

## Antibodies and DNA Probes Used To Analyze Variant Populations of the Indochina-1 Strain of *Plasmodium falciparum*

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Ten variant populations derived from the Indochina-1 strain of *Plasmodium falciparum* were analyzed by using (i) hyperimmune serum raised against some of these populations in squirrel monkeys and (ii) an oligonucleotide probe based on the *rep-20* sequence, which had previously been shown to be a useful marker of diversity. Although all 10 subpopulations had an identical fingerprint pattern on Southern blots probed with the oligonucleotide, thus demonstrating a homogeneous genetic makeup, they all had a different phenotype for erythrocyte-associated antigens, thus confirming serological variant-specific differences. Antibodies to erythrocyte-associated antigens were measured with a new technique including immunogold and silver enhancement. The results of this study indicate that antigenic variation can occur without major genomic reorganization.

The presence of new antigens on the surface of malaria-infected erythrocytes is a well-established phenomenon (reviewed in references 14 and 15). In *Plasmodium falciparum*, these antigens are present only during the second half of the 48-h intraerythrocytic cycle, are trypsin sensitive, and are believed to be associated with the parasite molecule PfEMP1 (17). There is a considerable diversity of erythrocyte-associated antigens from one *P. falciparum* isolate to another (10), and the expression of these antigens can be modulated in a given parasite population either by immune pressure or transfer from intact to splenectomized animals (13). This modulation of antigen expression has been termed antigenic variation, by analogy with the phenomenon described in African trypanosomes (21) and in *Plasmodium knowlesi* (4). The genes encoding for the erythrocyte-associated antigens of *P. falciparum* have not been identified, and consequently it is not known whether antigenic variation is related to a change at genomic level (e.g., point mutation or deletion) or to a differential phenotypic expression.

In this study, we compared 10 variant populations derived from the Indochina-1 strain of *P. falciparum* by using a combination of two different markers (antibodies and DNA probes). Variant-specific antibodies raised in hyperimmune squirrel monkeys were used to demonstrate diversity of erythrocyte-associated antigens, and DNA fingerprinting was used to examine the overall genetic background of the 10 populations studied. The *rep-20* sequence, which had previously been shown to be of value in the identification of different *P. falciparum* isolates (16), was used for DNA fingerprinting after Southern blotting. The objective of the study was to establish whether the serological differences observed between variant populations were accompanied by detectable differences in gene organization.

### MATERIALS AND METHODS

**Parasites.** The squirrel monkey-adapted strain of *P. falciparum*, Indochina-1, was used in this study. Variant populations of the original strain (INDO-A) were obtained by

isolating recrudescence populations that emerged either spontaneously or after challenge infections in immune animals (13); these variant populations were serially transferred either in intact animals (INDO-B/S<sup>+</sup>, INDO-C/S<sup>+</sup>, INDO-D/S<sup>+</sup>, INDO-E/S<sup>+</sup>, INDO-F/S<sup>+</sup>, and INDO-G/S<sup>+</sup>) or in splenectomized animals (INDO-A/S<sup>-</sup>, INDO-D/S<sup>-</sup>, and INDO-G/S<sup>-</sup>). The family tree of the Indochina-1 populations is given in Fig. 1, which shows the pattern of serial transfer and the derivation of the different variant populations. An in vitro culture of the Indochina-1 strain, established at the Centers for Disease Control (Atlanta, Ga.) with the original patient material after transfer in an *Aotus* monkey and maintained in the laboratory with standard procedures, was also examined in this study.

At a suitable level of parasitemia (2% infected erythrocytes or above), parasites were obtained by venipuncture of anesthetized infected monkeys; blood was collected either on heparin or acid-citrate-dextrose. Infected erythrocytes were cryopreserved in liquid nitrogen and thawed when required by using the technique of Diggs et al. (7). After retrieval from cryopreservation, thawed parasites were maintained in short-term culture to allow them to grow from the ring stage to the trophozoite-schizont stage (13).

**Animals and sera.** Male and female wild-caught squirrel monkeys (*Saimiri sciureus*) of Bolivian or Guyanan origin were used as a source of both infected erythrocytes and hyperimmune serum. Splenectomy was performed as previously described (8), and splenectomized animals were infected within a week after the removal of the spleen.

