Immunology, 1972, **23**, 503.

Antigenic Variation and Protective Immunity in *Plasmodium knowlesi* Malaria

G. A. Butcher and S. Cohen

Department of Chemical Pathology, Guy's Hospital Medical School, London, SE1 9RT

(*Received 2nd February* 1972)

Summary. Rhesus monkeys were immunized with defined strains and variants of *Plasmodium knowlesi* and their immunity on challenge was correlated with serum levels of schizont agglutinins and specific inhibitory antibody assayed by *in vitro* parasite culture. The results indicate that the inhibitory antibody provides a consistent index of immune status and probably represents the protective antibody which initiates specific immune reactions *in vivo*. The relationship between inhibitory and schizont agglutinating antibodies is discussed.

Inhibitory antibody is predominantly specific for those variants which have produced patent infections. However, antibody active against other variants is also present at lower titre and is associated with clinical immunity on challenge with such variants. The presence of this antibody could explain why *P. knowlesi* parasites, which arise by antigenic variation during the course of a chronic infection, produce mild parasitaemia in the host and yet are fully virulent in normal monkeys.

The occurrence of cross-immunization between variants and between some strains of *P. knowlesi* is encouraging from the point of view of malaria vaccine production. A preparation containing common antigens may induce a degree of clinical immunity comparable with that appearing during the course of chronic infection.

INTRODUCTION

Malarial immunity in many animal species, including the Rhesus monkey, becomes manifest very slowly during the course of the disease and is associated with persistence of the infecting organism as shown by the occurrence of periodic low-grade parasitaemia in clinically immune subjects and the induction of malarial relapse by splenectomy in immune animals. This form of non-sterilizing, relative immunity is thought to occur also in other protozoal infections and parasitologists have described it by such terms as 'premunition' and 'tolerance' (Sergent and Sergent, 1956). Several mechanisms may contribute to this phenomenon including the relatively poor immunogenicity of the plasmodium, the immunosuppressive effect of malarial infection (reviewed, Cohen and Butcher, 1971), the presence of enhancing antibody (Jerusalem, Weiss and Poels, 1971), and the ability of malaria parasites to evade the host's immune response by undergoing repeated antigenic variation (Brown and Brown, 1965).

A wide spectrum of antigenic variants of *P. knowlesi* is recognizable on the basis of the

Correspondence: Professor Sydney Cohen, Department of Chemical Pathology, Guy's Hospital Medical School, London, SE1 9RT.

schizont agglutination test described by Eaton (1938) which involves the interaction of antibody with antigen on the surface of those red blood cells which contain mature parasites (Brown and Brown, 1965). During chronic *P. knowlesi* infections each relapse was associated with a serologically distinct variant (Brown and Brown, 1965); in addition, infected animals challenged repeatedly with a given variant show progressive prolongation of the pre-patent period followed always by the appearance of a new variant as the patent infection (Voller and Rossan, 1969a). These observations suggest that antigenic variation is fundamental for parasite survival in the immunized host and constitutes the basic mechanism which underlies chronicity of infection (Brown, 1971). However, the ability of the infected host to limit proliferation of relapse variants known to be virulent in normal animals requires explanation. It has been suggested that chronic infection is associated with a variant-transcending immunity of undefined character (Brown and Brown, 1965; Voller and Rossan, 1969a; Brown, Brown and Hills, 1968) or that protective antibody is variant specific, but that common malarial antigens sensitize thymus-derived cells which enable the host to respond rapidly to new variants (Brown, 1971).

In a recent series of experiments we observed that serum from monkeys immune to *P. knowlesi* inhibited the cyclical proliferation of the parasite maintained *in vitro* (Cohen and Butcher, 1970, 1971; Cohen, Butcher and Crandall, 1969). A technique was used which gave near physiological multiplication rates in 24-hour cultures (Butcher and Cohen, 1971) and parasite growth was assessed by incorporation of ^{3}H-leucine into parasite protein. Pooled anti-sera had no effect upon the growth of intra-cellular parasites, but inhibited the cycle of growth which followed parasite division. This effect was not complement dependent and in the sera analysed was associated with IgG and IgM but not with IgA or IgE; the bivalent peptic fragments of IgG were inhibitory, but univalent Fab fragments were not (Cohen and Butcher, 1970). The similarity between malarial inhibitory antibody, which appeared to block cell reinvasion, and some viral neutralizing antibodies is apparent from these experiments (Cohen and Butcher, 1970).

In the present study this *in vitro* assay has been used to measure the specificity and levels of malarial inhibitory antibody in animals challenged with defined strains and variants of *P. knowlesi*. The results emphasize the importance of antigenic variation in promoting continued infection, and make clear the immunological mechanism which limits the proliferation of new variants as they arise.

MATERIALS AND METHODS

Animals

Rhesus monkeys (*Macaca mulatta*) of either sex weighing 2–4 kg were maintained in separate cages in a room artifically illuminated between 7.00 a.m. and 7.00 p.m.

Parasites

P. knowlesi parasites were obtained from the following sources:

(i) an anonymous Malayan strain isolated in 1964 from *Macaca fascicularis* and provided initially by Professor P. C. C. Garnham at the London School of Hygiene and Tropical Medicine (designated G);

(ii) a strain maintained at the Walter Reed Army Institute of Research, Washington and provided by Dr E. H. Sadun (designated W);

(iii) a strain maintained at the National Institute for Medical Research, London,

provided by Dr K. N. Brown and designated Nuri (Brown *et al.*, 1968).

Variants of the W strain were derived from a monkey (E1, Fig. 1) during repeated challenge according to the procedure described by Brown *et al.* (1968). These variants called W1, W2, W3 and W4 were serologically distinct by the schizont agglutination test and maintained their specificity unchanged after storage at −80° and during repeated passage through normal monkeys.

Storage of parasites

Parasites were stored as whole infected blood at −80° in an equal volume of 30 per cent glycerol in 0·9 per cent NaCl. During a series of experiments, parasites were maintained by weekly passage between uninfected monkeys.

Parasite counts

The first appearance of parasitaemia was assessed on thick blood films haemolysed in water and stained with Giemsa's solution. Parasite density was estimated from erythrocyte counts and the percentage of red cells parasitized was determined by counting up to 10^4 cells on thin blood films. Differential parasite counts were based upon classification of 100 parasitized cells observed on thin blood films stained with Giemsa's solution.

