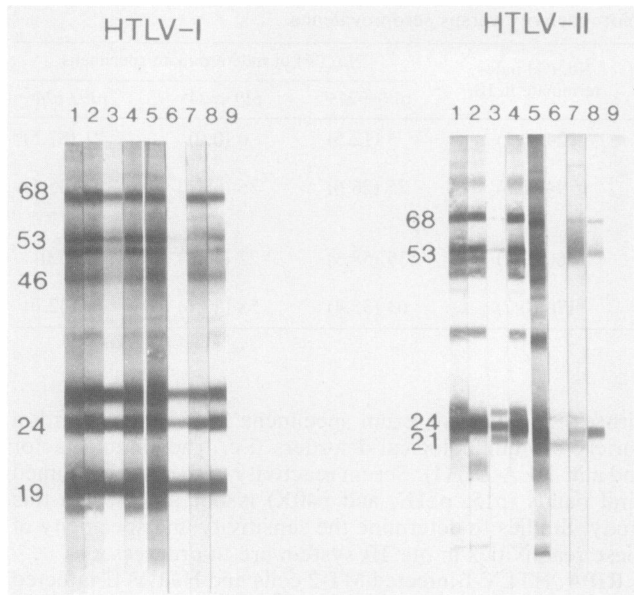

FIG. 1. Immunoblots of HTLV-diseased patients. Lanes 1 to 4, HTLV-I and HTLV-II immunoblot strips reacted against serum specimens from four adult T-cell leukemia patients; lanes 5 to 8, strips reacted against serum specimens from four spastic paraparesis patients. Note extensive cross-reactivity to both *gag* and *env* antigens of each virus. Lane 9 (both panels), Immunoblot strips reacted against serum specimen from a normal donor.

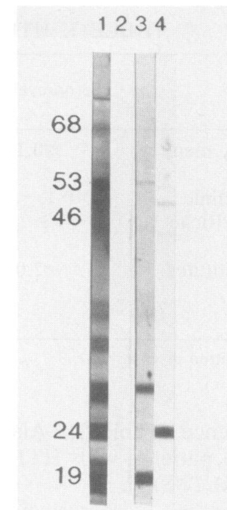

FIG. 2. HTLV-I-indeterminate immunoblots. Lane 1 and 2, Immunoblot strips reacted against positive control (ATLL patient) and negative control (normal donor), respectively; lane 3, typical p19-indeterminate serum reaction. Note less-intense band compared with that of positive control and presence of a band at approximately p28. This p28 band reaction is due to incomplete cleavage of MT-2 *gag* and contains p19 epitopes (19). Lane 4, Immunoblot strip showing pattern of indeterminate serum specimens reacting only against HTLV-I p24.

gag proteins on the IB strips. These specimens were categorized into three groups according to their reactivity with the two major *gag*-encoded proteins, p19 and p24: 57 (32.0%) were p19+ p24−, 58 (32.6%) were p19− p24+, and 63 (35.4%) were p19+ p24+ (Table 1). While these patterns were seen in nearly equal numbers among all the indeterminate serum specimens, their distribution was not equal among the low, intermediate, and high seroprevalence groups. The p19+ p24− was the most common indeterminate pattern (87.5%) in groups with low HTLV-I seroprevalence and the least common pattern (5.0%) in groups with high seroprevalence. In contrast, indeterminate specimens with p19+ p24+ were seen most frequently (58.3%) in the highest seroprevalence groups and least frequently (12.5%) in the lowest seroprevalence groups (Table 1).

Typically, p19+ p24− serum specimens reacted weakly at p19, compared with specimens from patients with ATLL or HAM/TSP or seropositive virus carriers (Fig. 2). To determine whether this reactivity represented early seroconversion to HTLV-I, sequential serum samples from four persons with IB reactivity at p19 only (low seroprevalence group) were tested. Samples were obtained at least yearly for periods of 3 to 5 years. None of the four samples seroconverted to other HTLV-I proteins; all maintained the p19-only pattern over the sampling period.

All these indeterminate serum specimens were further tested by RIPA, and 71 (39.9%) were confirmed positive. However, the probability that antibody to *env* proteins would be detected was different among the three indeterminate patterns: 44 of 63 (69.8%) of the p19+ p24+ sera, 27 of 58 (46.6%) of the p19− p24+ sera, and 0 of 57 of the p19+ p24− sera (Table 2). RIPA did not detect *gag* reactivity in any of the indeterminate samples that was not previously detected by IB.

To determine the ability of IB to distinguish between HTLV-I- and HTLV-II-positive sera, we tested serum specimens from individuals whose infection status was established by virus isolation or polymerase chain reaction or both. HTLV-I-positive serum specimens tested on both HTLV-I and HTLV-II IB strips showed extensive cross-reactivity between these two viral antigen preparations, especially with *gag* proteins (Fig. 2 and 3). In general, however, the intensity of reactivity was greater when the homologous antigen was used in the assay (i.e., HTLV-I serum with HTLV-I antigen). RIPA analysis of these sera revealed reactivity with *gag*- and *env*-encoded proteins from both HTLV-I- and HTLV-II-infected cell lysates.