Hyperimmune sera were obtained by infecting and repeatedly challenging the same animal by intravenous inoculation of a thawed cryopreserved stabilate of the appropriate parasite population. Some animals were repeatedly challenged with the same population, and others were challenged with more than one population.

**Immunogold-silver enhancement assay.** The assay used to demonstrate the presence of antibodies to erythrocyte-associated antigens is based on the same general principle as the surface immunofluorescence assay, originally described for *P. knowlesi* (12), with the difference that in the last step of the assay fluorescein isothiocyanate-labeled conjugate was

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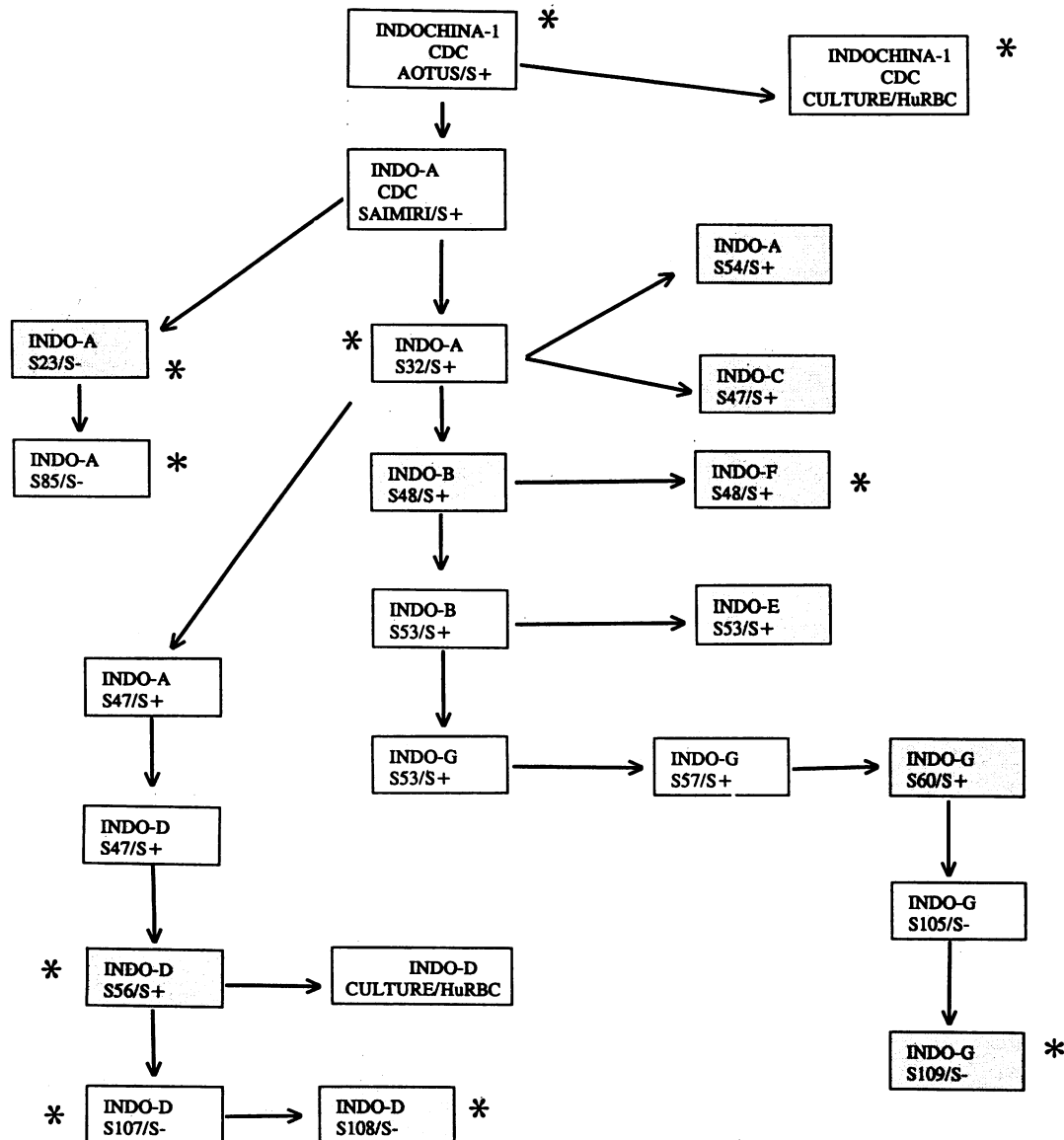


