

SURFACE ALTERATIONS OF ERYTHROCYTES IN *PLASMODIUM FALCIPARUM* MALARIA

Antigenic Variation, Antigenic Diversity,
and the Role of the Spleen*

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The understanding of antigenic diversity in *Plasmodium falciparum* is important in research aimed at the development of a vaccine against malaria. Previous studies (1–5) have demonstrated, with a variety of biochemical and immunological markers, that different geographical isolates could present distinct characteristics. None of these studies, however, has shown any diversity among the antigens exposed at the surface either of the parasite or the infected host cell, i.e., antigens that are most likely to play a role in the development of a protective immunity (6). Protective immunity against *P. falciparum* has been shown to have a strain-specific component (7–9).

We have examined the antigenic diversity of determinants expressed on the surface of squirrel monkey erythrocytes infected with *P. falciparum* using a technique of surface immunofluorescence. We found that three geographical strains express different surface determinants and that parasites of a given strain could undergo antigenic variation; and we describe a situation where the transfer of a clone of *P. falciparum* from a splenectomized into an intact squirrel monkey results in a switch from one set of erythrocyte-associated antigens to another. This novel mechanism of antigenic variation in *P. falciparum* is discussed in the context of the strain-specific antigenic diversity of this parasite and protective immunity.

Materials and Methods

Parasites. The Ugandan Palo Alto/PLF-3 strain, Gambian FCR-3 strain, and Indochina-1 strain of *P. falciparum*, which have been adapted to the squirrel monkey, *Saimiri sciureus* (10), have been used throughout these experiments. Parasites were obtained by venipuncture of anesthetized, infected monkeys. Infected blood was cryopreserved in liquid nitrogen and thawed when needed according to the method of Diggs et al. (11). Short-term cultures of fresh or thawed infected erythrocytes were performed as described by Trager and Jensen (12), except that fetal bovine serum (FBS) was substituted for human serum.

In view of the differences observed earlier between parasites maintained in intact (S⁺)¹ and

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¹ Abbreviations used in this paper: IFAT, indirect fluorescent antibody test; PMSF, phenylmethylsulfonyl-fluoride; S⁺, spleen intact; S⁻, splenectomized; SICA, schizont-infected cell agglutination; VAT, variant antigenic type.

splenectomized (S^-) animals (13), we maintained two different lines for each strain by serial transfer either in S^+ or S^- animals.

Clone B-11 was derived from PLF-3 (S^- line) in the following way: an in vitro culture of PLF-3 in human erythrocytes was established and grown for 5 mo in routine culture conditions (12); infected erythrocytes were diluted in medium to a final concentration of 10 parasites/ml and normal erythrocytes were added to obtain a 2.5% hematocrit; the suspension was distributed into a 96-well flat-bottomed microtiter plate, at 100 μ l/well, and cultivated at 37°C in a candle-jar; the medium was changed daily and fresh erythrocytes were added on days 5 and 11; on day 16, all cultures were screened for parasite growth using Giemsa-stained thin films. A single clone was isolated by this procedure and this population was subsequently re-cloned using the same technique. Three clones were isolated and one of these (PLF-3/B-11) was transferred into squirrel monkey erythrocytes in vitro and injected 2 d later into a splenectomized animal.

Animals and Immune Sera. Female Bolivian squirrel monkeys (South American Primates, Inc., Miami, FL) were used throughout this study. Splenectomy was performed as described earlier (10) and splenectomized animals were infected within 1 wk after splenectomy.

Fig. 1 gives details of the evolution of parasitemia in six monkeys after primary infection with different lines of PLF-3, the timing of recovery and challenge infections, and the follow-up of parasitemia after challenge with either homologous or heterologous populations. Fig. 2 shows the same in five monkeys infected with different lines of Indochina-1; secondary peaks were identified as new populations (Indo-B, Indo-C, Indo-D, Indo-E, Indo-F, and Indo-G). Challenge infections were performed by intravenous inoculation of a cryopreserved stabilate of the

