Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2008.03664.x

Salivary human immunodeficiency virus (HIV)-1-specific immunoglobulin A in HIV-1-exposed infants in Kenya

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Accepted for publication 17 March 2008
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Summary

Humoral immunity, and specifically immunoglobulin A (IgA) that is directed against human immunodeficiency virus (HIV)-1, may contribute to protection against HIV-1 acquisition at mucosal surfaces. HIV-1-specific IgA has been detected in genital tract secretions of HIV-1-uninfected commercial sex workers with HIV-1 exposure, and may be produced in parotid saliva by infants exposed orally to HIV-1 during delivery and breastfeeding. To explore this hypothesis, we collected saliva from 145 infants aged ≤ 6 months enrolled in a perinatal HIV-1 transmission study in Nairobi and from 55 control infants without HIV-1 exposure who were born to HIV-1-seronegative mothers. Among the 145 infants, 115 (79%) remained uninfected during the 12-month study period and 30 (21%) became HIV-1-infected during follow-up. Nine (8%) of the 115 HIV-1-exposed, uninfected infants had detectable levels of HIV-1 gp160-specific IgA compared with four (13%) of 30 infected infants and none of 55 control infants ($P = 0{\cdot}47$ and $P = 0{\cdot}03$ respectively). Among the nine HIV-1-exposed, uninfected infants with positive assays, median age was 1 month and none acquired HIV-1 during follow-up. We conclude that HIV-1-specific salivary IgA responses may be generated by very young infants exposed perinatally to maternal HIV-1. Mucosal responses would be an appropriate target for paediatric vaccines against breast milk HIV-1 transmission.

Keywords: immunoglobulin A, mother-to-child HIV-1 transmission, saliva

Introduction

Mother-to-child transmission of human immunodeficiency virus (HIV)-1 accounts for ~ 600 000 new infections annually in sub-Saharan Africa where infants are exposed to HIV-1 *in utero*, during delivery and through breastfeeding. In the absence of anti-retroviral prophylaxis, approximately 25–35% of infants will become infected with HIV-1 after exposure to virus in maternal blood, cervicovaginal secretions or breast milk. Understanding the reasons why approximately two-thirds of infants do not acquire HIV-1 may contribute significantly to paediatric HIV-1 prevention and vaccine research.

The majority of exposure to HIV-1 is across oral and gastrointestinal mucosa, and one proposed explanation is that infant immune responses in saliva provide protection against HIV-1 acquisition [1]. While immunoglobulin A (IgA), the major mucosal antibody, has been shown to neutralize HIV-1 *in vitro* and can be isolated from parotid saliva, the role played by IgA in protecting against oral exposure to HIV-1 has not been well defined [2–5]. Several studies have assayed saliva from HIV-1-infected adults for HIV-1-specific IgA, and one study included HIV-1-infected children [6–10]. Results have been variable, probably because of assay differences because both enzyme-linked immune assay and Western blot techniques have been used with a range of HIV-1 antigens [6–10]. However, despite technical variation, most studies have concluded that salivary HIV-1-specific IgA is present in a minority of HIV-1-infected individuals. It has been suggested that HIV-1-infected individuals do not properly generate HIV-1-specific IgA, either because the virus does not stimulate mucosal antibody responses or because the humoral response of the HIV-1-infected person is defective in some way.

Only one study has explored salivary IgA production among HIV-1-uninfected individuals with exposure to HIV-1, and this was carried out among adults, not children [3]. Purified IgA in saliva was assessed for HIV-1

neutralization activity and investigators found that 11 (73%) of 15 saliva specimens contained IgA capable of neutralizing HIV-1 [3]. While these results are encouraging for adults, young infants may not be as capable of generating HIV-1-specific humoral immune responses in oral secretions. Because secretory IgA is not transported actively across the placenta, levels are generally low to absent at birth and increase with age, achieving adult levels near 6–8 years [11]. In support of infant IgA responses are non-HIV studies demonstrating pathogen-specific salivary IgA antibodies against toxoplasmosis and influenza virus in saliva from young infants [11]. More compelling data come from a recent paediatric HIV-1 vaccine trial which found that a small proportion of infants who had been vaccinated with a recombinant canarypox virus (ALVAC) HIV-1 vaccine developed salivary HIV-1-specific IgA in response to this immunization [12].

