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Antigenic variation in *Plasmodium falciparum*

(malaria/*Plasmodium falciparum* erythrocyte membrane protein 1/cytoadherence)

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ABSTRACT **Antigenic variation of infectious organisms is a major factor in evasion of the host immune response. However, there has been no definitive demonstration of this phenomenon in the malaria parasite *Plasmodium falciparum*. In this study, cloned parasites were examined serologically and biochemically for the expression of erythrocyte surface antigens. A cloned line of *P. falciparum* gave rise to progeny that expressed antigenically distinct forms of an erythrocyte surface antigen but were otherwise identical. This demonstrates that antigenic differences on the surface of *P. falciparum*-infected erythrocytes can arise by antigenic variation of clonal parasite populations. The antigenic differences were shown to result from antigenic variation of the parasite-encoded protein, the *P. falciparum* erythrocyte membrane protein 1.**

Infectious organisms can evade the host immune response by expressing different antigenic forms of surface molecules. An important step in investigating these antigenic differences is to determine whether cloned organisms can change the antigenic phenotype of a specific surface molecule. This indicates that antigenic differences are due to "antigenic variation" rather than to the "antigenic diversity" created by multiple different stable alleles within a mixed population of organisms. For example, in the primate malaria, *Plasmodium knowlesi*, and the African trypanosome, *Trypanosoma brucei*, clonal parasite populations can undergo antigenic variation in surface molecules (1–5). In contrast, antigenic diversity in the S antigen, a non-surface antigen of *Plasmodium falciparum*, reflects a large number of different stable alleles (6), and the S antigen phenotype of cloned parasites is stable (R. Anders, personal communication).

Erythrocytes infected with the trophozoite and schizont stages of *P. falciparum* have parasite-determined antigens on their surface that are potential targets of host immune responses. One such erythrocyte surface antigen is a large, radioiodinatable protein known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (7–9). PfEMP1 is likely to be the same molecule as the protein "sequestrin," recently described by Ockenhouse and others (10). PfEMP1 appears to be the target antigen for antibodies that agglutinate infected erythrocytes and inhibit cytoadherence between infected erythrocytes and venular endothelium. These antibodies may have an important role in naturally acquired immunity to *P. falciparum* malaria, although field studies suggest that there are a large number of different antigenic forms of PfEMP1 (11).

We report here that a cloned parental line of *P. falciparum*, maintained *in vitro*, gave rise to clones that differed in their agglutination by clone-specific antisera and had antigenically distinct forms of PfEMP1. The clones were derived from the same parental clone and were identical with respect to all other genetic markers. We therefore conclude that antigenic change in *P. falciparum* can be generated by the mechanism of antigenic variation.

MATERIALS AND METHODS

Parasites. ItG2F6, a clone of a clone of a Brazilian isolate (Ituxi) was obtained from L. H. Miller (National Institutes of Health). Clones FAF6 and GBC6 were generated by limiting dilution from two different stabilates of ItG2F6, stabilates ItG2F6-F and ItG2F6-G, respectively. Stabilates F and G had been cultured independently for 6 months. All cultures were maintained as described (12).

Agglutination and Cytoadherence Assays. Agglutination and cytoadherence experiments were performed as described (12–18). Cytoadherence inhibition assays were performed by incubating infected erythrocytes with rabbit antiserum at 37°C for 1 hr, diluting cells to 1–2% hematocrit with RPMI/Hepes (GIBCO; BDH), and then performing cytoadherence assays in the usual way. In agglutination assays, rabbit serum was used at a 1:10 dilution, and in cytoadherence inhibition assays, rabbit serum was used at a 1:2 dilution.

Rabbit Antisera. Agglutinating antisera to stabilate ItG2F6-G ("anti-G" serum) or clone FAF6, which was derived from stabilate ItG2F6-F ("anti-F" serum), were prepared as described (12). Briefly, rabbit antisera were prepared by immunizing animals with washed, enriched (19), infected trophozoites and schizonts in Freund's complete adjuvant. Rabbits were given booster immunizations 4 and 8 weeks later with cells in Freund's incomplete adjuvant. Serum taken prior to immunization ("preimmune") was used as a negative control in agglutination and cytoadherence inhibition experiments and all sera were preabsorbed against uninfected group O erythrocytes by incubation with an equal volume of uninfected erythrocytes for 30 min at 37°C. Different aliquots of the same sera were used for each series of experiments. Antibodies to ItG2F6 and FC27 S antigens were prepared by immunizing rabbits with synthetic peptides in Freund's complete adjuvant (R. Anders, unpublished data).