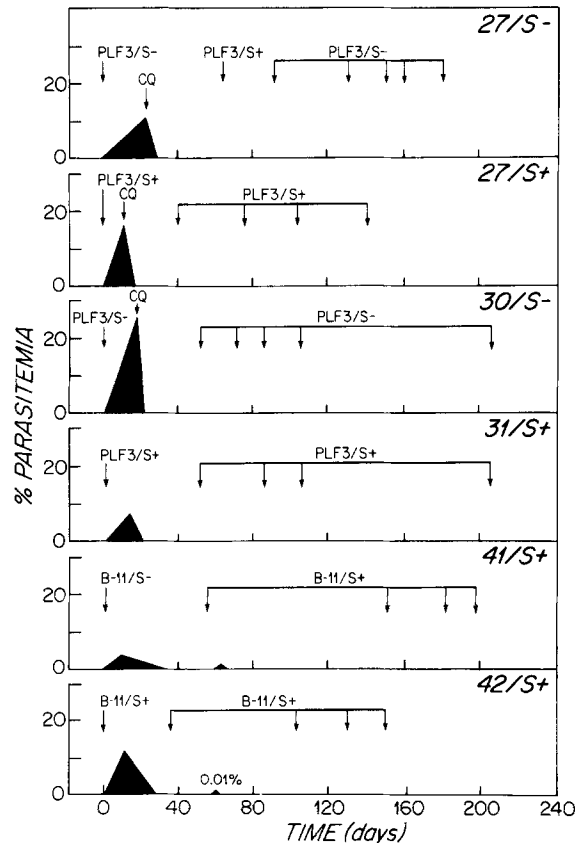


FIG. 1. Evolution of parasitemia and timing of challenge in various monkeys infected with different lines of the Ugandan Palo Alto strain of *P. falciparum*. Arrows indicate timing of infection, challenge with a specified population, or drug treatment (CQ, chloroquine, intramuscular).

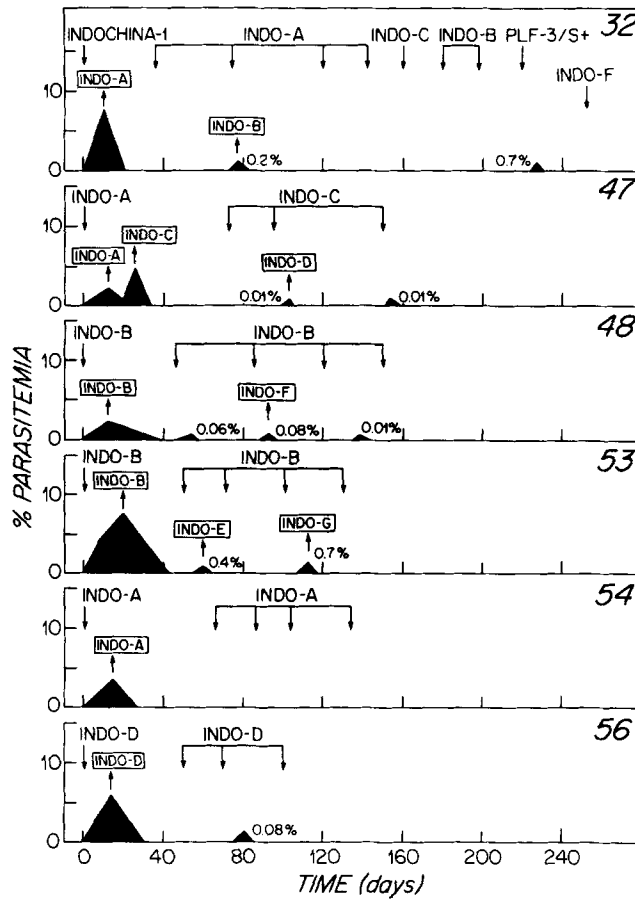


FIG. 2. Evolution of infection and timing of challenge in various monkeys infected with the Indochina-1 strain of *P. falciparum*. Arrows indicate the timing of infection, challenge, or drug-treatment (Q, quinine, intravenous). New populations isolated from recrudescence or secondary peaks are identified in brackets. When a secondary peak was observed, the maximum level of parasitemia is indicated.

appropriate population thawed as indicated above. Sera were taken before infection, after recovery, and 5–10 d after each challenge infection. Other monkeys were used in this study either for subinoculations or as a source of infected erythrocytes, but details on parasitemia are not given.

