CONCISE COMMUNICATION

In Vivo Switching between Variant Surface Antigens in Human Plasmodium falciparum Infection

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A semi-immune individual was retrospectively found to have maintained an apparently monoclonal and genotypically stable asymptomatic infection for months after clinical cure of a Plasmodium falciparum malaria episode. Before the attack, the individual had no antibodies to variant surface antigens (VSAs) expressed by an isolate (isolate A) obtained at the time of the episode or by a genotypically identical isolate (isolate B) obtained from the same individual 3 months later. Six weeks after the attack, a strong isolate A–specific VSA antibody response had developed in the complete absence of isolate B–specific antibodies. In contrast, plasma obtained 7 months after the attack contained high levels of VSA antibodies recognizing both isolates. This is the first direct evidence of in vivo switching between VSAs in human P. falciparum infection. Our results suggest that VSA switching is an important survival strategy of P. falciparum, enabling the parasite to persist despite protective, parasite-specific immune responses.

Individuals living in areas of endemic Plasmodium falciparum transmission gradually acquire protective immunity against malaria. The targets and mechanisms of this protection are not fully understood, but an increasing body of evidence indicates that antibodies directed against parasite-encoded variant surface antigens (VSAs) on the surface of infected red blood cells (iRBCs) play an important role [1–3]. P. falciparum erythrocyte membrane protein 1 (PfEMP1) is the dominant and best characterized VSA. These highly polymorphic proteins are encoded by the var gene family, which is present at 40–50 loci per genome [4]. var gene switching and ensuing changes in PfEMP1 expression have been demonstrated in vitro [5], and clonal antigenic variation with successive appearance of distinct VSA occurs in simian malaria parasites in vivo [6].

Individual clones of the P. falciparum parasite can maintain genotypically stable, asymptomatic blood-stage infections in human carriers for long periods in areas where mosquito transmission is essentially absent and clinical disease very rare [7, 8]. Precisely how the immune system can control parasite multiplication and keep parasitemia at a very low level for >1 year without eliminating the infection is not clear, but the phenomenon has been linked to the above-mentioned clonal antigenic variation [9]. However, so far, direct evidence of in vivo VSA switching in P. falciparum–infected humans has been lacking.

We studied a cohort of individuals living in an area of unstable, seasonal P. falciparum transmission in eastern Sudan. Among the cohort members, an individual was retrospectively found to have maintained a submicroscopic, apparently monoclonal, and genotypically stable P. falciparum infection for months after treatment of a malaria episode. We report here our analysis of antibodies specific for VSA expressed by 2 parasite isolates obtained from that individual at the time of clinical disease and 3 months later.

Methods

Study area and study population. This study was conducted in Daraweesh village, Gedaref State, Sudan, a study site used exten-
sively by us for more than a decade. The epidemiology of malaria in this area has been described in detail elsewhere [10]. In brief, the area is characterized by hypoendemic, unstable parasite transmission with marked seasonal variation, and essentially all malaria episodes occur during and shortly after the annual rains, between June and October. The remainder of the year is very dry, and the level of parasite transmission is undetectable.

Clinical surveillance and collection of blood samples. Thirty-seven individuals who contracted *P. falciparum* malaria during the 1996 transmission season were followed up regularly. Heparinized blood samples were collected from all of these individuals at the beginning of the study, before the 1996 transmission season. Additional samples were collected at the time that clinical *P. falciparum* malaria was diagnosed, 4–6 weeks after the attack, and at the end of the 1996 transmission season. Throughout the following dry season, venous blood samples were collected at monthly intervals, interspersed fortnightly by collection of filter-paper blood spots. For details, see [8].

Sample preparation. From all the heparinized samples, we separated plasma and RBCs by centrifugation (at 800 g for 10 min). Plasma was stored at −40°C, and the RBC pellet was snap-frozen and stored in N2, as described elsewhere [2].

DNA extraction and polymerase chain reaction (PCR) analysis. All blood samples and filter-paper samples were genotyped by PCR at the *msp1*, *msp2*, and *glurp* loci and sequenced so that the number of parasite clones could be estimated, as described elsewhere [8].

Parasites, in vitro culture, and preparation of late-stage iRBCs. In vitro *P. falciparum* cultures were established from cryopreserved iRBC. Immediately before assays were done, we labeled the cultures with ethidium bromide (40 μg/mL) and purified late-stage iRBCs (hemozoin-containing trophozoites and schizonts) by exposure to a high-gradient magnetic field, as described elsewhere [11].

