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Comparison of Immunofluorescence, Enzyme Immunoassay, and Western Blot (Immunoblot) Methods for Detection of Antibody to Human T-Cell Leukemia Virus Type I

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A total of 3,349 serum samples were screened by the immunofluorescence (IF) method for antibody to human T-cell leukemia virus type I (HTLV-I). Only 9 of 2,409 specimens from selected individuals, blood bank donors, patients with encephalitis-meningitis, and human immunodeficiency virus antibody-positive homosexual or bisexual men were reactive by IF. In addition, 940 serum samples from intravenous drug abusers were tested by IF and also by an HTLV-I enzyme immunoassay (EIA) method. Of these, 222 (24%) were positive for both HTLV-I and HTLV-II antigens by IF, and 191 of these 222 were also reactive in the HTLV-I EIA. Of the 31 IF-positive, EIA-negative serum samples, 20 exhibited optical density readings ≥70% of the positive cutoff in the EIA, and 29 samples reacted with 1 or more bands in the Western blot (immunoblot) test. An additional 10 specimens that were EIA negative reacted only with HTLV-I by IF. Differences in staining morphology and in reactions on HTLV-I and HTLV-II antigens before and after absorption of the serum specimens with HTLV-I- and HTLV-II-infected cell pellets revealed six distinct serological patterns by IF. These results indicate that infections by HTLV-I or by another closely related retrovirus(es) occur in California. Further studies utilizing statistically valid sampling methods are needed to estimate true prevalence rates among various groups. IF and Western blot tests should supplement the EIA method to maximize sensitivity and specificity of test procedures.

Human T-cell leukemia virus type I (HTLV-I) is a C-type retrovirus which has been isolated from patients with leukemia-lymphoma in the United States (6), Japan (5), the Caribbean (1, 10), Africa (9), and other geographical areas. HTLV-II, originally isolated from a patient with a T-cell variant of hairy cell leukemia (3), is closely related to, but distinct from, HTLV-I. Antibody studies have linked HTLV-I to some cases of tropical spastic paraparesis (8), and relatively high incidences of HTLV-I and HTLV-II antibodies, i.e., 9 and 18%, respectively, have been reported in serum samples from intravenous (i.v.) drug abusers in Queens, N.Y. (7).

To compare the immunofluorescence (IF), enzyme immunoassay (EIA), and Western blot (immunoblot) methods for HTLV-I antibody detection, we tested 3,349 serum samples from various populations.

MATERIALS AND METHODS

Specimen selection. Serum samples were from 1,224 individuals from juvenile halls, patients in clinics for prenatal and sexually transmitted diseases, and marriage license applicants from whom sera had previously been collected for a human immunodeficiency virus seroprevalence study; 366 blood bank donors; 456 patients with encephalitis or meningitis; 363 human immunodeficiency virus antibody-positive homosexual or bisexual men; and 940 i.v. drug abusers from 19 California counties. All specimens except those from the encephalitis-meningitis group were coded and thus unlinked from any personal identifiers.

IF. For the IF, an HTLV-I-transformed human T-cell line (MT-2) was obtained from Jay Levy, University of California, San Francisco, and the H9 uninfected human T-cell line was obtained from Robert Gallo, National Institutes of Health, Bethesda, Md. The cells were propagated in Dulbecco high-glucose medium containing 10% fetal bovine serum, 0.09% glutamine, 100 U each of penicillin and streptomycin per ml, and 1 μg of amphotericin B per ml and were split 1:3 once a week. Five days after the split, 40 ml of HTLV-I and 120 ml of H9 cell suspension were centrifuged at 500 × *g* for 5 min, the supernatant fluid was removed, and the cell pellet was reconstituted in phosphate-buffered saline containing 2% fetal bovine serum, sufficient to give almost a confluent monolayer when a drop was placed on a slide and observed microscopically. On each slide ten cell smears were made; the slides were air dried, fixed in acetone for 10 min at room temperature, and stored at −60°C. An HTLV-II-transformed cell line (Mo-T, ATCC CRL8066) was propagated as described above, and antigen slides were prepared in a similar manner.