FIG. 1. Genealogic tree of the Indochina-1 strain of *P. falciparum* from its source (an infected *Aotus* monkey and in vitro culture) through its various transfers in intact (S<sup>+</sup>) and splenectomized (S<sup>-</sup>) squirrel monkeys. The shaded boxes indicate the parasite populations used for immunological studies, and the asterisks indicate the parasite populations used for DNA fingerprinting studies.

replaced by a series of steps including the use of protein A-immunogold (to bind the antibody), silver enhancement (to visualize the presence of immunogold), and counterstaining with Giemsa (to visualize the parasite); the last two steps are performed on methanol-fixed thin films. The steps of the assay are summarized on Fig. 2.

Washed, unfixed infected erythrocytes (10  $\mu$ l) taken at the appropriate stage of parasite development (late trophozoites to schizonts at a parasitaemia of 1 to 5%) were first incubated for 1 h at 37°C in 100  $\mu$ l of a 1:10 dilution of squirrel monkey serum in RPMI 1640 medium (RPMI; Flow Laboratories) in a plastic round-bottomed 5-ml hemolysis tube. After incubation, the cells were washed in two changes of 3.5 ml of RPMI and then incubated for 30 min in 100  $\mu$ l of a 1:100 dilution of rabbit anti-squirrel monkey immunoglobulin. After another wash in RPMI, the cells were incubated for 30 min in a 1:25 dilution of protein A-labeled colloidal gold (5-nm diameter,

Ultragold; Ultra Ltd.) and then washed again in RPMI. At this stage, a drop of the small pellet of infected erythrocytes was transferred onto a clean microscope slide, and a thin film was made; the film was fixed for 1 min in methanol. The fixed thin film was covered with a solution of silver enhancement solution (Intense II; Janssen Biotech NV) as previously described (5); after 10 to 15 min, the thin film was washed three times with double-distilled water and stained with a 10% Giemsa solution in pH 7.2 staining buffer (BDH).

**DNA preparation.** After retrieval from cryopreservation, parasites were grown in vitro for 24 to 36 h, washed in 0.9% (wt/vol) NaCl in 10 mM Tris-HCl (pH 8)–1 mM EDTA, and resuspended in the same buffer. Parasites were freed from host cells by the addition of saponin to a final concentration of 0.1% and left on ice for 10 min. They were recovered by low-speed centrifugation, and the DNA was isolated with proteinase K-phenol extraction by standard methodologies

