Immunological Responses to *Plasmodium falciparum* in African Children and the Influence of Epstein-Barr Virus

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Cette these est dediée
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Introduction

Malaria has proved throughout history to be a significant threat to human health. Between 300 and 500 million clinical cases occur each year worldwide, approximately 2.7 million of which are fatal, primarily among children. Systematic control of malaria started after the discovery of the malaria parasite by Laveran in 1889 (for which he received the Nobel Prize for medicine in 1907), and the demonstration by Ross in 1897 that the mosquito was the vector of malaria. In endemic regions of Africa severe malaria due to *P. falciparum* represents the leading cause of morbidity and mortality, whereas *P. vivax* causes severe febrile illness especially in Asia and South America but very rarely leads to a fatal outcome, and *P. malariae* or *P. ovale* infections that are associated with very little morbidity at all (World Health Organization 1994, Delaunay et al 2001, Stevenson et al 2004). One peculiarity of *P. falciparum* is the ability of its intra-erythrocytic mature asexual stages, i.e. late trophozoites and schizonts, to mediate cytoadherence of infected erythrocytes to a range of receptors expressed on endothelial cells lining post-capillary venules or on syncytiotrophoblasts in the placenta, as well as autoagglutination and rosetting with uninfected erythrocytes (Magowan et al 1988, Biggs et al 1992, Rowe et al 1997). These processes enable *P. falciparum* to avoid filtration and splenic clearance via which mature forms of *P. vivax, P. ovale P. malariae* are commonly eliminated (David et al 1983, Dodoo D et al 2001), to sustain parasite multiplication and evasion of host immune responses, and to generate the sexual stages (gametocytes) to enable transmission to the female *Anopheles* mosquito vector (Carter et al 1988, Hayward et al 1999, Craig et al 2001). *P. falciparum* along with other plasmodia including *P. vivax, P. knowlesi, P. chabaudi,* and *P. fragile,* exhibits antigenic variation, also thought to represent a survival mechanism. Several parasite surface

antigens or parasite proteins inserted into the plasma membrane of the infected red blood cell are polymorphic and exhibit inter- or intraclonal antigenic diversity or variation, respectively (Newbold 1999). Antigenic variation allows the parasite to subvert variant surface antigen (VSA)-specific immune responses and to extend the duration of infection in the face of mounting VSA-specific host immunity (Staalsoe et al 2002). Several VSA and the gene families that encode them have been identified to date, including *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), RIFIN, STEVOR, CLAG, encoded by the gene families *var, rifin, stevor* and *clag*, respectively (Craig et al 2001). The best characterized of these VSA families is PfEMP-1 (Figure 1), an antigen that mediates parasite cytoadherence, agglutination and rosetting of erythrocytes, and therefore particularly important in the pathophysiology of severe malaria (Magowan et al 1988, Biggs et al 1992, Roberts et al 1992, Baruch et al 1995, Rowe et al 1997, Giha et al 1999, Smith et al 2000). PfEMP-1 molecules are physically anchored within so-called "knobs" at the infected erythrocyte membrane, and their extra-cellular portions have specificity for a variety of host receptors including thrombospondin (TSP), CD36, ICAM-1, VCAM-1, E-selectin, chondroitin sulfate A (CSA), hyalunoric acid (HA), complement receptor-1(CR1), CD31 and P-selectin (Berendt et al 1989, Baruch et al 1995, Rogerson et al 1995, Newbold et al 1997, Coppel et al 1998, Beeson et al 1999, Smith et al 2000). Although the acquisition of protective immunity is paralleled by acquisition of both cell-mediated and humoral responses to a long list of parasite antigens, a growing body of evidence points to a slow acquisition of malaria-protective immunity which correlates with a steady accumulation of antibodies to a broad repertoire of VSA specificities, in particular to PfEMP-1, since anti-VSA antibodies can efficiently block parasite invasion, prevent cytoadherence and agglutination, and opsonize infected erythrocytes to facilitate their uptake and killing by phagocytes, a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC) (Brown et al 1980-81, David et al 1983, Bull et al 1998-2000, Giha et al 1999, Craig et al 2001, Ofori et al 2002, Artavanis-Tsakonas et

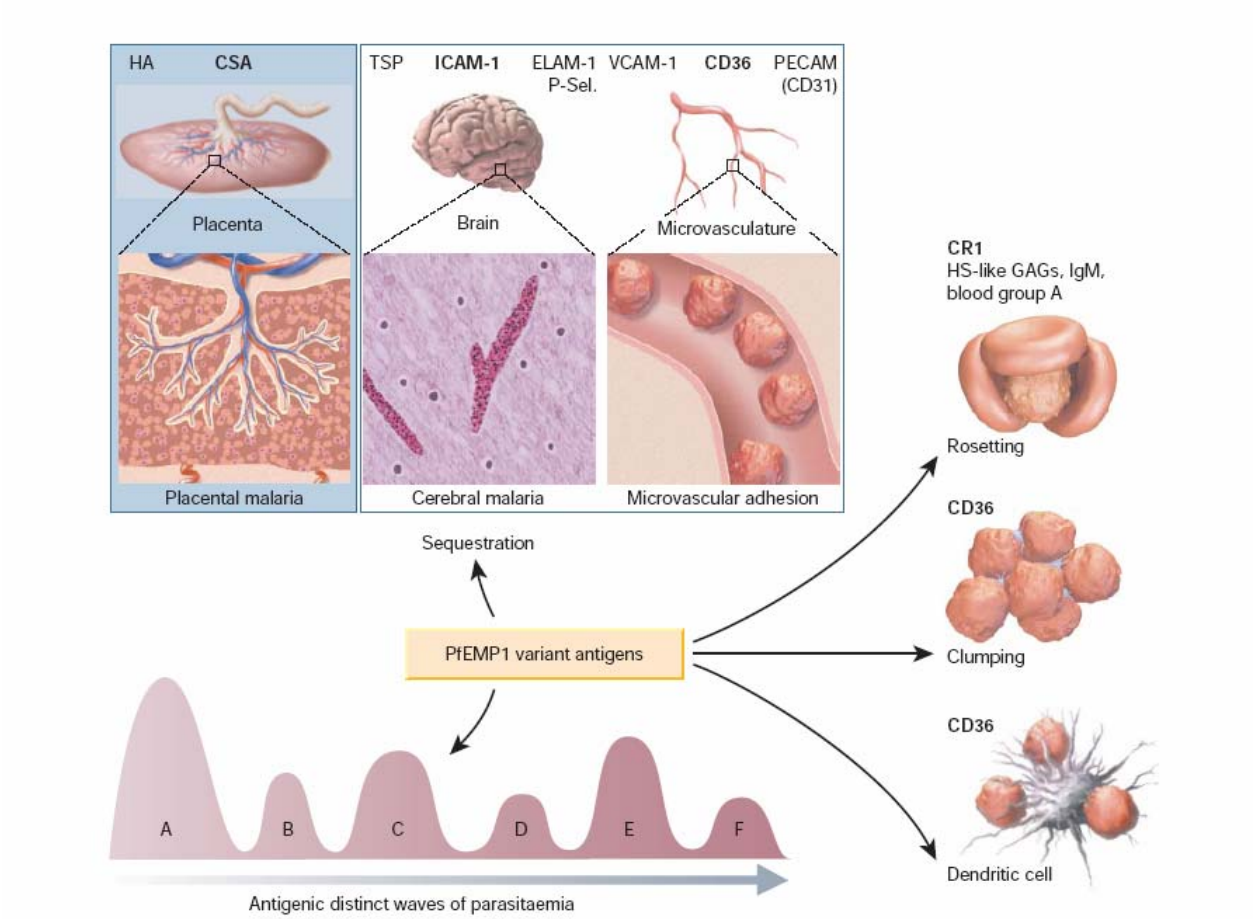


Figure 1. The variant antigen family of PfEMP1 is central to host–parasite interaction and pathogenesis. PfEMP1 expressed on the surface of mature RBCs infected with *P. falciparum* is involved with clonal antigenic variation and can bind to many host receptors through its multiple adhesion domains. The different properties of PfEMP1 - sequestration for evading spleen-dependent killing and antigenic variation for evading antibody-dependent killing - contribute to the virulence and pathogenesis of *P. falciparum* and are essential for survival of the parasite. Parasite sequestration in the brain and placenta contribute to the complications of cerebral malaria and placental malaria, respectively. Simultaneous binding to several receptors, binding of uninfected erythrocytes (rosetting), and clumping of infected erythrocytes through platelets are associated with the pathogenesis of malaria. Parasite-infected RBCs binding to dendritic cells downregulate the host immune response. HA, hyaluronic acid; TSP, thrombospondin; ELAM-1, endothelial/leukocyte adhesion molecule 1; P-Sel., Pselectin; ICAM-1 inter-cellular adhesion molecule-1, VCAM-1, vascular cell adhesion molecule 1; PECAM (CD31), platelet endothelial cell adhesion molecule 1; CR1, complement receptor 1; HS-like GAGs, heparin sulphate-like glycosaminoglycans; IgM, immunoglobulin M. **NATURE | VOL 415 | 7 FEBRUARY 2002 |**

al 2003, Jensen et al 2003). *P. falciparum* VSA-specific antibody responses are mediated by immunoglobulin G (IgG), and the expressed VSA type depends upon the host age and disease severity, as indicated in studies reporting that parasites infecting malarious non-immune children under five years old frequently exhibit VSA commonly and strongly recognized by IgG in the general population, whereas VSA expressed in older children and semi-immune adults are poorly recognized by specific IgG (Bull et al 1998-2000, Tebo AE et al 2002, Nielsen et al 2002 and 2004, Ofori et al 2002, Hviid et al 2004). These observations led to the concept of severe malaria-specific VSA (VSA_{SM}) commonly found during severe *P. falciparum* malaria in young and non-immune patients, and of uncomplicated malaria-specific VSA (VSA_{UM}) expressed during asymptomatic uncomplicated malaria in semi-immune older children and adults (Bull et al 1998-99, Nielsen et al 2002 and 2004, Ofori et al 2002, Staalsoe et al 2004). Hence, *P. falciparum*-infected erythrocytes expressing VSA_{SM} are frequent and highly recognized, whereas those expressing VSA_{UM} are rare and poorly recognized by IgG, independently of the geographical area or of the endemicity of malaria transmission (Aguiar et al 1992, Nielsen et al 2002 and 2004). Although the molecular biological and structural differences between both types of VSA remain unclear, VSA_{SM}-specific immunity is acquired more rapidly than immunity to VSA_{UM}, probably because they correspond to VSA that are preferentially expressed by most or all *P. falciparum* as they may maximize successful transmission to new hosts via efficacious mediation of cytoadherence, higher growth rates and avoidance of splenic clearance (Gupta et al 1999, Hviid et al 2004). This argument is supported by recent data describing VSA_{SM} as more virulent and antigenically more conserved (and cross-reactive), and VSA_{UM} as less virulent but more diverse (Aguiar et al 1992, Bull et al 1998-2000, Nielsen et al 2002 and 2004, Hviid et al 2004, Jensen et al 2004). Hence, the gradual acquisition of protective immunity to malaria appears to restrict the repertoire of VSA that is compatible with parasite survival and to force progressively *P. falciparum* VSA expression away from the VSA_{SM}-type towards VSA_{UM} (Bull

et al 1998-2000, Ofori et al 2002). The shift towards VSA_{UM} occurs as the strong selective advantage of VSA_{SM}-expressing parasites gradually disappears due to acquisition of VSA_{SM}-specific immunity (Hviid 2004). Thus, the switching and expression pattern of VSA on the erythrocyte surface is not random as was widely assumed, but is to a large extent regulated by the acquired anti-malarial immune status of the infected host (Bull et al 1998-2000, Gupta et al 1999, Nielsen et al 2002, Staalsoe et al 2002). Longitudinal studies have revealed that VSA expressed by *P. falciparum* parasites causing clinical malaria episodes are not recognized by the pre-existing VSA-specific IgG antibody repertoire, and the IgG response associated with the disease-causing VSA develops relatively shortly (2-3 weeks) after the symptomatic attack (Bull et al 2000, Ofori et al 2002). Taken together, all these findings led to the theory of "holes in the VSA-specific antibody repertoire", according to which only parasite strains expressing VSA for which there is no specific IgG in the antibody repertoire can proliferate and evade the host more rapidly, freely cytoadhere, sequester, and mediate the pathophysiological features associated with *P. falciparum* malaria (Bull et al 1998-2000, Ofori et al 2002). Following all these concepts, it might be possible to develop disease-ameliorating vaccines that protect against mortality and severe morbidity by accelerating acquisition of immunity to VSA_{SM}-expressing *P. falciparum* parasites and forcing VSA-expression away from VSA_{SM}. Arguably the most striking evidence in favor of a major protective role of VSA-specific antibodies has been observed in semi-immune women in endemic areas during their first pregnancy. A VSA-subtype called pregnancy-associated malaria VSA (VSA_{PAM}) has been described only in *P. falciparum* infected pregnant women, and has been associated with the malaria susceptibility observed in otherwise clinically immune women at the time of their first pregnancies (Brabin et al 1983, Staalsoe et al 2001). VSA_{PAM} mediates the sequestration of parasites in the placenta, and their expression decays rapidly with increasing gravidity and pregnancy duration (Fried et al 1998, Ricke et al 2000, Staalsoe et al 2001 and 2004, Duffy et al 2003). Thus, primigravid women have no pre-

existing anti-VSA_{PAM} IgG and will accordingly develop placental parasitemia that often leads to a clinical attack, even though the parasitemia detectable in the peripheral circulation may remain low, whereas multigravidae have no or only asymptomatic parasitemia because of the pre-existing anti-VSA_{PAM} IgG repertoire developed during earlier pregnancies (Staalsoe et al 2004).

Attempts to identify mechanisms underlying antibody-mediated premunition against *P. falciparum* blood stages have relied so far on the in vitro growth inhibition assay (GIA), and there is a need for more studies that reliably reflect the available in vivo findings. Despite conflicting data, in vitro studies led to the nowadays generally accepted ADCC-like mechanism referred to as antibody-dependent cellular inhibition (ADCI) of parasite growth via which protective, pre-existing antibodies attach to free merozoites and thereby promote monocyte-dependent anti-parasitic effector functions including phagocytosis of opsonized merozoites and release of parasititstatic mediators such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and nitric oxide (NO) (Druilhe et al 1987, Bouharoun-Tayoun et al 1990, Oeuvray et al 1994). ADCI–mediated *P. falciparum* growth inhibition involves the binding of cytophilic IgG isotypes to monocytes via the polymorphic low-affinity receptor FcγRIIA, but not via the high-affinity receptor FcγRI (Bouharoun-Tayoun et al 1995). Supportive studies showed that parasite-specific IgG1 and IgG3 mediate ADCI, whereas the non-cytophilic IgG2 and IgG4 isotypes elicit ADCI-blocking properties (Groux et al 1990, Bouharoun-Tayoun et al 1992 and 1995, Chumpitazi et al 1996, Shi et al 1999, Tebo et al 2001). However, IgG2 could also mediate ADCI, since this isotype binds to the H131 allelic form of FcγRIIA that is common in Africans exposed to *P. falciparum* (Parren et al 1992, Osborne et al 1994, Norris et al 1998, Aucan et al 2000). Furthermore, IgG2 has been shown to bind to the K^{127} allelic form of the newly described K/Q^{127} mutation within the FcγRIIA allelic form R131 (Norris et al 1998).

Although very limited in scope, a body of immunoepidemiological evidence was provided in the late 1980's to support the proposal that the oncogenicity of Epstein-Barr virus (EBV) leading to endemic Burkitt's lymphoma (eBL) might be greatly enhanced by previous or concurrent malaria infections. EBV-related or eBL was described for the first time in 1961 by the surgeon Denis Burkitt as a form of cancer affecting the jaws of young Gambian children (Burkitt 1958 and 1983). Burkitt made the crucial insight that the distribution of this common tumor may be influenced by climatic factors, notably temperature and elevation, and might be caused by a mosquito-born virus, or arbovirus (Burkitt 1961-4, Davies 1964). In 1964, the original discovery and description of EBV as the causative agent of eBL was made by Tony Epstein, Yvonne Barr and Burt Achong in freshly cultured BL cells obtained from excised tumor biopsies, by means of electron microscopy (Epstein et al 1964). Later on, Burkitt reported that the tumor he had described might result from interaction between a virus (es) and a "reticuloendothelial system altered by chronic and heavy infection by malarial or other parasites" (Burkitt 1969). Since that time, intriguing similarities between both life-threatening and usually concurrent diseases eBL and *P. falciparum* malaria have been established at both the epidemiological and immunopathological levels. eBL is endemic in sub-Saharan Africa and in Papua New Guinea, where *P. falciparum* is the predominant cause of malaria (Booth et al 1967, Klein 1978, Moss et al 1983, Stiller et al 1990). As such, the geographical distribution of EBV-related eBL and *P. falciparum* holoendemic malaria are identical (Edington et al 1963, Epstein 1984, Geser et al 1985, Facer et al 1989). In the sub-Saharan region of Africa, eBL appears to be the commonest childhood malignancy, since more than 44% of African children are EBV-seroconverted by 12 months of age and 80% by 2 years (Biggar et al 1978). It is also in the same region that severe anemia and cerebral malaria, the typical features of severe malaria due to *P. falciparum,* represent the leading cause of morbidity and mortality principally in children under the age of 5 years, and account for up to 2 million deaths per year (World Health Organization 1994,

Delaunay et al 2001). Acute *P. falciparum* infection in young African children leads to spontaneous transformation of EBV-infected B cells, *in vitro*, into proliferating colonies, suggesting a loss of control of the viral infection (Whittle et al 1984, Lam et al 1991). This finding was supported by dramatic alterations of EBV-specific CTL responses observed during acute and chronic *P. falciparum* malaria in individuals living in malaria endemic regions, similar to that observed in immnunosuppressed patients, which returned to normal several weeks following anti-malarial drug therapy (Moss et al 1983, Whittle et al 1984). The suppressed CTL control of EBV during acute malaria was then characterized by the massive reduction of the CD4+/CD8+ T cell ratio, resulting probably from impaired CD4+ T lymphocyte trafficking to the lymph nodes and/or from apoptosis (Troye-Blomberg et al 1983, Gunapala et al 1990, Whittle et al 1990). It should be noted that eBL is typically characterized by tumorigenic chromosomal translocation of the portion of chromosome 8 bearing the *c-myc* gene locus, to chromosome 14 near the enhancer of the immunoglobulin heavy-chain locus (Taub et al 1982, Klein 1986). Immunosuppression, polyclonal B-cell activation and germinal center hyperactivity are not only well-documented in EBV-associated diseases, but also during malaria, particularly in individuals with continuous exposure to *P. falciparum* infections, and are thought to increase the EBV load, and therefore the chance of chromosomal translocation and a state of B-cell neoplasia (Burkitt 1970, Katahaa PK 1984a). Based on this theory, Moss et al (1983) suggested for the first time that the immunosuppressive effects of malaria may be sufficiently severe to induce the reactivation of latent EBV infection, implying that even subclinical malaria can induce a significant level of immunosuppression, since the majority of adults living in malaria endemic areas carry low-grade *P. falciparum* parasitemia. As such, EBV infection early in life concomitantly with chronic malaria may trigger in some individuals carcinogenic processes that ultimately develop into eBL (Manson-Bahr et al 1987).

It is evident that in vivo data are required to explain how malaria brings about these changes; but there is also a question of primordial importance, which emerges from the interaction of these co-infecting pathogens in endemic areas and needs to be addressed:

May the cascade of immunological events generated during malaria-induced EBV reactivation be beneficial or deleterious for *P. falciparum*-specific humoral/cellular immunity in individuals from malaria-endemic regions?

EBV is a human gamma herpes virus infecting non-dividing cells including epithelial cells, but particularly resting B cells (Roizman et al 1981). It is the only known human lymphotropic virus with the ability to trigger the proliferation and immortalization of infected human B lymphocytes into lymphoblastoid cell lines (LCL) both in vivo and in vitro (Middleton et al 1991). EBV is ubiquitous, with life-long latent infections in more than 90% of the human population, and has been attributed, along with *P. falciparum* malaria, a major role in the pathogenesis of endemic Burkitt's lymphoma. The virus is associated with numerous human malignancies including Hodgkin's lymphoma, post-transplantation B-cell lymphoma, undifferentiated nasopharyngeal carcinoma (NPC), lymphoproliferative disorders (LPD) in immunosuppressed subjects, nasal Natural Killer (NK)- and T cell lymphomas (Young et al 1988, Su et al 1990, Pallesen et al 1993, Paya et al 1999, Peh et al 2003). At the time of infection with EBV, the viral genome persists in infected cells either as an episome or as integrated viral DNA (Figure 2), with only a limited number of EBV genes being expressed in the latently infected cells (Anagnostopoulos et al 1995, Decker et al 1996, Miyashita et al 1997). The lytic phase, characterized by the replication of the EBV genome, occurs more frequently in epithelial but rarely in B cells, as a result of EBV reactivation due, for example, to parasites or any concurrent immune deficiency-associated disease. Primary infection with EBV generally occurs early in childhood and is usually

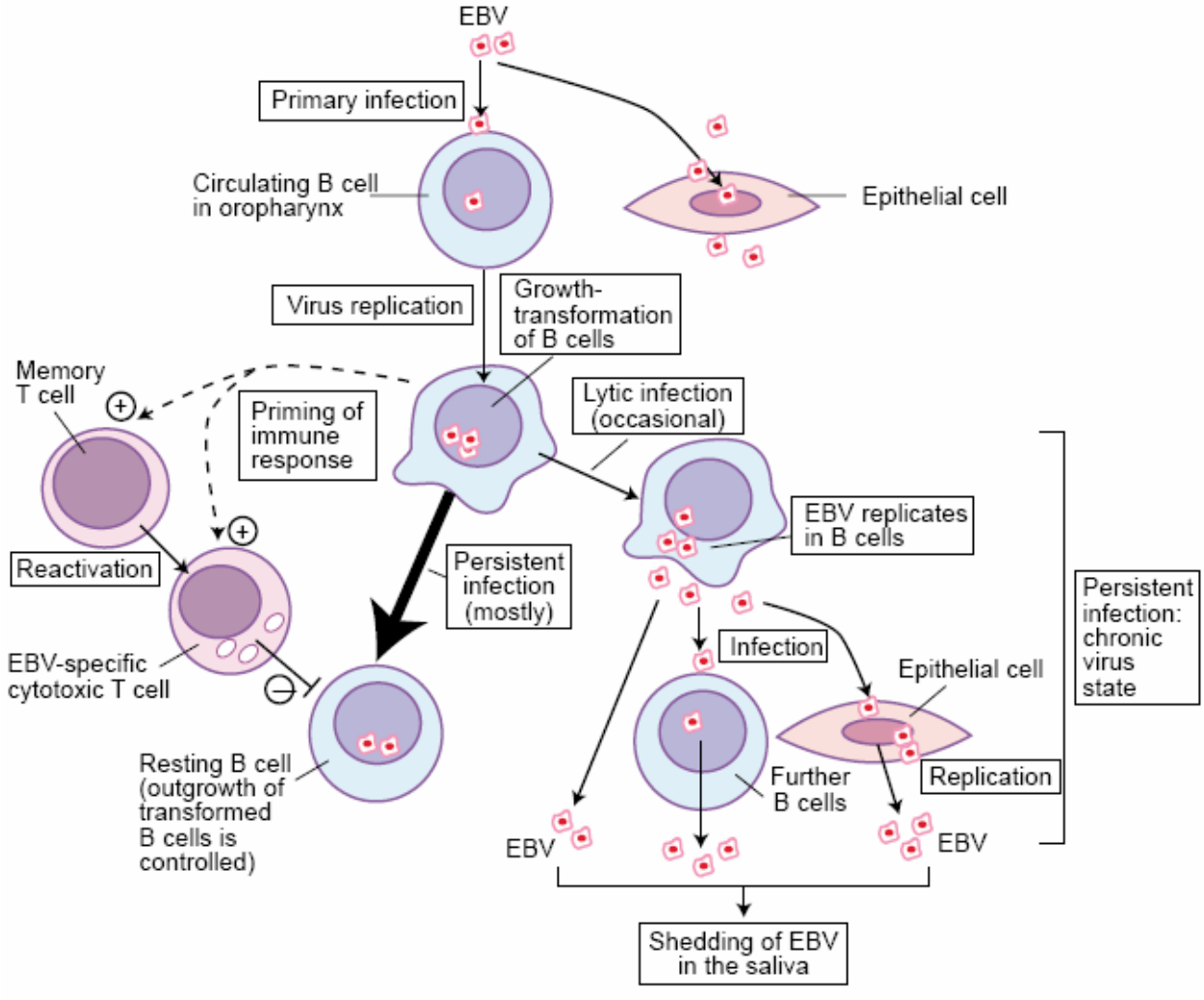


Figure 2: Epstein–Barr virus (EBV) infection in normal healthy virus carriers. Virus infection involves two cellular compartments: (1) B cells, where infection is predominantly latent and has the potential to induce growth-transformation of infected cells; and (2) epithelial cells, where infection is predominantly replicative. Although the exact mode of primary and persistent EBV infection and the relative contributions of B cells and epithelial cells are uncertain, recent data point to the B-cell compartment as the main mediator of primary as well as persistent infection. Following primary infection of B cells, a chronic virus carrier state is established in which the outgrowth of EBV-transformed B cells is controlled by an EBV-specific cytotoxic T lymphocyte response re-activated from a pool of virus-specific memory T cells. At certain sites, latently infected B cells can become permissive for lytic EBV infection. Infectious virus released from these cells can be shed directly into the saliva or might infect epithelial cells and other B cells. In this way a virus-carrier state is established that is characterized by persistent, latent infection in circulating B cells and occasional EBV replication in B cells and epithelial cells**. Expert Reviews in Molecular Medicine 11/2001**.

