VACCINATION-INDUCED VARIATION IN THE 140 kD MEROZOITE SURFACE ANTIGEN OF *PLASMODIUM KNOWLESI* MALARIA

By FRANCIS W. KLOTZ,* DIANA E. HUDSON,* HAYDEN G. COON,‡ AND LOUIS H. MILLER*

*From *The Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases; and ‡The Laboratory of Genetics, National Cancer Institute, National Institute of Health, Bethesda, Maryland 20892*

The asexual stages of malaria parasites multiply within erythrocytes and are released as merozoites to invade other erythrocytes. One goal of vaccines against this stage is to induce antibodies against merozoite surface antigens to block erythrocyte invasion. Antibodies can inhibit erythrocyte invasion in vitro (1) and provide protection against lethal infection in vivo (2).

A 140 kD merozoite surface protein of *Plasmodium knowlesi*, a monkey malaria, was identified as a potential vaccine candidate for this model system (3). Antibodies against this protein and its 143 kD precursor inhibit erythrocyte invasion in vitro (4, 5). Immunity to the 143/140 kD protein conferred partial protection in vivo in two of four monkeys immunized with purified antigens (5). Only these two animals had circulating antibodies that inhibited erythrocyte invasion in vitro. Parasites isolated from a protected animal, however, were no longer inhibited by immune sera in vitro. The 143/140 kD antigens disappeared and antigens of different molecular mass appeared (5). Since the challenge inoculum was not cloned (6) and had been maintained in monkeys for many years after isolation from an infected individual, we did not know if variants existed in the inoculum or, alternatively, if the parasites were able to vary this antigen rapidly. To resolve this question, we inoculated a cloned parasite into two monkeys immunized with the 143/140 kD proteins. We describe the disappearance of parasites bearing 143/140 kD proteins within less than one month in both monkeys, and the appearance of parasites bearing new antigens of 72–160 kD or no detectable antigen immunologically crossreactive with the original immunogen.

Materials and Methods

Parasites. The Malaysian H strain of *P. knowlesi* was used in this study (6). Two clones, Pk1(A^+) and Pk1(B^+)1^+, were derived from this parasite strain by micromanipulation (7). Pk1(B^+)1^+ was a clone derived from a variant population of the clone Pk1(A^+) (7).

To study the effect of immunity to the 143/140 kD antigens on *P. knowlesi*, we inoculated a *P. knowlesi* clone, Pk1(A^+), into two rhesus monkeys that had been immunized with the 143/140 kD antigens. They had previously been infected with uncloned *P. knowlesi* parasites (5) and cured of their infections before challenge.

Parasitized erythrocytes were maintained as cryopreserved stocks stored at −80°C until

use. For biochemical studies, asexual erythrocytic stage parasites were maintained in splenectomized monkeys. Monkeys were inoculated with 5×10^5 to 2×10^9 ring-stage parasites intravenously. Monkeys with life-threatening parasitemias (>10%) were treated with chloroquine hydrochloride (Aralen; Sterling Drug, Inc., New York).

Chronic *P. knowlesi* infection was induced in one monkey by administration of sufficient chloroquine (20 to 40 mg in a single dose) to control the infection without totally eliminating the parasites. After several such treatments a chronic infection ensued, characterized by periodic recrudescent parasitemias.

Metabolic Labeling of Schizont Antigens. Schizont-infected blood was collected at parasitemias between 1–10%. For metabolic labeling studies, blood was collected when the modal population among circulating schizonts had four to six nuclei. Parasite culture conditions and metabolic labeling were described previously (4, 5).

Labeled parasites were washed in serum-free RPMI 1640 and extracted in 1% Triton X-100, 10 mM Hepes, 50 μg/ml chymostatin, and 50 μg/ml leupeptin. All detergent extractions were conducted at 4°C. Triton-soluble proteins were stored at −70°C until use (5). Immunoprecipitations, SDS-polyacrylamide gels, and fluorography were performed as previously described (4, 5, 8). Molecular weight standards were obtained from Bio Rad Laboratories or Bethesda Research Laboratories, Gaithersburg, MD.

