Clearance of Circulating Epstein-Barr Virus DNA in Children with Acute Malaria after Antimalaria Treatment

Daria Donati,1 Eva Espmark,2 Fred Kironde,4 Edward Katongole Mbidde,4 Moses Kamya,5 Åke Lundkvist,2,3 Mats Wahlgren,3 Maria Teresa Bejarano,1,3 and Kerstin I. Falk2,3

1Center for Infectious Medicine, Karolinska University Hospital–Huddinge, 2Swedish Institute for Infectious Disease Control, and 3Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden; 4Department of Biochemistry and 5Faculty of Medicine, Makerere University, and 6Ugandan Cancer Institute, Kampala, Uganda

Children living in malaria-endemic regions have a high incidence of Burkitt lymphoma (BL), the etiology of which involves Plasmodium falciparum malaria and Epstein-Barr virus (EBV) infections. In the present study, we compared EBV DNA loads in plasma and saliva samples from Ugandan children with acute malaria (M+) at the time of diagnosis and 14 days after antimalaria treatment, children without malaria (M−), and children with BL. EBV DNA was detected, by real-time polymerase chain reaction, in 31% of the plasma and in 79% of the saliva samples from children in the M+ group. Antimalaria treatment led to clearance of plasma viral load in 85% of the cases but did not affect the levels in saliva. There was a significant difference in plasma EBV loads across the groups. The lowest levels were detected in samples from the M− group, increased levels were detected in samples from the M+ group, and levels reached the highest values in samples from children with BL. The same trend was evident in the frequency and levels of anti-BZLF1 antibodies, which is indicative of viral reactivation. In the M+ group, the positive plasma samples clustered around 7–9 years of age, the peak incidence of BL. The clearance of circulating EBV after antimalaria treatment suggests a direct relationship between active malaria infection and viral reactivation.

Malaria and Epstein-Barr virus (EBV) infection are recognized as cofactors in the genesis of endemic Burkitt lymphoma (BL), the most common pediatric cancer in equatorial Africa, which accounts for up to 74% of childhood malignancies [1]. EBV infects >95% of the human population. African children are infected early in life, and nearly all have seroconverted by 3 years of age, whereas, in affluent countries, primary infection is often delayed until adolescence [2]. After primary infection, EBV establishes a lifelong persistent infection, usually without adverse health consequences, that relies on a balance between viral latency, viral replication, and host immune responses. The impact of malaria infection on EBV reactivation remains unclear. Acute malaria impairs the EBV-specific immune responses, leading to an increased number of circulating EBV–carrying B cells [3, 4]. Whether this increase reflects proliferation of previously infected B cells or increased virus production and subsequent infection of bystander B cells or whether it reflects both has not yet been established. Recent evidence suggests that viral reactivation is common among children living in malaria-endemic areas. We have shown that children, but not adults, living in malaria-endemic areas have high levels of EBV DNA in serum, an indicator of active EBV replication [5]. Comparison of EBV DNA levels in blood from children living in 2 areas with different malaria-transmission intensities showed a correlation between viral load and the end-
micty of malaria [6]. In the present study, we analyzed the direct effect of Plasmodium falciparum infection on EBV persistence (i.e., the relationship between viral load and the course of malaria). We evaluated the occurrence and levels of EBV DNA in plasma and saliva samples from Ugandan children with acute malaria before and 14 days after antimalaria treatment. Viral loads were higher during the acute phase of malaria and dropped after effective treatment, suggesting that P. falciparum infection leads to viral reactivation. We also report that the viral load in plasma and saliva samples from children with BL was high. The increased viral burden, apparently elicited by acute malaria, may augment the risk of development of BL.