Surface Labeling, Immunoprecipitations, and Immunoblotting. Infected erythrocytes were surface radioiodinated using the lactoperoxidase technique (7, 9) and incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington; 10 μg/ml in phosphate-buffered saline) or with trypsin plus an excess of soybean trypsin inhibitor. Immunoprecipitations and immunoblots were performed as described (20, 21).

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Abbreviations: PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; MESA, mature-parasite-infected erythrocyte surface antigen.

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Southern Blot Analysis. Restriction enzyme digestion with *Acc* I and Southern analysis were performed as described (22, 23). Blots were hybridized with the mature-parasite-infected erythrocyte surface antigen (MESA; refs. 22, 24) and the isolate-specific marker 7H8/6 (23).

RESULTS

Clone-Specific Antisera Distinguish Two *P. falciparum* Clones. To investigate the mechanism for generating antigenic differences in a *P. falciparum* erythrocyte surface antigen, we raised an agglutinating antiserum (12) by immunizing rabbits with erythrocytes infected with trophozoites and schizonts of a cloned, cytoadherent stabilate, ItG2F6-G. This antiserum agglutinated and blocked cytoadherence of erythrocytes infected with the ItG2F6-G stabilate but caused minimal agglutination or cytoadherence inhibition of erythrocytes infected with an earlier ItG2F6 stabilate, ItG2F6-F. The two stabilates were derived from the same parental ItG2F6 clone but had been cultured independently for 6 months. The differences between the two stabilates in their agglutination and cytoadherence inhibition by the anti-G antiserum suggested that a change had occurred in an erythrocyte surface antigen during culture. The antigenic differences between the stabilates may also have reflected differences in growth rates between parasites with altered erythrocyte surface antigen phenotypes. A difference in growth rate would presumably have been required for the new phenotype to become predominant.

To confirm the change in erythrocyte surface antigen phenotype, we subcloned the F and G stabilates (16), prepared an anti-F serum, and performed crossover agglutination and cytoadherence inhibition experiments. Knobbed, cytoadherent clones FAF6 and GBC6 were obtained from the F and G stabilates, respectively. Anti-G serum strongly agglutinated and inhibited cytoadherence of GBC6-infected erythrocytes but caused minimal agglutination and cytoadherence inhibition of FAF6-infected erythrocytes (Table 1). Conversely, rabbit antiserum raised against FAF6 trophozoite-infected erythrocytes (anti-F) agglutinated and partially blocked cytoadherence of FAF6- but not GBC6-infected erythrocytes (Table 1). Thus, FAF6 and GBC6 had different erythrocyte surface antigens, as demonstrated by their agglutination and cytoadherence inhibition by homologous but not by heterologous antisera.

PfEMP1 Is the Target of Clone-Specific Agglutinating Antisera. To determine whether PfEMP1 differed antigenically between GBC6 and FAF6, we performed an immunoprecipitation analysis with the two parasite clones and the clone-specific antisera. PfEMP1 is identified biochemically by its accessibility to lactoperoxidase-catalyzed surface radioiodination, insolubility with Triton X-100, and cleavage when infected erythrocytes are incubated with low concentrations of trypsin (1–10 μg/ml) (9). We surface radioiodinated FAF6- and GBC6-infected erythrocytes and identified PfEMP1 with each clone. The PfEMP1 of FAF6 was specifically immunoprecipitated by anti-F serum but not by preimmune serum or by anti-G serum (Fig. 1). Conversely, the PfEMP1 of GBC6 was specifically immunoprecipitated by anti-G serum but not by preimmune serum or by anti-F serum (Fig. 1). Taken together, the immunoprecipitation results indicate that the PfEMP1 proteins of the two clones differ antigenically. The correlation of immunoprecipitation of PfEMP1 by clone-specific antisera that cause agglutination and inhibit cytoadherence supports the conclusion that PfEMP1 is the target antigen of agglutinating and cytoadherence-inhibiting antibodies.

