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Evaluation of the Human T-Cell Leukemia Virus Type I Seropositivity of Blood Donors by the Particle Agglutination Inhibition Test

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In the HTLV-I seroscreening of blood donor sera by gelatin particle agglutination (PA), more than 50% (55.6%) of the PA-positive sera were negative by immunofluorescence assay (IF). However, when donors were divided into age groups, there were increasing numbers of IF-positive/PA-positive donors with age. Among the PA-positive donors in the 50–64 age group, 65.9% were IF-positive compared to 16.0% in the 16–19 age group. The serological specificities of the IF-negative/PA-positive specimens were tested by using a newly developed PA inhibition (PAI) test. The HTLV-I specificity of the PAI test was confirmed by the observation that agglutinations with anti-HTLV-I p19 and gp21 monoclonal antibodies as well as IF-positive sera were specifically inhibited with HTLV-I preparations or HTLV-I-positive cell extracts and not with HTLV-I-negative cell extracts. Sixty of the 104 specimens collected randomly from the IF-negative/PA-positive donors were PAI-positive. The majority (80%) of such PAI-positive sera showed more than two bands of HTLV-I *gag*-encoded polypeptide, p19, p24, p28 and p53 on Western blotting. Some of the PAI-positive sera were also positive by enzyme immunoassay. These results indicate that at least some of the IF-negative/PA-positive donors possess HTLV-I-specific antibody and may be potential HTLV-I carriers who will become IF-positive at a later age.

Key words: HTLV-I seroscreening — Blood donor — PA-positive/IF-negative — PAI

Human T-cell leukemia virus type I (HTLV-I), a causal agent of adult T-cell leukemia/lymphoma (ATL)[1] and HTLV-I-associated myelopathy (HAM),[2] was shown to be prevalent among Japanese[3,4] and to be transmitted by blood transfusion.[5] While such sero-epidemiological findings were obtained mainly by immunofluorescence assay (IF),[6] a gelatin particle agglutination (PA) test[7] for the viral antibody screening has been introduced at Japanese Red Cross Blood Centers since 1986 to prevent transfusion-associated HTLV-I infection. The PA test was adopted as a mass screening method because it involved a single step, was rapid, and required no special equipment. Transfusion-associated seroconverted cases have been markedly reduced by the donor screening.[8–10] After the start of the HTLV-I seroscreening by PA, it was shown that a considerable number of PA-reactive sera were negative by IF.[9,10] Such IF-negative/PA-positive sera have been poorly characterized.

In this paper, we describe the serological specificities of the IF-negative/PA-positive sera. We developed the PA inhibition (PAI) test to examine the HTLV-I specificities of these sera. The results of the PAI test appeared to be HTLV-I specific and were highly concordant with those of the Western blotting (WB), which indicated that at least some of the IF-negative/PA-positive sera were HTLV-I specific.

MATERIALS AND METHODS

Serum samples Sera tested in this study were obtained from volunteer blood donors at Hokkaido Red Cross Blood Center during the period from April 1987 to March 1988. Serum samples from ATL and HAM patients were gifts from Drs. K. Sakurada and T. Togashi (Hokkaido University Hospital), respectively.

Monoclonal antibodies 2A2 was a monoclonal antibody to HTLV-I *gag*-encoded p19. F10,[11] a monoclonal antibody to *env* gene product gp21, was provided by Fujirebio, Tokyo.

Serological tests Donor sera were initially screened by the PA test using Serodia-ATLA (Fujirebio) with an automated pretransfusion testing system, PK7100 (Olympus Co., Tokyo) as described by Sekiguchi *et al.*[12] The reactive sera were further tested by the manual PA test according to Ikeda *et al.*[7] Repeatedly reactive sera were regarded as PA-positive. The PA-positive blood units were excluded from transfusion and subjected to further tests.