Measurement of plasma antibodies to VSAs by flow cytometry. Levels of plasma IgG with specificity for VSAs were measured by flow cytometry as described in detail elsewhere [11]. In brief, we sequentially incubated ethidium bromide–labeled iRBCs (2 × 10⁷ RBCs) with 5 μL of plasma (30 min), 0.4 μL of goat anti–human IgG (Dako) (30 min), and 4 μL of fluorescein isothiocyanate–conjugated rabbit anti–goat IgG (Dako) (30 min). Data were collected with a FACScan flow cytometer (BD PharMingen), using a gate on ethidium bromide–positive (and thus infected) RBCs. Flow cytometry data were analyzed by means of WinMDI 2.8 software (Flow Cytometry Core Facility, Scripps Research Institute; http://facs.scripps.edu/software.html).

Results

Retrospective analysis by PCR showed evidence of submicroscopic infections that persisted for extended periods after curative drug treatment in 12 of 37 of the cohort members. In 5 of these 12 subjects, the chronic infection was monoclonal and was identical to the disease-inducing infection (8); data not shown). We succeeded in establishing in vitro parasite cultures from the blood samples collected from 1 subject (AE5, a 21-year-old man) at the time of the clinical attack (23 October 1996; isolate A) and 3 months later (31 January 1997; isolate B, when the infection was only detectable by PCR). Genotyping of these 2 isolates yielded the expected PCR profile (figure 1).

Attempts to establish in vitro cultures from blood samples with submicroscopic infection obtained from the remaining 4 individuals were unsuccessful.

We next used flow cytometry to measure levels of VSA-specific IgG in plasma samples collected ~2 months (3 September 1996) before and 6 weeks (7 December 1996) and 7 months (1 June 1997) after the disease episode [11]. Before the malaria episode, we did not detect expression of IgG specific for VSA by the parasite isolate A (VSAₐ), which was collected at the time of the attack (figure 2A). However, 6 weeks after the clinical episode, VSAₐ IgG levels had increased several-fold (figure 2B), and they remained high 7 months later (figure 2C). This shows that the clinical episode was caused by a parasite expressing VSA to which the patient did not have preexisting IgG and that the malaria attack caused a rapid and sustained IgG response specifically recognizing the VSA (i.e., VSAₐ) expressed by the infecting isolate. These results are in complete agreement with those of previous studies [1, 12]. In marked contrast, no IgG specific for the VSA expressed by the genotypically identical parasite isolate B (VSAₐ), which was collected 3 months later, could be detected before the clinical episode (figure 2D), and only borderline reactivity was seen 6 weeks after the clinical episode.
attack (figure 2E). However, high levels of VSA-specific IgG levels were found in the plasma sample collected 4 months after the collection of isolate B (figure 2F).

Discussion

Results of animal experiments have long indicated that antigenic variation of VSAs is the basis for the persistence of malaria parasitemia despite the presence of a continuing immunological attack [6, 13, 14]. Furthermore, recent studies of human P. falciparum infections have indicated that acquisition of VSA-specific immunity can shape the VSA-expression pattern, probably by gradually narrowing the repertoire of VSAs compatible with parasite survival in the semi-immune host, which leads to a decrease in the virulence of the infection [2, 15]. Thus, acquisition of VSA-specific antibodies appears to be central to acquisition of protective immunity in humans. However, although antigenic variation is known to occur in clonal P. falciparum in vitro, only circumstantial evidence has been available, until now, that this also occurs in vivo in P. falciparum-infected humans. By documenting the progression in plasma antibody levels with specificity for VSA expressed by P. falciparum isolates obtained at different time points, we show directly that the antigenic phenotype of a genotypically stable and monoclonal P. falciparum infection can change in the course of a human infection, and that these changes can induce production of VSA-specific antibodies. Our data are the first, to our knowledge, to formally prove that the VSA-expression pattern changes with time during chronic malaria infections in humans, probably as a mechanism developed by the parasite to survive successive waves of immunological attack on parasite-encoded VSAs, allowing the maintenance of long-term, subclinical infections, similar to those described elsewhere in experimental simian infections [6, 13, 14].

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