Immunofluorescence. The technique for surface immunofluorescence using fresh, unfixed, infected erythrocytes had been previously used for the demonstration of variant antigens on the surface of *P. knowlesi*-infected erythrocytes (14). The modifications were briefly the following: 100 μ l of each serum dilution (1:5, 1:25, 1:125, and 1:250) was incubated in a hemolysis tube for 30 min at 37°C with 7.5–10 μ l of packed erythrocytes. After centrifugation for 5 min at 500 g (PR-6 Centrifuge; International Equipment Co., Needham Heights, MA), the pellet was washed twice in 3 ml medium and incubated in 100 μ l of a 1:100 dilution of the IgG fraction of a rabbit anti-squirrel monkey IgG for 30 min at 37°C. After two further washings, the pellet was incubated in 100 μ l of a 1:25 fluorescein-labeled goat anti-rabbit IgG (Nordic Immunological Laboratories, El Toro, CA) for 30 min at 37°C. After two final washings, a drop of the suspension was examined with a Leitz Orthoplan fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ). Erythrocytes infected with late developmental stages of *P. falciparum* can be recognized under bright field by the presence of refringent malarial pigment. Under ultraviolet

light, only pigment bearing erythrocytes show a rim-like membrane associated fluorescence when the assay is positive (Fig. 3). A reading was only considered negative if 20 trophozoite/schizont-infected erythrocytes showed no membrane fluorescence. Similarly, the percentage of fluorescent trophozoite/schizont-infected erythrocytes was determined by examining under fluorescent light the cells with pigment visible under bright field. Other fluorescent cells, including lymphocytes, free parasites, or lysed infected cells, are visible but were not considered in this study. The medium used for incubation and washing was RPMI 1640. Both the rabbit anti-squirrel monkey IgG and the fluorescein-labeled goat anti-rabbit IgG were adsorbed with normal squirrel monkey erythrocytes.

Indirect fluorescent antibody test (IFAT) using acetone-fixed antigen was performed as described by Voller and O'Neill (15), but using a double sandwich technique with the same antiserum and conjugate as above.

Treatment of Erythrocytes with Enzymes. Erythrocytes were incubated in 1 ml RPMI 1640 containing 0.1 mg/ml of trypsin (Sigma Chemical Co., St. Louis, MO), 0.1 mg/ml chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) or 25 U neuraminidase (Gibco Laboratories, Grand Island, NY) for 30 min at 37°C. Cells were washed three times in 3 ml RPMI 1640. Trypsin-treated cells were then incubated at 37°C for 15 min with soybean trypsin inhibitor (1 mg/ml; Millipore Corp., Bedford, MA) and chymotrypsin-treated cells were incubated for 15 min with phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., 10^{-4} M). After treatment with protease inhibitors, the cells were washed three times in 3 ml RPMI 1640.

Results

Strain-specific Antigenic Diversity. In looking for strain-specific diversity of erythrocyte-associated antigens, we found that the three lines of parasites tested differed in the antigenic determinants as detected by surface immunofluorescence (Table I). The convalescent sera gave a high specificity, but a low titer of antibodies (1:5); with these sera, it was possible to distinguish between the three different strains. The hyperimmune sera (C-4) gave higher titers (1:125), but a cross-reactivity between Palo Alto and FCR-3 was observed, while the Indochina-1 strain remained antigenically distinct.

Only the membranes of erythrocytes infected with late trophozoites or schizonts

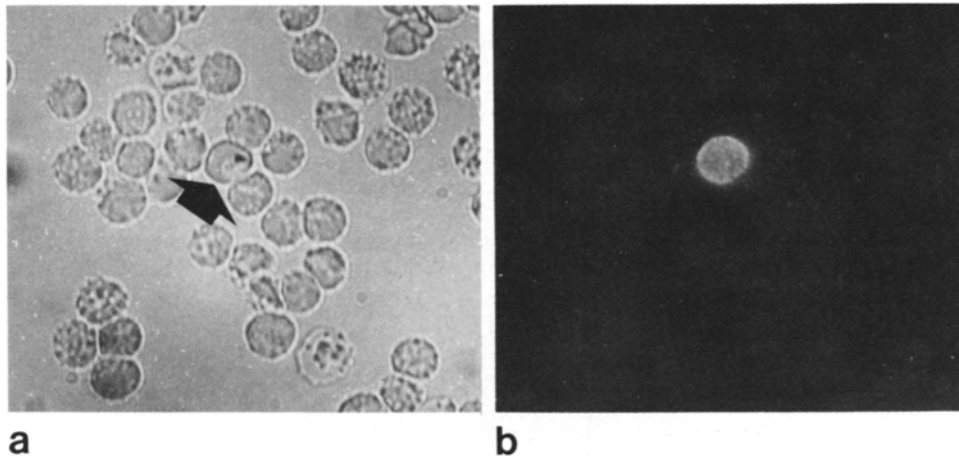


FIG. 3. Surface fluorescence of schizont-induced erythrocytes. Infected blood was incubated with immune squirrel monkey serum followed by rabbit anti-squirrel monkey IgG and rhodamine-labeled goat anti-rabbit IgG. The same field ($\times 630$) shows erythrocytes examined under bright field (a) and ultraviolet light (b). Arrows identify the cells with pigment detected in a which are fluorescent in b.

