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Exposure to Holoendemic Malaria Results in Elevated Epstein-Barr Virus Loads in Children

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Perennial and intense malaria transmission (holoendemic malaria) and Epstein-Barr virus (EBV) infection are 2 cofactors in the pathogenesis of endemic Burkitt lymphoma (eBL). In the present study, we compared EBV loads in children living in 2 regions of Kenya with differing malaria transmission intensities: Kisumu District, where malaria transmission is holoendemic, and Nandi District, where malaria transmission is sporadic. For comparison, blood samples were also obtained from US adults, Kenyan adults, and patients with eBL. Extraction of DNA from blood and quantification by polymerase chain reaction give an EBV load estimate that reflects the number of EBV-infected B cells. We observed a significant linear trend in mean EBV load, with the lowest EBV load detected in US adults and increasing EBV loads detected in Kenyan adults, Nandi children, Kisumu children, and patients with eBL, respectively. In addition, EBV loads were significantly higher in Kisumu children 1–4 years of age than in Nandi children of the same age. Our results support the hypothesis that repeated malaria infections in very young children modulate the persistence of EBV and increase the risk for the development of eBL.

Both Epstein-Barr virus (EBV) infection and perennial and intense exposure to *Plasmodium falciparum* malaria (holoendemic malaria) have been proposed to be requisite cofactors in the pathogenesis of endemic Burkitt lymphoma (eBL); how these 2 pathogens interact to drive the emergence of the malignant B cell clone remains unknown. Seminal studies by de-The et al. demonstrated that elevated titers of antibody against EBV viral capsid antigen (VCA) preceded the development of eBL [1]. On the basis of both this observation and others' data supporting a role for EBV as a cofactor in the etiology of eBL [2, 3], de-The proposed that perinatal infection with EBV results in a massive infection that increases the risk for eBL [4]. Morrow and Klein extended this hypothesis to propose that early infection with a high dose of EBV increases the number of EBV-infected B cells [5, 6]. That *P. falciparum* malaria infection increases the risk for eBL was suggested by Lam et al. [7], who demonstrated that the number of EBV-infected B cells was higher during an episode of malaria than after recovery. Thus, the consequence of repeated malaria infections and early EBV infection would be an increase in the number of EBV-infected B cells. However, little is known about the maintenance of persistence of EBV in children living in regions where malaria transmission is holoendemic.

EBV establishes a lifelong, latent infection in memory B cells [8]. The measurement of EBV DNA in peripheral blood by polymerase chain reaction (PCR)–based methods is a reliable indicator of the number of latently infected B cells [9]. In healthy, immunocompetent adults, the EBV load is stable from year to year and is typically

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<1 copy/10^5 peripheral blood lymphocytes (PBLs) (equivalent to <10 copies/μg of DNA from whole blood) [9, 10, 11]. EBV loads are more variable and can be >4000 copies/μg of DNA in immunosuppressed individuals and are frequently associated with clinical illnesses [9, 12, 13].

The majority of malaria morbidity and mortality occurs in children <5 years of age in areas where malaria transmission is holoendemic [14]. Acquisition of immunity to malaria is age dependent and occurs only after repeated infections [14]. In addition, children living in areas where malaria transmission is holoendemic often have chronic, asymptomatic infections characterized by malaria parasites in the blood [15]. In countries such as Kenya, a wide range of malaria transmission intensities exist, ranging from areas with low malaria risk and low prevalence of parasites in children <14 years of age (e.g., sporadic malaria) to areas where continual, repeated exposure to malaria occurs throughout the year and the prevalence of parasites in children is >50% (e.g., holoendemic malaria) [14, 16, 17]. The differences in malaria transmission intensity in geographically proximate areas allowed us to test the hypothesis that continual exposure to malaria infections, as occurs in areas malaria where transmission is holoendemic, shifts the balance of persistence of EBV toward higher numbers of infected B cells in children. Our data provide new insights into how differences in exposure to malaria alter the virus-host equilibrium and could potentially lead to emergence of a malignant B cell clone.