The IF test was carried out as outlined by Hinuma *et al.*[6] Ten μl of 1:5 diluted test serum was incubated with acetone-fixed MT-1[13] and/or MT-2[14] cells on the spots for 45 min at 37°C. After the incubation, the slide was washed and incubated further with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human-IgG(γ) or FITC-conjugated rabbit anti-human IgM(μ) (DAKOPATTS, Copenhagen) for 45 min at 37°C.

The PA inhibition test (PAI) For the PA inhibition test, a serum specimen was usually diluted to give fourfold concentration over the 'endpoint' condition. Twenty-five μl of the diluted serum was incubated with an equal volume of cell extract overnight at 4°C and the residual antibody activity was tested by the PA test. The cell extracts used for the inhibition were prepared from HTLV-I-producing (MT-2, HUT102[15] and OKM-2) and non-producing (CEM,[16] TALL-1[17] and EBV-Wa[18]) human lymphoblastoid cell lines. OKM-2, derived from an ATL patient, was provided by Dr. K. Miyamoto (School of Health Sciences, Okayama University). The cells were washed twice with phosphate-buffered saline (PBS), and a 10% cell suspension was disrupted with a sonicator (Heat Systems-Ultrasonics, New York) for 20 min at a setting of 4 in ice. The sonicated cells were then subjected to ultracentrifugation at 100,000g for 10 min in a Beckman TLA100.2 rotor. Cell extracts contained approximately 1 mg protein/ml. To analyze IF-negative/PA-positive sera, the cell extracts were used without any dilution. The preparation was stored at −80°C until use.

Western blotting (WB) Nitrocellulose strips with transferred HTLV-I proteins were provided by Fujirebio. The virus proteins were derived from TCL-Kan.[19] The strips were incubated with test sera for 1 h at room temperature. The binding of HTLV-I-specific antibodies was detected with biotinylated anti-human IgG(γ) (TAGO, California) or IgM(μ) (Vector Labs, Burlingame) and avidin-biotin-peroxidase complex technique as described in the supplier's manual. The sera showing three or more bands of HTLV-I *gag*-encoded polypeptide, p19, p24, p28 and p53 were regarded as positive. This criterion is based on the fact that, with the WB strips provided by Fujirebio, p19 and/or p24 bands often appear in HTLV-I seronegative samples.

Enzyme immunoassay (EIA) EIA was performed by using the HTLV-I preparation from TCL-Kan, a gift from Fujirebio, as the antigen. Each well of a microplate was coated with 100 μl of the viral antigen (ca. 0.4 μg protein/well) diluted with carbonate buffer (pH 9.6) overnight at 4°C and incubated with 1% bovine serum albumin (BSA)-PBS for blocking nonspecific binding sites. Donor serum diluted with BSA-PBS was added to the well and incubated for 2 h at room temperature. The microplate was then incubated with alkaline phosphatase-conjugated goat anti-human IgG(γ) or anti-human IgM(μ)(KPL, Maryland) for 1 h. Every incubation step was followed by sufficient washing with 0.05% Tween 20-PBS. Each well of the plate was finally incubated with *p*-nitrophenyl phosphate substrate for 30 min. The results were expressed as the value of absorbance at 405 nm.

Preparation of HTLV-I concentrates Virus particles were concentrated as described by Krichbaum-Stenger *et al.*[20] Virus particles in the culture fluid of MT-2 cell line were concentrated by centrifugation at 30,500g for 45 min. The virus concentrate was resuspended in the TNE buffer, and centrifuged further on discontinuous 30% to 45% sucrose density gradients at 89,400g for 2.5 h. The HTLV-I antigen preparations purified from TCL-Kan cell line were provided by Fujirebio.

RESULTS

Screening of blood donor sera for HTLV-I antibodies Of 201,372 donor sera tested by the PA test, 2,654 (1.32%) were reactive (Table I). The PA-positive sera were further examined by IF and 44.4%(1,178/2,654) were reactive. More than 50% of the PA-positive sera were IF-negative. When donors were divided into age groups, an increase of the PA-positive donors with age was noted. Also, there was an increasing number of IF-positive/PA-positive donors with age; 65.9% of the PA-positive donors in the 50–64 age group were IF-positive compared to 16.0% in the 16–19 age group.