In addition to the difference in antigenicity between the PfEMP1 proteins of FAF6 and GBC6, we also observed a difference in their electrophoretic mobility on SDS/polyacrylamide gels. The PfEMP1 of GBC6 (estimated M_r 250,000) migrated more slowly than the PfEMP1 of FAF6 (estimated M_r 240,000) when the proteins were electrophoresed in the same gel. Identical results were obtained in two independent experiments. Thus the antigenic differences between the proteins were accompanied by differences in their apparent molecular weights.

Both Antigenically Variant Clones Are Cytoadherent. The cytoadherence of these two clones to different host cells and to the putative cytoadherence receptors, thrombospondin and the leukocyte differentiation antigen, CD36, was also examined (Table 1). FAF6 and GBC6 bound strongly to C32 melanoma cells (1829 and 944 infected erythrocytes per 100 melanoma cells, respectively). However, FAF6 consistently showed stronger binding to human umbilical vein endothelial cells (249, 38, and 22 infected erythrocytes per 100 endothe-

Table 1. Agglutination and cytoadherence of ItG2F6 clones

Agglutination or cytoadherence	Clone FAF6*	Clone GBC6*
Agglutination with rabbit antisera†		
Anti-G (1:10)‡	Negative	+++
Anti-F (1:10)‡	+++	Negative
Preimmune	Negative	Negative
Cytoadherence§		
To melanoma cells	1829 (863–2081)	944 (699–1651)
To human umbilical vein endothelial cells¶	103 (22–249)	7 (2–15)
To thrombospondin	2800 (2030–3570)	2212 (1965–2530)
To CD36	4715 (1755–8460)	8442 (5010–14,170)
Inhibition of cytoadherence to melanoma cells‖		
Anti-G (1:2)	1645 (10)	186 (80)
Anti-F (1:2)	1138 (38)	1086 (−15)

*Parasitemias were 5–10% trophozoites.
†Negative, no discernible difference when compared to preimmune rabbit serum; +++, strongly positive (five or six clumps of >50 infected erythrocytes per field; ×20 objective). Similar results were obtained in three different experiments.
‡The titration endpoint of the rabbit antisera for variant-specific agglutination of the homologous clone was between 1:50 and 1:200.
§Results of at least three different experiments are presented as mean (range). Results are expressed as number of infected erythrocytes bound per 100 target cells or mm^2.
¶The ItG2F6 clone, $B8C^+$, bound 0, 1, and 3 infected erythrocytes per 100 endothelial cells in three different experiments, this serving as a negative control.
‖Results of two different experiments performed in duplicate with cytoadherence inhibition shown as a percentage in parentheses.

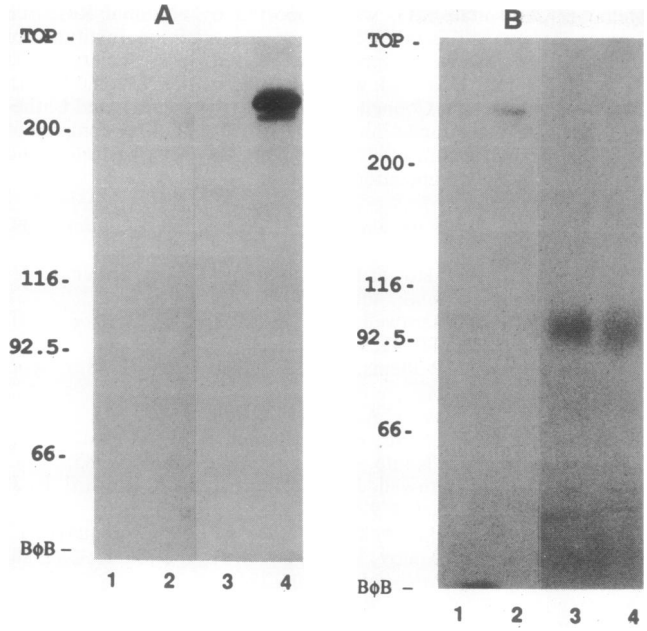

FIG. 1. Immunoprecipitation analysis of the surface radioiodinated proteins of ItG2F6 clones GBC6 and FAF6. (*A*) Immunoprecipitation with anti-F. (*B*) Immunoprecipitation with anti-G. Lanes 1 and 2, GBC6 parasites; lanes 3 and 4, FAF6 parasites. Molecular weight markers ($M_r \times 10^{-3}$) are shown on the left. BφB, bromophenol blue. Infected erythrocytes have been incubated with trypsin (lanes 1 and 3) or with trypsin plus inhibitor (lanes 2 and 4).

