

ANTIGENIC VARIATION OF CLONED  
*PLASMODIUM FRAGILE* IN ITS NATURAL HOST  
*MACACA SINICA*

Sequential Appearance of Successive Variant Antigenic Types

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Increasing knowledge in the field of primate and human malaria has shown the extent of the antigenic diversity that occurs in the asexual erythrocytic stages of *Plasmodium*. Such plasticity of the parasite's antigenic makeup could explain the slow development and incomplete nature of the protective immune response against malaria. Naturally occurring (S antigen [1], 195 kD antigen of *Plasmodium falciparum* [2]) and induced (HRP/K<sup>+</sup> antigen of *P. falciparum* [3, 4], 140 kD antigen of *P. knowlesi* [5]) antigenic polymorphism has been described. Evidence was at first acquired with an uncloned line of a primate malaria parasite, *P. knowlesi* in an experimental host, the rhesus monkey, indicating that antigenic variation was one of the mechanisms that contributed to the antigenic heterogeneity of the parasite (6). This was later confirmed with a cloned line of the parasite (7). Evidence was also obtained in favor of the existence of antigenic variation on the surface of *P. falciparum*-infected erythrocytes (8) in an experimental host, the squirrel monkey; however, this study was performed with uncloned parasites. In the present study, we have explored antigenic variation in a natural host-parasite association, *P. fragile* in the toque monkey. Using a cloned line of the parasite, we have demonstrated, that antigenic variation occurred during the spontaneous evolution of infection, and that the different variant antigenic types (VATs)<sup>1</sup> followed one another in a sequential order.

### Materials and Methods

*Animals.* The toque monkey *Macaca sinica*, the natural host of *P. fragile* in Sri Lanka was used in this study. Wild-caught animals, both male and female, 3–5 kg, were quarantined, screened for malaria, and those that had no previous exposure to malaria were used for experiments.

Splenectomies were performed using a standard sterile technique, taking care in identifying and removing all accessory spleens. Spleen-intact animals will be referred to as S<sup>+</sup>, and splenectomized animals as S<sup>-</sup> animals.

*Parasites.* A cloned strain of *P. fragile*, kindly provided by Dr. J. H. Leech (Malaria Division, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious

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<sup>1</sup>Abbreviations used in this paper: NMS, normal monkey serum; PE, parasitized erythrocyte; SIFT, surface immunofluorescence test; VAT, variant antigenic types.

Diseases, National Institutes of Health, Bethesda, MD) was used throughout this study. Two lines of the parasite were derived, each by three consecutive serial passages, one in  $S^+$  and the other in  $S^-$  animals. Splenectomized animals were inoculated within 6–12 d after splenectomy. The parasites were maintained either by cryopreservation (9) or by blood passage, the  $S^+$  line in spleen-intact animals, and the  $S^-$  line in splenectomized animals. Animals were infected by intravenous inoculation of parasitized erythrocytes. Infected blood was obtained by femoral puncture of anesthetized animals.

Short-term cultures of parasitized erythrocytes (PE) were maintained for one or two cycles by the candle-jar method (10) using normal toque monkey serum (NMS). Before culture, leucocytes were removed by passing infected blood through a column of CF<sub>11</sub> cellulose powder (Whatman Ltd., Kent, United Kingdom) (11).

*Hyperimmune Sera.* Three  $S^+$  animals were immunized as follows: Animals were infected with parasites from the  $S^+$  line, and once the parasitemia reached 1–5%, the infection was cured with chloroquine treatment. The animals were challenged by three intravenous inoculations of  $10^6$ – $10^7$  autologous live parasites at 2-wk intervals. Hyperimmune sera were collected 7–10 d after the last challenge. Hyperimmune sera were obtained from three  $S^-$  animals using the same immunization schedule but using parasites of the  $S^-$  line.

*Convalescent Sera.* Convalescent sera were collected from seven  $S^+$  animals during the course of parasitemia of untreated infections, after the primary parasitemia peak and after each recrudescence (Fig. 1). All sera were heat inactivated (30 min at 56°C). The IgG fraction was separated from sera using protein A–Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) (12).

*Surface Immunofluorescence Test (SIFT).* A modification of the technique for surface immunofluorescence (SIF) using fresh unfixed schizont-infected erythrocytes (13) was used as follows: 100  $\mu$ l of each dilution of whole serum or the IgG fraction (6–12 mg/ml) was incubated with 5  $\mu$ l of packed erythrocytes for 30 min, and centrifuged (MSE Scientific Instruments, Sussex, United Kingdom) for 5 min at 500 *g*. The pellet was washed twice in 100  $\mu$ l of medium and incubated with 100  $\mu$ l of a 1:20 dilution of fluorescein-conjugated IgG fraction of rabbit anti-monkey IgG (Cappel Laboratories, Cochranville, PA) for 30 min. All incubations were performed at room temperature (27°C). Culture medium RPMI 1640 containing 10% FCS was used for all dilutions and washing procedures. The cells were centrifuged as before and the pellet was washed twice in 100  $\mu$ l, and resuspended in 40  $\mu$ l of medium. A drop of this suspension was examined under  $\times 100$  magnification of a Leitz Diaplan Microscope. Infected erythrocytes were recognized under phase-contrast optics by the presence of malaria pigment. The indirect immunofluorescence test (IFT) using acetone-fixed thin films of schizont-infected erythrocytes as antigen (14) was also performed using the same sera and conjugate as above.

*Assay for Intraerythrocytic Growth Inhibition.* Infected erythrocytes containing ring stages (at  $\sim 5$  h of erythrocytic development) of the parasite were obtained from  $S^+$  and  $S^-$  animals that had synchronous infections, and cultured in vitro at a 10% hematocrit in culture medium containing 10% NMS. At 18–20 h of erythrocytic development the culture medium was replaced with one containing either immune serum at 5  $\mu$ l/ml or the IgG fraction of immune serum at 0.3–0.5 mg/ml, and the hematocrit was decreased to 2–3%. Thin blood films were made in duplicate from each culture plate 24 h later when parasites in normal culture medium had reached the schizont stage of development. The total parasitemia and the percentage of schizonts in each culture plate were assessed by microscopic examination of Giemsa-stained thin blood films.

