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Differences in the Epstein-Barr Virus gp350 IgA Antibody Response Are Associated With Increased Risk for Coinfection With a Second Strain of Epstein-Barr Virus

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Background. The Epstein-Barr virus (EBV) viral glycoprotein gp350 has been proposed as a candidate antigen for an EBV vaccine. However, the proposed formulations of these vaccines have not taken into account the presence of 2 unique EBV strains (EBV-1 and EBV-2) present in areas of high incidence of the EBV-associated cancer, Burkitt lymphoma.

Methods. In this study, we analyze the kinetics of EBV-1 and EBV-2 infection in an asymptomatic infant cohort from Kisumu, Kenya. We also analyzed the kinetics of the antibody response against 5 EBV antigens, gp350 (IgG and IgA), VCA (IgG), EBNA-1 (IgG), EAd (IgG), and Zta (IgG).

Results. We observed a high frequency of coinfection with both EBV types over time, with the only observable defect in the antibody response in infants coinfected being a significantly lower level of anti-gp350 IgA at peak response. Gp350 IgA levels were also significantly lower in coinfected infants 2.5 months postinfection and at the time of coinfection.

Conclusions. These results suggest that anti-gp350 IgA antibodies may be important for sterilizing immunity against secondary infection. These findings have implications for the development of an efficacious EBV vaccine to prevent both EBV-1 and EBV-2 infection in a population at high risk for Burkitt lymphoma.

Keywords. Epstein-Barr virus; primary infection; antibody.

Primary infection with Epstein-Barr virus (EBV) can occur early in childhood, generally resulting in asymptomatic infection, or later in adolescence, often presenting as acute infectious mononucleosis (AIM) [1]. By adulthood, infection is almost ubiquitous, with >95% of the global population EBV seropositive [2]. There are 2 strains of EBV, EBV type 1 (EBV-1) and type 2 (EBV-2). These 2 strains have distinct geographic distribution with EBV-1 found worldwide, while EBV-2 is more localized to regions within Africa [3–5] and an increased prevalence in human immunodeficiency virus (HIV)-positive populations [6].

Evaluation of the kinetics of the humoral response to primary EBV infection has focused primarily on the detection of IgG specific for 2 lytic antigens, the EBV viral capsid antigen (VCA) and the early antigen diffuse complex (EAd), and a latent antigen, Epstein-Barr nuclear antigen-1 (EBNA-1). In asymptomatic primary infection and AIM, the IgG response to VCA precedes the detection of the EBNA IgG [1, 7, 8]. Previous studies of asymptomatic infection have used these responses as clinical indicators of EBV infection, while focusing on the T-cell response [9, 10]. Interestingly, antibodies to EAd are indicative of viral reactivation and not sustained [11]. A more thorough analysis of the antibody response has been done following AIM, including the response against the viral gp350 [12].

The EBV glycoprotein, gp350, is the ligand for CD21 on the B cell, an interaction required for infection [13–16]. Antibodies to gp350 neutralize B-cell infection in vitro and correlate with the neutralizing ability of human serum [17]. This is most associated with gp350 IgG1 isotype [18]. Importantly, the level of gp350 antibodies are inversely correlated to AIM severity [19]. For these reasons, gp350 is a potential EBV vaccine candidate [20] with studies focusing on gp350 IgG. However, EBV is transmitted via saliva with primary infections occurring in the oral pharynx [21], suggesting a potential role for gp350 IgA antibodies in protection. Furthermore, the anti-gp350 response has not been analyzed in asymptomatic primary infection.

We have previously reported on a longitudinal study following EBV infection of infants from a malaria-endemic region of Kenya [22]. The majority of infants in this cohort were infected with EBV prior to 12 months of age, with multiple peaks of EBV viral load following primary infection [22, 23]. In this study, we sought to answer 2 important questions. First, in infants with multiple peaks of viral load, were they coinfected with a

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different strain of EBV and, second, what was the pattern of the gp350 IgG and IgA response and was this protective against a secondary infection.

METHODS

Study Design

Samples were collected from study participants from a malaria holoendemic region of Western Kenya, with a high risk for endemic Burkitt lymphoma. This study comprised a subset of 36 infants, previously described, having 3 or more episodes of detectable viral load [22]. The infants were born to HIV-negative mothers and therefore assumed to be HIV negative.

Comparison of EBV types in blood and saliva of adults and children was done using samples from a previous cross-sectional cohort in the same region of Kenya [24]. The adult population had an average age of 34 years (19.5–67.8 years) and was 91.2% female. The child population had an average age of 4.8 years (0.5–11.3 years) and was 41.0% female.