Invasion Inhibition Assay. Sera were tested for their effects on erythrocyte invasion as previously described (4, 5).

Antisera and Monoclonal Antibodies. The monoclonal antibodies and rhesus or rabbit antisera against the 143/140 kD proteins used in the present study were previously described (4, 5).

Results

Stability of the 143/140 kD Protein During Chronic P. knowlesi Infections. *P. knowlesi* parasitemia usually increases 10-fold each day until death in nonimmune rhesus monkeys. If the infection is repeatedly suppressed with antimalarial drugs, the animal becomes partially immune so that the untreated infection no longer reaches life-threatening parasitemias. After immunization with the 143/140 kD antigens, David et al. (5) found that an infected monkey developed a chronic infection. Parasites obtained 45 d after infection expressed new antigens immunologically crossreactive with the 143/140 kD antigens. In the present study, we determined whether the 143/140 kD antigens vary during chronic infection in the absence of specific immunity to the 143/140 kD proteins.

After inoculation of a nonimmune monkey with PK1(A^+), parasitemias reached 4.4, 14, and 3.3% during initial waves of parasitemia. These parasitemias were suppressed with subcurative doses of chloroquine. A chronic infection then ensued characterized by periodic recrudescences (Fig. 1). We compared the 143/140 kD proteins of the inoculum with a recrudescent isolate taken several months after infection.

Rabbit antiserum against the 143/140 kD proteins immunoprecipitated a 143/140 kD doublet from Pk1(A^+) and breakdown products of 88/86 and 60 kD (Fig. 2, lane *1*). These smaller fragments are occasionally observed after detergent extraction and immunoprecipitation. They may result from proteolysis during handling. The identical antigens were observed in parasites recovered from a chronic infection (Fig. 2, lane *2*). The 220 kD antigen (Fig. 2, lane *2*) was nonspecific, since it was also immunoprecipitated with nonimmune rabbit serum (data not shown). Previous studies had shown that processing of the 143 kD precursor to the 140 kD product molecule occurs late in schizogony (5). The intensity difference between the 143/140 kD antigens (Fig. 2, lanes *1* and *2*)

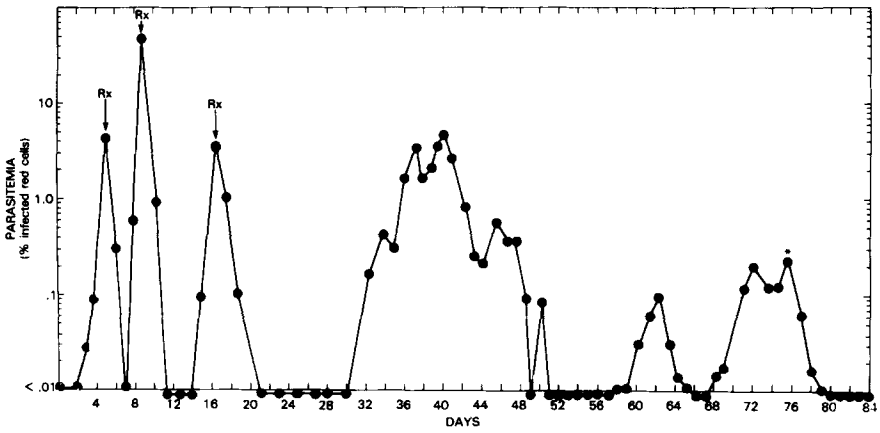

FIGURE 1. The course of parasitemia in a monkey inoculated with clone Pk1(A$^+$) of *P. knowlesi*. Chloroquine (*Rx*) was administered to suppress parasitemia. An isolate (asterisk) was collected on day 76 of infection.

reflects the degree of maturation of schizonts to merozoites during metabolic labeling (5). Thus, the 143/140 kD proteins were identical in the isolate recovered from the chronic infection and the original inoculum.