## Results

*Natural Infections of P. fragile in Macaca sinica.* *P. fragile* causes a nonlethal infection in its natural host the toque monkey *M. sinica*. Untreated infections consisted of a primary parasitemia peak ( $1.0 \pm 0.13\%$ ) and a series of smaller recrudescences in which the parasitemia did not exceed 0.3%. Periods of subpatency (parasitemia  $<0.004\%$ ) intervened between these parasitemia peaks (see Fig. 1, first graph).

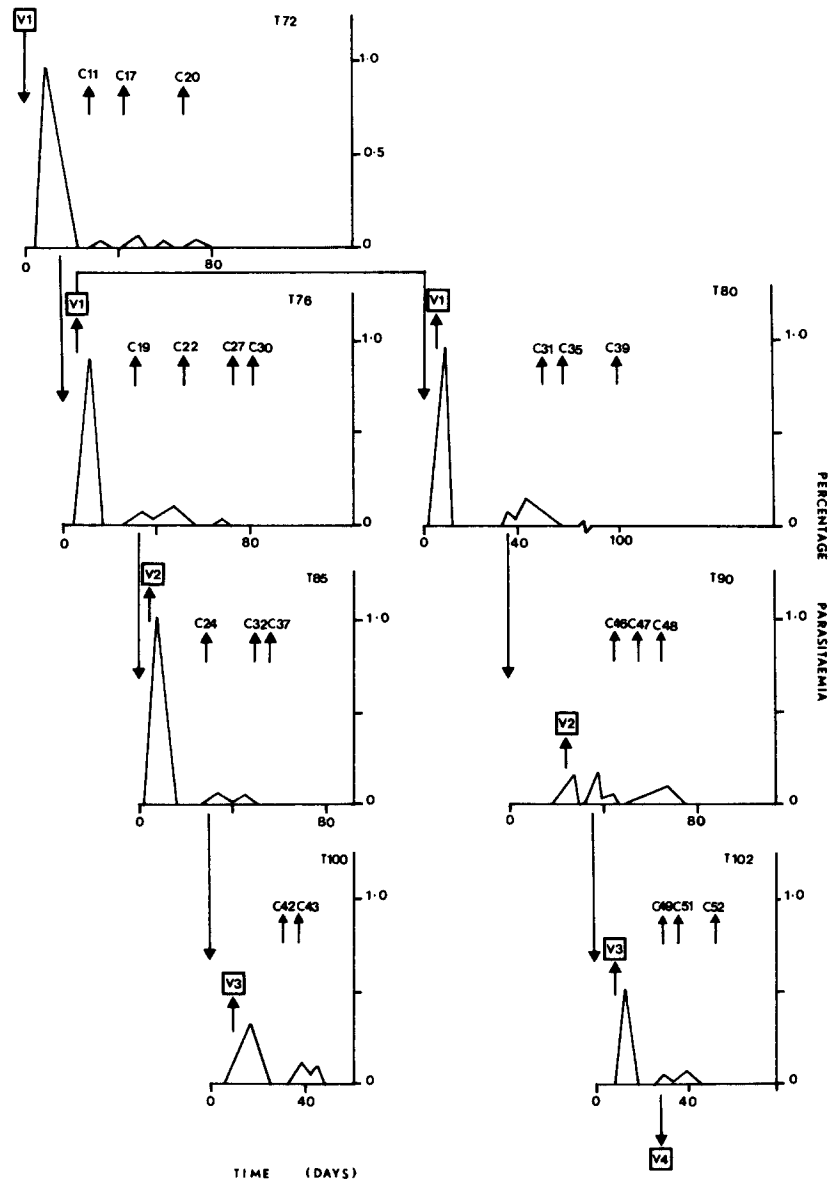


FIGURE 1. Sequential blood passage ( $\downarrow$ ) of the  $S^+$  line of *P. fragile* in  $S^+$  animals. The parasitemia was monitored daily in each animal, and sera (C 11–C 52) were collected at various times ( $\uparrow$ ) during the course of untreated infections. V1, V2, V3, and V4 indicate the variant antigenic type of each parasite population.

*Surface Antigens on Infected Erythrocytes.* Surface immunofluorescence of erythrocytes infected with mature stages (late trophozoites and schizonts) of the  $S^+$  line of the parasite was obtained with hyperimmune sera from  $S^+$  animals. Distinct, fine fluorescent dots were seen throughout the erythrocyte membrane; these dots were not confined to the area overlying the parasite (Fig. 2). >80% of the trophozoite- and schizont- infected erythrocytes (recognizable by the

