Detection of Antibodies to Human T-Cell Leukemia Virus Types 1 and 2 in Breast Milk from East Asian Women

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We investigated the incidence of human T-cell leukemia virus type I (HTLV-1) infection in a total of 17 regions in four countries, including 13 regions in Japan, as well as Korea (Seoul and Busan), China, and Vietnam, by testing breast milk using a particle agglutination assay (PA) and line immunoassay (LIA). Among 266 samples from Japan, 24 (9.0%) were positive on PA and 3 (1.1%) were positive on LIA. Among 50 samples from Seoul, 2 were positive on PA and 1 was positive on LIA. In contrast, all 50 samples from Busan were negative on both tests, suggesting the maldistribution of HTLV-1 infectants in South Korea. The numbers of positive samples were 2/91 on PA and 1/91 on LIA for China and 1/88 on both PA and LIA for Vietnam. In China, one sample with a high probability of HTLV-2 infection was identified by LIA and synthetic peptide enzyme-linked immunosorbent assay (ELISA). We examined HTLV-1 antibody in breast milk samples using commercially available test kits, suggesting the existence of HTLV-1 carriers in endemic areas in Southeast Asia and an HTLV-2 infectant in China. As a part of human ethno-epidemiological research, these results constitute valuable epidemiological data. Further studies on the sensitivity, specificity, and reliability of assays using antibodies to HTLV-1 and 2 in breast milk will be necessary for large-scale epidemiological surveys of HTLV infection.

Key words breast milk; human T-cell leukemia virus type II; line immunoassay; particle agglutination assay

Adult T-cell leukemia (ATL) is a malignant CD4-positive T-cell neoplasm caused by infection with human T-cell leukemia virus type I (HTLV-1). Because HTLV-1 is a retrovirus, its genomic RNA is incorporated into target cells, and DNA is incorporated into the host genome as a provirus *via* reverse transcriptase.^{1,2)}

The prevalence of ATL is 0.2–0.3%, and a route of infection that poses a particular issue is vertical transmission (mother–child transmission) *via* breast milk.^{2,3)} ATL is endemic in equatorial Africa, the Caribbean islands, Colombia, Brazil, south India, Papua New Guinea, northeast Australia, and among indigenous people living at the margins of the Andes plateau in South America; in Japan, the infection rate is highest in western Japan, particularly Kyushu.^{3–5)}

In addition to blood transfusions and sexual intercourse, HTLV-1 infection can be transmitted from mother to child *via* breast milk, with the cause of vertical transmission being infection *via* lymphocytes in breast milk.^{1–3)} Pregnant women who are carriers must switch to infant formula in order to prevent infection. HTLV-1 testing is required for all pregnant women in Japan, with the main test methods used comprising antigen or antibody testing of blood.⁴⁾ Recently, ATL provirus DNA testing using automated nucleic acid purifiers has also been investigated.⁶⁾ In developing countries, however, blood testing may not always be adequate.

HTLV-2 has also been isolated from individuals other than leukemia patients, and its association with disease remains

unclear. Although HTLV-2 carriers have been reported to be common in Central and South America, the regional distribution of HTLV-2 has not been investigated in detail.⁷⁾

In our preceding paper,⁸⁾ we reported for the first time that the gelatin particle agglutination (PA) method can be used to measure HTLV-1 antibody in breast milk, which can be sampled non-invasively and is the major source of infection. This PA method is comparatively easy to use as needed, in both the laboratory and the field. For confirmatory testing, HTLV-1 was examined using the InnogeneticsTM Inno-liaTM HTLV I/II Score. A combination of PA and line immunoassay (LIA) was used on breast milk.

The purpose of this study was to develop a screening assay for the detection of antibodies to HTLV-1/2 in breast milk from East Asian women and to further examine HTLV-1 and HTLV-2 incidence using the InnogeneticsTM Inno-liaTM HTLV I/II Score and synthetic peptide enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Specimens A total of 545 breast milk samples (Beijing, 91; Hanoi, 88; Seoul, 50; Busan, 50; Okinawa, 33; Nagasaki 28, Yamaguchi, 20; Okayama, 20; Kochi, 10; Hyogo, 20; Wakayama, 15; Kyoto, 20; Fukui, 20; Gifu, 20; Tokyo, 20; Miyagi, 20; and Hokkaido, 20) were collected between 2004 and 2010, and were archived in the Kyoto University Human Specimen Bank. Written informed consent was obtained from all participants. The bank project was reviewed⁹⁾ and approved

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by the Ethics Committee of the Kyoto University Graduate School of Medicine on 14 November 2003 (E25).