asymptomatic. In the event that primary EBV infection occurs in adolescence or adulthood, it can lead to infectious mononucleosis manifested by extremely elevated numbers of EBV-infected B cells in the peripheral circulation, all identified as non-proliferating and non-pathogenic resting memory cells (up to 50% of such are EBV-infected), followed by a vigorous activation and oligoclonal expansion of EBV-specific cytotoxic T lymphocytes (CTL, up to 50% of circulating CD8+ T cells) responsible for the atypical lymphocytosis (Wood et al 1967, Henle et al 1968, Tomkinson et al 1987, Callan et al 1996, Rickinson et al 1997). In vitro studies have shown that EBV-infected B cells produce large amounts of *P. falciparum*-specific antibody that inhibits parasite growth, as well as virus-specific antibody and heterophilic antibody in vitro (Brown et al 1986). Moreover, EBV-transformed B lymphocytes from malaria patients can produce antibodies against sporozoites as well as asexual erythrocytic stages of *P. falciparum* (Monjour et al 1983-84), whereas *P. falciparum* merozoite surface protein-1 (MSP-1) activates and maintains the outgrowth of B cells from EBV-positive individuals into lymphoblastoid cell lines (Kataaha et al 1984b). At the level of cellular immunity, it can be a priori expected, since erythrocytes lack MHC molecules, that cytotoxic lymphocytes have little or no role to play in protective immunity to malaria. However, it appears that the inhibition of parasite growth and development inside hepatocytes is mediated by NK and CD8+ CTL, and that parasite-killing mechanisms are mediated by T-helper (Th) 1-type inflammatory responses, whereas Th2-type cells mediate humoral immunity and regulate pro-inflammatory responses during malaria (Figure 3) (Mosmann et al 1989, Artavanis-Tsakona et al 2002, Tsuji et al 2003). Indeed, excessive production of inflammatory mediators such as nitric oxide, and INF-γ-induced secretion of pyrogenic IL-1, IL-6, and TNF-α by macrophages, is typical of severe malarial anaemia and cerebral malaria in non-immune individuals (Kern et al 1989, Chizzolini et al 1990, Kwiatkowski et al 1990, McGuire et al 1994, Richards et al 1997, Day et al 1999, Luty et al 1999, Dodoo et al 2002). Moreover, severe malaria is thought to be potentiated by

immunopathological mechanisms resulting from vigorous cell-mediated inflammatory responses to *P. falciparum* initiated by IL-12, a heterodimeric molecule comprising p40 and p35 chains that together constitute the biologically active IL-12p70 (Clark et al 1987, Trinchieri et al 2003). Experimental human infection studies have shown that plasma IL-12p40 and IFN-γ levels increase at the time of emergence of *P. falciparum* from the liver in malaria-naïve humans (Hermsen et al 2003). IL-12 is released from monocytes/macrophages, dendritic cells, B cells and other cell types, and plays a determinant role in the prognosis of malaria (Mosmann et al 1989, Crutcher et al 1995, Trinchieri et al 1998, Luty et al 2000, Malaguarnera et al 2002). Severe malaria is associated with low levels of plasma IL-12, which is in turn negatively associated with risk of infection, but positively with haemoglobin concentration (indicative for absence of malarial anaemia), and with IFN-γ and TNF-α production (Luty et al 2000, Perkins et al 2000, Malaguarnera et al 2002). EBV mimics human cytokine synthesis in order to enhance viral persistence. IL-12 was first described as a secretion product of EBV-transformed B-cell lines that activates NK cells and was accordingly termed NK cell stimulatory factor (NKSF) (Valiante et al 1992). EBV-induced gene 3 (EBI3), for example, is homologous to and shares 27% amino acid identity with IL-12p40 (Devergne et al 1996, Holscher et al 2004). EBI3 associates non-covalently with IL-12p35 to form IL-12p35/EBI3. Although the physiological relevance and biological function of IL-12p35/EBI3 remains uncertain, and the combination does not compete with IL-12p70 (IL-12-p40/p35) for the IL-12β1/β2 receptor (Devergne et al 1996-97, Devergne et al 2001). EBI3 also dimerizes with another molecule, p28, to form a cytokine now known as IL-27 (Devergne et al 2001, Pflanz et al 2002, Brombacher et al 2003). Monomeric EBI3 has an essential role in the growth and differentiation of Th2 type cells, and in the regulation of Th2-mediated immunopathology, whereas IL-27 (EBI3/p28) induces proliferation of naïve but not memory T cells, promotes Th1-differentiation, and synergizes with IL-12 to induce IFN-γ production in human NK cells and naïve CD4+T cells (Pflanz et al 2002,

Holscher et al 2004). These contrasting features between monomeric EBI3 and dimeric EBI3/p28 (IL-27) suggest a dual role of EBI3 (Devergne et al 1996).

To avoid tissue damage mediated by excessive pro-inflammatory responses, immuno-regulatory cytokines are also actively produced during infection. Effective production of anti-inflammatory cytokines has been linked with protection from severe malaria and development of clinical immunity, since they can modulate the pathophysiological activities of pro-inflammatory cytokines. As such, IL-10 (also termed cytokine secretion inhibitory factor) and transforming-growth factor (TGF)-β play a key role in limiting cellular responses (Figure 3). Both molecules are produced by macrophages, as well as by activated T and B cells (Gazzinelli et al 1992, Howard et al 1992, Omer et al 2000, Bogdan et al 2003, Stevenson et al 2004). Thus, severe *P. falciparum* malaria in African children has been linked with low levels of TGF-β and IL-10 relative to TNF-α, whereas an early and sustained burst of TGF-β reduces pro-inflammatory responses, and is associated with reduced mortality (Wenisch et al 1995, Kurtzhals et al 1998, Othoro et al 1999, Omer et al 2000, Perkins et al 2000). IL-10 appears to reduce parasite-killing responses and therefore severe pathology during malaria, but paradoxically allows parasite persistence in the host and increased transmission to the mosquito vector (Gazzinelli et al 1992, Howard et al 1992). Decreased levels of IL-10 have been associated with increased risk of severe malarial anaemia in African children and cerebral malaria in adult Vietnamese patients (Kurtzhals et al 1998, Day et al 1999). Conversely, after malaria chemotherapy malaria patients have high frequencies of IL-10-expressing CD4+ cells (Winkler et al 1998-99). IL-10 is also important during EBV infection. EBV secretes a human IL-10-like peptide termed BCRF-1 or viral IL-10 (vIL-10), that shares more than 84% amino-acid sequence homology and most of the biological functions of human IL-10 (hIL-10) including the suppression of pro-inflammatory cytokines (most notably IL-2, IL-12, IFN-γ, IL-18 and TNF-α), potent B cell growth-promoting activity, inhibition of T cell proliferation and macrophage costimulatory activity (Hsu et al 1990,

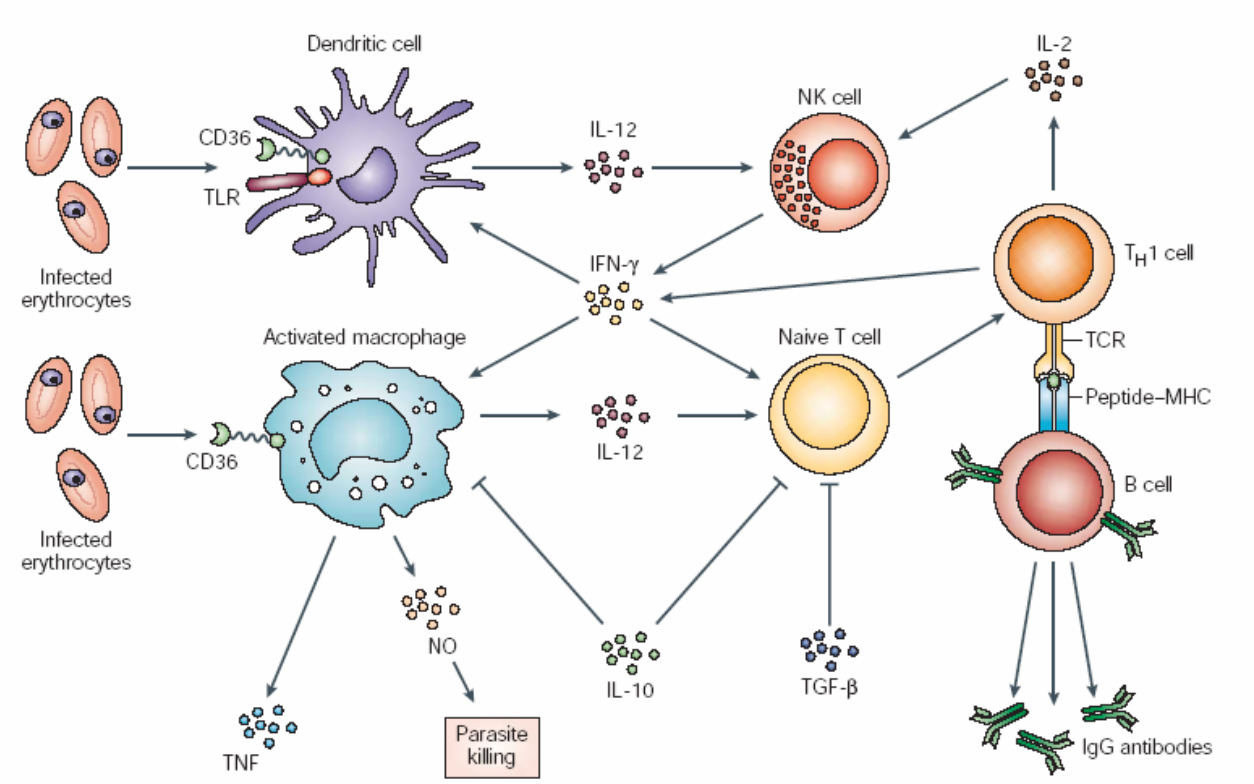


Figure 3. Linking innate and adaptive immunity to blood-stage malaria. Possible regulation of adaptive immunity to blood-stage malaria by cytokines produced by cells of the innate immune response. In response to parasite ligands recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon-γ (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen — the primary site of immune responses against blood-stage *Plasmodium* parasites. Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86 and adhesion molecules and the production of cytokines including interleukin-12 (IL-12). IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of T helper 1 (T_H1) cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive $CD4^+$ T cells. IL-2 produced by antigen-specific T_H1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response. Cytokines such as IL-10 and transforming growth factor (TGF)-β negatively regulate both innate and adaptive responses. NO, nitric oxide; TCR, T-cell receptor; TNF, tumour-necrosis factor. **Nature Reviews Immunology 4; 169-180 (2004). INNATE IMMUNITY TO MALARIA**

Moore et al 1990, Mosmann et al 1991, Vieira et al 1991, Rousset et al 1992). In addition, EBV triggers the production of both vIL-10 and hIL-10 by EBV-infected lymphoblastoid cells, thereby enhancing B cell proliferation and abrogating the CTL-mediated inhibition of EBV-infected B cell outgrowth (Burdin et al 1993, Bejarano et al 1998). It appears that vIL-10 is responsible, at least partly, for the successful invasion of EBV into human B cells and for the latent state of EBV infection (Miyazaki et al 1993, Bejarano et al 1998, Muller et al 1998). Hence, vIL-10 has suppressive effects on the expression of the cell-surface adhesion molecule ICAM-1, and can interact with cell-surface glycosaminoglycans such as heparin and heparin sulphate (Salek-Ardakani et al 2000 and 2002). Interestingly, it is known from malaria studies that heparan sulfate molecules expressed on endothelial cells interact with PfEMP-1 to mediate cytoadhesion of parasite-infected or rosetting of uninfected erythrocytes, whereas expression of ICAM-1 on cerebral endothelium and its recognition by PfEMP-1 molecules probably contributes to the development of cerebral malaria (Berendt et al 1989, Turner et al 1994, Rogerson et al 1999, Vogt et al 2003).

Aim

Factors underlying the susceptibility to severe malaria are still poorly understood, although the immunopathological effects of pro-inflammatory cytokines are clearly implicated (Brasseur et al 1983, Kern et al 1989, Ringwald et al 1991, Luty et al 1999-2000). Acquisition of immune-mediated clinical protection in malaria is certainly associated with the development of a broad repertoire of antibody responses to *P. falciparum* blood-stages antigens including VSA (Brasseur et al 1990, Bull et al 1998, Tebo et al 2002, Cabrera et al 2004). Whether concurrent EBV infection may influence the production of protective antibodies to *P. falciparum*-derived VSA, for example, or may modulate parasite antigen-specific cellular responses and/or play a role in susceptibility to severe malaria, are the questions addressed in the work presented here.

Design and Methods

To achieve our aim, we used blood and plasma samples collected from Gabonese children during a longitudinal immuno-epidemiological, study in an area with hyperendemic and perennially-transmitted *P. falciparum* malaria. The study was carried out between 1995 and 2002 at the Albert Schweitzer Hospital in Lambaréné, where the annual entomologic inoculation rate is estimated to be ~50 infective bites per person per year (Wildling et al 1995, Sylla et al 2000). Children older than 6 months presenting with severe *P. falciparum* malaria were admitted to hospital, and for each such case an age-, gender- and provenance-matched control presenting with mild *P. falciparum* malaria was enrolled as soon as possible afterwards. A detailed description of the participants, inclusion and exclusion criteria used, treatment given, clinical in-home surveillances, haematological and biochemical methods used, and ethical approval has been published elsewhere (Kun et al 1998). Blood/plasma samples were collected on 5 occasions: i) at admission to hospital just prior to administration of malaria chemotherapy, referred to as the acute phase (60 samples); ii) one month post-admission, referred to as convalescence (60 samples); iii) at the time of the first post-treatment reinfection (the first thick blood smear containing *P. falciparum* parasites detected during either active or passive surveillance following discharge from hospital); iv) at least 6 months post-admission when the children were parasite-free for the preceding 6 weeks, referred to as healthy phase I (60 samples); v) at least 24 months post-admission, referred to as healthy phase II (82 samples), when the children were asymptomatic and parasite-free as determined both by microscopical examination of Giemsa-stained thick blood smears and by ELISA-based screening for *P. falciparum*-specific histidine-rich protein II (as described in the materials and methods section of

publication I). Plasma samples from 21 clinically healthy semi-immune Gabonese adults over 18 years of age, living in Lambaréné, and from 50 healthy non-exposed German adults with no history of malaria, were included as positive and negative controls, respectively. Detection of IgG antibody responses to *P. falciparum* VSA expressed by a panel of 6 heterologous *P. falciparum* isolates (Cys002, Cys007, Cys030, Cys035, Cym030 and Cym033), previously adapted to *in vitro* parasite culture according to the standard method of Trager and Jensen (1977), was performed using standard flow cytometric assay as described in materials and methods section of publications I and II. For analytical evaluation of the antibody activity recorded for each plasma sample, IgG responses specific for *P. falciparum* VSA were expressed as the Mean Fluorescence Intensity (MFI) of antibody bound to infected erythrocytes (iE), which was calculated as follows: MFI = (MFI iE test-MFI uE test)-(MFI iE NIP-MFI uE NIP), where NIP refers to non-immune plasma (German), and uE refers to uninfected Erythrocytes. NIP samples were used to establish a threshold value of positivity for each IgG isotype and isolate, such that a test sample was considered as responder when its MFI value was greater than the mean plus two standard deviations of the MFI value of NIP (see Tables 1 and 2 in publication I).
For EBV analyses, DNA was extracted according to standard methods (Qiagen blood kits) from peripheral blood and plasma samples collected in the acute, convalescent and healthy (6 months later) phases. DNA-based quantitation of EBV activity was carried out by real-time PCR, using primer sets specific for the EBV-polymerase gene (as target) and for the albumin gene (as endogenous control). The techniques and statistical methods applied are described in detail in publication III.

Results

Publication I: Immunoglobulin G Isotype Responses to Variant Surface Antigens of *Plasmodium falciparum in* Healthy Gabonese Adults and Children during and after Successive Malaria Attacks.

Flow cytometry-based measurements of *P. falciparum* VSA-specific plasma IgG isotype antibody responses of healthy Gabonese adults were compared with those of Gabonese children during two consecutive malaria episodes (acute phase and first post-treatment reinfection) and when they were healthy. As shown in FIG.1A and 2A of the paper, a broad similarity in the magnitude of isolate-specific IgG isotype responses (MFI) was discernible between the adults and children. Analyses of cumulated MFI values of responses to all 6 *P. falciparum* isolates revealed that anti-VSA IgG3, followed by IgG2, predominated in healthy Gabonese adults (FIG. 1B) whereas in healthy children, anti-VSA IgG3 and IgG4 were the highest (FIG. 2B). Qualitative IgG isotype responses, defined as the isolate recognition rate (IRR), i.e. the proportion of isolates for which a given individual had MFI values above the threshold of positivity (TABLE 2). The highest IRR of healthy Gabonese adults were recorded for anti-VSA IgG2 and IgG3 (FIG. 1C), whereas the highest IRR in healthy II children was recorded for anti-VSA IgG3 (~70%, FIG. 2C). As shown in TABLE 3, the ratios of IgG3:IgG1 and of IgG3:IgG4 were significantly greater in adults than in children, whereas the IgG3:IgG2 ratio did not differ between the groups. Segregation according to the clinical status of children revealed that the IgG3:IgG4 ratio was almost 2-fold higher in adults compared with the severe malaria group (Table 3).

We also investigated temporal changes in anti-VSA IgG responses according to the children's clinical status at admission (FIG.3). We compared paired samples corresponding to a total of 18

pairs of children at admission and first reinfection, and of 33 pairs at each healthy phase. In samples from the two consecutive malaria episodes, the amounts of anti-VSA IgG2 and IgG3 remained unchanged (FIG.3B-C), whereas the anti-VSA IgG1 in both clinical malaria groups, and anti-VSA IgG4 in the severe-malaria group only, were significantly increased in samples from the first reinfection compared to admission (FIG.3A: IgG1 *P*=0.025 for mild and *P*=0.028 for severe malaria; FIG. 3D: IgG4 *P*=0.009 for severe malaria). When children were healthy the levels of anti-VSA IgG1 and IgG2 were unchanged at similarly low levels (FIG. 4A-B), whereas anti-VSA IgG3 increased significantly over time in the mild malaria group (FIG. 4B, *P*=0.002), while there was a trend for enhanced anti-VSA IgG4 responses in the severe malaria group (FIG. 4D, *P*=0.069). Qualitatively, the anti-VSA specific IgG1 IRR increased from admission to first reinfection in both groups, but only significantly so in the severe malaria group (FIG. 5A, *P*=0.007), while the anti-VSA specific IgG4 IRR in the mild group decreased from the level seen at admission (fig. 5D, *P*=0.034), and anti-VSA specific IgG2 and IgG3 IRR remained similar in both clinical groups, unchanged during both malaria episodes (FIG. 5B-C). In the healthy-phase samples, however, the anti-VSA specific IgG3 IRR increased while the anti-VSA specific IgG4 IRR decreased significantly in the mild malaria group (FIG. 6C, IgG3 *P*=0.046; FIG. 6D, IgG4 *P*=0.016). Anti-VSA IgG1- and IgG2 IRR did not change (FIG.6A-B).

Publication II: IgG isotype responses to erythrocyte surface-expressed antigens of *Plasmodium falciparum* predict protection from malaria in African children.

In the second part of the study, the origin of the six heterologous *P. falciparum* isolates used for screening of anti-VSA activity was taken into account, as well as the malaria histories of the children, in order to investigate whether the profile of VSA-specific IgG isotype responses to parasite isolates with different origins is a determinant factor in the susceptibility to *P.*

falciparum malaria. The 6 heterologous isolates were thus grouped according to their origin into VSA_{SM}-isolates, designating those collected in a separate study from Gabonese infants with severe malaria (Cys002, Cys007, Cys030, Cys035), and VSA_{UM}-isolates designating those obtained from Gabonese children with uncomplicated (mild) malaria (Cym030 and Cym033). VSA_{ALL} here indicates comparisons of cumulated responses to all 6 isolates. This part of the study was restricted to comparisons of flow cytometry-based assessments of anti-VSA IgG isotype responses (MFI) measured in the acute, convalescent and healthy (6 month) phases in a total of 60 Gabonese children, half of whom presented with severe and the other half with mild malaria. Figure 2 shows the profile of convalescent-phase anti-VSA IgG isotype antibody responses for each *P. falciparum* isolate. The patterns differ among the isolates, with some similarities noticeable between Cys035 and Cys007 (Figure 2A-B), or between Cys030, Cys002, Cym030 and Cym033 (Figure 2C-F). In subsequent analyses, the IgG isotype-specific MFI for the four VSA_{SM} and the two VSA_{UM}-isolates were cumulated separately and compared according to children's clinical presentation and histories. As illustrated in Figure 1, anti-VSA_{UM} and anti-VSA_{SM} IgG1 responses were similarly low in the acute phase (figure 1A), whereas anti-VSA_{SM} IgG3 responses predominated in both malaria-groups (Figure 1C), and anti-VSA_{SM} IgG2 responses were significantly higher in the severe group (Figure 1B). In the convalescence, anti-VSA_{SM} and anti-VSA_{UM} IgG1 increased significantly and predominated in both groups (Figure 1A), while anti-VSA_{UM} IgG2 and anti-VSA_{SM} IgG3 increased significantly in the mild, but anti-VSA_{SM} IgG2 decreased in the severe-malaria group (Figure 1B-C). MFI-values for anti-VSA_{SM} and anti-VSA_{UM} IgG4 remained similar and constant from the acute to the convalescent phase (Figure 1D). The healthy phase was characterized by a decline of IgG1 and IgG3 responses specific for both VSA_{UM} and VSA_{SM} in both groups, while anti-VSA_{SM} IgG4 responses increased significantly in the severe malaria group (Figure 1A, C-D). Temporal changes in cytophilic (IgG1+IgG3) versus non-cytophilic (IgG2+IgG4) IgG isotype anti-VSA responses

were also examined. As shown in Table 1, anti-VSA IgG3 and IgG1 responses predominated in both groups in the acute and convalescent phases respectively and anti-VSA IgG4 responses in the severe malaria group in the healthy phase. Since active two-weekly follow-up surveillance indicated that those admitted with severe malaria had significantly shorter delays to their first post-treatment reinfection and a significantly increased frequency of malaria attacks compared with their mild malaria counterparts (Lell et al 1999, Luty et al 1999), survival analyses using the Cox's proportional hazards model were performed to determine whether the prospective convalescent phase measurements of IgG isotype responses with specificity for VSA_{UM} and/or VSA_{SM} isolates we made could predict protection from malaria in these children. These analyses showed, as expected, a significant association of malaria clinical status (mild versus severe) with time to first post-treatment reinfection in the study population (Table 2A), and revealed that this association was reflected specifically within the subgroup of children with responses to VSA_{UM} isolates (Table 2B). In addition, analyses of cumulated MFI for all 6 isolates (VSA_{ALL}) indicated a trend toward longer delay to first reinfection with increasing anti-VSA_{ALL} and anti-VSA_{UM} IgG1 responses (Table 2A, VSA_{ALL}: P=0.059; Table 2B, VSA_{UM}: P=0.057). In the context of malaria reinfection intervals, a significant association was also found between clinical status of donors and shorter malaria reinfection intervals in the whole population (VSA_{ALL}, P=0.021), and after segregation for responses to either VSA_{UM} (P=0.004) or VSA_{SM} isolates (P=0.006). Moreover, VSA_{ALL} and VSA_{UM}-specific IgG1, but also VSA_{SM}-specific IgG4 responses displayed significant associations with longer intervals between reinfections (IgG1: P<0.001; IgG4: P=0.006). Since the evaluation of healthy-phase anti-VSA IgG isotype responses as retrospective measures for malaria episodes was also relevant for the study, correlation analyses with the number of reinfections between collection of the convalescent and healthy phase samples were performed using Spearman rank test, but revealed no association with patients' clinical status, nor with anti-$VSA_{ALL,}$ $VSA_{SM\ or}$ VSA_{UM} IgG isotype responses (data not shown).