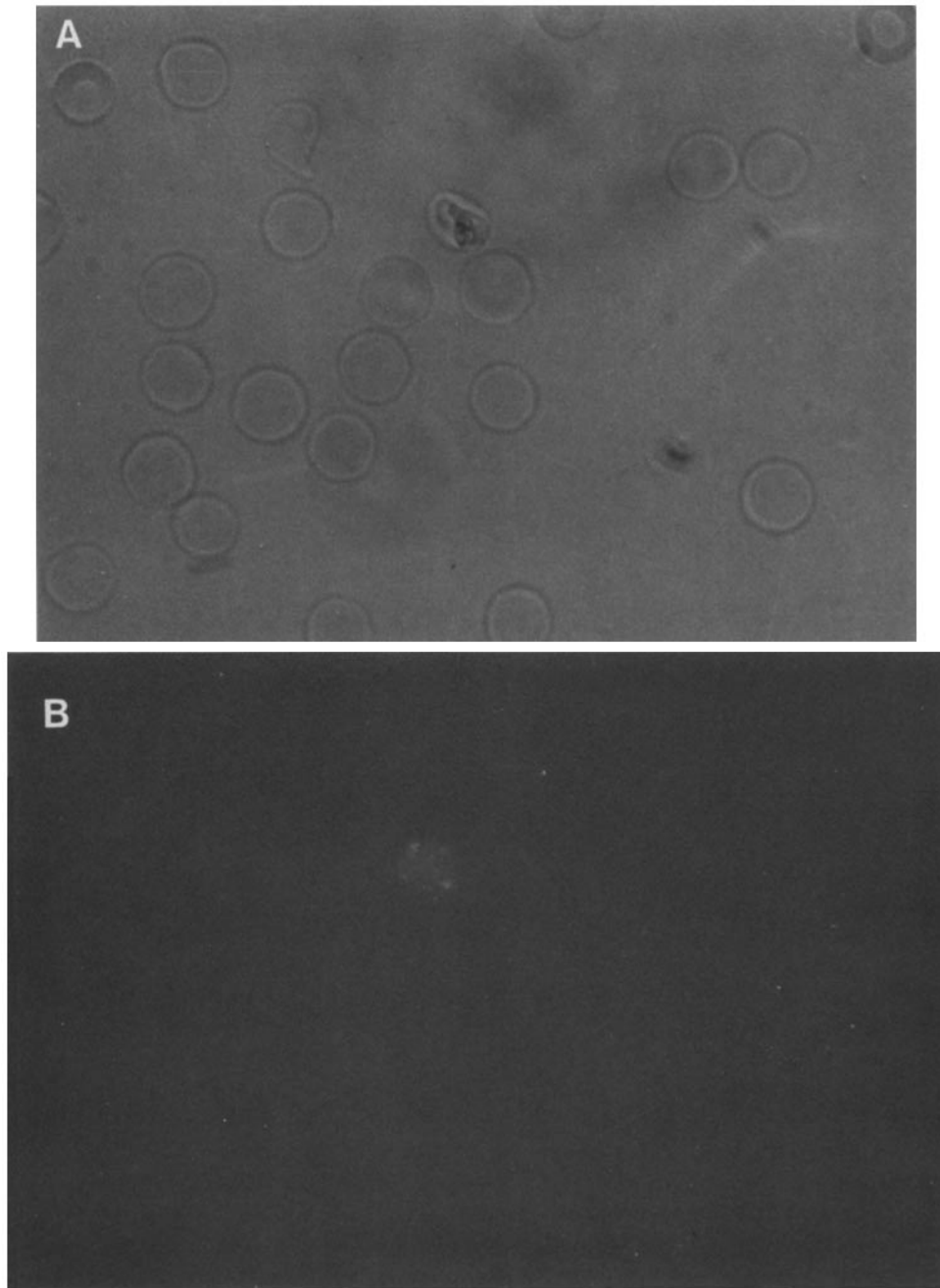


FIGURE 2. Surface immunofluorescence of PE of the  $S^+$  line of the parasite. Infected erythrocytes containing late trophozoites and schizonts were incubated with  $S^+$  hyperimmune serum followed by fluorescein-conjugated rabbit anti-monkey IgG. A pigment-containing late trophozoite/schizont-infected erythrocyte in an unfixed erythrocyte suspension examined under normal light (A) and the same field ( $\times 1,250$ ) under ultraviolet light (B), where the infected erythrocyte shows surface immunofluorescence.

TABLE I  
*Surface Immunofluorescence Titers of Distinct Parasite Populations  
 in Three S<sup>+</sup> Animals with Convalescent Sera*

Serum		Parasites from primary parasitemia peak of animal:		
Animal	Code	T72	T76	T85
T72	C 11	+	+	-
T76	C 19	+	+	-
T85	C 24	-	ND	+

\* +, Positive at a titer of 1:5; -, negative at a titer of 1:5.

visible pigment) gave a positive SIF, up to a titer of 1:125 of S<sup>+</sup> hyperimmune serum.

*Differences in Antigenicity in Parasites Obtained from Primary and Secondary Parasitemia Peaks.* The antigenic type of parasite populations of the primary parasitemia peak and the first recrudescence were investigated using the technique of surface immunofluorescence of PE with immune sera specific to each distinct parasite population. Parasites from the first recrudescence were studied by passaging them into a naive nonimmune animal and thus obtaining immune serum specific to the antigenic type of the recrudescence parasite population.

PE from the primary parasitemia peak of T72 were passaged into T76, and from the first recrudescence of T76 into T85 (Fig. 1). The SIF results of live unfixed PE from the primary parasitemia peak of T72, T76, and T85 with convalescent sera obtained after the primary peak of these animals C 11, C 19, and C 24 are shown in Table I. Convalescent sera C 11 and C 19 of animals T72 and T76, respectively, reacted positively with PE of primary parasitemia peaks of both animals, but neither sera reacted with PE of the primary peak of T85, indicating that the antigenic types of primary peaks of T72 and T76 were identical. This demonstrated that when PE were passaged into a naive nonimmune animal they retained their antigenic type during the primary parasitemia peak in the latter host.

Further, convalescent serum C 24 of animal T85, which reacted positively with PE of the primary parasitemia peak of T85, did not react with PE of the primary peak of T72 (Table I). Because parasites of the primary peak of T85 were those passaged from the first recrudescence of T76, these results demonstrate that surface antigens of PE of the first recrudescence (of T76) were different from those of PE of the primary peak. Thus, this cloned parasite undergoes antigenic variation during the course of an untreated natural infection.

