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Maternal Transmisson of HTLV-1 Other than through Breast Milk: Discrepancy between the Polymerase Chain Reaction Positivity of Cord Blood Samples for HTLV-1 and the Subsequent Seropositivity of Individuals

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We used a nested polymerase chain reaction (PCR) to diagnose HTLV-1 carriers. The DNA isolated from the nuclear extract obtained from frozen whole blood was found appropriate for PCR study both qualitatively and quantitatively. The use of freshly frozen whole blood made the field work much easier, and the use of a nuclear extraction procedure allowed DNA isolation in just 4 microcentrifuge tubes. We could not attain sufficient sensitivity to detect a single molecule with single-step PCR, but nested PCR was confirmed to detect a single molecule/reaction. All samples of the seropositive group including 94 blood donors, 66 mothers, and 13 children were positive in the nested PCR, while none of the seronegative group, including 198 blood donors and 285 children, was positive. Although 18/717 (2.5%) cord blood samples obtained from babies born to carrier mothers were PCR-positive, none of 5 formula-fed children tested who had been PCR-positive in the cord blood gave evidence of infection later on. Furthermore, all of 4 seropositive infected children who were formula-fed had been PCR-negative in their cord blood. The results are not consistent with intrauterine infection, but suggest the presence of a perinatal or postnatal infection route other than through breast milk.

Key words: Cord blood — HTLV-1 — Maternal transmission — Milk-borne infection — PCR

Human T-lymphotropic virus type 1 (HTLV-1[7]), the first human retrovirus to have been isolated,[1-3] is closely linked with serious diseases, such as adult T-cell leukemia (ATL)[2,4,5] and tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM).[6,7] The former is almost always fatal within 2 years, and the latter disables the patients with prolonged natural courses. Unfortunately, it is not currently possible to cure the diseases or to prevent HTLV-1 carriers from developing these diseases. The only way available to prevent these diseases is breaking the cycle of HTLV-1 endemy.

These diseases cluster in areas in which HTLV-1 is endemic, including southwestern Japan,[8,9] the Caribbean basin,[10] and Africa.[11] HTLV-1 is heavily endemic in Nagasaki, the westernmost Prefecture (population: 1.5 million) of Japan.[12,13] The prevalence of carriers in Nagasaki is approximately 10% in older persons, and 4% in pregnant women.[13] The annual incidence of ATL in Nagasaki is 60–100 cases, and that of TSP/HAM is computed as less than 10.[14,15]

The main route of HTLV-1 transmission has been established as maternal, specifically via milk.[13,16] The incidence of maternal transmission is 20–30%.[13] In 1987, we started an ATL Prevention Program (APP, Nagasaki) to screen HTLV-1 carriers among pregnant women, and to persuade carrier mothers to refrain from breast feeding. Maternal transmissions were remarkably decreased to the level of 3% in formula-fed children (children raised with artificial milk feeding).[17] To elucidate the route of the remaining transmissions, it became essential to analyze again the babies born to carrier mothers.

Our previous studies on cord blood cells using cell cultures followed by indirect immunofluorescence tests (IF) failed to find evidence of infection in 227 children born to carrier mothers.[13,16] However, the classic method for detection of HTLV-1-infected cells by culture is cumbersome, and dependent on less sensitive assays.[18] Recently, Saito *et al.* reported the presence of HTLV-1-infected cells in cord blood of children born to carrier mothers by using polymerase chain reaction (PCR).[19] The PCR is potentially extremely sensitive, and has been

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[7] Abbreviations: HTLV-1, human T-lymphotropic virus type 1; APP, ATL Prevention Program; EDTA, N,N′-1,2-ethanediylbis[N-(carboxymethyl)glycine]; Et-Br, ethidium bromide; IB, immunoblot; IF, immunofluorescence test; mo, months; PA, gelatin particle agglutination test; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; RNase, ribonuclease; WBC, white blood cells.

used for the diagnosis of HTLV-1 infection.[20] However, the regular single-step PCR has not been sensitive enough to detect every seropositive adult carrier. Therefore, we formulated a PCR system with sufficient sensitivity, and studied blood samples obtained from babies born to carrier mothers to elucidate the putative alternative pathway of HTLV-1 transmission.