The serum samples were screened on HTLV-I slides at a 1:10 dilution by the IF method, as previously described (2). Those that reacted with HTLV-I were tested on the HTLV-II slides. Specimens were considered positive if they reacted with both antigens. The uninfected H9 cells served as nonspecific controls. Any specimen that reacted with all the cells was absorbed with an H9 cell pellet and retested (2). Samples that still stained nonspecifically after absorption were excluded from the study.

EIA. The HTLV-I EIA (Cellular Products, Inc. [CPI], Buffalo, N.Y., and E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) were performed according to the directions of the manufacturer, except that the test was not repeated with reactive serum samples that were also positive by IF.

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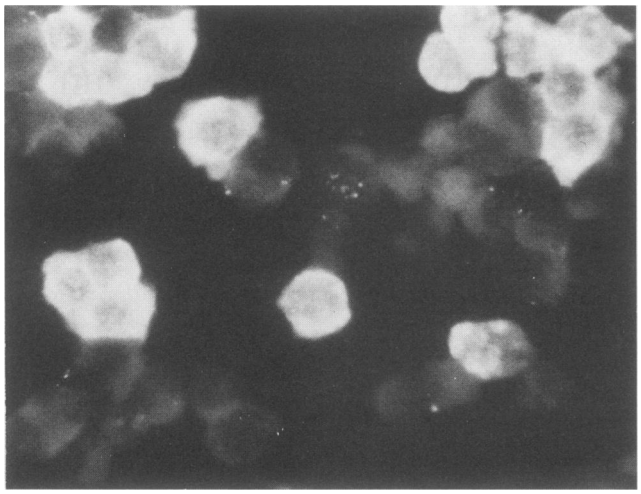

FIG. 1. Diffuse cytoplasmic staining of a reactive human serum sample on HTLV-I-infected cells.

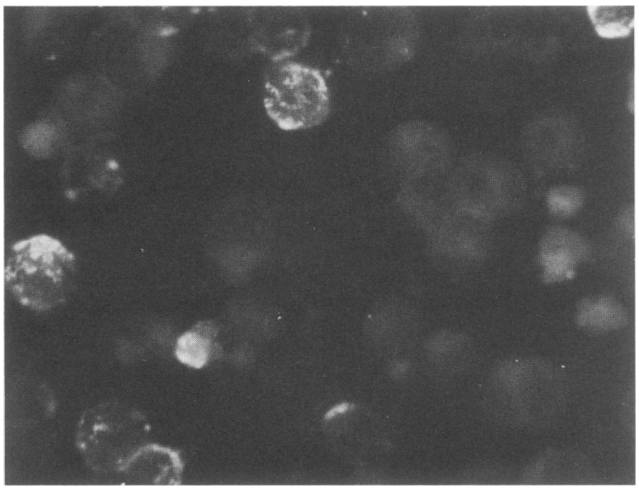

FIG. 2. Particulate cytoplasmic staining of a reactive human serum sample on HTLV-I-infected cells.

Western blot. The HTLV-I and HTLV-II Western blot antigens were supplied by Hillcrest Biologicals, Inc., Cypress, Calif., and the test was performed as described by Tsang et al. (11), except that 2-mercaptoethanol was used in the disruption mixture and the antigen was boiled for 3 min.

RESULTS

Specimens were first screened by the IF method. Only eight samples from persons in the HIV seroprevalence study (seven persons from the sexually transmitted disease clinics and one from the premarital group) and one sample from the encephalitis-meningitis group were positive, and none from the blood donor or human immunodeficiency virus-positive homosexual or bisexual groups were reactive. However, 222 (24%) of the 940 serum specimens from i.v. drug abusers were IF positive on both HTLV-I and HTLV-II antigens, and reactive specimens were found in inhabitants from 16 of the 19 counties screened. Of these 222, 198 exhibited diffuse cytoplasmic staining reactions on the infected cells (Fig. 1), and 24 specimens stained particulate cytoplasmic antigen in the HTLV-I cells (Fig. 2). Ten other specimens reacted with particulate antigens in the HTLV-I antigen but were negative with the HTLV-II antigen.