In this prospective cohort study we explored whether HIV-1-exposed, uninfected infants make immune responses in saliva after natural challenge with maternal breast milk and cervicovaginal secretions containing HIV-1. We conducted a comprehensive analysis of salivary HIV-1 gp160-specific IgA and IgG in 145 HIV-1-exposed infants under 6 months of age who were tested quarterly for 12 months for HIV-1 acquisition. Our primary objective was to determine whether detection of salivary HIV-1-specific IgA was associated with a reduced risk of infant HIV-1 infection intrapartum and during follow-up. The study was also designed to define the prevalence and correlates of this local immune response in saliva obtained from the HIV-1-uninfected infants exposed orally to HIV-1.

Methods

Recruitment and follow-up

Pregnant women attending Nairobi City Council clinics were invited to participate in a perinatal HIV-1 transmission cohort that has been described elsewhere [13,14]. Briefly, women were eligible if they were ≥ 18 years of age, < 32 weeks' gestation and planned to live in Nairobi for 1 year after delivery. Study participants received counselling regarding infant feeding options and oral zidovudine prophylaxis was initiated at 34–36 weeks gestation in accordance with the Thai short-course regimen. Within 48 h of birth, neonatal blood was obtained by venipuncture to determine HIV-1 infection status. Saliva was obtained at birth using six Dacron swabs soaked with saliva from the buccal mucosa, and these were placed into conical tubes for processing as described below.

Mothers and their infants were seen and examined at the clinic postpartum at 2 weeks and then monthly until month 12. At months 1, 3, 6, 9 and 12, infant blood was obtained to determine infant HIV-1 infection status using HIV-1 DNA filter paper assays, and at months 1, 3 and 6 infant saliva specimens were collected. Saliva collection in breastfeeding infants was performed at least 1 h after breastfeeding to minimize maternal breast milk contamination of salivary samples. Maternal blood specimens were collected for HIV-1 RNA polymerase chain reaction (PCR) assays and CD4 T cell count at 32 weeks' gestation and delivery, and breast milk was collected at week 2 and month 1 postpartum.

Saliva processing and HIV-1-specific IgA and IgG determination

Saliva specimens were maintained on ice until initial processing. Saliva fluid was expressed from the swabs and placed in 0·22 μm centrifuge filter tubes with 1·5 ml phosphate-buffered saline (PBS), and centrifuged at 1500 ***g*** for 30 min. To reduce degradation of Igs, 10 μl of protease inhibitor 4-(2-aminoethyl)-benzenesulphonylfluoride was added at 100 mM. Samples were stored at −80°C until HIV-1-specific IgA and IgG quantitative enzyme-linked immunosorbent assays (ELISAs) were performed, as described previously [7,15].

Briefly, for detection of HIV-1-specific IgA, Immulon-2 round-bottomed microtitre plates were coated overnight at 4°C with recombinant HIV-1 IIIB gp160 (1 μg/ml). Plates were washed and incubated for 1 h at 37°C with twofold dilutions of positive control [pooled sera from HIV-1-infected individuals (initial concentration of 40 μg/ml)], test saliva starting at 1 : 4, or negative control [pooled sera from HIV-1-uninfected individuals (40 μg/ml)] diluted in serum diluent (5% skimmed milk in PBS with 0·1% Tween-20, pH 7·4 and 0·01% Thimerosal). Plates were again washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-human IgA (Kirkegaard & Perry, Gaithersburg, MD, USA) in serum diluent containing 50 μg/ml purified IgG. Plates were washed and substrate was added for 15 min at 37°C and the reaction was stopped with 1·0 M phosphoric acid.

Maternal HIV-1 viral load in plasma and breast milk

HIV-1 RNA viral load was determined in maternal plasma and breast milk supernatant using the Gen-Probe HIV-1 vival load assay (Gen-Probe, Inc., San Diego, CA, USA), a transcription-mediated amplification method sensitive for detection of Kenyan HIV-1 subtypes A, C and D [16–18]. Plasma and breast milk were tested at a volume of 1–500 μl and the lower limit of detection was three HIV-1 copies/assay, with copies/ml calculated based on the volume of fluid tested [18].