PARTICIPANTS, MATERIALS, AND METHODS

To compare EBV loads in a group of children with different exposures to malaria, recruitment of participants and collection of samples were conducted in 2 epidemiologically distinct areas of western Kenya. The first site was located in a region where malaria transmission intensity is holoendemic. This site was in Nyanza Province, Kisumu District, in the sublocation of Kanyawegi (here referred to as "Kisumu"), which is situated on the shore of Lake Victoria. The second study site was located 150 km northeast of Kisumu in the highlands in Rift Valley Province, Nandi District, in the sublocation of Kipsamoite (here referred to as "Nandi"). Malaria transmission in this area is unstable and associated with periodic outbreaks of malaria morbidity. We collected blood samples in August 2002 from children 1–14 years of age, with a roughly equal representation of children 1–4, 5–9, and 10–14 years of age from each study site. Blood samples from Kenyan adults were obtained from Kisumu; those from patients with eBL were obtained from Nyanza Provincial General Hospital, Kisumu; and those from US adults were obtained from Case Western Reserve University (CWRU), Cleveland, Ohio.

Approval for the present study was obtained from the Kenya Medical Research Institute National Ethical Review Committee and the Institutional Review Board for Human Studies at University Hospitals of Cleveland, CWRU, University of Michigan, and SUNY Upstate Medical University. Written, informed consent was obtained from participants; in the case of minors, consent was obtained from guardians of study participants.

Blood samples were collected in sodium heparinzed tubes and transported to the CWRU/Kenyan Medical Research Institute laboratory, which is located at the Center for Vector Biology and Control Research in Kisumu, for processing the same day. Plasma was used for measurement of EBV-specific antibodies by use of an ELISA (Daimedix). Two hundred microliters of blood was collected in EDTA for DNA extraction. DNA was extracted from 200 μL of whole blood by use of a Qiagen DNAeasy kit (Qiagen), in accordance with the manufacturer's protocol. Primers and probes were designed by use of Primer Express software (version 2.0; PE Applied Biosystems), to detect a 70-bp region of the EBV BALF5 gene [18]. The real-time quantitative (RTQ) PCR cycle used was as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The TaqMan Master mix (PE Applied Biosystems) was used for all reactions. To generate a standard curve, we extracted DNA from the Namalwa BL cell line (ATCC CRl-1432), which contains 2 integrated copies of the EBV genome per cell. The correlation coefficient obtained by linear regression analysis was $R^2 = 0.98$. We also analyzed samples for β-actin as a positive PCR control, using commercially available probes and primers (PE Applied Biosystems). EBV load was normalized to the number of β-actin copies and then calculated on the basis of copies of EBV genome per microgram of DNA. Only samples with a positive β-actin signal were used for further analysis.

RESULTS

The demographic characteristics of the study populations are shown in table 1. With the exception of the percentage of children who were found to be positive for parasitemia, no significant differences were observed between study sites. Seventy-seven percent of Kisumu children had *P. falciparum* parasites in their blood, which is consistent with holoendemic malaria transmission (e.g., prevalence of parasites in children is >50%). In contrast, 16% of Nandi children had *P. falciparum* parasites in their blood, which is consistent with the low prevalence of parasites in children <14 years of age and is typical of sporadic malaria transmission. For all parasite-positive children, there was no evidence of anemia or fever, indicating that these were asymptomatic infections. The differences in prevalence of parasites confirmed that children living in these regions experienced different intensities of malaria transmission.

Because our objective was to determine the effect of continual malaria exposure on EBV load, we first needed to determine the frequency of EBV infection for each study site. In addition, we wanted to determine whether any children were experiencing a

Table 1. Demographics of study population for measurement of Epstein-Barr virus (EBV) load in children in Nandi and Kisumu districts, Kenya.

District, characteristic	Age group, years			
	1–4	5–9	10–14	All ages
Kisumu				
EBV seropositive	32 (94)	37 (100)	35 (100)	104 (98)
Mean hemoglobin level, g/dL	9.72	12.25	12.51	11.53
Malaria-positive smear[a]	26 (77)	27 (73)	29 (83)	82 (77)
Mean body temperature, °C	36.8	36.8	36.9	36.8
Total	34 (32)	37 (35)	35 (33)	106 (100)
Nandi				
EBV seropositive	36 (92)	50 (100)	41 (100)	127 (98)
Mean hemoglobin level, g/dL	12.18	12.82	13.29	12.77
Malaria-positive smear	3 (8)	7 (14)	11 (26)	21 (16)
Mean body temperature, °C	37.21	37.2	37.1	37.2
Total	39 (30)	50 (38)	41 (32)	130 (100)

NOTE. Data are no. (%) of children, unless otherwise noted.