The CPI EIA test was performed on the 940 i.v. drug abuser samples, and 191 of these were also positive by the CPI EIA (Table 1). Of these EIA-reactive serum samples, 147 were tested by the Du Pont EIA method, and, with this test, 105 were positive. Those 31 specimens that gave discrepant results between the IF and the CPI EIA methods were tested by Western blot and by the Du Pont EIA. Table 2 illustrates IF, EIA, and Western blot reactions with 19 samples that gave a diffuse staining pattern by IF but were negative by the CPI EIA. Eighteen of these were also nonreactive in the Du Pont EIA. Of the 19, 17 were Western blot reactive, and 15 had CPI EIA ratios ≥0.70. The Du Pont EIA ratios were usually lower than ratios for the CPI reactions.

An attempt was made to distinguish antibody reactions due to HTLV-I infection from those due to HTLV-II. A 1:10 dilution (0.2 ml) of each of 15 serum samples which exhibited diffuse cytoplasmic staining on both antigens by IF and which were reactive by EIA and by Western blot was mixed with the cell pack from 10 ml of HTLV-I or HTLV-II cell suspension and was incubated horizontally at room temperature on a shaker for 1 h. The cells were centrifuged, and the IF test was performed using the absorbed serum samples.

TABLE 1. Comparison of HTLV-I EIA and IF results on 940 serum samples

IF result	Result (no.) by EIA:	
	Positive	Negative
Positive[a]	191	31
Negative	0	718

[a] Reactive with both HTLV-I and HTLV-II antigens.

TABLE 2. IF, EIA, and Western blot reactions on HTLV-I IF-positive, EIA-negative serum samples

Specimen No.	IF[a]		EIA ratio[b]		Mol wt by Western blot[c] on HI
	HI	HII	CPI	Du Pont	
1	2	4	0.98	0.63	24
2	2	3	0.98	0.80	19, 24
3	1	2	0.96	1.07	19, 24
4	2	1	0.90	0.22	
5	2	3	0.89	0.92	24
6	2	2	0.88	0.56	24
7	4	3	0.84	0.45	24, 46
8	1	3	0.82	0.29	24
9	1	2	0.81	0.50	24
10	1	3	0.81	0.90	24
11	2	3	0.80	0.59	19, 24
12	2	2	0.79	0.41	19, 24
13	1	2	0.73	0.15	24
14	2	3	0.72	0.36	24
15	1	3	0.71	0.53	19, 24
16	1	3	0.69	0.29	
17	2	3	0.60	0.40	24
18	1	1	0.51	0.31	24
19	3	3	0.27	0.24	24

[a] Degree of fluorescence (1 to 4+ diffuse staining); HI, HTLV-I antigen; HII, HTLV-II antigen.
[b] OD of specimen/OD cutoff.
[c] Reactive protein band. Molecular weight is given in thousands.

TABLE 3. IF results of IF-, Western blot-, and EIA-positive serum samples absorbed with HTLV-I- and HTLV-II-infected cell pellets