Infant HIV-1 infection status

Infant plasma specimens were tested using the same Gen-Probe HIV-1 vival load assay (Gen-Probe, Inc.) and defined as positive if > 50 copies/assay and > 100 copies/ml of HIV-1

RNA were detected. Infant filter paper specimens were assayed for HIV-1 DNA using PCR as described elsewhere [19]. Infants were considered HIV-1-infected if they had (i) a positive filter paper DNA or plasma RNA assay on two consecutive dates or (ii) a single positive filter paper or plasma RNA assay if this was the last available sample.

Human immunodeficiency virus-1-unexposed controls

To confirm the specificity of HIV-1-specific IgA results in this cohort, HIV-1-seronegative mothers and their infants were recruited to provide control specimens. Women presenting to a Nairobi City Council Clinic with infants between the ages of 6 weeks and 12 months were invited to participate. After women had provided written informed consent, they were interviewed with a standard questionnaire to assess risk factors and blood was obtained from both mother and infant. Saliva collection and processing were the same as described above. Maternal HIV-1 status was determined using HIV-1 ELISA and only HIV-1-seronegative mother–infant pairs included. Infant HIV-1 status was confirmed as negative with HIV-1 RNA PCR and HIV-1-specific IgA assays were performed as described above for the main study. Written informed consent was obtained from all study participants. This study received ethical approval from the institutional review boards of the University of Washington and the University of Nairobi and was conducted according to the guidelines set forth by the United States Department of Health and Human Services.

Statistical analyses

Correlates of infant HIV-1-specific IgA production were defined using χ^2 and Fisher's exact tests for dichotomous variables and independent *t*-tests for continuous variables. Fisher's exact test was also used to determine whether there was a statistically significant difference in the rate of HIV-1-specific IgA positivity for study *versus* control infants. Regression analyses were performed to evaluate the association between salivary IgA detection and risk of infant HIV-1 acquisition during follow-up.

Results

Cohort description

Saliva specimens were collected from 145 infants enrolled in a perinatal HIV-1 cohort that has been described previously [13,14]. Of the 145 infants, 115 (79%) remained HIV-1-uninfected during follow-up, 30 (21%) acquired HIV-1 infection during the 12-month study period, 19 (13%) infants died and 12 (8%) were lost to follow-up. Median maternal CD4 count and HIV-1 viral load at 32 weeks' gestation were 469 cells/μl [interquartile range (IQR) 340, 615] and 4·7 $\log_{10}$ copies/ml (IQR 4·1, 5·1) respectively. Women received zidovudine before delivery for a median duration of 3·3 weeks (IQR 0·7, 4·6) and 130 (90%) of the 115 infants were delivered by spontaneous vaginal delivery at a median maturity of 40 weeks (IQR 38, 40) and birth weight of 3·0 kg (IQR 2·8, 3·4). One hundred and twenty-two (84%) women chose to breastfeed their infants and median breast milk HIV-1 RNA viral load was 2·4 $\log_{10}$ copies/ml (IQR 2·0, 3·3) for the 117 women who provided samples at 1 month postpartum. Clinical mastitis was diagnosed in nine (7%) of these breastfeeding women.

Detection of HIV-1 gp160-specific IgA and IgG in saliva

In total, 356 HIV-1 gp160-specific IgA and IgG assays were performed on specimens collected from the 145 HIV-1-exposed infants at the following time-points: 130 assays at birth; 123 at 1 month of age; 79 at 3 months; and 24 at 6 months (Table 1). Among the 115 HIV-1-exposed, uninfected infants, HIV-1 gp160-specific IgA was detected in 10 (3·5%) of 286 saliva samples and in nine (8%) infants, as one infant had HIV-1-specific IgA detected at two study visits. Among the 30 HIV-1-infected infants, four (13%) had a positive assay during follow-up (Table 1). This proportion was not significantly different to the 9% prevalence described above for HIV-1-exposed, uninfected infants in the cohort ($P = 0{\cdot}47$). The greatest number of infants positive for HIV-1-specific IgA occurred at 1 month of age, the visit when the majority of specimens were

Table 1. Infant saliva specimen collection and proportion positive for human immunodeficiency virus (HIV-1) gp160-specific immunoglobulin A (IgA) and IgG.