In a previous study with the same cloned parasites of *P. knowlesi*, we characterized a variant antigen unrelated to the 143/140 kD proteins. This antigen, called schizont-infected cell agglutination antigen (SICA),[1] is expressed on the surface of infected erythrocytes. Antibody against one SICA type induced the parasite to express other SICA types (7). The 143/140 kD antigens were unchanged in parasites that had varied the erythrocyte surface antigen (data not shown).

We conclude that the 143/140 kD *P. knowlesi* proteins remain unchanged in chronic infections and during antigenic variation in another protein, SICA.

Effects of Immunity to the 143/140 kD Proteins on Expression of these Proteins in Surviving Organisms. Two monkeys (1 and 4) that had been immunized with the 143/140 kD proteins, and a control monkey were inoculated with cloned parasites, Pk1(A$^+$). The control monkey developed a typical fulminant infection and was cured of malaria (Fig. 3). The immunized monkeys controlled the primary parasitemia. After 3 wk, monkey 4 developed a fulminant infection and was cured. The other monkey (monkey 1) developed a chronic infection with multiple recrudescences of parasitemia occurring over several months, and was then drug cured.

Antibodies against the 143/140 kD proteins inhibit erythrocyte invasion in vitro by merozoites of the parental clone, Pk1(A$^+$). The antibodies had no effect on invasion by parasites recovered on day 30 in monkey 4 or by parasites recovered on day 87 during the chronic infection in monkey 1 (Table I). Normal invasion despite antibodies against the 143/140 kD proteins correlated with change in the 143/140 kD proteins in these parasites.

Parasite isolates were obtained during several peaks of parasitemia in the two monkeys (two for monkey 4 and five for monkey 1). Metabolically-labelled parasite antigens obtained from each isolate were immunoprecipitated with rabbit

[1] *Abbreviation used in this paper:* SICA, schizont-infected cell agglutination antigen.

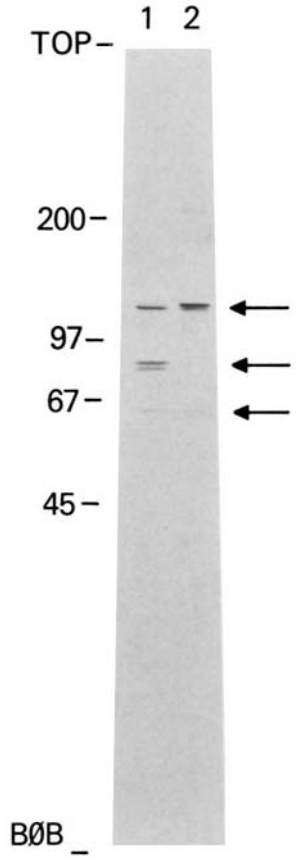

FIGURE 2. Fluorograph of SDS-PAGE (5–15% linear polyacrylamide gradient) of [^{35}S]-methionine-labeled antigens immunoprecipitated by rabbit antiserum against the 143/140 kD antigens. Clone Pk1(A$^+$) (lane *1*), the parasite inoculated into the monkey described in Fig. 1, was compared with the isolate obtained on day 76 (lane *2*). Arrows mark the 143/140 kD proteins and their cleavage products. *BϕB*, bromophenol blue dye front. Molecular mass shown in kD.

antiserum against the 143/140 kD proteins. Parasites recovered 8 d after challenge from monkey 1 retained the original 143/140 kD antigens. The 143/140 kD proteins had disappeared by day 30 (data not shown). New proteins were immunoprecipitated from parasites recovered from days 59, 87, and 102. Proteins immunoprecipitated from parasites recovered on day 87 (Fig. 4) are identical to those immunoprecipitated on days 59 and 102 (data not shown). The new antigens reactive with rabbit antiserum against the 143/140 kD proteins had molecular masses of 160/155, 115/113, and 87/85 kD (Fig. 4). In monkey 4, the 143/140 kD proteins are present on day 8 and had disappeared by day 30.