Specimen no.	IF[a]						Mol wt by Western blot on:[b]		EIA ratio[c]
	HI	HII	HTLV-I		HTLV-II		HTLV-I	HTLV-II	
			HI[d]	HII	HI	HII			
1	4 (160)	4	−	2	4	4	19, 24, 37, 46, 55	ND	4.56
2	3 (40)	4	−	3	4	4	19, 24, 37, 46, 55	ND	4.23
3	4 (640)	3	−	4	4	4	19, 24, 37, 46, 55	ND	4.00
4	4	1	−	3	−	1	19		2.37
5	2	4	−	1	1	2	24	21, 24, 37, 46, 55	2.33
6	2	1	−	4	−	−	19		2.02
7	1	3	−	1	4	3	24	21, 24, 37, 42, 46, 55	1.73
8	4	3	2	4	4	4	24	21, 24, 37, 42, 46, 55	1.23
9	1	2	−	−	3	−	24	24	1.21
10	2	4	−	3	3	3	24, 37, 46, 55	21, 24, 37, 42, 46, 55	1.20
11	1	2	−	1	4	3	19, 24, 37, 46, 55	21, 24, 37, 42, 46, 55	1.14
12	2	1	−	−	2	−	24	24	1.14
13	4 (2,560)	4	4 (−)[e]	4 (4)	2 (−)[e]	2 (2)	19, 24, 37, 46, 55	ND	6.77
14	4 (10,240)	4	4 (4)	4 (4)	3 (2)	3 (4)	19, 24, 37, 46, 55	ND	4.15
15	4 (640)	4	3 (2)	4 (4)	4 (4)	4 (4)	19, 24, 37, 46, 55	ND	5.23

[a] Degree of fluorescence (1 to 4+ diffuse staining); HI, HTLV-I antigen; HII, HTLV-II antigen; −, negative. Numbers in parentheses are reciprocals of dilution titers.
[b] Reactive protein band. Molecular weight is given in thousands. ND, Not done.
[c] OD of specimen/OD cutoff.
[d] Sera absorbed with HTLV-I cell pellet.
[e] Absorbed with cells from four pellets.

Absorption with HTLV-I-infected cells reduced or eliminated the staining reaction on the HTLV-I antigen of the first 12 samples but did not affect the staining intensity of the last 3 specimens (Table 3). Absorption with HTLV-II did not appreciably affect the fluorescence on either antigen. Nine samples were tested by Western blot with the HTLV-II antigen. Specimens 4 and 6, the only two which reacted with the single band p19 of HTLV-I were negative with the HTLV-II antigen, but the other seven reacted with as many as or more bands with HTLV-II than with HTLV-I. Antibody titers for HTLV-I were determined by IF on specimens 1, 2, 3, 13, 14, and 15. Specimens 13, 14, and 15, which had the highest titers, were absorbed with four times the number of cells originally used and retested to see if the previous inability to absorb out the reaction with HTLV-I was due to high serum titers and insufficient absorption material. The reaction on HTLV-I of specimen 13 was not repeated, but the reactions of specimens 14 and 15 were not affected.

Because the atypical particulate staining exhibited by 34 of the serum samples might have been due to antibody to a cross-reacting antigen of another retrovirus, these specimens were tested by Western blotting, employing both antigens, and were also absorbed with infected cells as described above and reacted by IF on HTLV-I- and HTLV-II-infected slides (Table 4). Specimens 1 to 24 fluoresced with both HTLV-I and HTLV-II antigens. Absorption with HTLV-I cells reduced or eliminated HTLV-I reactions with specimens 1 to 20 but did not affect the staining of specimens 21 to 24. Absorption did not appreciably affect the fluorescence on HTLV-II. Most of these 24 samples reacted with more bands in the Western blot with the HTLV-II antigen than with the HTLV-I antigen. Twelve were EIA positive and five others exhibited EIA ratios of ≥ 0.70. In contrast, samples 25 to 34, which were HTLV-II negative by IF, all exhibited very low EIA ratios. One was reactive only with HTLV-I by blot, one was reactive with both antigens, and three reacted only with the HTLV-II antigen. Absorption with HTLV-I cells reduced the IF reaction on HTLV-I with the first four specimens but did not affect the staining intensity of the last six. Antibody titers on three samples from each of the four staining patterns did not vary significantly, and titer differences did not appear to explain absorption differences.

DISCUSSION

When the IF method for antibody determinations is used, the serum samples are reacted with antigen-positive cells and also with uninfected cells from the same cell line and passage level as the antigen as a specificity control. An HTLV-I-transformed cell line, MT-1, which contains approximately 5% infected and 95% uninfected cells, has been used successfully for HTLV-I IF tests (4). Because the MT-2 cells that we employed were 100% infected, they were mixed with uninfected H9 cells, which served as one control. All samples reacting with the HTLV-I antigen were also tested against HTLV-II-infected cells as a further control for samples that might stain nonspecifically with the MT-2 but not with the H9 cells. A total of 222 serum specimens reacted with both antigens and were considered IF positive.