Publication III: Epstein-Barr viral reactivation persists at a high frequency in young African children with a history of severe *Plasmodium falciparum* malaria

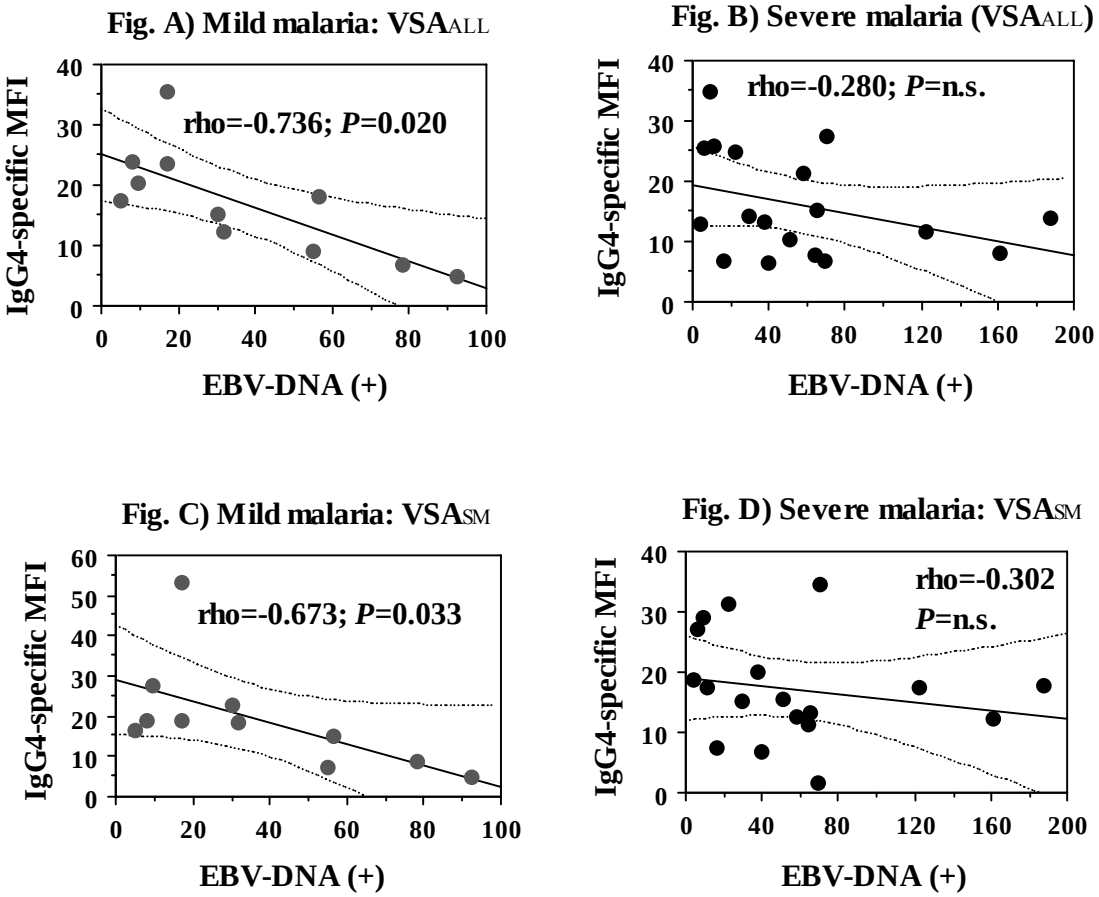
The basic question of the role of EBV co-infection in the immunity to and outcome of *P. falciparum* infection in African children was addressed in this part of the study. Molecular investigations were performed using whole blood and plasma samples collected in the acute, convalescent and healthy phases from the same 60 Gabonese children monitored in publication II. The mean age of the participants was 52 [13-101] months. Preliminary tests for DNA quality and PCR efficacy revealed that whole blood was a much better source of DNA than plasma for real-time PCR analyses, in terms of sensitivity, amplifiability and reproducibility (Table 1 and 2). Therefore, only whole blood-derived DNA was used to quantify EBV polymerase gene expression over the time course of the study. Amplification data were analyzed by the standard method of relative quantitation (see material and methods section of publication III). As shown in Figure1A and 2A, EBV gene expression increased in the acute phase, and then decreased significantly in the convalescent and healthy phases in the mild malaria group. In the severe malaria group EBV gene expression remained constant even after malaria parasite clearance (Figure 1B and 2A). Comparisons between the clinical groups revealed that EBV gene load in the healthy phase was significantly higher in the severe compared with the mild malaria group (Figure 2A). Similar analyses restricted to EBV DNA-positive (EBV DNA^+) individuals showed a decline of EBV-gene load between the acute and healthy phases in both clinical groups (Figure 2B). Analyses of temporal changes in the proportion of EBV DNA^+ individuals showed that the frequency of EBV DNA^+ individuals increased in the severe but decreased in the mild group over the time course of the study (Table 1), and further revealed a significant difference between the groups in the healthy phase (Table 1). The different patterns (profiles) of EBV-gene expression occurring in the population subsequent to acute *P. falciparum* malaria were also investigated. As shown in Table 3, the more common patterns P1 and P2 were characterized by

the complete absence and by the persistence of EBV DNA, respectively, in the convalescent and healthy phases, while patterns P3 and P4 were rare events in both clinical groups. P1 was more frequent than P2 in the mild group, whereas P2 predominated in the severe group (mild, P1 vs. P2: 45% vs. 29%; severe, P1 vs. P2: 54% vs. 25%; $P<0.05$ by Chi-square test). We also assessed the influence of EBV co-infection on susceptibility to malaria both at the epidemiological and the immunological levels. An inverse correlation between age and EBV gene load was observed at all time-points, and was particularly pronounced in the healthy phase ($P<0.001$, by Spearman rank test). EBV DNA^+ individuals, consequently, were younger in age (given in months) than those without EBV DNA (acute: 43[32] vs 52[44], $P=0.033$; convalescence: 39[26] vs 58[44], $P=0.005$; healthy: 33[24] vs 57[40], $P=0.001$; by Mann-Whitney test). Malaria-related parameters such as parasitaemia, haematocrit and parasite clearance time did not show any significant association with EBV DNA load in the acute phase (data not shown). We also made prospective analyses using convalescent-phase EBV-gene load and showed an inverse association of EBV-gene load with time to first malaria reinfection (Figure 3A, $\rho=-0.571$ and $P=0.003$; by Spearman rank test), but a positive association with the number of malaria reinfections, especially in EBV-DNA^+ children (Figure 3B, $\rho=0.542$ and $P=0.006$; by Spearman rank test).

In the context of the influence of EBV co-infection on immunological responses, the plasma concentrations of different cytokines (IL-10, IL-12p70, IL-12p40, TNF-α and IFN-γ) were considered. These analyses revealed a trend for higher plasma IL-10 levels in EBV DNA-free compared to EBV DNA^+ individuals (Figure 4A), but no association between EBV DNA load and plasma levels of TNF-α, IL-12p40 (Figure 4B) and IFN-γ (data not shown) in the acute phase. However, acute-phase levels of IL-12p70 were significantly higher in EBV DNA-free compared to EBV DNA^+ children in the severe, but not in the mild malaria group (Figure 4A). In addition, regression analyses showed an inverse correlation between acute-phase levels of

IL-12p70 and EBV DNA-load in the severe group only (ρ=-0.498 and P=0.017; by Spearman rank test). The healthy phase was characterized by significantly higher levels of IL-12p40 and TNF-α (Figure 4B), by a trend to higher levels of IFN-γ (P=0.069, by Mann-Whitney rank test), in EBV DNA^+ compared to EBV DNA-free children, and by a positive association of EBV DNA load with IL-12p40 levels (ρ=0.485 and P<0.001; by Spearman rank test). Healthy phase levels of IL-10 remained low and unchanged in the presence of EBV (data not shown).

One of the main objectives of this work was to determine the influence of concurrent EBV infection on IgG isotype responses specific to *P. falciparum* VSA (expressed by 6 isolates Cys002, Cys007, Cys030, Cys035, Cym030, Cym033), known to mediate clinical immunity to *P. falciparum* malaria. Prospective analyses using convalescent-phase measures revealed inverse associations of the magnitude of EBV-gene expression with anti-VSA_{ALL} and with anti-VSA_{SM} IgG4 responses only in EBV-DNA^+ individuals (anti-VSA_{ALL} IgG4: ρ=-0.492, *P*=0.009; anti-VSA_{SM} IgG4: ρ=-0.480, *P*=0.010; Spearman rank test). These associations were solely attributable to EBV-DNA^+ children in the mild malaria group (mild: anti-VSA_{ALL} IgG4: ρ=-0.736, *P*=0.020; anti-VSA_{SM} IgG4: ρ=-0.673, *P*=0.033; Spearman rank test). In the context of IgG antibody responses to VSA_{UM} isolates, similar prospective analyses using convalescent-phase measures revealed a positive association between anti-VSA_{UM} IgG2 and EBV-gene load (anti-VSA_{UM} IgG2: ρ=0.332, *P*=0.031; by Spearman rank test), again attributable to those in the mild malaria group (anti-VSA_{UM} IgG2: ρ=-0.473, *P*=0.036; by Spearman rank test). Representative regression plots for convalescent-phase anti-VSA_{ALL} and anti-VSA_{SM} IgG4 responses relative to EBV-DNA load in children presenting with mild or severe malaria are illustrated on the next page.



Figures: Association between EBV-DNA load and anti-VSA_{ALL} or anti-VSA_{SM} IgG4 responses in patients with mild **(A and C,** respectively**)** or severe malaria **(B and D,** respectively**).** Convalescent phase values were taken as prospective measures. Regression plots show malariometric parameters (y axes) plotted against EBV DNA loads (x axes) for those with blood samples EBV DNA+, and with 95% confidence intervals represented as dotted lines. since each DNA-sample was amplified in triplicate in 384-wells plate, a sample was defined as EBV-DNA (+) positive if EBV DNA was detectable in at least 2 out of 3 wells, and the corresponding threshold cycle $C_T \leq 39$ (see materials and methods section for details). The non-parametric Spearman rank test was used to test for associations, where $p<0.05$ in conjunction with $-0.25<\rho(rho)>0.25$ was considered as significant. MFI mean fluorescence intensity; n.s not significant; VSA_{ALL} all 6 *P. falciparum* isolates; VSA_{SM} isolates from severe malaria outpatients.

Discussion

The present study was initiated in order to investigate anti-*P. falciparum* immunological responses in young African children who are at an age when they are acquiring anti-malarial immunity, and the potential of concomitant EBV-infection to influence the process of acquisition of these malaria-protective immunological responses. Using large groups of African adults and children, this study reports a detailed investigation of the specific reactivity of IgG antibody subclasses to the polymorphic *P. falciparum*-derived VSA inserted into the membranes of infected erythrocytes, and describes the evolution of anti-VSA IgG isotype responses as a function both of young African children's clinical presentation at inclusion into the study and of their subsequent reinfection profiles. IgG isotype responses assessed in this study were directed to the VSA expressed by a panel of six locally collected heterologous parasite isolates, each known to be composed of several antigenically distinct parasite strains. Distinct from studies of individuals' responses to VSA expressed by homologous parasite isolates, i.e., by the parasites causing a given malaria episode, the fundamental questions addressed in assessments here concerned changes in individuals' overall anti-VSA IgG antibody isotype responses and the ability of these individuals to mount such responses, in order to enable comparisons, in the case of children, between groups of individuals who presented with malaria of differing levels of severity, and also between healthy children and adults.

The profiles of anti-VSA IgG responses to the panel of heterologous isolates that were used lacked any discernible isotype-specific pattern, both in samples from healthy adults and healthy children (publication I). Hence, no anti-VSA IgG isotype-specific pattern could be associated with malaria clinical status of children (publication II). This lack of IgG isotype-specific pattern

may be assigned to the intrinsically highly polymorphic nature of VSA and hence the diversity of the potential epitopic specificities of these antibodies that they induce, given that PfEMP-1 is currently the principal target of the antibodies detected by the cytometric methods employed here and the isolates used comprise multiple strains, each of which could be assumed to be expressing distinct VSA. In the profile of anti-VSA IgG responses observed in adults, the prevalence of IgG3 and IgG2 observed in adults in terms of the isolate-specific rate of recognition (IRR) is rather unexpected. The finding contrasts with the reported predominance of IgG1 in the profile observed in healthy Papua New Guinean adults (Piper et al 1999). As a note of caution, that study reported results as percentages of positive cells rather than the MFIs we used here, making direct comparisons invalid. The preferential induction of IgG3 responses to various *P. falciparum* asexual-stage antigens, including merozoite surface protein 1 (MSP-1), -2, and -3 and parasite glycosylphosphatidylinositols, is a well-described phenomenon (Oeuvray et al 1994, Ferrante et al 1997, Cavanagh et al 2001, Boutlis et al 2003). This preference seems to be associated with the degree of polymorphism associated with the target antigen or epitope thereof, an observation with which in vitro antibody induction assays are largely concordant (Garraud et al 2002). However in children, anti-VSA IgG3 responses predominate in the acute phase, followed by IgG2 and then IgG4 antibodies, independently of their clinical presentation (publication II). The data thus strongly indicate that cross-reactive antibodies are a prominent feature of the profile of anti-VSA IgG responses induced by *P. falciparum* malaria episodes in young African children and in adults with high and perennial levels of exposure to infection. These findings are consistent with a recent study indicating that the IgG isotype response to VSA expressed by homologous parasite isolates in Kenyan children who presented with uncomplicated *P. falciparum* malaria is predominantly, although not exclusively, composed of IgG3 antibodies (Kinyanjui et al 2003). Several lines of evidence support the concept that severe and life-threatening *P. falciparum* malaria occurring frequently in young non-immune children is

associated with virulent parasites expressing restricted and antigenically conserved VSA subset (VSA_{SM}), whereas parasites expressing less virulent and more diverse VSA (VSA_{UM}) dominate infections in semi-immune older children and adults (Bull et al 2000, Nielsen et al 2002, Hviid et al 2004). Although the molecular characterization of these different subsets of VSA remains to be elucidated, the panel of heterologous *P. falciparum* parasites used here was segregated into VSA_{SM} and VSA_{UM}-expressing isolates according to their source of availability (from Gabonese pediatric cases presented with severe and from those with mild malaria respectively). The data revealed that in both clinical groups of children, acute phase responses of IgG3, IgG2 and IgG4 prevailed for VSA_{SM}-expressing parasites but were very low for VSA_{UM}-expressing isolates. These findings are consistent with the conclusion drawn by others that acquisition of antibody specificity for VSA_{SM} isolates is more rapid and the recognition higher than for parasites expressing VSA_{UM} (Bull et al 2000, Nielsen et al 2002, Gupta et al 1999). Hence, this study is the first to clearly describe the chronology and the sequence of the acquisition of anti-VSA IgG isotype antibodies, and their association with protection from clinical malaria. The predominance of IgG3 antibodies during symptomatic malaria in children, and in healthy adults, implies that IgG3 antibodies exhibit the most diverse "VSA-specific repertoire" of all the isotypes and that, as a cytophilic isotype, they may be the first to be acquired very early during infection with *P. falciparum*, presumably contributing to control of parasitemia via, for example, blockade of parasitized erythrocyte cytoadherence to endothelial cells, activation of the complement system, and opsonization involving interactions with Fcγ receptors on monocytes/macrophages and possibly NK cells leading to ADCI and/or ADCC, resepctively (Tebo et al 2002). Following treatment of symptomatic malaria, and particularly in the absence of infection, IgG3 responses declined significantly. Such a temporally-related decrease in the amount of anti-VSA IgG3 antibodies in the absence of parasite antigen-mediated stimulation might be expected, since IgG3 has the shortest half-life (ca. 8 days) of all the IgG isotypes. The prevalence of anti-VSA_{SM}-

specific IgG3 after parasite clearance in the group of children with a history of severe malaria, paralleling that of anti-VSA_{SM} IgG4 and anti-VSA_{SM} IgG2, confirms that anti-VSA_{SM} IgG responses dominate in non-immune children susceptible to severe clinical malaria (Bull et al 2000, Ofori et al 2002, Nielsen et al 2004). IgG2, the most prevalent anti-VSA isotype next to IgG3 during symptomatic malaria episodes, particularly in children with severe malaria, appears to be important in the early steps of control of parasitemia. These findings imply skewing of IgG subclass-switching towards protective IgG2 responses to plasmodial antigens in these individuals. Since IgG2 is also one of the predominant anti-VSA antibody isotypes in adults, these data suggest that IgG2 and IgG3 may synergize to enhance and sustain anti-malarial protection. Such a putative synergistic effect is substantiated by the similarity between IgG2 and IgG3 responses to the VSA_{UM} and VSA_{SM} parasite subsets during asymptomatic carriage of malarial parasites (publication II). The specificity of IgG2 antibodies for plasmodial antigens deserves further comment, since its remains a subject of some controversy. The data are at variance with studies reporting on the one hand the association of anti-plasmodial IgG2 responses with high risk of developing severe malaria in Kenyan children, and showing on the other hand that purified IgG2 antibodies block the ability of purified cytophilic antibodies to inhibit parasite growth in vitro (Groux et al 1990, Ndungu et al 2002). The model of IgG2-mediated protection proposed in the current study is consistent with an earlier study showing that maternally transmitted parasite antigen-specific IgG2 responses have malaria-protective effects in Cameroonian infants, in whom they were associated with a reduced risk of *P. falciparum* infection (Deloron et al 1997). The model also agrees well with the association between levels of parasite antigen-specific IgG2 and resistance to *P. falciparum* recently shown in a study conducted in Burkina Faso, which was attributed to the high prevalence in the study population of a polymorphism in the monocytes' FcγRIIA that results in an abnormally high affinity for Fcγ2 (Aucan et al 2000). The prevalence of the above-mentioned FcγRIIA mutation is very high

in the Gabonese population, (~75% allele frequency; F. Ntoumi, personal communication), which may suggest an influence on the development of IgG2 responses. Defining a clear role for anti-VSA IgG2 antibodies nevertheless awaits further study.

The boosting of IgG1 responses to VSA_{UM} predominantly and to VSA_{SM} in the convalescent phase in both groups of children represents persuasive evidence for a protective function of antibodies of the major cytophilic IgG isotype directed to the VSA expressed by heterologous parasite isolates of a particular sub-type, which is also substantiated by the elevated anti-VSA IgG1 responses found in children during the first post-treatment reinfection (publication I). These observations are consistent with the results of numerous studies reporting that the levels of IgG antibodies with specificity for VSA expressed by heterologous parasite isolates are enhanced in the post-infection period (Iqbal et al 1993, Giha et al 1999, Bull et al 2002, Ofori et al 2002, Chattopadhyay et al 2003). The particularly outstanding observation of this study concerns the strong association of anti-VSA_{UM} IgG1 responses with clinical protection manifest by significantly prolonged intervals between malaria attacks. Recent in vitro studies showed that in a *P. falciparum* clone (3D7) expressing VSA_{UM} with a small minority of VSA_{SM}, expression of the dominant VSA_{UM} variant switched to VSA_{SM} in the presence of IgG from African children (Staalsoe et al 2003, Jensen et al 2004). Moreover, expression of VSA_{SM} is somehow preferred by parasites infecting non-immune hosts and the sequence of appearance of VSA is not random, as already suggested by others (Nielsen et al 2002). Following these arguments, one could thus speculate on the potential of IgG1 to drive VSA expression switching away from the rare, less recognized VSA_{UM}-type toward the frequent more recognized VSA_{SM}-type. This concept also emphasizes the putative selective advantage of VSA_{SM}-expressing isolates in non-immune individuals (Hviid et al 2004).

A molecular explanation for the prominence of the magnitude of IgG4 responses to VSA in healthy adults, and to VSA_{SM} particularly in healthy children, is hampered by the lack of

appropriate studies defining the functional specificities of this antibody subclass (Hviid et al 2003). Non-cytophilic IgG4 antibodies are reported to interfere with the parasite growth inhibition mediated by cytophilic isotypes *in vitro* and may therefore act as 'blocking' antibodies *in vivo* (Groux et al 1990). In the study described here the children who presented with severe malaria lacked effective immune responses capable of suppressing the growth of the parasites responsible for their condition. Clearly, however, the data indicate that high levels of IgG4 anti-VSA antibodies with a particular VSA_{SM} specificity are beneficial rather than detrimental, as substantiated by the significant association of anti-VSA_{SM} IgG4 found with prolonged intervals between malaria attacks. Although speculative, anti-VSA IgG4 antibodies may function by interfering with cytoadherence of infected erythrocytes on endothelial cell receptors, but their specificity remains a paradox. IgG4 isotype constitute 5% of the IgG in adults' serum, compared to 65% for IgG1, 25% for IgG2 and 6% for IgG3, but antibody activity of the different IgG isotypes is not necessarily distributed in this proportion. For reasons related to differences in affinity but also in avidity, the number of IgG4 antibody molecules required for agglutination is higher than that required for agglutination by total IgG antibodies. Hence, based on the observation that IgG1 antibodies are most prominent in individuals with limited antigenic stimulation, studies have shown that prolonged exposure to immunogens can skew responses towards IgG4 (Aalberse et al 1983a and b, Iskander et al 1981). Following these arguments, the data presented here is therefore in agreement with the long-held view that the acquisition and maintenance of immunity to malaria is related to repeated exposure to *P. falciparum* as highlighted in the follow-up surveillance of children with a history of severe malaria (Lell et al 1999, Luty et al 1999), and also raise the possibility of temporally-related IgG-isotype switching from IgG1 toward IgG4 isotype induced by the persisting and virulent VSA_{SM}-expressing parasites in non-immune children. This is substantiated by the observation that only ~40% of semi-immune adult Gabonese have IgG1 whereas almost 100% have IgG2 and IgG3 responses

with specificity for VSA_{UM} parasites, while ~75% have IgG4 responses with specificity for the VSA_{SM} isolates (Cabrera G, unpublished observations). Thus, the data are consistent with the idea of a gradual acquisition of protective immunity shifting expression away from the VSA_{SM}-repertoire towards the VSA_{UM}-subtype, as VSA_{SM}-specific immunity is acquired, thus pointing to a nonrandom closure of the holes (Bull et al 1999). How anti-VSA IgG4 antibodies exert their malaria-protective effects remains to be elucidated.

In the attempt to address the question of whether a concomitant infection with EBV may potentially contribute to the susceptibility to severe *P. falciparum* malaria in children living in areas where malaria is hyperendemic and the transmission perennial, reactivation of EBV-genome in the same cohort of children was monitored over the same previously characterized malaria episodes. The choice of whole blood-based real-time PCR as a useful method to quantify the load of EBV DNA in the blood of these children was made on the basis of its extremely low detection limits (sensitivity of approximately 10 genomic EBV DNA copies or approximately 1 EBV-infected cell), its high specificity and precision and reproducibility, its ability to detect PCR inhibitors such as heparin possibly present in clinical probes, to correct for variations in amplification efficiencies between different samples, and to control for false-negative results (Beutler et al 1990, Kimura et al 1999, Lo et al 1999, Stevens et al 1999 and 2001). Serological assays using antibodies to EBV viral capsid antigen (EBV-VCA) or early antigen (EBV-EA) are not reliable in immunosuppressed individuals, and PBMC or B cell-based real-time PCR does unfortunately not give information about loads of EBV DNA probably present in serum, as is the case in patients with EBV-associated malignancies (Lo et al 1999, Kimura et al 2000, Pratesi et al 2003, Fan et al 2004). Hence, due to inter-laboratory differences in DNA sample type, DNA isolation methods and/or EBV DNA detection assays, there is a lack of appropriately standardized settings for detection and quantitation of EBV DNA-specific real-time PCR signals.

The increase in EBV DNA during the acute phase of *P. falciparum* malaria, followed by a significant decline during the post-treatment phase is in agreement with earlier studies in African children with malaria, but also with studies of immunosuppressed patients or in those presenting other EBV-related malignancies (Moss et al 1983, Whittle et al 1984 and 1990, Ragona et al 1986, Gunapala et al 1990, Lam et al 1991). Original studies on the topic of malaria-related EBV reactivation conducted in the Gambia showed that spontaneous proliferation of EBV-infected lymphocytes in Gambian children increased during acute and chronic *P. falciparum* malaria attacks, but then significantly decreased in convalescence (Whittle et al 1984 and 1990, Lam et al 1991). It is widely known that acute *P. falciparum* malaria is associated with T cell lymphopenia due to the sequestration of activated T cells to the site of inflammation (Wells et al 1979, Chougnet et al 1992, Hviid et al 1997). These cells, involving populations of both CD4+ and CD8+ T cells, are engaged in the immune response taking place at the sites of infection and, after anti-malarial drug therapy, re-emerge to comprise a transitory state of lymphocytosis in the peripheral circulation, before immunological homeostasis is re-established during convalescence (Chougnet et al 1992, Hviid et al 1997, Kemp et al 2002). Although speculative, it is likely that as a result of concomitant EBV infection, the immunosuppressive effects of *P. falciparum* malaria acutely impairs the normally efficient cytotoxic T cell surveillance of EBV replication, leading to uncontrolled EBV reactivation followed by the expansion of an EBV-infected B cell population. This is reflected in the data by the high EBV DNA loads present in the peripheral circulation during the acute malaria episodes, returning to minimal levels, particularly in the mild malaria group, after gradual re-emergence of EBV-specific cytotoxic T cells along with elimination of malaria parasites. The most outstanding and somewhat unexpected observation in this part of the study was the contrasting profile of evolution of EBV DNA loads in the children that presented with severe malaria. In that group, peripheral EBV DNA loads persisted at high levels and in a higher proportion of children throughout the study period, compared to their

counterparts with mild malaria. The difference in EBV DNA loads between the groups was particularly significant in the healthy infection-free phase, but also in the convalescent phase where 54% of children who presented with severe malaria could not control the burden of circulating EBV DNA after the acute malaria attack, compared to 45% of children in the mild malaria group who were free of EBV DNA in the post-treatment period. This is the first study showing evidence of differential resolution of EBV reactivation according to the clinical severity of *P. falciparum* malaria. The implication is of a possible dysfunction of EBV control mechanisms in children susceptible to severe *P. falciparum* malaria, possibly predisposing them ultimately to malignancies such as Burkitt's lymphoma. As a possible cause of this differential control of EBV, anti-malarial chemotherapy could be excluded, since both groups of patients received the same anti-malarial drug regimens - consisting of sulfadoxine-pyrimethamine - for treatment of reinfections detected during active in-home follow-up. As we reported earlier, active surveillance showed that, as a group, those who presented with severe malaria were more susceptible to malaria attacks subsequently (Lell et al 1999, Luty et al 1999). The data presented here show that high EBV DNA loads were associated with greater numbers of malaria attacks, and with shorter delays to first post-treatment reinfection with *P. falciparum* in the whole study cohort. Further analyses taking into account their clinical malaria presentation status indicated that these associations were only attributable to children presenting with severe malaria (data not shown). Similarly, the clear age dependency of the presence of EBV DNA observed in the whole study cohort was only attributable to children with severe malaria, and coincided with the age-dependent acquisition of protective immunity to severe malaria described in earlier studies (Premji et al 1995). A possible explanation is that the recovery of effective cell-mediated immune control of EBV seen in the majority of individuals presenting with mild malaria is abrogated in children suffering frequent repeated *P. falciparum* infections, especially so in those that presented with severe malaria at inclusion into the study. The age-dependent acquisition of

anti-EBV activities is an indication that the putative defects in cell-mediated immune control of EBV in children that presented with severe malaria are temporal and reversible.

