Background. To investigate how intense Plasmodium falciparum infection predisposes to Epstein-Barr virus (EBV)–positive Burkitt lymphoma (BL), we analyzed the effect of acute malaria on existing EBV-host balance.

Methods. EBV genome loads in peripheral blood mononuclear cells were assayed by quantitative polymerase chain reaction, and EBV-specific CD8<sup>+</sup> T cell responses were assayed by interferon-γ enzyme-linked immunospot assay.

Results. Gambian children, from whom samples were obtained during an acute malaria attack and again up to 6 weeks later, had extremely high viral loads, reaching levels that in the United Kingdom are seen only in patients with infectious mononucleosis. Gambian control subjects (children and adults with no recent history of malaria) had lower median viral loads, although they were still >10-fold above the median for healthy UK adults. Limited experiments with EBV epitope peptides (restricted through the HLA-B*3501 and HLA-B*5301 alleles) also suggested an impairment of virus-specific CD8<sup>+</sup> T cell function in children with malaria, but only during acute disease.

Conclusions. Acute malaria is associated with sustained increase in EBV load and, possibly, a transient decrease in EBV-specific T cell surveillance. We infer that the unusually high set point of virus carriage in P. falciparum–challenged populations, allied with the parasite’s capacity to act as a chronic B cell stimulus, probably contributes to the pathogenesis of endemic BL.

Epstein-Barr virus (EBV) is usually acquired as a subclinical infection in childhood and thereafter persists as a largely asymptomatic passenger in the B lymphocyte pool, maintained under host T cell control [1]. However, this same virus has potent B cell growth-transforming ability and, in T cell–immunocompromised patients, EBV-transformed cells can grow out as malignant lymphoproliferative lesions [2]. The virus is also associated with 2 other B cell malignancies, Hodgkin lymphoma and Burkitt lymphoma (BL), which are etiologically more complex and arise independently of frank immune impairment [1, 3]. BL is particularly interesting, because the tumor occurs in 3 epidemiologically distinct forms. Endemic BL is the most common cancer of childhood in equatorial areas of Africa and New Guinea and is consistently EBV-genome positive [4]. Elsewhere, sporadic BL occurs in children at a low to intermediate incidence and with 15%–85% of tumors being EBV positive, depending on the geographic area. A third form of BL occurs in HIV-infected individuals, with incidence rates far above even that of the endemic tumor; this form, called AIDS-BL, typically appears as an early manifestation of AIDS and, in Western patient cohorts, is EBV positive in 30%–40% of cases [4]. Epidemiological evidence strongly implicates high exposure to Plasmodium falciparum malaria as a second risk factor in endemic BL [4, 5]. Debate continues as to how malarial infection might predispose the host to virus-positive disease, by acting as a second chronic stimulus to the B cell system and/or by suppression of EBV-specific T cell surveillance [6, 7].
One approach to exploring these issues is to compare the EBV-host balance in residents of adjacent, but climatically different, areas in which malaria is or is not holoendemic. In one such study, healthy adults from malarial areas had significantly weaker T cell–mediated regression of B cell transformation in cultures of experimentally infected peripheral blood mononuclear cells (PBMCs) [8]. This regression assay, although complex in its component cell interactions, ultimately measures the ability of the donor’s EBV-specific CD8⁺ T cell response to expand in vitro and kill autologous virus-transformed B cells [9]. More recently, others have used pools of commonly recognized EBV CD8 epitope peptides to challenge donor PBMCs ex vivo in interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assays. Children from malarial areas (screened blindly without HLA typing) had fewer positive responses to the peptide panel than did control children from nonmalarial areas, although in only 1 of the 3 age groups tested (5–9 years) [10]. In a parallel study, the EBV genome load in whole blood was significantly higher in children from malarial areas, but this time only in the 1–4-year-old age group [11].