Clones of Parasites from Monkey 4. The isolate recovered on day 30 from monkey 4 during the first recrudescence (hereafter called V1) was cloned by micromanipulation and inoculated into five monkeys. Parasites from the five clones were recovered from each of the five monkeys. Antigens from each isolate

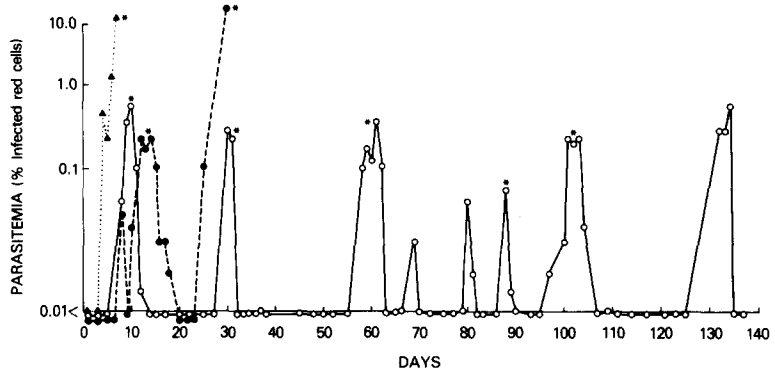

FIGURE 3. The course of parasitemia in monkeys inoculated with clone Pk1(A+). Control monkey (*triangles*) and two monkeys previously immunized with the 143/140 kD proteins (monkey 1, *open circles* and monkey 4, *filled circles*). An asterisk marks the time points at which a parasite isolate was collected.

TABLE I

Effect of Immune Sera on Erythrocyte Invasion by a Clone of P. knowlesi, Pk1(A+), and Two Uncloned Variants (V1 and V2) of this Clone

Monkey	Serum	Source of schizonts for invasion		
		Pk1(A+)	V1*	V2*
1	Preimmune‡	28.1§	10.3	32.6
	Immune	4.2	10.8	35.5
4	Preimmune	23.4	8.6	30.6
	Immune	4.6	9.7	31.0

* V1 was obtained from monkey 4 on day 30 of infection. V2 were obtained from monkey 1 on day 87 of infection.

‡ Preimmune sera refers to sera collected before immunization with purified antigens. Immune sera were collected after repeated immunizations (6).

§ Numbers are percentage of newly-invaded erythrocytes.

were metabolically labeled and immunoprecipitated with rabbit antiserum against the 143/140 kD proteins. Two clones (V1a and V1b) expressed proteins of 76 and 72 kD and three clones (V1c, V1d, V1e) expressed no protein crossreactive with rabbit antiserum against the 143/140 kD proteins (Fig. 5). Proteins of >300, 210, 100, and 45 kD are background bands, as they are seen in immunoprecipitates with preimmune sera (Fig. 5).

To determine whether the new 76/72 kD antigens of clones V1a and V1b retained all antigenic determinants identified in the parental molecules by monoclonal antibodies, immunoprecipitations were performed using a panel of monoclonal antibodies against the 143/140 kD proteins. The 76 kD protein was immunoprecipitated by two monoclonal antibodies (1005-B7 and 1019-C3) and not by three others (1001-E12, 1002-6E8, and 1038-6D3). Two other clones, V1c and V1d, which did not react with rabbit antiserum against the 143/140 kD proteins expressed no new antigens reactive with any of the monoclonal antibodies.