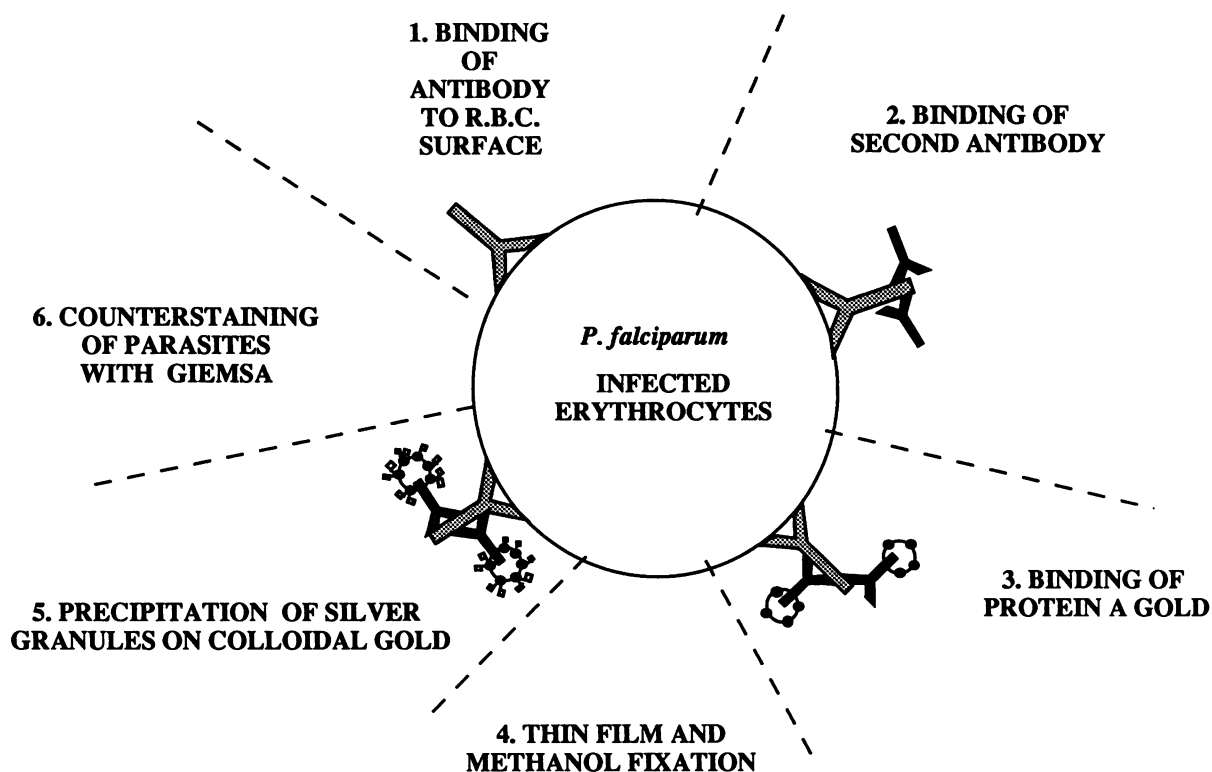


FIG. 2. Principle of the immunogold-silver enhancement technique, including the sequence of steps used in the assay.

(18). One cryopreserved vial, containing 125  $\mu$ l of infected cells at a parasitemia of 2% or above, was generally sufficient to prepare DNA for fingerprint analysis.

**Radiolabeled *rep-20* probe.** The *rep-20* oligonucleotide (Fig. 3) was radiolabeled with  $^{32}$ P at the 5' end by using T4 polynucleotide kinase. A 1- $\mu$ g sample of the oligomer was incubated in the presence of 50 mM Tris-Cl (pH 7.5)–10 mM MgCl<sub>2</sub>–5 mM dithiothreitol–30  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]dATP (specific activity, 3,000 Ci/mmol) with 10 U of T4 polynucleotide kinase at 37°C for 30 min. The labeled probe was separated from the free label by purification through Sephadex G50.

**Fingerprint analysis.** DNA from various populations was digested to completion with the restriction enzyme *Hae*III with the conditions recommended by the manufacturer (Boehringer Mannheim). Fragments were separated on a 1% agarose gel and Southern blotted onto nitrocellulose for fingerprint analysis with the radiolabeled *rep-20* probe.

The nitrocellulose filter was prehybridized for several hours in a solution consisting of 5 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$  Denhardt solution, 50  $\mu$ g yeast tRNA per ml, and 0.1% (wt/vol) sodium dodecyl sulfate at 37°C. After blocking was complete, the probe was added at a concentration no greater than 50 ng/ml, and the filter was incubated overnight at 37°C. The filter was washed

in 2 $\times$  SSC–0.1% sodium dodecyl sulfate at 42°C and exposed to preflashed X-ray film at –70°C.

The fingerprint analysis was performed after Southern blotting as previously described (16) but with the radiolabeled *rep-20* probe.

## RESULTS

**Immunogold-silver enhancement assay.** Figure 4 shows the typical aspect of immunogold-silver enhancement labeling with black silver grains covering the surface of infected cells. The Giemsa-stained parasites can be easily identified within the cell (which is a useful feature for reading the assay).

