Immune Pressure Selects for *Plasmodium falciparum* Parasites Presenting Distinct Red Blood Cell Surface Antigens and Inducing Strain-specific Protection in *Saimiri sciureus* Monkeys

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Summary

The passive transfer of specific antibodies to a naive splenectomized *Saimiri sciureus* monkey infected with the Palo Alto FUP/SP strain of *Plasmodium falciparum* resulted in the emergence of parasites resistant to the transferred antibodies. Molecular typing indicated that the original and resistant parasites were isogenic. *Saimiri* monkeys primed with original parasites were fully susceptible to a challenge by the resistant ones, and vice versa. This absence of crossprotection indicates that strain-specific determinants would be the major targets of protective immunity developed in these monkeys. Phenotypic analysis showed that the surface of the infected red blood cells differed in both lines. Original parasites formed rosettes, autoagglutinated, presented characteristic knobs at the surface of the infected red blood cell, and did not agglutinate in the presence of a pool of human immune sera. In contrast, the resistant parasites did not form rosettes, did not spontaneously autoagglutinate, presented abnormal flattened knobs, and formed large aggregates in the presence of a pool of human immune sera. The presence of strain-specific determinants at the surface of the resistant parasites was confirmed by surface immunofluorescence and agglutination using homologous *Saimiri* serum. Neither the original nor the resistant parasites cytoadhered to an amelanotic melanoma cell line, suggesting that cytoadherence and agglutination can be dissociated. These results indicate that parasites that differ by the antigens exposed at the surface of the red blood cell induce strain-specific immunity. Furthermore they show that rosetting and nonrosetting parasites differ in their antigenic properties and do not crossprotect.

The important role played by IgG antibodies in controlling *Plasmodium falciparum* blood-stage infections has been established by passive immunization of children suffering from malaria using immunoglobulins prepared from immune adults (1–3). Passive transfer of immunity against blood-induced *P. falciparum* malaria could also be achieved in the nonhuman primate *Saimiri sciureus* using immune monkey IgG. Complete clearance of the infection was observed using unfractionated IgG administered over several days (4). Heterologous transfer of human antibody preparations in *Aotus* or *Saimiri* monkey resulted in an important reduction of parasitaemia (5, 6). These observations indicated that these primates were precious experimental hosts for dissecting the protective immune response to *P. falciparum* blood stages. In *S. sciureus*, a direct correlation could be established between the level of antibodies promoting phagocytosis of infected erythrocytes in vitro, and the ability to clear infections in vivo (7, 8). Further analysis has however been hampered by the lack of reagents defining *S. sciureus* immunoglobulin (Ig) isotypes. This led us to generate a panel of mouse mAbs directed against *S. sciureus* Ig (9, 10). The specific anti-*P. falciparum* IgG could be separated using some of these mAbs into antibodies providing protection by passive transfer and nonprotective antibodies (11). These mAbs reacted with the Ig light chain (11). Isolation and screening of mAbs was pursued, in order to obtain reagents that we hope would define specific Ig isotypes.

In the study reported here, we used a previously undescribed mAb, 2H8F1, reacting with a subpopulation of total Ig, to fractionate antibodies from a pool of *P. falciparum Saimiri* immune sera. The protective capacity of the 2H8F1$^+$ and of the 2H8F1-depleted fractions was analyzed by passive transfer in naive infected recipients. 2H8F1$^+$ Ig did not protect the monkeys. Passive transfer of the 2H8F1-depleted antibody prep-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/01/0283/13 $2.00
Volume 181 January 1995 283–295

aration controlled but did not clear the infection, resulting in the emergence of a peak of escape parasites, resistant to the transfused antibodies. Challenge of monkeys previously infected with original or resistant parasites indicated that there was no crossprotection between both types of parasites. Molecular typing demonstrated that resistant parasites were isogenic with the parasites originally injected for all single copy-loci investigated and for most repetitive sequences analyzed, apart from some telomeric regions. Resistant parasites presented a rearranged red blood cell surface, and exposed a distinct set of antigenic determinants. The absence of crossprotection indicates that the determinants exposed on the surface of the infected red blood cell are major targets of the immune effectors involved in protection in this experimental model.

Materials and Methods

mAb 2H8F1. mAb 2H8F1 (IgG1/K) was isolated in our laboratory in 1989, as described in Fandeur et al. (10). Briefly, BALB/c mice were injected with an IgM-enriched antibody fraction prepared from a pool of *Klebsiella pneumoniae*–immune *Saimiri* sera by boric acid precipitation, and chromatography on a Sepharose CL4B (Pharmacia LKB, Uppsala, Sweden) column. After two consecutive intravenous injections of 20 and 30 μg of monkey antibodies at 20-d intervals, spleen cells were collected and fused with the myeloma cell line P3U1. The clones producing anti-*Saimiri* Ig were screened by a conventional radioimmunoassay.

mAb 2H8F1 is still incompletely characterized. It defines a *Saimiri* Ig population precipitable by 2% boric acid. The epitope recognized is conformational, as deduced from its liability in presence of SDS, and not detected on human Igs. Antibodies, affinity purified on mAb 2H8F1 migrate on SDS-polyacrylamide gels with an apparent molecular weight of 160 kD, they are disrupted by β-mercaptoethanol into subunits of 51 and 24 kD, and react with *Staphylococcus aureus* protein A. These findings indicate that the 2H8F1$^+$ Ig is of IgG isotype. The kinetics of production and persistance of the specific 2H8F1$^+$ antibodies raised to *P. falciparum* parasites, is that of a typical IgG response (data not shown).

Antibody Fractions. Antibody fractions were prepared as follows: a pool (37.5 ml) of immune sera (PI)[1], highly protective by passive transfer, was prepared using sera collected from animals protected against *P. falciparum* by repeated injections of living Uganda Palo Alto *P. falciparum* strain propagated in *Saimiri* monkeys (FUP/SP) parasites at monthly intervals (sera were collected between the third and seventh parasite boosts). The PI was clarified by filtration over a 0.22-μm membrane, incubated at 56°C for 30 min, and diluted vol/vol with Tris-buffered saline, pH 8.0, (10 mM Tris-HCl, 0.14 M NaCl) containing 10 mM EDTA (TBS-EDTA). For fractionation, 15 ml aliquots of the diluted PI were mixed with an equal volume of an immunoadsorbent prepared by coupling mAb 2H8F1 to CNBr-activated Sepharose 4B (Pharmacia LKB) as recommended by the supplier. Two to three passages on immunoadsorbent allowed complete adsorption of the 2H8F1$^+$ Ig contained in the PI. 2H8F1-depleted IgG were further purified from the unbound material by adsorption/elution on a protein A–Sepharose column (Pharmacia LKB). Ig retained on mAb 2H8F1 or protein A columns were eluted with glycine-HCl (0.2 M, pH 2.5), and immediately neutralized using Tris 2 M (0.1 ml of neutralizing solution per ml of elution buffer). 12 mg 2H8F1$^+$ Ig (OD 280 nm), were collected in a 410 ml final volume of elution/neutralizing buffer. 2H8F1$^+$ Ig were concentrated by osmotic pressure using polyvinylpyrrolidine (Sigma Chemical Co., St. Louis, MO). After concentration, dialysis and filtration, 26 ml of a 100-μg/ml (Bio-Rad protein assay; Bio-Rad Labs, München, Germany) solution containing 2H8F1$^+$ Ig were recovered. Because of the very low serum concentration in 2H8F1$^+$ Ig, albumin contaminated sightly the eluted 2H8F1$^+$ fraction. As this was a minor contaminant, it was neglected in the quantification of the 2H8F1$^+$ antibodies transfused to monkeys S1410 and S1417. The Ig preparations were extensively dialyzed against glucosed saline (2.5% glucose, 0.45% NaCl). 200 μl to 1 ml aliquots were stored at −20°C until use. Analysis on SDS-polyacrylamide gels indicated that contamination by other serum protein was minimal and that the Ig were intact. Indirect immunofluorescence titer on air-dried parasites was 1/80 for 2H8F1$^+$ antibodies and 1/640 for the 2H8F1-depleted fraction.