*Sequential Appearance of Variant Parasite Antigens.* The appearance of VATs during an untreated infection was studied in two sets of experiments. In one, parasites were passaged from the primary parasitemia peak of T72 into T76, from the first recrudescence of T76 into T85 as described before, and from the first recrudescence of T85 into T100 (Fig. 1). Similarly another parallel set of experiments were initiated by passaging parasites from the primary peak of T76 into T80 (Fig. 1). The parasites from the first recrudescence of T80 was passaged into T90 and from the first recrudescence of T90 into T102. The SIF results of live unfixed PE from the primary parasitemia peak of each animal with conva-

TABLE II  
*Surface Immunofluorescence Titers of Live Unfixed Schizont-infected Erythrocytes Obtained from the Primary Parasitemia Peak of Six S<sup>+</sup> Animals with Antisera of Seven S<sup>+</sup> Animals Collected at Various Times during the Course of Infection*

Serum		Surface immunofluorescence titers (reciprocals) for antigens:						
Animal	Code	T72	T80	T85	T90	T100	T102	T102*
T72	C 11	5	25	0 <sup>‡</sup>	0	0	0	ND
	C 17	25	125	25	5	0	0	ND
	C 20	125	250	125	25	25	25	ND
T76	C 19	5	5	0	0	0	0	0
	C 22	25	125	5	5	5	0	0
	C 27	125	125	25	25	5	25	0
	C 30	ND	ND	ND	ND	ND	ND	5
T80	C 31	5	5	0	0	0	0	0
	C 35	25	125	5	5	0	0	0
	C 39	125	125	25	25	25	25	5
T85	C 24	0	0	5	5	0	0	0
	C 32	0	0	25	5	5	5	0
	C 37	0	0	25	25	5	25	5
T90	C 46	0	0	5	ND	0	ND	0
	C 47	0	0	25	ND	5	ND	5
	C 48	0	0	125	ND	125	ND	5
T100	C 42	0	0	0	0	5	5	0
	C 43	0	0	0	0	25	25	5
T102	C 49	0	0	0	ND	5	ND	0
	C 51	0	0	0	ND	25	ND	5
	C 52	0	0	0	ND	125	ND	25

All sera, C 11–C 52 (see Fig. 1) were tested at four dilutions 1:5, 1:25, 1:125, and 1:250.

\* 1R parasites obtained from the first recrudescence of T102.

‡ 0, Negative at a titer of 1:5.

lescent sera collected after the primary peak and each recrudescence are shown in Table II.

Convalescent sera C 11, C 19, and C 31 of animals T72, T76, and T80, respectively, reacted specifically in SIF with PE from the primary parasitemia peaks of T72 and T80, but not with that of any other animal presented in Fig. 2 (Table II) indicating that the antigenic types of the primary peaks of T72, T76, and T80 were identical, and that they differed from other antigenic types. This antigenic type was designated as VAT V1, and convalescent sera were defined as V1 variant-specific sera.

Convalescent sera C 24 and C 46, of animals T85 and T90, respectively, reacted with PE of the primary peaks of both animals, but not with PE from the primary peaks of other animals. Thus the antigenic types of the primary peaks of T85 and T90, which were parasites passaged from the first recrudescence of T76 and T80, respectively, were identical, and differed from the VAT V1. This new VAT of the primary peaks of T85 and T90 was designated VAT V2.

In animals T100 and T102, their convalescent sera (C 42 and C 49, respectively) reacted with PE of the primary peaks of both animals, but not with VAT V1 or V2. Thus the PE of the primary peak of T100 and T102, which were parasites passaged from the first recrudescence of T85 and T90, respectively, had the same VAT, which was different from VAT V1 and V2 and was designated VAT V3.

The PE of the first recrudescence of animal T102 consisted of a distinct VAT that did not react positively with VAT V1-, V2-, or V3-specific sera, but reacted positively with its own convalescent serum, C 51. This VAT was designated VAT V4.

In these two sets of experiments the same sequential order of appearance of VATs were detected, VAT V1 followed by V2, followed by V3, followed by V4. These results demonstrate that, in natural untreated infections, parasites of the primary parasitemia peak and of each recrudescence contain different VATs, and that they appear in a definite sequential order. This order of appearance is maintained when the sequence is interrupted by passaging a VAT in the middle of the sequence.

Convalescent sera obtained from these animals at various times during the course of parasitemia and in relation to the appearance of recrudescences were tested by SIF with parasites of known VATs V1, V2, and V3 (Table II). The series of convalescent sera obtained from each animal reacted with the VATs corresponding to the order of appearance of variants, e.g., in T76, in which the primary peak was shown to be VAT V1, C 19, the first convalescent serum reacted only with PE of VAT V1. The second convalescent serum, C 22, which was obtained after the appearance of the first recrudescence, reacted with VAT V1 and also with VAT V2. A later convalescent serum C 27, which was obtained after the appearance of the second recrudescence, reacted with VAT V1, V2, and also with V3, corroborating the evidence that during the course of parasitemia in the host, parasites undergo antigenic variation in a sequential order of VAT V3 following V2, and V2 following V1.

*Spleen Dependency of Variant Antigens.* Schizont-infected erythrocytes of S<sup>+</sup> and S<sup>-</sup> lines of the parasite were tested by surface immunofluorescence with hyperimmune sera obtained from three S<sup>+</sup> and S<sup>-</sup> animals. Infected erythrocytes of the S<sup>+</sup> line gave a positive surface immunofluorescence with hyperimmune sera from all three S<sup>+</sup> immune animals and the IgG fraction of these sera, but did not react with hyperimmune sera or the IgG fraction of hyperimmune sera from any of the three S<sup>-</sup> animals (Table III). Infected erythrocytes of the S<sup>-</sup> line did not react with hyperimmune sera from either S<sup>+</sup> or S<sup>-</sup> immune animals.

IFT using acetone-fixed thin films of PE as antigen, which detect both surface as well as internal antigens, gave similar titers of 1:2,560 and 1:5,120 with hyperimmune sera from S<sup>+</sup> and S<sup>-</sup> animals, respectively, indicating that the antibody response to immunizing infection was quantitatively comparable in both S<sup>+</sup> and S<sup>-</sup> animals. These immunofluorescence data indicate that surface antigen(s) can only be detected in the S<sup>+</sup> line of the parasite.