Specificity of the PA inhibition test The inhibition tests were carried out in order to evaluate the specificity of the PA tests. The antigens used for the inhibition test included the sonicated cell extracts from HTLV-I-producing or non-producing cell lines and virus concentrates from the culture fluid. Monoclonal antibodies to p19 and gp21 were incubated with the antigens, and residual

Table I. HTLV-1 Seropositive Blood Donors Screened by PA and IF

Assay	Age (yr)					Total
	16–19	20–29	30–39	40–49	50–64	
PA	306/34,845[a]	573/58,569	717/47,709	583/33,818	475/26,431	2,654/201,372
	(0.88)	(0.98)	(1.50)	(1.72)	(1.80)	(1.32)
IF[b]	49/306	181/573	305/717	330/583	313/475	1,178/2,654
	(16.0)	(31.6)	(42.5)	(56.6)	(65.9)	(44.4)

a) Number of positive samples/number of samples tested (positive percentage).
b) FITC-conjugated anti-human IgG(γ) was used as the second antibody. Tested on MT-2 cells.

antibody activities were tested by means of the PA tests. Agglutination was specifically inhibited by the HTLV-I preparations or the HTLV-I-positive cell extracts and not by the HTLV-I-negative cell extracts (Fig. 1). Cell extracts from MT-2, HUT102 and OKM-2 inhibited the PA reaction of monoclonal anti-gp21 (F10) at dilutions of 1:4, 1:8 and 1:16, respectively, and also inhibited the reaction of anti-p19(2A2) at dilutions of 1:16, 1:2 and 1:1, respectively. Agglutination with IF-positive serum samples from blood donors, ATL and HAM patients was also specifically inhibited by the HTLV-I antigens (Fig. 1). In the case of serum from an ATL patient, the PA reaction was inhibited by MT-2 extract diluted 1:4, HUT102 diluted 1:8 and OKM-2 diluted 1:16. The results indicate that agglutination by PA represents the HTLV-I-specific reaction and that the PAI can detect viral envelope as well as core antigens.

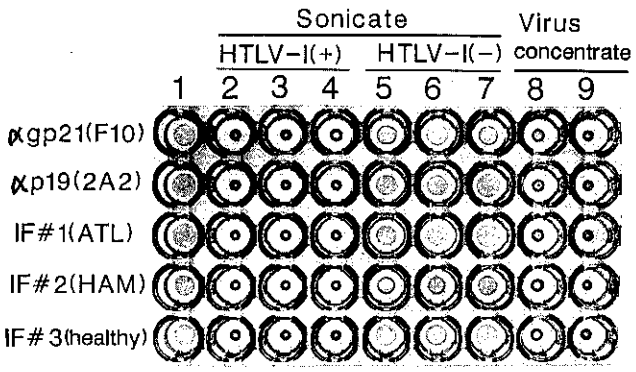

Fig. 1. Specificity of the PA inhibition test. 1, without antigen; 2, MT-2; 3, HUT102; 4, OKM-2; 5, CEM; 6, TALL-1; 7, EBV-Wa; 8, HTLV-I (MT-2); 9, HTLV-I (TCL-Kan).