Parasites and Immune Passive Transfers. On day 0, four splenectomized naive *S. sciureus* monkeys (S1410, S1417, S1393, and S1413) were injected with 45 × 10^6 parasites derived from the 93rd blood passage of the Palo Alto strain of *P. falciparum* (FUP/SP), which is fully adapted to the *Saimiri* monkey (12, 13). Passive transfers were initiated 1 h after inoculation. 2H8F1$^+$ Ig were administered for 6 d to S1410 and S1417 at a 200-μg daily dose, which is approximatively the amount present in 0.5 ml of PI, and S1413 was treated for 21 d with 2H8F1-depleted IgG, at a 700-μg daily dose. Finally, control monkey S1393 was transfused with a placebo made of glucosed saline. On day 60, animals were challenged with 20 × 10^6 thawed cryopreserved parasites derived from S1413.

Molecular Typing of Parasites. DNA samples were prepared by phenol/chloroform extraction according to standard methods (13). For Southern-blot analysis (14), 2–5 μg of DNA were digested with an excess of enzyme following the manufacturer's recommendations and size-fractionated by agarose gel electrophoresis. Gels were treated and blotted onto nylon membranes (Hybond N; Amersham International, Little Chalfort, UK), and finally incubated with the appropriate ^{32}P-radiolabeled probes (nick-translation kit; Boehringer Mannheim, Mannheim, Germany). The pPF11.1-5', 96tR/GBP130, SAgV1, KAHRP, pPF rep20, and *P. berghei* telomer probes have been described (13, 15). The Pf60.1 probe is a 313-bp fragment recently isolated in our laboratory, defining a large multigene family present in the *P. falciparum* genome (15a).

Agglutination and Surface Immunofluorescence Assays. The sera used for these assays were: (*a*) an anti-original FUP/SP strain (O) antiserum, i.e., the pool of sera collected at ~5-d intervals, from day 30–60 after the infection with O parasites from monkeys 1410, 1417, and 1393; (*b*) an anti-resistant FUP/SP strain (R) antiserum, obtained similarly from monkey 1413; (*c*) a pool of hyperimmune *Saimiri* sera, collected from 15 monkeys that had experienced one or more infections by the IPC/Ray (8) and/or FUP/SP strain and had been recently challenged with O or R parasites; (*d*) a pool of five human sera, collected from adults living in a holoendemic Senegalese village, Dielmo.

Monkey parasitized-blood samples were collected on heparin, washed twice in saline, and once in RPMI (GIBCO BRL, Gaithersburg, MD) containing 20% FCS (RP-S). For the antibody-mediated agglutination assay (16), 5 μl packed cells volume was mixed with 45 μl of diluted serum in RP-S containing 20 μg/ml ethidium bromide, to give a final hematocrit of 10%. In some experiments, heparin (heparin sodium salt grade II from porcine intestinal mucosa; Sigma Chemical Co.) was added to obtain a final concentration

[1] *Abbreviations used in this paper:* FUP/SP, Uganda Palo Alto strain of *P. falciparum* propagated in *Saimiri* monkeys; O parasites, original FUP/SP strain; PfHRPI, knob-associated histidine-rich protein I; PI, pool of immune *Saimiri* sera; R parasites, FUP/SP strain that is resistant to antibodies directed against O parasites; RP-S, saline/RPMI.

ranging from 1 to 100 IU/ml. The preparations were gently rotated (20 rpm) at room temperature for 30 min before being examined under ultraviolet illumination on a microscope slide under a cover slip (Leitz laborlux). For surface immunofluorescence (16), fresh living parasites were incubated with the various dilutions of sera as above, except that ethidium bromide was omitted, and that incubations were done for 1 h. After several washings in RP-S, infected red blood cells were further incubated for 1 h with an anti-human Ig FITC conjugate (Institut Pasteur), diluted 1/25 in RP-S. Red blood cells were pelleted and extensively washed before being examined for fluorescence. Occasionally a 1/50 volume of 2H8F1 ascite was mixed together with the monkey serum during incubation with the parasites. In those cases, antibodies bound to the erythrocyte surface were detected by using an anti-mouse IgG FITC conjugate (affinity purified antibodies; Sigma Chemical Co.) adjusted to a 1/20 dilution. The slides for agglutination and immunofluorescence were randomly labeled and examined in a blind manner.

Rosetting and Autoagglutination. Rosetting (17) and antibody-independent trophozoite agglutination (autoagglutination) (18, 19) of infected erythrocytes were examined on blood samples collected from infected animals. The percentage of infected erythrocytes forming rosettes was used as an index of rosetting. To facilitate counting, parasitized erythrocytes were prepared in RP-S containing ethidium bromide, and examined under ultraviolet illumination as for the agglutination assay, as well as for the presence of visible malarial pigment. The autoagglutination phenotype was estimated on Giemsa-stained thin blood films, so as to avoid doubtful positive reactions resulting from the marked rosetting phenotype that is sometimes associated with parasites under examination.

Cytoadherence to Melanoma Cells. The binding of infected erythrocytes to C32 melanoma cells subcultured in Lab-Tek (Miles Laboratories, Naperville, IL) was examined essentially as described (20). Infected red blood cells were either used directly after collection from the monkey or after maturation in vitro for 24–48 h. Parasitized blood was washed three times in RPMI and adjusted to 5% hematocrit in RP-S so that the parasitaemia never exceeded 10%. After incubation with melanoma cells for 1 h at 37°C, the coverslips were washed gently with RPMI. Cells were finally fixed with 2% glutaraldehyde in PBS, stained with 5% Giemsa for 15 min and examined microscopically. At least 250 melanoma cells were counted per Lab-Tek chamber.

Electron Microscopy. Parasitized blood was fixed for electron microscopy as described (21). Thin sections were cut on an Ultracut E ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) and observed, after staining, on an electron microscope (H600, Hitachi, Tokyo, Japan).

PCR. Genomic DNA and cDNA were used as templates for in vitro amplification of knob-associated histidine-rich protein I (PfHRPI) sequences in FUP/SP parasites. Total RNA was extracted by the guanidium-hot phenol method (22) and reverse transcribed essentially as described elsewhere (23) by using random hexamers to initiate first strand cDNA synthesis. PCR experiments were performed in a final volume of 50 μl of a mixture containing 0.2 mM dNTP, 2 μM each of sense and antisense HRPI oligonucleotide primers (see sequence in the legend of Fig. 7), 2 U of *Taq* DNA polymerase in appropriate buffer. After 30 rounds of amplification, PCR products were analyzed by gel electrophoresis.