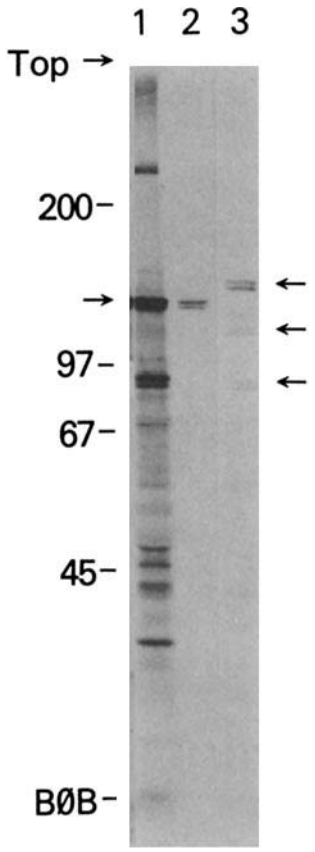

FIGURE 4. Fluorograph of SDS-PAGE (5–12.5% linear polyacrylamide gradient) of [^{35}S]-methionine-labeled antigens immunoprecipitated by rabbit antiserum against the 143/140 kD proteins. Lane *1*, Pk1(A$^+$), the parasite inoculated into monkey 1 (see Fig. 3); lane *2*, parasites isolated from monkey 1 on day 8; lane *3*, parasites isolated from monkey 1 on day 87. Arrows identify the specific immunoprecipitates and the 143/140 kD proteins. *BϕB*, bromophenol blue dye front. Molecular mass shown in kD.

The 140 kD protein of parental clone Pk1(A$^+$) is readily radiolabeled with ^{125}I on intact merozoites as described previously (3). The 140 kD protein was missing from the surface radioiodinated merozoites from the five clones and no new proteins were labeled. Immunoprecipitation with antisera against the 143/140 kD proteins failed to precipitate any proteins from the clones (data not shown). Despite the presence of [^{35}S]methionine labeled antigens in clones V1a and V1b, no radioiodinated antigens were detected. It is conceivable that the 76 kD protein is not transported to the merozoite surface or once transported to the merozoite surface, the 76 kD protein fails to attach. It is also possible that the 76 kD protein lacks an accessible tyrosine for iodination.

Invasion of Erythrocytes by Parasites Apparently Lacking the 143/140 kD Proteins. Parasite clone V1c expresses no detectable 143/140 kD proteins. We sought to determine if the absence of these proteins would alter the erythrocyte ligand requirements for invasion. Duffy blood group–negative human erythrocytes and chymotrypsin-treated human erythrocytes are refractory to invasion