Viral Load and EBV Typing

DNA from whole blood was extracted and cell-associated EBV DNA was quantified by quantitative polymerase chain reaction (qPCR) as described previously [22]. EBV typing was performed on the EBV-positive samples. The EBNA-3c region was amplified by PCR as previously described [25]. PCR products were analyzed by 2% agarose gel containing 5% ethidium bromide. The PCR product for EBV-1 is 153 base pairs and EBV-2 is 246 base pairs. Single-type infection was defined as only 1 EBV type at any timepoint and coinfection was detection of both EBV types at any timepoint, though not necessarily concurrently.

Antibody Detection

Anti-VCA IgG, anti-EBNA-1 IgG, anti-EAd IgG, and anti-Zta IgG responses were detecting by Luminex assay, as previously described [22, 26]. Briefly, 20 μg of antigen peptide was coupled to 1×10^6 carboxylated microspheres. Antigen-specific IgG was detected by incubating 1000 microspheres with a 1:100 dilution of plasma. After washing, phycoerythrin-conjugated goat anti-human IgG was added and at least 75 microspheres were analyzed for mean fluorescence intensity (MFI) for each EBV antigen.

gp350 IgG and IgA antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). We coated 96-well plates overnight at 4°C with 1 μg/mL recombinant gp350 peptide (amino acid 4–450; Immune-tech, New York, NY). This peptide contains the N-terminal region involved in CD21 binding and the epitope for the monoclonal neutralizing antibody [27]. Plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour at 37°C. Plasma, diluted 1:100 in 0.05% Tween-20, 3% BSA in PBS, was then incubated for 1 hour at 37°C. Plates were washed with 0.05% Tween-20 in PBS. A 1:10 000 dilution of goat anti-human IgG-horseradish peroxidase (HRP) or 1:2000 goat anti-human IgA-HRP in 3% BSA in PBS was added and incubated for 1 hour at 37°C. Plates were washed and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 10 minutes before addition of 1M H_3PO_4. OD_{450} was read on a Versamax microplate reader.

Measles and tetanus toxoid IgG were measured by ELISA. Plates were coated with 0.5 μg/mL tetanus toxoid (List Biological Laboratories, Campbell, CA) and 5 μg/mL measles grade 2 antigen (Microbix Biosystems, Mississauga, Canada) in 50 mM carbonate/bicarbonate coating buffer, pH 9.6 for 2–4 hours at room temperature. Plates were washed with PBS with 0.05% Tween-20 and blocked overnight in 5% nonfat milk in 1× PBS. After washing, 50 μL of plasma dilutions in blocking buffer were incubated for 2–4 hours at room temperature. Following another wash, a 1:5000 dilution of HRP conjugated AffiniPure donkey anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated for 2 hours at room temperature. After washing, TMB substrate was added. Following an 8-minute incubation, 50 μL of 1M H_3PO_4 was added. OD_{450} was read using a plate reader (BioTek μQuant, Gen5 software).

Mean OD_{450} was calculated by taking the mean of duplicate values for each plasma sample and subtracting the mean OD_{450} of 4 replicate wells that had only sample buffer plus 3 times the standard deviation.

Statistical Analysis

The statistical analysis of peak responses was performed using Graphpad Prism version 6.0 (La Jolla, CA). Comparisons of antibody response between EBV single-type infected and coinfected groups were done using the Mann-Whitney test, due to only 13 samples in the coinfected group. A P value ≤ .05 was considered statistically significant.

Piecewise linear models were fit to log transformed OD_{450} values for gp350 IgA and IgG to determine differences in response between single-type and coinfected infants [28]. Because the antibody response was characterized by a rapid antibody increase followed by a leveling off (seen through smoothing splines, Supplementary Figure 1), our objective was to estimate these slopes separately. Piecewise linear models allow for slopes to differ before and after the breakpoint (where antibody response over time levels off), allowing for hypotheses related to infection type and antibody response to be tested. The "segmented" package in R (R Foundation for Statistical Computing, Vienna, Austria) was used to estimate an individual breakpoint for each outcome, using the combined infection type data, allowing for the same breakpoint but different slopes based on infection type [29, 30]. A piecewise linear mixed model was used to account for correlation among repeated measures from the same subjects in SAS 9.4 (SAS Institute, Cary, NC) with a spatial power covariance structure (due to irregular repeated measurements and differences in age at first infection) [28]. Time was normalized to zero at first detection of virus via PCR. The final models included a binary factor for infection type, continuous time, a

binary indicator for before and after the breakpoint, and interactions between these 3 variables to estimate slopes prior to and after the breakpoint. Age at baseline was also included. Estimate statements in SAS were used to test for differences in infection type via *t* test. Final plots were generated using R.