Table 1 shows that all 10 variant populations express a different erythrocyte-associated malaria antigen on the surface of infected squirrel monkey erythrocytes. Interpretation of results is clearest when the typing serum used has been produced by immunization with a single variant population (e.g., serum 85 A/S<sup>–</sup>, which recognizes only the erythrocyte-associated antigens of INDO-A/S<sup>–</sup>). The results are more complex when the squirrel monkey has been successively exposed to more than one population, as was the case with monkey 32. In this case, serum 32i (taken after the first infection and challenge with the INDO-A/S<sup>+</sup> population) recognizes only INDO-A/S<sup>+</sup>, whereas serum 32ii (taken after a series of heterologous challenges with INDO-B/S<sup>+</sup>, INDO-C/S<sup>+</sup>, and INDO-F/S<sup>+</sup>) recognizes all of the populations to which the animal had been exposed. It is of interest to note that serum 32ii does not recognize all the variant populations of Indochina-1, a result that is consistent with the fact that there is no cross-reaction between the different variant populations and that antibodies can be detected only in response to the immunizing challenge.

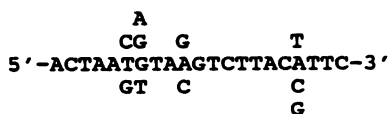


FIG. 3. Structure of the *rep-20* oligonucleotide.