Specimens 25 to 34 (Table 4) did not fluoresce with the HTLV-II antigen. Thus, the reactions of these sera may be considered nonspecific, but 6 of these 10 reactions were not reduced by absorption with MT-2 cells, contrary to what would have been expected if the staining had been due to nonspecific binding of the sera to these cells. In addition, these 10 sera were also positive on slides made from an HTLV-I-infected Molt-3 cell line established in this laboratory, and 5 of the 10 reacted with either HTLV-I or HTLV-II by Western blot.

The majority of IF-positive serum samples (198 of 222) reacted with multiple cytoplasmic antigens in both the HTLV-I- and the HTLV-II-infected cells, and the overall appearance was that of diffuse staining, although particulate fluorescence was often visible also. The highest EIA ratios and the greatest number of band reactions in the Western blot were found in the diffuse staining group (Table 3). The particulate staining samples reacted with fewer antigens in

TABLE 4. IF, Western blot, and EIA reactions on serum samples exhibiting particulate staining patterns in the HTLV-I IF test

Specimen no.	IF[a]						Mol wt by Western blot[b] on:		EIA ratio[c]
	HI	HII	HTLV-I		HTLV-II		HI	HII	
			HI[d]	HII	HI	HII			
1	2P (160)	3	–	3P	2	1	24	24, 37, 42, 55	1.44
2	2P	3	–	3P	4	2	QNS	QNS	1.34
3	1P (160)	2	–	1P	2	2	24, 37	24	1.31
4	2P (160)	3	–	3P	3	3	19, 24, 37, 46, 55	21, 24, 37, 42, 46, 55	1.23
5	1P	2	–	2P	3	3	24	21, 24	1.19
6	2P	2P	–	1P	2	1	24	24, 42, 46	1.14
7	3P	4	–	2P	2	2	QNS	QNS	1.11
8	4P	2	1P	3P	4	3	19, 24	24	1.08
9	2P	2	–	1P	2	2	24	21, 24, 37, 46	1.05
10	1P	3	–	2P	3	3	24	24, 37, 46	1.03
11	2P	1P	1P	2P	2	1	24	21, 24	1.00
12	1P	3	–	1P	2	1	24	24	0.99
13	4P	2	–	3P	3	3	24, 37, 46	24, 37, 42, 46, 55	0.97
14	1P	3P	–	3P	4	3	24, 37, 46, 55	24, 37, 42, 46, 55	0.90
15	±P	2	–	1P	4	4	24	24, 37, 42, 46, 55	0.85
16	2P	3	–	–	3	2	24, 37, 46, 55	21, 24, 37, 42, 46, 55	0.78
17	1P	3	–	1P	2	3	24	24	0.65
18	1P	3	–	2P	3	3	24	QNS	0.65
19	2P	3P	–	2P	3	2	24	24, 37, 42, 46	0.58
20	2P	3	–	1P	2	2	24	24	0.20
21	2P (640)	3	3P	4P	3	3	24	21, 37, 42, 46	1.06
22	1P (160)	2	1P	2P	2	2	24	24	0.59
23	4P (2,560)	2	4P	4P	3	2	24	24	0.48
24	2P	4	3P	1P	3	3			0.26
25	4P (640)	–	–	4P	–	–	19		0.18
26	3P (160)	–	–	+P	–	–			0.11
27	2P (640)	–	–	3P	–	–			0.05
28	3P	–	1P	3P	–	–			0.04
29	4P	–	4P	3P	–	–			0.08
30	4P (640)	–	3P	4P	–	–		24	0.06
31	4P (160)	–	3P	3P	–	–		24	0.09
32	2P (640)	–	3P	3P	–	–		24	0.17
33	1P	–	2P	2P	–	–	19, 24	24	0.06
34	1P	–	2P	1P	–	–			0.24

[a] Degree of fluorescence (1 to 4+ diffuse or particulate [P] staining); HI, HTLV-I antigen; HII, HTLV-II antigen; –, negative. Numbers in parentheses are reciprocals of dilution titers.
[b] Reactive protein bands. Molecular weight is given in thousands. QNS, quantity not sufficient.
[c] OD of specimen/OD cutoff.
[d] Sera absorbed with HTLV-I cell pellet.

the IF and the Western blot tests; the highest EIA ratio in this group was 1.44 (Table 4).