MATERIALS AND METHODS

Screening of pregnant women and follow-up of their children Over 90% of pregnant women in the Prefecture (approximately 16,000 births per year) voluntarily join APP, Nagasaki. Commercial laboratories undertake the primary screening for anti-HTLV-1 using a gelatin particle agglutination (PA) kit [Serodia-HTLV-1[21] (Serodia-ATLA[22] until early 1990); FujiRebio, Tokyo]. Approximately a half of them visited for follow-up at the ages of 6, 12, 18, 24 and/or 36 mo at 17 designated pediatric departments in the Prefecture.

Confirmation of serological tests The confirmation tests on all samples were performed at the Department of Bacteriology using PA paired with IF targeting on a 1:4 mixture of HTLV-1-producing MT-2[23] and uninfected CEM[24] cells. Indeterminates were subjected to immunoblots (IB) using MT-2 cell lysate as an antigen. The sera with signals at p19, p24 and p28 bands were scored as positive.[25] The prevalence rate among pregnant women was approximately 4% after confirmation.

Immunological criteria for determination of seropositivity may cause discrepancies among researchers. In our routine screening of approximately 10,000 samples in 1991, we scored 472 confirmed positives (PA- and IF-positives, except 4 cases) and 45 IB-negative intermediates (PA- or IF-positives). The latter included 29 IF-positive but PA-negative cases, and 16 IF-negative but PA-positive cases. There was no IB-positive but PA-negative sample. There were 4 IB-positive samples which were IF-negative but PA-positive.

Cord blood samples obtained from 717 babies born to carrier mothers were supplied by hospitals or clinics distributed in the Prefecture. Blood samples of 298 formula-fed children collected by the designated pediatric units were used in this study.

Control blood samples for PCR Blood donors screened at Red Cross Nagasaki Blood Center served as positive or negative controls, including 94 seropositive carriers with ages of 16–63, and 100 and 98 seronegative donors with ages of 16–20 and 40–64, respectively. Blood samples obtained at the follow-up study from 66 carrier mothers (age range: 23–50) were also used as positive controls. The samples had remained coded until all PCR results were obtained.

Blood sampling Since heparin inhibits PCR,[26] most blood samples were anti-coagulated with EDTA, and freshly frozen in the form of whole blood at −20°C. Some samples were frozen after separation of plasma and cells.

Cells and DNAs We used 2 cultured cell lines: MT-2 containing 8 HTLV-1 genomes per cell,[23] and uninfected CEM.[24] As control DNAs, we used a purified plasmid DNA of an HTLV-1 clone, pMT2,[27] and a salmon sperm DNA (Sigma D1626, St. Louis, MO). The concentration of DNA was estimated by measuring OD260 and the molecular size for the plasmid DNA, and the number of cells for MT-2 DNA. The sources of template DNAs, cloned or MT-2 genomic, or of carrier DNAs, yeast or human, did not influence the sensitivity of the PCR to amplify the *gag* or *pX* region of HTLV-1 DNA.

Preparation of cellular or nuclear extracts We tested the following 3 methods to prepare the template DNA.

(1) Mononuclear cells were isolated from 5 ml of heparinized blood by the Ficoll-Conray method.[28] After 2 washings, the cell pellet was lysed and digested in 2.5 ml of a proteinase K buffer containing 10 m*M* Tris-HCl, pH 8.2, 1 m*M* EDTA, 150 m*M* NaCl, 0.45% Tween 20, and 0.12 mg/ml proteinase K at 55°C for 2 h or at 37°C for 16 h.[29]

(2) To obtain cell extracts of white blood cells (WBC), frozen whole blood (1 ml) was thawed, and hemolyzed with 10 ml of 1% ammonium acetate buffer. After 2 washings, the cell pellet was lysed and digested as described above. The sensitivity of PCR remained low even after the digestion of excess cellular RNA by 0.1 μg/ml RNase I at 37°C for 30 min (see "Results").

(3) To obtain nuclear extracts of WBC, a thawed 1-ml sample was centrifuged at 13,000*g* for 60 s, and 0.5 ml of the supernatant was replaced with a lysis buffer containing 0.32 *M* sucrose, 10 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, and 1% Triton X-100.[30] After centrifugation at 13,000*g* for 20 s, and 2 washings with 1 ml each of the lysis buffer, the nuclear pellet was digested in 0.5 ml of the proteinase K buffer.