PATIENTS, MATERIALS, AND METHODS

Children with malaria were recruited at the Assessment Centre, Mulago National Referral Hospital, Kampala, Uganda, from October to December, which was after the second rainy season of 2002. Malaria is seasonally mesoendemic in Kampala [7] and occurs with 2 major peaks following the rainy seasons. Children with BL were recruited from the Ugandan Cancer Institute, Kampala. Blood and saliva samples were collected from 43 children with acute noncomplicated malaria (M⁺) on the day of recruitment (M⁺ day 0), from the same children 2 weeks after they had received antimalaria treatment (M⁺ day 14) (age range, 2–15 years; mean age, 6.9 years), from 41 children with mild infections unrelated to malaria (M⁻; age range, 2–14 years; mean age, 6.3 years), and from 26 children with BL (age range, 3–10 years; mean age, 6.4 years). Acute malaria was defined as P. falciparum parasitemia (>2500 asexual parasites/μL). At enrollment, M⁺ children received antimalaria treatment consisting of sulfadoxine (25 mg/kg) and pyrimethamine (1.25 mg/kg) (Fansidar; Roche) on day 0 plus amodiaquine (Camoquine; Park-Davis) (25 mg/kg in 3 divided doses) on days 0, 1, and 2 [8]. Some children received paracetamol and/or an anticough mixture. Parasitemia was undetectable in the convalescent children (M⁺ day 14). Children who received any antimalaria treatment within 72 h before the examination were excluded. The M⁻ control group consisted of children who visited the clinic with a variety of symptoms unrelated to malaria (no parasites were detected in their blood smears). BL was diagnosed by the presence of a tumor mass and histological analysis. Staging was performed according to the Ziegler and Magrath classification. The study protocols were approved by the ethics committees of Makerere University Faculty of Medicine, the Uganda National Council of Science and Technology, and the Karolinska Institutet. Written, informed consent was obtained from guardians of study participants.

Venous blood (2–5 mL) was collected in tubes containing EDTA and was centrifuged to obtain the plasma fraction. All plasma samples analyzed contained IgG antibodies against EBV viral capsid antigen and EBV nuclear antigen–1, which is indicative of previous, but not primary, EBV infection. Sali-va was obtained by the following method: children chewed a sponge for 1 min, then the sponge was washed in 1 mL of PBS. All samples were processed the same day and stored at −20°C until analysis. DNA was extracted from plasma and saliva samples using the QIAamp Blood kit (Qiagen), in accordance with the manufacturer's instructions. EBV DNA was quantified by real-time polymerase chain reaction (PCR) using primers and probes designed to detect a 65-bp region of the EBV LMP-1 gene, as described elsewhere [9]. The albumin gene was quantified using a 139-bp fragment of the human gene [10]. PCR mixtures consisted of TaqMan universal PCR mix, primers, and probes (Applied Biosystems). The cycling profile reaction was 50°C for 2 min, 95°C for 10 min, and 45 times at 95°C for 15 s and 60°C for 1 min. The EBV load is expressed as the log number of EBV DNA copies per milliliter. Log transformation was performed after a value of 1 was added to all samples, to make all values positive. To clarify whether the EBV genomes were free DNA or were included in viral particles, plasma samples (50 μL) were treated with 2 doses of 5 U of DNaseI (Promega) for 1 h at 37°C. Stop buffer was then added, and the mixture was incubated for 10 min at 65°C. DNA was then extracted using the QIAamp Blood kit (Qiagen). To control the effectiveness of DNaseI treatment, the human albumin gene was amplified, treated, and measured in parallel with EBV DNA.

BZLF1 was amplified with specific primers (forward, 5'-GTCGGATCCGACCCAAAACATCGACTTCTGA-3'; reverse, 5'-CATGGAAATCTTAGAAATTTAAGAGATCCT-3'). PCR products were cloned into the pCR4 TOPO vector, and the entire cDNA sequence was directly subcloned into the pTrcHis2 TOPO expression vector (Invitrogen). TOP 10 One-Shot cells (Invitrogen) transformed with the expression vector were induced to express recombinant protein with 1 mmol/L isopropyl-β-thio-

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EBV Load in Children with Malaria

Figure 1. Epstein-Barr virus (EBV) DNA loads in children with acute malaria, before and after antimalaria treatment. A, EBV DNA load quantified by real-time polymerase chain reaction in saliva and plasma samples from children with acute malaria, before (day 0) and after (day 14) antimalaria treatment. Vertical lines depict median values, with 25th- and 75th-percentile values represented by the bottom and top edges of lines, respectively. Each circle represents the EBV load of one patient. Neg/tot, no. of negative samples/total no. of samples. *P < .01. B, Change in EBV load for each child before (day 0) and after (day 14) antimalaria treatment. The continuous line represents no change in EBV load, the dashed lines represent a decrease in EBV load, and the dotted lines represent an increase in EBV load.