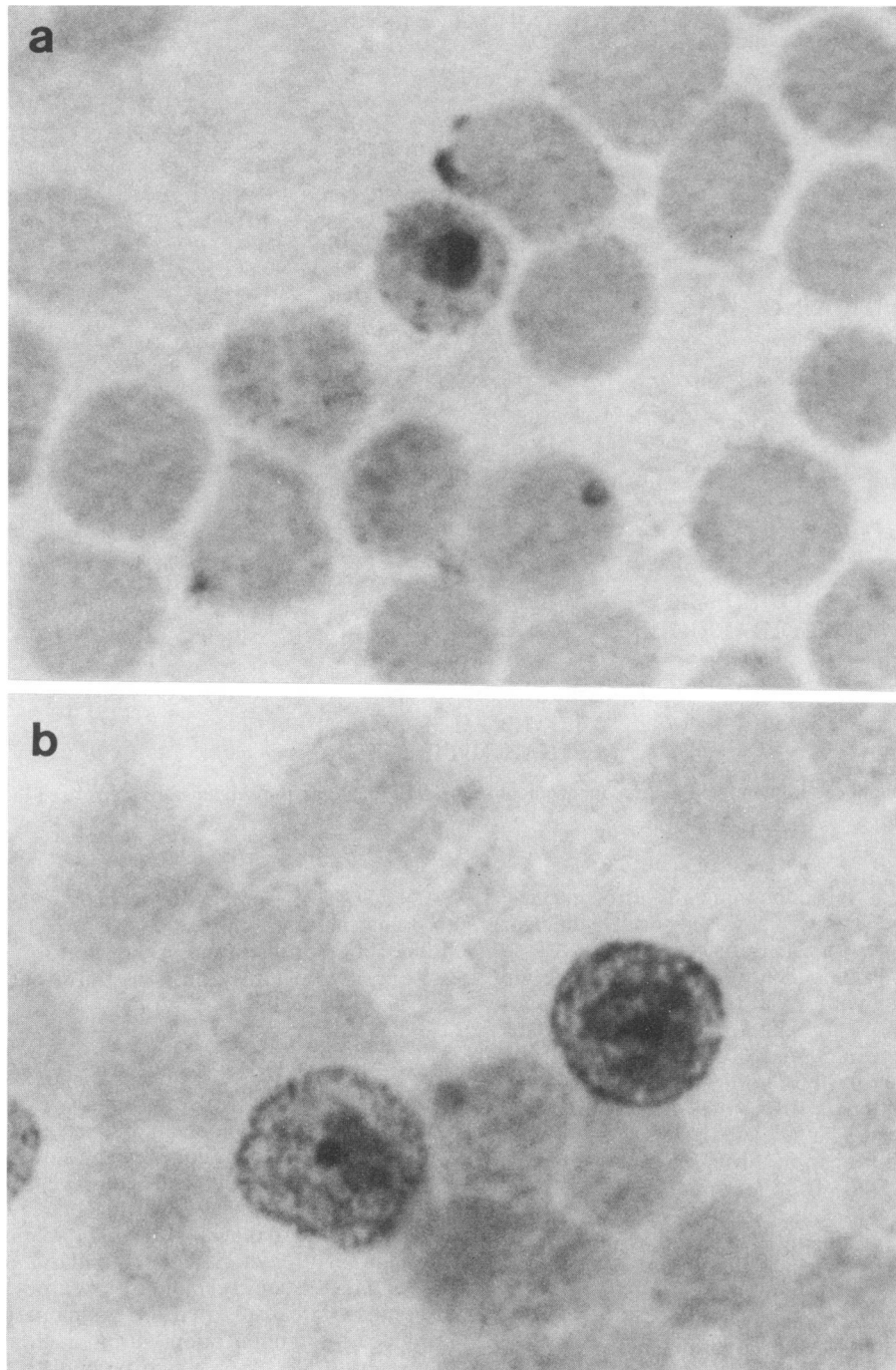


FIG. 4. Characteristic aspect of infected blood processed in the immunogold-silver enhancement technique, showing a negative sample (a), where only the Giemsa-stained parasite is visible, and a positive sample (b), where the surface of the infected erythrocyte is covered by numerous refringent silver grains. The parasite population used for these images was INDO-A/S<sup>+</sup> incubated with preimmune serum from a naive monkey (a) or with immune serum 32i (b) (100 $\times$  objective, oil immersion).

Although it is possible to quantify the assay either by estimating the number of silver grains per cell (which provides a measurement of the intensity of positive versus negative reactions and allows comparisons to be made between tests) or by counting the number of immunogold-positive infected erythrocytes versus the total number of infected erythrocytes (which provides a measurement of the

size of the parasite population that reacts with a particular antibody), neither method is practicable routinely or on a large scale. In most positive cases, only 60 to 85% of late-stage infected erythrocytes showed silver grains on their surfaces. This suggests either the presence of subpopulations that do not express erythrocyte-associated antigens, the presence of antigens not recognized by the test serum, or

TABLE 1. Results of the serotyping of erythrocyte-associated antigens with the immunogold-silver enhancement technique<sup>a</sup>

Typing serum	Serotyping results with the following parasites:									
	A/S <sup>+</sup> (S54)	A/S <sup>-</sup> (S23)	B/S <sup>+</sup> (S48)	C/S <sup>+</sup> (S47)	D/S <sup>+</sup> (S56)	D/S <sup>-</sup> (S108)	E/S <sup>+</sup> (S53)	F/S <sup>+</sup> (S48)	G/S <sup>+</sup> (S60)	G/S <sup>-</sup> (S109)
32i (A/S <sup>+</sup> )	+	-	-	-	-	-	-	-	-	-
32ii (A/S <sup>+</sup> , B/S <sup>+</sup> , C/S <sup>+</sup> , F/S <sup>+</sup> )	+	-	+	+	-	-	-	+	-	-
48 (B/S <sup>+</sup> )	-	-	+	-	-	-	-	-	-	-
56 (D/S <sup>+</sup> )	-	-	-	-	+	-	-	-	-	-
85 (A/S <sup>-</sup> )	-	+	-	-	-	-	-	-	-	-
108 (D/S <sup>-</sup> )	-	-	-	-	-	+	-	-	-	-
109 (G/S <sup>-</sup> )	-	-	-	-	-	-	-	-	-	+
47 (A/S <sup>+</sup> , B/S <sup>+</sup> , C/S <sup>+</sup> )	+	-	+	+	-	-	-	-	-	-
105 (G/S <sup>+</sup> , G/S <sup>-</sup> )	-	-	-	-	-	-	-	-	+	+
75 (control)	-	-	-	-	-	-	-	-	-	-

<sup>a</sup> The different parasite populations used are identified by shaded boxes in the family tree of the strain (Fig. 1); for each typing squirrel monkey serum used, the population of parasite used for infection and subsequent challenges is given within parentheses.

a problem inherent to the technique (i.e., the antibody, protein A-immunogold, or silver grains may be washed off). There is an obvious difference between immunogold-positive cells and immunogold-negative cells, with well over 200 silver grains per positive cell compared with fewer than 20 silver grains per negative cell (in most cases, fewer than 5). Uninfected erythrocytes or erythrocytes infected with ring-stage parasites do not exhibit any silver grains above the background level.