Like most viruses infecting humans, EBV exhibits a multiplicity of mechanisms to subvert the immune system and ensure its survival. The data presented here concerning alterations in the plasma profiles of certain anti-VSA IgG antibodies that are related to the simultaneous presence of EBV DNA in peripheral blood suggest that the loss of control of EBV replication may influence antibody responses considered important for anti-plasmodial immunity. In this context, the association of EBV DNA load with increasing levels VSA_{UM}-specific IgG2 responses, but with decreasing IgG4 responses to VSA_{ALL} and VSA_{SM} in the mild group is of particular interest. Molecular or immunological explanations for these associations remain a challenge, since studies clarifying the role and specificity of IgG2 and IgG4 in EBV and in plasmodial infections are still lacking. However, it is likely that during concomitant infections, EBV can skew IgG isotype switching away from cytophilic ADCC-efficient IgG3 and IgG1 which exhibit diverse cross-specificities for VSA epitopes, towards the non-cytophilic IgG2 subclass, which can potentially act as "blocking antibodies". EBV could thus delay or prevent the removal of parasitized red cells, alter the chronology of the sequential IgG-isotype specific acquisition of "VSA epitope specificities", and thereby lead to maintenance of parasitemia at a level that favors the *P. falciparum*-induced immunsuppression of EBV-specific T cell control. This concept is substantiated by the conclusion drawn from an earlier Gambian study that in individuals living in malaria endemic regions, even subclinical malaria can induce a significant level of immunosuppression leading to EBV reactivation (Moss et al 1983). Protective anti-VSA_{SM} IgG4 responses, as demonstrated by their association with longer intervals between malarial reinfections, are also reduced by concomitant EBV infection probably because, as shown in publication II, IgG4 antibodies are predominant in VSA_{SM}-specific recognition after remission from clinical malaria, and may therefore contribute to the removal of undetectable sub-clinical

levels of parasites which potentially maintain EBV reactivation. This theory needs further investigation.

Another strategy evolved by EBV to escape host immunity is the production of a viral homologue of human IL-10 as well as induction of the production by EBV-infected B cells of the IL-12p40 homologue, EBI3, a component of the heterodimeric immunomodulatory cytokine IL-27 (Devergne et al 1996, Brombacher et al 2003). It could be also demonstrated in this study that the loss of control of EBV replication may influence cytokine responses considered important for anti-plasmodial immunity. Most notable, in this context, is the significantly lower concentration of biologically active IL-12p70 detected in the plasma of children presenting concomitantly with severe *P. falciparum* malaria and EBV DNA, since IL-12 is thought to play a prominent role in initiation of early Th1-mediated control of plasmodial infection (Luty et al 2000, Malaguarnera et al 2002, Stevenson et al 2004). The significantly elevated levels of IL-12p40 detected in plasma of children with EBV DNA when they were otherwise healthy and parasite-free may also have consequences for subsequent host-parasite interactions since the p40 homodimer has known immunomodulatory activities (Brombacher et al 2003). The association between the presence of EBV DNA and significantly enhanced levels of plasma TNF-α found in children when they were healthy and parasite-free is consistent both with our own earlier findings and with the known effects of EBV on constitutive B cell cytokine activity (Rochford et al 1997, Perkins et al 2000, Spender et al 2001). Viral persistence may be directly linked to virus-mediated enhancement of TNF-α activity since this cytokine is known to be required for survival of plasma cells that themselves represent one of the primary reservoirs for latent EBV (Cassese et al 2003).

The study reported here has helped to extend the knowledge in the context of immunology of malaria in young African children and adults, and of the interactions at the clinical and immunological levels between a ubiquitous virus and a protozoan with enormous public health impact. Others have reported evidence of clinically important interactions between a viral

infection common in childhood in Africa - hepatitis B virus - and *P. falciparum* (Thursz MR 1995). Determining the nature and extent of these cross-species influences remains a challenge to both immunologists and vaccinologists with an interest in developing or improving control measures for the diseases associated with poverty in Africa.

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Personal contribution

I declare that following practical works were carried out on my own:

Paper I:

- Part of parasite culture**,**
- flow cytometric measurements of IgG isotype responses from plasma samples collected from Gabonese children in the acute, first reinfection, convalescent, and healthy phase (I)
- flow cytometric measurements of IgG isotype responses from plasma samples collected from nonimmune German donors
- Detection of parasite isolate polyclonality by means of merozoite surface antigens (MSP-1/2)-based PCR-genotyping
- Contribution in writing the paper: part of this paper was used by Cabrera G. for his doctoral thesis

Paper II and III:

- All technical aspects,
- statistical analyses
- writing of the manuscripts

Prof. P. G. Kremsner and PhD. Adrian J. F. Luty supervised all the works related to publications I, II and III in the Institute of Tropical Medicine, Eberhard Karls University of Tuebingen (Tuebingen, Germany), and PhD. Dieter Kube supervised the work related to publication III in the Center for Internal medicine, Department of Hematology and Oncology, Georg-August-University of Goettingen (Goettingen, Germany).

List of publications

I. Immunoglobulin G isotype responses to variant surface antigens of *Plasmodium falciparum* in healthy Gabonese adults and children during and after successive malaria attacks. Cabrera G, **Clarisse Yone (equal contributor)**, Tebo AE, van Aaken J, Lell B, Kremsner PG, Luty AJ. Infect Immun. 2004 Jan; 72(1): 284-94.

II. Immunoglobulin G isotype responses to erythrocyte surface-expressed variant antigens of Plasmodium falciparum predict protection from malaria in African children. **Clarisse L.R.P. Yone**, Kremsner PG, Luty AJ. Infect Immun. 2005 Apr; 73(4):2281-7.

III. Epstein-Barr viral reactivation persists at a high frequency in young African children with a history of severe *Plasmodium falciparum* malaria. **Clarisse L.R.P. Yone**, Dieter Kube, Peter G. Kremsner for the 1/95-C study team and Adrian J.F. Luty. Infect Immun. Under review.

Curriculum Vitae

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Education

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1994-2001	Biology studies at the Friedrich-Alexander-University of Erlangen, Erlangen, Germany
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2000/1	Diploma-thesis on "Association of polymorphisms in Interleukin-4 receptor Alpha gene with rheumatoid Arthritis"
1998	Pre-research project on "Investigation of the responsiveness to CD28-Stimulation of freshly isolated CD4-T-memory cells from healthy donors and Individuals with rheumatoid Arthritis
1996	Pre-diploma degree (Vordiplom) in Biology
1992-1993	German language school leaving certificate (PNDS) in Chemnitz, Germany
1984-1991	High school "College Privé de la Retraite" and "Lycee General Leclerc", in Cameroon
1991	High school leaving certificate (Abitur)
1978-1983	Primary school "Ecole Publique Mixte de Messa" in Cameroon

Presentations, Meetings, Memberships

- Poster at the 53nd Annual Meeting of American Society of Tropical Medicine and Hygiene in Miami Beach, Florida, USA, on "Does Epstein-Barr Virus co-infection influence the outcome of *Plasmodium falciparum* malaria in African children?". **Yone C**, Kube D, Kremsner PG, Luty AJF. November 2004
- Lecture and Poster at the 11th biennial EBV-Symposium in Regensburg, Germany, on "Does Epstein-Barr Virus co-infection influence the outcome of *Plasmodium falciparum* malaria in African children?" **Yone C**, Kube D, Kremsner PG, Luty AJF. September 2004
- Lecture at the Joint Malaria and Spring Meeting of the British Society of Parasitology in Chester, England, on "Epstein-Barr viral activation in *Plasmodium falciparum* malaria". **Clarisse Yone**, Dieter Kube, Peter G. Kremsner and Adrian J.F. Luty. April 2004
- Member of the British Society of Parasitology since March 2004
- Lecture-abstract at the 52nd Annual Meeting of American Society of Tropical Medicine and Hygiene in Philadelphia, Pennsylvania, USA, on "Impact of Epstein-Barr virus infection on humoral responses during *Plasmodium falciparum* infection in Gabonese children". **Yone C**, Kube D, Kremsner PG, Luty AJF. December 2003
- Lecture at the 3rd MIM Pan-African Conference in Arusha, Tanzania, on "Evolution of IgG isotype-mediated immune responses against variant surface antigens of *Plasmodium falciparum* in Gabonese children". G. Cabrera, **C. Yone**, A.E.Tebo, P.G. Kremsner and A.J.F. Luty. November 2002
- Poster at the MIM Pan-African Conference in Arusha, Tanzania, on "Impaired Development of Variant Surface Antigen-specific Antibody Responses in Gabonese Children with Severe *Plasmodium falciparum* Malaria". **Clarisse Yone,** Anne E. Tebo, Peter G. Kremser and Adrian J.F. Luty. November 2002
- Poster at the 30th Conference of German Society of Immunology in Düsseldorf, Berlin, on "A novel polymorphic haplotype in the human interleukin-4 receptor alpha chain (CD124)". **Clarisse Yone**, Alla Skapenko, Hendrik Schulze-Koops. November 2000

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Immunoglobulin G Isotype Responses to Variant Surface Antigens of *Plasmodium falciparum* in Healthy Gabonese Adults and Children during and after Successive Malaria Attacks

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We assessed immunoglobulin G (IgG) isotype responses with specificity for the variant surface antigens (VSA) of heterologous *Plasmodium falciparum* isolates by using flow cytometry and plasma from healthy Gabonese adults and from children during and after two consecutive malaria episodes. The individual isolate-specific antibody profiles differed markedly in terms of their isotype content but were similar for healthy adults and healthy uninfected children. In healthy adults, IgG3 and IgG2 responses were the highest, while in healthy children, IgG3 and IgG4 predominated. A transiently elevated IgG1 response was observed during the second of two successive malaria episodes in children, signaling *P. falciparum* infection-induced cross-reactive anti-VSA responses. Our findings highlight the prominence of IgG3 in the overall profile of these responses but also indicate a marked age-related increase in the prevalence of anti-VSA antibodies of the classically noncytophilic IgG2 isotype, possibly reflecting the high frequency of the histidine-131 variant of FcγRIIA in the Gabonese population.

The existence of clonally variant surface antigens (VSA) of *Plasmodium falciparum* that are inserted into the membranes of infected erythrocytes was first demonstrated 2 decades ago (25). A number of subsequent studies have shown that VSA comprise targets of antibody responses that are enhanced with age and are associated with protection from malaria (9, 11, 16, 20, 27, 38, 47, 55). Such associations have been reported in the context of responses to VSA expressed by both autologous and heterologous parasite isolates, which may contribute to the putatively rapid acquisition of immunity to malaria (24). *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) is considered to be the principal target of anti-VSA antibodies, although rifin proteins, a second, polymorphic, parasite-derived family of antigens that are inserted into the infected erythrocyte membrane, also induce antibody responses in exposed populations (2, 7, 31, 32, 42). Studies incorporating longitudinal components have shown that, in most but not all cases, antibodies with specificity for the VSA expressed by the autologous parasites causing a given malaria attack are infrequent or absent prior to the attack but are enhanced and sustained posttreatment (10, 13, 21, 26, 37, 46). In contrast, the profile of antibody responses to the VSA expressed by heterologous parasite isolates shows no such consistent pattern during and after a malaria attack, although the responses have been shown to be elevated in a proportion of individuals in all longitudinal studies reported to date (9, 13, 21, 26, 46). Subclinical pediatric *P. falciparum* infections are associated with raised levels of antibodies that interact with the VSA of heterologous isolates (9). Such findings, along with the known antibody-mediated recognition of VSA expressed by parasites from distant geographical regions, perhaps point to a predominance of variant specificity over cross-reactivity in these responses that is rather less marked than has been conjectured (3, 6, 43).

The cytophilic immunoglobulin G (IgG) isotypes, IgG1 and IgG3, are known to mediate the in vitro phagocytosis of *P. falciparum*-infected erythrocytes (23, 54), but studies incorporating measurements of IgG isotype-mediated responses directed to *P. falciparum* VSA are scarce. Piper and colleagues (50), by using flow cytometric techniques, reported a predominance of IgG1 antibodies with specificity for VSA of heterologous isolates in the sera of Papua New Guinean (semi-immune) adults, with smaller amounts of IgG3 and negligible amounts of IgG2 and IgG4. Kenyan children with uncomplicated malaria, on the other hand, display a predominantly IgG3-mediated antibody response to VSA of autologous isolates in parallel with the expected IgM response (27). In the study presented here, we investigated and compared the profiles of IgG isotype antibodies with specificity for the VSA expressed by a panel of locally collected heterologous parasite isolates from Gabonese adults and children. The latter comprised participants in an extended longitudinal study, thus allowing comparisons within and between groups that had differing initial clinical presentations as well as subsequent infection histories (33, 34).

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MATERIALS AND METHODS

Study site. A study, with the reference code 1/95-C, was initiated in 1995 at the Albert Schweitzer Hospital in Lambaréné, Gabon, a site in equatorial central Africa where malaria is hyperendemic on account of the perennial transmission

TABLE 1. Typical isotype-specific fluorescence intensity values obtained with plasma samples from healthy Gabonese children and adults and used for calculation of MFI values

Cell type (dot plot quadrant)[b]	Fluorescence intensity			
	Healthy child[a]		Healthy adult	
	IgG1	IgG3	IgG1	IgG3
Double-stained cells (upper right quadrant)	54.25	57.05	23.14	55.53
Nonparasitized stained cells (lower right quadrant)	36.80	45.87	11.45	16.20
Mean[b]	17.45	11.18	11.69	39.33

[a] Plasma samples were from the healthy II time point.
[b] Cells were obtained from the upper and lower right quadrants of a dot plot obtained from flow cytometric determinations. Means are as defined in Materials and Methods.

of *P. falciparum* (59). The estimated annual entomologic inoculation rate for Lambaréné is about 50 infective bites/person/yeast (52).

Ethical clearance. Ethical clearance for the study was given by the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné. Children were included in the study after informed consent was obtained from the parent or guardian.

Study design. Details of the study design, patient enrollment, care, and treatment given have been described elsewhere (28, 29, 34). Briefly, 100 children presenting with severe malaria were admitted to the hospital, and an equal number presenting with mild malaria were included. The latter were pair matched to children with severe malaria by gender, age, and area of residence. Children were included in the severe-malaria group if they had a *P. falciparum* parasitemia of >1,000/μl, were older than 6 months, were not homozygous for hemoglobin S, had severe anemia (<5 g of hemoglobin/dl) and/or hyperparasitemia (>250,000 parasites/μl), and had or did not have other signs of severe malaria, for example, loss of consciousness, hypoglycemia, lactic acidosis, or respiratory distress (58). The level of consciousness was determined by using the Blantyre coma score (58). Mild malaria was defined as a parasitemia of between 1,000 and 50,000 parasites/μl on admission, no schizontemia, <50 circulating leukocytes containing malarial pigment/μl, >8 g of hemoglobin/dl, >50 platelets/nl, <12 leukocytes/nl, <3 mM lactate, and >50 mg of glucose/dl of blood. Exclusion criteria for the mild-malaria controls were signs of severe malaria, concomitant acute infection, prior hospitalization for any reason, and intake of antimalarial agents within the preceding week.

Plasma samples. The plasma samples that were used were selected from those collected during the 1/95-C study from the children presenting with severe and mild malaria whose mean age (± standard error) was 47 (±3) months.

We focused on samples from four different time points: (i) the point of acute infection at admission (immediately prior to treatment), (ii) the first posttreatment reinfection, (iii) the healthy phase at least 6 months after admission (healthy I) when the child was aparasitemic for the preceding 6 weeks as confirmed by the examination of Giemsa-stained thick blood smears prepared during the routine fortnightly follow-up home visits undertaken as part of the study, and (iv) the healthy phase at least 24 months after inclusion (healthy II) when children were again known to be asymptomatic and aparasitemic through home visits and the examination of thick blood smears.

A panel of 21 positive-control plasma samples from clinically healthy Gabonese adults was tested in parallel. These donors were all over 18 years of age, were residents of Lambaréné, and consequently had had life-long continuous exposure to *P. falciparum* infection. They are thus considered representative of the semi-immune population in the study area. *P. falciparum* parasitemia in these individuals was not assessed but, if present, could be assumed to be minimal.

Screening for *P. falciparum* infection by the PfHRPII ELISA. In order to verify the parasitological status of individuals at the healthy time points, the levels in plasma of the *P. falciparum* histidine-rich protein II (PfHRPII) antigen, present during or for a short period immediately following the termination of an active *P. falciparum* infection, were determined with a commercially available monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA), which was used according to the manufacturer's instructions (Malaria AG Celisa; Cellabs, Brookevale, Australia).

Parasite isolates and culture. A panel of six *P. falciparum* isolates was used. These were obtained from patients enrolled in a separate outpatient study conducted in 1997 at the Albert Schweitzer Hospital. Isolates with reference codes *Cys002*, *Cys007*, *Cys030*, and *Cys035* were obtained from children presenting with severe malarial anemia, while isolates *Cym030* and *Cym033* were obtained from children presenting with mild malaria. All cases were confirmed monoinfections with *P. falciparum*, and all were shown by routine standardized merozoite surface antigen-based PCR genotyping techniques to be polyclonal, each with at least three different strains (C. Yone, unpublished observations). Details of the methods used for the collection and culture of parasites have been described elsewhere (55). Briefly, peripheral venous blood was centrifuged and the erythrocytes obtained were spin washed twice. Pellets containing infected erythrocytes were then cryopreserved in liquid nitrogen for subsequent in vitro adaptation. Primary isolates were adapted to in vitro culture according to the method of Trager and Jensen (56). Briefly, cells were resuspended in complete medium supplemented with 10% heat-treated, prescreened nonimmune AB^+ serum (from the blood bank of the University Hospital, Tübingen, Germany) and were then incubated in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Fresh O^+ erythrocytes depleted of lymphocytes (University Hospital, Tübingen, Germany) were periodically added. Isolates were initially expanded over a short period of 8 to 10 multiplication cycles (48 h), after which identical stabilates of cultures containing mostly asexual ring forms were cryopreserved for subsequent culture and use in cytometric assays (see below).

Flow cytometric measurement of IgG antibody isotype responses with specificity for *P. falciparum*-infected erythrocyte surfaces. Details of the methods, including the flow cytometric (fluorescence-activated cell sorting) assay employed, have been described elsewhere (50, 55). The following additional procedures were used here: trophozoite-infected erythrocytes (T-IE) were washed in RPMI 1640 and resuspended in phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA) at 10 to 15% parasitemia. Fifty microliters of the T-IE suspension was then transferred to round-bottomed 96-well tissue culture plates (Costar, Corning, N.Y.), and 50 μl of plasma samples previously diluted 1:50 in PBS–1% BSA were added to each well. Negative- and positive-control plasma samples were included in each assay, comprising, respectively, plasma samples from 31 European donors resident in Germany with no history of contact with *Plasmodium* species and plasma samples from 21 healthy African adults resident in Lambaréné. After 30 min of incubation at room temperature, the plates were spin washed three times with PBS–1% BSA at 1,000 rpm for 2 min. Fifty microliters of mouse anti-human IgG at a 1/100 dilution or mouse anti-human IgG1, IgG2, IgG3, or IgG4 (SkyBio, Wyboston, Bedford, United Kingdom) at a 1/50 dilution was added to the wells, and the plates were again incubated for 30 min at room temperature, followed by spin washing as described above. Finally, a fluorescein isothiocyanate-coupled goat anti-mouse IgG antibody (Southern Biotech, Birmingham, Ala.), containing 50 μg of ethidium bromide/ml, was added at a 1/100 dilution. Plates were incubated for a further 30 min and washed as described above. Samples were assayed by fluorescence-activated cell sorting immediately after the double staining with a FACScan (Becton Dickinson, Heidelberg, Germany) using CellQuest 3.3 software. An event was defined as the passage of one cell through the cytometer optical system. We counted 10,000 events per sample, and the geometric mean of the fluorescence intensities was calculated. The mean fluorescence intensity (MFI) was defined as the difference between the geometric mean of the fluorescence emitted by the T-IE and the geometric mean of the fluorescence emitted by the noninfected erythrocytes (background).

Statistical analyses. Analyses were performed by using StatView for Windows 5.0.1 (SAS Institute Inc., Cary, N.C.) running on Windows XP (Microsoft Corp., Redmond, Wash.). Pairwise comparisons of continuous variables were performed with the nonparametric Wilcoxon sign rank test, and for unpaired comparisons the Mann-Whitney U test was applied. The level of significance was set at a two-tailed P value of <0.05.

RESULTS

Sample sizes. Plasma samples from 21 healthy semi-immune Gabonese adults were used. From the cohort of Gabonese children, we analyzed a total of 57 plasma samples from children in the acute phase, 60 samples from children at the first reinfection, 58 samples from children at the first healthy phase, and 82 samples from children at the second healthy phase. The smaller numbers of individuals available for pairwise comparisons at the different time points are explained at the appropriate points in the following subsections.

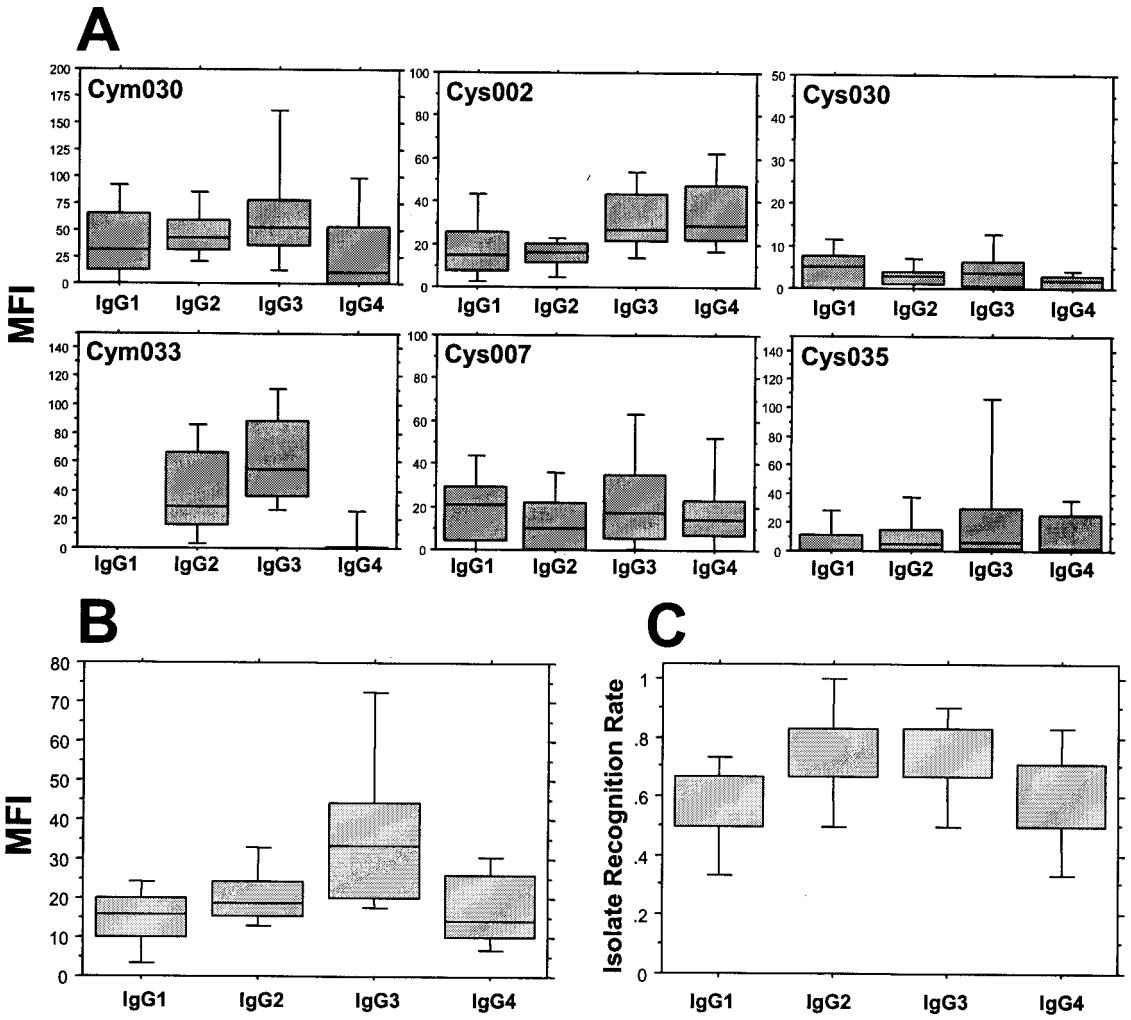

FIG. 1. Healthy semi-immune Gabonese adults' IgG isotype antibody responses to the VSA expressed by a panel of six heterologous *P. falciparum* isolates. (A) MFIs of individual isolate-specific profiles; (B) cumulated MFIs of isotype-specific responses; (C) cumulated isotype-specific IRRs. Box plots illustrate the medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles.