DNA isolation DNA was isolated from each lysate by successive treatments with phenol, phenol/CIA and CIA. The isolated DNA was precipitated with 2 volumes of ethanol, dissolved in 10 m*M* Tris-HCl, pH 8.3, and 1 m*M* EDTA (TE buffer), and freeze-stocked at the concentration of 100 μg/ml in the TE buffer (1 OD260=50 μg/ml).[19]

Primers for PCR and probes Primer and probe oligonucleotides were synthesized by the phosphoramidite method on a synthesizer (Applied Biosystems 380B, Foster City, CA) (Table I). The base positions and sequences of the primers and probes are based on the published sequences of lambda ATK-1.[31] As a representative of genomic DNA, we used a primer pair

Table I. Base Positions and Sequences[a] of Primer and Probe Oligonucleotides in the HTLV-1 Proviral Genome

Target region	Primer or probe[b]		Base (5′-3′) position	Base (5′-3′) sequence
U3	Primer	+	99–123	ACTAAGGCTCTGACGTCTCCCCCCG
	Probe	+	188–207	TCAGACCTCCGGGAAGCCAC
	Primer	−	348–324	CTGAACTGTCTCCACGCTTTTATAG
U5	Primer	+	601–625	GTTCTGCGCCGTTACAGATCGAAAG
	Probe	+	641–660	TTTCATTCACGACTGACTGC
	Primer	−	755–730	TGTGTACTAAATTTCTCTCCTGAGAG
gag	Primer	+	1259–1279	TTAAGCAAGAAGTCTCCCAAG
	Primer	+	1301–1320	TTATGCAGACCATCCGGCTT
	Probe	−	1380–1361	GCAAAGGTACTGCAGGAGGT
	Primer	−	1440–1421	TTCGGCCTCTGATATAAGGC
	Primer	−	1420–1401	TATCTAGCTGCTGGTGATGG
pol	Primer	+	3082–3105	CCCTCCCATGAGGACCTACTACTA
	Probe	−	3147–3124	CAACCCATGGGAGATTAGGGAAGC
	Primer	−	3219–3196	ATTGGGTGAAATTATCTGCCCTAG
env	Primer	+	6116–6139	GCGGTACCGGTGGCGGTCTGGCTT
	Probe	−	6196–6173	GGACATGGAGCCGGTAATCCCGCC
	Primer	−	6264–6241	ACTATTGCTTGAGTTAACTGGGAA
pX	Primer	+	7302–7326	CCCACTTCCCAGGGTTTGGACAGAG
	Primer	+	7341–7360	ACCCAGTCTACGTGTTTGGA
	Probe	−	7420–7401	ATGTAGGCGGGCCGAACATA
	Primer	−	7504–7481	CTGTAGAGCTGAGCCGATAACGCG
	Primer	−	7460–7441	TGATCTGATGCTCTGGACAG
PDGF-A	Primer	+	711–730	TCCGCAAATATGCAGAATTA
	Primer	−	1159–1142	AGAACATGGGCGAGGTAT

a) The base positions and sequences of the primers and probes are based on the published sequences of lambda ATK-1.[31]
b) "+" designates the genomic strand and "−" complementary to the genomic strand.

(nt: 711–730, 1159–1142) of the human *PDGF-A*-chain gene (Table I).[32]

PCR Unless otherwise specified, we used 1 μg of target DNA in a 50-μl cocktail containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* each of dATP, dCTP, dGTP and TTP, 1 μM each of primers, and 1.25 units of Taq DNA polymerase (AmpliTaq: Perkin Elmer Cetus, Norwalk, CT).[33] Programmable incubators (PC-500, ASTEC, Fukuoka; TSR-300, Iwaki, Tokyo) were operated for 30 cycles: each involved denaturation at 94°C for 2 min (3 min on the first cycle), annealing at 60°C for 2 min, and extension at 72°C for 2 min. Because the temperature shift curves in the proximity of a setting are dependent on the make of incubators, we set the timer to start at 0.5°C proximity to the setting to equalize the conditions for different incubators. Since an increasing amount of carrier DNA made the PCR less sensitive, we fixed the amount of input DNA at 1 μg/reaction.

For the nested PCR,[34,35] a 5-μl aliquot of the PCR product for 30 cycles using an outer pair of *gag* or *pX* primers was amplified further for 10–30 cycles using the inner pair of primers. Thirty cycles of PCR on the second run was optimal. Although 1/10 of the primers remaining in the first run will be transferred into the second run, we confirmed that the optimal concentration of the primer in the first run is 1 μM.