DISCUSSION

Immunoblots from persons with HTLV-I-associated disease (ATLL or HAM/TSP) were not difficult to interpret. The serum samples of these patients were higher in HTLV-I antibody titers than those from seropositive asymptomatic persons and, when tested by IB, exhibited consistent reactivity to *gag* p19, p24, and p53 and *env* gp46 and/or gp61/68 proteins. In contrast to our experience with immunoblots from HAM/TSP and ATLL patients, the HTLV-I seroprevalence studies revealed many individuals with indeterminate IB results, necessitating the further evaluation of their serum

TABLE 2. RIPA analysis of serum specimens with indeterminate pattern of reactivity on immunoblot

IB reactivity pattern	No. (%) of indeterminate serum specimens in IB	No. (%) positive after RIPA
p19+p24+	63 (35.4)	44 (69.8)
p19−p24+	58 (32.6)	27 (46.6)
p19+p24−	57 (32.0)	0 (0.0)
Total	178	71 (39.9)

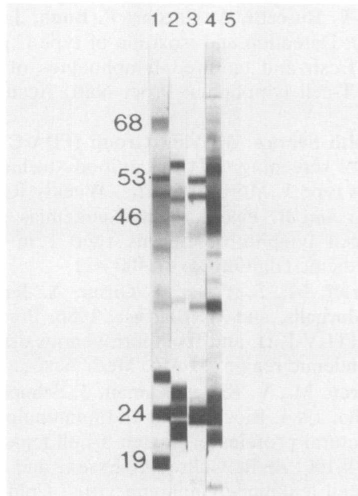

FIG. 3. Comparison of reactivities of HTLV-I- and HTLV-II-infected persons by using immunoblot assay. Lanes 1 and 2, Serum specimen from HTLV-I-infected patient reacted against HTLV-I and HTLV-II immunoblot strips, respectively; lanes 3 and 4, serum specimen from HTLV-II-infected patient reacted against HTLV-I and HTLV-II immunoblot strips, respectively; lane 5, serum specimen from normal donor reacted against HTLV-I immunoblot strip.

specimens by RIPA. All of the 178 IB-indeterminate serum specimens were *gag* reactive only; we found no specimens with antibody to only *env* glycoproteins by IB. The absence of specimens testing repeatedly reactive by HTLV-I EIA with only *env* reactivity on IB suggests a relative insensitivity to detect *env* reactivity in these tests or a true absence of this pattern of seroreactivity in the groups tested. However, 196 serum specimens randomly chosen from the highest seroprevalence group and tested by IB and RIPA, without initial screening by EIA, revealed no specimen with only *env* reactivity (Khabbaz et al., in press).

We found that RIPA detected *env* reactivity in 40% of IB-indeterminate serum specimens. The probability that *env* reactivity would be detected by RIPA, however, depended on the pattern of IB *gag* reactivity. Nearly 70% of the IB-indeterminate serum specimens with antibody to both p19 and p24 had *env* reactivity after RIPA testing and were then interpreted as positive specimens. The probability of detecting antibody to *env* decreased to less than 50% when reactivity to only p24 was seen on IB and dropped to zero when p19 reactivity alone was seen.

Titration studies of HTLV-I-infected blood donors indicated that IB was superior to RIPA for detecting antibodies to *gag* proteins (1). However, since the *gag* proteins of HTLV-I and HTLV-II contain proportionally less cysteine or methionine residue (19) (the amino acids we radiolabeled for RIPA), this apparent difference in sensitivity may be lessened by radiolabeling an amino acid present in greater quantity in *gag* proteins. The ability of RIPA to detect antibody to *env* in a significant number of the indeterminate serum specimens that were *gag*-only reactive by IB could be due to the dilution at which the specimens are tested in RIPA (1:10), dilutions impractical for IB (because of increased background), or because sera the specimens are reacted with HTLV-I antigen prior to denaturation by sample buffer.

In groups with low HTLV-I seroprevalence (blood donors, hemophiliacs, and gay men), p19-only IB reactivity was seen most often; in contrast, 95% of the indeterminate serum samples from high seroprevalence groups (prostitutes and IVDU) had reactivity to p24, with or without reactivity to p19. These data suggest the importance of the *gag* p24 reactivity in defining seropositive criteria for HTLV-I.

The specificity of HTLV-I p19-only IB reactivity needs further study. We found this IB pattern predominate in the lowest HTLV-I seroprevalence group—p19-only reactivity remained constant in longitudinal studies and a low frequency of this pattern of IB-indeterminate reactivity remained constant in high HTLV-I seroprevalence groups. Taken together, these data suggest that p19-only reactivity is not a reliable marker of HTLV-I infection and may represent nonspecific reactivity to HTLV-I or cellular antigens in the IB test. Palker et al. (12) reported the synthesis of a monoclonal antibody to p19, which in addition to reacting with HTLV-I p19, also reacted with a variety of human cells and tissues uninfected with HTLV-I. This reactivity may be due to antibody to cellular antigens that share epitopes with HTLV-I p19 or may represent cross-reactivity with a closely related retrovirus.

HTLV-I IB with p19-only reactivity apparently is not due to infection with HTLV-II, since the reactions of these serum specimens remained unresolved with HTLV-II IB (unpublished data). Our data suggest that IB and RIPA reactivity with both HTLV-I and HTLV-II antigens does not clearly distinguish between these viral infections. Reactivity of known HTLV-II serum specimens was greater, however, with HTLV-II antigens in the test.

Continued definition of immunoreactivity to HTLV-I or HTLV-II infection and improved serologic techniques to identify these viral infections are critical to future population-based studies of HTLV-I or HTLV-II infection. Currently, the only available methods to clearly distinguish between these viral infections involve labor-intensive techniques (e.g., virus isolation and polymerase chain reaction) (3, 9). In this regard, improvements in antigen preparations (e.g., synthetic peptides and recombinant viral antigens) used in specific serologic tests are needed to distinguish between HTLV-I and HTLV-II infections.

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