Another approach is to study the EBV-host balance in children during and after an acute malaria attack. Such an approach has shown that acute malaria was associated with a transient weakening of T cell control, as measured by a regression assay [12, 13], and a transient increase in latently infected cell numbers in the blood, as measured by the spontaneous outgrowth of EBV–transformed B cells from T cell–depleted PBMC preparations [14]. However, the magnitude of these effects could not be accurately determined by the semiquantitative culture assays available at the time. Since then, the question of EBV-specific T cell responses in children with acute malaria and in those who have recovered from malaria has not been pursued using contemporary methods. Furthermore, recent findings concerning viral genome load in such children are difficult to interpret, because the assays were conducted either only in plasma [15], where the viral DNA is present in degraded fragments of unknown origin [16], or in whole blood (plasma plus cells), using an assay of limited sensitivity so that viral signals were detectable only in a fraction of study subjects [17]. In the present study, we have accurately determined the EBV load in PBMCs of children both during and after acute malaria, comparing the load values with those in appropriate control subjects. We then selected some individuals who, on the basis of their known HLA class I type and their known EBV strain, could be screened for IFN-γ ELISPOT responses against relevant CD8 epitope peptides.

**METHODS**

**Donors.** We studied 57 Gambian children (aged 3–14 years) presenting with symptomatic *P. falciparum* malaria (fever, nausea, headache, and temperature >37.5°C) and blood parasite numbers of 2–800 (mean, 170) per high-powered microscope field. They attended the Medical Research Council (MRC) outpatient clinic in Fajara, The Gambia, which serves urban, peri-urban, and rural areas. Treatment routinely was chloroquine and sulfadoxine-pyrimethamine (Fansidar), with quinine used in severe or relapsed cases. Control Gambian cohorts from the same areas were 40 age-matched children and 25 adults (aged 25–40 years), all asymptomatic with no parasitemia and no recent history of malaria. Malaria endemicity in The Gambia varied from hypoenemic to mesoendemic at the time of study. The reference UK cohorts were 14 patients with acute infectious mononucleosis (IM) (aged 18–30 years) who had primary EBV infection and 25 healthy EBV-seropositive adults (aged 25–40 years) with no history of IM. PBMCs were prepared, and aliquots were either snap-frozen as cell pellets for EBV genome load assays or cryopreserved as cell suspensions for subsequent ELISPOT assays. Some children with malaria and healthy Gambian adults underwent HLA typing. All samples were obtained with informed consent, as required by the Gambian Government/MRC Laboratories Joint Ethics Committee and the South Birmingham Local Research Ethics Committee.

**Viral load.** EBV genome load was assayed by quantitative real-time polymerase chain reaction (PCR), as described elsewhere [18, 19], and results were expressed as EBV genome copies per 1 × 10⁶ PBMCs. All data were plotted and statistically analyzed using GraphPad Prism software (version 4; GraphPad).

**EBV typing and epitope sequencing.** Virus types present in PBMC samples were determined by nested PCR amplification of a type-specific region of Epstein-Barr nuclear antigen (EBNA) 3C [20, 21]. Epitope sequences were determined as described in table 1.

**ELISPOT assays.** As described elsewhere [22], PBMCs were screened for reactivity in overnight IFN-γ release assays using the HLA-B*3501–restricted epitope peptides EPLPQGQLTAY (designated EPL, from the EBV lytic-cycle protein BamHI Z leftward open reading frame [BZLF1]), YPLHEQHGM (designated YPL, from EBNA3A), and HPVGEADYFEY (designated HPV, from EBNA1) and the HLA-B*5301–restricted epitope peptide HPVGGEADYF (designated HPV⁵, from EBNA1) [23]. Because the overlapping HPV and HPV⁵ epitopes can cross-react in ELISPOT assays, we tested individuals who were either B*3501 positive or B*5301 positive but not individuals with both alleles.