TABLE I
Detection of Strain-specific Differences Using Surface Immunofluorescence

| | Anti-PLF-3 (31/S ⁺) | | Anti-FCR-3 (36/S ⁺) | | Anti-Indochina-1 (32/S ⁺) | |
|---------------------------------|------------------------------------|-------|------------------------------------|-------|--|-------|
| | R | C-4 | R | C-4 | R | C-4 |
| PLF-3 (Uganda) | 1:5 | 1:125 | 0 | 1:125 | 0 | 0 |
| FCR-3 (The Gambia) | 0 | 1:125 | 1:5 | 1:125 | 0 | 0 |
| Indochina-1 (Vietnam/Kampuchea) | 0 | 0 | 0 | 0 | 1:5 | 1:125 |

Three strains were tested with their respective antisera. For each immune monkey, antibody titers are given for convalescent serum (R) and serum taken after the 4th challenge (C-4) infection.

TABLE II
Comparison of S⁺ and S⁻ Lines Using Surface Immunofluorescence

| | Anti-PLF-3/S ⁺ | | | | Anti-PLF-3/S ⁻ | | | | Anti-Indochina/S ⁺ | | |
|---------------------------------|---------------------------|-------|-------------------|-------|---------------------------|-------|-------------------|-------|-------------------------------|-------------------|-----|
| | 31/S ⁺ | | 29/S ⁺ | | 30/S ⁻ | | 27/S ⁻ | | 32/S ⁺ | 54/S ⁺ | |
| | R | C-4 | R | C-4 | R | C-4 | R | C-4 | R | C-4 | R |
| PLF-3/S ⁺ line | 1:5 | 1:125 | 1:5 | 1:125 | 0 | 0 | 0 | 1:125 | 0 | 0 | 0 |
| PLF-3/S ⁻ line | 0 | 0 | 0 | 0 | 1:5 | 1:125 | 1:5 | 1:125 | 0 | 0 | 0 |
| Indochina-1/S ⁺ line | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1:5 | 1:125 | 1:5 |
| Indochina-1/S ⁻ line | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The S⁺ and S⁻ lines of PLF-3 and Indochina-1 were compared by surface immunofluorescence, using their respective antisera. For each immune monkey, antibody titers are given for convalescent serum (R) and serum taken after the 4th challenge (C-4) infection.

show a rim-like fluorescence (Fig. 3); normal erythrocytes or erythrocytes infected with ring-stage parasites do not show any fluorescence.

The results were identical with infected erythrocytes present in the peripheral bloodstream, infected erythrocytes maintained *in vitro* for 24–40 h (to allow maturation from the ring to the trophozoite/schizont stage) or with infected erythrocytes that had been cryopreserved (11) and subsequently grown *in vitro* for 24 h.

In contrast to surface immunofluorescence, IFAT using acetone-fixed, infected erythrocytes as antigen detects the presence of antibodies against a variety of malarial antigens, not necessarily localized on the surface of the cell, and does not show any strain specificity. This assay demonstrates that all sera have comparable antibody titers (1:80 for convalescent sera; 1:1,280–1:2,560 for hyperimmune sera), which shows that there is no difference in the level of antibody production in intact or splenectomized animals. Sera from animals with a patent parasitemia were always negative by surface immunofluorescence, although they had already detectable titers in the IFAT.

This demonstrates that surface immunofluorescence can be used to examine erythrocyte-associated antigens in *P. falciparum*-infected cells and to distinguish between different populations.

Modulation by the Spleen. Table II shows that different antigens are expressed on the surface of S⁺ and S⁻ lines, both for the PLF-3 and the Indochina-1 strains. The results obtained with monkey 27/S⁻, infected first with the S⁻ line and then challenged with the S⁺ line, show that a single challenge with a different phenotype is sufficient to induce a lasting immunity to this phenotype. This stresses the importance of selection

TABLE III
Spleen-modulated Antigenic Variation

| | Successive monkeys | | | | | | |
|---------------------|--------------------|-------------------|--------|-----------------------------|--------|-------------------|-------------------|
| | 28/S ⁻ | 35/S ⁻ | Day 8 | 40/S ⁺ Day 14 | Day 20 | 42/S ⁺ | 45/S ⁻ |
| Anti-S ⁻ | +(90%) | +(90%) | +(90%) | +(45%) | - | - | +(80%) |
| Anti-S ⁺ | - | - | - | +(40%) | +(90%) | +(90%) | +(20%) |

Follow-up of surface IFAT using serum 30/S⁻ and 31/S⁺ in five serial transfers of clone PLF-3/B-11 in splenectomized and intact squirrel monkeys. Positive means a titer of 1/125 and negative means no fluorescence at 1/5. The percentage indicates the number of pigment-containing erythrocytes that presents a rim-like fluorescence under ultraviolet light.

of S⁺ and S⁻ lines by serial transfer before attempting differential serotyping; in earlier studies (13), where this was not done, results were less clear-cut.