Analysis of the amplified products by PCR A 10-μl aliquot of the reaction mixture was electrophoresed in Tris/borate/EDTA buffer, pH 8.3,[29] on a composite gel containing 2.0% NuSieve/1.0% SeaKem ME agarose (FMC, Rockland, ME). DNA was transferred onto a Zeta Probe nylon membrane (BioRad Lab., Richmond, CA), and hybridized at 37°C for 18 h with the oligonucleotide probe labeled by ^{32}P-γ-dATP at the 5′ end, in a buffer containing 5×SSC, 7% SDS, 20 m*M* NaH_2PO_4, pH 7.0, 10×Denhardt's solution, 10% dextran sulfate, and 20 μg/ml salmon sperm DNA. The membrane

was then washed to a final stringency of 0.5×SSC at 42°C for 20 min, followed by exposure to Konica X-ray film (Konica, Tokyo) with double layers of Kodak X-Omatic regular intensifying screen (Kodak, Rochester, NY) at −80°C.

RESULTS

Frozen whole blood as the DNA source for PCR Because most of the field clinics are not equipped with a centrifuge, and transport of the sample may take several days, we chose to obtain whole blood freshly frozen. The paired WBC counts on fresh and freeze-thawed samples were the same within the limits of statistical error (data not shown). Frozen blood without anticoagulants was not suitable for quantitative recovery of WBC. Although the total amount of DNA isolated from 1 ml of blood varied in the range of 10–50 μg, the amounts obtained by the 3 methods of preparation (see "Materials and Methods") were the same for a given individual within the limit of statistical error (data not shown). These data indicated that DNA of WBCs can be quantitatively obtained from frozen whole blood.

Eeach DNA sample was diluted in serial 2-fold dilutions, and subjected to 30 cycles of PCR to multiply the *PDGF-A*-chain gene. The minimum template DNA required for a positive signal was 2.5 ng, or 400 molecules, for both DNAs from freshly prepared mononuclear cells (method 1; Fig. 1A) and freeze-thawed nuclear extracts (method 3; Fig. 1C), in contrast to 10 ng for the DNA from freeze-thawed cellular extracts even after RNase digestion (method 2; Fig. 1B). Thus, DNA isolated from freeze-thawed nuclear extracts (method 3) was found suitable for PCR, both qualitatively and quantitatively.

The maximum sensitivity of the single-step PCR Using optimal conditions derived from preliminary testing of PCR conditions (see "Materials and Methods"), the sensitivities of the single-step PCR were compared at 50 cycles of amplification using *U5*, *gag*, *pol*, *env* or *pX* primer sets. By ethidium bromide (Et-Br) staining, the detection levels of the template DNA were 1,000, 100, 1,000, 100,000, and 100 molecules/reaction, respectively. Hybridization with the respective ^{32}P-probes increased the sensitivity 10- to 100-fold in most target regions (Fig. 2, Table II). However, the sensitivity never reached

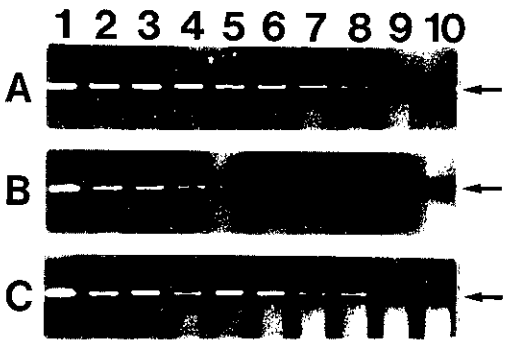

Fig. 1. Quantitative recovery of DNA from a frozen whole blood sample. DNAs isolated from WBC of a human individual by 3 methods (see text for details) were subjected to single-step PCR for 30 cycles with primers targeted on *PDGF-A*-chain gene. Et-Br staining. (A) Mononuclear cell DNA isolated from the fresh blood, and (B) whole cell DNA and (C) nuclear DNA isolated from the frozen blood. Amounts of the input human DNA in lanes: 1, 1,000; 2,100; 3, 50; 4, 25; 5, 12.5; 6, 10; 7, 5; 8, 2.5; 9, 1.25, and 10, 1 ng/reaction. Total DNA was adjusted to 1 μg/reaction by adding salmon sperm DNA.