Anti-VSA IgG antibody isotype responses of healthy Gabonese adults and children. The results of the PfHRPII ELISA confirmed the results of microscopy, namely, that none of the children had active *P. falciparum* infections at either of the healthy-phase time points (data not shown). A typical example of the patterns of isotype-specific anti-VSA activity that we detected, expressed as MFIs, is given in Table 1, showing that the background level of binding to nonparasitized erythrocytes was frequently higher in samples from healthy children than in samples from adults.

The IgG isotype profiles of anti-VSA antibody responses detected in plasma samples from healthy Gabonese adults and children (in this case, samples from healthy phase II individuals) are illustrated, respectively, in Fig. 1 and 2. The isolate-specific profiles showed no discernible pattern: IgG isotype responses to individual isolates varied both in the magnitude of anti-VSA antibodies of each isotype and in the relative predominance of any given isotype. For three of the parasite isolates tested (*Cym030*, *Cym033*, and *Cys035*), the highest level in adults was seen with IgG3 antibodies (Fig. 1A). In contrast, IgG1 antibodies were marginally the highest in adults' responses to two of the other parasite isolates (*Cys007* and *Cys030*) but were undetectable in the response to isolate *Cym033*. The profiles seen with healthy children's samples were broadly similar to the adults', except for the relative predominance of IgG1 in the children's response to isolate *Cym030* and a similarly predominant IgG4 response to isolate *Cys002* (Fig. 2A).

Of particular interest in the adults' profile, the classically noncytophilic IgG2 and IgG4 anti-VSA responses were frequent and were notable for the fact that (i) the levels of IgG2 detected were equivalent to those of, for example, IgG1 and (ii) although IgG4 responses were for the most part the weakest, in the profile of responses to isolate *Cys002*, they were detected at a level equivalent to that of IgG3 and were markedly higher in this case than in that of either IgG1 or IgG2 (Fig. 1A).

The cumulated anti-VSA IgG isotype responses (medians of arithmetic means derived from pooled data) of healthy adults and children to all six isolates are shown in Fig. 1B and 2B.

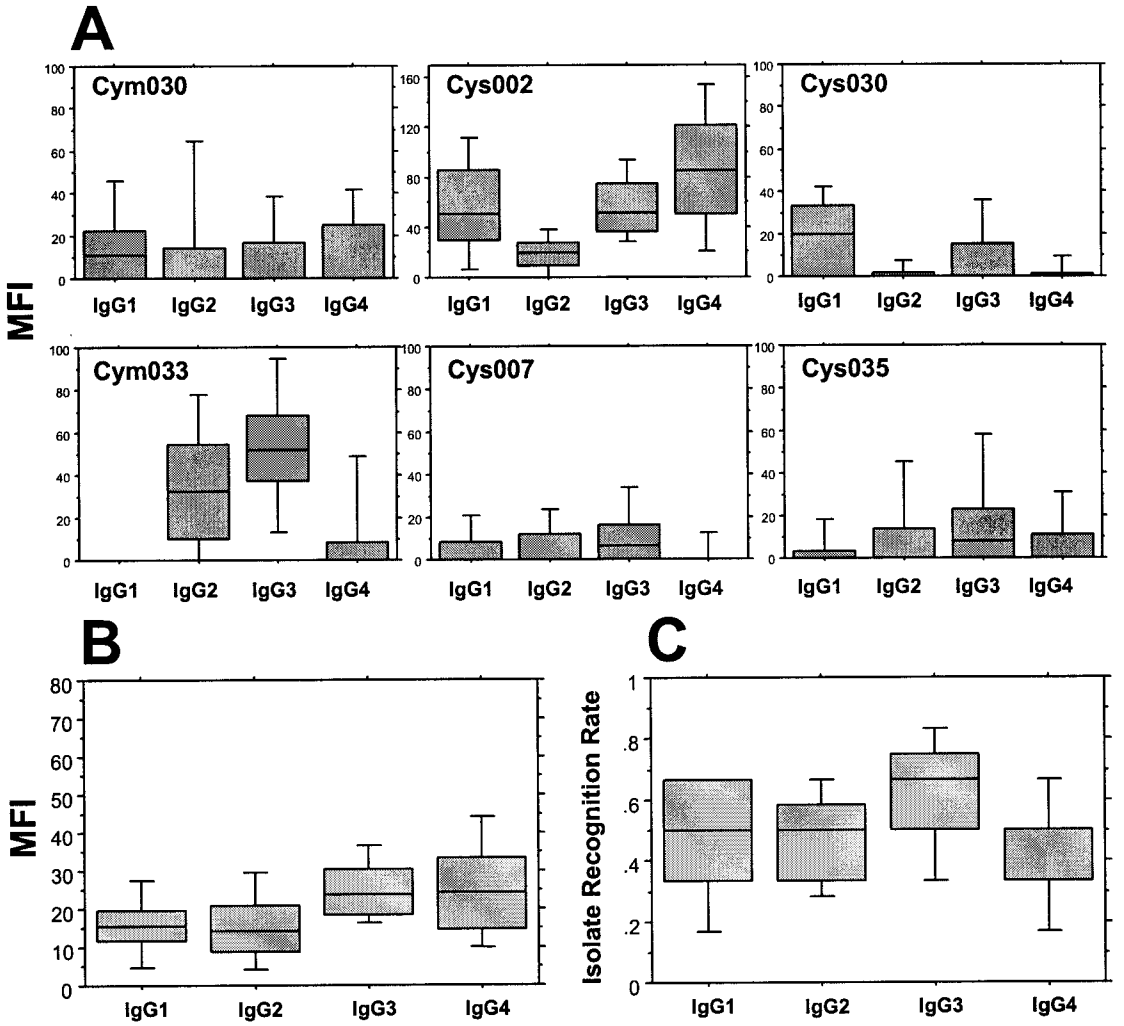

FIG. 2. Healthy Gabonese childrens' IgG isotype antibody responses to the VSA expressed by a panel of six heterologous *P. falciparum* isolates. (A) MFIs of individual isolate-specific profiles; (B) cumulated MFIs of isotype-specific responses; (C) cumulated isotype-specific IRRs. Box plots illustrate medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles. Data are pooled from all children at the healthy II time point and without segregation according to malaria history, and the MFIs of nonresponders were considered to be 0.

These pooled data show that, in healthy adults, antibodies of the IgG3 isotype predominate at a level significantly higher than those of the other isotypes (versus values for IgG1 or IgG4, P was <0.001, and versus values for IgG2, P was <0.005, as determined by the Wilcoxon rank test), with an overall hierarchy of the magnitude of anti-VSA responses as follows: IgG3 > IgG2 > IgG1 = IgG4 (Fig. 1B). In healthy children, there was no clear IgG isotype predominance: the levels of IgG3 and IgG4 were similar, and both were higher than the levels of either IgG1 or IgG2 (Fig. 2B).

In order to make qualitative comparisons of anti-VSA antibody responses, an isolate recognition rate (IRR) was determined. The IRR was based on an initial segregation into responder or nonresponder subgroups according to the thresholds determined for each isotype with each isolate (Table 2). The IRR was then defined as the proportion of isolates from the panel for which antibodies from a given individual showed specific binding above the threshold, as illustrated in Fig. 1C and 2C. The IRRs for IgG2 and IgG3 were similar in adults and were significantly higher than those for either IgG1 ($P < 0.01$) or IgG4 ($P < 0.03$) (Fig. 1C). In healthy children, the highest IRR (~70%) was recorded for IgG3, with lower-level recognition (~50%) for IgG1, IgG2, and IgG4 (Fig. 2C). Over-

TABLE 2. Threshold values of MFIs of isolate-specific IgG isotype anti-VSA responses

Isolate[a]	Threshold MFI[a] for:			
	IgG1	IgG2	IgG3	IgG4
Cym030	5.5	8.8	9.1	8.0
Cym033	4.4	1.9	6.2	2.6
Cys002	0.0	3.9	7.9	8.8
Cys007	10.5	10.9	5.1	6.4
Cys030	4.6	0.0	6.1	0.4
Cys035	0.0	9.5	6.5	3.2

[a] Thresholds were defined as the geometric mean plus 2 standard deviations of the MFIs of responses of a panel of samples from nonexposed Germans.

TABLE 3. Comparison of the ratios of IgG isotype antibodies of healthy Gabonese adults and children with specificities for VSA of a panel of six heterologous *P. falciparum* isolates

Isotypes compared	Population[b]	Clinical group	Anti-VSA isotype ratio			*P* value[a]
			Median	25th percentile	75th percentile	
IgG3/IgG1	Adults		2.650	1.833	3.315	
	Children	All	1.605	1.018	2.228	0.002
		Mild malaria	1.427	1.001	2.228	0.002
		Severe malaria	1.537	1.075	2.213	0.007
IgG3/IgG2	Adults		1.630	0.968	2.553	
	Children	All	1.726	1.194	3.035	NS
		Mild malaria	1.684	1.325	2.978	NS
		Severe malaria	1.911	1.096	3.116	NS
IgG3/IgG4	Adults		1.890	1.345	4.012	
	Children	All	1.388	0.762	1.967	0.002
		Mild malaria	1.392	0.823	2.504	0.031
		Severe malaria	1.160	0.732	1.676	<0.001

[a] Determined by the Mann-Whitney U test for differences between adults and children. NS, not significant.
[b] Children's plasma samples were taken at the healthy II time point.

all, the range of IRRs seen with adults' samples (60 to 80%) was higher than that seen with children's (50 to 70%).

Relative amounts of IgG isotype antibodies in healthy adults and children with specificity for VSA of heterologous *P. falciparum* isolates. Since IgG3 was the predominant isotype detected in the adults' anti-VSA antibody profile, we used the magnitude of the IgG3 response as a reference value to determine ratios with respect to the other isotypes in order to make comparisons of their relative amounts in healthy adults and children, as illustrated in Table 3. These analyses showed that IgG3 predominated over the other anti-VSA isotypes in both adults and children but that the amounts of IgG3 relative to both IgG1 and IgG4 were significantly greater in adults than in children regardless of the latter's history of malaria. The IgG3/IgG2 ratios in adults and children were similar. The most marked difference observed concerned the IgG3/IgG4 ratio in children with a history of severe malaria, in whom the ratio was close to unity compared with the almost double ratio of IgG3 to IgG4 in adults. The different ratios observed in children did not differ significantly after segregation and comparison according to their history of malaria.

Infection-related and temporally related changes in the profile of anti-VSA IgG isotype responses in Gabonese children. We measured anti-VSA IgG isotype responses in paired samples from a total of 18 individuals (8 in the mild- and 10 in the severe-malaria groups) at the admission and first reinfection time points and from a total of 33 individuals (18 in the mild- and 15 in the severe-malaria groups) at the healthy I and healthy II time points. The age at admission and the gender distribution of these subgroups did not differ from those of the entire group of children (data not shown). The median times to the first reinfection in those individuals with a history of mild and severe malaria were, respectively, 35 and 15 weeks (ranges, 4 to 53 weeks and 6 to 37 weeks). Their cumulated responses to all six heterologous isolates at the different infection and healthy time points are illustrated, respectively, in Fig. 3 and 4. At admission, the levels of all four isotypes in the two groups were similar, with IgG3 responses predominant (Fig. 3). At the first reinfection, the levels of IgG2 and IgG3 remained unchanged compared to their levels at admission (Fig. 3B and C), but the level of IgG1 showed a consistent and significant increase in both groups (Fig. 3A) and the level of IgG4 increased significantly at the first reinfection only in the severe-malaria group (Fig. 3D). A comparison of healthy-phase samples revealed a trend towards increased cytophilic-isotype (IgG1 and IgG3) responses in both groups over time but a significant increase only in IgG3 responses in the mild-malaria group (Fig. 4A and C). The noncytophilic IgG2 and IgG4 responses remained unchanged over time but with a trend towards enhanced IgG4 responses among those with a history of severe malaria (*P* was 0.069 in a comparison of values for healthy I- and healthy II-phase samples as determined by a Wilcoxon signed-rank test) (Fig. 4B and D). Of further interest, in both groups, the levels of IgG4 responses in healthy-phase samples were equivalent to (IgG3) or higher than (IgG1) those of the cytophilic isotypes at the same time points (Fig. 4A, B, and D). IgG4, furthermore, was the only isotype for which healthy-phase levels were noticeably higher than those recorded during acute infection (Fig. 3D and 4D).

Isolate recognition rates in Gabonese children. The IRRs at the different time points for children segregated according to clinical presentation are illustrated in Fig. 5 and 6. The IRRs mediated by IgG1 increased from admission to the first reinfection in both groups, significantly so in those with severe malaria (Fig. 5A). The levels of recognition mediated by either IgG2 or IgG3 were stable in both groups, but at the first reinfection assessment, IgG4-mediated recognition had declined significantly in the mild-malaria group from the level seen at admission (Fig. 5B-D). In healthy-phase samples, the only significant changes in IRRs concerned children with a

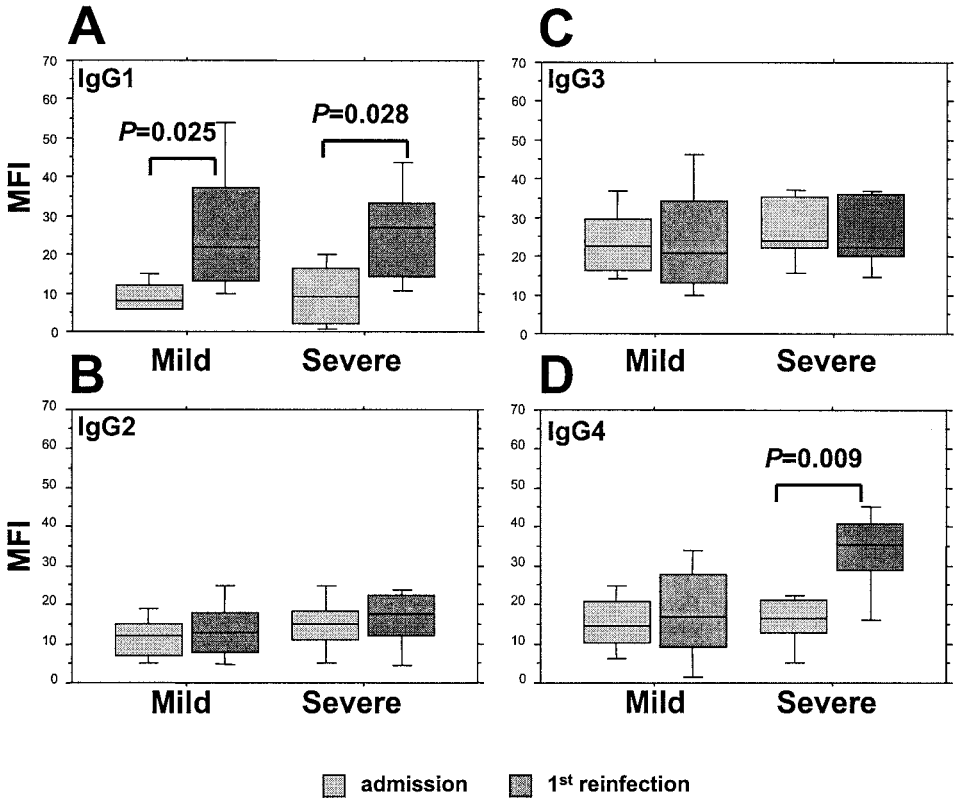

FIG. 3. Comparisons of the IgG isotype (IgG1[A], IgG2 [B], IgG3 [C], and IgG4 [D])-specific anti-VSA antibody profiles of matched groups of Gabonese children during two successive malaria episodes, segregated according to the severity of the admission episode. Box plots illustrate medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles, of cumulated MFIs of responses to the panel of six heterologous *P. falciparum* isolates.

history of mild malaria in whom IgG3-mediated recognition increased over the period of the study; a corresponding decline in IgG4-mediated recognition was observed (Fig. 6C and D).

DISCUSSION

This is the first study to report a detailed investigation, using large groups of African adults and children, of IgG antibody isotype responses with specificity for the polymorphic parasite VSA inserted into the membranes of infected erythrocytes. We assessed the activities directed to the VSA expressed by a panel of six locally collected heterologous parasite isolates, each known to be composed of several antigenically distinct parasite strains. These assessments were thus designed to address questions concerning changes in individuals' overall anti-VSA antibody isotype responses. They were also designed to serve as a measure of the ability of these individuals to mount such responses in order to enable comparisons, in the case of children, between groups of individuals who presented with malaria of differing levels of severity. These questions are somewhat distinct from those addressed by studies of individuals' responses to VSA expressed by homologous parasite isolates, i.e., by the parasites causing a given malaria episode.

In samples from both healthy adults and healthy children, the profiles of anti-VSA IgG responses to the panel of heterologous isolates that we used lacked any discernible isotype-specific pattern. We conclude that this lack of a pattern reflects the intrinsically polymorphic nature of VSA and hence the diversity of the potential B-cell epitopes that they may contain. The current consensus view is that PfEMP-1 is the principal target of the antibodies detected by the cytometric methods employed here, but the epitopic specificities of these antibodies can only be a matter of speculation, especially given that the isolates used comprise multiple strains, each of which could be assumed to be expressing distinct VSA. Another aspect of particular note concerns the detection of the anti-VSA antibodies of all four IgG isotypes, in the case of some isolates at very similar levels, which is consistent with an earlier report (23). The antibody response to crude parasite antigen preparations commonly comprises a mixture of all four IgG isotypes, but the response to defined *P. falciparum* asexual-stage antigens is usually more restricted and is dominated by cytophilic isotypes (4, 18, 41, 57). In the profile that we observed here, IgG3 antibodies did predominate, although they were equivalent to IgG4 in children in terms of magnitude and equivalent to IgG2 in adults in terms of the IRR. These findings contrast with the reported predominance of IgG1 in the profile observed in healthy Papua New Guinean adults (50). As a note of caution, that study reported results as percentages of positive cells rather than the MFIs that we used here, making direct comparisons invalid. The preferential induction of IgG3 responses to various *P. falciparum* asexual-stage antigens, including merozoite surface protein 1 (MSP-1), -2, and -3 and par-

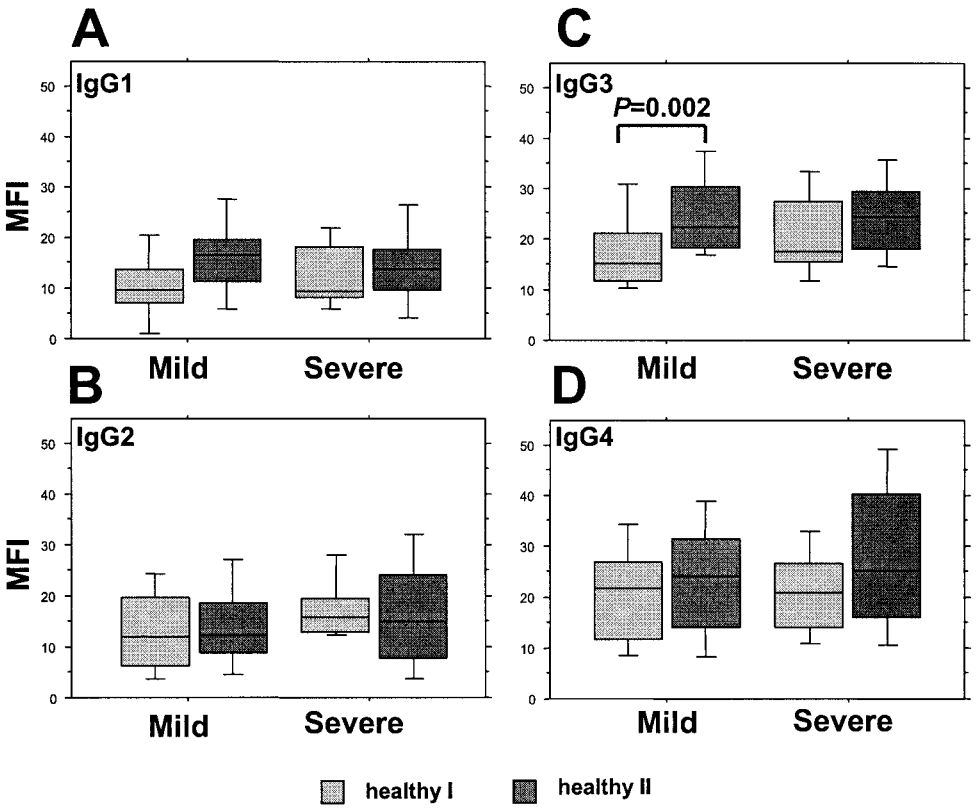

FIG. 4. Comparisons of the IgG isotype (IgG1 [A], IgG2 [B], IgG3 [C], and IgG4 [D])-specific anti-VSA antibody profiles of matched groups of Gabonese children at successive healthy-phase time points, segregated according to the severity of the admission episode. Box plots illustrate medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles, of cumulated MFIs of responses to the panel of six heterologous *P. falciparum* isolates.

asite glycosylphosphatidylinositols, is a well-described phenomenon (8, 12, 17, 45). This preference seems to be associated with the degree of polymorphism associated with the target antigen or epitope thereof, an observation with which in vitro antibody induction assays are largely concordant (19).

The prominence of IgG2 that we observed in the adults' anti-VSA antibody profile deserves further comment, since the role of IgG2 antibodies with specificity for plasmodial antigens remains a subject of some controversy. A recent study conducted in Burkina Faso showed an association between levels of parasite antigen-specific IgG2 and resistance to *P. falciparum*, which the authors attributed to the high prevalence in the study population of a polymorphism in the monocytes' FcγRIIA that results in an abnormally high affinity for Fcγ2 (5). This finding implies an influence on IgG isotype switching that allows for the preferential induction of protective IgG2 antibody responses to plasmodial antigens in these individuals. Protective effects have also been attributed to maternally transmitted parasite antigen-specific IgG2 responses in Cameroonian infants, in whom they were associated with a reduced risk of *P. falciparum* infection (14). Conversely, antiparasitic IgG2 responses were associated with a higher risk of developing severe malaria in Kenyan children, and purified IgG2 antibodies have been shown to block the ability of purified cytophilic antibodies to inhibit parasite growth in vitro (23, 41). The prevalence of the above-mentioned FcγRIIA mutation in the Gabonese population is very high (~75% allele frequency; F. Ntoumi, personal communication), so an influence on IgG2 responses could be envisaged. We have found evidence of an association between anti-VSA IgG2 responses and protection from malaria (C. Yone et al., unpublished observations). On the other hand, the level of IgG2 (and of IgG4) in the profile of the IgG isotype responses of our child study cohort is negligible compared to levels of other defined parasite asexual-stage antigens (A. J. F. Luty, unpublished observations), and the level of semi-immune serum-mediated phagocytosis of *P. falciparum*-infected erythrocytes is apparently unaffected by the amount of anti-parasite IgG2 in samples from Gabonese adults (54). Defining a clear role for anti-VSA IgG2 antibodies therefore awaits further study.

We can offer no clear explanation for the prominence of IgG4 in the anti-VSA responses of both adults and children to one of the heterologous isolates we used, which was itself obtained from a child with severe malaria. Repeated exposure to immunogens can skew responses towards IgG4, raising the possibility that a cross-reactive epitope expressed on the "common" VSA associated with severe malaria may be responsible for the pattern that we observed here (1, 10, 44). At the molecular level, IgG4 responses are commonly, but not exclusively, directed to carbohydrate epitopes, but the well-described *P. falciparum* VSA (PfEMP-1 and rifins) are not known to be glycosylated. Peptide-induced IgG4 responses have nev-

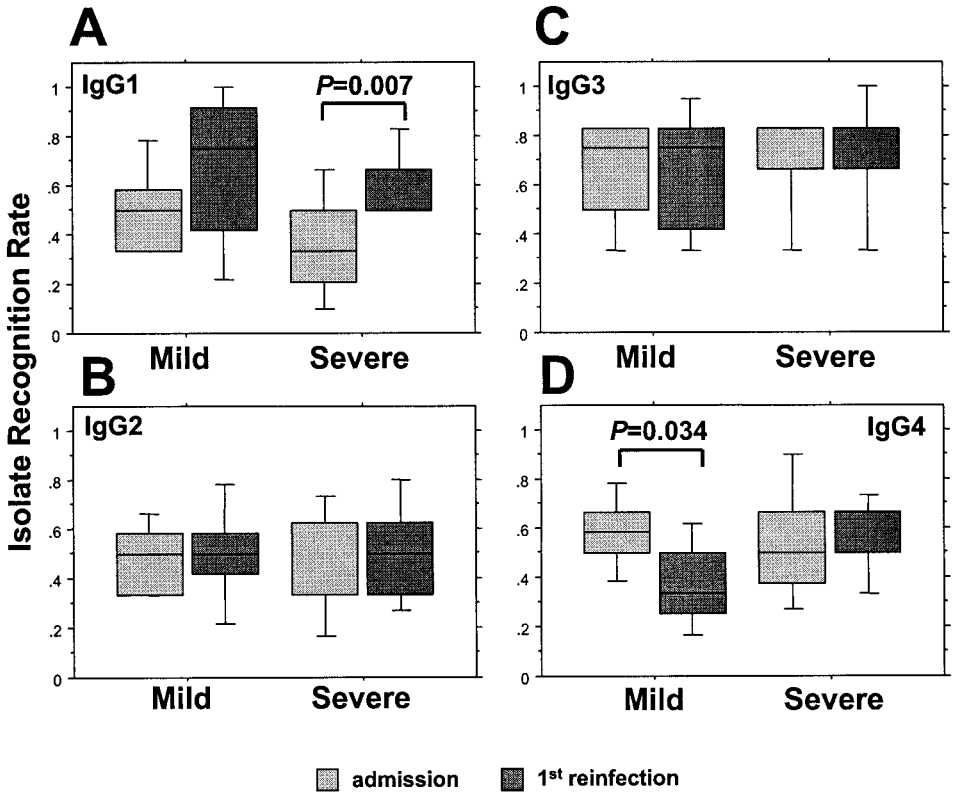

FIG. 5. The IgG isotype (IgG1 [A], IgG2 [B], IgG3 [C], and IgG4 [D])-specific IRRs of matched groups of Gabonese children during two successive malaria episodes, segregated according to the severity of the admission episode. Box plots illustrate medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles, of proportions of the panel of the six heterologous *P. falciparum* isolates that were recognized.

ertheless been reported for other nonplasmodial parasite infections, and antiplasmodial IgG4 antibodies are functionally important because of their ability to block the activities of cytophilic isotypes (5, 23, 51). In this context, the relatively enhanced IgG4 anti-VSA responses observed here in children, both during and after a malaria episode and especially in children with a history of severe malaria, merit a more detailed investigation. These findings reiterate the need, already evoked by others, for comprehensive analyses of antibody activities with defined specificities in different epidemiological settings (L. Hviid, T. Staalsoe, M. A. Nielsen, and T. G. Theander, Letter, Infect. Immun. **71:**2296, 2003.).