Results

Fractionation of Antibody and Passive Transfer. mAb 2H8F1, a mouse mAb reacting with a minor population of *Saimiri* Ig, was isolated as described in Materials and Methods. It was used to fractionate Ig from a pool of FUP-SP-immune *Saimiri* sera by affinity-chromatography in order to examine the protective properties of the Ig identified by this mAb. 2H8F1$^+$ Ig and 2H8F1-depleted antibodies were prepared and their antiparasitic activity was determined in vivo by passive transfer into naive recipient monkeys, that had been infected with the standard FUP/SP strain (thereafter called "Original" or O parasites). Results are shown in Fig. 1. S1393 (control monkey received placebo) and S1417 (injected with the 2H8F1$^+$ Ig) developed virulent infections, with parasitaemia reaching 25% on day 13 and 20% on day 7, respectively, and requiring quinine treatment. Similarly, a high parasitaemia was observed in S1410 (transfused with 2H8F1$^+$ Ig), and reached 12.6% on day 11. Infection continued for 7 d at a parasitaemia oscillating between 9 and 16%, and the animal had to be treated with quinine because of a severe anaemia. Over the first 6 d of antibody treatment of S1413 using 2H8F1-depleted antibodies, the parasites were maintained at low densities (<0.02%). Because of persisting circulating parasites, passive transfer was carried on beyond day 6, and under this treatment, parasitaemia was maintained under 1% until day 12. Some of the parasites circulating during this period had an abnormal morphology. The parasitaemia then increased regularly despite continued daily injections, reaching 11% by day 16. The animal recovered spontaneously from its infection by day 21. The standard dose required in passive transfer to cure infection, as determined for the FUP/SP isolate is usually 5–20 mg total IgG (11, 24) delivered over a 5-d period, a regimen ensuring full protection if the initial parasitaemia does not exceed 1%. The incomplete efficiency of the 2H8F1-depleted Ig and the insensitivity of the emerging parasites to the transfused Ig suggested that these parasites differed from those inoculated in S1413, or in other words that parasites resistant to transfused antibodies (thereafter called "Resistant" or R parasites) had been selected. To test this hypothesis, the monkeys were challenged with the parasites collected from S1413.

Challenge Experiment with R Parasites. In the experimental infection of *Saimiri* monkeys with FUP/SP parasites, immunity to FUP/SP parasites (the O type) is usually obtained after a single drug-cured, high parasitaemia infection (25–27). Hommel et al. (27) reported that the animals receiving a homologous challenge 2–5 mo after a primary Palo Alto episode developed very low or no parasitaemia. This was confirmed by Roussilhon et al. (26) and was further illustrated in recent challenge experiments summarized in Table 1, where most monkeys immune to O parasites resisted a homologous challenge by large doses of parasites. As shown in Table 1, homologous challenge after a delay of several months is not invariably followed by protection, especially if the interval between infections is long (e.g., monkeys S90079 and S90072, which were challenged 6 mo after their primary infection). Monkey S90065, which did not resist homologous challenge after 2 mo, is one of the rare exceptions to the consistent protection afforded by O parasites against an early homologous challenge. Upon rechallenge 4 mo later, this animal again developed an infection, indicating that it

Table 1. *Characteristics of Primary and Secondary Infections Using O and R Parasites*

Monkeys	First infection				Interval	Second infection				Outcome
	Parasite type	Inoculum	Maximum parasitaemia (at day)	Day of treatment		Parasites	Inoculum	Maximum parasitaemia (at day)	Day of treatment	
		× 10^6			*d*		× 10^6			
S1393	O	45	25% (13)	13	60	R	20	33% (8)	8	No protection
Si9203	O	50	28% (7)	7	63	R	20	20% (12)	12	No protection
Si9204	O	1	18% (10)	10	98	R	20	22% (11)	11	No protection
S90077	O	20	18% (10)	10	193	R	7	20% (19)	19	No protection
S90094	O	20	27% (10)	10	193	R	7	21% (15)	15	No protection
Si9202	R	50	19% (9)	9	63	O	20	6% (12)	9/12*	No protection
Si9201	R	1	27% (16)	16	98	O	20	18% (11)	11	No protection
S1417	O	45	20% (7)	7	60	R	20	31% (7)	7	No protection
S1410	O	45	16% (13)	18	60	R	20	4% (6)	–	Protection
S1413	O + R	45	11% (16)	–	60	R	20	0.2% (4)	–	Protection
S90071	O	20	26% (12)	12	180	O	20	2% (14)	–	Protection
S90034	O	20	25% (10)	10	180	O	20	0.8% (14)	–	Protection
S90079	O	20	22% (12)	12	180	O	20	18% (18)	19	No protection
S90049	O	20	16% (6)	6	270	O	20	1% (14)	–	Protection
S90065	O	20	13% (8)	8	61	O	20	25% (13)	13	No protection
S90072	O	20	33% (11)	11	180	O	20	18% (12)	12	No protection
S90122	O	20	33% (11)	11	180	O	20	2% (16)	–	Protection
S90055	R	20	16% (13)	13	95	R	20	0.1% (45)	–	Protection

Absence of cross-protection between FUP/SP O and R parasites, contrasting with protection against homologous challenge. The parasite type and number of parasites inoculated, day of maximum parasitaemia and day of treatment are indicated. Animals were splenectomized. In all cases, the first inoculation resulted in a typical FUP/SP virulent infection (12) requiring drug treatment to avoid animal death. Challenges were done at the indicated interval after primary infections, by injecting homologous or heterologous parasites, as indicated. No protection refers to the development of a virulent infection, that had to be controlled by drug cure. Protection indicates either the absence of parasites or a mild infection, spontaneously controlled by the animal, with a parasitaemia below 3%.

* Challenge of Si9202 by O parasites was followed by the development of a fulminant infection. The animal was treated by passive transfer of 0.3 and 0.5 ml of anti-O antiserum, administered on days 9 and 12, respectively. This treatment cured the infection, indicating that the O parasites multiplying in this monkey had retained their O characteristics.

may have an unusual sensitivity to *P. falciparum* infection. Apart from these rare cases, available evidence indicates that the monkeys infected here should resist a challenge by O parasites. The outcome of the challenge with R parasites is shown in Fig. 1. The monkeys showed marked differences in susceptibility. Monkey S1413, from which the parasites originated, was completely protected. Conversely, animals S1393 and S1417, which had developed fulminating O infections, developed virulent R infections. S1410, which had experienced a prolonged and fluctuating infection with O parasites, developed an infection with R parasites that self-cured later on. The lack of crossprotection between the two lines was confirmed by infecting several monkeys with O or R parasites and by challenging them with homologous or heterologous parasites. As predicted, homologous challenge showed a high level of protection, whereas heterologous infection always resulted in uncontrolled fulminant parasitaemias (see Table 1). This indicated that the characteristics of the R parasites were stable. This further indicated that R parasites elicited a specific protection, distinct from the specific protection induced by the O parasites, and hence that both parasite lines were antigenically distinct.