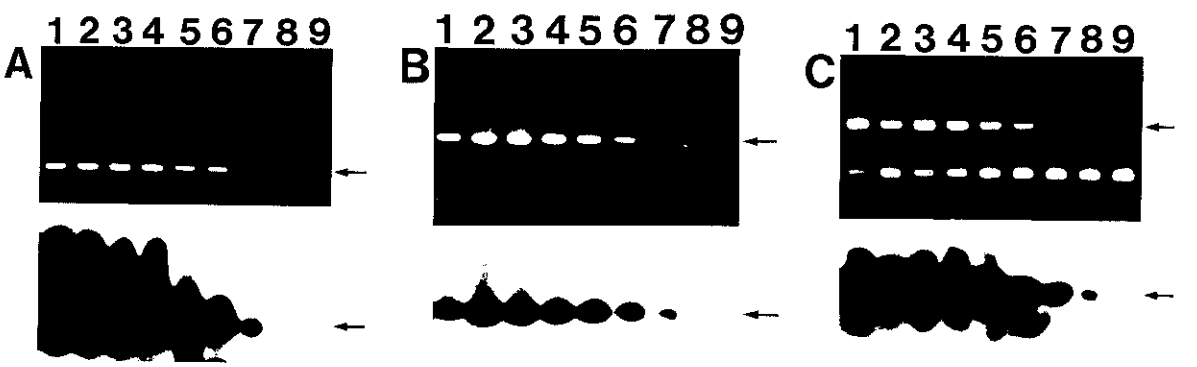

Fig. 2. Sensitivity of the single-step PCRs at 50 cycles using *U5* (A), *gag* (B) and *pX* (C) primers. Upper panel of each figure, Et-Br staining; Lower panel, hybridization with the respective ^{32}P-probes. The amounts of template DNA in lanes: 1, 10^8; 2, 10^7; 3, 10^6; 4, 10^5; 5, 10^4; 6, 10^3; 7, 10^2; 8, 10^1; and 9, 10^0 molecules/reaction.

the level of 1 molecule/reaction even after the hybridization. The *U3* primer set failed to give a specific signal.

Sensitivity of the nested PCR The nested PCR can detect a single molecule/reaction.[35] To confirm the sensitivity of our system, ten aliquots, which contained pMT2 and salmon sperm DNA in concentrations of 1 molecule and 1 μg/reaction, respectively, were subjected to nested PCRs using the *gag* or *pX* primer pairs (Fig. 3). The positive rates, 5/10 and 7/10 in *gag* and *pX* regions, respectively, agreed with the expected frequency of positives computed by Poisson distribution, $P(\geq 1)=0.63$. Thus, our nested PCR has been confirmed to detect a single template molecule/reaction. To maintain the sensitivity in each batch of nested PCRs, we used 2 tubes of positive controls, each of which contained the target DNA at the concentration of 2 molecules/reaction. If both of the positive controls in the batch failed to give a positive signal, we discarded the results.

Consistency of the nested PCR and serology in adults Although several reports using single-step PCR failed to pick up some seropositive individuals,[20] the nested PCR was claimed to pick up every seropositive carrier.[35] To compare the sensitivity of our nested PCR with that of serology, we tested 160 seropositive blood samples including 94 blood donors, and 66 mothers. Every one was positive in the nested PCR using both *gag* and *pX* target regions (Table III). In contrast, none of 198 seronegative donors, consisting of 100 in the young group and 98 in the older group, was PCR-positive in either *gag* or *pX* region. These findings suggested that the results of nested PCR and serology in adults are consistent with each other.

Consistency of serology and the nested PCR in formula-fed children We reported that refraining from breast feeding by carrier mothers decreased the maternal infection significantly on the basis of serology.[17] However, there remains a possibility that children may not seroconvert even after infection. We tested 376 samples from 298 formula-fed children born to carrier mothers at the ages of 1–8 with the nested PCR as well as serology (Table IV). None of 285 seronegative children was PCR-positive in either *gag* or *pX* region. The same 13 children were picked up both by serology and the nested PCR,