RESULTS

To evaluate the direct effect of malaria infection on EBV persistence, we analyzed the EBV DNA load in plasma and saliva samples from children with acute malaria before and after they received antimalaria treatment. EBV DNA was detected in 31% (13/42) of the plasma and in 79% (34/43) of the saliva day-0 samples. EBV DNA was detected in only 5% (2/38) of the plasma and in 93% (38/41) of the saliva day-14 samples (figure 1A). Paired analysis of EBV levels in saliva and plasma samples for each patient before and after treatment showed that, although there was a profound reduction of EBV plasma levels, the respective levels in saliva were unaffected. Antimalaria treatment led to clearance of plasma viral load in 85% (11/13) of M+ children (P < .01, Wilcoxon signed rank test) (figure 1A and 1B). These results demonstrate a direct relationship between circulating EBV level and acute P. falciparum malaria infection.

The proportion of children with detectable EBV in plasma was significantly different between the 3 groups (P < .001, Kruskal-Wallis test) (figure 2). In the M- and BL groups, EBV DNA was found in 18% (7/40) and 89% (23/26) of the samples, respectively. The difference between the M- and the M+ groups was significant, considering the group size (P = .05, Kruskal-Wallis test). There was a progressive increase in the plasma levels of viral DNA from the M- group to the M+ group and
Figure 2. Epstein-Barr virus (EBV) DNA loads in children with acute malaria (M+), children without malaria (M−), and children with Burkitt lymphoma (BL). The EBV DNA load was quantified by real-time polymerase chain reaction in saliva and plasma samples from M+ children, M− children, and children with BL. Vertical lines depict median values, with 25th- and 75th-percentile values represented by the bottom and top edges of lines. Each circle represents the EBV load of one patient. Neg/tot, no. of negative samples/total no. of samples. * ; **.

from the M+ group to the BL group (figure 2). Plasma levels were significantly higher (P = .05, Kruskal-Wallis test) in the M− group (median, 0 copies/mL; mean, 33,693 copies/mL) than in the M+ group (median, 0 copies/mL; mean, 114 copies/mL). Consistent with the findings of previous reports [11, 12], children with BL had significantly higher viral loads (median, 243,228 copies/mL; mean, 1,101,966 copies/mL) than did M− children (P < .01, Kruskal-Wallis test). In contrast to the variation in plasma, there were no significant differences between the proportion and levels of EBV DNA in saliva samples between the groups. The highest saliva EBV DNA levels were in children with BL (median, 1,105,875 copies/mL; mean, 26,695,775 copies/mL) (figure 2).

We characterized the state of EBV in 3 plasma samples with the highest viral load from each of the M+, day-0, and BL groups. DNaseI removed the detectable plasma viral DNA from the samples (data not shown), indicating that the EBV DNA found in plasma was in a naked form. Inside intact virions, viral DNA is protected from DNase digestion by the viral protein capsid coat, whereas naked DNA is susceptible to degradation by the enzyme. A precise estimation of the nature of the EBV DNA could not be done in these samples, since the freezing and thawing had probably affected the state of viral genomes. This kind of determination must be performed using fresh material.

Previous studies have shown a direct correlation between EBV reactivation and the levels of anti-BZLF1 antibody, an immediate/early transcription factor of the EBV replicative cycle [13]. We therefore used the presence of anti-BZLF1 IgG antibodies as an indicator of EBV replication. In the M− and BL groups, but not in the M+ group, there was a correlation between the presence of anti-BZLF1 and EBV in the plasma samples. However, the overall difference between the proportion of positive samples in all groups was significant. Positive samples were classified into 4 groups on the basis of their BZLF1-specific IgG antibody levels. Paired comparison of the proportion of positive samples and antibody levels revealed a significant difference between the M− group and the other 2 groups (P < .001); the proportion in the M+ group (74%) was twice as high as that in the M− group (34%). Among the positive samples, the fraction with high antibody titers was 3-fold higher in the M+ group than in the M− group. Children with BL had the highest proportion (87%) and levels of anti-BZLF1 IgG antibodies (P = .05) (figure 3). Antimalaria treatment did not alter the proportion or the levels of seroreactivity to BZLF1 (P = .165, Wilcoxon test); this lack of effect may relate to the half-life of IgGs. The data suggest that, in the same geographical region, the frequency of EBV productive replication is higher among children with acute P. falciparum infection than it is among children who are not actively infected with the malaria parasite.

Because malaria morbidity is age dependent and malaria immunity is gradually acquired [14], we analyzed the relationship between viral load, age, and sex. No correlation was found between these variables, although, in the M+ group, the presence of EBV DNA clustered in samples from children 7–9 years of age.