As the FUP/SP O isolate injected was uncloned, the escape R parasites could obviously derive from two distinct types of selection. They could result from outgrowth of a minor parasite population presenting distinct antigenic determinants, but not abundant enough in the O isolate to elicit a specific immune response. If so, in view of the extended genetic polymorphism of *P. falciparum* parasites (28, 29), the R parasites should be easily distinguishable by molecular typing. Alternatively, the R parasites could derive from selection of mutants or emergence of variants among an otherwise homogeneous population, and hence should present the same genetic background as the O parasites (30). We there-

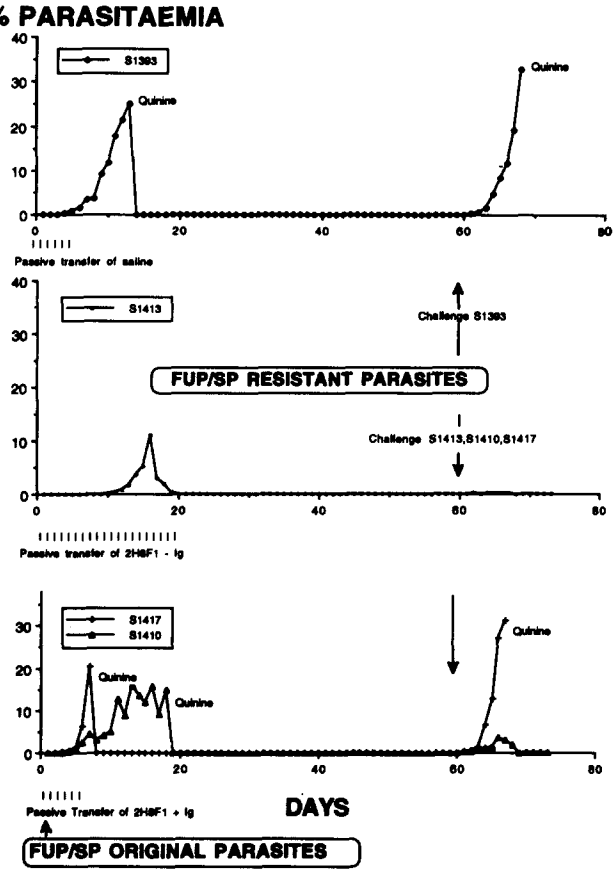

Figure 1. Passive transfer of the antibody fractions and challenge using R parasites. Four splenectomized naive monkeys, S1393, S1413, S1410, and S1417, were inoculated on day 0 with the Palo Alto FUP/SP strain of *P. falciparum*. Passive transfer of Ig fractions was initiated immediately thereafter. S1393 used here as a control was injected with saline; S1413 received 21 daily doses of 700 μg each of the 2H8F1-depleted Ig preparation; S1410 and S1417 received 6 daily injections of 200 μg each of affinity-purified 2H8F1$^+$Ig. On day 60, the animals were challenged with R parasites collected on day 17 from S1413.

fore carefully genotyped the O and R parasites using a variety of probes.

Molecular Typing of Parasites. In *P. falciparum* parasites, the strain-specific genetic background is readily visualized using repetitive probes (15, 30–32). Fig. 2 compares the restriction patterns for O and R DNAs digested with various enzymes, and hybridized to probes specific for well-documented makers for diversity, derived from the pPF11.1, 96 tR, SAgV1, and HRPI/KAHRP genes (15). The parasite lines could not be differentiated on this basis, even by probing with pPf11.1 repeats known to be of value in detecting restriction polymorphism (33), as illustrated in Fig. 2, lanes *C* of the gels, where a distinct strain has been used. This was confirmed by DNA fingerprinting using the highly repetitive probe pPFrep20 (34) (Fig. 3 *B*), previously shown to be highly sensitive in detecting genomic diversity (15, 30–32), as well as using the telomer-specific repeats (35) (Fig. 3 *C*) and Pf60.1, a repetitive probe recently isolated in our laboratory (15a) (Fig. 3 *A*). The pPFrep20- and telomer-specific patterns observed were remarkably similar, apart from a few differences, indicated on the figure. As the chromosome ends are the sites of frequent rearrangements, the differences observed are not unexpected. The similarity of O and R parasites was further substantiated by analysing several polymorphic loci by PCR. Several regions of the genes coding for merozoite surface proteins MSP1 or MSP2 were amplified as indicated in (15, 36). The fragments obtained from O and R parasites did not differ by size and had identical DNA sequences (data not shown). The data therefore indicated that O and R parasites had quite similar genetic backgrounds and could be regarded as isogenic. This suggested that escape R parasites had arisen by selection of mutants or variants of the O parasites.

Phenotypic Typing of Parasites. As the major effector mechanism used to eliminated parasites in *Saimiri* monkeys is phagocytosis of the red blood cell (7, 8), involving recognition of antigens exposed on the surface of the infected red blood cell, we compared the phenotype of the O and R red blood cell surface for several characters. The results are summarized in Table 2.

Rosette formation (17) was examined for ex vivo blood samples parasitized with O and R parasites, and the percentage of parasitized erythrocytes forming rosettes (two or more attached uninfected red blood cells) was determined. As indicated in Table 2, 62 ± 4% of erythrocytes infected with O parasites formed rosettes (two example are shown in Fig. 4 *A*). This confirmed the observation of Tourneur et al. (37) using this strain. In contrast, rosettes were not observed on the erythrocytes infected with R parasites (not shown). An additional difference was that erythrocytes infected with O, but not with R parasites, autoagglutinated, as shown in Fig. 4 *B*. This phenomenon, already observed by Hommel et al. (19) on an early passage of the same strain of parasites, was difficult to quantify precisely. It was nevertheless clear that this was restricted to erythrocytes infected with late trophozoites, while, as reported by Roberts et al. (18), mature shizonts and young stages did not autoagglutinate.

We then looked for the presence of specific antigenic determinants at the surface of erythrocytes infected by O or R parasites using antibody-mediated agglutination and indirect immunofluorescence assays. Experiments were conducted with several immune sera of monkey or human origin. Representative positive results of both assays are shown in Fig. 5, *A–F* and data is summarized in Table 2. R-parasitized erythrocytes were strongly agglutinated by all sera tested, including a pool of human immune sera, except by the anti-O antiserum. Conversely, O parasites were poorly to not agglutinated by any antiserum, even by the homologous anti-O antiserum. A pool of *Saimiri* hyperimmune sera agglutinated unambiguously the O parasites when tested at a 1/5 dilution, but did not agglutinate significantly at higher dilutions. The weakly positive reactions indicated in Table 2 were difficult to differentiate from autoagglutination. The high propensity of the O-parasitized erythrocytes to form rosettes could obviously prevent access of the antibodies to the surface of the infected red blood cell and sterically inhibit efficient antibody-dependent agglutination. To investigate this possibility, several agglutination experiments were performed in

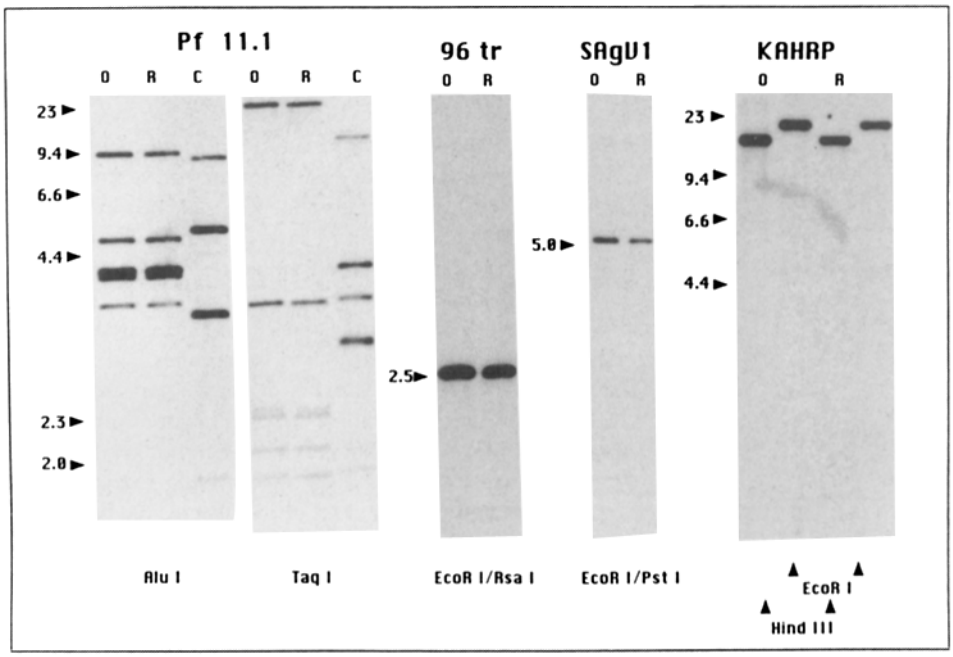

Figure 2. Southern analysis of DNAs from O FUP/SP (lanes *O*), R FUP/SP (lanes *R*), or from culture-adapted (unrelated) FUP/CP (lanes *C*) parasites (15) after digestion with various restriction enzymes. Hybridization was done using nick-translated pPF11.1-5', 96 tR, SAgV1, or KAHRP probes, as indicated. Molecular weight markers, as indicated on the left, were HindIII-digested λ DNA.