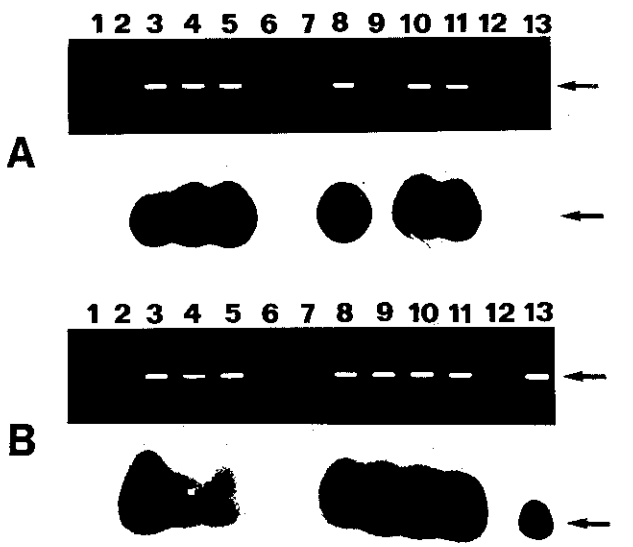

Fig. 3. A single template molecule detected by the nested PCR directed to *gag* (A) or *pX* (B) gene. The amount of template DNA in lanes: 1, H_2O; 2, carrier DNA only; 3, 10; and 4–13, average 1 molecule/reaction.

Table II. Sensitivities of the Single-step PCR of HTLV-1 with 50 Cycles of Amplification

Target region	Minimum no. molecules per tube	
	Et-Br staining	^{32}P hybridization
U5	1,000	100
gag	100	100
pol	1,000	100
env	100,000	1,000
pX	100	10
U3	non-specific amplification	

Table III. Consistency of Serology and the Nested PCR in Adults

Serological status	Group	Age (yr)	No. tested	No. positive		% +ve
				gag	*pX*	
Positive	Blood donors	16–63	94	94	94	100
	Mothers	23–50	66	66	66	100
	subtotal		160	160	160	100
Negative	Blood donors	16–20	100	0	0	0
	Blood donors	40–64	98	0	0	0
	subtotal		198	0	0	0

Table IV. Consistency of Serology and the Nested PCR in Children Born to Carrier Mothers

Age (yr)	No. tested	Ab (+)[a] PCR (+)[b]	Ab (+) PCR (−)	Ab (−) PCR (+)	Ab (−) PCR (−)	% +ve
1.0	103	5	0	0	98	4.9
1.5	88	1	0	0	87	1.1
2.0	56	3	0	0	53	5.4
≥3.0	51	4	0	0	47	7.8
Total	298	13	0	0	285	4.3

a) Anti-HTLV-1 screening was performed with Serodia-HTLV-I. Positive samples were subjected to confirmation tests including IF and IBs.
b) Results of the nested PCRs using *gag* and *pX* regions as targets agreed with each other on each sample.

Table V. Discrepancy of HTLV-1 Proviral DNA in the Cord Blood Cells vs. Carrier Status of Children Born to Carrier Mothers

Case no.	Cord blood		Age tested			
			6 mo		≥12 mo	
	Ab	PCR	Ab	PCR	Ab	PCR
1	+	+	−	nt[a]	−	−
2	+	+	−	nt	−	−
3	+	+	nt	nt	−	−
4	+	+	+	−	−	nt
5	+	+	+	−	nt	nt
6	+	−	−	nt	+	+
7	+	−	nt	nt	+	+
8	+	−	+	nt	+	nt
9	+	−	−	nt	+	nt

a) nt: not tested.

and every positive child was both *gag*- and *pX*-positive. Thus, our nested PCR picked up every seropositive child, but not a single case out of the seronegative children.

Cord blood of children born to carrier mothers The presence of HTLV-1-infected cells in cord blood of children born to carrier mothers has been used as a marker of intrauterine infection. The data obtained by the less sensitive culture technique have been controversial, either negative[17, 36] or positive.[37, 38] We tested 717 cord blood samples of children born to carrier mothers with our nested PCR, and found 18 positive cases (2.5%): every positive case was positive for both *gag* and *pX* regions. Further, each sample was seropositive for the maternal antibody.

The presence of HTLV-1 in the cord blood is not directly correlated with the intrauterine infection To determine whether a baby with PCR-positive cord blood is infected, the PCR data on cord blood samples and on the follow-up blood samples were compared. Among 18 formula-fed cases whose cord blood were positive by the nested PCR, blood samples from follow-up studies were available in 5 cases (Table V). Of these 5 cases, 3 cases (Cases 1–3) were both PCR- and sero-negative at the ages of 12 or 18 mo. Case 4 was seropositive but PCR-negative at 6 mo, and seronegative at 12 mo. Case 5 was seropositive but PCR-negative at 6 mo. Thus, we could not find any carrier child who had been PCR-positive in the cord blood. Three children were seronegative at 6 months without DNA samples, and 10 other children did not show for the follow-up study.