In the case of PLF-3, each of the S⁺ and the S⁻ specificities remained stable over successive transfers in intact or splenectomized monkeys, whereas the S⁺ line of the Indochina-1 strain underwent antigenic variation (see below), a feature that complicates the comparison of S⁺ and S⁻ specificities in the latter strain. The S⁻ line of PLF-3 was cloned *in vitro* and one of the clones (PLF-3/B-11) was reintroduced into an S⁻ squirrel monkey. This clone was recognized by serum 30/S⁻, which indicates that the S⁻ specificity had remained stable over long-term *in vitro* cultivation.

To demonstrate the role of the spleen in this antigenic expression, we transferred clone B-11 from a splenectomized to an intact animal. Table III gives the results of this critical experiment. In the S⁺ animal, the S⁻ specificity was still found on day 8, and surface antigens then progressively switched from the S⁻ to the S⁺ specificity between days 14 and 20. In the reverse experiment (i.e., when clone B-11/S⁺ was transferred from an intact to a splenectomized animal), only a partial switch from the S⁺ to the S⁻ specificity had occurred (80% parasites showed the S⁻ specificity) by the time parasitemia reached its terminal peak, at which point the experiment was interrupted. The fact that the S⁻ specificity can be found on erythrocytes of intact animals indicates that the antigenic difference between S⁺ and S⁻ lines does not relate to some peculiar characteristic of erythrocytes in splenectomized animals.

Effect of Enzyme Treatment. When parasites had developed from rings to trophozoites/schizonts in culture, infected blood was treated with neuraminidase, chymotrypsin, or trypsin and the unfixed cells were tested by IFAT. Treatment with trypsin or chymotrypsin completely abolished membrane-associated fluorescence. Neuraminidase did not affect the results of IFAT.

To ensure that this result was not an effect of proteolytic enzymes on immune serum, even after inhibition of soybean trypsin inhibitor or PMSF, we tested the serum dilution that had been incubated with the enzyme-treated cells and performed a control IFAT with untreated cells. No difference in antibody titer could be detected, which confirmed that the protease treatment altered the antigen rather than the antibody.

Antigenic Variation of the Indochina-1 Strain. When we examined the reactivity in monkey 32/S⁺ infected with Indochina-1 of surface determinants on parasitized cells taken at the primary peak of parasitemia (Indo-A) and at a secondary peak (Indo-B) obtained after challenge of this animal with Indo-A and subsequently transferred into

a new animal (48/S⁺), these two populations proved to be antigenically distinct. Further secondary or recrudescent peaks were isolated as shown in Fig. 2: Indo-C and Indo-D from monkey 47, which had been infected with Indo-A and challenged with Indo-C; Indo-E, and Indo-F, and Indo-G from monkeys 53 and 48, which had both been infected with Indo-B.

Table IV shows the reactivity of different sera taken from monkeys infected and challenged with populations A, B, C, D, and F against the seven different variant antigenic types (VAT) that have emerged from Indochina-1. These sera reacted neither with the Indochina/S⁻ line nor the S⁺ and the S⁻ lines of PLF-3. These results show that in all monkeys, the serum from convalescent animals (R) always reacted specifically with the VAT inoculated. Later challenges with the same VAT increased the antibody titer to this VAT, but cross-reactions started to appear once new VAT had emerged. In monkey 47, for example, the initial inoculum was Indo-A and the convalescent serum was specifically directed against Indo-A, although at this time Indo-C had already emerged; a later serum (after challenge with Indo-C) recognized both A and C as well as B (although no infection with B had been detected). Further challenges with Indo-C increased the titer to C, but not to A or B. None of the sera taken from monkey 47 recognized VAT D, E, or F. Similarly, serum C-9 of monkey 32, which had been infected and challenged with A, B, C, and F, recognized all these VAT with different antibody titers, but did not recognize Indo-D or E (to which the animal has not been exposed). In contrast, the convalescent serum (R) and the serum taken after the fourth challenge with Indo-A (C-4) were specific to VAT A.