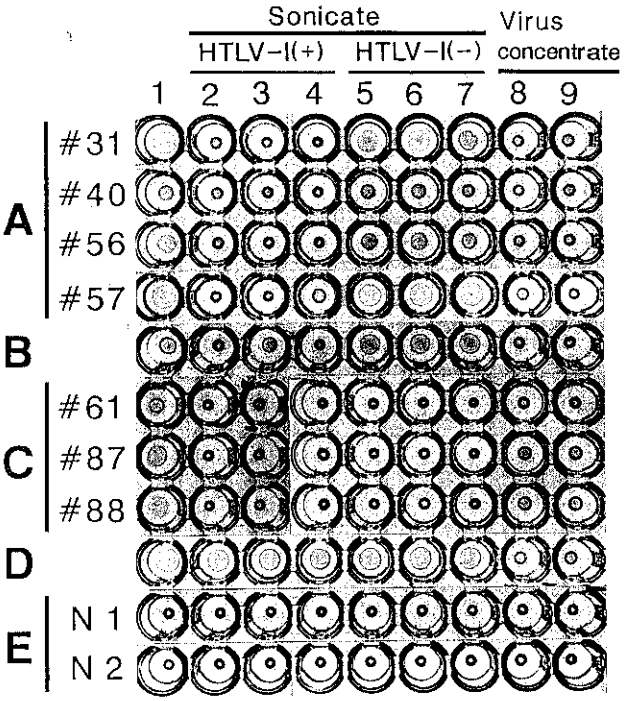

Fig. 2. The PA inhibition test with IF-negative/PA-positive samples. A, positive; B, equivocal; C and D, negative PAI. E, PA negative. See the legend to Fig. 1 for identification of inhibition antigen used.

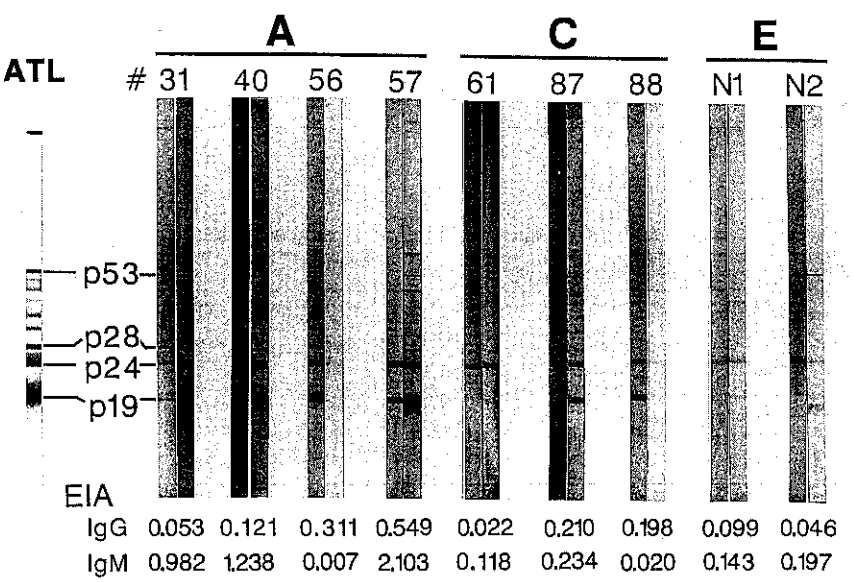

Fig. 3. WB patterns and EIA reactivities of IF-negative/PA-positive samples. PAI-positive (A), PAI-negative (C) and PA-negative donor samples (E) were analyzed by WB and EIA as described in "Materials and Methods." Of each pair of strips on WB, the left and the right present IgG and IgM reactions, respectively. EIA results are expressed by the value of absorbance at 405 nm.

The IF-negative/PA-positive sera from blood donors were subjected to the PAI tests. As shown in Fig. 2, the sera from PA-positive donors could be divided into three groups according to the results of the PAI test. The agglutination was inhibited by the HTLV-I-positive cell extracts in the first group (positive PAI, Fig. 2 pattern A), by one or two HTLV-I-positive cell extracts in the second group (equivocal PAI, Fig. 2 pattern B) and by HTLV-I-negative cell extracts or showed no inhibition in the third group (negative PAI, Fig. 2 patterns C and D). The agglutination of PA-positive sera was inhibited by the HTLV-I concentrates regardless of PAI patterns.