**RESULTS**

**EBV genome load in healthy Gambian adults.** Figure 1 shows the EBV genome load in PBMCs of 25 healthy EBV-seropositive adult donors in The Gambia versus 25 healthy age-
matched EBV carriers in the United Kingdom, all expressed as viral genome copies per $1 \times 10^6$ PBMCs. Although both cohorts displayed a wide range of values, the Gambian donors had significantly elevated virus loads overall, with a median (850 genome copies/$1 \times 10^6$ PBMCs) >10-fold higher than that seen in the United Kingdom (79 genome copies/$1 \times 10^6$ PBMCs). For reference, 14 patients with acute IM from the United Kingdom had a median load of 6280 genome copies/$1 \times 10^6$ PBMCs. Thus, healthy Gambian adults tend to carry EBV at unusually high levels, with values in 10 of 25 individuals that, in the United Kingdom, are normally seen only in acute IM.

**EBV genome load in Gambian children with acute malaria.**

We then studied 57 Gambian children (aged 3–14 years) presenting with acute malarial symptoms and, for comparison, an age-matched control group of 40 children resident in the same area but with no recent history of malaria. All children were detectably EBV positive, consistent with epidemiological evidence that all acquire EBV within the first 2 years of life [24, 25], and again a wide range of viral loads was observed within each group (figure 2A). However, although the median value for control children was very similar to that for healthy Gambian adults, viral loads in children with malaria were significantly higher by 5–6-fold, with a median of 4780 genome copies/$1 \times 10^6$ PBMCs. When the data on both the malarial and control groups were analyzed according to age (figure 2B), in both groups viral loads tended to be higher in younger (3–5-year-old) than in older (6–8- or 9–14-year-old) subjects. Although these age-dependent differences were not statistically significant, a similar trend has been noted before among healthy children living in a malarial area [11]. That the children with malaria showed the same age-dependent trend implies that, at all ages, acute malarial infection increases the preexisting EBV load.

Thirty-eight of the children with malaria were tested again 4–6 weeks later, at which time 35 were parasite free and asymptomatic. Figure 3A summarizes the data on these 35 fully recov-
ered subjects. There was no significant difference in the range of EBV loads seen during acute disease and after treatment, with median values of 4930 and 4090 genome copies/10^6 PBMCs, respectively. Figure 3B shows the individual results at the 2 time points, using a >2-fold change as an arbitrary threshold; 15 children showed a >2-fold decrease in EBV load, 15 showed a <2-fold overall change (“unchanged”), and 5 showed a >2-fold increase. Changes over time were not obviously related to the absolute virus load at disease presentation.

Analysis of EBV strains in the Gambian population. As a prelude to assays of EBV epitope–specific CD8+ T cell immunity in study subjects, we sought to identify resident EBV strains. This was important because there are 2 broad types of EBV [20], distinguished by polymorphisms in genes that encode many of the known CD8 epitopes [23]. If, as reported for some African populations [1], type 2 strains are as prevalent as type 1 strains in The Gambia, that would mean that epitopes currently defined on the basis of work in type 1 virus–infected white donors would be inappropriate for many Gambian subjects. Resident EBV strains in our study groups were determined by amplifying viral DNA in PBMC samples across a polymorphic region of the gene for EBNA3C and hybridizing with type-specific probes. Figure 4 shows representative results to illustrate how individuals carrying type 1 strain, type 2 strain, or both types of virus can be identified. Table 2 summarizes the overall results from Gambian donors (children with malaria and healthy adults) compared with patients with IM and healthy adults from the United Kingdom. Type 2 EBV strains were detected more frequently in Gambian than in UK donors (11/42 vs. 3/43), but usually alongside a coresident type 1 strain. Overall, therefore, we found that 90% of Gambian donors carried type 1 EBV, usually as the only detectable virus type.

However, because epitope sequences can also differ between geographically distinct type 1 virus strains [26, 27], we selected 3 immunodominant epitopes (EPL from BZLF1, YPL from EBNA3A, and HPV from EBNA1) that had originally been defined in type 1 viruses from white subjects origin but were restricted through HLA-B*3501, a common HLA allele in the Gambian population; it is interesting that the 11mer HPV epitope also contains a 9mer epitope (HPV*) restricted through another common Gambian allele, HLA-B*5301 [28]. Some 40 Gambian virus strains were sequenced across each epitope, and the overall results are summarized in table 1. The EPL epitope was perfectly conserved in all viruses analyzed, and the YPL epitope.