TABLE IV
Antigenic Variation in the Indochina-1 Strain

| Parasite population | 32/S ⁺ | | | 47/S ⁺ | | | 48/S ⁺ | | 56/S ⁺ | | 54/S ⁺ | | 53/S ⁺ | |
|---------------------|-------------------|-------|-------|-------------------|------|-------|-------------------|-------|-------------------|------|-------------------|------|-------------------|------|
| | R | C-4 | C-9 | R | C-1 | C-3 | R | C-3 | R | C-3 | R | C-2 | R | C-2 |
| Indo-A | | | | | | | | | | | | | | |
| Saimiri 32 | 1:5 | 1:125 | 1:125 | 1:5 | 1:25 | 1:25 | 0 | 0 | 0 | 0 | 1:5 | 1:25 | 0 | 0 |
| Saimiri 54 | 1:5 | 1:125 | 1:125 | 1:5 | 1:25 | 1:25 | 0 | 0 | 0 | 0 | 1:5 | 1:25 | 0 | 0 |
| Indo-B | | | | | | | | | | | | | | |
| Saimiri 48 | 0 | 0 | 1:25 | 0 | 1:25 | 1:25 | 1:5 | 1:125 | 0 | 0 | 0 | 0 | 1:5 | 1:25 |
| Saimiri 53 | 0 | 0 | 1:25 | 0 | 1:25 | 1:25 | 1:5 | 1:125 | ND* | ND | ND | ND | 1:5 | 1:25 |
| Indo-C | | | | | | | | | | | | | | |
| Saimiri 47 | 0 | 0 | 1:25 | 0 | 1:25 | 1:125 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Indo-D | | | | | | | | | | | | | | |
| Saimiri 56 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1:5 | 1:25 | 0 | 0 | 0 | 0 |
| Indo-E | | | | | | | | | | | | | | |
| Saimiri 53 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Indo-F | | | | | | | | | | | | | | |
| Saimiri 48 | 0 | 0 | 1:5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Indo-G | | | | | | | | | | | | | | |
| Saimiri 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Populations Indo-A-G were compared using various immune sera from animals infected and challenged with population A (32/S⁺ and 54/S⁺), population B (48/S⁺ and 53/S⁺), or population D (56/S⁺). The animal 47/S⁺ had been infected with population A, had developed a recrudescence with population C, and was further challenged with C. Sera R, C-1, C-2, C-3, C-4, and C-9 refer respectively to sera taken after recovery or after one to nine challenge infections. Serum C-4 of monkey 32/S⁺ was taken after four challenge infections with population A, while serum C-9 was taken after further challenge with B, C, and F (see Fig. 1 for details of immunization schedule). Only four dilutions (1:5, 1:25, 1:125, and 1:250) were tested for all sera.

* Not determined.

These results indicate that the molecules exposed at the surface of erythrocytes infected with *P. falciparum* are capable of antigenic variation under immune pressure.

Antigenic Variation of the Palo Alto Strain. With this strain, spontaneous recrudescence peaks were not observed and secondary peaks after homologous challenge were rare. Antigenic variation was observed after passive transfer of immune serum.

An intact monkey infected with PLF-3/S⁺ was treated with 1.5 ml of anti-S⁺ serum (31/S⁺/C-4) when parasitemia had reached 0.4%; parasitemia was totally suppressed 48 h later and remained negative for 9 d; a recrudescence peak (0.35% parasitemia) was observed 15 d after passive transfer and this population proved to be of the S⁻ phenotype, i.e., positive by surface immunofluorescence with anti-S⁻ serum. In this intact monkey, PLF-3 had switched from the S⁺ to the S⁻ specificity after passive transfer of anti-S⁺ serum. In this intact monkey, PLF-3 had switched from the S⁺ to the S⁻ specificity after passive transfer of anti-S⁺ serum.

A splenectomized monkey infected with clone B-11/S⁻ was treated with 1.5 ml of anti-S⁺/S⁻ serum (27/S⁻/C-6) when parasitemia reached 7.2%; 24 h later, the parasitemia had dropped to 1.7%, but progressively increased thereafter. A second peak (14.2% parasitemia) was observed 10 d after passive transfer and this population was not recognized by any of the anti-S⁻ or anti-S⁺ sera tested, and was consequently considered to be a new VAT of clone B-11/S⁻. This experiment shows that antigenic variation can occur in a cloned population.