the presence of increasing doses of heparin, reported to disrupt rosettes (38), in particular those formed by FUP/SP parasites (37). Heparin treatment was however unable to promote antibody-dependent agglutination of O parasites at any concentration (results not shown). Surface immunofluorescence was negative on O parasites, whatever the serum used. This is contradictory with the positive reaction described by Hommel et al. (27). We suppose that this is due to a lower sensitivity of our detection technique, as positive immunofluorescence on original FUP/SP parasites was detected by Jouin et al. using the more sensitive FACScan® assay (Becton Dickinson & Co., Mountain View, CA) (39). In contrast, R parasites produced a strong surface immunofluorescence, as shown in Fig. 5 *E*. Importantly, 2H8F1$^+$ antibodies reacted with the surface of R-infected erythrocytes, as shown in Fig. 5 *F*.

We next determined the cytoadherence, HRPI and knob phenotypes of O and R parasites. In these splenectomized monkeys, O and R late stages were consistently observed in the peripheral blood. For both parasite types, neither the

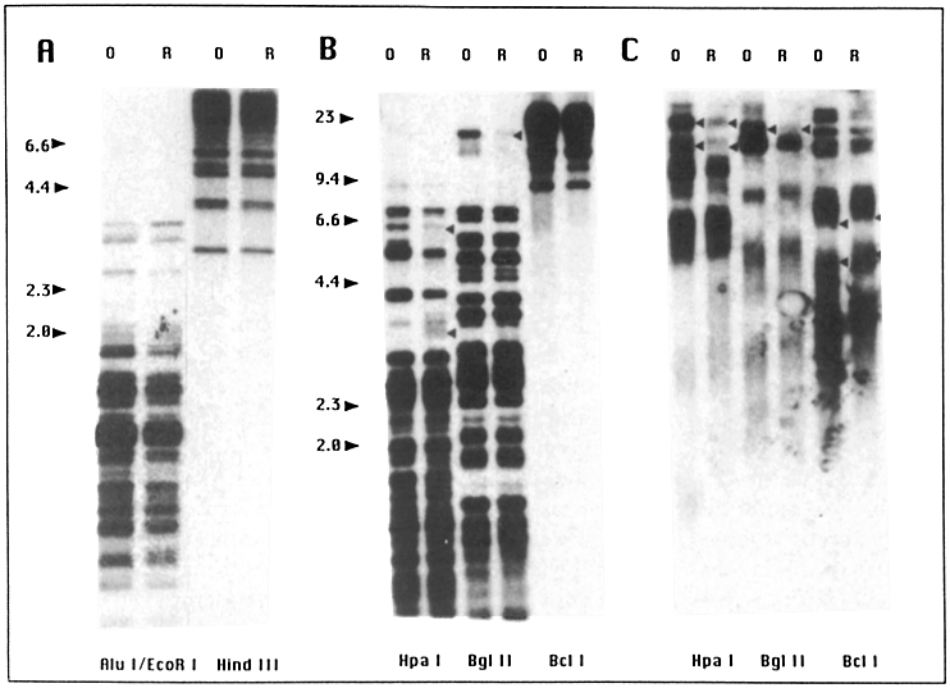

Figure 3. Autoradiographs of Southern blots of O (lanes O) and R (lanes *R*), FUP/SP DNA preparations restricted with various enzymes and hybridized to Pf60.1 (*A*), pPFrep20 (*B*) or *P. berghei* telomer (*C*) probes. Differences between both preparations are indicated by *arrows*. Molecular weight markers on the left are HindIII-digested λ DNA.

Table 2. *Phenotypes of FUP/SP O and R Parasites*

Phenotype		FUP/SP O Parasites	FUP/SR R Parasites
Rosette formation		62 ± 4%	0%
Autoagglutination		+ +	–
Agglutination			
Anti-R	1/5	+ (b)	+ + + (d)
	1/10	– (a)	+ + + (e)
	1/50	+ (b)	+ + + + + (d)
Anti-O	1/5	+ (b)	– (d)
	1/10	– (a)	– (d)
	1/50	± (b)	– (d)
Pool human	1/10	+ (c)	+ + + + (e)
	1/100	– (c)	+ + (e)
Pool *Saimiri*	1/5	+ + + (b)	+ + + + (d)
	1/10	+ (a)	nd
	1/50	– (b)	+ + + + + (d)
Surface immunofluorescence			
Anti-R	1/5	– (a)	– (d)
	1/50	– (a)	– (d)
Anti-O	1/5	– (a)	– (d)
	1/50	– (a)	– (d)
Pool Saimiri	1/5	– (a)	+ + + (d)
	1/10	– (a)	+ + + (d)
Cytoadherence		–	–
mAb 89 reactivity by indirect immunofluorence		+	+ + +
Knobs		Normal	Abnormal

Aggluination and surface immunofluorescence assays were done using a pool of immuno sera from *Saimiri*, a pool of immune sera from adults living in an endemic area, anti-O or anti-R *Saimiri* antisera, obtained by infection with the relevant strain followed by drug cure, as indicated in Materials and Methods.

Score used for agglutination: –, negative reaction; + / –, weakly positive reaction; +, weakly positive reaction (agglutinates <5 infected-cells [IC]); + +, weakly positive reaction (5 IC < agglutinates < 10 IC); see Fig. 6 for moderately positive (+ + +), positive (+ + + +), and strongly positive (+ + + + +) reactions.