[a] *Plasmodium falciparum* parasitemia was determined by thick blood smear analysis by use of Geimsa staining and microscopic examination [37].

primary infection that could account for unusually elevated EBV loads. Plasma was analyzed for the presence of EBV antibodies by use of an EBV-specific ELISA, to detect IgG to EBV nuclear antigen, IgM and IgG to EBV VCA, and IgG to EBV early antigen D complex. We observed that the seroprevalence of EBV was the same between Nandi and Kisumu children: 98% of the samples tested were positive for EBV infection (table 1). The 5 EBV-seronegative children were excluded from further analysis. None of the study participants had evidence of primary EBV infection (positive for VCA IgM) (data not shown).

EBV load was measured by RTQ-PCR in EBV-seropositive children and calculated as copies of EBV per microgram of DNA. At both study sites, regardless of malaria transmission intensity, we observed a high frequency of children in whom EBV load was readily detectable (68.3% in Nandi and 75.0% in Kisumu). The EBV load data were highly skewed to the right, and, thus, log transformation was performed after adding a value of 0.5 to samples that had <1 copy/μg of DNA, to make all values positive before performing the log transformation. We omitted the EBV load data on 1 Kisumu child whose standardized load was >3 SD above the mean. The EBV loads for individual participants are shown in figure 1. We observed a higher mean $\pm$ SE EBV load (log EBV copies per microgram of DNA) in Kisumu children (2.10 ± 0.38), compared with that in Nandi children (1.00 ± 0.29). We also determined the EBV loads in EBV-seropositive US adults (-1.98 ± 0.46), Kenyan adults (0.34 ± 0.56), and children with eBL (5.69 ± 1.31), who served as reference populations. We submitted the log-transformed EBV load data to a 1-way analysis of variance (ANOVA) with a priori contrasts. There was a significant overall difference in EBV load across groups ($P < .001$), with a significant linear trend contrast of increasing EBV load in groups, in the following order: US adults, Kenyan adults, Nandi children, Kisumu children, and children with eBL ($P < .001$).

Because malaria morbidity is age dependent and malaria immunity is gradually acquired [19], we wanted to determine whether there was an age-related difference in EBV load between Kisumu children and Nandi children. We categorized the children according to age (1–4, 5–9, and 10–14 years) and submitted their EBV load data to a 2-factor ANOVA, crossing age group and region (Nandi vs. Kisumu). The analysis revealed a significant age-by-region interaction ($P < .05$), indicating that the association between age and EBV load differed for children from the 2 regions. Figure 2 illustrates this effect: EBV loads in Kisumu children 1–4 years of age were significantly higher than those in Nandi children 1–4 years of age. However, by 5–9 years of age, the mean EBV load decreased and remained at the same level as in the children 10–14 years of age. In contrast to the high EBV loads in Kisumu children 1–4 years of age, the EBV load remained the same across all age groups in Nandi children. In addition, ANOVA of the mean EBV load in each age group yielded a significant difference in the means of Kisumu versus Nandi children 1–4 years of age ($P < .01$). No significant differences in EBV load were observed between other age groups.

DISCUSSION

The interaction between EBV and holoendemic malaria has long been postulated as a requirement for the development of eBL, but the effect of holoendemic malaria on persistence of EBV in healthy children living in regions where malaria transmission is holoendemic has not been investigated. In the present study, we compared EBV loads in a cohort of children

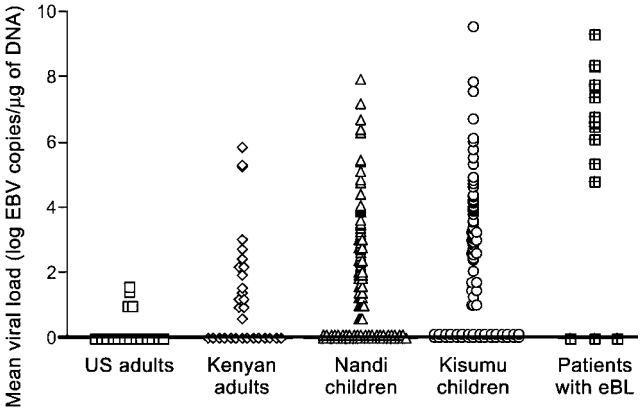

Figure 1. Epstein-Barr virus (EBV) loads in different populations. EBV load was determined by real-time quantitative polymerase chain reaction for US adults ($n = 17$), Kenyan adults ($n = 31$), Nandi children ($n = 127$), Kisumu children ($n = 96$), and patients with endemic Burkitt lymphoma (eBL) ($n = 15$). Values obtained were log transformed, and the mean ± SE EBV load for each group was calculated. Each object represents the EBV load obtained from an individual.

living in 2 regions of Kenya with differing malaria transmission intensities. We found significantly higher EBV loads in children 1–4 years of age living in a region where malaria transmission is holoendemic, compared with those in children of the same age living in a region where malaria transmission is sporadic. This is the same age group of children in whom malaria morbidity and mortality is the most severe [14] and precedes the age at which the majority of eBL cases, which are found primarily in children 5–7 years of age, emerge [20, 21].