	HIV-1-exposed uninfected infants ($n = 115$)			HIV-1-infected infants ($n = 30$)		
Infant age at saliva collection	Number of specimens collected	HIV-1-specific IgA positive (%)	HIV-1-specific IgG positive (%)	Number of specimens collected	HIV-1-specific IgA positive (%)	HIV-1-specific IgG positive (%)
Birth	102	1 (1%)	89 (87%)	28	1 (4%)	20 (71%)
1 month	97	6 (6%)	80 (82%)	26	1 (4%)	22 (85%)
3 months	65	0	17 (26%)	14	2 (14%)	8 (57%)
6 months	22	3 (13·5%)	2 (9%)	2	0	0
Total	286	10 (4%)	189 (66%)	70	4 (6%)	50 (71%)

Table 2. Characteristics of 13 human immunodeficiency virus (HIV-1)-exposed infants with detectable salivary HIV-1 gp160-specific immunoglobulin A (IgA).

	Age at saliva collection	Feeding mode	HIV-1 gp160-specific IgA titre	Maternal CD4 count (cells/μl)	Maternal plasma HIV-1 RNA (log_{10} copies/ml)	Breast milk HIV-1 RNA (log_{10} copies/ml)
HIV-1-uninfected						
3	<48 h	BF	1:7	511	3·2	2·0
7	6 months	FF	1:8	415	4·1	n.a.
8	1 month	FF	1:5	300	5·1	n.a.
17	6 months	BF	1:13	596	4·8	3·2
33	1 month	BF	1:48	317	4·9	2·3
39	1 month	BF	1:4	346	4·5	2·1
47	<48 h	BF	1:4	587	3·9	1·9
86	1 month	BF	1:12	611	3·8	2·1
125	1 month	BF	1:12	873	n.a.	1·9
Median	1 month		1:13	511	4·3	2·1
HIV-1-infected						
5	<48 h	BF	1:54	284	4·4	2·3
27	3 months	BF	1:7	78	5·7	4·6
62	1 month	FF	1:5	720	5·7	n.a.
93	3 months	BF	1:4	382	5·0	3·8
Median	3 months		1:18	333	5·4	3·8

BF, breastfeeding; FF, formula feeding; n.a., not available.

collected. The highest proportion of positive assays was at the 3-month visit for HIV-1-infected infants and at the 6-month visit for exposed, uninfected infants (~ 14% for both).

We next compared prevalence of positive salivary HIV-1-specific IgA responses among the three groups: (i) 115 HIV-1-exposed, uninfected infants; (ii) 30 HIV-1-infected infants; and (iii) 55 HIV-1-uninfected, unexposed infants enrolled as controls. We found that infected and uninfected infants with HIV-1 exposure were significantly more likely to have a positive IgA response than control infants with no exposure. None of the IgA assays performed on saliva samples from the 55 control infants were positive compared with 8% of exposed, uninfected infants and 13% of infected infants ($P = 0{\cdot}03$ and 0·01 respectively). Thus, the likelihood of a positive salivary IgA assay was significantly higher among infants with exposure to HIV-1 and infected infants than among infants born to HIV-1-seronegative mothers, confirming the specificity of the assay.

Human immunodeficiency virus-1-specific IgG was measured in all specimens assayed for IgA and was detected in 238 (83%) of the 286 saliva samples. HIV-1-specific IgG assays were positive at least once in 107 (93%) of 115 exposed, uninfected infants and in 26 (87%) of 30 HIV-1-infected infants. Among the HIV-1-exposed, uninfected infants, the proportion with positive assays decreased over time, as one would expect for antibodies transferred passively *in utero*. Eighty-seven per cent of samples collected at month 1 were positive compared with only 26% at the month 3 visit and 9% at month 6 ($P > 0{\cdot}05$ for both comparisons) (Table 1).