Different monkeys were used as parasitized cells donors in independent experiments. (*a*) O parasites from Si9203, parasitaemia 4.6%; (*b*) O parasites from Si9203, parasitaemia 28.0%; (*c*) O parasites from Si9065, parasitaemia 6.3%; (*d*) R parasites from Si9202, parasitaemia 3.6%; (*e*) R parasites from Si9202, parasitaemia 18.9%.

trophozoites and schizonts collected directly from the monkeys nor those obtained after 24 or 48 h maturation in vitro showed any significant cytoadherence to the melanoma cells (using fixed or fresh C32 cells), whatever the donor monkeys used (not shown). This confirmed the observation of David et al. (40). The presence of the HRPI protein was examined by using mAb 89 (41) by IIF and Western blotting. R parasites gave a positive fluorescence up to a 1/1,600 dilution, whereas O parasites were uniformly negative at dilutions above 1/400 (data not shown). Furthermore, even at a low dilution, there was an obvious difference in fluorescence intensity between R and O parasites, the O parasites giving a weak signal. We also looked for HRPI expression by reverse PCR in both parasite types. We chose HRPI-specific primers located in regions flanking the intron (42), so that fragments derived from cDNA amplification could be readily identified due to their smaller size. Fig. 6 shows the result for HRPI amplification using either genomic DNA (1,400 bp) or random reversed mRNA

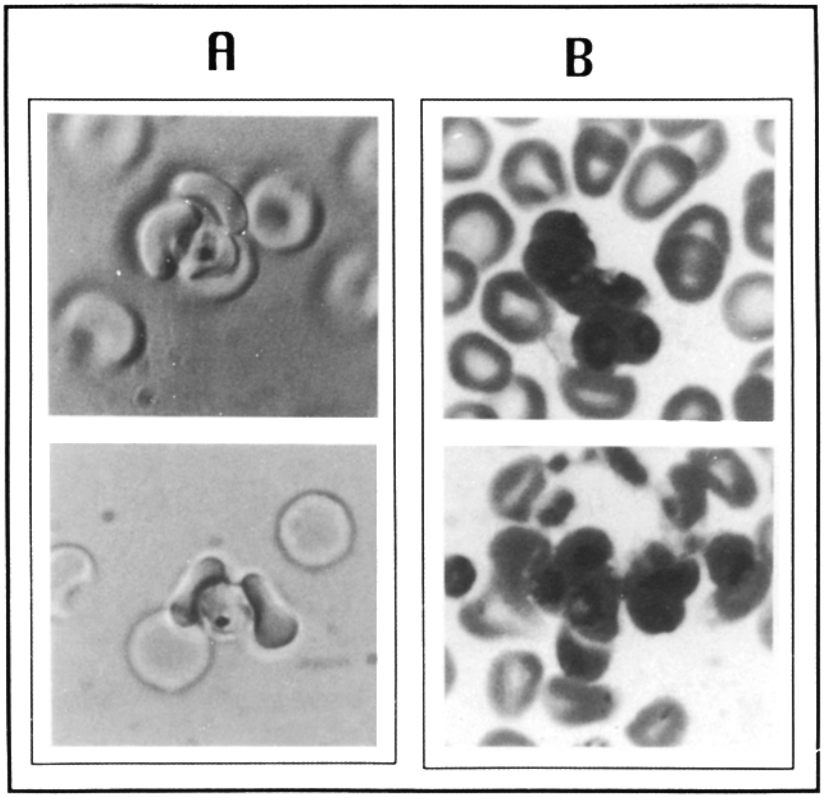

Figure 4. Rosette formation (*A*) and spontaneous antibody-independent trophozoite agglutination (*B*) in FUP/SP O parasites collected from splenectomized *Saimiri* monkeys.

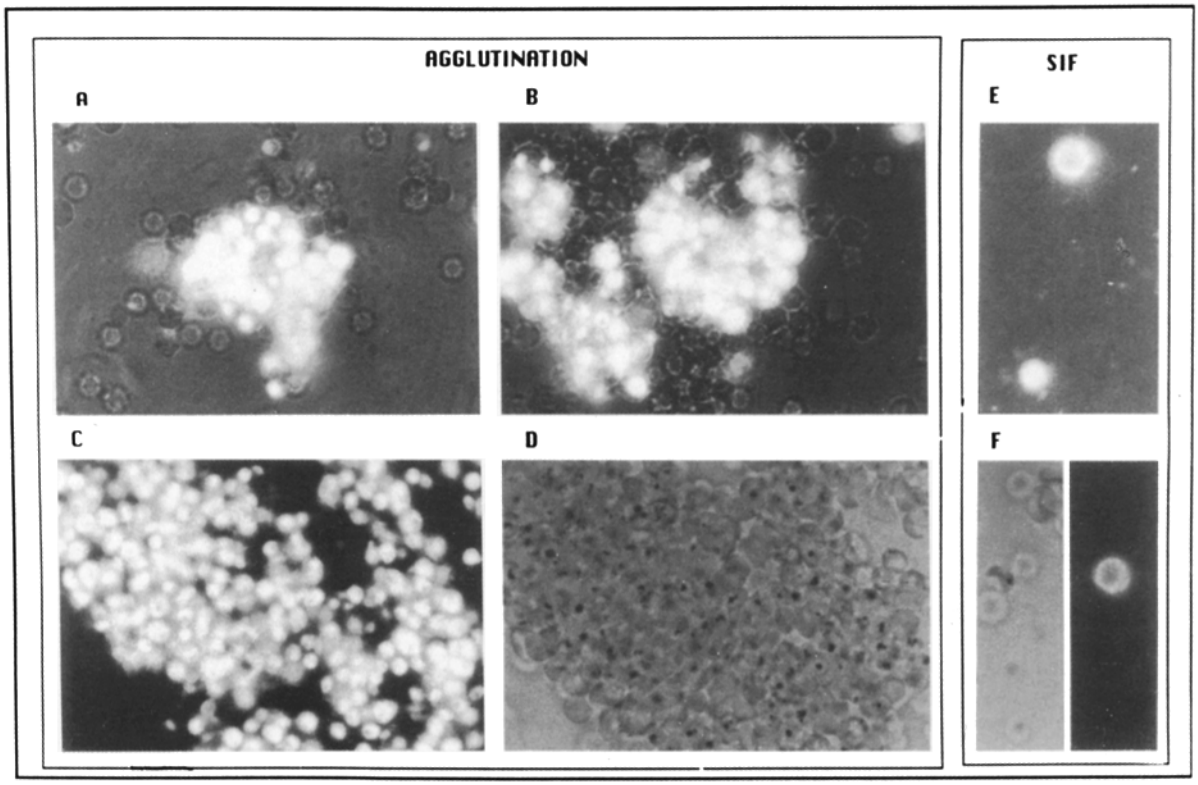

Figure 5. Parasite-infected cell agglutination and surface immunofluorescence assays using FUP/SP R parasites. (*A–C*) typical agglutinates, stained by ethidium bromide and examined under UV illumination. (*A*) Agglutination obtained using *Saimiri* anti-R antiserum diluted 1/10, recorded as moderately positive (+++). (*B*) Agglutination obtained using a pool of human hyperimmune sera diluted 1/10, recorded as positive (++++). (*C*) Agglutination obtained using *Saimiri* anti-R antiserum diluted 1/15, recorded as strongly positive (+++++). *D* shows the same field as *C*, but under bright field illumination. (*E*) Strongly positive surface immunofluorescence (+++), obtained by incubating a pool of *Saimiri* immune sera at a 1/5 dilution, with FUP/SP R parasites, and detected using an anti-human Ig FITC conjugate. (*F*) Same as in *E*, detected using mAb 2H8F1, and an anti-mouse Ig FITC conjugate (see Materials and Methods). On the left, the same field is shown under bright field illumination.