Experimental procedure

Experimental monkeys were infected with known numbers of parasites (usually 10^4) of defined strain or variant specificity by i.v. injection of a dilution in phosphate buffered saline of blood from an infected donor. Three–5 days after patency, or 12 days after inoculation when infections did not become patent, monkeys were given a single dose of chloroquine (5 mg per kg i.m. injection) and repeated doses of sulphadiazine (10 mg per kg i.p. injection daily for at intervals by cardiac 5 days or 30 mg per kg i.p. injection daily for 3 days). They were bled puncture or from the inguinal vein. Blood for storage or culture was collected in heparinized syringes and samples of anti-serum were defibrinated with glass beads and stored at −80°.

Monkey E1 (Fig. 1, Table 1) was infected with 10^4 parasites of the W strain (designated variant W1). When parasitaemia reached 10 per cent, 10^2 W1 parasites were transferred to a normal monkey (D1) and E1 was cured. When parasitaemia in D1 reached 10 per cent, E1 was re-challenged with 10^4 W1 parasites and D1 was in turn cured. The parasites which became patent in E1 after this second challenge were serologically distinct from W1 because they did not react with anti-W1 serum in the schizont agglutination test. They were designated W2. This sequence of passage through normal donors, (D1, D2, D3 and D4) and re-challenge with the passage variant, led to the appearance in monkey E1 of four distinct variants W1–W4 (Fig. 1). Using this procedure, E1 was challenged twice with W1 and once with W2 and W3, respectively, during a period of about 12 weeks. Sera collected at intervals were assayed for schizont infected red cell agglutinin (SICA) titres and levels of inhibitory antibody.

Variant specific antisera (Table 2) were obtained from the monkeys (D1–D4) which had been injected with 10^2 parasites of a given variant and cured on the third to fifth day of patency (see above). These animals were bled 30 days after the injection of parasites.

Monkey E2 (Table 3) was challenged five times with 10^4 W1 parasites and twice with 10^4 W2 derived from E1 as described above. Sera collected at intervals during 240 days were assayed for SICA and inhibitory titres.

Monkey E4 (Table 4) was challenged three times with W2 and sera collected over 6 weeks were assayed for inhibitory antibody.

Monkey E3 (Table 5) was re-challenged five times with 10^4 W1 parasites and then twice with 10^4 W4 parasites derived originally from E1. Sera collected during 216 days were assayed for SICA and inhibitory titres.

Assessment of clinical immunity.

Each inoculum of *P. knowlesi* parasites administered to experimental animals was given simultaneously to a normal control monkey. In the latter, the pre-patent period with the doses of parasites used, 10^2–10^4, was 3–4 days, the variant which became patent was the same as that inoculated, and the infection showed a rapidly fatal course unless terminated by drug therapy. Clinical immunity to challenge in the experimental monkeys was manifest as:

(i) spontaneous control of parasitaemia;

or (ii) prolongation of the pre-patent period for 1 or more days; in all instances tested (see Results). This was associated with the appearance of a variant of *P. knowlesi* serologically distinct from that used for challenge;

or (iii) absence of detectable parasitaemia for 2 weeks after challenge.

Culture of parasites

This was carried out as previously described (Butcher and Cohen, 1971) except that the concentration of amino acids was doubled to correspond with that in Eagle's medium. Parasite growth was assessed as described by Cohen *et al.* (1969) by measuring the incorporation of either ^{3}H-leucine or ^{3}H-methionine into parasite protein during culture for 24 hour. The average *in vitro* multiplication rate in flasks containing preinfection serum in the present series of experiments was 5·2 for W1, 5·5 for W2, 6·0 for W3 and 5·4 for the W4 isolate of *P. knowlesi*.

Assay of inhibitory antibody

Volumes of immune anti-serum (0·001–0·40 ml) were added to duplicate flasks containing 3-ml culture medium and 0·25 ml Rhesus red cells of which about 1 per cent were initially infected with a defined strain or variant of *P. knowlesi*. Protein bound radioactivity was measured after 24-hour culture as previously described (Cohen and Butcher, 1970). In most instances the entire contents of the flask was precipitated with trichloroacetic acid to avoid sampling errors associated with parasite agglutination. Mean radioactivity values at 24 hour in flasks containing anti-sera were expressed as per cent of those in flasks containing normal sera; this value is referred to as per cent normal growth (A) and 100-A is called per cent inhibition. Since the first intracellular cycle of parasite growth is not affected by antibody (Cohen *et al.*, 1969), the maximum inhibition measured by the above technique is about 70–80 per cent.

Schizont infected red cell agglutination (SICA) titres were measured by the method of Brown and Brown (1965). Schizont infected cells were isolated and washed by centrifugation and suspended in phosphate buffered saline (pH 7·4) containing 1 per cent normal Rhesus monkey serum; all suspensions were added to dilutions of sera in agglutination trays and titres were read macroscopically after 2–3 hour at room temperature.

RESULTS

1. ISOLATION AND SPECIFICITY OF VARIANTS

A single monkey (E1, Fig. 1, Table 1) challenged repeatedly with W strain parasites (see Materials and Methods) developed four consecutive infections which were serologically distinct and designated W1, W2, W3 and W4. Each variant was isolated from E1 by passage through a normal Rhesus monkey and these monkeys (D1–D4) were cured when parasitaemia reached about 10 per cent (see Materials and Methods). About 4 weeks after infection and 3 weeks after cure, the sera of these monkeys contained schizont agglutinins which were specific for the immunizing variant and did not react with the other variants (Table 2).

2. THE IMMUNE RESPONSE TO *P. knowlesi* INFECTION

The immune response of Rhesus monkeys to variants of the W strain of *P. knowlesi* was assessed by measurement of inhibitory antibody, SICA titres and the clinical response to infection (see Materials and Methods). One group of four animals (D1–D4) experienced a single infection terminated by drug cure and later these monkeys were challenged with the same or with different variants of the same stain (Table 2). Another four monkeys (E1–E4) experienced repeated infections, terminated by drug cure and induced with the same or with homologous variants (see Materials and Methods). The results for these animals appear individually in Tables 1 and 3–5, and the course of parasitaemia after challenge in Figs 3, 5 and 7. The data are considered collectively, below, in relation to specific aspects of the immune response to *P. knowlesi* infection.