RESULTS

Study Population

We have previously reported on this infant cohort from Kisumu, Kenya [22, 31]. Of the original 68 infants evaluated, we identified 36 that had multiple peaks of EBV viral load in the blood within the first 30 months of age (Figure 1A and Supplementary Figure 2) [22]. The mean age of EBV infection in the 36 infants was 7.8 months (0.48 standard error of the mean [SEM]) and not significantly different than the mean age of EBV infection in the entire cohort (7.3 months; 0.33 SEM). Gender was also similar to the full cohort (38.8% male and 42.6% male, subset and full cohort, respectively).

Because EBV-2 has previously been detected in Kenya [3–5], we reasoned that the peaks of viral load could be due to secondary infection with a different EBV strain or virus reactivation. To test the EBV genotypes in the blood, we used PCR to distinguish EBV type based on a deletion in the EBNA-3c gene (Figure 1A). This PCR was performed on all EBV-positive samples. In total, we had an average of 6.4 EBV-positive timepoints per infant. At the initial detection of EBV in the blood, 22 infants were EBV-1–positive (61.1%), 11 were EBV-2–positive (30.6%), and 3 were coinfected (8.3%).

When EBV type was analyzed over the entire study, 12 infants remained EBV-1–positive at all timepoints (33.3%), 8 were only EBV-2–positive (22.2%), while 16 had evidence of EBV-1 and EBV-2 coinfection at any timepoint (44.4%; Figure 1B). While viral load peaks in infants with only 1 EBV type could be due to virus reactivation, 13 out of 36 infants (36.1%) showed direct evidence of secondary infection with a different EBV type. Importantly, there was no difference in the mean age of primary infection between infants with a single EBV type (7.46 months) and coinfected (7.89 months) (P = .6654). The frequency of malaria infection, as detected by qPCR, was not significantly different between the 2 groups (single type 3.2 episodes, 0.4 SEM; coinfection 3.6, 0.5 SEM). Therefore, we sought to analyze the EBV-specific antibody response to understand this lack of sterilizing immunity against secondary infection.

To determine whether this level of coinfection was unique to this cohort, we analyzed the EBV type in the saliva and blood from older children and adults in a different cohort from the same region of Kenya [24]. In the blood of adults, EBV-1 was detected in 4 (11.8%), EBV-2 in 1 (2.9%), coinfection in 20 (58.8%), and no detectable EBV in 9 (26.5%). In the blood of children, we detected EBV-1 in 7 (11.5%), EBV-2 in 1 (1.6%), coinfection in 34 (55.7%), and no detectable EBV in 19 (31.2%) (Figure 1C). In the adult saliva, we observed 3 participants EBV-1–positive (8.8%), 4 participants EBV-2–positive (11.8%), 15 participants coinfected (44.1%), and 12 had no detectable EBV (35.3%). In the children's saliva, we observed 13 EBV-1–positive children (21.3%), 4 EBV-2–positive (6.56%), 23 coinfected (37.7%), and 21 had no detectable EBV (34.4%) (Figure 1D). The high prevalence of EBV-2 in this population was consistent with our observations from the infant cohort.

Reduced Anti-gp350 IgA Antibodies at Peak Response in Coinfected Infants

The kinetics of the EBV antibody response was measured for 34 of the 36 original infants. Two were excluded due to the lack of available plasma samples (study identity number [ID] 30 and 36). We compared the peak EBV antibody responses in infants that were infected with a single detectable EBV type (n = 19) to coinfected infants (n = 13); initially coinfected infants were excluded (study ID 34 and 35). First, we evaluated the peak levels of VCA IgG, EBNA-1 IgG, EAd IgG, and Zta IgG following primary EBV infection. These data were generated using a Luminex bead based array and the results are expressed as MFI for antibody levels. As shown in Figure 2A, we observed no differences in peak IgG to any of the measured EBV antigens.

gp350 is the proposed EBV vaccine candidate and antibody levels in serum have been correlated to neutralization, in vitro [17, 20]. Therefore, we evaluated gp350 IgG and gp350 IgA present in infant plasma. We compared infants with a single EBV type to those that acquired a secondary infection with a different EBV type. The mean peak magnitude of these antibody responses for the group infected with a single EBV type was an OD_{450} of 1.15 for gp350 IgG and an OD_{450} of 1.76 for gp350 IgA. For the infants acquiring a secondary infection, there was a mean OD_{450} of 1.02 for gp350 IgG and an OD_{450} of 1.03 for gp350 IgA (Figure 2B). Interestingly, the peak magnitude of anti-gp350 IgA but not the anti-gp350 IgG antibodies was significantly lower in infants that were coinfected compared to those with a single type (P = .049).