The influence of the spleen on the expression of surface antigens was further explored by transferring parasites of the S<sup>-</sup> line into S<sup>+</sup> animals, and of the S<sup>+</sup> line into S<sup>-</sup> animals. A large inoculum ( $2-5 \times 10^9$ ) of ring-infected erythrocytes of the S<sup>-</sup> line were inoculated intravenously into each of four S<sup>+</sup> animals so that

TABLE III  
*Surface Immunofluorescence Titers of PE of the S<sup>+</sup> and S<sup>-</sup> Lines of the Parasite with S<sup>+</sup> and S<sup>-</sup> Hyperimmune Sera*

Immunized animal	Serum	Antigen	
		PE of the S <sup>+</sup> line	PE of the S <sup>-</sup> line
T43/S <sup>+</sup>	1 24	1:125	0*
T47/S <sup>+</sup>	1 25	1:125	0
T63/S <sup>+</sup>	1 51	1:125	0
T34/S <sup>-</sup>	1 11	0	0
T60/S <sup>-</sup>	1 40	0	0
T64/S <sup>-</sup>	1 41	0	0

Sera were used at four dilutions: 1:5, 1:25, 1:125, and 1:250.

\* 0, Negative at a titer of 1:5.

the parasitemia became patent in the recipient S<sup>+</sup> animals immediately after inoculation. 48 h later, ring stage parasites from the next developmental cycle were obtained, cultured in vitro up to the schizont stage and the SIFT was performed with S<sup>+</sup> hyperimmune sera. Surface antigens were detected on >80% of schizont-infected erythrocytes, indicating that the switch in expression of surface antigens had occurred within two erythrocytic cycles when nonexpressing parasites of the S<sup>-</sup> line were transferred to an S<sup>+</sup> animal.

Similarly, four splenectomized animals were each inoculated with  $2.5 \times 10^8$  i.v. ring-infected erythrocytes of the S<sup>+</sup> line. Ring-infected erythrocytes obtained from each developmental cycle in the recipient S<sup>-</sup> animals were cultured in vitro up to the schizont stage and the SIFT was performed with S<sup>+</sup> hyperimmune serum. In three of the recipient S<sup>-</sup> animals, surface antigens were still detectable in 80–95% of schizont-infected erythrocytes during the second and third developmental cycles. In the fourth animal, the number of schizont-infected erythrocytes positive for surface immunofluorescence had dropped to 50% by the third developmental cycle. Gradually both the percentage of PE positive for surface immunofluorescence and the intensity of the fluorescence decreased in all animals. By the seventh developmental cycle in the S<sup>-</sup> host only 20% of PE, and by the 24th developmental cycle <10% of PE were positive for SIF.

*Antigenic Variation in the S<sup>-</sup> Line of the Parasite.* The course of infection of the S<sup>-</sup> line of the parasite in the splenectomized host consisted of a high primary parasitemia peak (parasitemia  $21.6 \pm 9.1\%$ ; mean  $\pm$  SE) followed by successive peaks of low parasitemia; here, unlike in the case of S<sup>-</sup> animals, the peaks were not always separated by periods of subpatency but by very low patent parasitemias. Surface antigens were not expressed on infected erythrocytes of the S<sup>-</sup> line of the parasite, but as mentioned earlier, when transferred into a naive nonimmune S<sup>+</sup> animal, surface antigens were expressed in the recipient S<sup>+</sup> animal by the second erythrocytic cycle (parasitemia in the first cycle was too low to perform the SIFT). This phenomenon of switching from nonexpression to expression of surface antigens within two erythrocytic cycles when transferred from an S<sup>-</sup> to an S<sup>+</sup> animal was used to explore antigenic variation in the S<sup>-</sup> animal, as follows.

Two sets of experiments were initiated by inoculating two splenectomized animals, T148 and T156, with parasites of the S<sup>-</sup> line (Fig. 3A) from a single



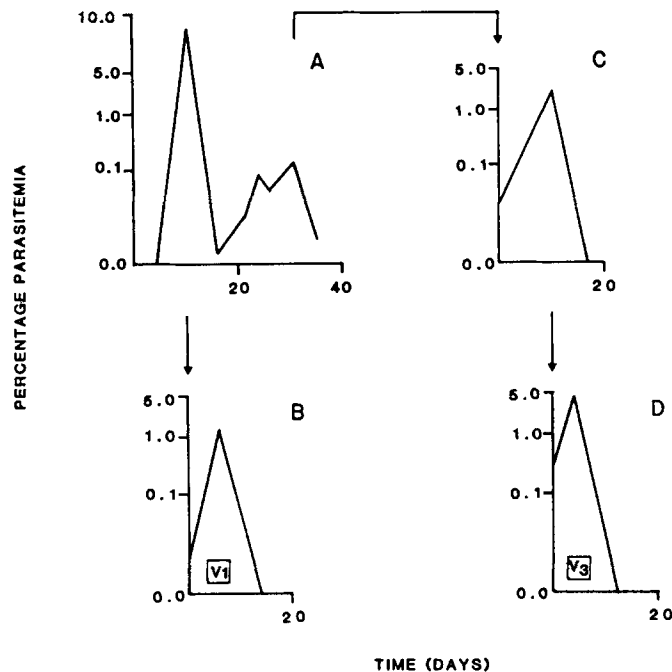


FIGURE 3. Passage of PE from the primary parasitemia peak and recrudescences of infected  $S^-$  animals to naive  $S^+$  animals. Two  $S^-$  animals, T148 and T156 (A), were inoculated intravenously with cryopreserved parasites of the  $S^-$  line. PE from the primary peak of these two  $S^-$  animals was passaged ( $\downarrow$ ) into two  $S^+$  animals, T140 and T163 (B). PE from the second recrudescence of the two  $S^-$  animals were passaged each through two  $S^-$  animals, T149 and T158, respectively (C) into two  $S^+$  animals, T142 and T161, respectively (D). The VAT expressed in the  $S^+$  animals are indicated in the squares ( $V_n$ ).

cryopreserved source. Parasites from the primary parasitemia peak of the two  $S^-$  animals were passaged into two naive nonimmune  $S^+$  animals (Fig. 3B), T140 and T163, respectively, using a large inoculum so that the infection became patent immediately in the recipient animals. The SIFT was performed on PE from the primary parasitemia peak of the recipient  $S^+$  animals, using variant-specific sera. PE from both animals only reacted with VAT V1-specific serum and not with VAT V2- or V3-specific sera. Thus, parasites from both animals had the potential, when transferred into an  $S^+$  animal, to switch to an expression characteristic of VAT V1.

