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Patterns of Anti-HIV IgG3, IgA and p24Ag in Perinatally HIV-1 Infected Infants

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SUMMARY **The antibody patterns of HIV-1 IgG3, IgG and IgA and of HIV-1 p24 antigen were investigated in Thai infants born to mothers infected with HIV-1. In the 17 HIV-1 infected infants, anti-HIV antibodies were detected continuously over a period of 15-18 months and a high level of specific IgG3 subclass was observed. Anti-HIV IgA could be detected at 6 months of age whereas p24Ag was detected at 2 months. In 79 uninfected infants, maternal anti-HIV IgG gradually decreased over 9 months whilst specific IgG3 decayed rapidly during the first 6 months. Both p24Ag and anti-HIV IgA were not found in these uninfected infants. Thus, the disappearance of anti-IgG3 subclass antibodies within 6 months can predict whether infants are uninfected whereas the persistence of anti-HIV IgG and IgG3 subclass antibodies, the production of anti-HIV IgA antibody and the presence of p24Ag appear as an adjunct to the diagnosis of HIV vertical transmission. The necessary assays are relatively simple and could be performed individually.**

In addition to sexual contact or infection through HIV contaminated blood, mother-to-child transmission of HIV can also occur.[1] A number of HIV assays for early detection of infection in infants born to HIV-1 positive mothers have been developed.[2] In general, anti-HIV antibody can be detected within 20-25 days after infection whereas the presence of serum p24Ag can be found 5 days earlier than specific antibody.[3,4] However, serological diagnosis by standard anti-HIV testing in infants is difficult due to the problem of maternal specific IgG crossing the placenta to their offspring, which may persist for 15-18 months.[5,6] The detection of specific IgA and IgM, which do not cross the placenta, has been suggested as a serological diagnostic tool in pediatric infection.[7,8] Analysis of HIV-1 specific IgG subclasses showed that IgG3 can be a marker to support a diagnosis of infection in children born to HIV infected mothers due to a much shorter half life of about one-third of the other IgG subclasses.[9,10] Detection of core (p24) antigen in serum is an alternative approach for the diagnosis of vertical transmission. Its sensitivity can be improved by performing the dissociation of the p24 antigen-antibody immune complex either by acid hydrolysis or heat treatment[11,12] Other approaches are ELISPOT and IVAP (*in vitro* antibody production) assays, which detect HIV-1 specific antibody-producing B-cells based on the secretion of HIV-1 specific antibodies *in vitro*.[13] However, these assays are unreliable in infants under 3 months of age due to the immaturity of B-cells in the neonate and the potential carry-over of maternal antibodies which attach to the B-cells of the infant. Polymerase chain reaction (PCR) and viral culture from peripheral blood mononuclear cells (PBMC) are recommended for the early diagnosis in neonatal HIV-1 infection.[14,15] The detection of plasma HIV-1 RNA and proviral HIV DNA in infected cells are also used as early diagnosis of vertically acquired transmission.[2,16] However, these molecular techniques are expensive and need professional experience to perform, and virus culture is laborious and lengthy. In addition, a meta-analytic evaluation has shown that the positive predictive value of PCR in neonates was 55.8% compared to 83.2% in older infants, in particular for those with a low risk of perinatal transmission.[17] We therefore decided to evaluate the serological patterns of HIV-1 parameters, including anti-HIV IgG, IgG3, IgA and p24Ag, in

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HIV IgG, IgG3, IgA and p24Ag, in sequential sera/plasma of infants born to HIV-1 infected mothers in Thailand.

MATERIALS AND METHODS

Subjects

These cohorts were a substudy of the Collaborative Perinatal HIV Transmission Study from Bamrasnaradura Infectious Diseases Hospital and Siriraj Hospital during the period 1994-1997. Blood samples were collected from 96 HIV-1 infected mothers at one visit, and from their infants on different occasions for up to 15-18 months (at 2, 4, 6, 9, 12 and 15 or 18 months). HIV infection in the neonate was diagnosed if HIV-1 DNA in PBMC was positive by PCR on at least 2 occasions, and/or HIV-specific antibody persisted until 15-18 months.