Six distinct staining patterns were seen when the IF method was employed: (i) diffuse staining of HTLV-I and HTLV-II which was reduced on HTLV-I when the serum was absorbed with an HTLV-I cell pellet, (ii) diffuse staining which was not reduced by absorption (Table 3), (iii) particulate staining of both antigens which was reduced by HTLV-I cell absorption, (iv) particulate staining of both antigens which was not reduced, (v) particulate staining of HTLV-I but not of HTLV-II which was reduced by HTLV-I cell absorption, and (vi) HTLV-I-only staining which was not reduced by cell absorption (Table 4).

We found that the IF-positive sera stained more brightly with the MT-2 antigen than with two other HTLV-I-infected cell lines, MT-4 and HTLV-I-infected Molt-3. However, most of the diffusely staining sera showed more intense fluorescence with the HTLV-II than with the HTLV-I antigen, those sera that reacted with HTLV-I in a particulate pattern usually exhibited diffuse staining when they were HTLV-II positive, and the pattern 3 particulate stainers reacted with more bands in the Western blot test with the HTLV-II than with the HTLV-I antigen. Because the serum samples had originally been screened by IF employing the HTLV-I antigen and had been reacted with HTLV-II only if they were positive with HTLV-I, it is not known whether there were any HTLV-II-positive, HTLV-I-negative sera in this study.

The absorption tests indicated that antibody responses were more closely related to HTLV-I than to HTLV-II in groups 1 and 3. Absorption did not reduce the staining of sera in group 2 and group 4; and the sera from groups 5 and 6 did not react with the HTLV-II antigen and exhibited extremely low EIA ratios, and 5 of the 10 were Western blot negative. The multiplicity of staining reactions appeared to be sporadically distributed throughout the state, and there was no correlation of any of the reactions with a particular ethnic group or sex.

The IF method was found to be more sensitive than the EIA in detecting antibody to HTLV-I, and the CPI EIA was more sensitive than the Du Pont EIA, but it is difficult to assess the specificity of the IF, or any other test, on sera from i.v. drug abusers with no disease. However, only 9 of 2,409 samples from the other populations examined, who were presumably of low HTLV-I risk, were IF reactive. Thus, it would appear that the relatively high positivity rate

found by this method in the i.v. drug abuser group is a real phenomenon. Most of the IF-positive, EIA-negative samples gave optical density (OD) readings of ≥70% of cutoff in the CPI EIA. Lowering the cutoff would have caused these specimens to be detected, although 10 sera that were negative by IF were also in this range. Of the 30 IF-positive serum samples tested by Western blot with both HTLV-I and HTLV-II, 19 reacted with only the p24 band of the HTLV-I antigen. Of the 19, 12 reacted with multiple bands with the HTLV-II antigen. The specificity of single-core-band reactions in the Western blot is suspect because of the false-positive reactions experienced in the human immunodeficiency virus type 1 blot with p17, p24, and p55 core proteins. Use of the HTLV-II antigen may be helpful in determining the specificity of single-band reactions with HTLV-I. The Western blot was performed only on IF-reactive specimens, so the prevalence of positive blot reactions with IF and with EIA-negative sera could not be determined.

The results of this survey indicate that a portion of the California i.v. drug abuser population has been exposed to a retrovirus that shares antigens with HTLV-I and HTLV-II but that further studies are needed to determine both the specificity of the antibody reactions and the prevalence rates among various populations.

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