The broad similarity in the isolate-specific profiles of the responses seen with samples from healthy children and adults suggests that antibody responses to a particular isolate, and thus, by implication, to any given VSA type, are rather stable over time. The most notable age-related changes in the overall profile of the anti-VSA IgG isotype responses that we observed here concern the decline in the relative prominence of IgG4 in children, with a corresponding increase in IgG2-mediated isolate recognition in adults, whereas IgG3 responses, as noted earlier, were sustained at similar levels. A multitude of factors can enhance or suppress Ig secretion, including monocyte- and T-cell-derived cytokines, such as tumor necrosis factor alpha, gamma interferon, interleukin-10 (IL-10), and IL-12, which are known to be associated with the acquisition of antimalarial immunity and/or with pathogenesis during *P. falciparum* infection (15, 22, 30, 35, 39, 40, 49). In this context, the profound effect that IL-10, for example, has on the pattern of isotype switching in naïve B cells may have far-reaching implications for the development of antiplasmodial antibody responses, given the dramatically increased levels of this cytokine associated with acute malaria episodes in African children (30, 35, 36, 48, 53).

An association between *P. falciparum* infection and enhanced IgG antibody responses to VSA expressed by heterologous isolates has been reported in several different studies (9, 13, 21, 26, 46). In the study presented here, IgG1 responses to VSA expressed by heterologous isolates displayed a clear, albeit transient, infection-related enhancement. Interestingly, this increase was seen only in samples taken at the time of the first posttreatment malaria attack, not in those taken during the admission episode. We speculate that this pattern may be a reflection of the chronology of IgG isotype switching events during a malaria episode, since IgG1 is, ontologically, the first isotype to be produced in response to any given protein antigen; in vitro experiments have shown that the switching of human B cells from IgG1 to IgG2 or IgG3 production requires several rounds of division (53). The follow-up surveillance that formed an integral part of this study inevitably resulted in the detection and treatment of reinfections at a stage in their evolution earlier than the admission episode at inclusion, which might therefore favor the detection of putatively earlier IgG1 anti-VSA responses in the reinfection samples. Whatever

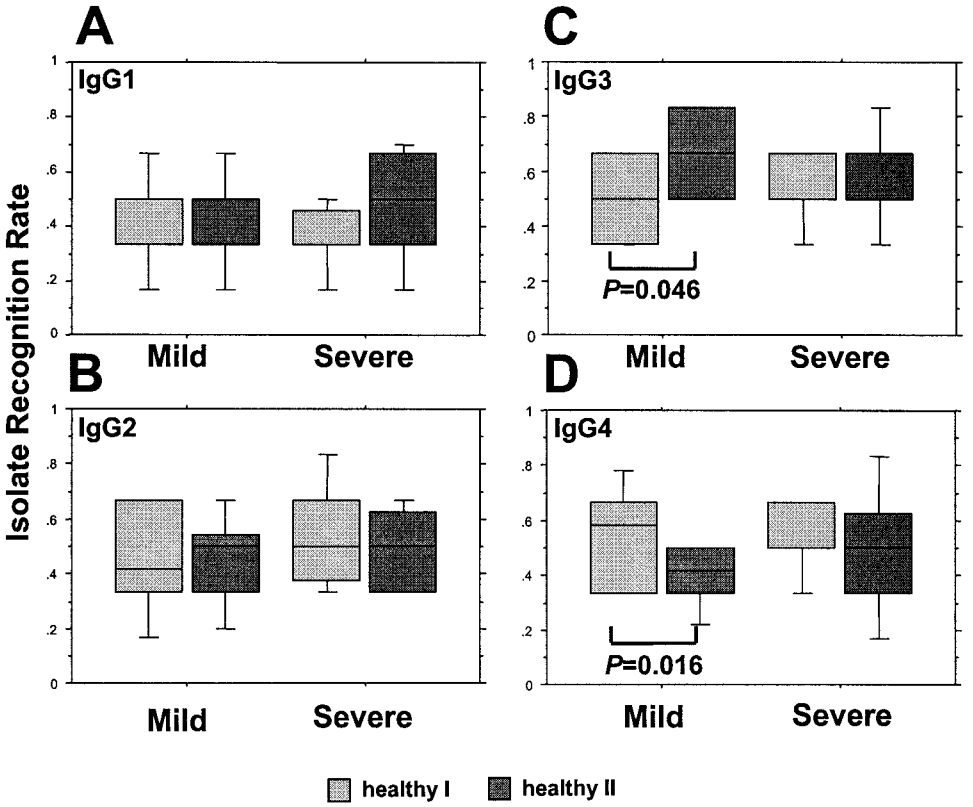

FIG. 6. The IgG isotype (IgG1 [A], IgG2 [B], IgG3 [C], and IgG4 [D])-specific IRRs of matched groups of Gabonese children at successive healthy-phase time points, segregated according to the severity of the admission episode. Box plots illustrate medians with the 25th and 75th percentiles, and I bars indicate the 10th and 90th percentiles, of proportions of the panel of six heterologous *P. falciparum* isolates that were recognized.

the reasons for these particular findings, the clear implication is that cross-reactive antibodies can be induced by *P. falciparum* VSA during malaria attacks in African children who have perennial exposure to infection. Such cross-reactivity may be more common than was previously thought to be the case (13, 43; Hviid et al., letter).

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Immunoglobulin G Isotype Responses to Erythrocyte Surface-Expressed Variant Antigens of *Plasmodium falciparum* Predict Protection from Malaria in African Children

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We assessed immunoglobulin G (IgG) isotype responses to variant surface antigens (VSA) expressed on parasite-infected erythrocytes of a panel of heterologous isolates during and after acute episodes in groups of Gabonese children presenting with either mild or severe *Plasmodium falciparum* malaria. In the acute and convalescent phases IgG3 and IgG1 anti-VSA antibodies, respectively, predominated. In the absence of infection, the levels of both cytophilic isotypes waned, while those of IgG4 increased, particularly in those admitted with severe malaria. Prospective analyses showed significantly longer delays between malaria attacks associated both (i) with increasing IgG1 responses with specificity for VSA of isolates from children with mild malaria and (ii) with increasing IgG4 responses with specificity for VSA of isolates from children with severe malaria. These findings imply that the predictive value of prospectively measured cross-reactive VSA-specific IgG antibodies with respect to protection against malaria in African children depends both on their isotype and on their fine specificity.

A mounting body of evidence supports the idea that antibody responses directed to *Plasmodium falciparum* variant surface antigens (VSA) inserted into the surface membranes of infected erythrocytes (iE) contribute to the acquired immune protection against malaria caused by this protozoan parasite (2, 9, 13, 26, 36). The VSA described to date include *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) (33) and the rifins (1, 10, 22). Adhesion of iE to vascular endothelial receptors via these VSA is thought to play a role in the pathogenesis of malaria (8, 27). Anti-VSA antibodies may serve to prevent these adherent interactions, thereby leading to removal of iE in the spleen, and/or to opsonize iE for uptake by phagocytes (14, 37). Such antibody-based protective mechanisms form the basis of a cumulative-exposure model in which the acquisition and maturation of these responses over time leads to the establishment of an antibody repertoire with broad specificity covering the range of VSA expressed by a given parasite population (15). Refinements of this model based on the profiles of antibody-mediated recognition of VSA expressed by diverse isolates suggest the existence of putative rare and common variants associated with mild and severe malaria, respectively (3, 4, 28).

Opsonization of iE presupposes the generation of cytophilic immunoglobulin G (IgG) antibody isotypes in the anti-VSA antibody repertoire, but there are few published data concerning this topic. IgG1 antibodies predominate in the responses of semi-immune Papua New Guinean adults to the VSA expressed by heterologous parasite isolates, in contrast to the profile observed in Gabonese adults, in which IgG3 is predominant (6, 31). We were therefore interested to know whether African children exposed to intense and perennial transmission of *P. falciparum* exhibit a similar isotypic profile of anti-VSA IgG antibodies. Data from a small-scale Kenyan study have, in addition, suggested that children who are susceptible to severe malaria may display altered dynamics of anti-VSA antibody responses, which is in accord with our own recent report (5, 36). Here we addressed this question further through comparison of the IgG isotype profiles of anti-VSA antibodies in Gabonese children with differing outcomes of infection in terms of the clinical severity of *P. falciparum* malaria. For this purpose we used flow cytometric techniques with plasma samples taken at different times either during or after a malaria episode in a cohort of age- and gender-matched Gabonese children who presented with either mild or severe malaria in order to assess changes in the profiles of IgG isotype antibodies directed to the VSA expressed by a panel of six (two putatively rare and four common) heterologous *P. falciparum* isolates. Our own published work has indicated differences in susceptibility to *P. falciparum* infection in terms of both significantly shorter delays to the first reinfections and significantly higher annual malaria attack rates in the group of children who presented with severe rather than mild malaria in this study (23, 24). We therefore also sought associations between these particular parameters and appropriate prospective measures of the children's immune responses, here represented by their convalescent-phase anti-VSA IgG antibody isotype activity.

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MATERIALS AND METHODS

Study site. The study was conducted at the Albert Schweitzer Hospital in Lambaréné, Gabon. The hospital is situated in an area where malaria is hyperendemic and caused predominantly by *P. falciparum* and where transmission is perennial, with an estimated annual entomological inoculation rate of ~50 (34, 39).

Ethical clearance. Ethical clearance for the study was obtained from the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. Informed consent for inclusion into the study was obtained from the parents or guardians of each participating child.

Study design. The study population comprised a subgroup within a matched-pair cohort study of 200 Gabonese children, half of whom presented with severe malaria and half of whom presented with mild malaria due to *P. falciparum*. Inclusion into the study occurred in the period 1995 to 1997. For the purposes of the assessments described here, a subgroup of 60 children, i.e., 30 matched pairs, was selected on the basis of the availability of plasma samples from these individuals at all three study time points (see below). These children's ages ranged from 13 to 101 months, with a mean of 52 months. Severe malaria cases were matched to mild malaria controls according to their age, gender, and provenance. Severe malaria was defined as severe anemia (hemoglobin of <50 g/liter) and/or hyperparasitemia (>250,000 parasites/μl, corresponding to >10% infected erythrocytes) with or without other signs of severe malaria. Samples from children with severe malaria who received blood transfusions were not included. Mild malaria was defined as a parasitemia of between 1,000 and 50,000 parasites/μl of blood, hemoglobin of >8 g/dl, glycemia of >50 mg/dl, and no signs of severe malaria. Children were excluded if they had either concurrent acute infection, previous hospitalization for malaria, intake of antimalarials during the week preceding admission, or any chronic diseases or malnutrition. Details of patient care and treatment have been given elsewhere (20, 21).

Plasma samples. The plasma samples used here were isolated from undiluted venous blood taken on three separate occasions: (i) on the day of admission to the hospital, just prior to administration of antimalarial chemotherapy (here referred to as the acute-phase sample); (ii) 1 month posttreatment (referred to as the convalescent-phase sample); and (iii) at least 6 months posttreatment, when the children had no clinically obvious infection and had been aparasitemic for the preceding 6 weeks, as determined during the active surveillance at 2-week intervals undertaken in the participants' homes following discharge from the hospital (here referred to as the healthy-phase sample). The active in-home surveillance referred to above allowed identification of reinfections (new infections or malaria episodes) through examination of routinely prepared and Giemsa-stained thick blood smears. Children diagnosed with malaria (defined as any *P. falciparum* parasitemia with a rectal temperature of >38°C or clinical symptoms) during this follow-up period were given standard antimalarial treatment with sulfadoxine-pyrimethamine. The time to first reinfection was defined as the time from admission until the time when the first thick blood smear containing parasites was detected.

Plasma samples from 30 nonimmune German adults and from 21 semi-immune Gabonese adults resident in Lambaréné were included as negative and positive controls, respectively.

Parasite isolates and culture. Six *P. falciparum* isolates collected from Gabonese children recruited in a separate outpatient study carried out during 1997 at the Albert Schweitzer Hospital were used. The reference isolates designated Cys002, Cys007, Cys030, and Cys035 (here referred to as VSA_{SM}) were obtained from children with severe *P. falciparum* malaria, and Cym030 and Cym033 (VSA_{UM}) were obtained from children with mild *P. falciparum* malaria. All isolates were confirmed microscopically as monoinfections with *P. falciparum*, and all were shown by routine standardized merozoite surface protein-based PCR genotyping techniques to be polyclonal, each with at least three different strains (C. Yone, unpublished observations). Details of the methods used for collection and culture of parasites have been given elsewhere (35). Briefly, peripheral venous blood was centrifuged, and the erythrocytes obtained were spin washed twice. Pellets containing infected erythrocytes were then cryopreserved in liquid nitrogen for subsequent in vitro adaptation.

Primary isolates were subsequently adapted to in vitro culture according to the method of Trager and Jensen (38). Briefly, cells were resuspended in complete medium supplemented with 10% heat-treated, prescreened, nonimmune AB+ serum (from the blood bank of the University Hospital, Tübingen, Germany), and were then incubated in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Fresh O+ erythrocytes depleted of lymphocytes (University Hospital, Tübingen, Germany) were periodically added. Isolates were initially expanded over a short period of 8 to 10 48-h multiplication cycles, after which identical stabilates of cultures containing mostly asexual ring forms were cryopreserved for later use in cytometric assays (see below).

Flow cytometric measurement of *P. falciparum*-infected erythrocyte surface-specific IgG isotype responses. Detection of IgG with specificity for the surface of *P. falciparum* trophozoite-infected erythrocytes was performed by using a flow cytometric assay described in detail elsewhere (31, 35). Briefly, iE were enriched by flotation on Plasmagel (Fresenius, Louviers, France) and were then tested for their capacity for binding to the endothelial receptor CD36 expressed on an amelanotic melanoma cell line (C32MC). Binding of iE of each isolate was shown to be maintained at a consistently high level, indicating no loss of the cytoadherent phenotype. iE were then sequentially incubated for 30 min at room temperature with test or control plasma samples diluted 1:50 in phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA), followed by mouse anti-human IgG1, IgG2, IgG3, or IgG4 monoclonal antibody (SkyBio Limited, Wyboston, Bedford, United Kingdom) diluted 1:50 in PBS–1% BSA and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, Ala.) diluted 1:100 in PBS–1% BSA and containing 0.5 μg of ethidium bromide per ml. Cells were spin washed twice with PBS–1% BSA after each incubation. Finally, iE were resuspended in PBS and analyzed on a FACScan flow cytometer with CellQuest software (Becton Dickinson, Heidelberg, Germany).

Sample and data analysis. Samples were segregated on the flow cytometer into iE and uninfected erythrocytes (uE) by using forward- and side-scatter parameters, and a gate defining fluorescing (ethidium bromide-stained) cells further segregated parasite-infected cells. Counting 10,000 events per sample and using the geometric mean of the emitted fluorescence intensity (MFI), the amounts of individual IgG isotypes specifically bound to the surface of iE were estimated by application of the formula MFI = (MFI iE test − MFI uE test) − (MFI iE NIP − MFI uE NIP), where NIP represents a pool of nonimmune (German) plasma samples. A threshold value of positivity was established for each IgG isotype and isolate by using the panel of plasma samples from nonexposed Germans, such that test samples were considered anti-VSA IgG isotype responders when the MFI calculated with the equation above was greater than the mean plus two standard deviations of the values obtained with these control samples.

Statistical methods. Data were analyzed by using the Statview and STATA software programs. For paired and unpaired comparisons of continuous variables, the nonparametric Wilcoxon sign rank and Kruskal-Wallis or Mann-Whitney U-test were used, respectively. Contingency tables with continuity corrections were used to compare proportions within and between groups. Correlations between continuous variables were assessed with the nonparametric Spearman rank test corrected for ties, where a rho value of >0.25, concomitant with a P value of <0.05, was considered significant. Survival analyses, using the Cox proportional hazards model, were used to analyze the relationship between IgG isotype anti-VSA antibodies and time to first posttreatment reinfection. MFI values for each IgG isotype for all isolates or separately for VSA_{SM}- or VSA_{UM}-specific antibodies were entered into this model. For this purpose, 49 reinfections recorded in 57 subjects were included. The Cox proportional hazards model with multiple failure events was used to analyze the relationship between IgG isotype anti-VSA antibodies and the delay between reinfections in each individual. For this, a recorded total of 381 reinfections during a total follow-up of 258 years (mean incidence, 1.5 infections per person per year) was included. In both cases, clinical status at admission (severe or mild malaria) was included in the model as a confounding variable. The level of statistical significance in all cases was set at a P value of <0.05.

RESULTS

Quantitative comparison of IgG isotype responses to heterologous *P. falciparum* VSA: within- and between-group comparisons. In order to compare the anti-VSA antibody responses within and between the two groups of children with differing clinical presentation at admission, the MFIs of individual IgG isotype responses to the two VSA_{UM} heterologous parasite isolates and to the four VSA_{SM} were separately pooled for each child (Fig. 1). In acute-phase responses of both groups, VSA_{SM}-specific IgG3 antibodies were the highest, while VSA_{SM}-specific IgG2 responses at this time were significantly higher in the severe malaria group than in the mild malaria group (Fig. 1B and C). In the convalescent phase the

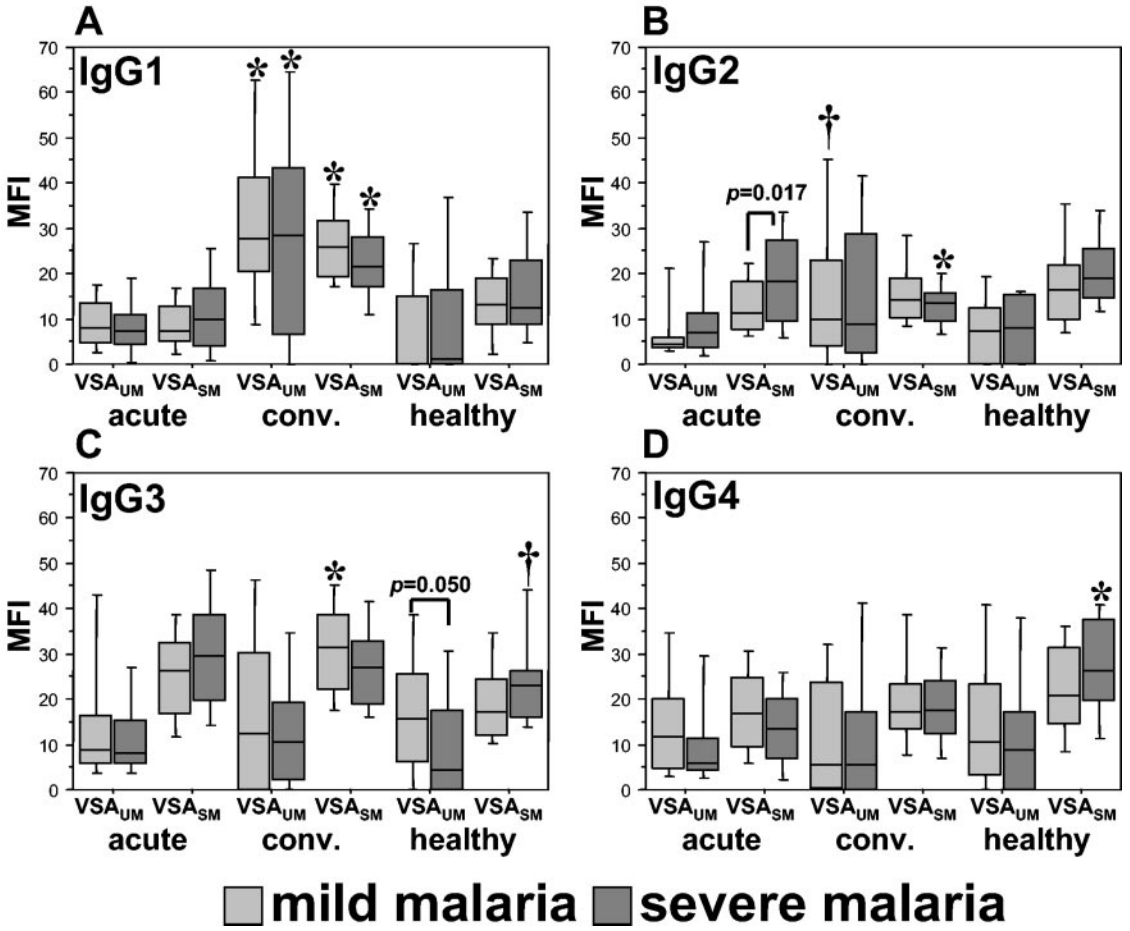

FIG. 1. Magnitudes of IgG isotype responses to heterologous *P. falciparum* VSA. Temporal changes in VSA-specific IgG1 (A), IgG2 (B), IgG3 (C), and IgG4 (D) responses, calculated for each individual as the pooled VSA_{UM}-specific or VSA_{SM}-specific MFI from assays with six heterologous *P. falciparum* isolates, in the acute, convalescent (conv.), and healthy phases and segregated according to children's clinical presentation at admission are shown. Box-whisker plots represent medians with 25th and 75th percentiles and whiskers for 10th and 90th percentiles of the mean MFI. *P* values are derived from the Wilcoxon sign rank test for paired comparisons. *, $P < 0.001$ for within-group comparison with both other time points. †, $P < 0.001$ for within-group comparison with acute phase.

VSA_{UM}- and VSA_{SM}-specific IgG1 responses of both groups were significantly higher than the corresponding activities detected in the acute phase (Fig. 1A). At the same time, IgG2 anti-VSA_{UM} and IgG3 anti-VSA_{SM} responses in the mild malaria group increased significantly, but IgG2 anti-VSA_{SM} antibodies in the severe malaria group declined (Fig. 1B and C). In healthy-phase samples of both groups, cytophilic (IgG1 and IgG3) isotype activity of both VSA_{UM} and VSA_{SM} specificities declined, while IgG2 and IgG4 VSA_{SM}-specific responses increased significantly in the severe malaria group (Fig. 1).

Changes in the ratios of cytophilic to noncytophilic isotypes are shown in Table 1. The observed profiles emphasize the relative predominance of cytophilic IgG3 and IgG1 anti-VSA antibodies in the acute and convalescent phases, respectively, in both groups of children and of IgG4 responses in particular in the healthy-phase profile of children in the severe malaria group.

The profiles of convalescent-phase anti-VSA IgG antibody isotype responses with specificity for the individual isolates are illustrated in Fig. 2. No obvious isolate-specific pattern is discernible. For certain isolates e.g., Cym033, Cys030, and Cym030 (Fig. 2C, D, and F), the activity of cytophilic isotypes appears to be relatively greater than that of noncytophilic isotypes, but this pattern did not apply to both groups in all cases; e.g., there was reduced IgG3 activity in the severe malaria group with respect to Cym033 and Cym030 (Fig. 2C and F). No particular isotype-specific predominance was discernible in the profiles of responses with specificity for the VSA_{SM} isolates Cys035 and Cys007 (Fig. 2A and B).