On the other hand, all of 4 seropositive children with the ages of 12 mo or over, who had been formula-fed and

whose cord blood samples were available for the study (Cases 6–9), were PCR-negative in the cord blood. Thus, none of the cases with available blood samples both at birth and at the ages of 12 mo or over gave consistent results by PCR and/or serology, unless the case had been PCR-negative in the cord blood and remained seronegative. The results are not consistent with the conjecture that most of the infections in formula-fed babies are via the intrauterine route, but suggest the presence of a perinatal or postnatal infection route other than through breast milk.

DISCUSSION

Refraining from the breast feeding by carrier mothers successfully reduced the maternal transmission of HTLV-1 to the level of 3% as determined by serology.[17] The residual level of infection means that we must consider an alternative pathway of HTLV-1 transmission other than through breast milk, and the possibility of delayed seroconversion which should leave a sizable number of seronegative carriers. In our experience, most seroconversions occur within the first 12 mo, though there are a few children who seroconvert even after 2 yr (S. Hino, unpublished data). The seropositive rate remained constant from 12 mo through 18 yr.[39] If delayed seroconversion beyond the age of 3 is common, there should be a sizable fraction of seronegative carriers. Introduction of a potentially more sensitive method, PCR, has made it possible to analyze these questions with a new method. We have optimized the conditions of PCR, and used the technique to examine the above possibilities.

Before we started PCR, we sought to simplify the method of sample transport and DNA isolation. We decided to use freshly frozen whole blood to overcome the lack of equipment in the field. We found that DNA prepared from the nuclear fraction of thawed whole blood was adequate to prepare template DNAs for PCR both qualitatively and quantitatively. The nuclear extraction efficiently reduced the contamination with cellular RNA, which apparently inhibits the PCR reaction. Furthermore, the nuclear extraction method was the least time-consuming among the 3 methods tested, and could conveniently be finished using only 4 1.5-ml microcentrifuge tubes. This enabled us easily to investigate many samples collected in the field.

Most reports using single-step PCR failed to detect every seropositive carrier. Potentially, PCR can detect a single target molecule/reaction. However, reports specifically attesting its sensitivity have been rare.[35] Although the population of infected cells in healthy HTLV-1 carriers has been estimated to be of the order of 10^{-3},[40] that in HIV carriers can be well below 10^{-3}.[41] If a population of 10^{-4} of CD4+ cells amounting to 10% of WBC is infected, 1 μg of DNA in a PCR tube should contain only 2 HTLV-1 proviral molecules. Thus, it seems important to ensure sufficient sensitivity of PCR to detect 1 molecule/reaction. We tried maximize the sensitivity of the single-step PCR. However, we could not find conditions giving sufficiently high sensitivity.

The positive rates of nested PCR with limiting dilution were consistent with the expected rates computed by Poisson distribution, and confirmed that our nested PCR was detecting a single template molecule/reaction for both *gag* and *pX* regions. We believe that simultaneous tests on at least 2 distant regions on the genomic map are important to ensure the presence of whole viral genome. All the samples we collected gave consistent results on the nested PCR with *gag* and *pX* regions. Sensitivity to detect 1 target molecule in 2×10^5 cells seems to be acceptable for the diagnosis of HTLV-1 carriers, since every seropositive case of 94 donors and 66 mothers was PCR-positive. These results in turn imply that the fraction of HTLV-1-infected cells in healthy carriers is usually over 0.05% of CD4+ cells according to the theorem of DeMoivre-Laplace, consistent with the previous report by Watanabe *et al.*[40]

Various epidemiological surveys in the endemic areas have shown a discrepancy of seroprevalence between young and older persons: for example, the values in Nagasaki are approximately 1 and 10 %, respectively.[13] The discrepancy has been explained by two alternative possibilities: a cohort effect, or delayed seroconversion. Although the explanation based on the cohort effect is more widely accepted,[13, 39, 42, 43] several reports have stressed the presence of seronegative carriers, especially at young ages,[44] based on the concept of delayed seroconversion.