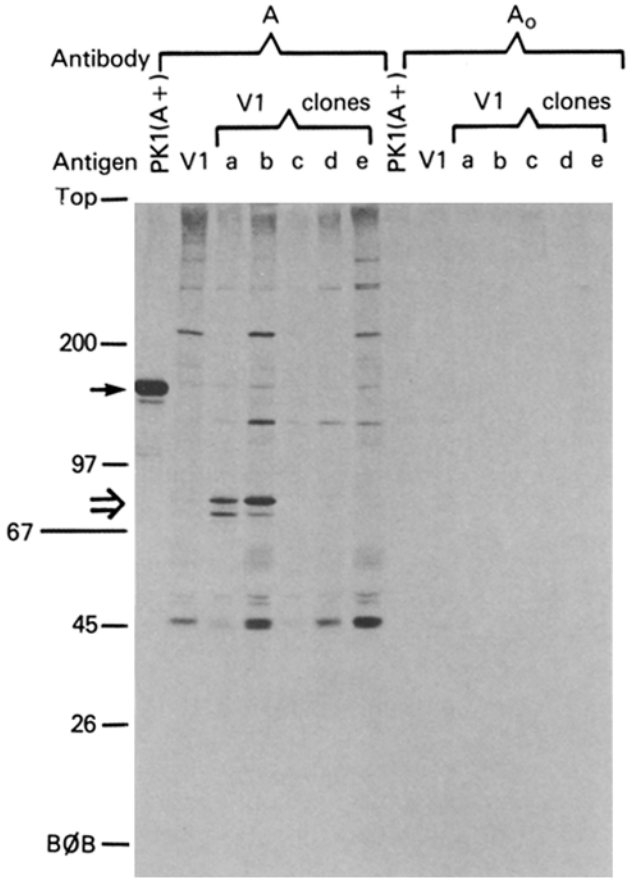

FIGURE 5. Fluorograph of SDS-PAGE (5–12.5% linear polyacrylamide gradient) of [^{35}S]-methionine-labeled antigens from Pk1(A$^+$), V1 and five clones from V1. The letter A denotes immune rabbit serum against the 143/140 kD antigens, A_o, preimmune serum. Arrows identify specific immunoprecipitates. *BϕB*, bromophenol blue dye front. Molecular mass shown in kD.

by *P. knowlesi* (9). We found Duffy-negative and chymotrypsin-treated human erythrocytes were refractory to invasion by clone V1c. Trypsin-treated human erythrocytes are equally invaded by *P. knowlesi* merozoites from Pk1(A$^+$) or V1c. Therefore we can identify no differences in requirements for invasion between the variant V1c and those described previously.

Discussion

Under immune pressure, cloned parasites underwent rapid change, such that antibody no longer blocked their invasion of erythrocytes. This report describes these events and begins to explore the biochemical basis for the parasite's evasion of the immune response. Two monkeys had been immunized with a 140 kD merozoite surface protein (5). This protein was chosen for vaccination trials because monoclonal antibodies against this protein inhibited invasion in vitro (4). The 143/140 kD proteins were also chosen because of their presumed stability in vivo. The 143/140 kD proteins are unchanged during chronic infection over several months duration.

In the present study, the monkeys were inoculated with cloned parasites. The initial wave of parasitemia reached a peak of ~3.5% and then fell to undetectable levels. During the next wave of parasitemia in both monkeys, the 143/140 kD proteins had disappeared. The only previous description of variation in antigenic type from cloned malaria parasites was the SICA antigen of *P. knowlesi* (7) and its probable analogue in *P. falciparum* with uncloned parasites (10). With antibody against the SICA antigen of the inoculum, the SICA antigen changes rapidly to a new type. During the initial rise in parasitemia, new variants appear, and parasitemia increases as rapidly in immune animals as in animals without anti-SICA antibody (7). Thus, anti-SICA antibody probably induces variation; selection does not appear to be involved with antigenic variation of SICA types. In contrast, the 140 kD merozoite protein does not vary during the first wave of parasitemia. The variants are first observed in the second wave of parasitemia. Because antibodies against the 143/140 kD proteins inhibit invasion, selection of variants is the likely mechanism. If the parasitemia during the first wave reaches a peak of 3% in a 3-kg monkey, there are $\sim 2 \times 10^{10}$ parasites from which to select mutants. The variant antigens differ in molecular mass (165–76 kD), and have lost some or all epitopes recognized by monoclonal antibodies against the 143/140 kD antigens. It is unknown whether the variants have new antigenic epitopes or if some clones express a unique antigen.

Although we do not know the function of the 143/140 kD proteins of *P. knowlesi*, it is surprising that parasites apparently lacking the merozoite surface protein (V1 c,d,e) can invade in vitro and multiply effectively in vivo. The 143/140 kD proteins may not be involved in invasion and may not be critical for parasite survival. Alternatively, unrecognized mutations may have replaced the function of the 143/140 kD proteins. For example, there is variation in the requirement for erythrocyte ligands among various isolates of *P. falciparum* (11). Some require sialic acid on the erythrocyte for invasion; others can invade effectively through sialic acid–independent mechanisms. In the present study, we could detect no differences in the ligand requirements for invasion by variant parasites, although some undetected alteration in the invasion sequence may have occurred.