(i) *Response to the first infecting variant*

Variant-specific inhibitory antibody measured by *in vitro* assay became detectable 2–3 weeks after inoculation of W1, W2 and W3 (Tables 2 and 4) and levels rose during the month following a single infection: however, a single infection with W4 produced no detectable inhibitory response. In animals infected repeatedly with either the same (Tables 3, 4 and 5) or different (Table 1) variants, levels of inhibitory antibody against the initial infecting variant increased progressively for 6–10 weeks; a level of 50 per cent inhibition (using 0·1 ml of serum for assay) was observed after 5–6 weeks (Fig. 2). SICA tests became positive at about the time when inhibitory antibody was first detected, but titres varied considerably among individual animals (Fig. 2).

Clinical immunity to challenge with the first infecting variant was observed 60 days after a single infection (Table 2, D2) and after 2–3 weeks with repeated infections (Tables 1, 4 and 5). Immunity was manifest as a lengthening of the pre-patent period and SICA tests showed that this was associated with the appearance in the blood of a variant distinct from that used for challenge (e.g. Table 1, day 16).

(ii) *The response to subsequent challenge with homologous variants*

A single infection induces slight immunity to the corresponding variant as mentioned above, but not to other variants tested after about 4 weeks (Table 2, D1, D3 and D4). Repeated infections at shorter intervals, on the other hand, e.g. with W1 followed by W2 and W3, induced clinical immunity to all but the first infection as manifest by the increasing length of the pre-patent period (Fig. 3) and the appearance of variants distinct

TABLE 1

DATA FOR EXPERIMENTAL MONKEY E1

Challenge*					W1		W2		W3		W4	
Day	Variant	Pre-patent (days)†	Patent Variant	Serum day	SICA titre‡	Per cent Inhib.§	SICA titre	Per cent Inhib.	SICA titre	Per cent Inhib.	SICA titre	Per cent Inhib.
0	W1	0	W1	0	0	0	0	0	0	0	0	0
				7	0	0	0	0	0	2	0	0
16	W1	1	W2									
				23	250	18	0	0	0	22	0	0
32	W2	2	W3									
				43	250	31	1250	45	0	0	0	1
67	W3	3	W4	67	1250	64	250	67	50	63	10	6
				82	1250	71	6250	66	50	76	10	7
105	W1	9										
				118								9
156	W1	−ve										
				173								7
175	W1	3										
				184			250	49				0
197	W1	−ve		197								2
212	W1	−ve										
232	W1	9		232								0
				244			250	63				7

* 10^4 Parasitized cells i.v., except for challenges on days 105, 175, 212 and 232 when 10^6 parasitized cells i.v. were given.

† Prolongation of pre-patent period beyond normal monkey. Negative (−ve) indicates that parasites did not become patent within 2 weeks of challenge.

‡ A SICA titre of $0 = < 1/2$.

§ Inhibition based on 24-hour radioactivity measurements (see Materials and Methods) using 0·3 ml serum per culture flask.

TABLE 2

DATA FOR DONOR MONKEYS (D1 – D4) USED FOR PASSAGE OF SPECIFIC VARIANTS ORIGINATING IN EXPERIMENTAL ANIMAL E1 (SEE TABLE 1)

Donor	Challenge*		Pre-patent (days)	Serum day	W1		W2		W3		W4	
	Day	Variant			SICA titre	Inhib. (per cent)	SICA titre	Inhib. (per cent)	SICA titre	Inhib. (per cent)	SICA titre	Inhib. (per cent)
D1	0	W1	0									
				9	0	11	0	4	0	18	0	0
	29	W3	0	29	1250	17	0	0	0	0	0	0
D2	0	W2	0									
				9	0	16	0	8	0	27	0	0
				29	0	0	250	37	0	0	0	0
				45			50	32				
	60	W2	2									
				65			50	46				
D3	0	W3	0									
				8	0	10	0	15	0	0	0	12
				30	0	0	0	0	1250	48	0	0
	61	W1	0									
D4	0	W4	0									
				8	0	7	0	0	0	16	0	0
				30	0	0	0	0	0	0	250	0
	73	W1	0									

The sera collected on day 29 or 30 were used as variant specific reagents for the SICA test.
* 10^2 Parasitized cells i.v. (See also legend Table 1).

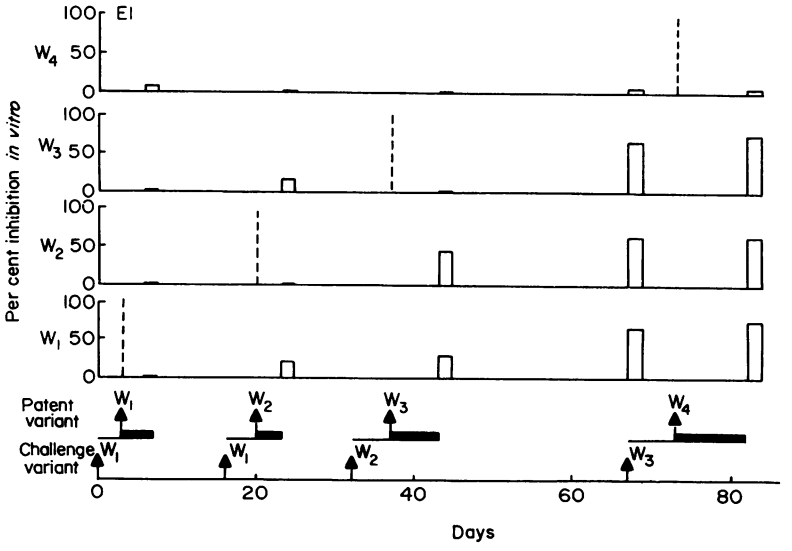

FIG. 1. Diagram showing the identity of challenge and patent variants, duration of parasitaemia (solid blocks) and levels of inhibitory antibody specific for variants W1–4 (open blocks) in monkey E1. Vertical dotted lines indicate the day on which each variant was first patent. Data for inhibitory antibody represent assays using 0·3 ml serum per culture flask.