Prospective assessment of associations between convalescent-phase anti-VSA IgG isotype responses and reinfections. Follow-up surveillance showed that children in the severe malaria group had significantly shorter delays to their first post-treatment malaria attack and significantly higher malaria attack rates than their matched counterparts with mild malaria (23, 24). Here, therefore, we performed survival analyses to test the extent of the association between a prospective measure, convalescent-phase anti-VSA IgG antibody isotype responses, and protection from malaria, using Cox's proportional hazards model to determine their influence on either the time

TABLE 1. Temporal changes in the ratios of cytophilic and noncytophilic IgG isotype antibodies with specificity for VSA of heterologous *P. falciparum* isolates in groups of Gabonese children segregated according to the clinical severity of malaria at admission

Ratio	Clinical group	MFI[a] in:			P[b]		
		Acute phase	Convalescent phase	Healthy phase	Acute vs convalescent	Convalescent vs healthy	Acute vs healthy
IgG1/(IgG2 + IgG4)	Mild	0.656 (0.391–1.026)	1.854 (1.425–2.404)	0.780 (0.451–1.165)	<0.001	<0.001	NS[c]
	Severe	0.577 (0.334–1.183)	1.791 (1.075–2.412)	0.661 (0.377–1.180)	<0.001	<0.001	NS
IgG3/(IgG2 + IgG4)	Mild	1.770 (1.13–2.444)	1.393 (1.160–2.343)	1.000 (0.623–1.975)	NS	0.075	NS
	Severe	1.960 (1.22–2.548)	1.517 (1.057–1.922)	0.931 (0.778–1.100)	0.061	0.013	<0.001
IgG2/(IgG1 + IgG3)	Mild	0.463 (0.336–1.104)	0.534 (0.334–0.813)	0.970 (0.543–1.589)	NS	0.010	NS
	Severe	0.918 (0.652–1.174)	0.660 (0.398–0.790)	0.969 (0.640–1.424)	0.005	0.002	NS
IgG4/(IgG1 + IgG3)	Mild	1.040[d] (0.684–1.623)	0.554 (0.428–0.750)	1.090 (0.664–1.921)	0.002	<0.001	NS
	Severe	0.751 (0.420–0.977)	0.527 (0.352–0.873)	1.516 (0.929–1.947)	NS	<0.001	<0.001

[a] Values given are medians (interquartile ranges) of ratios of anti-VSA antibody isotype MFIs.
[b] P values derived from Wilcoxon sign rank test for paired comparisons.
[c] NS, not significant.
[d] $P = 0.017$ (mild versus severe).

to first posttreatment reinfection or the interval between reinfections observed in each child, controlled for clinical presentation status (mild or severe malaria). As expected, reinfection outcomes were found to be significantly influenced by clinical status (Table 2). Consideration of anti-VSA IgG isotype responses without regard for their specificity in this model revealed an independent but nonsignificant trend towards a longer delay to first reinfection with increasing magnitude of IgG1 activity (hazard ratio; 0.968, 95% confidence interval [CI], 0.935 to 1.001; $P = 0.059$) but no such influence for any other IgG isotype (data not shown). Segregation of responses according to their specificity for isolates expressing either VSA_{UM} or VSA_{SM} showed that the trend referred to above was solely attributable to IgG1 antibodies with specificity for VSA_{UM} (hazard ratio 0.987; 95% CI, 0.974 to 1.000; $P = 0.057$), while also revealing separate and independent trends towards associations of IgG2 anti-VSA_{UM} antibodies with longer delays to first reinfection and of IgG3 anti-VSA_{SM} antibodies with shorter delays to first reinfection (hazard ratio, 1.023; 95% CI, 0.997 to 1.049; $P = 0.082$). The results of the

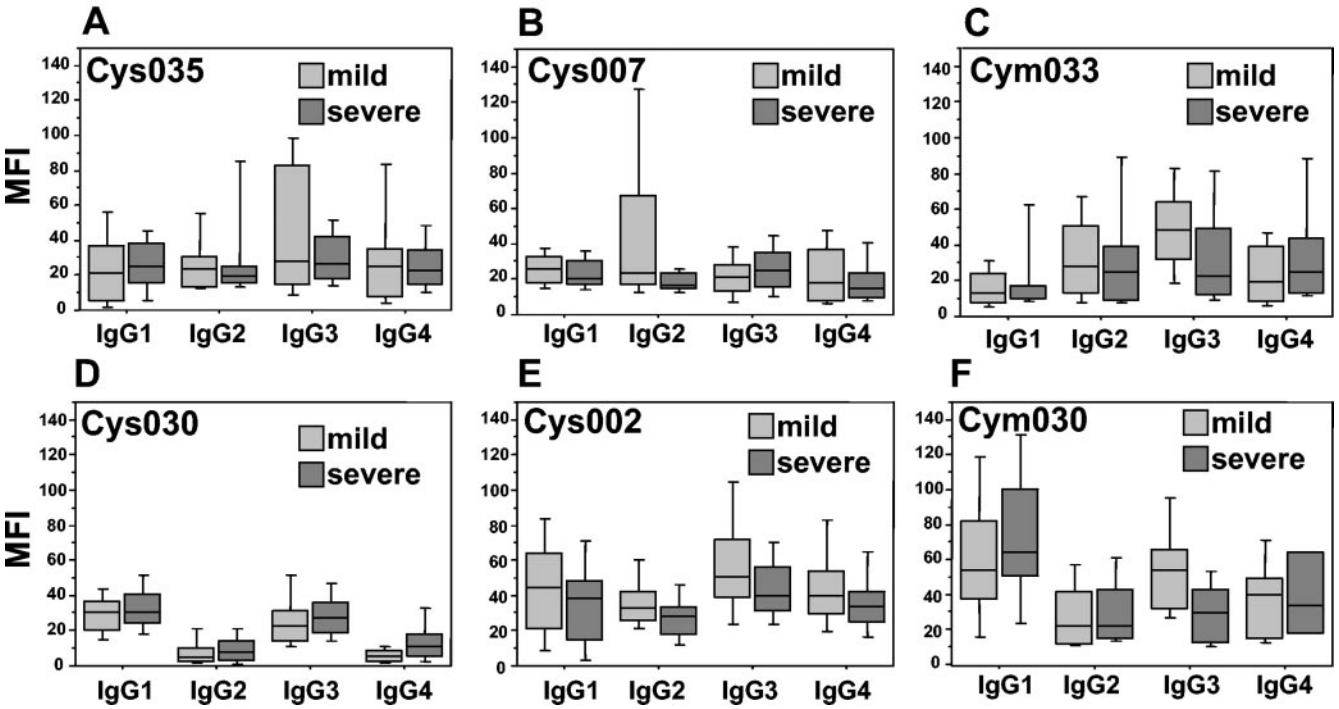

FIG. 2. Profiles of convalescent-phase IgG isotype responses to heterologous *P. falciparum* VSA, segregated according to children's clinical presentation at admission. Box-whisker plots represent medians with 25th and 75th percentiles and whiskers for 10th and 90th percentiles of the mean MFI, excluding nonresponders. Responses to the four VSA_{SM} isolates (A, B, D, and E) are illustrated separately from those to the two VSA_{UM} isolates (C and F).

TABLE 2. Survival analysis, using Cox proportional hazards model with multiple failure events, of the interval between reinfections for convalescent-phase IgG isotype anti-VSA antibody responses

Group[a]	Clinical status or isotype	Hazard ratio	95% CI	*P*
α-VSA_{ALL}	Clinical status	1.306	1.041–1.638	0.021
	IgG1	0.979	0.968–0.991	<0.001
	IgG2	0.998	0.982–1.014	0.807
	IgG3	1.001	0.989–1.013	0.888
	IgG4	0.997	0.983–1.012	0.725
α-VSA_{UM}	Clinical status	1.371	1.105–1.701	0.004
	IgG1	0.990	0.985–0.995	<0.001
	IgG2	0.998	0.991–1.004	0.510
	IgG3	0.996	0.989–1.004	0.316
	IgG4	1.006	0.999–1.012	0.115
α-VSA_{SM}	Clinical status	1.369	1.093–1.713	0.006
	IgG1	0.993	0.981–1.005	0.269
	IgG2	1.000	0.988–1.013	0.999
	IgG3	1.008	0.997–1.019	0.139
	IgG4	0.984	0.973–0.995	0.006

[a] Analyses considered the MFI of anti-VSA responses to all six heterologous isolates together (α-VSA_{ALL}) or after segregation into those with specificity for the two isolates expressing VSA_{UM} or for the four isolates expressing VSA_{SM}.

analyses of the interval between reinfections are shown in Table 2. Here, a highly significant association between longer intervals and IgG1 anti-VSA antibodies was found (hazard ratio, 0.979; 95% CI, 0.968 to 0.991; $P < 0.001$), and this was attributable exclusively to the antibodies with specificity for VSA_{UM} (hazard ratio, 0.990; 95% CI, 0.985 to 0.995; $P < 0.001$) (Table 2). The same analyses revealed a statistically significant association of extended intervals between reinfections and increasing levels of IgG4 anti-VSA_{SM} antibodies (hazard ratio, 0.984; 95% CI, 0.973 to 0.995; $P = 0.006$) (Table 2).

Retrospective assessment of the influence of reinfections on healthy-phase anti-VSA antibody responses. Since the first and subsequent posttreatment reinfections in many individuals occurred in the interval between collection of the convalescent- and healthy-phase samples, we determined the potential influence of reinfections during this period on the profile of healthy-phase anti-VSA antibody IgG isotype activity by assessment of correlations (Spearman rank), using the number of reinfections as a continuous variable, or by direct comparison between groups (Mann-Whitney) following dichotomization according to the presence or absence of reinfection. No statistically significant associations were detected by either test either for the cohort as a whole or when the cohort was segregated according to clinical presentation status with respect to individual IgG isotype activity with specificity for either VSA_{UM} or VSA_{SM} (data not shown).

DISCUSSION

We describe here the evolution of IgG isotype antibody responses to the VSA expressed by heterologous *P. falciparum* isolates as a function both of young African children's clinical presentation at inclusion into the study and of their subsequent reinfection profiles. We specifically excluded samples from children diagnosed with severe malarial anemia who received blood transfusions as part of their supportive treatment in order to avoid the potential confounding effects of passively transferred antibodies in these analyses.

The data are presented as geometric MFIs and are therefore not directly comparable to those of a recently published Kenyan study, which are expressed as the proportion of infected erythrocytes positive for bound antibody (18). The results of the latter study indicated that the IgG isotype response to the VSA expressed by homologous parasite isolates, in children who presented with uncomplicated *P. falciparum* malaria, is composed predominantly, although not exclusively, of IgG3 antibodies. Our data showing that levels of IgG3 anti-VSA antibodies were the highest in samples taken in the acute phase of the infection are consistent with that finding. IgG3 antibodies are the predominant isotype in the profile of responses of healthy semi-immune adult Gabonese, with specificity for the VSA expressed by the same panel of heterologous parasite isolates (6). Where our data diverge from those of the Kenyan study is in the profile of posttreatment (convalescent-phase) anti-VSA responses, in which, in our study, the IgG1 anti-VSA response was clearly predominant (Fig. 1A). This observation is consistent with the results of numerous studies that have reported enhancements of the levels of IgG antibodies with specificity for the VSA expressed by heterologous parasite isolates in the postinfection period (5, 7, 12, 16, 29). Our data thus strongly imply that cross-reactive antibodies are a prominent feature of the profile of anti-VSA responses induced by *P. falciparum* malaria episodes in young African children with high and perennial levels of exposure to infection. This further substantiates our own observations that in some members of the same cohort, IgG1 antibody responses with specificity for VSA of heterologous parasite isolates are also elevated during the first posttreatment malaria episodes that they experienced (6).

The significant decline of the level of IgG3 and enhancement of that of IgG4 anti-VSA responses observed when children were healthy and parasite free are aspects of particular interest in the data we present here. Since IgG3 has the shortest half-life (ca. 8 days) of all of the IgG isotypes, a temporally related decrease in the amount of such antibodies in the absence of parasite antigen-mediated stimulation might be expected. Noncytophilic IgG4 antibodies are reported to interfere with the parasite growth inhibition mediated by cytophilic isotypes in vitro and may therefore act as "blocking" antibodies in vivo (14). Clearly, however, the data we present here indicate that high levels of IgG4 anti-VSA antibodies with a particular VSA_{SM} specificity are beneficial rather than detrimental, in the sense that they are associated with prolonged intervals between malaria attacks. We speculate that they may function by interfering with cytoadherence via blockade of infected erythrocyte-endothelial cell ligand-receptor interactions, but their specificity remains a paradox. IgG4 antibodies are commonly thought to be directed to carbohydrate epitopes, but there is no evidence for carbohydrate epitopes as components of either PfEMP-1 or rifins. Since our own study has shown that IgG4 antibodies represent only a relatively minor component of the anti-VSA response repertoire of healthy semi-immune adults, we conclude that repeated exposure nevertheless results in a change in the clinico-physiopathological relevance of the different IgG isotypes (6). Age-related

switches in IgG isotype activity with specificity for polymorphic determinants have been noted in the profile of at least one other asexual-stage antigen (35).

The particularly outstanding observation of this study concerns the strong association between high convalescent-phase anti-VSA_{UM} IgG1 responses and clinical protection as manifest by significantly prolonged intervals between malaria attacks. This represents persuasive evidence for a protective function of antibodies of the major cytophilic IgG isotype directed to the VSA expressed by heterologous parasite isolates of a particular subtype. Such infection-induced cytophilic antibodies could mediate their effects via targeting of determinants expressed by *P. falciparum* VSA, leading to blockade of infected erythrocyte cytoadherence to endothelial cells, and/or opsonization, leading to phagocytosis through interactions with Fcγ receptors on phagocytic cells (36). The putative principal target of anti-VSA antibodies, PfEMP-1, is known to contain conserved epitopes that are recognized by antibodies from African children and adults (11, 30). Whether these or other epitopes of PfEMP-1, or even of other VSA such as the rifins, are the targets of the protective IgG1 isotype responses that our study has revealed remains to be determined.

Based on coding sequences and chromosomal positional parameters, it has been proposed that PfEMP-1 *var* genes can be segregated into groups encoding variants with greater or lesser degrees of complexity and that the clinical severity of malaria may reflect preferential expression of members of a particular subgroup of these genes (17, 32). We have not determined the precise molecular identity of the PfEMP-1 variants expressed by our panel of isolates, although we do know that they comprise multiple strains (C. Yone, unpublished observations) and also that the donors were young children, with mean ages of 25 and 40 months for the VSA_{UM} and VSA_{SM} donors, respectively. Since putatively rare and common VSA variants are thought to be preferentially expressed in older and younger children, respectively, primarily reflecting differences in the level of acquired immunity (3, 4, 28), we conclude that in the absence of detailed molecular characterization such a distinction cannot be definitively applied to our panel of isolates. Nevertheless, only ~40% of adult Gabonese have IgG1 responses, whereas almost 100% have IgG2 and IgG3 responses with specificity for the two VSA_{UM} isolates and ~75% have IgG4 responses with specificity for the VSA_{SM} isolates of our panel (G. Cabrera, unpublished observations). These observations serve to emphasize both the age-related changes and the apparent differences in the pattern of IgG isotype antibodies induced predominantly by variants expressed by parasite isolates with different origins, differences that presumably lie at the epitope level. Self-evidently, in the study described here the children who presented with severe malaria lacked effective immune responses capable of suppressing the growth of the parasites responsible for their condition. Despite the relatively greater susceptibility to malaria and the relatively poorer persistence of parasite antigen-specific antibodies within this particular group (19, 23–25), the findings we present here nevertheless suggest that an ability to produce larger amounts of anti-VSA antibodies with specificity for determinants expressed by heterologous parasite isolates is associated with a benefit to some of these children in the form of a degree of protection from malaria.

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Epstein-Barr viral reactivation persists at a high frequency in young African children with a history of severe *Plasmodium falciparum* malaria

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Running title: EBV and *P. falciparum* malaria

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Abstract

EBV and *Plasmodium falciparum* have overlapping distributions and are thought to have causal interactions, particularly concerning the etiology of Burkitt's lymphoma. Using real-time PCR we quantified EBV DNA in the blood of Gabonese children with either mild or severe *P. falciparum* malaria both before and after anti-malarial treatment. After treatment the proportion with EBV DNA declined in the mild but increased in the severe malaria group. EBV DNA loads were consistently highest in the latter group, significantly so when they were healthy and parasite-free, at which time the proportion with EBV DNA was significantly higher (67% vs 39%, p=0.013). Children with the highest EBV DNA loads had shorter delays to their first *P. falciparum* reinfections and suffered more malaria attacks. Pre-treatment plasma IL-12p70 levels were lower in those with EBV DNA, and EBV DNA was detected in a higher proportion of under-five year olds. Our findings confirm EBV reactivation during acute *P. falciparum* malaria but show also that (i) EBV activity persists at a higher frequency in children with a history of severe malaria and (ii) high peripheral blood EBV DNA loads are associated with enhanced susceptibility to *P. falciparum* malaria and with altered pro-inflammatory cytokine activity.

Introduction

Causative interactions between Epstein-Barr virus (EBV) and *Plasmodium falciparum* have long been suspected, and since the original discovery of EBV with cultured Burkitt lymphoma (BL) cells **[13]** a handful of studies have investigated the possible associations between *P. falciparum* and EBV, primarily with a view to establishing a causal link between malaria and BL. The geographical distribution of BL substantially overlaps that of *P. falciparum*, the predominant cause of malaria in sub-Saharan Africa **[12,14,15,19].**

Epstein-Barr virus (EBV) is a ubiquitous and persistent human herpes virus that is carried by more than 90% of the world's population, preferentially infecting B lymphocytes and epithelial cells **[8,45].** Primary EBV infection is usually asymptomatic but can cause infectious mononucleosis, progressing in some individuals to lymphoid or epithelial malignancies. The latter include BL, the commonest childhood malignancy in sub-Saharan Africa where it may account for over 50% of all childhood malignant tumors. Whittle and colleagues **[54]** first observed that, in patients with acute malaria, outgrowth of spontaneous lymphoblastoid cell lines is enhanced to levels comparable to those seen in infectious mononucleosis but declines to background levels after treatment, thus suggesting that *P. falciparum* malaria is associated with expansion of EBV-positive B cells in the blood **[23,29]**. Cytotoxic T lymphocyte (CTL)-mediated control of EBV is defective in children with acute *P. falciparum* malaria **[54,55]**. The efficacy of the EBV-specific CTL response is also impaired in chronic malarial infections **[41]**.

Severe malaria, although a relatively rare event in terms of the proportion of children in whom such complications arise, nevertheless accounts for up to 2 million deaths per year, most of whom are young children **[3,20].** Host-related factors that underlie the susceptibility to severe malaria are poorly understood but are numerous and include genetic as well as immunological components **[36]**. An effect of concomitant viral infection on the outcome of *P. falciparum* infection has been reported for hepatitis

B **[52]**. Whether a similar association exists between EBV co-infection and the outcome of *P. falciparum* infection is not known. Appropriately timed pro-inflammatory cellular immune responses, mediated by cytokines such as IL-12, TNF-α and IFN-γ, are thought to be pivotal in controlling *P. falciparum* **[1,35,37,50].** Pathogenesis leading to severe malaria, conversely, may be related to an inability to tightly control exuberant pro-inflammatory cytokine activity **[2,10,28,32,40,44].** Modulation of cellular responses may occur via EBV-mediated induction of EBI3, a secreted IL-12p40-related protein that is a component of the IL-12 family member IL-27 **[4,9].** On the other hand, the preferential infection of B cells by EBV allied to the loss of cytotoxic T cell control during acute *P. falciparum* malaria has led to the suggestion that anti-plasmodial antibody production may be altered by EBV co-infection **[5,23,38,39].** Since antibodies with specificity for asexual *P. falciparum* blood stage parasite antigens, in particular, represent a major component of acquired anti-malarial immunity, concurrent viral infection-related changes to the profile of such antibodies may have consequences for the outcome of infection **[11,18,21]**. Therefore, using samples collected as part of a longitudinal matched-pair case-control study of severe malaria in Gabonese children living in an area with hyperendemic and perennial malaria transmission, we determined EBV DNA loads in peripheral blood both during and after acute *P. falciparum* malaria attacks in an attempt to define the possible contribution of EBV reactivation to susceptibility to and/or protection against malaria in such a population, and to assess possible associated changes in immunological parameters.

Materials and methods

Study site. A study, designated 1-95/C, was initiated in 1995 at the Albert Schweitzer Hospital in Lambaréné, Gabon, situated in an area of equatorial central Africa where malaria is hyperendemic and *P. falciparum* is perennially transmitted **[53].** The estimated annual entomologic inoculation rate in the area is 50 **[51].**

Ethical clearance. The ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné approved the study. Children were included after obtaining informed consent from the parent/guardian.

Study subjects and samples. Plasma and whole blood samples from 60 children (a sub-set of 200 original participants in the 1-95/C study) were used, selected as a function of their availability at three different time-points from 30 matched pairs of children i.e. from 30 children who presented with severe and 30 with mild *P. falciparum* malaria, the latter matched to the former for age, gender and area of residence. Their mean age was 52 [range: 13-101] months. Samples from children who received blood transfusions were not used. Samples used were collected on three separate occasions: i) acute phase, at admission to the hospital, immediately before treatment; ii) convalescence, one month post-treatment; iii) healthy phase, at least 6 months post-treatment when children were healthy and parasite-free for the preceding 6 weeks, as determined during the active two-weekly surveillance undertaken in the participants' homes following discharge from the hospital. The absence of sub-microscopical parasitemia in healthy phase samples was confirmed through the use of a commercially-available ELISA for detection of PfHRPII antigen, as previously described **[6].** Active in-home surveillance identified reinfections (new infections/malaria episodes) through examination of routinely-prepared and Giemsa-stained thick blood smears. Children diagnosed with malaria (defined as any *P. falciparum* parasitemia with rectal temperature >38°C or clinical symptoms) during this follow-up period received standard anti-malarial treatment with sulfadoxine-pyrimethamine. The time

to first reinfection was defined as the time from admission until the time of detection of the first thick blood smear containing parasites. Incidence density rates (IDR) i.e. annual reinfection or malaria attack rates were estimated by calculating the ratio between the number of reinfections detected and the follow-up observation in years, corresponding to a maximum of four years' surveillance. Details of the study participants, inclusion criteria, definitions of severe and mild malaria, clinical care and treatment given have been described in detail elsewhere **[26,27,30,31].**

Immunological assessments. Plasma cytokine profiles were assessed in acute and healthy phase samples from all those available in the 1-95/C study, and were the subject of an earlier publication that describes the methods used in detail **[32].** Data corresponding to the 60 individuals included in the sub-study described here were used to assess the influence, if any, of EBV activity on plasma cytokine profiles.

DNA Extraction. DNA was extracted using the QIAmp Blood Kit (Qiagen, Hilden, Germany), either from plasma or whole blood, as recommended by the manufacturer. A final elution volume of 100µl was obtained from each sample.

Real-Time Quantitative PCR. Real-time quantitative PCR comprises continuous optical monitoring of a fluorogenic PCR reaction **[22,33,34].** TaqMan methodology was used and data collected and analyzed using an ABI Prism 7900H (PE Applied Biosystems, Cheshire, UK). Primers and probe were derived from the EBV polymerase gene as described previously **[17,42],** using specific primers: upstream: 5'-AGT CCT TCT TGG CTA GTC TGT TGA C-3'; downstream: 5'-CTT TGG CGC GGA TCC TC-3', and fluorogenic probe 5'(FAM)-CAT CAA GAA GCT GCT GGC GGC C-(MGB) 3'. Albumin gene-specific primers were used as endogenous control: upstream: 5'-TGG CAA GCT GAG TCT CCC TAA-3', downstream: 5'-CTC TCC TTC TCA GAA AGT GTG CAT AT-3', and fluorogenic probe 5'(VIC)-TGC TGA AAC ATT CAC CTT CCA TGC AGA-(TAMRA)3'.

The dual-labeled fluorogenic probes were located between the PCR primers, and contained a 3'-

blocking phosphatase group to prevent extension during PCR. EBV- and albumin-specific probes were synthesized by PE Applied Biosystems. EBV primers were purchased from Thermo-Hybaid (Ulm, Germany), and albumin primers from IBA Nucleic Acids Synthesis (Göttingen, Germany). PCR reactions were performed in 10µl volumes containing 1X TaqMan® Universal PCR Master Mix, 200nM (EBV) or 300nM (albumin) primer/probe combination, and 4.5µl DNA. Thermal cycling was carried out with 2min initial incubation at 50°C, 10min enzymatic activation at 95°C, 50 cycles for 15sec denaturation at 95°C and 1min annealing/extension at 60°C. For optimization and sensitivity determination, replicates of 10-fold dilutions of DNA from the BL cell lines BL-36 were tested; 6 replicates of each of the 3 highest dilutions were assayed. Diluted standard DNA (mutu), endogenous albumin control, and water blank samples were run in parallel in each PCR plate. Amplification data for EBV and albumin were summarized as amplification plots of fluorescence intensity against the threshold cycle (C_T) of a given PCR reaction. C_T is defined as the cycle number at which the fluorescence signal is first recorded as statistically significant above the threshold or background limit. The more DNA templates present at the beginning of the reaction, the fewer number of cycles required to reach the C_T. The protocol included 10-fold dilutions of DNA isolated from an EBV-positive cell line 'mutu' (standard DNA) to construct a standard curve, three EBV-negative lymphoma cell-lines (BL-2, L-428 and BL-41) and two other EBV-positive cell lines (BL-36, BL-74) as controls for the specificity of the EBV-primers (see below). Amplification reactions for target and endogenous genes were run in separate tubes. Reproducibility of the real-time PCR was assessed by including DNA isolated from anti-CD3 antibody-stimulated whole blood samples. All reactions were carried out in triplicate in 384-well plates. Samples were defined as negative if EBV DNA was detectable in only one or no well of the triplicates i.e. $C_T \geq 40$ in at least two wells.

Relative quantitation of EBV gene expression. As the absolute quantity of standard DNA was unknown, we applied the "relative quantitation method of Standard Curve" following the

manufacturer's instructions (PE Applied Biosystems) to calculate the EBV genome load Q, which expresses the relative input amount of EBV DNA detected in the test samples. This method has the advantage of being consistently applicable and to correct automatically for differences in the PCR efficiency. Briefly, we constructed a standard curve of the C_T values from the 10-fold diluted standard samples against standard units arbitrarily given to express the respective dilutions. The following standard units were given to decreasing dilutions: 100; 10; 1; 0.1; and 0.01. From the standard curve, the standard input amount of the EBV and albumin DNA in the unknown samples was calculated separately using the software SDS 2.0. Then, we determined the average of standard input amounts for EBV (Q_{EBV}) and albumin (Q_{Alb}) obtained from triplicate reactions of each sample. Q_{EBV} was then normalized relative to Q_{Alb} by calculating the ratio $NormQ_{EBV} = Q_{EBV} / Q_{Alb}$. Finally, we arbitrarily chose and defined a calibrator as $NormQ_{calib}$ (a test sample with the lowest Norm Q_{EBV}) and we determined EBV DNA load Q upon normalization relative to the calibrator: $Q = Norm\ Q_{EBV} / Norm\ Q_{calib}$.