Under selective pressure where antibody against a particular protein leads to death of the parasite (e.g., through vaccination), there may be loss of certain antigenic determinants. Such events could occur from expression of other analogous genes within a repertoire, or from multiple point mutations, rearrangements, nonsense mutations, or deletions within a gene. The chance for survival of such mutants is increased if the gene is not crucial for survival.

Summary

Immunity to 143/140 kD schizont antigens of a monkey malaria, *Plasmodium knowlesi*, provides partial protection to lethal malaria infection in rhesus monkeys challenged with uncloned parasites. To determine the capacity of a cloned parasite to generate variants of the 143/140 kD antigens, immunized monkeys were challenged with a clone of *P. knowlesi*. Parasites recovered 8 d after inoculation with a cloned parasite retained the 143/140 kD antigens. Parasites recovered 30 d after challenge had undergone changes in the 143/140 kD

antigens. Antibodies that block erythrocyte invasion in vitro of the inoculum parasites did not inhibit invasion of erythrocytes by two isolates recovered from the immunized monkeys. An isolate from one monkey recovered on day 30 contained clones expressing new 76/72 kD antigens reactive with rabbit antiserum against the 143/140 kD proteins, and other clones expressing no antigens crossreactive with antisera against the 143/140 kD proteins. An isolate from another monkey obtained 59 d after challenge expressed new antigens of 160/155, 115/113, and 87/85 kD. Using monoclonal antibodies, we found that epitopes were lost from the variant proteins, but we were unable to determine whether new epitopes had appeared. We conclude that clones of *P. knowlesi* can rapidly vary antigenic determinants on the 143/140 kD proteins in animals immunized with these antigens.

We thank Mr. Louis Koontz and Mr. Douglas Seeley for technical assistance. We also thank Mrs. Wilma Davis and Mrs. Brenda Martin for excellent editorial assistance.

Received for publication 3 September 1986 and in revised form 23 October 1986.

References

1. Cohen, S., G. A. Butcher, and R. B. Crandall. 1969. Action of malarial antibody in vitro. *Nature (Lond.).* 223:368.
2. Cohen, S., I. A. McGregor, and S. C. Carrington. 1961. Gamma globulin and acquired immunity to human malaria. *Nature (Lond.).* 192:733.
3. Johnson, J. G., N. Epstein, T. Shiroishi, and L. H. Miller. 1981. Identification of surface proteins on viable *Plasmodium knowlesi* merozoites. *J. Protozool.* 28:160.
4. Miller, L. H., P. H. David, D. E. Hudson, T. J. Hadley, R. L. Richards, and M. Aikawa. 1984. Monoclonal antibodies to a 140,000 m.w. protein on *Plasmodium knowlesi* merozoites inhibit their invasion of rhesus erythrocytes. *J. Immunol.* 132:438.
5. David, P. H., D. E. Hudson, T. J. Hadley, F. W. Klotz, and L. H. Miller. 1985. Immunization of monkeys with a 140 kilodalton merozoite surface protein of *Plasmodium knowlesi* malaria: Appearance of alternate forms of this protein. *J. Immunol.* 134:4146.
6. Chin, W., P. G. Contacos, G. R. Coatney, and H. R. Kimball. 1965. A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science (Wash. DC).* 149:865.
7. Barnwell, J. W., R. J. Howard, H. G. Coon, and L. H. Miller. 1983. Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. *Infect. Immun.* 40:985.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
9. Miller, L. H., S. J. Mason, J. A. Dvorak, M. H. McGinniss, and I. K. Rothman. 1975. Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science (Wash. DC).* 189:561.
10. Hommel, M., P. H. David, and L. D. Oligino. 1983. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity and the role of the spleen. *J. Exp. Med.* 157:1137.
11. Mitchell, G. H., T. J. Hadley, M. H. McGinniss, F. W. Klotz, and L. H. Miller. 1986. Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: Evidence for receptor heterogeneity and two receptors. *Blood.* 67:1519.