Anti-HIV IgG detection

Anti-HIV antibody, mainly IgG, was determined using a one step assay from Organon (Vironostika HIV Uni-Form II *plus* O, Netherlands) based on a double sandwich principle. Briefly, diluted serum (100 µl, 1:20) was simultaneously incubated with HIV-1/2 coated onto a microtiter strip and HIV-1/2 conjugated with an enzyme peroxidase (POD) bead at 37° C for 1 hour. After 6 washes, a substrate was added and the reaction was stopped with 2N H_2SO_4 after 10 minutes. Color reaction was measured at 450/630 nm. The criteria for HIV-1 positivity were determined as recommended in the kit. The average cut-off level for anti-HIV antibodies using the kit was found to be 0.102 optical density (OD) units.

Anti-HIV IgG3 subclass detection

Anti-HIV IgG3 determination was done by modifying the Organon kit based on an indirect ELISA principle. Briefly, diluted serum (100 µl, 1:10) was added onto a HIV1/2 antigen strip at room temperature and incubated overnight instead of only 1 hour, due to the relatively small amount of IgG3 in blood. After 4 washes, bound antibody was determined by anti-IgG3 antibody conjugated with biotin (100 µl, 1:100, Zymed) at 37°C for 2 hours. After the final washes, the strip was incubated with streptavidin labeled with enzyme alkaline phosphatase (100 µl, 1:100, Sigma) at 37°C for another 2 hours. The color was developed by a substrate (p-nitrophenyl phosphate, 100 µl, 5 mg tablet in 10 ml ethanolamine, Sigma) for 20 minutes. The reaction was stopped with 3M NaOH and measured at 405/630 nm. Subsequently, anti-HIV IgG3 antibody was determined as positive if the serum gave an optical density greater than the cut-off value ([mean of a panel HIV-1 negative normal controls + 3SD] x 2) based on 22 negative sera. The average cut-off for anti-HIV IgG3 was found to be 0.50 OD units.

Anti-HIV IgA detection

Anti-HIV-IgA was also investigated in parallel to IgG3, using the same Organon ELISA kit reagents. This assay was developed based on the capture IgA principle. Briefly, anti-IgA (100 µl, 10 µg/ml, Dakopatt in 20 mM carbonate buffer, pH 9.6) was coated on a microtiter strip (F16 maxisorb, Nunc) at room temperature overnight. After 3 washes, the strip was blocked with blocking buffer (100 µl; washing solution containing 5% skimmed milk powder) at 37°C for 1 hour. After a washing step, a diluted serum (100 µl, 1:10) was added and incubated at 37°C for 2 hours. After this step, the procedure was followed as recommended by the kit reagents. Following 6 washes, bound IgA was determined by an HIV-1/HIV-2 antigen bead conjugated with POD enzyme at 37°C for 2 hours. After the washing step, the colour was developed by adding a substrate and the reaction was stopped with 2 N H_2SO_4. Then, the color intensity was measured at 450/630 nm. Anti-HIV IgA was determined as described above for anti-HIV IgG3. The average value for the anti-HIV IgA cut-off was found to be 0.202 OD units.

p24Ag detection

The plasma sample was pretreated with heat under 7 mM sodium dodecyl sulphate (SDS) at 96°C for 4 minutes in order to dissociate the immune complex. Subsequently, free p24Ag was determined using a commercial kit (Organon, Vironostika, Netherland) based on an antibody double sandwich assay.

RESULTS

Because of the widespread use of anti-retroviral therapy, the rate of vertical transmission has decreased considerably. Thus, only 17 out of 96 infants born to HIV-1 mothers were diagnosed with perinatal infection in this study. A total of 42 blood samples were obtained from HIV-1 infected infants at 2, 4, 6, 9, 12 and 15 months (n = 12, 8, 7, 3, 11 and 1 samples, respectively). Of the remaining 79 uninfected subjects, 387 samples were obtained at 2, 4, 6, 9 12, 15 and 18 months (n = 76, 64, 56, 55, 71, 55 and 9 samples, respectively).