Correlates of salivary HIV-1-specific IgA among HIV-1-exposed, uninfected

Detailed characteristics of the 13 HIV-1-exposed infants with positive assays are provided in Table 2. HIV-1-specific IgA was detected at a median titre of 1 : 13 (range 1 : 4–1 : 48) among the exposed, uninfected infants and a median titre of 1 : 18 (range 1 : 4–1 : 54) among infected infants (Table 2). Most infants were breastfed, with only three (23%) never breastfeeding per maternal report, and 11 (85%) of 13 infants were born by spontaneous vaginal delivery.

To explore correlates of salivary IgA responses among uninfected infants, we compared the nine HIV-1-exposed, uninfected infants with positive assays to the 106 with negative assays. We found no significant differences in maternal or infant characteristics (Table 3). A similar number of infants were breastfed, premature and low birth weight, and maternal CD4 T cell count and HIV-1 RNA levels in plasma and breast milk were no different between the two groups (Table 3).

Risk of mother-to-child HIV-1 transmission

To examine the risk of acquiring HIV-1 during follow-up within the cohort, we compared those with and without a positive IgA response and determined whether the presence of HIV-1-specific IgA was associated with protection from infant HIV-1 infection. For this analysis, the 136 infants who were HIV-1-uninfected at birth were included. We found that none of the nine infants with salivary HIV-1-specific IgA acquired HIV-1, while 21 (16·5%) of the 127 infants who

Table 3. Comparison of 115 human immunodeficiency virus (HIV-1)-exposed, uninfected infants with and without detection of salivary HIV-1-specific immunoglobulin A (IgA).

Characteristic	Salivary HIV-1-specific IgA not detected ($n = 106$) Median (IQR) or number (%)	Salivary HIV-1-specific IgA detected ($n = 9$) Median (IQR) or number (%)	*P*-value
Maternal			
Age (years)	24 (22–27)	23 (21–27)	0·96
$CD4^+$ T cell count at 32 weeks gestation (cells/μl)	469 (340–620)	511 (331–604)	0·93
HIV-1 RNA viral load at 32 weeks' gestation (log_{10} copies/ml)	4·6 (4·0–5·0)	4·3 (3·8–4·9)	0·44
Breast milk HIV-1 RNA levels 1 month postpartum (log_{10} copies/ml)[†]	2·25 (1·95–3·04)	2·1 (1·9–2·3)	0·27
Zidovudine prophylaxis duration (weeks)	3·6 (1·3–4·75)	3·6 (0·9–5·25)	0·89
Spontaneous vaginal delivery	97 (92%)	8 (89%)	0·79
Clinical mastitis	9 (10%)	0 (n.a.)	0·31
Infant			
Birth weight (kg)	3·0 (2·8–3·4)	3·2 (2·9–3·3)	0·55
Maturity (estimated gestational age at delivery)	40 (38–40)	39·5 (38·3–40·0)	0·81
Breastfed	89 (84%)	7 (78%)	0·75
Duration of breastfeeding (months)	5 (3–9)	7 (7·0–7·5)	0·49
Duration of follow-up (days)	366 (363–368)	367 (320–369)	0·80

[†]Ninety-one of the 96 breastfeeding women provided specimens. IQR, interquartile range; n.a., not available.

did not have salivary HIV-1-specific IgA became HIV-1-infected during the course of follow-up (odds ratio = 1·2; 95% confidence interval 0·3–6·2; $P = 0·8$).

Discussion

A vaccine designed to prevent mother-to-child HIV-1 transmission should, ideally, induce an immune response at mucosal surfaces where transmission occurs. In this study, we hypothesized that infant exposure to maternal HIV-1 during delivery and breastfeeding was similar to immunization against HIV-1 via the oral route and would result in salivary HIV-1-specific IgA that would provide some degree of protection against intrapartum and breast milk HIV-1 acquisition. While we did not find HIV-1-specific salivary IgA to be associated with a significantly lower risk of HIV-1 in our cohort, we did detect IgA in a significantly greater number of exposed, uninfected infants compared with our control population. Overall, 8% of HIV-1-uninfected infants in the study tested positive for HIV-1-specific IgA at one time-point, and all HIV-1-exposed infants with IgA responses remained uninfected during 1 year of follow-up. Furthermore, infants were very young at the time of specimen collection; all were under 6 months of age and median age was 1 month.