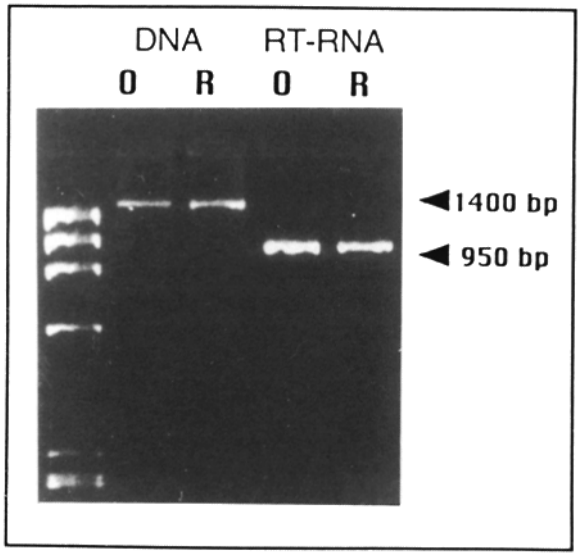

Figure 6. Analysis of PCR products generated using HRPI-specific primers from genomic DNA or reversed-transcribed RNA from FUP/SP O (lanes *a*) or R (lanes *b*) parasites. The marker shown on the left hand side is HaeIII-digested PhiX174 DNA. The primers used derived from the previously published HRPI sequence from the knob-producing FCR3 strain (42) and were as follows: HRPI-5'CCGGATCCATGAAAAGTTTTAAGAACAA and HRPI-3'TGAATTCCCTGCACCATGGGGTGGGAC. Underlined artificial BamHI and EcoRI sites were introduced.

(950 bp) as templates. It is difficult to estimate the exact quantity of HRPI-specific RNA in both preparations by this technique. The results nevertheless indicated that undoubtedly the gene was expressed in both parasite types. It is possible that the differences visualized by using mAb 89 in immunofluorescence could be linked to a lower affinity of mAb 89 for the O HRPI gene product. This interpretation has to be confirmed by sequencing the gene region encoding the epitope binding to mAb 89 (43).

The data altogether indicated significant differences of the red blood cell surface in both parasite types. We therefore examined the ultrastructure of both parasite types by electron microscopy. Representative images are shown in Fig. 7, and indicate that the O-parasitized erythrocyte presented knobs, consistent with the data of Hommel et al. (19) and Gysin et al. (44). The number of knobs was relatively low, but their structure was normal. In contrast, the R-parasitized erythrocytes presented abnormal knobs. The electron-dense material was condensed in patches (a similar number as in the O parasites), but there were no protrusions. The surface was flat throughout.

Discussion

In the course of a passive transfer experiment, planed to evaluate the efficacy of antibodies affinity-purified on mAb 2H8F1, we observed the emergence of R parasites resistant to the 2H8F1-depleted antibodies transfused and presenting a rearranged red blood cell surface. Hommel et al. (27) have previously described that a single injection of unfractionated antiserum was followed by a phenotype switch of antigens exposed on the surface of the infected red blood cell. This switch, detected as a modification of the surface immunofluorescence serotype, occurred in the ancestral *Saimiri*-adapted Palo Alto strain PLF-3—from which the FUP/SP O parasites were derived by numerous successive passages in *Saimiri* monkeys (13)—in a spleen-intact monkey, and for the Palo Alto B11 clone in a splenectomized animal. We have also observed the emergence of escape parasites in monkeys infected with the 89F5 cloned line derived from FUP/SP (original) parasites (13) and daily transfused with subcurative doses of unfractionated specific anti FUP/SP O IgG. The phenotype of the 89F5 escape parasites is still to be analysed in detail, but the genotype could not be distinguished from that of R parasites (data not shown). A switch in the surface antigens was also observed by Hommel et al. (27) upon homologous challenge of immune *Saimiri* monkeys with the Indochina 1 strain (27). The results reported here expand upon these original observations and furthermore indicate that parasites that present distinct red blood cell determinants induce a strain-specific immunity. To our knowledge, this is the first report of a direct role for erythrocyte surface antigens in eliciting a potent protective and sterilizing immunity against malaria. To date, serological diversity of these antigens has been mostly regarded as allowing a chronic infection with low-grade, recrudescent parasitaemia (27, 45). We show here that they may be major targets of the immune effectors involved in parasite clearance.

The Palo Alto FUP/SP O parasites used in the experiments described here were not clonal. Genotyping using a panel of probes well-documented for their ability to detect polymorphism (15, 30–34), indicated that resistant and original parasites were isogenic for all genetic markers analyzed, except for a few differences in the restriction profiles of some subtelomeric and telomeric sequences. These regions are prone to frequent recombinations and rearrangements, mainly documented during in vitro cultivation (reviewed in reference 46), the significance of which is still unclear. Therefore minor differences such as those observed here in the context of identity of all other pPFrep20 and telomer restriction fragments and of the other genomic markers analyzed do not invalidate the conclusion of isogeny. Furthermore, as already noted above, escape parasites with an R-type genotype have been selected from the 89F5 cloned line (data not shown). The number of R parasites present in the 4.5×10^7 inoculum of O parasites could be estimated by extrapolating the growth curve observed in monkey 1413. Assuming a constant growth rate and a constant haematocrit during the course of infection, this figure was around 4.5×10^5, i.e., 1% of the inoculum. As imprecise as this calculation may be, such a high proportion is unlikely to be due to mutation and is highly consistent with the report by Roberts et al. (18) that switching of the infected red blood cell surface phenotype occurs spontaneously at a high frequency (about 2% per generation). We conclude that the R parasites represented a small fraction in the inoculum, that has been selected after elimination of the bulk of O parasites by the antibodies reacting with the dominant population.