In seroreverted or HIV-1 uninfected infants, the rate of maternal specific IgG antibodies showed a gradual decline over the first 9 months (from 99% to 91%), and then a rapid decrease between 12-18 months (27%, 2% and 0% at 12, 15 and 18 months, respectively) as shown in Table 1. A rapid decrease

Table 1 HIV parameters in infants born to mothers infected with HIV-1

	Results at month (%)						
	M2	**M4**	**M6**	**M9**	**M12**	**M15**	**M18**
HIV-1 uninfected children (n = 79)							
Anti-HIV	99% (75/76)	94% (60/64)	93% (52/56)	91% (50/55)	27% (33/71)	2% (1/55)	0% (0/9)
Anti-HIV IgG3	66% (50/76)	59% (38/64)	36% (20/56)	27% (15/55)	19% (13/69)	9% (5/55)	0% (0/9)
Anti-HIV IgA	0% (0/76)	0% (0/64)	2%* (1/56)	2% (1/55)	0% (0/71)	0% (0/55)	0% (0/9)
HIV-1 infected children (n = 17)							
HIV Ag	57% (4/7)	57% (4/7)	71% (5/7)	67% (2/3)	80% (4/5)		
Anti-HIV	100% (12/12)	100% (8/8)	100% (7/7)	100% (3/3)	100% (11/11)	100% (1/1)	
Anti-HIV IgG3	92% (11/12)	71% (5/7)	86% (6/7)	100% (3/3)	82% (9/11)	100% (1/1)	
Anti-HIV IgA	8% (1/12)	13% (1/8)	42% (3/7)	33% (1/3)	46% (5/11)	100 (1/1)	

*Weakly reactive at M6 and M9 but disappeared in sequential blood samples

Table 2 Predictive values, sensitivity and specificity of anti-HIV-1 IgG3 and/or IgG assay(s) in HIV-1 vertical transmission*

	Results at month (%)						
	M2	**M4**	**M6**	**M9**	**M12**	**M15***	**M18***
Anti-IgG assay							
Positive predictive value	16	12	13	6	33	100	
Negative predictive value	100	100	100	100	100	100	100
Sensitivity	100	100	100	100	100		
Specificity	1	6	7	9	54	98	100
Anti-IgG3 assay							
Positive predictive value	18	12	23	17	41	17	
Negative predictive value	96	93	97	100	97	100	100
Sensitivity	92	71	86	100	82		
Specificity	34	41	64	73	81	91	100
Both anti-IgG and IgG3 assays							
Positive predictive value	14	10	14	8	26	22	
Negative predictive value	96	94	98	100	98	100	100
Sensitivity	96	86	93	100	91		
Specificity	16	22	34	40	62	94	100

*Vertically HIV infected subject after 12 months was only 1 sample

in specific IgG3 subclass antibodies in seroreverted infants was observed. The rate detection of IgG3 subclass antibodies was 66%, 59%, 36%, 27%, 19%, 9% and 0% (Table 1), with a median antibody level of OD = 0.96, 0.68, 0.53, 0.46, 0.38 0.34 and 0.32 (cut-off = 0.5, Fig. 1), at 2, 4, 6, 9, 12, 15 and 18 months, respectively. Thus, IgG3 gave a high negative predictive value (93-100%) as early as the first 2 months with a specificity of 64% after the first 6 months (Table 2). Whereas in HIV-1 infected infants, the presence of anti-HIV antibodies persisted over the whole 15-18 months (Table 1). This small number of infected infants showed specific IgG3 subclass antibodies at a rate of 80-100% with a high signal (OD $\geq$ 1) as shown in Fig. 1. In addition, specific IgG3 subclass antibodies were also analyzed in the related

infected mothers. No difference in the level of anti-HIV IgG3 was observed in non-transmitting and transmitting mothers (mean OD = 0.89 *vs* 0.81).

The production of HIV-1 IgA in infected infants was age dependent (8%, 13%, 42%, 33% 46% and 100% at 2, 4, 6, 9, 12 and 15 months, respectively) as shown in Table 1 and Fig. 2. Antibody to HIV IgA was absent in seroreverted infants, except in one case that showed a low signal reactivity between 6-9 months, which disappeared again later on. Like that of specific IgG3, the level of HIV-1 specific IgA was similar in non-transmitting and transmitting mothers (OD = 0.53 *vs* 0.66).

Owing to the large volume of serum requirement for p24Ag detection, only 7 samples from infected infants were available in this assay. The presence of p24Ag was detected as early as the first 2 months with a rate of 57%, 57%, 71%, 71% and 80% at 2, 4, 6, 9 and 12 month, respectively (Table 1).