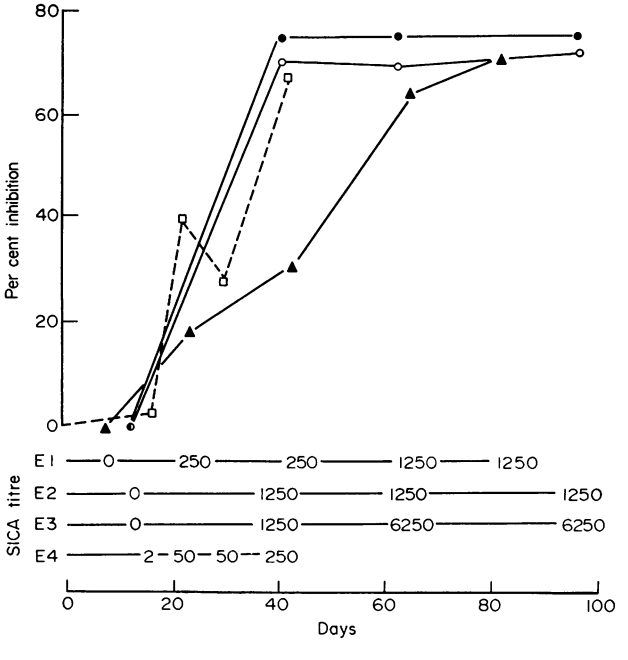

FIG. 2. Inhibitory antibody and SICA levels in response to the first infecting variant which was W1 (solid lines) in monkeys (▲) E1, (○) E2 and (●) E3, and W2 (broken line) in (□) E4. The data are for assays using 0·1 ml serum in E2, E3 and E4 and 0·3 ml serum in E1.

TABLE 3

DATA FOR EXPERIMENTAL MONKEY E2

Challenge*			Serum (day)	W1					W2					W4				
				Per cent inhibition†					Per cent inhibition†					Per cent inhibition†				
Day	Variant	Prepatent (days)		0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre	0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre	0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre
0	W1	0																
13	W1	0	13	0	0			0	0	0	0	0	0	0	0	0	0	0
41	W1	0‡	41	63	71			1250	18		22	42	0	1	0	16	20	0
63	W1	5	63	67	70			1250	7		38	55	0		4	20	33	0
97	W1	4	97	53	72			1250	10		48	75	0	0	0	18	36	0
			123	56	66			6250	1	23	53	73	0	0	1	11	35	0
126	W2	8																
			133		66				0	20	58	78	50		9	15	22	
			139		67				27	63	83	84	1250			12	22	
			146		67				43	63	80	82	1250			18		
			151		71				45	58	82	85	6250	0	4	12	35	0
			160		68				30	58			1250					
			165						25	60	82	84	1250					
167	W2	9																
			180		63													
188	W1	3	188												0	6	2	0
			216								80	88			5	0	28	
			242							62								10

* 10^4 Parasitized cells i.v.

† Figures show per cent *in vitro* inhibition with various doses of serum.

‡ Parasitaemia controlled spontaneously (Fig. 5). (See also legend Table 1.)

TABLE 4

DATA FOR EXPERIMENTAL MONKEY E4

Challenge*			Serum (day)	W2					W4		
Day	Variant	Pre-patent (days)		Per cent inhibition†				SICA titre	Per cent inhibition†		
				0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)		0·10 (ml)	0·20 (ml)	0·40 (ml)
0	W2	0	16	6	3	4	17	2			
22	W2	2	22	12	41	46	65	50	0	0	0
			30	11	28	31	39	50			
42	W2	4	42	33	69	69	70	250	3	2	2

* 10^4 Parasitized cells i.v.

† Figures show per cent *in vitro* inhibition with various doses of serum. (See also legend Table 1).

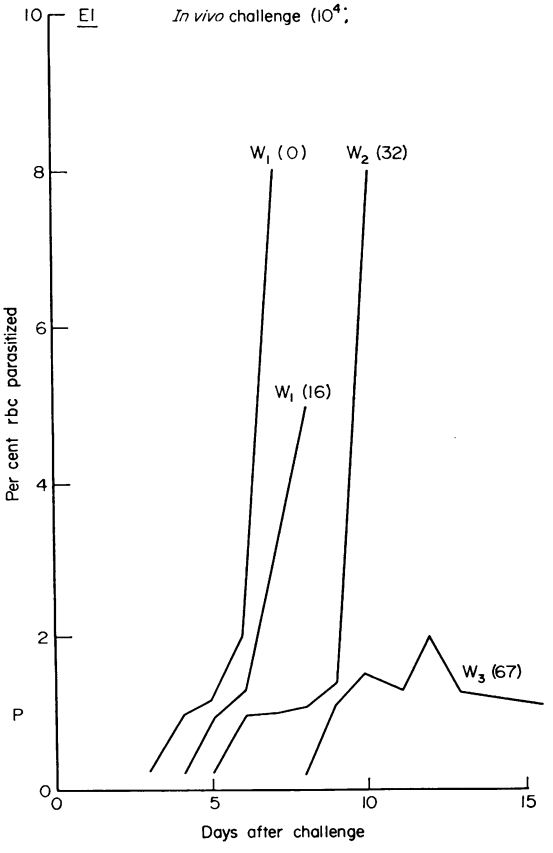

FIG. 3. Course of parasitaemia in monkey E1 after successive challenge with 10^4 (see Table 1) *P. knowlesi* parasites (variants W1, W2 and W3). Figures in parentheses represent day of challenge (see Table 1).

from those used for challenge (Table 1). The antibody response induced by these successive infections appeared to be variant specific as judged by the SICA test and the measurement of inhibitory antibody at a single dose level (Fig. 1 and Table 1); both tests became consistently positive only after the corresponding variant had appeared in the blood (about 20–30 days after patency). However, as shown in Fig. 4, the inhibitory antibody level to the first infecting variant (W1) increased relatively slowly compared with the response to subsequent challenge variants (W2 and W3) which reached 50 per cent inhibition after about 3 weeks. This response could not be attributed to differences in immunogenicity, since W1 (in E2 and E3) and W2 (in E4) induced closely similar antibody responses when given as first infections (Fig. 2). SICA tests showed variable titres to W1, 2 and 3 in monkey E1 and the responses to first and subsequent infections

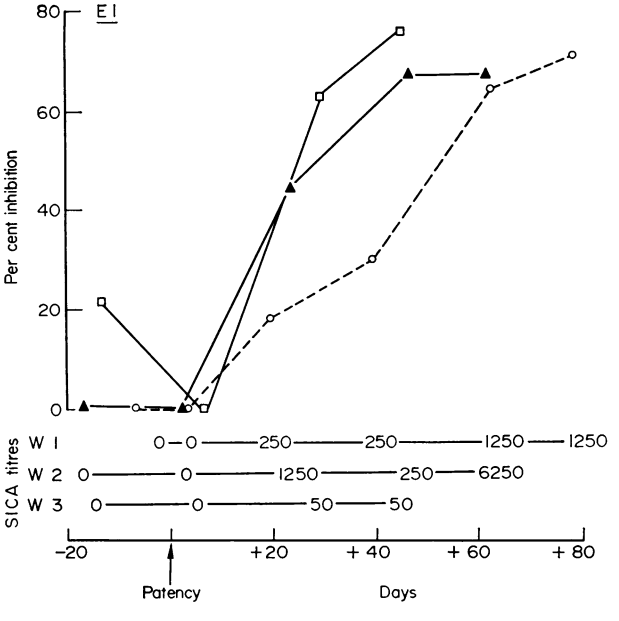

FIG. 4. Levels of specific inhibitory antibody and schizont agglutinins after patency of the first infecting variant, (○) W1 (broken line), and subsequent challenge variants, (▲) W2 and (□) W3 (solid lines), in monkey E1. Inhibitory antibody assays were made with 0·3 ml of serum per culture flask.