Children with BL served as an internal reference for the study. Altogether, their plasma and saliva viral loads were the highest of the 3 groups tested and at least 10 times higher than those in the M+ group. The levels of EBV DNA in saliva reached 9 billion copies/mL (figure 2) and were significantly higher in
and high without anti-BZLF1 IgG Burkitt lymphoma (BL). Bar fractions indicate the percentage of samples 

2 pathogens. To assess the relationship between 
P. falciparum 
malaria. We found high levels of circulating EBV DNA loads in children during and after an episode of acute malaria infection and EBV reactivation, we compared EBV loads and proportion of samples with anti-BZLF1 were significantly higher among M+ children. Since EBV DNA is usually not detected in the plasma of healthy individuals [16, 17], its presence is interpreted as an indicator of viral reactivation [16, 18]. High levels of anti-BZLF1 are also associated with increased EBV DNA loads in blood and correlate with high titers of antibodies characteristic of EBV reactivation [13].

The mechanisms that may lead to B cell expansion and/or viral reactivation during P. falciparum malaria are not well understood. Our recent identification of a polyclonal B cell activator in P. falciparum is of relevance in this context. We have established that infected erythrocytes (IEs) and the cysteine-rich interdomain region–1α (CIDR1α) of the P. falciparum erythrocyte membrane protein–1 (PIEMP1) interact with B lymphocytes, inducing activation and immunoglobulin secretion [19]. Of note, it was recently demonstrated that plasma-cell differentiation initiates EBV replication in vivo [20]. The hyperglobulinemia that characterizes human malaria suggests that IEs and malarial parasite antigens, such as CIDR1α, can induce B cell proliferation and plasma-cell differentiation. Most likely, therefore, the plasma-cell differentiation induced by malaria results in viral reactivation. Moreover, the immunoglobulin-binding capacity of CIDR1α and other P. falciparum antigens [19, 21] could induce viral replication in the way that anti-immunoglobulin and rheumatoid factor induce viral production in circulating EBV–carrying B cell lines [22, 23].

The clearance of circulating EBV DNA after antimalaria treatment may have resulted from elimination of the parasite and from a direct effect of the drugs on B cells—Fansidar is known to inhibit lymphocyte proliferation [24]. Of note, however, in a previous study, treatment with another compound (chloroquine) reduced, in a related way, the number of circulating EBV–carrying B cells [3]. Although malaria treatment cleared the plasma EBV load in nearly all M+ children, the levels of virus in saliva samples were not significantly affected and did not vary between the different groups. After primary infection, EBV can be found in 3 different compartments: the oral cavity, blood lymphocytes, and the blood cell–free fraction. Characterization of viral strains in these compartments during primary infection indicates that, despite the possible exchange among them, infection in the oral cavity appears to be self-renewing and independent of replenishment from peripheral lymphocytes [25]. The apparent lack of impact of P. falciparum

Figure 3. Levels of anti-BZLF1 antibodies in the different groups. Anti-BZLF1 IgG levels were estimated by ELISA and compared between children with acute malaria (M*), children without malaria (M+), and children with Burkitt lymphoma (BL). Bar fractions indicate the percentage of samples without anti-BZLF1 IgG (black) and with low (white), medium (light gray), and high (dark gray) levels.

boys (median, 3,120,050 copies/mL; mean, 40,169,228 copies/mL) than in girls (median, 207,414 copies/mL; mean, 966,279 copies/mL) (P = .02, Mann-Whitney U test). Given the higher incidence of BL among boys, we recruited 17 boys and 6 girls in this group. There was a correlation between EBV load and BL disease progression (figure 4)—the increase in EBV load was more evident in the plasma samples. Although most of the studied BL tumors were located in the oral cavity, there was no correlation between plasma or saliva viral load and site of the tumor (data not shown).

DISCUSSION

Despite the well-established relationship between endemic P. falciparum malaria infection and EBV infection in the genesis of BL, little research has examined the interaction between these 2 pathogens. To assess the relationship between P. falciparum malaria infection and EBV reactivation, we compared EBV loads in children during and after an episode of acute P. fal- ciparum malaria. We found high levels of circulating EBV DNA in plasma samples from children with acute malaria, and, interestingly, in 85% of the cases, the EBV loads dropped to undetectable levels after antimalaria treatment.