**Fingerprinting of parasite DNA with the *rep-20* probe.** The DNA extracted from different populations of the Indochina-1 strain (INDO-A/S<sup>+</sup>, INDO-A/S<sup>-</sup>, INDO-B/S<sup>+</sup>, INDO-C/S<sup>+</sup>, INDO-D/S<sup>+</sup>, INDO-D/S<sup>-</sup>, INDO-E/S<sup>+</sup>, INDO-F/S<sup>+</sup>, INDO-G/S<sup>+</sup>, and INDO-G/S<sup>-</sup>) was analyzed on Southern blots with the radioactive oligonucleotide probe. There was no detectable difference in the pattern observed with the different variant populations (whether S<sup>+</sup> or S<sup>-</sup>), except for INDO-D/107S<sup>-</sup> and INDO-D/108S<sup>-</sup>, for which an extra band is visible at 4 kb (Fig. 5). However, the Indochina-1 pattern was quite different from the previously described pattern of the Palo Alto/PLF3 strain of *P. falciparum* (16) (comparative data not shown). The pattern of the Indochina-1 strain established in culture from the original isolate from an *Aotus* monkey had a fingerprint pattern that was substantially different from those of all 10 in vivo-maintained populations of Indochina-1 and that of the PLF3/Palo Alto strain.

## DISCUSSION

The immunogold-silver enhancement technique for the detection of erythrocyte-associated surface antigens of *P. falciparum* is a substantial improvement over existing techniques, e.g., the surface immunofluorescence technique (12), the schizont-infected cell agglutination test (22), or the microagglutination test (19). With comparable sensitivities and specificities, both the surface immunofluorescence and the immunogold-silver enhancement techniques yield more information than agglutination techniques, because they allow the examination of the reactivity of individual infected cells rather than populations of infected cells. Although agglutination techniques are simpler to perform, they also require larger numbers of cells at a high rate of infection. The examination of cells on a stained thin film, as introduced in the immunogold-silver enhancement technique, has an advantage over the other techniques in that it allows a more careful examination of the results, a delayed examination

(not possible with live infected cells in suspension), and the possibility of keeping the slide as a record. The problem of evaluating negative tests, which is difficult in the surface immunofluorescence test because infected cells can only be recognized by the presence of refringent malaria pigment visible under a bright field (requiring the operator to switch from UV light to the bright field for each field examined), was solved in the immunogold-silver enhancement technique by the use of a Giemsa counterstain, which shows infected cells whether they are covered with silver grains or not.

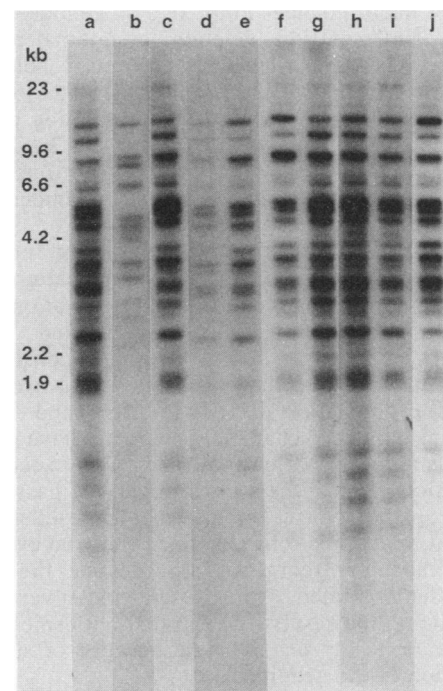


FIG. 5. DNA fingerprinting with the *rep-20* probe. Shown is a Southern blot of the DNA of the following populations treated with *Hae*III endonuclease (lanes): a, Indochina-1, *Aotus*/S<sup>+</sup>; b, Indochina-1, in vitro culture in human erythrocytes; c, INDO-A/S23/S<sup>-</sup>; d, INDO-A/S32/S<sup>+</sup>; e, INDO-F/S48/S<sup>+</sup>; f, INDO-D/S56/S<sup>+</sup>; g, INDO-A/S85/S<sup>-</sup>; h, INDO-D/S107/S<sup>-</sup>; i, INDO-D/S108/S<sup>-</sup>; j, INDO-G/S109/S<sup>-</sup>. Each of the isolates used for DNA fingerprinting was identified by an asterisk on the family tree of Indochina-1 (Fig. 1).