The elevated EBV loads observed in Kisumu children 1–4 years of age are striking, since they are similar to those reported for some organ-transplant patients and are more typical of a chronic high-viral-load infection than of a persistent low-viral-load infection [9, 13, 22] and suggest that recurrent malaria infections affect either the establishment and/or the maintenance of EBV latency. Three possible but not exclusive mechanisms could exist. Malaria causes polyclonal B cell activation [23]; therefore, the higher EBV load in the children 1–4 years of age in the region where malaria transmission is holoendemic could reflect an indirect expansion of EBV-infected B cells. In support of this, Lam et al. [7] demonstrated that children with acute malaria had a higher frequency of EBV-infected B cells than did children who had recovered from malaria. In that study, analysis of healthy children was not performed, so it is not known whether the data were comparable to the population in general. An alternative explanation is that EBV-specific immunity is suppressed. Whittle et al. demonstrated that, during an episode of acute malaria, spontaneous outgrowth of EBV-transformed B cells occurred at a greater frequency in children suffering from acute malaria [24], which suggests impaired EBV-specific immunity. Reduced T cell responses could lead to higher EBV loads, as is seen in both patients after transplant and patients with AIDS [13, 22]. Studies in Kenya to test these possibilities are ongoing. The final possibility was originally raised by de-The [4], who suggested that perinatal infection with EBV, as occurs in African countries, could result in a massive primary infection. Given that there is a difference in EBV load between the cohorts of children with divergent levels of exposure to malaria, our data do not support the hypothesis per se that perinatal infection results in higher EBV loads. However, it is possible that concurrent malaria infection or malaria infection preceding EBV infection could result in elevated EBV loads, possibly because of expansion of the target B cell population.

The question remains whether the EBV loads we observed in Kisumu children 1–4 years of age are indicative of an emergent pathological state. In one study, it was argued that healthy US children had 1 EBV genome/μg of peripheral blood mononuclear cell (PBMC) DNA (range, 0–35 EBV genomes/μg of PBMC DNA) [25]; thus, anything above that threshold would be considered pathological. Values from these and other studies [18, 26, 27] are difficult to compare with our data, since several methods have been used to define EBV load, including competitive PCR (cPCR), in situ hybridization, and RTQ-PCR. More recently, Wadowsky et al. [9] directly compared several methods for quantifying EBV load and also the source of EBV DNA (whole blood, plasma, or PBLs). They found that com-

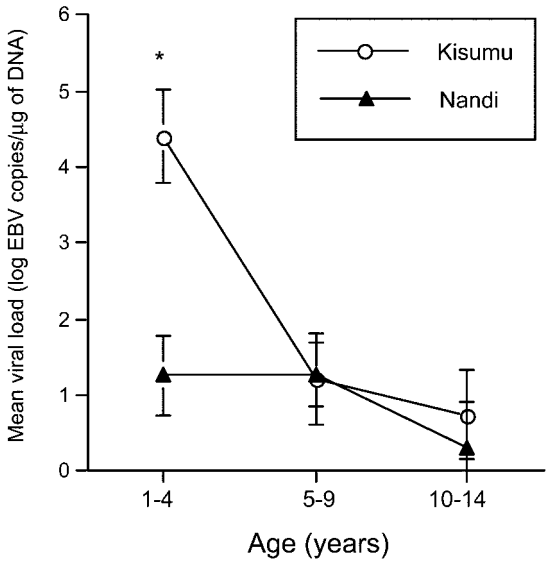

Figure 2. Comparison of Epstein-Barr virus (EBV) loads in children of different age groups from Nandi and Kisumu districts. Children were categorized into 3 age groups (1–4, 5–9, and 10–14 years), and the mean EBV load for each age group was determined. Error bars indicate SE. $^*P < .01$.