Discussion

The data presented in this paper demonstrate the antigenic complexity of the surface determinants exposed on *P. falciparum*-infected erythrocytes. The evidence for the existence of major alterations of the surface of erythrocytes infected with malaria parasites stems from various experimental approaches, such as the observation of morphological changes (16), the biochemical detection of new determinants (17-18), the study of the cell surface either with lectins (19) or with antibodies (10, 14, 20-21), and the binding of infected cells to endothelial cells (22). All these studies have one common feature, that surface alterations occur at the trophozoite/schizont stage (i.e., the second half of the 48-h developmental cycle), while no alterations are detectable on earlier forms. In the present study we have confirmed and expanded earlier observations showing that new antigenic determinants could be detected on the surface of erythrocytes infected with such late developmental stages of *P. falciparum* by using surface immunofluorescence with immune squirrel monkey serum. Surface immunofluorescence allows a direct examination of the surface of individual infected cells, which means that blood containing a low percentage of late stages (as low as 0.05%) can be tested without prior concentration and that a heterogeneous sample, containing more than one serotype, can be identified by the relative percentage of fluorescent and nonfluorescent pigment-containing cells. It is of interest to recall, in the context of antigenic diversity of surface antigens, that extensive serotyping of variant antigens in African trypanosomes only became possible when a surface IFAT was introduced to replace the neutralizing and agglutination tests used in earlier studies (23). We have demonstrated that different parasite populations could be effectively serotyped with this method. For instance, three geographical strains of *P. falciparum*, one from Uganda, one from The Gambia, and the other from Vietnam/Kampuchea, proved to have distinct serotypes. The cross-reaction between FCR-3

and PLF-3, which appears after homologous challenge, could suggest that, besides a strain-specific antigen, the parasite can express cross-reactive antigens that are less immunogenic than the strain-specific ones. This, however, is in contradiction to the fact that different antigenic types of the same strain (whether S⁺ and S⁻, or A and B) do not appear to express such a cross-reactive antigen.

Although strain diversity of malarial antigens had been suggested by earlier studies (1-5), the existence of an antigenic diversity of surface determinants had not before been unequivocally demonstrated in *P. falciparum*. The biochemical nature of this kind of surface alteration on infected erythrocytes is not yet known and its origin (i.e., parasite-made or modified host component) remains a subject for speculation. The fact that surface immunofluorescence becomes negative after protease treatment suggests that the molecule recognized by immune serum might be a protein.

Acquired immunity to *P. falciparum* has a distinct strain-specific component. For instance, studies in neurosyphilitic patients infected with *P. falciparum* for therapeutic purposes have shown that these patients could be reinfected more than once with either homologous or heterologous strains, but that a fourfold-higher parasitemia was observed in heterologous infections (8). Comparable results have been obtained in splenectomized chimpanzees (24) and in owl monkeys (9). If the strain-specific antigenic determinants present on the surface of infected erythrocytes are a target for immune mechanisms, it is essential to determine the extent of their diversity. In this study, we have shown that these determinants were not only strain specific, but capable of antigenic variation within a given strain. Antigenic variation is a process by which an infectious organism gains a selective advantage by changing its antigenic profile, thus avoiding elimination by the host's immune system. In malaria, variation has been demonstrated in antigens exposed on the surface of *P. knowlesi* schizont-infected erythrocytes using either the schizont-infected cell agglutination (SICA) test (21) or surface immunofluorescence (14), but had not been unambiguously demonstrated in other malaria parasites.

We have shown that antigenic variation of the determinants exposed on the surface of *P. falciparum*-infected erythrocytes could be induced by a variety of environmental pressures. First, antigenic variation can be modulated by the presence or the absence of the spleen. With two of the strains studied, we found a difference in the surface antigens of parasitized erythrocytes in S⁺ or S⁻ line squirrel monkeys. The transfer of a cloned population from an S⁻ into an S⁺ monkey resulted in a progressive switch from one type of antigen to the other and this switch was reversible. The role of the spleen in the modulation of antigenic determinants on *P. falciparum*-infected erythrocytes resembles in some ways what has been described when *P. knowlesi* is transferred from intact into splenectomized rhesus monkeys (25), where a SICA⁺ organism becomes SICA⁻. In contrast to *P. knowlesi*, both the S⁺ and the S⁻ lines of *P. falciparum* express surface antigens (which are serologically different), while only the SICA⁺ populations of *P. knowlesi* seem to express such antigens.