PA Screening tests were performed using 545 breast milk samples and a commercially available SERODIA HTLV-1 test kit (Fujirebio Inc., Tokyo, Japan) for *in vitro* diagnosis.¹⁰⁾ The kit includes HTLV-1 antigen-coated gelatin particles that agglutinate in the presence of HTLV-1 antibody in human serum or plasma. Test samples (25 μ L) and positive control serum were prepared by 2-fold dilution up to 1:512. After an equal volume of sensitized particles was added, reactions were visually interpreted in duplicate. The agglutination patterns were interpreted according to the following criteria for an antibody titer of 1/8: negative (–), particles concentrated in the shape of a button with a smooth round outer margin; inconclusive ($\pm$), particles concentrated in the shape of a compact ring with a smooth round outer margin; positive (+), peripheral agglutination of the particles in a definite large ring with a rough multiform outer margin; and strongly positive (++), a film of agglutinated particles spread out uniformly on the bottom of the well.

LIA PA-positive breast milk was assayed for the presence of HTLV-1 antibodies using INNO-LIATM HTLV I/II Score assays (Innogenetics N.V., Gent, Belgium), which were originally designed for testing serum or plasma. Milk samples (100 μ L) were incubated in troughs containing LIA strips at 25°C overnight for 16 h. This incubation was followed by three washing steps with washing buffer before the addition of an alkaline phosphatase anti-human immunoglobulin conjugate. Samples were then incubated for 30 min at 25°C. Three

washing steps were again performed, followed by incubation with the chromogen 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for 30 min at 25°C. The results were compared to a positive control.

Synthetic Peptide-ELISA (sp-ELISA) LIA-positive breast milk was assayed by sp-ELISA¹¹⁾ using three synthesized peptides (corresponding to the Gag p19 protein 100 to 130 aa, the Env gp46 protein 175 to 199 aa, and the Env gp46 288 to 317 aa) derived from HTLV-1 genome sequence ATK-1. The peptide corresponding to the region of the Env gp46 protein 175 to 199 aa (gp46-175) allowed the discrimination of HTLV-1/2 antibodies.

RESULTS AND DISCUSSION

We carried out preliminary screening of HTLV-1 antibody using the PA method with a total of 545 samples: 266 from 13 locations in Japan, and 279 samples from China, Vietnam, and South Korea (Seoul and Busan). Of these, 29 samples showed positive or inconclusive patterns of agglutination. LIA of these 29 samples revealed that three were indeterminate for HTLV-1 infection, four were inconclusive, and two were positive. Of the two positive samples, one from China also indicated positivity for HTLV-2, which is an extremely interesting result. Table 1 shows the overall results for PA and LIA. In the agglutination reaction, 516 of the 545 (94.7%) breast milk samples were negative. Confirmatory testing was required for 29 samples (5.3%), comprising 11 in which particles formed a compact ring with a smooth outer margin ($\pm$), eight in which

Table 1. Screening Assay for Antibodies to HTLV-1 in Breast Milk Samples Using PA and LIA

Country	Sampling area	Number of samples	Positive on PA	Positive on LIA
Japan	Okinawa	33	7	0
	Nagasaki	28	3	0
	Yamaguchi	20	0	0
	Okayama	20	0	0
	Kochi	10	0	0
	Hyogo	20	3	0
	Wakayama	15	0	0
	Kyoto	20	0	0
	Fukui	20	0	0
	Gifu	20	0	0
	Tokyo	20	0	0
	Miyagi	20	3	2
	Hokkaido	20	8	1
Japan total		266	24	3
China	Beijing	91	2	1
			$\pm^a)$	$-\ ^a)$
			$\pm^b)$	$\pm^b)$
Korea	Seoul	50	2	1
			$+\ ^c)$	$+\ ^c)$
			$\pm^d)$	$-\ ^d)$
	Busan	50	0	0
Vietnam	Hanoi	88	1	1
			$\pm^e)$	$\pm^e)$
Total		545	29	6