We next analyzed the timing of the antibody response from initial virus detection to peak response. The average time to peak antibody response for infants infected with a single type of EBV occurred 10.9 (gp350 IgG), 9.4 (gp350 IgA), 9.6 (VCA IgG), 11.8 (EBNA-1 IgG), 9.1 (EAd IgG,), and 7.3 (Zta IgG) months following first PCR detection of virus (Figure 2C). For infants with a coinfection, the average time to peak response was 11.1 (gp350 IgG), 12.4 (gp350 IgA), 10.8 (VCA IgG), 13.2 (EBNA-1 IgG), 8.5, (EAd IgG), and 7.9 (Zta IgG) months following first detection of virus. There was no significant difference in the time to the peak response between infants infected with 1 EBV type compared to infants with coinfection (Figure 2C).

Single-Infected and Coinfected Infants Have no Difference in Response to Vaccine Antigens

Infants in Kenya are vaccinated with tetanus toxoid at 6, 10, and 14 weeks of age and later vaccinated against measles at 9 months

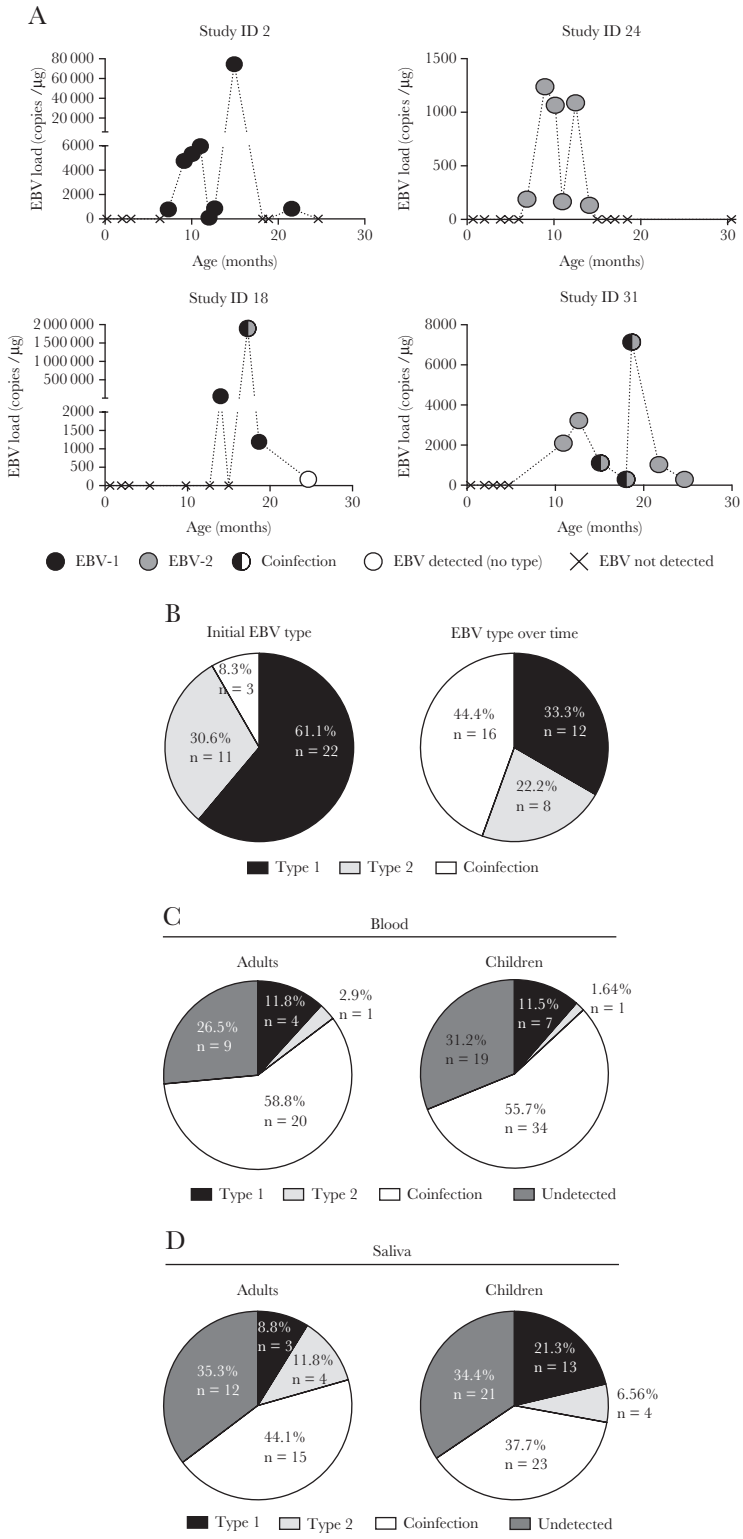

Figure 1. *A*, Four representative time courses from 4 individual infants showing viral load (copies/µg) and Epstein-Barr virus (EBV) type over time (months). Time courses for all 36 infants are shown in Supplementary Figure 2. Black (EBV-1), gray (EBV-2), black/gray (coinfection), white (EBV detected, no type), and X (no EBV detected). *B*, Compiled data for all 36 infants. The left chart represents the EBV type at initial detection of virus. The chart on the right represents the EBV type at all time points for each individual infant over time. EBV types in (*C*) blood or (*D*) saliva from a cohort of Kenyan adults (n = 34) and children (n = 61).