We then transferred PE from the second recrudescence of T148 and T156 each through another  $S^-$  host (T149 and T158, respectively) (Fig. 3C) into an  $S^+$  host, T142 and T161, respectively (Fig. 3D). The intermediate  $S^-$  hosts T149 and T158 were used to amplify the number of parasites of the recrudescences, so that a large inoculum could be passaged into the  $S^+$  hosts, enabling the parasitemia to become patent immediately. The SIFT was performed on PE from the primary peak of the two recipient  $S^+$  animals T142 and T161 (Fig. 3D) using variant-specific sera. PE from T142 and T161 reacted only with VAT V3-specific serum and not with antisera of any other specificity, indicating that PE from the second recrudescence of the  $S^-$  animal T148 and T156 had varied in its potential VAT from V1, through the second recrudescence, to V3. Thus mechanisms leading to antigenic variation do occur in the  $S^-$  animal although

TABLE IV  
*Effect of Immune Serum on Intraerythrocytic Development of Parasites of the S<sup>+</sup> and S<sup>-</sup> Lines*

Serum	Percentage of schizonts*	
	PE of the S <sup>+</sup> line	PE of the S <sup>-</sup> line
M 20	66.00 ± 6.33	67.00 ± 3.79
I 25	25.00 ± 10.00	63.33 ± 3.29
I 40	62.27 ± 2.68	64.33 ± 4.38

Early trophozoites of S<sup>+</sup> and S<sup>-</sup> lines were cultured with S<sup>+</sup> (I 25) and S<sup>-</sup> (I 40) hyperimmune sera at 5 µl/ml and with normal monkey serum (M 20). The percentage of schizonts was assessed after 24 h.

\* Mean ± SE of three experiments.

the variant antigens do not appear either to be expressed on the surface of PE (S<sup>+</sup> or S<sup>-</sup> hyperimmune sera do not react with PE of the S<sup>-</sup> line) or occur internally (variant-specific antibodies cannot be detected in S<sup>-</sup> hyperimmune sera).

*Effect of Hyperimmune Serum on the Intraerythrocytic Development of S<sup>+</sup> and S<sup>-</sup> Lines of the Parasite.* The effect of hyperimmune sera of S<sup>+</sup> and S<sup>-</sup> animals on the intraerythrocytic development of S<sup>+</sup> and S<sup>-</sup> lines of the parasite was assessed in in vitro cultures to investigate the possible significance in protective immunity of these spleen-dependent surface antigen(s). The results of these experiments, summarized in Table IV, demonstrate that S<sup>+</sup> hyperimmune serum had an inhibitory effect on the intracellular development of the S<sup>+</sup> line of the parasite, arresting development at the late trophozoite stage (Fig. 4), resulting in a 62% reduction in the number of schizonts, as compared with development in normal monkey serum. This S<sup>+</sup> hyperimmune serum however had no effect on the intraerythrocytic development of parasites of the S<sup>-</sup> line. Further, S<sup>-</sup> hyperimmune serum had no effect on the development of parasites of either the S<sup>+</sup> or S<sup>-</sup> line.

*Effect of Variant-specific Sera on the Intraerythrocytic Development of VATs of Parasites of the S<sup>+</sup> Line.* The effect of variant-specific sera on the intraerythrocytic development of two VATs, V1 and V3 of the S<sup>+</sup> line of the parasite, is shown in Table V. Development of parasites of VAT V1 was specifically inhibited by V1 variant-specific serum C 72, but not by V3 variant-specific serum C 43. Also, the development of parasites of VAT V3 was specifically inhibited by V3 variant-specific serum, but not affected by V1 variant-specific serum. The IgG fraction separated from the variant-specific sera had the same specific growth-inhibitory effects as the respective sera.

The S<sup>+</sup> hyperimmune sera I 51 and I 25, which reacted by SIF with both VATs V1 and V3 and the IgG purified from these sera inhibited the intraerythrocytic development of both VAT V1 and V3.

### Discussion

The natural infection of the toque monkey by *P. fragile* includes at first the appearance of a major peak of parasitemia followed by a chronic evolution of the infection in the form of several successive smaller parasitemia peaks. Immunofluorescence using unfixed erythrocytes in suspension has shown that antigens