could not be distinguished (Fig. 4). It is evident that the initial infection with W1 sensitized monkey E1 for rapid inhibitory antibody responses associated with specific immunity to W2 and W3.

This phenomenon was further investigated in monkey E2 (Table 3) which was challenged five times during 100 days with W1 and twice during the next 70 days with W2. After the second challenge with W1, clinical immunity was manifest by spontaneous control of parasitaemia and increasing length of the pre-patent period (Fig. 5). When challenged with W2 on day 126, the pre-patent period was prolonged 8 days beyond the normal and parasitaemia was spontaneously controlled; the second W2 challenge on day 167 induced only transient parasitaemia. It is significant that the SICA titres for W2 remained negative throughout the period of W1 challenge and rose to 1250 within 2 weeks after W2 challenge (Table 3). This indicates that none of the variants induced in this

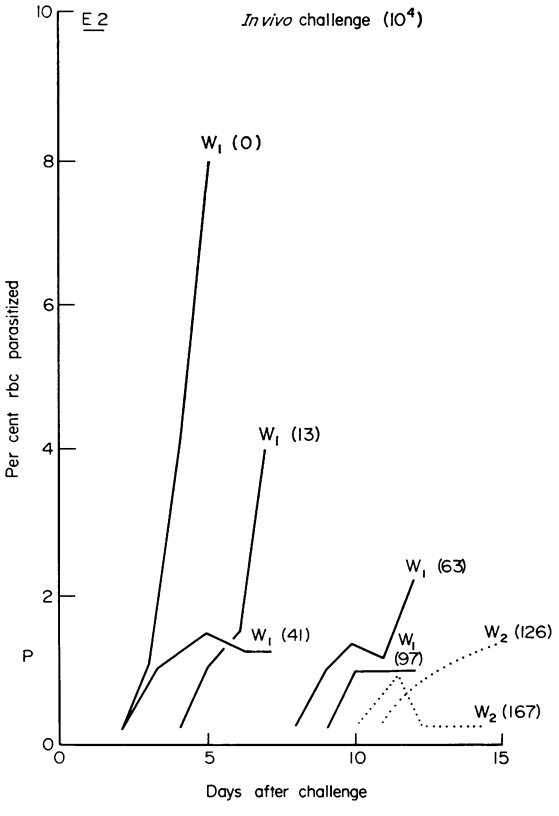

FIG. 5. Course of parasitaemia in monkey E2 after successive challenges with *P. knowlesi* variants, W1 (five times) and W2 (twice). Figures in parentheses represent day of challenge (see Table 3).

animal by W1 corresponds to W2, since positive SICA tests for W2 occur 1–3 weeks after W2 patency (Tables 1 and 2). Nevertheless, repeated challenge of monkey E2 with the W1 variant induced a significant inhibitory antibody response to W2 by the 6th week after infection (Table 3 and Fig. 6). The level of inhibitory antibody increased steadily to about 1/40th the level of anti-W1 by day 123. Nine days after W2 challenge, the titre of inhibitory antibody against W2 had risen rapidly to a level approximating that of anti-W1 (Fig. 6) and the SICA test for W2 became positive for the first time.

Repeated challenge with W1 did not induce schizont agglutinins against W4, but inhibitory antibody for the latter variant was detectable after 6 weeks; the level then increased but remained below that for W2 (Table 3). The question as to whether W1 challenge sensitizes for a clinically effective immune response to W4 was investigated in another experimental animal. Monkey E3 was challenged five times during 100 days with W1 and twice during the next 50 days with W4 (Table 5). Successive W1 challenges again led to a progressive lengthening of the pre-patent period from 2 to 7 days while the fifth challenge did not induce a patent infection (Fig. 7). Subsequent challenge with W4 produced parasitaemia after a delay of 1 day; the SICA test showed that the patent variant was not W4 and that schizont agglutinins to this new variant appeared (titre

TABLE 5

DATA FOR EXPERIMENTAL MONKEY E3

Challenge*			Serum (day)	W1					W2					W4				
Day	Variant	Pre-patent (days)		Per cent inhibition†					Per cent inhibition†					Per cent inhibition†				
				0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre	0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre	0·01 (ml)	0·10 (ml)	0·20 (ml)	0·40 (ml)	SICA titre
0	W1	0																
13	W1	3	13	0	0			0	0		0	0	0	0	0	0	0	0
41	W1	1	41	61	75			1250	7		27	46	0	6	11	20	29	0
63	W1	4	63	56	75			6250	0		25	28	0	0	10	18	29	0
97	W1	NEG	97	61	75			6250	8		24	37	0	2	3	21	52	0
			123	56	75			6250	0	15	26	36	0	0	0	19	32	0
124	W4	1‡			79													2
			146										2	6	22			
			151							32	54	69		5	20	20	39	10
152	W4	2																
			165							20	41	52				24	42	50
			174		72						58	74	2		20	25	31	
			180												29	33	50	50
188	W1	4	188										2					50
			196											11	16	24	36	50
			216							19	36	53						

* 10^4 Parasites.

† Figures show per cent *in vitro* inhibition with various doses of serum.

‡ The patent variant was not W4. (See also legend Table 1.)

1250) by day 151; a second W4 challenge at this time was associated with prolongation of the pre-patent period by 2 days and spontaneous control of parasitaemia (Fig. 7).

W1 challenge induced, as in monkey E2, high levels of inhibitory antibody against this variant and lower levels against W2 and W4, which were associated with negative SICA tests indicating that these variants had not been patent. The SICA test became positive for W4 about 3–4 weeks after challenge with W4. The level of inhibitory antibody against W4 remained relatively low even after the second W4 challenge (Fig. 8) and this contrasts with the response to W2 challenge in monkey E2 (Fig. 6).