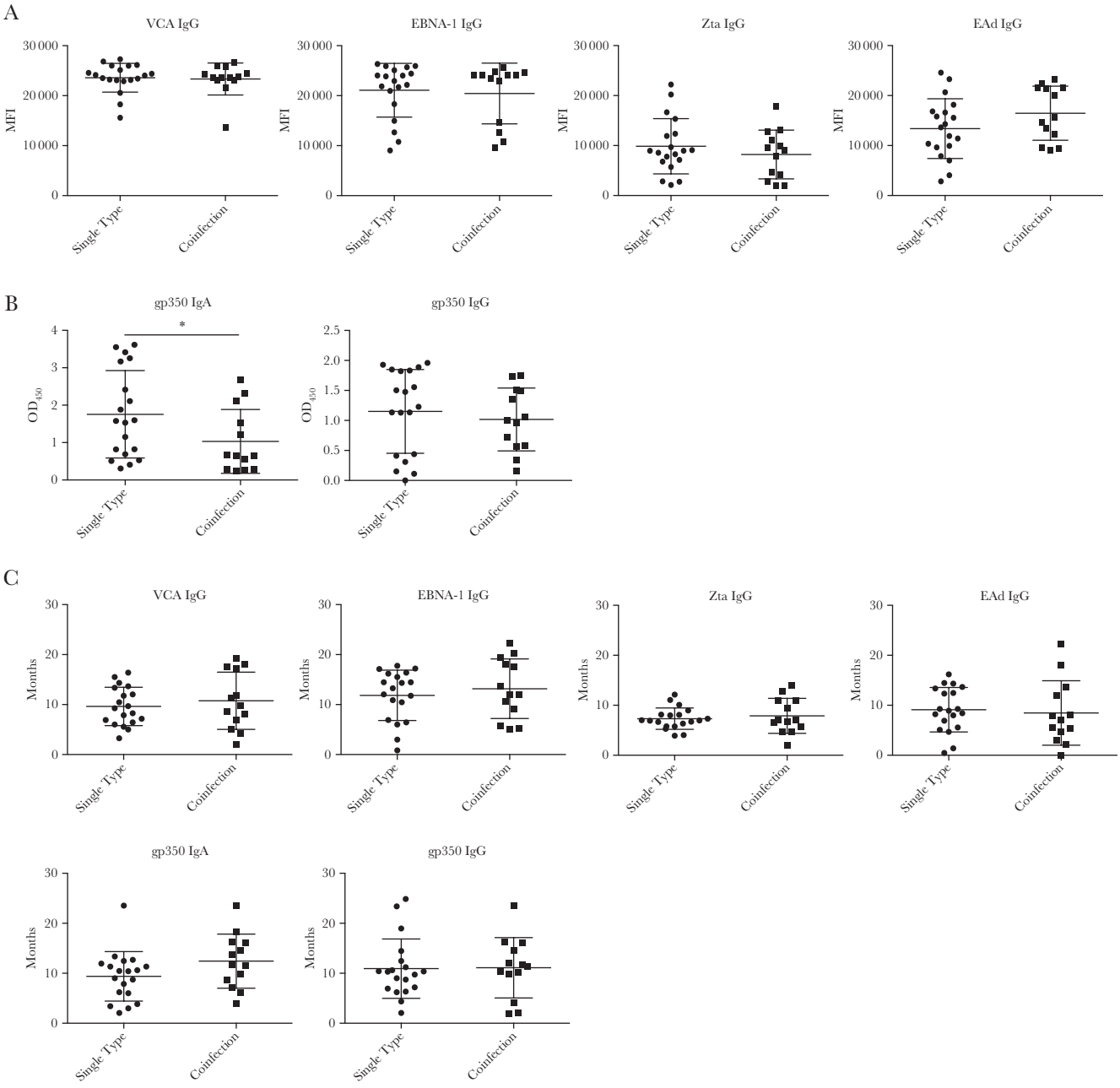

Figure 2. *A*, Analysis of the magnitude of the antibody response at the peak level postinfection measured by Luminex, comparing single-type infected infants (n = 19) with coinfected infants (n = 13; VCA IgG $P = .821$; EBNA-1 IgG $P > .999$; EAd IgG $P = .238$; Zta IgG $P = .677$). *B*, Analysis of gp350 antibody response by ELISA, comparing single infected (n = 19) to coinfection (n = 13; gp350 IgA $P = .049$; gp350 IgG $P = .430$). *C*, Analysis of the time from initial EBV detection to peak response comparing infants infected with a single type (n = 19) with infants showing coinfection (n = 13) in months (gp350 IgG $P = .570$; gp350 IgA $P = .094$; VCA IgG $P = .630$; EBNA-1 IgG $P = .603$; EAd IgG $P = .367$; Zta IgG $P = .858$). * = $P \leq .05$. Abbreviations: EAd, early antigen diffuse complex; EBNA, Epstein-Barr nuclear antigen-1; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; gp350, glycoprotein 350; MFI, mean fluorescence intensity; VCA, viral capsid antigen.

of age as per the World Health Organization Expanded Program in Immunization. We analyzed the antibody response to these vaccines to determine if there was any defect in the non-EBV associated immune response in the coinfected infants. We analyzed plasma samples at 12 and 24 months of age for IgG responses against tetanus and measles by ELISA (Figures 3A and 3B, respectively). We observed no statistically significant difference between the single-type infected and coinfected infants, at either timepoint, suggesting no difference in the IgG response against non-EBV antigens.

Modeling Anti-gp350 Antibody Response in Asymptomatic Infants

Because the only observable difference in the antibody response between infants infected with a single type of EBV compared to

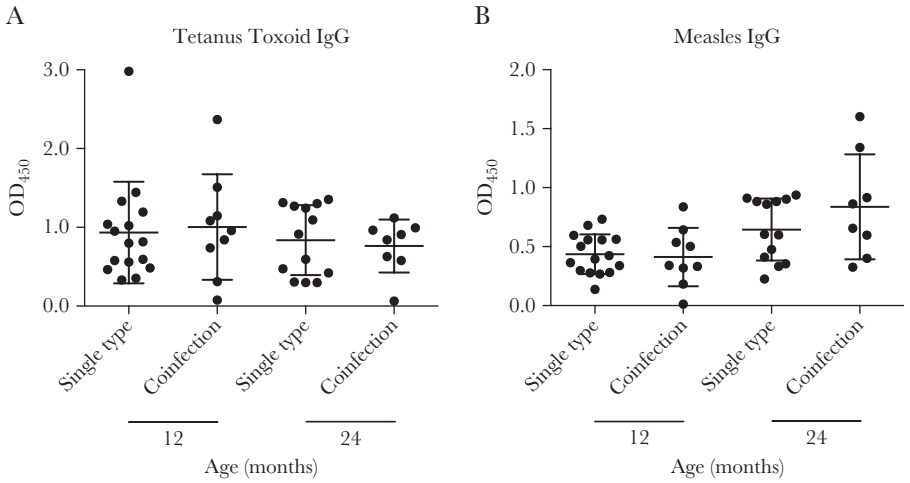