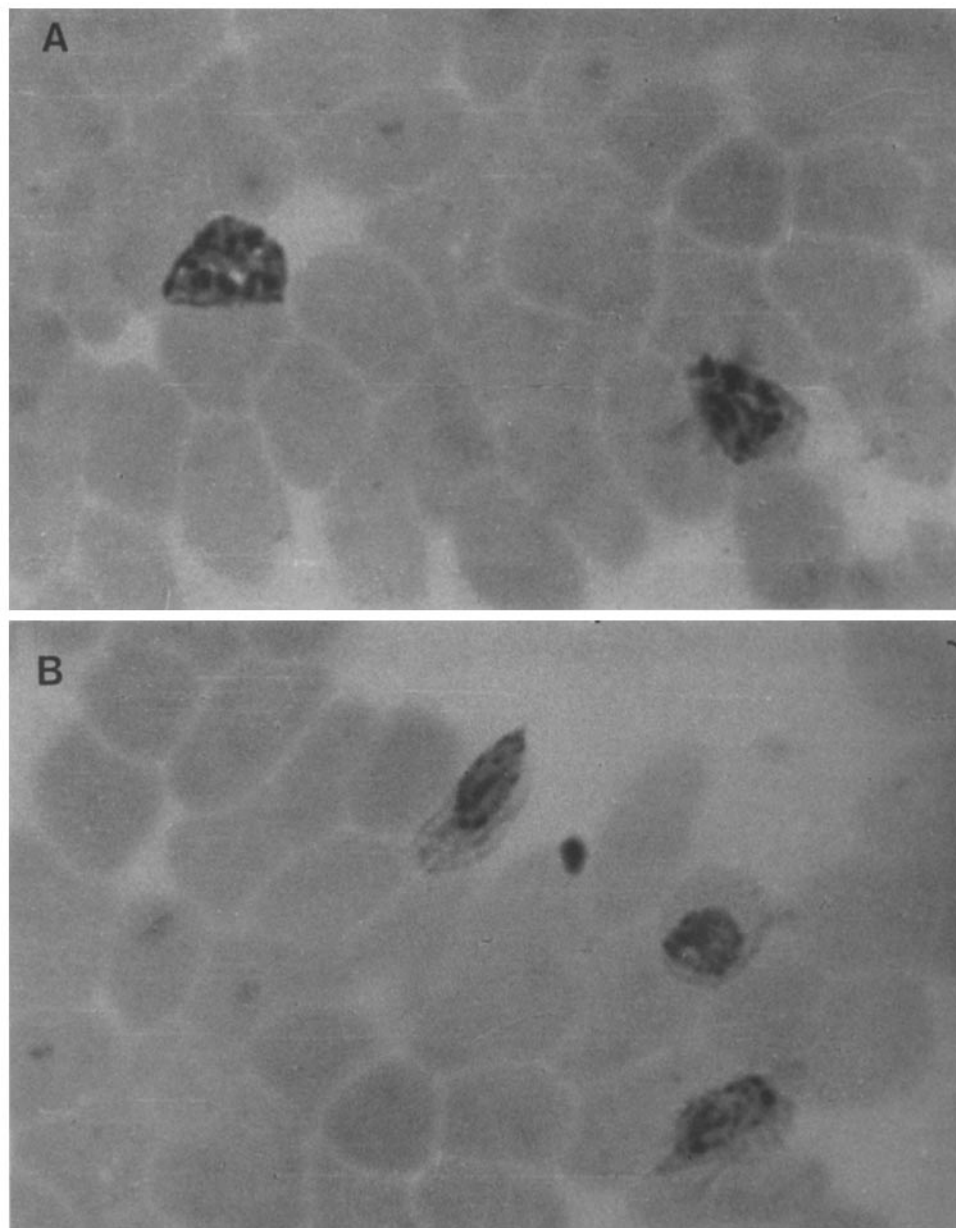


FIGURE 4. Inhibition of intracellular growth of parasites of the  $S^+$  line by immune serum. PE containing early trophozoite stages (18–20 h of growth; 1–2% parasitemia) of  $S^+$  line were cultured in  $S^+$  hyperimmune serum I 25 and  $S^-$  hyperimmune serum I 40 at 5  $\mu$ l/ml. The percentage parasitemia and percentage of schizonts were assessed after 24 h by microscopic examination of Giemsa-stained thin blood films. Parasites developed into schizont stage in cultures containing  $S^-$  hyperimmune serum (A) and growth was arrested at late trophozoite stage in cultures containing  $S^+$  hyperimmune serum (B).  $\times$  2,500.

are recognized by immune serum on the surface of the erythrocytes infected with more mature stages of the parasite. These antigens undergo variation, each successive peak of parasitemia being characterized by a different variant antigenic

TABLE V  
*Effect of Variant-specific Sera on Intracellular Development of Two VATs  
of the S<sup>+</sup> Line of the Parasite*

VAT	Exp.	Percent inhibition* of parasite development							
		C 72		C 43		I 51		I 40	
		Serum	IgG	Serum	IgG	Serum	IgG	Serum	IgG
V1	I	39.98 ± 3.48 <sup>†</sup>	37.10 ± 4.89	-16.04 ± 6.46	-1.39 ± 2.69	49.69 ± 3.46	44.16 ± 4.16	9.91 ± 3.17	3.39 ± 3.16
	II	34.17 ± 2.73	24.46 ± 1.16	-5.96 ± 2.75	-3.36 ± 1.64	40.44 ± 2.54	50.01 ± 4.24	-6.84 ± 3.41	-4.31 ± 2.63
V3	III	6.41 ± 2.47	4.76 ± 4.27	55.75 ± 4.67	62.15 ± 1.72	49.37 ± 1.44	59.18 ± 1.36	6.23 ± 1.15	8.37 ± 2.81
	IV	ND	ND	49.17 ± 7.91	ND	35.67 ± 5.57	ND	-3.33 ± 3.16	ND

Sera were used at 5  $\mu$ l/ml with NMS M 20; C 72, V1-specific; C 43, V3-specific; I 51, S<sup>+</sup> hyperimmune serum; I 40, S<sup>-</sup> hyperimmune serum.

\* Percent inhibition = 100  $\times$  (percent schizonts in NMS or IgG) - (percent schizonts in test serum or IgG)/(percent of schizonts in NMS or IgG).

<sup>†</sup> Mean  $\pm$  SE, n = 6.

type (VAT). The appearance of the successive VATs occurs in a sequential manner, following the same order in different sets of animals. This constitutes the first example of a sequential expression of antigens in a malaria parasite; it indicates that in *P. fragile* antigenic variation is not the result of random mutations selected by antibody. In cloned trypanosomes, variant surface antigens are known to appear in a sequential manner during the course of an infection (15-17). However, in contrast with the situation in trypanosomes where the mechanisms of antigenic variation have been extensively studied (reviewed in 18), the mechanisms of antigenic variation in *Plasmodium* remain largely unknown.