![Figure 3](image1.png)

**Figure 3.** Epstein-Barr virus (EBV) genome loads in Gambian children with acute malaria before and after treatment. A, EBV genome loads in patients with acute malaria compared with those in the same patients 4–6 weeks after treatment (n = 38); the median values for the 2 time points (solid horizontal lines) were 4930 and 4090 EBV genome copies/10^6 peripheral blood mononuclear cells (PBMCs), respectively. B, Comparison of EBV genome loads in matched acute disease and posttreatment samples. With a 2-fold change considered to be the threshold value, 15 patients showed a decreased EBV load, 15 showed an unchanged load, and 5 showed an increased load.

![Figure 4](image2.png)

**Figure 4.** Polymerase chain reaction (PCR) typing analysis of Epstein-Barr virus (EBV) strains isolated from Gambian donors. Type-specific Epstein-Barr nuclear antigen (EBNA) 3C sequences were PCR amplified from samples from 12 representative Gambian children with acute malaria by means of common nested 5′ and 3′ primers and then probed with a type 1 (upper panel [T1]) or type 2 (lower panel [T2]) EBNA3C probe. B95-8 and Ag876 viruses served as type 1 and 2 controls, respectively. Note that the type 1 probe also shows some cross-hybridization with the type 2 PCR product. Donors RN41–46, RN48, RN50, and RN51 carry type 1 virus only; donor RN47 carries type 2 virus only; and donors RN49 and RN52 carry both virus types. NTC, no-template control.
tope was conserved in most type 1 virus strains. By contrast, the HPV and HPV9 epitopes carried a Glu to Asp change at position 4 in more than half of the Gambian type 1 strains analyzed; this did not appear to affect immunogenicity, however, because in the ELISPOT assays epitope responses were observed in individuals carrying either viral sequence (data not shown).

EBV-specific CD8\(^+\) T cell responses in Gambian adults and children with malaria. We first focused on 9 Gambian and 6 UK adult donors, all of whom were HLA-B*3501 positive and carried epitope-positive type 1 virus strains. These were screened in ex vivo IFN-\(\gamma\) ELISPOT assays against EPL, YPL, and HPV epitope peptides. The results, expressed as IFN-\(\gamma\) spot-forming cells per 1 \(\times\) 10\(^6\) PBMCs, are shown in figure 5. Although there were no significant differences, for all 3 epitopes the median level of response was lower in the Gambian donors, particularly with the EPL epitope.

Then, from the group of children from whom samples were obtained during acute malaria and 4 – 6 weeks later, we selected those carrying a type 1 virus and positive for either the B*3501 or the B*5301 allele. Given the very small volumes of blood taken from children with malaria, B*3501-positive PBMCs had to be screened against a pool of the HPV, YPL, and EPL peptides, whereas B*5301-positive PBMCs were screened only against the HPV\(^+\) peptide. Healthy adult donor PBMCs from individuals matched through 1 of the above 2 alleles were included as internal controls. Some children with malaria had high background responses in ELISPOT assays, which reduced the number of informative subjects to 3 with the B*3501 allele and 2 with the B*5301 allele. As shown in figure 6A, all 5 children had weak ELISPOT responses during the acute phase compared with adult controls; however, in 4 children, responses for the posttreatment samples had increased to within the adult donor range. EBV loads in these 4 subjects had either fallen at the time of the second blood sampling (RN36 and RN58) or showed no significant change (RN27 and RN37). Note that the fifth child (RN26) was one of the rare study subjects (3/38) who still had parasitemia at follow-up; in this subject, the lack of improvement in T cell reactivity was coincident with a marked increase in EBV load.

**DISCUSSION**

This work analyzes the effect of acute malaria on the EBV-host balance by means of ex vivo assays of viral genome load and epitope-specific CD8\(^+\) T cell memory that are more quantitative than the cell culture methods used in other studies [8, 12–14]. The results indicate that children with acute malaria do experience an elevation of EBV load in the blood and suggest that this is accompanied by a reduction in functional EBV-specific CD8\(^+\) T cells. However, whether these 2 phenomena—T cell impairment and increased EBV load—are causally linked remains to be determined.