Figure 3. Analysis of the IgG response to (*A*) tetanus toxoid and (*B*) measles vaccination. OD_{450} values were compared between single-type infected and coinfected infants at 12 months (n = 16 and n = 9, respectively) and 24 months of age (n = 13 and n = 8, respectively; 12 months tetanus toxoid $P = .677$ and measles $P = .792$; 24 months tetanus toxoid $P = .645$ and measles $P = .500$.

those infected with multiple types was a diminished gp350 IgA magnitude at the peak of the response in those that acquired a secondary infection, we wanted to evaluate these responses longitudinally using a piecewise linear model (Figure 4). The piecewise linear model generates 2 slopes, representing 2 distinct phases of the response. These 2 slopes meet at a breakpoint, which might indicate the change from the first to the second phase of the response over time. The gp350 IgA and gp350 IgG models were analyzed at 0, 2.5, 5, 6.4 (average time of coinfection) 10, and 15 months postinfection. The models were also compared at the breakpoint, 8.4 or 6.7 months postinfection, for IgA and IgG, respectively. We also compared the slope of the line, which represents the rate of change in antibody levels, before and after the breakpoint. We observed no statistically significant difference in any comparison performed for gp350 IgG (Table 1). However, for gp350 IgA, we observed that the coinfected infants had log(IgA) levels that were significantly lower than the single-type infected infants at 5 months and at the average age of coinfection (6.4 months; $P = .03$ and $P = .033$, respectively) and marginally significant lower levels at 2.5 months and the breakpoint (8.4 months; $P = .058$ and $P = .052$, respectively). Interestingly, there was no difference in the slopes of the line, either before or after the breakpoint (Table 1). This suggests that the anti-gp350 IgA response was diminished in infants prior to secondary infection with a different EBV type.