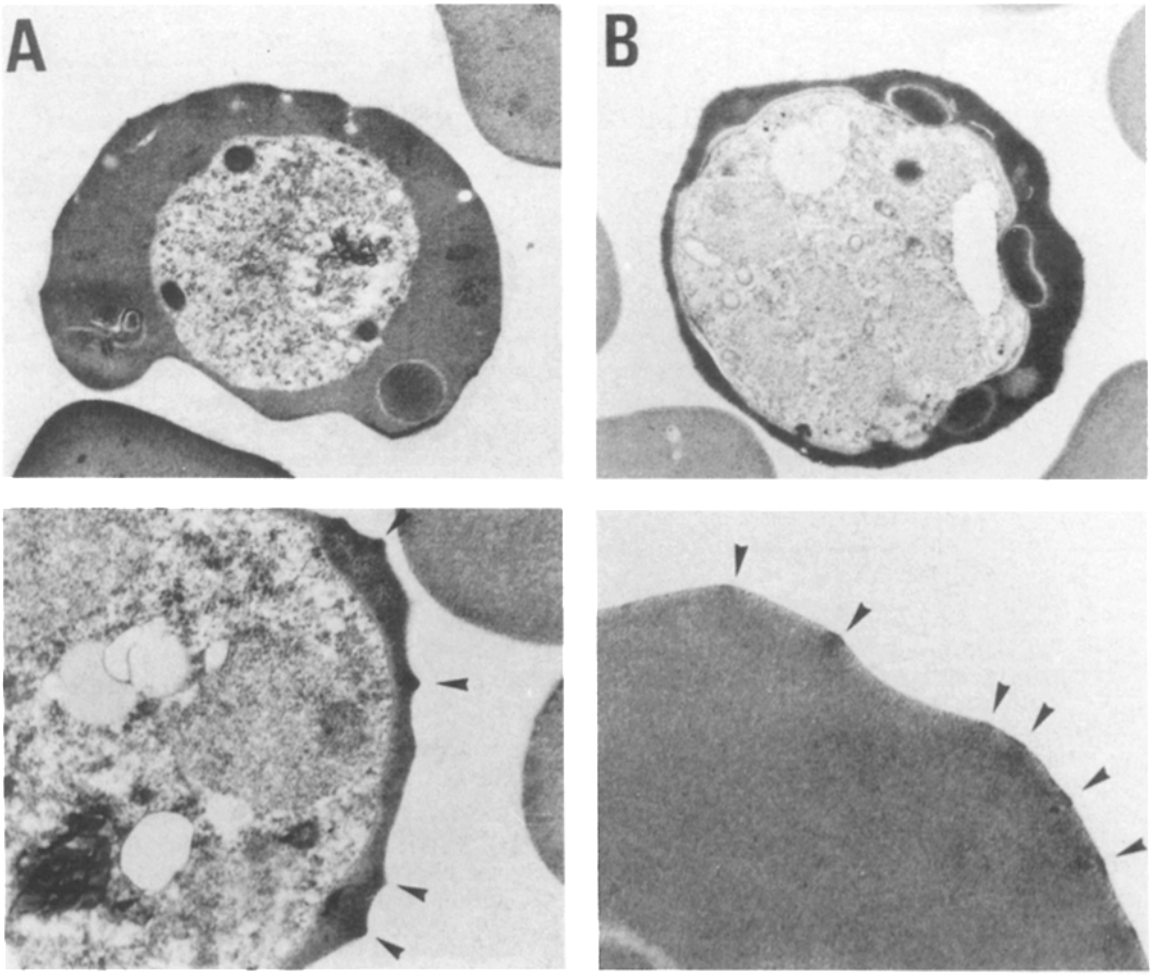

Figure 7. Electron microscopy showing typical knobs on *Saimiri* erythrocytes infected with FUP/SP O parasites (*A*) and the absence of protrusions on FUP/SP R parasitized erythrocytes (*B*). Deposits of electron dense material beneath the bilayer are indicated by *arrows*.

Phenotypic analysis showed that the surface of the infected red blood cell differed in O and R parasites. Antibody-dependent agglutination of O parasites was weak with most sera tested, including human sera, apart from the hyperimmune *Saimiri* pool which agglutinated the O-infected red blood cells significantly, albeit with a low titer. Surface immunofluorescence was negative. On the contrary, R parasites presented a strong surface immunofluorescence and formed large agglutinates in the presence of homologous and, interestingly, of human serum, indicating that the neoantigens involved in agglutination of R-infected monkey erythrocytes are immunogenic in the course of human infections. The markedly reduced agglutinability of O parasites by the human serum pool, the low titer of agglutinating antibody in the hyperimmune *Saimiri* pool and the small size of the agglutinates in the presence of anti-O antiserum indicate that O parasites may express the relevant antigens at a significantly lower level than the R parasites. Alternatively, these reactions may identify the few R(?)-parasitized red blood cells expressing the target antigens in the overall O population, resulting in lower titers and aggregates of small size. Newbold et al. (47) reported recently that the agglutination reaction involved predominantly variant-specific determinants. There is evidence that the variant antigen named the erythrocyte membrane protein 1 (PfEMP1), is the parasite receptor for cytoadherence, and that distinct antigenic types present different cytoadherence phenotypes (18, 48). However, as reported for parasites propagated in splenectomized animals (40), neither R nor O FUP/SP ex vivo parasites cytoadhered to melanoma cells, even after in vitro maturation, and for both lines, trophozoites and schizonts were observed in the peripheral blood. This does not rule out that some late stages would be sequestered in the deep vasculature. However, as agglutination was assayed using circulating trophozoites and schizonts, the cytoadherence and agglutination phenotypes can be dissociated and hence the agglutinogen may be distinct from the surface ligand involved in cytoadherence.

The selective pressure exerted here, using polyclonal, polyspecific Ig resulted in eliminating knob-positive, rosette-forming and auto-agglutinating parasites. The R parasites were altered for the three characters, which, according to Roberts et al. (18), are independent. The significant change

in the erythrocyte surface does not necessarily imply that the number of genetic loci affected is large. The architecture of the membrane of *P. falciparum* infected erythrocytes is obviously complex. Neoantigens include multiple parasite proteins (49) as well as modified host proteins (50). It is possible that by eliminating or perturbating one or two components, the entire structure was totally altered and accessibility to the antigens profoundly modified. Hommel et al. (19) showed that autoagglutination occurs through the knobs, which are concentrated in the regions of cell to cell association. Rosette formation by the Palo Alto FUP strain (analogous to our O parasites) has already been documented by Tourneur et al. (37), who also reported that mAb 89, an anti-HRPI antibody, as well as *Saimiri* hyperimmune sera were able to disrupt in vitro–formed rosettes. In vivo, this probably precedes elimination of the infected erythrocytes. Interestingly, the R parasites presented an altered knob morphology and an altered, possibly increased, expression of the knob-associated histidine-rich protein HRPI. It is still unclear whether the actual target of mAb 89 in rosette disruption is the HRPI protein itself, reported to be under the red blood cell membrane (41), or a crossreacting antigen of lower molecular weight (51). In that context, it is important to note that in humans, a strong correlation has been found between the presence of antibodies able to disrupt rosettes and protection against cerebral malaria (52). The data reported here show that the immunity raised to parasites that are unable to form rosettes does not protect against rosette-forming parasite strains and suggest that unless some form of immunity transcending strain-specificity is mounted upon hyperimmunization, only a specific subset of *P. falciparum* strains may be able to elicit an immune response protecting against cerebral malaria.

In monkeys, as in humans, protection is achieved by specific IgG (1–6) but the relative importance of various isotypes for development of efficient immunity is still unclear (7, 8, 11, 53). Passive transfer experiments performed in humans showed that transfused antibodies that eliminated the majority of the parasites, were unable to completely clear the infection (1–3). Escape parasites, however, were fully susceptible to the same Ig preparations (3). In the *Saimiri* monkey, while the unfractionated immune IgG invariably conferred sterile protection and provided sufficient amounts are injected for several consecutive days (11, 24), a single injection resulted in selecting parasites presenting a different red blood cell surface specificity (27). This suggests that limiting amounts of antibody do not permit elimination of a parasite subpopulation. Similarly, the 2H8F1-depleted Ig used here did not provide complete clearance, resulting in the outgrowth of R parasites. An obvious hypothesis would be that 2H8F1$^+$ Ig are necessary for elimination of R parasites. Consistent with this interpretation is the lack of protection by 2H8F1$^+$ Ig against an O inoculum. The precise role of 2H8F1$^+$ Ig however is still unclear, as different outcomes were observed in the animals that received this fraction. One monkey had a fulminant infection, whereas the other one had a prolonged infection at a high parasite density, a very unusual outcome in this experimental model. The 2H8F1$^+$ antibodies constitute a minor proportion of total Ig in a *Saimiri* serum. We do not know whether mAb 2H8F1, which does not react with human Ig, identifies a specific immunoglobulin class or subclass or whether it identifies a common idiotype. We have shown here that the 2H8F1$^+$ Ig react with the surface of the R-parasitized red blood cells. An intriguing possibility would be that the variant determinants located on the surface of infected-erythrocytes elicit an immune response restricted to this specific immunoglobulin type.

We thank Prof. L. Pereira da Silva for constant interest and suggestions. We are indebted to D. Taylor for her gift of the anti-PfHRPI mAb 89. We also thank S. Bonnefoy for frequent help and discussions; A. Scherf for providing C32 cells; K. Hinterberg for providing the HRPI oligonucleotides; as well as P. David and C. Roth for their valuable help and discussions; and V. Snewin and C. Grillot-Courvalin for helpful suggestions.

This work was supported by grant 92 S0032 from the Ministère de la Recherche et de l'Espace and grant 931302 from the Institut National de la Santé et de la Recherche Médicale.

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Received for publication 28 April 1994 and in revised form 1 August 1994.

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