Besides the known inhibitory effect of acute malaria on EBV-specific T cell responses [3, 4, 15], there are different, but not exclusive, mechanisms by which acute malaria could increase the viral load affecting the B cell compartment: (1) expansion of EBV–carrying B cells; (2) induction of apoptosis in the infected B cell pool, with consequent release of virus/viral DNA; or (3) increase of viral replication. Although we acknowledge the possibility of the first mechanism, the present study was performed on plasma samples and could not address it. The DNAse sensitivity of plasma viral DNA suggests that it is released from dying infected cells, probably as a result of protracted B cell activation. However, the present data also suggest that malaria plays a role as an enhancer of viral replication. Evidence for this assumption is provided by the presence of elevated plasma viral loads in the M+ children, by its clearance after antimalaria treatment, and by the lower frequency and level of circulating EBV among M− children. Furthermore, the levels and proportion of samples with anti-BZLF1 were significantly higher among M+ children. Since EBV DNA is usually not detected in the plasma of healthy individuals [16, 17], its presence is interpreted as an indicator of viral reactivation [16, 18].
infection on the salivary compartment, where reactivation of latently infected B cells seems to occur in healthy carriers [20, 26], could imply that acute malaria does not alter the compartment normally associated with EBV persistence. On the other hand, it suggests that malaria elicits viral reactivation in a distinct yet undefined compartment, leading to a pathological condition reflected by the presence of EBV in the plasma. The question remains whether this reflects a changed migration pattern or abnormal B cell proliferation imposed by malaria or whether it reflects both. Possible sources of circulating EBV are mucosal lymphocytes, lymph nodes, and the spleen. During the course of malaria, the most likely source of circulating viral DNA is the spleen, where IEs are trapped and where B cells represent ~40% of the splenocytes. Thus, splenic B cells may be activated by malarial parasite antigens not only presented on the IEs but phagocytosed and presented as soluble antigens or as immune complexes by dendritic cells in the presence of cytokines, T cell help, and costimulatory signals. This hyperstimulation would lead to reactivation of latently infected B lymphocytes present in or migrating to the spleen and enhanced viral production. Moreover, acute malaria may contribute to B cell death/apoptosis, leading to release of viral DNA. These assumptions are supported by the clearance of circulating viral DNA after antimalaria treatment. The contribution of malaria to viral reactivation could be further amplified by the known inhibitory effect of malaria on EBV-specific T cell responses [4, 15]—in healthy individuals, the viral load seems to be controlled by cytotoxic T lymphocytes directed against lytic, rather than latent, viral proteins [27].

We and others recently reported that children living in malaria-endemic areas have an increased viral load, compared with adults living in the same area or healthy European children [5, 6]. In the present study, the proportion of children with detectable EBV DNA in plasma was higher in the M+ group than in the M− group; however, the size of the sample may limit the generalization of this finding. The plasma levels of EBV DNA in M+ children were higher than those in patients with acute EBV infection [16, 17] or patients who received organ transplants [28], using comparable methodology. Whether this reflects a pathological state or a difference in the maintenance of EBV persistence remains to be defined. The high viral load in plasma samples from M+ children may have resulted from an early age of primary EBV infection combined with other chronic infections, such as malaria, which is prevalent in equatorial Africa. The relationship between the relative risk of development of BL and the presence of antibodies to lytic EBV antigens [29] should be considered, since EBV reactivation is a factor that contributes to tumor development. In the present study, the levels of viral DNA did not correlate with age. Nonetheless, in the M+ group, elevated plasma EBV loads clustered in samples from children around 6–10 years of age, the peak age incidence of BL [30]. The lack of correlation between viral load and age contrasts with the findings of Moorman et al., [6] who found higher viral loads in children 1–4 years of age living in a malaria-holoendemic region of Kenya. This difference may be explained by the sample size and the lower endemicity of malaria in Kampala, the location of the present study.

The observed high levels of EBV DNA in children with BL and their relation to the clinical stage is in line with previous reports that indicated that, in EBV-associated malignancies, there is a correlation between viral DNA plasma levels and dis-
ease activity [17, 31]. The viral load was at least 30 times higher in the children with BL than in the M+ children. These results, together with the correlation to clinical stage, suggest that the origin of viral DNA is tumoral.

In conclusion, we have shown that *P. falciparum* malaria infection leads to increased levels of circulating EBV. The plasma EBV load is cleared after antimalaria treatment, suggesting that malaria parasite infection has a direct effect on the maintenance of EBV persistence, increasing viral reactivation. Moreover, these results provide further insights into the mechanisms by which malaria contributes to the development of BL.

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