DISCUSSION

Since the first description of the second strain of EBV, few studies have characterized the natural history of EBV-2. This is the first longitudinal study to evaluate the acquisition of EBV type following primary infection in infants in a region where both

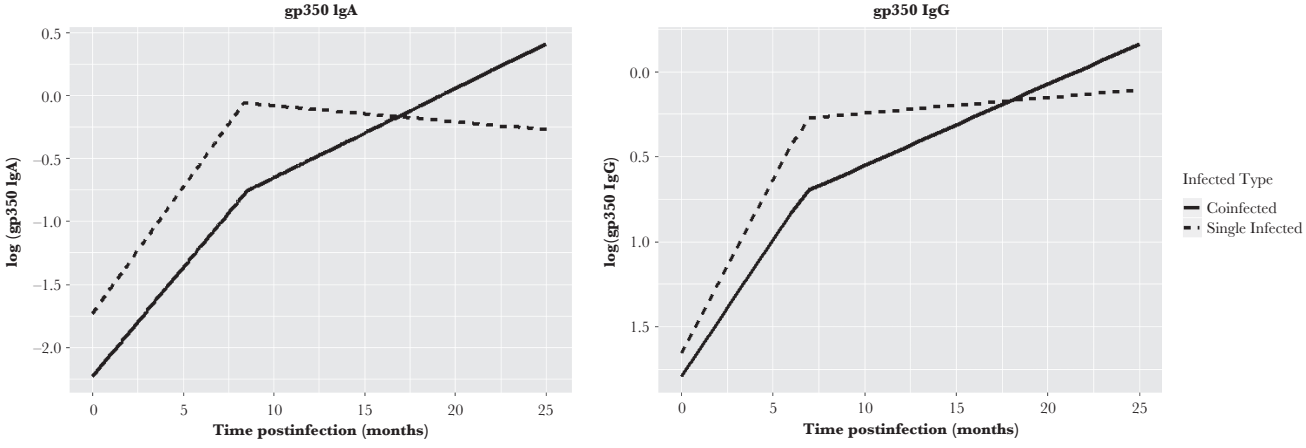

Figure 4. Piecewise linear model of log transformed enzyme-linked immunosorbent assay (ELISA) data for glycoprotein 350 (gp350) IgA and gp350 IgG. Time was normalized to 0 as initial detection of Epstein-Barr virus by polymerase chain reaction.

Table 1. Longitudinal Comparison of the gp350 Antibody Response in Single Type and Co-infected Infants

	gp350 IgA			gp350 IgG		
	Single Type (95% CI)	Coinfection (95% CI)	*P* Value	Single Type (95% CI)	Coinfection (95% CI)	*P* Value
0 mo[a]	−1.94 (−2.75 to −1.13)	−2.44 (−3.35 to −1.53)	.166	−2.39 (−3.15 to −1.63)	−2.58 (−3.38 to −1.68)	.672
2.5 mo[b]	−1.23 (−1.61 to −0.86)	−1.8 (−2.25 to −1.35)	.059	−1.15 (−1.50 to −0.80)	−1.39 (−1.82 to −0.97)	.373
5 mo[c]	−0.73 (−1.09 to −0.38)	−1.36 (−1.79 to −0.92)	.030*	−0.64 (−0.99 to −0.28)	−0.99 (−1.43 to −0.55)	.217
Average time of coinfection (6.4 mo)[d]	−0.45 (−0.83 to −0.07)	−1.11 (−1.58 to −0.64)	.033*	−0.35 (−0.75 to 0.05)	−0.76 (−1.25 to −0.28)	.192
Breakpoint (IgA 8.4 mo; IgG 6.7 mo)[e]	−0.05 (−0.50 to 0.39)	−0.76 (−1.32 to −0.20)	.052	−0.29 (−0.70 to 0.12)	−0.72 (−1.22 to −0.22)	.190
10 mo[f]	−0.07 (−0.47 to 0.33)	−0.64 (−1.14 to −0.15)	.078	−0.24 (−0.60 to 0.10)	−0.55 (−0.97 to −0.13)	.203
15 mo[g]	−0.13 (−0.49 to 0.23)	−0.28 (−0.77 to 0.21)	.182	−0.20 (−0.54 to 0.14)	−0.31 (−0.77 to 0.14)	.248
Slope before breakpoint[h]	0.20 (0.13 to 0.27)	0.18 (0.09 to 0.26)	.653	0.20 (0.14 to 0.27)	0.16 (0.08 to 0.24)	.420
Slope after breakpoint[i]	−0.01 (−0.06 to 0.04)	0.073 (0.00 to 0.15)	.079	0.01 (−0.03 to 0.05)	0.05 (−0.01 to 0.11)	.300

Estimate statements were generated using SAS and reported with a 95% confidence interval (CI). * = $P \leq .05$. Single Infection, n = 19; Co-infection, n = 13.