Analyses of PAI-positive sera by WB and EIA The PA-positive sera which were examined in Fig. 2 were subjected to WB analysis. The WB strips were incubated with serum specimens and the bands were detected with biotinylated anti-human IgG and IgM. As shown in Fig. 3, the *gag*-specific bands, p53, p28, p24 and p19 were observed in PAI-positive sera (i.e. #31, #40, #56 and #57 in Fig. 2). When the intensity of the band corresponding to p53, p28, p24 or p19 was equal to that of the ATL specimen, the band was regarded as positive. PAI-negative sera (i.e. #61, #87 and #88 in Fig. 2) could not be regarded as WB-positive according to the criteria described in "Materials and Methods," although p24 and/or p19 bands were observed. Antibodies corresponding to p19 and p24 bands in the PAI-negative sera may not be responsible for specific agglutination. The results also suggest that the virus concentrate contains nonviral components and some of the positive PA reactions are caused by virus nonspecific reaction.

The donor sera examined in Fig. 2 were further tested by EIA. The three PAI-positive sera (#31, #40 and #57) showed high reactivities compared with PAI- or PA-negative sera on EIA for IgM. An additional PAI-positive serum (#56) reacted on EIA for IgG (Fig. 3). These results were in good accordance with the observations on WB analysis and demonstrated that some PAI-positive sera contain IgM-type antibodies reacting with the HTLV-I preparation used.

PAI and WB analyses for IF-negative/PA-positive sera One hundred and four specimens were collected randomly from the IF-negative/PA-positive donors and analyzed by means of PAI and WB. The negative IF reactivities of these sera were confirmed with two different test cells, MT-1 and MT-2. Sixty of the 104 IF-negative/PA-positive sera showed positive PAI. The majority (more than 80%) of the PAI-positive and -equivocal sera were regarded as WB-positive (Table II). In contrast, none of the PAI-negative sera was positive by WB according to the criteria described in "Materials and Methods." Thus, the results of the PAI and WB were highly concordant.

The correlation of the polypeptide bands of WB with serological findings is shown in Table III. The sera from IF-positive blood donors, ATL and HAM patients were exclusively reactive to p53 and p28 of the HTLV-I *gag*-encoded proteins. The PAI-positive sera were also reactive to p53 and p28, whereas very few PA- or PAI-negative sera were reactive to these proteins. The band corresponding to p28 was observed with only one of the PAI- and PA-negative sera. The p24 band was observed in most of the sera regardless of IF and PA reactivities.

Table II. Comparison of PAI and WB Results on 104 Samples with IF-negative/PA-positive Results

PAI	No.	WB	
		Positive	Negative
Positive	60	48 (80)[a]	12 (20)
Equivocal	33	27 (82)	6 (18)
Negative	11	0 (0)	11 (100)
Total	104	75	29

a) Number of positive samples (percentage).

Table III. Frequency of HTLV-I *gag*-encoded Bands as Detected by WB in PAI-positive Samples

	IF-positive[a]	IF-negative			PA
	PAI positive	positive	PAI equivocal	negative	negative
p53	25 (100)[b]	43 (72)	24 (73)	0	0
p28	23 (92)	33 (55)	13 (39)	1 (9)	0
p24	25 (100)	60 (100)	33 (100)	10 (91)	6 (100)
p19	25 (100)	58 (97)	29 (88)	9 (82)	2 (33)
No. tested	25	60	33	11	6

a) All the IF-positive samples from patients (HAM and ATL) and blood donors were PAI-positive.
b) Number of positive samples (percentage).

Both PAI-positive and -negative sera were equally reactive to p19. Thus, when the WB strips from Fujirebio were used, the p53 and p28 bands were preferentially observed with not only IF-positive but also PAI-positive sera. The p24 and p19 bands were not helpful in discriminating HTLV-I specificity.