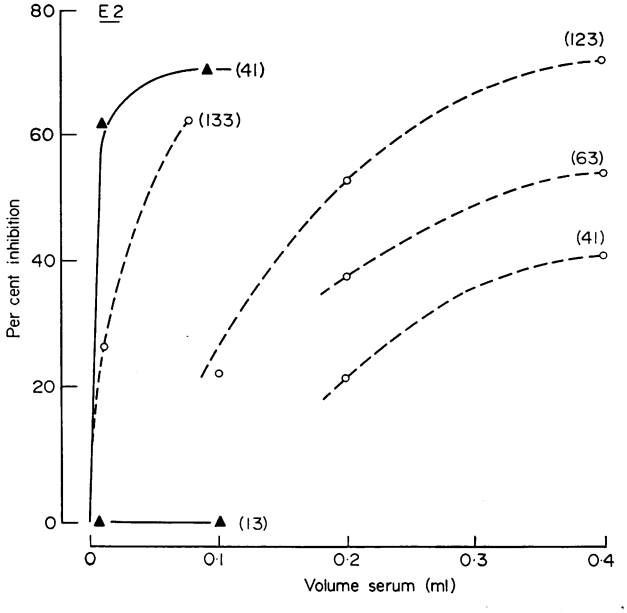

FIG. 6. Dose response data for inhibitory activity present in serum of monkey E2 assayed against *P. knowlesi* variants, W1 (solid line) and W2 (broken line). Figures in parentheses represent days on which sera were collected (see Table 3).

(iii) *The response to challenge with homologous strains*

The occurrence of cross-immunity between strains of *P. knowlesi* was investigated in six monkeys which had been exposed to chronic G-strain infections for 98–1010 days (Table 6). These animals, on challenge with the G-strain, showed prolongation of the pre-patent period or complete absence of parasitaemia. Their pooled sera gave a SICA titre of 4096 and when tested *in vitro*, at a dose of 0·3 ml, showed 61 per cent inhibition of the G-strain (Cohen and Butcher, 1970).

Two animals, M65 and G50 (Table 6) which had been immunized against the G-strain showed evidence of immunity when challenged with 10^4 W-strain parasites (variant W1); a spontaneous loss of parasitaemia occurred on the 5th day of patency in one animal and in the other the pre-patent period was prolonged 5 days beyond that of the control animal. Their sera, taken immediately before challenge with W1, gave negative SICA tests against this variant but showed 43–47 per cent inhibition of growth when tested in culture at a dose of 0·3 ml of serum per flask. Two other animals immunized with the

G-strain (G56 and G59, Table 6) were fully susceptible on challenge with another variant of the W-strain (W4); their pre-challenge sera gave negative SICA and inhibitory tests against W4. A further two animals immunized against the G-strain (G53 and G54, Table 4) were fully resistant to the Nuri strain when challenged *in vivo*; their pre-challenge sera showed SICA titres of 50 and 250 and gave 40–52 per cent inhibition of *in vitro* growth of the Nuri strain.

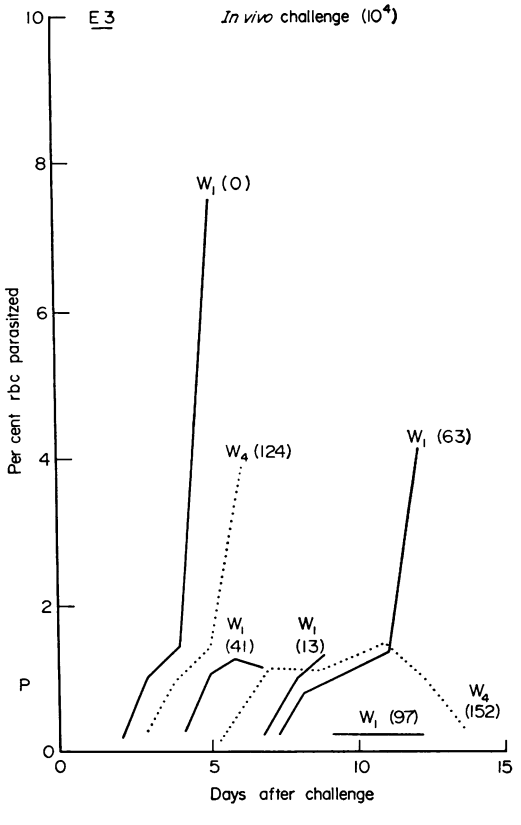

FIG. 7. Course of parasitaemia in monkey E3 after successive challenges with *P. knowlesi* variants, W1 (five times) and W4 (twice). Figures in parentheses represent day of challenge.

DISCUSSION

Chronic malarial infection provides a potent stimulus for Ig synthesis (Cohen, McGregor and Carrington, 1961). Much of this Ig appears to be non-specific, but malarial antibodies can be demonstrated in the serum of immune individuals by several serological tests, including precipitation, agglutination, opsonization, antibody fluorescence and complement fixation (reviewed, Sadun, 1966). These antibodies may cross-react with various plasmodial species whereas immunity to malarial infection is largely species specific. In addition, there is poor correlation between the immune status of an individual and observed levels of these malarial antibodies. Nevertheless, protective antibody has been demonstrated by passive transfer tests in monkey, human and rodent malarias (Brown,

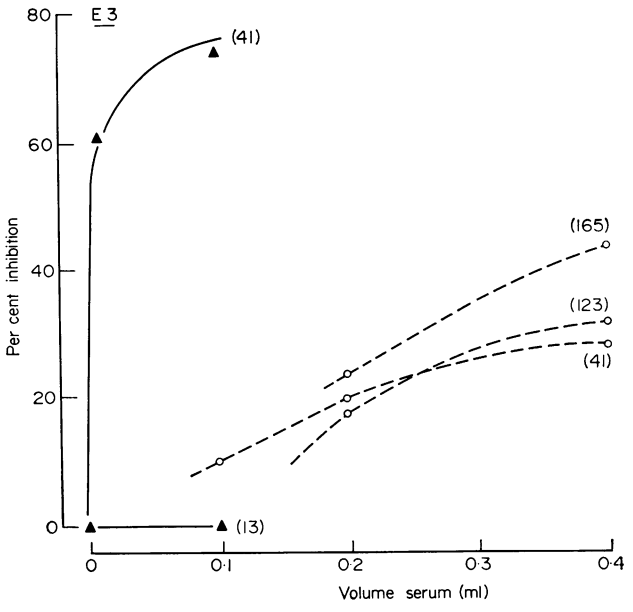

FIG. 8. Dose response data for inhibitory activity present in serum of monkey E3 assayed against *P. knowlesi* variants, W1 (solid line) and W4 (broken line). Figures in parentheses represent days on which sera were collected (see Table 5).