Statistical methods. Data were analyzed using Statview™. For paired comparisons of continuous variables the non-parametric Friedman and Wilcoxon sign-rank tests were used and for unpaired comparisons the Kruskal-Wallis and Mann-Whitney U-tests were used. The MacNemar test was used to compare proportions within and between groups. Statistical significance in all cases was set at a two-tailed $p<0.05$. Correlations between continuous variables were assessed with the non-parametric Spearman rank test corrected for ties where rho (ρ)>0.25 with $p<0.05$ was considered significant.

Results

Sensitivity and reproducibility of EBV DNA-specific real-time PCR

The sensitivity of the real-time PCR was assessed by comparing EBV DNA expression after amplifying DNA from plasma and whole blood samples. As shown in Table 1, EBV DNA was detected in 51% whole blood samples (58% in the severe, 44% in the mild group), but in only 6% plasma samples (10% in the severe, 2% in mild the group) collected in the acute and convalescent phases. Those with plasma EBV DNA also had whole blood EBV DNA, and comprised individuals expressing the highest EBV DNA loads (data not shown). C_T values of EBV DNA from unstimulated (C_{Tunst}) and from anti-CD3-stimulated (C_{Tstim}) whole blood samples were also compared and found to be similar regardless of the source (data not shown). In addition EBV DNA was detected in all EBV-positive (BL-36, BL-74), but not in EBV-negative cell lines (BL-2, L-428, BL-41) nor in the water control. Taken together, these preliminary tests confirmed that peripheral blood is more suitable than plasma for highly sensitive and reproducible DNA-based real-time PCR quantitation of EBV.

Profiles of EBV DNA loads in peripheral blood pre- and post-treatment for *P. falciparum* malaria

On the basis of our preliminary tests we screened DNA isolated from whole blood samples only **[48,49].** Quantitatively, EBV DNA loads in both groups of children tended to decrease over time, such that the lowest levels were observed in healthy phase samples (Figures 1,2). This decline was statistically significant only in those that presented with mild malaria, and consequently EBV DNA loads were found to be significantly higher in healthy phase samples of those that presented with severe malaria (Figure 2A, p=0.019 by Wilcoxon test). Comparison of the EBV DNA profiles in the sub-groups of children whose blood was EBV DNA^+ revealed similar loads in the acute and convalescent phases both within and between the groups but a statistically significant decline in both

groups when the children were healthy and parasite-free (Figure 2B).

Table 1 illustrates the proportions of EBV DNA^+ individuals in the study groups at different time-points. The proportion of children with EBV DNA^+ blood samples increased over time in the severe, but decreased in the mild malaria groups such that, in the healthy phase, a significantly higher proportion of children in the severe malaria group were EBV DNA^+ (Table 1). This pattern was further emphasized when comparing the evolution of EBV DNA positivity, showing that almost twice as many children in the severe versus the mild malaria group persisted with EBV DNA in their peripheral blood in both convalescent and healthy phases (Table 2).

Influence of age on EBV DNA profiles

Regardless of time-point and clinical presentation status, children with EBV DNA^+ peripheral blood were significantly younger than those EBV DNA^- (median ages[interquartile ranges] in months of those DNA^+ vs those DNA^- in acute, convalescent, healthy phases, respectively: 43[32] vs 52[44], p=0.033; 39[26] vs 58[44], p=0.005; 33[24] vs 57[40], p=0.001; all by Mann-Whitney U test). This association was evident in the statistically significant inverse correlation observed between age and EBV DNA load, especially in the healthy phase (ρ= -0.469, p<0.001, Spearman Rank test). At the latter time-point, the proportion of EBV DNA^+ under-five year olds was over twice that of those aged five or more (24/37 [65%] vs 5/18 [28%], p=0.021 by χ^2 contingency table analysis with continuity correction).

Association between EBV DNA status and susceptibility to malaria

Acute phase measures such as parasitemia, hematocrit and parasite clearance time did not vary according to the presence or absence of EBV DNA and showed no association with the magnitude of EBV DNA loads (data not shown). Survival analyses using prospective measures based on the

presence or absence of EBV DNA in convalescent phase samples did not reveal any influence on times to first post-treatment reinfection (data not shown). Similarly, incidence density rates of reinfection did not differ significantly between those EBV DNA^+ and those EBV DNA^- (data not shown). A significant inverse association was detected, however, between the convalescent phase EBV DNA load and times to first reinfection when considering only the sub-group of EBV DNA^+ individuals (Figure 3A). The magnitude of the convalescent phase EBV DNA load was also found to be significantly positively associated with the number of malaria attacks (reinfections) detected in these children in the interval between the convalescent and healthy phase sampling (Figure 3B).

Association between EBV DNA and plasma cytokine concentrations

In acute phase samples there was a non-significant trend for higher IL-10 levels in EBV DNA^- children (Figure 4A). At the same time IL-12p70 was found at a significantly higher concentration in plasma of EBV DNA^- children in the severe but not in the mild malaria group (Figure 4A), and the levels of this cytokine in the former group consequently displayed a significant inverse association with EBV DNA load (ρ=-0.498, p=0.017, by Spearman rank test). The acute phase levels of TNF-α, IFN-γ (data not shown) and of IL-12p40 were similar regardless of the presence or absence of EBV DNA (data not shown and Figure 4A,B). In healthy phase plasma samples, the levels of both TNF-α and IL-12p40 were significantly higher in children with EBV DNA compared to those without (Figure 4B), and the levels of the latter cytokine showed a strong positive association with EBV DNA load (ρ=0.485, p=<0.001, by Spearman rank test). Healthy phase plasma levels of IL-10 and IFN-γ were low and did not differ according to EBV DNA status (data not shown).

Discussion

We conducted the study presented here in order to determine the nature and extent of the interaction between a ubiquitous viral infection, EBV, and the malaria parasite *P. falciparum* in young African children living in an area where the latter is transmitted perennially at a high level. Specifically we wished to characterize the extent to which EBV DNA is detectable during acute *P. falciparum* malaria in such children, and to investigate the degree, if any, to which this viral parameter might be associated with either the severity of the clinical outcome of and/or the susceptibility to *P. falciparum* infection. We used a quantitative DNA-based real-time PCR method, previously described to be robust and applicable for the quantitation of EBV DNA **[17,24,34].** We found, in agreement with others, that this method is a highly efficient, sensitive and reproducible method for assessment of EBV DNA in whole blood-derived DNA **[24,48,49].**

Our study is in good accord with the original studies on the topic of malaria-related EBV reactivation conducted in the Gambia, in that it confirmed that acute *P. falciparum* malaria episodes in young African children are associated with increased peripheral blood EBV DNA loads that decline in the absence of plasmodial parasites **[29,54,55].** In keeping with the conclusions drawn from the Gambian studies, we interpret our findings as a direct indication of the kinetics of expanding and contracting populations of circulating EBV-infected B cells. As a result of our study design we also revealed entirely novel and somewhat unexpected aspects in the context of perceived interactions between the two pathogens. These concern the difference in long-term persistence of EBV reactivation related to the clinical severity of the malaria episode: viral DNA detectable in the peripheral circulation persists at a higher level and in a higher proportion of individuals with a history of severe rather than mild malaria. As we reported earlier, active surveillance showed that, as a group, those who presented with severe malaria were more susceptible to malaria attacks subsequently **[30,31].** The data presented here show that high EBV DNA loads were associated with greater numbers of malaria attacks. We

speculate, therefore, that the recovery of effective cell-mediated immune control of EBV seen in the majority of individuals presenting with mild malaria is abrogated in children suffering frequent repeated *P. falciparum* infections, especially so in those that presented with severe malaria at inclusion into the study. The clear age dependency of the presence of EBV DNA we detected here nevertheless suggests that the influence of these deleterious effects declines as children get older.

Viral infections are known to affect the human immune system to benefit their survival via a multiplicity of pathways. EBV exhibits an array of potentially subversive mechanisms that include the production of a viral homologue of human IL-10 as well as induction of the production by EBV-infected B cells of the IL-12p40 homologue, EBI3, a component of the heterodimeric immunomodulatory cytokine IL-27 **[4,9,16].** The data we present here concerning alterations in the plasma profiles of certain cytokines that are related to the simultaneous presence of EBV DNA in peripheral blood suggest that the loss of control of EBV replication may influence cytokine responses considered important for anti-plasmodial immunity. Most notable, in this context, is the significantly lower concentration of biologically active IL-12p70 detected in the plasma of children presenting concomitantly with severe *P. falciparum* malaria and EBV DNA, since IL-12 is thought to play a prominent role in initiation of early Th1-mediated control of plasmodial infection **[32,35,50]**. The significantly elevated levels of IL-12p40 detected in plasma of children with EBV DNA when they were otherwise healthy and parasite-free may also have consequences for subsequent host-parasite interactions since the p40 homodimer has known immunomodulatory activities **[4].** The association between the presence of EBV DNA and significantly enhanced levels of plasma TNF-α found in children when they were healthy and parasite-free is consistent both with our own earlier findings and with the known effects of EBV on constitutive B cell cytokine activity **[44,46,47].** Viral persistence may be directly linked to virus-mediated enhancement of TNF-α activity since this cytokine is known

to be required for survival of plasma cells that themselves represent one of the primary reservoirs for latent EBV **[7].**

The study reported here has helped to extend the knowledge, in the context of malaria in young African children, of the interactions at the clinical and immunological levels between a ubiquitous virus and a protozoal pathogen with an enormous public health impact. Others have reported evidence of clinically important interactions between a viral infection common in childhood in Africa - hepatitis B virus - and *P. falciparum* **[52].** In residents of sub-Saharan African countries, the degree to which the modulation of immune responses affects the outcome of infection with multiple pathogens present at birth and/or throughout early life is a largely neglected topic. Determining the nature and extent of these cross-species influences remains a challenge to both immunologists and vaccinologists with an interest in developing or improving control measures for the diseases associated with poverty in Africa.

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Figure legends

Figure 1. Individual profiles of EBV DNA loads detected in the peripheral blood during and after acute clinical episodes in individual Gabonese children presenting with either mild (A) or severe (B) *P. falciparum* malaria.

Figure 2. Kinetics of EBV DNA loads for groups segregated according to clinical presentation at admission either for all individuals (A) or only for those with EBV DNA^{+} samples (B), where box-plots represent medians, with 25th and 75th percentiles, and whiskers for 10th and 90th percentiles of EBV DNA loads, excluding outliers. Convalescent phase (conv.) samples were taken 1 month post-treatment, and healthy phase samples at least 6 months post-treatment. †/* $p<0.05$ (Friedman test) for decline in EBV DNA load over time-course of study.

Figure 3. Associations between EBV DNA load and either the delay in weeks to the first post-treatment *P. falciparum* reinfection (A), or the number of reinfections between convalescent and healthy-phase sampling (B). Regression plots show malariometric parameters (y axes) plotted against EBV DNA loads (x axes) for those with blood samples EBV DNA^{+}, and with 95% confidence intervals represented as dotted lines. The non-parametric Spearman rank test was used to test for associations, where $p<0.05$ in conjunction with $\rho>0.25$ was considered as significant.

Figure 4. Correlation between EBV DNA status and either acute phase plasma levels of the cytokines IL-10 and IL-12p70 in children segregated according to clinical presentation (A) or acute/healthy phase plasma levels of TNF-α and IL-12p40 in unsegregated groups (B). Box-plots represent medians, with 25th and 75th percentiles, and whiskers for 10th and 90th percentiles of cytokine concentrations measured by ELISA. * $p=0.022$, ** $p=0.028$, † $p<0.001$, all by non-parametric Mann-Whitney U test.

Table 1. Proportion of whole blood or plasma samples containing EBV DNA detected by real-time PCR

Group	Time-point	n*	% EBV DNA$^+$
Severe	**Acute**	27 (30)	52 (13)
	Convalescence	28 (30)	64 (7)
	Healthy	27 (nd)**	67†
Mild	**Acute**	28 (30)	46 (3)
	Convalescence	27 (30)	41 (0)
	Healthy	28 (nd)	39

* n: total number of whole blood (or plasma) samples tested at each time-point.

** nd.: not done (plasma samples not tested).

† McNemar $p<0.05$ for comparison of healthy phase % EBV DNA$^+$ in severe versus mild groups

Table 2 Profiles of EBV DNA detection in whole blood subsequent to acute *P. falciparum* malaria

Profile*	Acute	Conv.	Healthy	Mild (%)	Severe (%)
1	+/-	-	-	13 (45)	7 (25)
2	+/-	+	+	8 (29)	15 (54)
3	+/-	+	-	3 (11)	2 (7)
4	+/-	-	+	2 (7)	3 (11)
nd**				3 (11)	1 (4)

* The different profiles (P1, 2, 3 and 4) were determined as a function of the EBV DNA status in convalescent and healthy phases

** nd: not determined (donors with one or two missing samples)

Figure 1

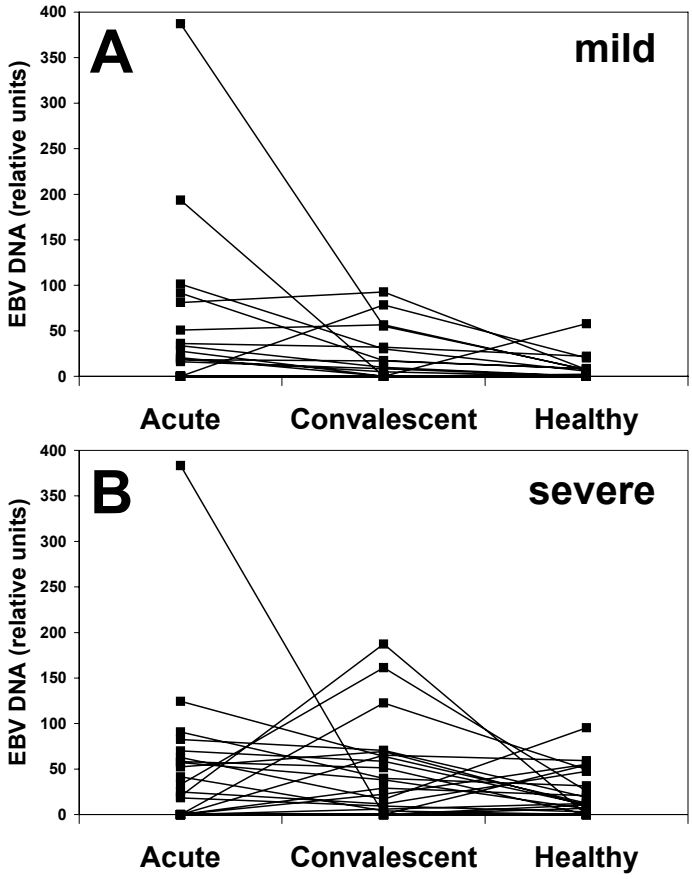

A
mild
EBV DNA (relative units)
400
350
300
250
200
150
100
50
0
Acute
Convalescent
Healthy
B
severe
EBV DNA (relative units)
400
350
300
250
200
150
100
50
0
Acute
Convalescent
Healthy

Figure 4

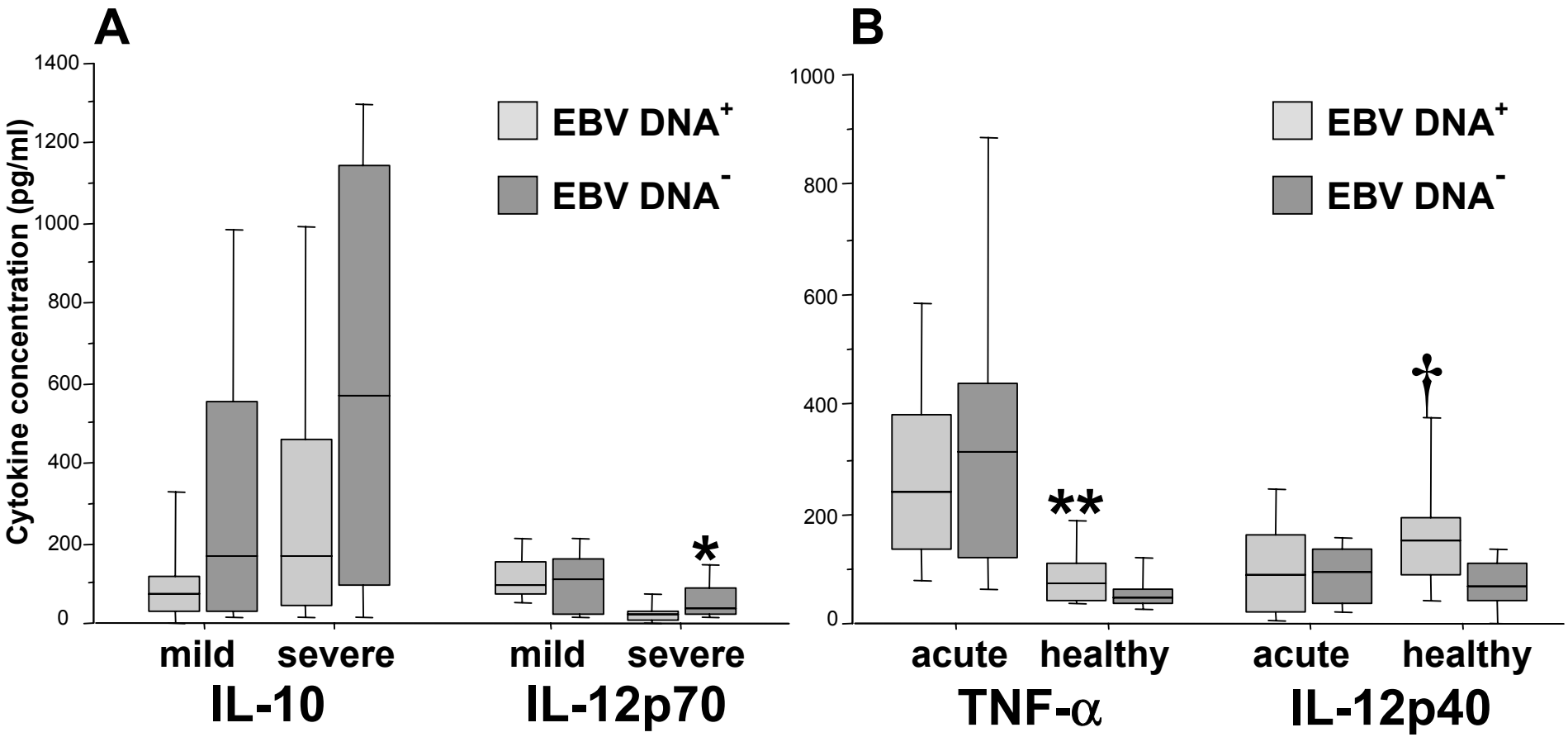

A
B
Cytokine concentration (pg/ml)
1400
1200
1000
800
600
400
200
0
EBV DNA+
EBV DNA-
mild
severe
mild
severe
IL-10
IL-12p70
*
1000
800
600
400
200
0
**
acute
healthy
acute
healthy
TNF-α
IL-12p40

Figure 3

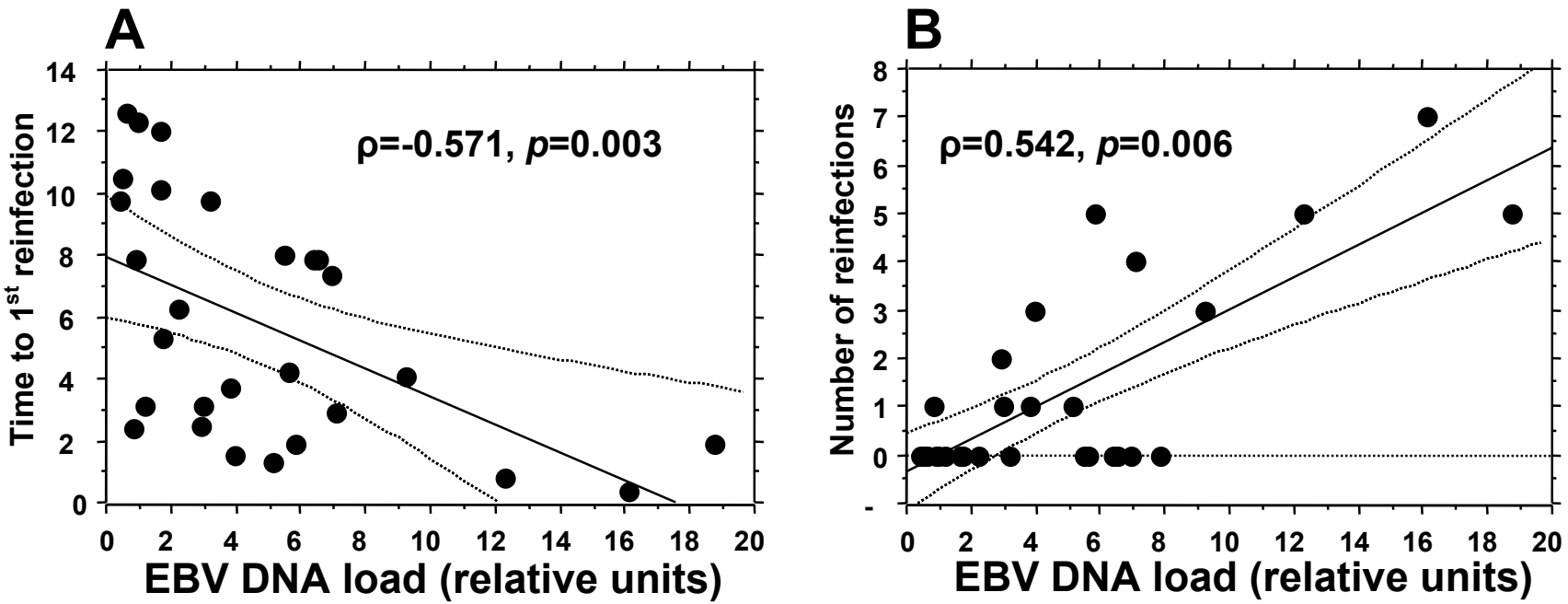
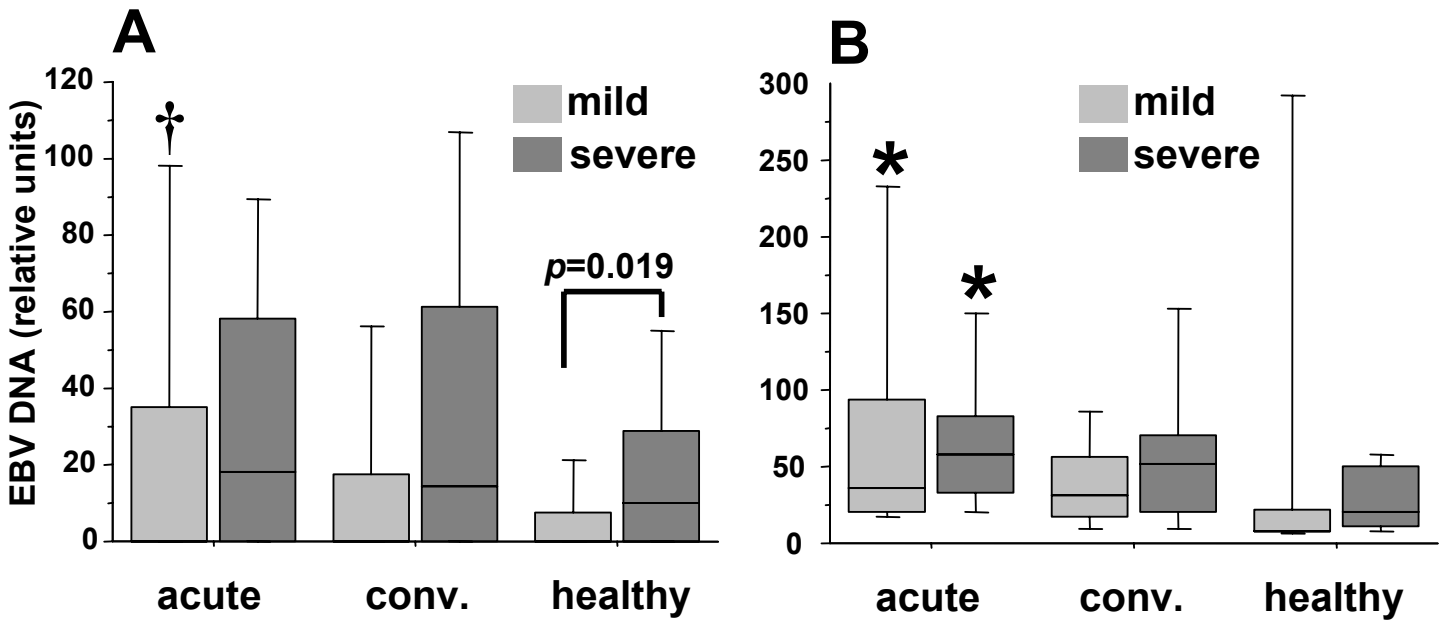

A
Time to 1st reinfection
ρ=-0.571, p=0.003
EBV DNA load (relative units)
B
Number of reinfections
ρ=0.542, p=0.006
EBV DNA load (relative units)

Figure 2


A
B
EBV DNA (relative units)
mild
severe
p=0.019
acute
conv.
healthy
0
20
40
60
80
100
120
50
150
200
250
300
*
†

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