If the discrepancy between young and older persons is based on delayed seroconversion, approximately 9% of young adults in Nagasaki should have been seronegative carriers. Furthermore, if 8% of children born to carrier mothers are destined to be carriers as a consequence of intrauterine infections,[19] approximately 20 children from 285 babies born to carrier mothers should have been seronegative carriers. We could not find anyone who was PCR-positive but seronegative among 100 seronegative young donors resident in the highly endemic Nagasaki area, and 285 seronegative formula-fed children born to carrier mothers. On the other hand, if there are frequent horizontal infections caused by seropositive adults, seronegative carriers should be more common in older persons. None of 98 seronegative blood donors in the age range of 40–63 was PCR-positive. Thus, the data are inconsistent with the idea of delayed seroconversion. Even though we can not rule out the possibility of seronegative carriers completely, the data strongly suggest

that seronegative carriers are rare, if any, provided the infected cell population of the seronegative carriers is below 0.005% of CD4+ cells in contrast to the estimated population in the seropositive carriers of over 0.05%.

In 1983, Komuro *et al.* reported an HTLV-1-positive culture of cord blood without showing the total number of samples tested,[37] and Satow *et al.* recently obtained a positive rate of 2/40.[38] However, neither Hino *et al.*[13,17] nor Ando *et al.*[36] could find any positive culture in 227 and 35 cord blood samples, respectively. We feel that the discrepancy is based on the immunological criteria employed. Our definition of a positive culture is that the culture contains cluster(s) of IF-positive cells to both a human reference carrier's serum and a monoclonal antibody against gp21E,[45] but not to a negative reference human serum.[17] This is based on our experience that most IF-positive cells grow in clusters, and that certain cord blood cultures of babies born to non-carrier mothers reacted non-specifically with anti-HTLV-1 serum including monoclonal antibodies.

Saito *et al.*[19] reported that 3/40 cord blood samples were positive with a single-step PCR having sufficient sensitivity to detect several target molecules/reaction. The same 3 formula-fed babies were culture-negative on the cord blood, but PCR-positive at the age of 3 to 10 mo. They explained the discrepancy of results by culture and PCR in terms of different sensitivity of the assays, and suggested intrauterine infection as the alternative route for formula-fed children. However, they did not include the serological status of these children, the common marker to diagnose HTLV-1 carriers. To evaluate their results, we applied our nested PCR to blood samples obtained from children born to carrier mothers.

In our study, we detected HTLV-1 proviral DNA from 2.5% (18/717) of the cord blood samples. The discrepancy between HTLV-1 detection in the cord blood by culture and the nested PCR is best explained by the sensitivity difference of the assys; of the order of 10^{-3} infected cells/WBC vs. 10^{-5}, respectively. The presence of HTLV-1 in the cord blood may suggest intrauterine infection. However, none of 5 cases with available blood samples in the follow-up study was PCR-positive at ages of 6 mo or over. The antibodies at 6 mo of Cases 4 and 5 in Table V were in all probability maternal, since 38/185 (20%) of children born to carrier mothers are still seropositive (S. Hino, unpublished). Briefly, we could not find a baby who had been PCR-positive in the cord blood and showed any sign of infection.

Moreover, we tested 4 formula-fed children known to be carriers by serology and PCR at 12 mo of age, for whom cord blood samples were available. None of them had PCR-positive cord blood. None of these infected children had a history of abnormal labor course, such as delayed labor, perinatal bleeding, etc. Although the number of cases tested is small, these results strongly argue against the concept that the presence of HTLV-1 in the cord blood is the hallmark of intrauterine infection.

There are several possibilities which can explain the PCR-positive cord blood of unifected babies. Laboratory contamination is possible. However, in our case every sample was coded until all the data were finalized, and we are not experiencing obvious problems due to laboratory contamination. The second is contamination with maternal blood, in the office or in the laboratory. This is almost impossible to negate because of the extremely high sensitivity of the PCR. More frequent blood sampling from babies is desirable, but it has not been feasible in our study. The third is that we may be detecting maternal cells without infectivity. Loss of infectivity by cell death is hard to accept, since even irradiated cells are infectious in cocultivation.[46] Since passive transfer of anti-HTLV-1 effectively neutralizes infection in rabbits,[47] maternal infected cells passed by intrauterine transfer may be efficiently neutralized by simultaneously transferred maternal antibodies.

If most formula-fed babies who had HTLV-1 in the cord blood can remain free from infection by HTLV-1, perinatal or postnatal transmission will be a better candidate for the pathway of infection, rather than intrauterine transmission. Further studies are needed.

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