TABLE 6

MONKEYS IMMUNE TO *P. knowlesi* (G-STRAIN) CHALLENGED WITH *P. knowlesi* W OR NURI STRAINS

Animal	Immunization with G-strain			Challenge			Pre-challenge serum	
	Duration (days)	Times immunized	Days since last infect.	Strain (variant)	No. parasites	Pre-patent (days)	Inhibition per cent§	SICA titre
M65*	1010	16	49	W1	10^4	0‡	43	0
G50*	98	3	14	W1	10^5	5	47	0
G56*	460	5	140	W4	10^6	0	0	0
G59*	300	2	140	W4	10^6	0	0	0
G53*	280	4	41	Nuri	10^4	−ve	52	250
G54†	252	4	49	Nuri	10^4	−ve**	40	50

* Infections with G-strain in these animals were controlled when necessary with mepacrine 6 mg/kg i.m. injection daily for 4 days.

† Infections with the G-strain in this animal was controlled when necessary with chloroquine 7·5 mg/kg followed by four injections of 2·5 mg/kg daily.

‡ On 5th day patency M65 showed spontaneous decrease in parasitaemia.

§ Based on 24-hour radioactivity values; sera tested at dose of 0·3 ml per flask containing 0·25 ml red cells (initial parasitaemia 1 per cent).

** Viability of the inoculum used for this monkey was not tested in a non-immune monkey. (See also legend Table 1).

1969). Such tests are cumbersome and there has consequently been a great need for a dependable *in vitro* technique for the detection of protective malarial antibody. The schizont infected red cell agglutination (SICA) test (Brown and Brown, 1965) and the *in vitro* assay of inhibitory antibody (Cohen *et al.*, 1969) have been proposed for this purpose. The present study in which both were employed allows these tests to be assessed as indicators of the immune status of the serum donor.

The specific inhibitory antibody and SICA titres both correlate with immune status: (i) in animals immune to one strain of *P. knowlesi* but fully susceptible to a variant of another strain, when both tests are negative for that variant (Table 6, G56, G59); (ii) in repeatedly challenged animals immune to the challenge variant, when both tests are positive for that variant. However, SICA tests are quantitatively unsatisfactory as compared with inhibitory antibody assays, since the former titres differ considerably with individual variants and do not consistently distinguish the responses to first and subsequent challenge infections (Fig. 4). In addition, there are circumstances where clinical immunity occurs with positive tests for inhibitory antibody but negative SICA tests. This was observed as follows: (i) animals immune to the G-strain of *P. knowlesi* had negative SICA tests, but high levels of inhibitory antibody against W1 and were clinically immune on challenge with W1 (Table 6); (ii) animals repeatedly immunized with W1 had negative SICA tests for W2 or W4 but their sera contained inhibitory antibody against both and they were clinically immune when challenged with either (Tables 3 and 5). These results show that the *in vitro* measurement of inhibitory antibody provides a consistently reliable index of the host's immune status and probably measures the protective antibody which initiates specific immune processes *in vivo*.

The relationship between the antibodies responsible for inhibition and those mediating schizont agglutination has not been established. Both have the same overall specificity, since inhibitory antibody is directed predominantly against specific variants identified by schizont agglutination. Although there are instances, mentioned above, in which inhibitory antibody is detectable in the absence of schizont agglutinins, the antibodies active in the two tests might be identical: (i) if the sensitivity of the inhibition test were greater than that of the agglutination test; this is unlikely as equally high levels of inhibition were observed with both negative and positive agglutination (e.g. Table 6); (ii) if surface determinants were distributed on some strains or variants in such a way that these were inhibited but not agglutinated by specific antibody. Alternatively, different antibodies might mediate the two tests; (i) if the Ig classes differ in their activity for SICA and inhibitory tests; this is unlikely since IgG and IgM are active in both tests (Cohen and Butcher, 1970); (ii) if agglutinating and inhibitory antibodies were directed against different specific antigens; it is likely, for example, that inhibitory antibodies are directed against merozoite antigens (Butcher and Cohen, 1970) and agglutinating antibodies against different determinants on the surface of red cells containing schizonts. The isolation and characterization of agglutinating and inhibitory antigens should establish their relationship.

Evidence for the appearance of antigenically distinct parasite populations during the course of the asexual erythrocytic cycle has been put forward for several species of malaria. The phenomenon has been described in *P. berghei* infections of mice (Cox, 1959; Wellde and Sadun, 1967), in the monkey malarias, *P. knowlesi* (Brown and Brown, 1965) and *P. cynomolgi bastianelli* (Voller and Rossan, 1969b), and more recently in the human malaria, *P. falciparum* (Voller, 1971). The schizont agglutination test (Eaton, 1938) has

revealed a very wide spectrum of variants of *P. knowlesi* (Brown and Brown, 1965) and their successive appearance seems responsible for persisting infections in semi-immune animals (Brown and Brown, 1965; Voller and Rossan, 1969b). However, certain observations require explanation (Cohen and Butcher, 1971; Brown, 1971): (i) spontaneous relapses in chronic *P. knowlesi* infections are usually well controlled by the host and parasitaemia rarely rises above 1 per cent; nevertheless, the relapse variant produces a rapidly fatal infection if inoculated into an unimmunized normal recipient; (ii) after repeated challenge with a single variant of *P. knowlesi*, Rhesus monkeys are resistant to several other variants (Voller and Rossan, 1969a). These findings have prompted the view that malarial infection induces a variant-transcending immunity which inhibits, but does not entirely prevent, the multiplication of new variants (Brown and Brown, 1965; Voller and Rossan, 1969a; Brown *et al.*, 1968). The specificity and nature of this broad spectrum response were undetermined. More recently, Brown (1971) has postulated that the protective antibody response is entirely variant specific, but certain surface antigens common to all variants sensitize thymus-derived lymphocytes; these act as 'helper' cells in antibody production and enable the host to respond more quickly to new variants and control their proliferation.