The study of erythrocyte-associated antigens of the different Indochina-1 populations with the immunogold-silver enhancement technique provides evidence of the extent of variation that can be generated in a given population of *P. falciparum*. Not only are all seven S<sup>+</sup> populations different (which confirms previous results obtained by surface immunofluorescence [13]), but the three S<sup>-</sup> populations were different both from the S<sup>+</sup> variants and from each other. This new finding is important because it is different from what happens in both the *P. knowlesi*- and the *Plasmodium fragile*-macaque models, in which the parasites found in splenectomized animals do not express any erythrocyte-associated surface antigen (1, 9). The present study shows that the S<sup>-</sup> phenotype in *P. falciparum* involves the expression of a specific antigen. The observation that all 10 phenotypes are entirely different, without any evidence of cross-reactivity, is of importance because of similar observations previously reported with different geographical *P. falciparum* isolates (11); the studies also show that animals infected in sequence with different populations can develop a cumulative response to all of these isolates, an observation which is of importance when interpreting the results from field studies where the patient exposure to different *P. falciparum* phenotypes is not documented.

Restriction enzyme DNA fingerprinting has previously been shown to be of use in differentiating isolates of *P. falciparum* with probes based on repetitive DNA sequences exhibiting restriction fragment length polymorphism, such as *rep-20* (2, 20). Because these *P. falciparum*-specific 21-bp repeat sequences occur in blocks near telomeres (they may represent up to 1% of the total genome), restriction enzyme DNA fingerprinting can be used with accuracy to provide evidence for major changes in the overall genome organization (e.g., point mutation, deletion, or internal rearrangement of the DNA by movement of transposable genetic elements or by exchange of chromosome ends [6]). It has already been established that the fingerprinting pattern is very different between different isolates (16, 20, 22) but is stable in a given cloned isolate (PLF3/Palo Alto) maintained in vitro or in vivo by serial transfer over a number of years (3).

The fact that all 10 variant populations of Indochina-1 exhibit an essentially identical fingerprinting pattern is interesting, particularly because the parent population had not been cloned before the variants were isolated. The only variation observed in the strains examined consisted of a single extra band of 4 kb that was observed in two samples of the same population (INDO-D/107S<sup>-</sup> and INDO-D/108S<sup>-</sup>) (Fig. 1). This change appears to have occurred spontaneously and is not correlated with any specific antigenic variation but may be a consequence of passaging the parasite in splenectomized monkeys. The results obtained indicate that the populations examined show no evidence of major chromosomal reorganization, despite the variable phenotype demonstrated by the immunogold-silver enhancement technique; the results suggest that antigenic variation in *P. falciparum* may occur in the absence of substantial reorganization of the genome.

The results described here may be examined in the context of earlier studies of passive transfer of immunity with serum from squirrel monkeys that were immune to three different variant populations of Indochina-1 (INDO-A/S<sup>+</sup>, INDO-B/S<sup>+</sup>, and INDO-D/S<sup>+</sup>) (11). Passive transfer results were suggestive of a correlation between acquired immunity and the presence of antibodies to the homologous erythrocyte-associated antigen phenotype. The new data on the charac-

terization of the different uncloned Indochina-1 populations (i.e., different phenotypes but an otherwise unchanged genotype) reinforce the conclusion of the passive transfer experiments; namely, that erythrocyte-associated antigens play a significant role in acquired immunity to malaria.

The difference observed between the in vitro culture material and the 10 variant populations obtained in vivo in the squirrel monkey may have a number of possible explanations, including the possibility that different populations may have been selected by establishing the original isolate in vitro or in the squirrel monkey, as well as a possible laboratory contamination (a frequent problem in the long-term maintenance of laboratory strains).

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