Our data support the hypothesis that HIV-1-specific humoral immunity can be generated in response to HIV-1 exposure and complement existing data describing mucosal antibody responses after vaccination with HIV-1 vaccines [12,20–23] and responses in HIV-1-infected individuals [6–10]. Among adults, heterosexual transmission is responsible for the majority of HIV-1 transmission, with acquisition occurring across genital tract mucosa. Several studies have documented production of HIV-1-specific secretory IgA in cervicovaginal secretions of commercial sex workers who remain uninfected despite repeated HIV-1 exposure [3,24–26]. While the cross-sectional design of such studies makes it difficult to determine whether these responses were associated with reduced HIV-1 acquisition, the findings suggest that HIV-1-specific humoral immunity can be induced in the female genital tract through HIV-1 exposure. Oral and intranasal immunization of animal models has also been shown to induce high titres of secretory IgA antibodies capable of HIV-1 neutralization in saliva and other mucosal secretions [22,23]. Furthermore, among 18 newborns who received the ALVAC HIV-1 vaccine, four (22%) developed HIV-1-specific IgA responses in saliva at 12 and 24 weeks post-vaccination [12]. While the proportion of infants with positive assays was relatively low, these results also support that salivary HIV-1-specific IgA can be elicited in infants by immunizing neonates, an important observation as exposure to at-risk infants after birth occurs across oral mucosal surfaces from breastfeeding.

We observed a low prevalence (8%) of IgA responses among HIV-1-exposed, uninfected infants in this cohort. We believe our low prevalence is an accurate representation of salivary antibody levels among exposed infants and not the result of poor assay sensitivity. Even among HIV-1-infected adults HIV-1-specific IgA prevalence is reported to be low, with IgA in parotid saliva being less than in either male or female genital secretions [27]. Another consideration is that maternal breast milk containing HIV-1-specific IgA may have contaminated infant specimens. We recognized this limitation prior to study initiation and ensured that saliva specimens were collected at least 1 h after last breastfeeding

to minimize risk of contamination. We also had two non-breastfeeding children who were not infected with HIV-1 and yet had a salivary response at 6 months of age, presumably as a result of HIV-1 exposure *in utero* or during delivery. This supports further that these are infant, rather than maternal breast milk, responses. In addition, we observed a decrease over time in the proportion of exposed, uninfected infants with detectable HIV-1-specific IgG in saliva. This is consistent with waning of passively transferred maternal IgG antibodies in infants during the first months of life and argues against breast milk contamination of saliva specimens, which would have resulted in sustained levels of salivary HIV-1-specific IgG.

We also considered the possibility that positive IgA responses in infants resulted from transplacental transfer of maternal IgA in the setting of placental inflammation with subsequent leakage into saliva [28]. Several studies have attempted to measure HIV-specific IgA in HIV-uninfected infants born to HIV-seropositive mothers [29–33]. In the largest of these, positive HIV-specific IgA responses in infant serum were extremely low, ranging from undetectable to less than 0·5% [30,33]. Other smaller studies have detected HIV-specific antibodies in cord blood obtained from uninfected infants born to HIV-infected mothers, which would support low-level diffusion of IgA antibodies; however, this could also represent infant IgA production resulting from HIV-1 exposure *in utero*, consistent with our current hypothesis [29,31,32].

In summary, this comprehensive study of salivary HIV-1-specific IgA among HIV-1-exposed infants tested 356 samples from 145 infants, 115 of whom were HIV-1-uninfected at the time of specimen collection. While we detected IgA in only a small subset of infants, our findings provide some evidence that natural HIV-1 exposure via the oral route can stimulate a humoral immune response in infants younger than 6 months of age and this may enhance understanding of how children are protected against mucosal exposure to HIV-1.

Acknowledgements

This research was funded by US National Institutes of Health (NIH) grants R01 HD23412 and K23 HD41879. C. Farquhar received support from NIH grant K23 HD41879 and C. Farquhar, R. Bosire and P. Otieno were scholars in the AIDS International Training and Research Program supported by the NIH Fogarty International Center grant D43 TW00007. G. John-Stewart is an Elizabeth Glaser Pediatric AIDS Foundation (EGPAF) Scientist and D. Mbori-Ngacha had an EGPAF Leadership Award.

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