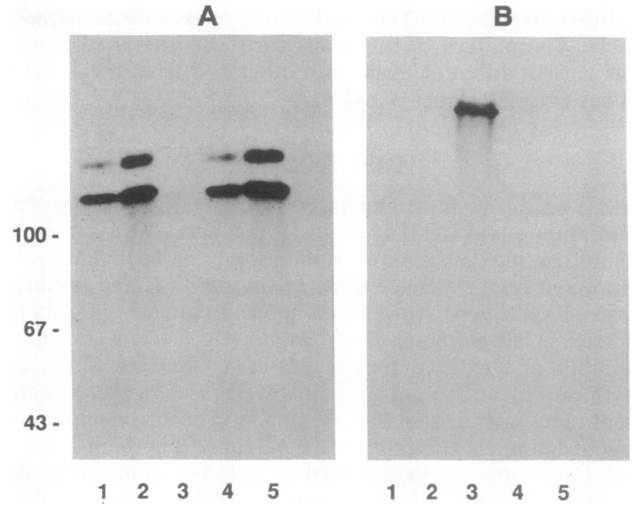

FIG. 2. Western blot analysis showing the S antigen of FAF6 and GBC6. (*A*) Immunoblot of culture supernatants probed with an ItG2F6 S-antigen-reactive rabbit antiserum. FAF6 and GBC6 are shown in lanes 1 and 4, and lanes 2 and 5, respectively. An unrelated isolate, FC27, is shown in lane 3 as a control. (*B*) The same filter probed with an antiserum raised to the FC27 S antigen. Molecular weight markers ($M_r \times 10^{-3}$) are shown on the left.

lial cells in three different experiments) than GBC6 (15, 4, and 2 infected erythrocytes per 100 endothelial cells in three different experiments). Another ItG2F6 clone, B8C$^+$, which served as a negative control in these experiments, bound 0, 1, and 3 infected erythrocytes per 100 endothelial cells in three different experiments. FAF6 and GBC6 bound to thrombospondin (2800 and 2212 infected erythrocytes per mm^2, respectively) and to CD36 (4715 and 8442 infected erythrocytes per mm^2, respectively).

Antigenically Variant Clones Are Otherwise Identical. That FAF6 and GBC6 clones were derived from the same parental clone (ItG2F6) was supported by chromosome analysis using pulsed-field gradient electrophoresis (data not shown). The variant clones and parental clone stabilates (ItG2F6-F and ItG2F6-G) had identical karyotypes except that, as previously reported, the parental clone stabilates contained a subpopulation of parasites with a chromosome 2 deletion, which included the knob-associated histidine-rich gene (16). A deletion of a larger chromosome was also present in both clones and parental clone stabilates, which to our knowledge has only been observed in ItG2F6 (J. Thompson, personal communication). To confirm that FAF6 and GBC6 were otherwise genetically identical, a number of loci were examined. FAF6 and GBC6 were identical with respect to the most polymorphic malaria protein known, the S antigen (Fig. 2). Immunoblotting with a rabbit antiserum to the ItG2F6 S antigen revealed a protein doublet in both ItG2F6 clones (Fig. 2*A*, lanes 1, 2, 4, and 5). This antiserum did not react with the S antigen of another *P. falciparum* isolate, FC27 (Fig. 2*A*, lane 3). In contrast, an antiserum specific for the FC27 S antigen reacted with a protein of M_r 200,000 in FC27 (Fig. 2*B*, lane 3) but did not react with either of the ItG2F6 clones (Fig. 2*B*, lanes 1, 2, 4, and 5).