Japanese data were reported by Matsubara *et al.*⁸⁾ Year of collection and age of sample donors were as follows: a) Sep. 2008, 27; b) Feb. 2009, 26; c) Jan. 2010, 31; d) Feb. 2010, 31; e) Sep. 2008, 19.

there was peripheral agglutination of the particles in a clear large ring with a rough multiform outer margin (+), and 10 in which a film of agglutinated particles was spread out uniformly on the bottom of the well (++). Table 1 also shows the donor locations of these 29 breast milk samples: seven from Okinawa, three from Nagasaki, three from Hyogo, three from Miyagi, and eight from Hokkaido were positive. Samples from all other Japanese regions were negative. Five samples (1.8%) from the other countries required confirmation, with two from Beijing, one from Hanoi, and two from Seoul being positive.

HTLV-1 and HTLV-2 were examined using the Innogenetics™ Inno-lia™ HTLV I/II Score using 10-fold quantities of samples according to the manufacturer's standard protocol. As shown in Table 1, two samples were positive and four had a deferred pattern. As shown in Fig. 1, a positive pattern for HTLV-2 was observed in one sample from China, and the sample did not react to gp46-175, suggesting that the donor may be an HTLV-2 carrier. Although it is difficult to distinguish between HTLV-1 and HTLV-2 using the PA method, Berini *et al.*¹²⁾ reported that the PA sensitivity of HTLV-2 in the blood of carriers is equal to that of HTLV-1.

The incidence of HTLV-1 infection is high in western Japan, particularly in Kyushu,^{3-5,13)} with some cases also found in Hokkaido,¹⁴⁾ and it has also been reported in equatorial Africa, the Caribbean islands, Colombia, Brazil, south India, Papua New Guinea, northeast Australia, and among indigenous people living at the margins of the Andes plateau in South America, which represent endemic areas of the virus.²⁾ As shown Table 1, with the exception of Japan, HTLV-1-endemic areas are often in developing countries, where blood testing may not be feasible. The PA method uses a freeze-dried product that is prepared when required, and determination is made by diluting the sample and visually observing agglutination images; thus, it can easily be used for preliminary screening and is effective for use in developing countries.

It seems that the PA positivity ratio in breast milk is higher than that in the blood, but the results of the LIA method in the present study were equivalent to those obtained with blood samples. Thus, although the PA method using breast milk is useful as a first screen, follow-up testing is necessary.

The sp-ELISA method uses ELISA-coated synthetic peptides corresponding to the immunodominant regions of

HTLV-1 structural proteins,¹¹⁾ and for this study we used p19 gag protein (100 to 130 aa), gp46 protein (175 to 199 aa), and gp46 (288 to 317 aa). The peptide corresponding to the region of the Env gp46 protein, 175 to 199 aa (gp46-175), allows the discrimination of HTLV-1/2 antibodies.

HTLV-2 has been isolated from individuals other than leukemia patients, and its association with disease remains unclear. It has also been found in locations including Central and South America,⁷⁾ Sweden,¹⁵⁾ Spain,¹⁶⁾ and Brazil,¹⁷⁾ and its roots are believed to lie in the indigenous peoples of areas such as the Florida peninsula of the United States, the Yucatan peninsula of Mexico, and Panama.⁵⁾ In this study, it was interesting that we detected an HTLV-2-positive infant in China.

In countries such as Japan, the United States, France, and the Netherlands, blood donations are routinely checked for a variety of infectious diseases. Recently, China¹⁸⁾ and Korea¹⁹⁾ have reported epidemiological analyses of HTLV-1 and -2 infection, but in other Asian countries, screening has only been established in the last few years, if at all.²⁰⁾ High-performance antibody screening, such as that on donated blood, is required to assess the status of HTLV infection and to prevent further infection. While it is useful to demonstrate the validity of screening breast milk as opposed to blood, it is important not only to facilitate simpler screening but also highly accurate screening. Further studies on the sensitivity, specificity, and reliability of assays using antibodies to HTLV-1 and -2 in breast milk are necessary for large-scale epidemiological surveys of HTLV infection.

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Fig. 1. Positive LIA Reactions of Milk Samples

C, control; PC, positive control; S, milk sample (HTLV-2 (±)).

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