DISCUSSION

Soon after the adoption of the PA method for HTLV-I screening, it was noticed that there were a considerable number of IF-negative/PA-positive sera. Similar observations have been reported by others.[9, 10] The majority of IF-negative/PA-positive sera were not reactive in IgG-EIA (Yano, unpublished). In the PA test, IgM-type as well as IgG-type antibodies are detectable, whereas IgM type antibodies are not monitored with usual EIA test kits. In a transfusion-associated seroconverted case, IgM-type antibodies could be demonstrated by EIA in the specimens of the PA-positive/IgG-EIA negative period (Yano, unpublished). Also, some of the IF-negative/PA-positive donor sera contained IgM-type antibodies reactive in EIA and WB, as shown in this paper. More than half of the IF-negative/PA-positive sera contained antibodies which were detectable by the use of PAI and WB. Thus the IF-negative/PA-positive reaction could not be regarded merely as nonspecific.

The PA inhibition test described in this study appeared to detect HTLV-I specific antibodies. The PA reactivities of the anti-p19 and -gp21 monoclonal antibodies were specifically inhibited by the HTLV-I positive cell extracts and the virus concentrates. The PA reactivities of sera from IF-positive individuals were also specifically inhibited by the HTLV-I preparations or the HTLV-I-positive cell extracts. However, in some of the IF-negative/PA-positive sera, agglutination was inhibited by HTLV-I-negative cell extracts. The HTLV-I concentrates also inhibited the agglutination of these PAI-negative sera. The PA reactivities of these PAI-negative sera are likely to be directed to nonviral components on the sensitized gelatin particles. It has been reported that the purified virus preparation almost always contain nonviral components.[21–26] Such nonviral components may be responsible for the inhibition of PAI-negative sera. The nature of the p19 or p24 band which was often observed with PAI-negative sera in WB analysis is not clear. It may not be responsible for agglutination because the PA was inhibited by the HTLV-I-negative extracts. Alternatively, the p19 or p24 band with the PAI-negative sera may not be derived from HTLV-I and may be due to crossreactive antibodies as suggested by Schüpbach *et al.*[27]

The results of the PAI were highly concordant with those of WB. The minor differences between PAI and WB was probably due to the different characteristics of the two assays. The PA test could detect not only anti-core but also anti-envelope reactions, whereas the WB used in this study mainly detected the anti-core reactions. The patterns of WB may be interpreted differently if a different antigen preparation was used for the nitrocellulose strips. The band corresponding to p24 was observed with PA-negative sera in this study. Schüpbach *et al.*[27] also reported the prevalence of p19- and p24-reactive antibodies among apparently HTLV-I-free Swiss population. However, the p24 band was regarded as HTLV-I specific when different strips were used for WB analysis.[28]

The present methods for HTLV-I carrier screening are designed to identify antibodies to the virus rather than the virus itself. While HTLV-I or HTLV-I antigens can be induced from the cells of IF-positive individuals, trials to induce the viral antigens from the cells of IF-negative individuals were unsuccessful. Moreover, Okochi *et al.*[5] reported that transfusions of IF-negative blood did not cause seroconversion in spite of the fact that some IF-negative/PA-positive blood seems to contain HTLV-I specific antibodies, as shown in this study. A possible interpretation of these facts is that the viral proteins may be constantly produced while the viral replication is deficient in some IF-negative/PA-positive individuals. They may be potential HTLV-I carriers and become IF-positive at a later age. In accordance with this, the incidence of IF-positive carriers in PA-positive donors increased with age. Alternatively, the IF-negative/PA-positive antibodies are directed to nonviral components which are activated by the viral genomes or crossreactive to viral proteins. HTLV-I is known to contain the genome which *trans*-activates transcription.[29] Also, some of the HTLV-I proteins are known to contain epitopes crossreactive to human tissues.[30] In any event, identification of HTLV-I or its genome in the cells by a more sensitive and specific method seems to be necessary for direct identification of HTLV-I carriers.

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