**Table 2. Typing of Epstein-Barr virus isolates from UK and Gambian donors.**

<table>
<thead>
<tr>
<th>Origin of donors</th>
<th>EBNA3C type</th>
</tr>
</thead>
<tbody>
<tr>
<td>United Kingdom</td>
<td>1 2 1 + 2</td>
</tr>
<tr>
<td>The Gambia</td>
<td>3 4 7</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of donors. Results are based on the analysis of 17 healthy adult control subjects and 26 patients with infectious mononucleosis from the United Kingdom as well as 9 healthy adults and 33 children with malaria from The Gambia. Typing was done using polymerase chain reaction primers and probes specific for type 1 and 2 alleles of Epstein-Barr nuclear antigen (EBNA) 3C.

Figure 5. CD8\(^+\) T cell responses to the HLA-B*3501-restricted BamHI Z leftward open reading frame (BZLF) 1 (designated EPL), Epstein-Barr nuclear antigen (EBNA) 3A (designated YPL), and EBNA1 (designated HPV) peptide epitopes in B*3501-positive adults. Interferon-\(\gamma\) enzyme-linked immunospot assays were used to measure the response to each epitope for 9 healthy Gambian and 6 UK adult donors. Data are reported as spot-forming colonies per 1 \(\times\) 10\(^6\) peripheral blood mononuclear cells (PBMCs) and were adjusted for the background responses seen in unstimulated cells. Horizontal lines indicate median values.
Focusing first on viral load assays, we found that, compared with long-term virus carriers in the United Kingdom, healthy Gambian adults harbor unusually high viral loads in PBMCs, often reaching values within the range found in patients with IM. That healthy Gambian children (naturally exposed to *P. falciparum* but with no recent history of clinical malaria) had similar results implies that high viral burdens are set relatively early during childhood and then are maintained for life. Comparisons between virus carriers in the 2 countries must be interpreted with caution, however, because the age at primary infection may also influence the viral load set point, and comparatively few individuals in the United Kingdom acquire EBV during infancy. That said, even if early exposure to the virus does predispose to a high viral load, it seems likely that any such effect will be magnified if the child is also subject to recurrent malarial infection.

Thus, we found that EBV loads are elevated a further 5–6-fold in the PBMCs of children with acute malaria. This supports the original finding from long-term spontaneous transformation assays [13, 14] that virus-infected B cell numbers are increased in children with the disease. It has been suggested that the surface immunoglobulin-binding domain of *P. falciparum* membrane protein 1, expressed on the surface of infected erythrocytes, may increase viral loads by inducing latently infected B cells into the lytic cycle in vivo [29, 30]. However, just as in IM B cell cultures [31], spontaneous transformation in malarial B cell cultures again requires reactivation from latency into the lytic cycle and the production of infectious virus in vitro [14]. This strongly suggests that the high virus genome loads in malarial blood reflect increased numbers of latently infected B cells and not ongoing virus replication. Total B cell numbers are slightly raised in the blood during acute disease [12], and it will be interesting to determine whether the memory B cell subset, which EBV selectively colonizes in vivo [32], is preferentially expanded during the disease process. Our data also show that, contrary to previous suggestions [14], viral loads remain high for at least 4–6 weeks after the acute disease episode. The increase in virus-infected B cell numbers in the blood, therefore, does not appear to be all that transient, and it will be necessary to follow up patients for longer periods after malaria to see how long the effect endures.