FAF6 and GBC6 were also compared for restriction fragment polymorphisms using two extensively polymorphic probes, MESA and 7H8/6. All unrelated isolates so far examined by Southern analysis with MESA or 7H8/6 probes have been different (R. Coppel, personal communication; ref. 23). FAF6 and GBC6 showed identical *Acc* I restriction fragment length polymorphisms (Fig. 3). When probed with MESA, a 16.2-kilobase (kb) fragment was evident in FAF6 and GBC6 (Fig. 3*A*, lanes 1 and 2). In the unrelated clone, 3D7, a 14-kb fragment was present and in another unrelated clone, D10, a 17.5-kb fragment was evident (Fig. 3*A*, lanes 3 and 4). When probed with 7H8/6, 17 identical fragments were apparent in FAF6 and GBC6 (Fig. 3*B*, lanes 1 and 2). In

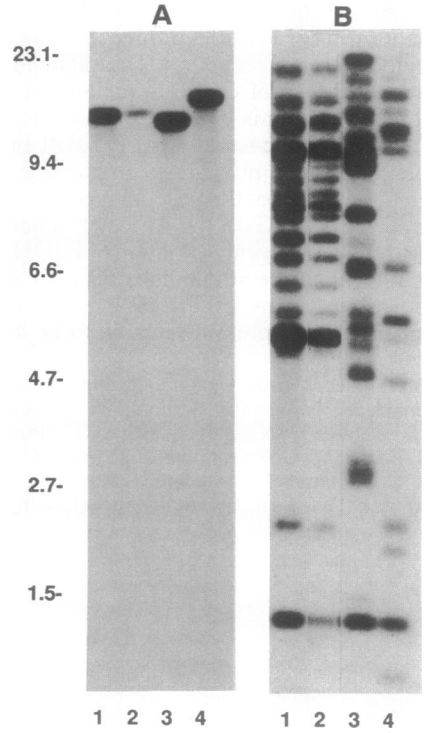

FIG. 3. Southern analysis of FAF6- and GBC6-restricted DNA. FAF6 and GBC6 genomic DNA digested with *Acc* I is shown in lanes 1 and 2, respectively. Two unrelated clones, 3D7 and D10, are shown in lanes 3 and 4, respectively. (*A*) Filter probed with MESA. (*B*) Filter probed with the isolate-specific marker 7H8/6. Size markers (kilobases) are indicated on the left.

contrast, 3D7 and D10 showed 19 and 15 fragments, respectively. The pattern of fragments for these unrelated clones was clearly different from each other and from the ItG2F6 clones (Fig. 3*B*, lanes 3 and 4).

DISCUSSION

In this study we have generated agglutinating antisera that differentiate two ItG2F6 clones (FAF6 and GBC6), thereby demonstrating antigenic variation of a parasite molecule expressed on the surface of *P. falciparum*-infected erythrocytes. Experiments with *P. falciparum* parasites in Saimiri monkeys, led Hommel *et al.* (25) to suggest that antigenic variation may explain the changes in *P. falciparum*-infected erythrocyte surface antigens observed *in vivo*. Our experiments demonstrate that this phenomenon occurs *in vitro*. Our conclusion relies critically on the evidence that the ItG2F6-F and -G variants are in fact derived from the same clone. As ItG2F6 was a clone of a clone and the F and G variants were studied in clones derived from this, the only alternative to a common origin is that one (or both) of the ItG2F6 stabilates was a contaminant. We have used the most sensitive tests available to exclude this possibility by demonstrating that FAF6 and GBC6 both secreted the ItG2F6 S antigen and that restriction enzyme digests of FAF6 and GBC6 genomic DNA, hybridized with probes for the highly polymorphic markers, MESA and 7H8/6, were identical.

The antigenically variant molecule expressed by the ItG2F6 clones was shown to be the parasite-encoded erythrocyte surface protein PfEMP1. The finding of differences in cytoadherence in clones with antigenically distinct forms of PfEMP1 is in keeping with previous findings (7–9, 20) that PfEMP1 is likely to be involved in cytoadherence. If PfEMP1 does mediate cytoadherence, it is likely that there are functional domains in the molecule that are conserved as well as domains that vary antigenically. The differences in cytoadherence observed in the variant clones would be explained if certain variant forms of PfEMP1 influence cytoadherence to particular cytoadherence receptors. The slight enhancement (15%) of cytoadherence of GBC6 to melanoma cells in the presence of anti-F serum is of uncertain significance.

The finding of antigenic variation of PfEMP1 suggests that this molecule may play a pivotal role in immune evasion by *P. falciparum*, similar to the role played by the variant surface glycoprotein (VSG) of the African trypanosomes. As is the case with VSG, antigenic variation of PfEMP1 occurred *in vitro* and in the absence of immune pressure. The number of possible variant forms of PfEMP1 and the extent of variation under immune pressure remain to be determined.

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