parable results were obtained whether EBV DNA was quantified by TaqMan PCR, as we have done, or by cPCR. Moreover, they provided a comparison of EBV load values obtained by use of different methodologies, allowing a more direct comparison of EBV load data. They proposed grouping EBV loads into 4 groups that correlate with EBV loads found in different populations. EBV loads in group III and IV are considered to be elevated above those found in healthy, immunocompetent individuals (>4000 copies EBV DNA/mg), on the basis of data obtained from monitoring EBV load in transplant patients. Using this EBV load grouping for comparison, we observed an age- and malaria exposure–dependent trend, with patients with eBL having the highest proportion of EBV loads indicative of disease (group III or IV) and no group III or IV EBV loads in either US or Kenyan adults. What is notable is the number of children in the present study with no evidence of clinical disease who had EBV loads considered to be pathological (4% of Kisumu children). No correlation with the levels of malaria parasitemia was found (data not shown), suggesting that, in our study participants, point-prevalence asymptomatic parasitemia did not directly affect EBV load.

We analyzed EBV load in DNA extracted from whole blood. This method is advantageous since it requires smaller sample size and less handling, compared with isolation of lymphocytes from blood, both of which are critical considerations when doing field-based studies. Others have validated this method and have found that measurement of EBV load from whole blood was comparable to isolation of PBLs [9] and preferable for routine clinical monitoring [28]. A disadvantage of this assay is its inability to distinguish whether the elevated EBV load is due to an increase in the number of EBV-infected B cells within the peripheral blood, a higher number of EBV copies per infected B cell, or both. Of note, however, is the study by Rose et al. [22], who found that the elevated EBV loads in pediatric organ transplant patients were due to an increase in the number of EBV-infected cells and not to an increase in the number of EBV copies per infected B cell.

In our cohort, there were only 5 children—all <4 years of age—who were EBV seronegative, confirming results of previous studies that demonstrated a high rate of EBV infection among young children in Africa [29, 30]. Of the children who were EBV seropositive, EBV load was readily detectable, regardless of malaria exposure, in >66% of study participants. This contrasts with reports of EBV loads in the peripheral blood of healthy US or European children—<15% of EBV-seropositive children had detectable levels of EBV DNA [31], and 90% of healthy EBV-seropositive blood donors were found to be negative for EBV by PCR [32]. One possibility is that perinatal infection with EBV, as occurs in Africa, might result in a higher EBV load set point. Thus, the pattern of persistence of EBV is clearly different in this setting, regardless of the effect of malaria, and it suggests that establishment of EBV latency might be differentially regulated in regions where primary infection frequently occurs in young children, such as Africa.

The differences in EBV load between Nandi children and Kisumu children also mirror the differences in incidence of eBL between these 2 regions. Kisumu is in Nyanza Province, whereas Nandi is in Rift Valley Province. On the basis of a 10-year retrospective analysis of eBL cases in Kenya [33], we have found that there is a 6-fold higher annual incidence of eBL in Nyanza Province than in Rift Valley Province. What is unknown is whether there are differences in EBV strains between these regions that might constitute an alternative explanation for the differences in increased risk for eBL and higher EBV load in young Kisumu children. There are 2 EBV strains, types A and B. Within these 2 types, other polymorphisms exist. Examination of the EBV strain in Burkitt lymphoma (BL) tumors of Kenyan origin did not reveal any differences between the viral strain found in the general population in which BL is endemic and the viral strain isolated from the tumors [34]. In other studies, no associations between viral subtypes, geographical origins of patients, and clinical presentation have been noted [35]. Moreover, the geographical distribution of eBL in Africa is more consistent with exposure to holoendemic malaria than with the spread of a particular viral strain [36]. Nonetheless, studies are needed to determine whether there are differences in the circulating strains of EBV in these distinct geographic regions, to exclusively rule out the possible contribution of a viral strain to the increased incidence of eBL in Nyanza Province.

We observed significantly higher EBV loads in patients with eBL, compared with those in all other study groups. This is in agreement with the findings of Stevens et al. [28], who demonstrated that patients with eBL from Malawi had higher EBV loads, compared with those in control subjects. It should be noted that, in that study, control subjects were not matched for age and were close relatives (usually mothers). Elevated EBV loads are predictors of EBV-associated lymphoproliferative disorders that occur in immunocompromised patients [27, 28], so, perhaps, this is true for eBL as well. Since eBL is a systemic disease, it is also possible that the elevated EBV loads in these children indicate tumor cells rather than latently infected B cells.

In summary, significantly elevated EBV loads were found in children 1–4 years of age living in a region in Kenya where malaria transmission is holoendemic. It remains to be determined whether recurrent malaria infections affect either the establishment or maintenance of EBV latency. We propose that this high viral burden, indicative of the number of infected B cells in the peripheral blood, increases the risk for development of eBL.

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