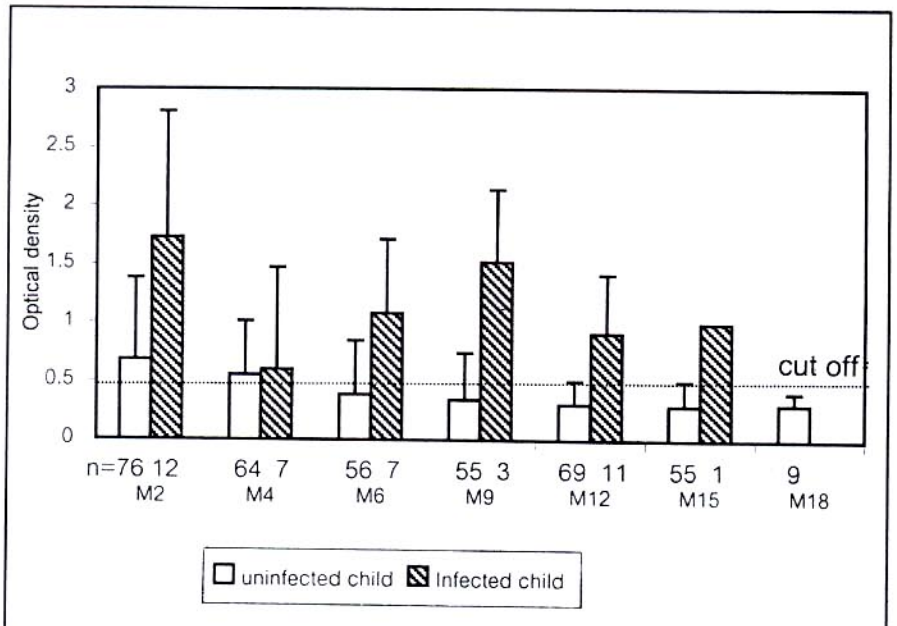

Fig. 1 Pattern of median anti-HIV IgG3 subclass antibodies in infants born to mothers infected with HIV-1.

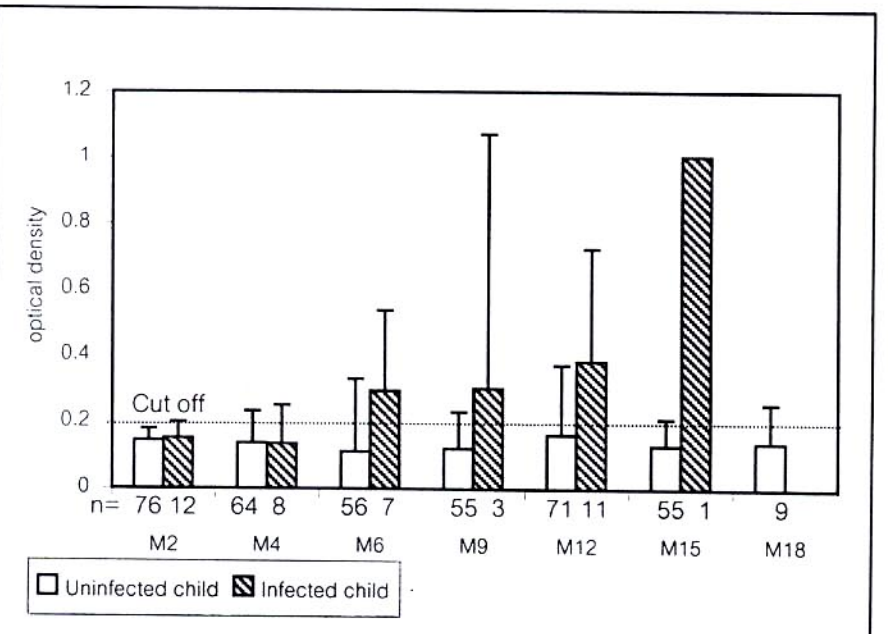

Fig. 2 Pattern of median anti-HIV IgA antibody in infants born to mothers infected with HIV-1.

DISCUSSION

The rate of mother to child transmission of HIV-1 has markedly decreased after making anti-retroviral therapy accessible for a large part of the population in Thailand.[18-19] Early diagnosis of infected newborns is required for their optimal care. In this study, we attempted to develop a simple diagnostic strategy to be used as an adjunct for assessment of perinatal infection, which can be performed in most laboratories. Besides HIV p24Ag, detection of anti-HIV IgG3 subclass antibodies and specific IgA antibody were performed in parallel using anti-HIV material from the same diagnostic kit as described in the Materials and Methods section.