The presence of the spleen has been shown to play a major role in the expression of malarial antigens on the surface of infected erythrocytes. In the case of *P. falciparum* in the squirrel monkey, a given cloned parasite expresses different antigens on the surface of the erythrocyte in a spleen-intact and in a splenectomized animal (8). During the infection of the rhesus monkey by *P. knowlesi*, the expression of the variant antigens on the surface of the schizont-infected erythrocyte (schizont-infected cell agglutination [SICA] antigens) is dependent on the presence of the spleen; parasites from a splenectomized host no longer express detectable SICA antigen on the surface of schizont-infected erythrocytes (7). This is comparable to what we have shown to occur in the infection of the toque monkey by *P. fragile*, where antigens can no longer be detected on the surface of erythrocytes in splenectomized animals. The observation that antibody recognizing the surface of infected erythrocytes from intact animals are not found in hyperimmune sera from three splenectomized animals indicates that VAT antigens are not only not expressed on the surface of the erythrocyte but are either nonimmunogenic or absent from any other part of the parasitized erythrocyte in the splenectomized animal. Thus the parasite exists as two distinct phenotypes, S<sup>+</sup> and S<sup>-</sup>, in relation to the expression of surface antigens. The fact that they can switch from nonexpression to expression of surface antigens within two erythrocytic cycles when transferred from an S<sup>-</sup> to an S<sup>+</sup> host environment favors the idea that the spleen modulates the expression of these antigens rather than that the two phenotypes are the outcome of the selection of mutant populations.

The absence of expression in the S<sup>-</sup> line of the parasite contrasts with the

observation that events leading to changes in the VAT can occur in the splenectomized animals. When a splenectomized animal is inoculated with S<sup>-</sup> parasites of potential VAT V1 (these were parasites of the nonexpressing S<sup>-</sup> line, which when transferred to an S<sup>+</sup> animal express VAT V1), a chronic infection occurs, made of successive peaks of low-grade parasitemia as in a spleen-intact animal except that antigens are not expressed on the surface of the infected erythrocyte. When parasites are isolated from the third peak of parasitemia of the splenectomized animal and inoculated into a spleen-intact animal, antigens are reexpressed on the surface of the infected erythrocytes isolated from the first peak of parasitemia (within two parasite cycles), and these antigens are shown to be of VAT V3 type. This shows that, although the presence of the spleen is necessary for the expression of the variant antigen(s) on the surface of the infected erythrocyte, the process(es) leading to variation takes place in the absence of the spleen.

Intraerythrocytic development of *P. fragile* can be inhibited by antibody and appears to be associated with the presence of antigens on the erythrocyte membrane. Morphological evidence of growth inhibition is clear-cut, from total arrest of growth to abnormal pycnotic forms resembling crisis forms. Inhibitory effects of antibody on growth are not due to nonspecific toxicity, and indeed appear to be associated with the presence of antigens on the erythrocyte membrane in as much as antibody has no effect on the growth of parasites from splenectomized animals, which do not express antigen(s) on the surface of the infected erythrocyte, and inhibitory effects of antibody were variant specific. This suggests that it is the binding of antibody to variant antigens present on the surface of the infected erythrocytes that is responsible at least in part for inhibition of parasite development. Though several non-antibody-mediated mechanisms (19, 20) are known to inhibit intraerythrocytic growth of Plasmodial species, an antibody-mediated mechanism leading to arrest of intraerythrocytic growth (as for example by the interaction of antibody with antigens on the surface infected erythrocytes), has not been previously described.

Despite a striking immune-evasive parasite mechanism of antigenic variation, which we have demonstrated here, there is also evidence for host protection in *P. fragile*, in that antibodies in hyperimmune serum from animals repeatedly exposed to infection can react with the different VATs and block parasite development of all VATs tested; and in natural infections, parasitemias are eventually controlled and maintained at subpatent levels. It is possible that, after repeated exposure to parasites, certain antigens on the infected erythrocyte membrane can elicit an immune response capable of transcending antigenic variation, or that there is a limited repertoire of VATs in *P. fragile*, to all of which the host eventually acquires immunity.

### Summary

The course of infection of *Plasmodium fragile* in its natural host, the toque monkey *Macaca sinica*, consists of a primary peak of parasitemia followed by several distinct, successive peaks of lower parasitemia. In the S<sup>+</sup> host, the late intraerythrocytic asexual developmental stages of *P. fragile* induce the expression of antigens on the surface of infected erythrocytes, which could be detected using the technique of surface immunofluorescence.

Immunofluorescence using unfixed erythrocytes in suspension has shown that antigens are recognized by immune serum on the surface of the erythrocytes infected with more mature stages of the parasite. These antigens undergo variation, each successive peak of parasitemia being characterized by a different variant antigenic type (VAT). The appearance of the successive VATs occurs in a sequential manner, following the same order in different sets of animals. This constitutes the first example of a sequential expression of antigens in a malaria parasite; it indicates that, in *P. fragile*, antigenic variation is not the result of random mutations selected by antibody.

Parasite-induced antigens on the surface of infected erythrocytes could not be detected in the S<sup>-</sup> host. However, when nonexpressing parasites from the S<sup>-</sup> host were transferred by blood passage into a naive S<sup>+</sup> animal, they began to express antigens on the surface of infected erythrocytes within two erythrocytic cycles. We have demonstrated that the ability of S<sup>-</sup> parasites to switch to a particular VAT when passaged into a S<sup>+</sup> animal changes during the course of an infection in the S<sup>-</sup> animal, indicating that, although surface antigens are not expressed, the processes leading to antigenic variation occurs even in the S<sup>-</sup> host.

Antibodies directed against these surface antigens inhibit the growth of intra-erythrocytic parasites. The growth inhibition effects of antibodies are also variant specific, indicating that these variant surface antigens are functionally important for parasite survival.

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