Turning to the effects of malaria on EBV-specific T cell immunity, early work with regression assays had shown a weakening of responses in children with acute malaria, followed by a recovery soon after resolution of symptoms [12]. One possible explanation was that this transient impairment reflected a loss of CD4+ T cell help for in vitro expansion of the virus-specific CD8+ T cells that effect regression; another was that circulating virus-specific CD8+ T cells themselves were either absent or functionally impaired during acute disease [13]. Our aim in the present was to address this latter possibility by means of ex vivo IFN-γ ELISPOT assays of memory T cell responses to epitope peptide stimulation. A recent study [10] used this approach to study EBV-specific CD8+ T cell responses in healthy children from areas of Kenya with different levels of malaria exposure, testing donors of unknown HLA type with pools of 4 lytic and 7 latent EBV epitope peptides. Response rates were reduced in children from areas in which malaria was holoendemic, but only in 1 of the 3 age groups tested. It is difficult to know whether such a result reflects genuinely impaired immunity or chance underrepresentation of children with relevant HLA alleles and/or with relevant EBV strains in the test group. Here we attempted a dif-
different strategy, focusing on a small number of specific epitopes and testing individuals known to have the HLA-restricting allele for those epitopes and known to carry a virus strain in which the epitope sequence is conserved. This approach improves the interpretability of results but restricts the number of individuals who can be tested.

We focused on responses restricted through 2 HLA alleles, B*3501 and B*5301, that are common in the Gambian population and that present peptide epitopes proved by sequencing to be antigenically conserved in the virus strains carried by our study subjects. Preliminary assays showed that epitope responses tended to be weaker in Gambian adults than in their UK counterparts. This is again consistent with a tipping of the virus-host balance in favor of the virus in populations with high P. falciparum exposure [8]; certainly, it appears that the high viral loads seen in such individuals do not lead to an increase in functional EBV-specific T cell numbers in the blood. Extending this approach to children with malaria, the number of successfully screened patients was small, but in each case the numbers of CD8+ T cells reactive to the viral epitopes were low during the acute phase; furthermore, in the 4 patients whose parasitemia resolved after treatment, T cell responses recovered to enter the normal adult range within 4–6 weeks. This suggests that the transient loss of T cell responses seen originally in regression assays of donors with malaria [12] reflects, at least in part, either a reduction in the number of virus-specific CD8+ T cells in the blood or the functional impairment of such circulating cells. Studies using EBV epitope–specific tetramers should help distinguish between these possibilities, as in other clinical situations in which the EBV-host balance is disturbed [33].

We conclude that intense exposure to P. falciparum might predispose to EBV-positive BL, first by creating conditions in which very high EBV loads are stably established in the circulating B cell pool and second by further increasing those loads during and for some time after an acute malaria episode. Acute malaria also appears to compromise the number of functional EBV-specific CD8+ T cells in the blood, but this effect may be transient and reversed on resolution of symptoms. Taken together, these 2 findings raise the possibility that, during malarial infection, impaired T cell surveillance may not be the main factor influencing virus-infected B cell numbers in the blood. A similar situation has been observed in which an existing EBV carrier state is disturbed by another agent predisposing to BL risk, HIV. Thus, within the first year of HIV infection, the number of latently infected B cells in the blood rise to a higher set point without any evidence of general T cell impairment nor any detectable change in EBV-specific T cell numbers or functional capacity [34].

The pathogenesis of BL involves deregulation of the MYC gene (which encodes the c-Myc protein) as the key oncogenic event. HIV infection, like malaria, induces polyclonal activation of the B cell pool [35, 36]. In HIV infection, this leads to a persistent generalized lymphadenopathy with highly expanded germinal centers [37], the B cell compartment in which the MYC/immunoglobulin translocation may occur [38]. Chronic P. falciparum infection may predispose to endemic BL in an analogous way, although the much lower incidence of the endemic tumor compared with AIDS-BL [4, 39] suggests that malaria will be a weaker germinal center stimulus. By contrast, the EBV loads reported here in children with malaria are considerably higher than those seen when HIV infection causes resetting of the EBV-host balance [34]. Thus, any B cell in which a relevant MYC/immunoglobulin translocation has occurred would have a much higher chance of either having or subsequently acquiring EBV infection in an individual infected with malaria than in an HIV carrier. This could explain why endemic BL almost always presents as an EBV-positive disease, whereas AIDS-BL, at least in the developed world, may less frequently have that advantage; it would mean that, in many AIDS-BL cases, the additional changes complementing MYC gene deregulation must be selected through an EBV-independent route.

References


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