The use of standard antibody-body assays for the diagnosis of perinatal HIV-1 infection in infants is distorted by transplacentally acquired maternal IgG antibodies. Indeed, passively acquired maternal antibodies in the child can be detected up to 15-18 months after birth; more than 50% of maternal antibodies decay by 6-9 months and 95% of uninfected or seroreverted children clear maternal antibodies by 12 months.[5] Analysis of HIV-1 specific IgG subclasses in children born to infected mothers generally show the highest reactivity with the IgG1 and IgG3 subclasses to envel-

ope peptides.[10] IgG3 has a much shorter half life than other IgG subclasses, resulting in the clearance of IgG3 in one-third of the time taken for IgG1 (14 weeks *vs* 7-18 months), *i.e.* 91% HIV-1 specific IgG3 have disappeared by 6 months.[9]

From our findings, anti-HIV antibody levels remained quite stable in uninfected infants (91%) up to 9 months, which reflects the high sensitivity of the third generation ELISA methods used presently. Anti-HIV IgG3 subclass antibodies in this uninfected group decreased to 36% with a low signal (mean OD = 0.48) at 6 months, whereas 86% of infected infants had a high signal (mean OD = 1.18) until 15 months. The specificity of IgG3 subclass antibodies appear to be higher than specific IgG as early as 2 months up to 6 months (34% vs 1% at 2 months and 64% vs 7% at 6 months) with a negative predictive value of IgG3 as high as 96% at 2 months as shown in Table 2.

Previous reports have shown maternal antibodies to the V3 region to be lost within 2-4 months after birth, and antibodies in infected infants are produced within 5 months.[6,20] In this study, levels of antibody to V3 in infants born to HIV-1 infected mothers decreased (titers of 100-200) within 1-2 months after birth. Subsequently, this V3 antibody increased (titers of 400-3,400) in those infants who were infected, but disappeared and became undetectable in seroreverted infants within 12 months (data not shown).

In general, measurement of IgM is recommended for the early diagnosis of viral infection in vertical transmission. Upon transmission of HIV-1 *in utero* or during birth, the infant first produces IgM antibodies to the p24 gag protein whose levels are transient and persist in only 10-20% of infected infants.[8] HIV IgA, which also cannot cross the placenta, seem to be a useful serological tool to diagnose HIV-1 infection in perinatal transmission.[7] Although the advantage of specific IgA detection lies in the availability of a simple and rapid assay, its sensitivity is age dependent, *i.e.* only 20-80% in infected infants are positive before 6 months of age, and false positive result may occur in uninfected infants.[2,7] Our findings showed that anti-HIV IgA was observed in less than 50% of infected infants in the first 6-12 months. In this study, the rate and optical density of IgA detection were recorded. This low result might be due to the fact that the HIV-1 subtype prevailing in Thailand is subtype E. In order to address this issue, we analyzed specific IgA in sera from HIV-1 subtype B infected individuals using the same ELISA kit. It was found that subtype B sera (n = 40) gave a more marked signal than subtype E (n = 81) to the prepared HIV-1/HIV-2 antigen which was derived from non-E subtype (mean OD = 1.11 *vs* 0.59, unpublished data).

In addition to anti-HIV, p24Ag testing has been recommended for early diagnosis during the "window period" from exposure-to-seroconversion of HIV-1 as early as 5 days post primary infection.[3,4] In perinatally infected infants, the high T-lymphocyte population found in the neonatal period might result in a rapid increase in viral replication, such that p24Ag becomes detectable very early, especially if augmented by immune complex dissociation through either acidification or heat denaturation.[11,12] Although the number of tests was limited in this study, our findings showed that heat treatment to dissociate the immune complex help to detect p24Ag in 70-80% of infected infants at 6-12 months.

In summary, our data shows that the disappearance of HIV IgG3 subclass within the first 6 months can be an earlier parameter than anti-HIV detection to indicate that infants born to HIV infected mothers are not infected. This parameter can be an alternative assay to support a molecular technique, which is very specific. In addition, the use of these parameters including the persistence of anti-HIV IgG3 subclass antibodies, the production of anti-HIV IgA antibody and the presence of p24Ag can be used as an adjunct for the diagnosis of vertical transmission of HIV. These assays are, technically, relatively simple and could therefore be developed individually from routine kits. Their low cost (approximately 100 Baht or 2.5 US dollars) makes them particularly attractive to use as an adjunct to diagnosis where molecular laboratory support is unavailable.

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