Second, with the Indochina-1 strain, the infection of immune monkeys with the homologous VAT produced a secondary peak of different variant specificity and, on one occasion, a new variant was isolated from a spontaneous recurrence peak. Six new antigenic determinants were thus isolated so far. The fact that variation has been observed in an uncloned population could mean that the variant populations have emerged from minor subpopulations present in the initial inoculum and selected by

immune pressure. The serial emergence of at least three generations of different variant populations and the absence of regression to the initial population after transfer to a naive animal suggests that there is a large repertoire of VAT available, a feature that favors other mechanisms, such as mutation/selection or adaptive phenotypic change.

Third, with the Palo Alto strain, where spontaneous recurrent secondary peaks do not usually occur, the antigenic switch could be induced by passive transfer of immune serum. The fact that PLF-3 of phenotype S⁺ can switch to an S⁻ phenotype in an S⁺ animal after exposure to anti-S⁺ immune serum demonstrates that the presence of antibodies alone can represent a selective pressure similar to the presence or absence of the spleen in the modulation of antigenic expression. This confirms that spleen-modulation is essentially a mode of antigenic variation. The difference in cytoadherence properties observed between S⁺ and S⁻,² might be the stimulus rather than the consequence of spleen-modulated variation. The antigenic variation of clone B-11/S⁻ in an S⁻ monkey, after passive transfer of immune serum, shows that Palo Alto has a wider variation potential than the S⁺/S⁻ switch and that variation can occur in a cloned population. It is not clear why there should be a difference in the mechanisms of antigenic variation between Palo Alto and Indochina-1, but it is possible that the multiple drug resistance of the latter strain might give it a more plastic genetic make-up.

The existence of antigenic variation raises the issue of how to define strains of *P. falciparum*. The existing methods are inadequate and will need to be reconsidered, taking into account a number of taxonomic parameters, such as geographical origin, drug resistance, isoenzyme type, and serotype.

The antibody response to different populations presents some informative features. The fact that convalescent sera are highly specific to the inoculum could have important practical applications. For instance, if we suppose that many different serotypes exist in a given geographical area, sera taken from patients after their first infection with malaria should have a special affinity for that particular strain. In certain instances, we have already been able to confirm this notion in clinical malaria, where the serum taken from a patient after recovery specifically recognized the strain isolated from this patient a few weeks earlier (M. Hommel, unpublished material). The fact that a monkey exposed to a variety of different VAT still does not recognize all VAT of that particular strain suggests that the notion of a "chronic serum" that would recognize all existing VAT, such as has been described in the case of *P. knowlesi* (26), might not apply to *P. falciparum* infections in squirrel monkeys. It is conceivable, however, that the situation might be different in a more susceptible host (e.g., man or *Aotus*), where the parasite could move quickly from one VAT to another and where the host would thus rapidly become immune to a wide range of VAT. This would explain why antigenic variation had never been recognized before in *P. falciparum*.

The presence of strain-specific antigenic determinants on the surface of *P. falciparum*-infected erythrocytes that induce the production of specific antibodies in immune individuals is a feature that will have to be examined in epidemiological studies in man, particularly in view of the potential role of these antigens in acquired immunity.

² David, P. H., M. Hommel, L. H. Miller, I. Udeinya, and L. D. Oligino. Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of infected erythrocyte cytoadherence. Manuscript submitted for publication.

Antigenic variation and/or strain-specific diversity might explain the long time-span that individuals living in hyperendemic malarious areas require to become immune (27). For instance, successive waves of parasitemia could be explained either by superimposed infection with a different strain or by the recurrence of a previous infection after antigenic variation. The observation that the reinfection of an immune animal with the homologous strain can produce a parasitemia with an antigenically different population is of particular importance in this context.

Summary

The surface of erythrocytes infected with late developmental stages of *Plasmodium falciparum* is profoundly altered and new antigenic determinants can be detected by surface immunofluorescence using immune squirrel monkey serum. The expression of these parasite-specific antigenic determinants on the surface of the host erythrocyte can be modulated by the presence or absence of the spleen and by immune pressure.

An antigenic switch occurred when a cloned population of the Ugandan Palo Alto strain of *P. falciparum* was transferred from a splenectomized into an intact monkey and this switch was reversible. In another strain (Indochina-1), we showed that the parasites isolated during secondary and recrudescence peaks expressed erythrocyte-associated surface antigens different from the parasites isolated during the primary infection; six variant antigenic types distinct from the original population were isolated in this way.

The passive transfer of immune serum can induce antigenic variation and this can occur in a cloned parasite.

The various mechanisms of antigenic variation in *P. falciparum* are discussed in the context of strain-specific diversity and the role of antigenic diversity in acquired immunity.

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