The *in vitro* assays recorded in this study show that inhibitory antibody is predominantly variant specific. Four weeks after a single infection of 3–5 days duration, followed by cure, inhibitory antibody was detectable only against the infecting variant. Similarly, in monkey E1 exposed successively over a period of 9 weeks to variants W1, W2 and W3, specific inhibitory antibody was consistently positive only after the corresponding variant had become patent. However, when animals were repeatedly challenged with a single variant, W1, inhibitory antibody against other variants, e.g. W2 and W4, became detectable after the sixth week at levels corresponding to about 2 per cent of that for W1.

The presence of inhibitory antibody directed against a variant other than that used for challenge might indicate that this variant had appeared in the host as a result of antigenic variation. This seems unlikely in the present instances because such inhibitory antibody was associated with negative SICA tests for the corresponding variants (Table 3 and 4). Brown and Brown (1965) have shown that patent infections consistently evoke specific schizont agglutinins in easily measurable titre and the present data illustrate the same fact. It appears therefore that chronic malarial infection induces the synthesis of inhibitory antibody active against variants which the host has never harboured. Such activity could be attributed to low-affinity cross-reacting antibody but, since levels increase with repeated challenge, it is likely that parasites within a strain carry common antigenic determinants. The fact that distinct strains of *P. knowlesi* may cross-immunize for one another (Table 6) also increases the probability that variants within a strain carry common protective antigens. Direct support for this is provided by the finding that W1 specific inhibitory antibody can be completely absorbed by soluble W1 antigen extracts and partly absorbed by W2 extracts (our unpublished results). Whether such common antigens occur on all variants of a strain is not known, but the present results are consistent with such a distribution and so is the rarity of severe relapse in chronically infected Rhesus monkeys.

It is apparent that the resistance of monkeys to relapse variants arising during chronic infection and the cross-immunity observed after single variant challenge can be correlated with the presence of inhibitory antibody active against variants not previously patent. Such antibody is detectable about 6 weeks after the first infection and may, by limiting the

multiplication rate of most new variants as they arise, account for the progessive lengthening of the pre-patent period characteristically observed with repeated challenge. However, the significance of this latter phenomenon remains uncertain because the mechanism of new variant induction by antibody has not been elucidated. Some new variants stimulate a rapid secondary response in sensitized animals (e.g. W2, Fig. 6), but this is not invariable (e.g. W4, Fig. 8). The reason for the apparently weak immunogenicity of W4 in all situations in which it was tested is not understood.

Despite the wide antigenic variability manifest within individual strains of the *P. knowlesi* parasite, the occurrence of cross-immunization between variants is encouraging from the point of view of vaccine production. A vaccine which contains cross-sensitizing antigens may be expected to induce a degree of clinical immunity similar to that observed after repeated challenge or during the course of chronic infection. Such antigen preparations of *P. knowlesi* are at present being tested for their immunizing properties in this laboratory.

ACKNOWLEDGMENTS

We thank Mr E. D. Dennis, Miss Susan Wanstall and Mrs Patricia Tatchell for skilled technical assistance. This work was supported by grants from the Medical Research Council, London and the World Health Organization.

REFERENCES

BROWN, I. N. (1969). 'Immunological aspects of malaria infection.' *Advanc. Immunol.*, **11**, 267.

BROWN, I. N., BROWN, K. N. and HILLS, L. A. (1968). 'Immunity to malaria: the antibody response to antigenic variation by *Plasmodium knowlesi*.' *Immunology*, **14**, 127.

BROWN, K. N. (1971). 'Protective immunity to malaria provides a model for the survival of cells in an immunologically hostile environment.' *Nature* (*Lond.*), **230**, 163.

BROWN, K. N. and BROWN, I. N. (1965). 'Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*.' *Nature* (*Lond.*), **208**, 1286.

BUTCHER, G. A. and COHEN, S. (1970). 'Schizogony of *Plasmodium knowlesi* in the presence of normal and immune sera.' *Trans. Roy. Soc. trop. med. Hyg.*, **64**, 470.

BUTCHER, G. A. and COHEN, S. (1971). 'Short-term culture of *Plasmodium knowlesi*.' *Parasitology*, **62**, 309.

COHEN, S. and BUTCHER, G. A. (1970). 'Properties of protective malarial antibody.' *Immunology*, **19**, 369.

COHEN, S. and BUTCHER, G. A. (1971). 'Serum antibody in acquired malarial immunity.' *Trans. Roy. Soc. trop. med. Hyg.*, **65**, 125.

COHEN, S., BUTCHER, G. A. and CRANDALL, R. B. (1969). 'Action of malarial antibody *in vitro*.' *Nature* (*Lond.*), **223**, 368.

COHEN, S., MCGREGOR, I. A. and CARRINGTON, S. C. (1961). 'Gamma globulin and acquired immunity to human malaria.' *Nature* (*Lond.*), **192**, 733.

COX, H. W. (1959). 'A study of relapse *Plasmodium berghei* infections isolated from white mice.' *J. Immunol.*, **82**, 208.

EATON, M. D. (1938). 'The agglutination of *Plasmodium knowlesi* by immune serum.' *J. exp. Med.*, **67**, 857.

JERUSALEM, C., WEISS, M. L. and POELS, L. (1971). 'Immunologic enhancement in malaria infection (*Plasmodium berghei*).' *J. Immunol.*, **107**, 260.

SADUN, E. H. (1966). Ed. 'Research in Malaria.' *Milt. Med. Suppl.*, **131**, 847.

SERGENT, E. and SERGENT, E. (1956). 'History of the concept of relative immunity or 'premunition' correlated to latent infection.' *Indian J. Malariol.*, **10**, 53.

VOLLER, A. (1971). 'Variant specific schizont agglutinating antibodies in human malaria (Plasmodium falciparum) infections in Aotus.' *Trans. Roy. Soc. trop. med. Hyg.*, **65**, 2.

VOLLER, A. and ROSSAN, R. N. (1969a). 'Immunological studies on simian malaria. III. Immunity to challenge and antigenic variation in *P. knowlesi*.' *Trans. Roy. Soc. trop. med. Hyg.*, **63**, 507.

VOLLER, A. and ROSSAN, R. N. (1969b). 'Immunological studies with simian malarias. I. Antigenic variants of *Plasmodium cynomolgi bastianelli*.' *Trans. Roy. Soc. trop. med. Hyg.*, **63**, 46.

WELLDE, B. T. and SADUN, E. H. (1967). 'Resistance produced in rats and mice by exposure to irradiated *P. berghei*.' *Exp. Parasitol.*, **21**, 310.