[a,b,c,d,e,f,g] $\log(OD_{450})$.

[h,i] $\log(OD_{450})$/month.

types of EBV are common. Our study revealed a high prevalence of coinfection with both EBV types and evidence that a primary infection with one EBV strain did not prevent infection with the second strain. Importantly, we found that secondary infection occurred in infants with a lower level of anti-gp350 IgA antibodies, but not anti-gp350 IgG, suggesting that coinfection occurred in infants that failed to mount an adequate IgA immune response to gp350, the EBV glycoprotein critical for viral entry in B cells.

In this study, we analyzed the EBV genotype at each EBV-positive timepoint for 36 infants that were shown to have multiple peaks of viral load in a previous analysis [22]. These infants could have also been characterized as "poor controllers", defined as having an EBV-positive timepoint 3 months or longer after primary infection [32]. Interestingly, we observed a high frequency of coinfection with both types of EBV (44.4%). In contrast, a cohort in the UK showed only 6% coinfection; however, the frequency of EBV-2 is much lower in this population [33]. Coinfection was much higher, (50%–60%) in an HIV-positive cohort from the Netherlands, consistent with a higher frequency of EBV-2 in HIV infection [6]. An earlier study in Kenya and Papua New Guinea that relied on lymphoblastoid cell line (LCL) outgrowth, rather than PCR-based methods, showed no evidence of coinfection [5]. However, higher frequencies of coinfection are observed when PCR methods are used, rather than tumor samples or LCLs [34].

Serum antibody titers against gp350 are correlated to the ability of serum to neutralize EBV infection of B cells [17]. In this study, we observed a reduced level of gp350 IgA levels at peak response in infants coinfected with 2 EBV types, compared to those that were infected with a single EBV type, but no difference in gp350 IgG levels. We hypothesize that the diminished gp350 IgA could be due to differences in exogenous factors, such as transforming growth factor β-1, B-cell activating factor, or a proliferation-inducing ligand (APRIL), which drive IgA class switching [35]. In a study of EBV-seropositive healthy adults or patients with AIM from Canada, there was no detection of anti-gp350 IgA antibodies in the serum [36]. However, we observed this response in all infants tested. gp350 IgA antibodies have been shown to enhance the infection of polarized epithelial cells, but neutralize B-cell transformation [37]. Interestingly, elevated anti-gp350 IgA antibodies have been shown to be a risk factor for nasopharyngeal carcinoma [38]. These antibodies have been suggested to have a potential pathogenic role in epithelial cell infection and diseases of this cell type [39]. However, our data suggest they can be helpful in protection against coinfection. This suggests a role for serum IgA antibodies for protection against B-cell infection, but a less-protective role in the mucosa, where EBV may first infect epithelial cells.

A comparable longitudinal study analyzed the kinetics of the anti-EBV humoral immune response in American college students with AIM [12]. They reported a 333-day delay after symptom onset before peak gp350 IgG response by ELISA. We observed almost identical timing for the development of peak anti-gp350 IgG levels in the plasma (334.5 days postinfection). Another study focusing on AIM showed a biphasic response [19], which was not consistently observed in our study, potentially because we only evaluated antibody levels through 3 years. Furthermore, we observed more incidences of recurring viral load, compared to what is typically seen post-AIM [40]. EBNA-1 responses have been shown to be delayed due to limited access for class II MHC processing and delayed $CD4^+$ T-cell responses [41]. Consistent with this, a previous longitudinal cohort of college-aged adults showed a delayed response for EBNA-1 IgG compared to VCA IgG, similar to what we see in our study [10]. Overall, we observed the pediatric antibody response to be relatively similar to that of adults with AIM for VCA IgG, gp350 IgG, and EBNA-1 IgG.

We cannot rule out that all infants were initially infected with both EBV types and that detection of a second EBV type at a later time represented a new infection rather than reactivation from

latency from the initial infection. However, it is likely that EBV in circulation in healthy children is indicative of cell-associated virus and therefore a latent infection not a replicating infection. A limitation to our study is that we do not have sufficient samples from infants to distinguish these possibilities. We did observe a boosted antibody response against EAd and Zta in some infants, potentially indicative of reactivation [26]. However, this was not consistent among study participants. Another limitation of this study was the small sample size, which made it difficult to detect differences in the slope of the response that were evident post-breakpoint in anti-gp350 IgA.

The EBV gp350 protein is a target for an EBV vaccine [20]. As EBV is transmitted through saliva, an IgA response is likely to be critical in protection against infection. This is supported by our data where we found a significantly lower level of anti-gp350 IgA in infants that acquire infection with a second strain of EBV. The role of gp350 IgA antibodies to protect against secondary infection and a potential role in the development of an efficacious EBV vaccine should be further explored.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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