INTRODUCTION

Some parasitic protozoa can survive and multiply in host animals which show high levels of parasiticidal antibody developed as a result of infection (Mesnil & Brimont, 1909; Coggeshall & Kumm, 1937; Remington et al., 1961; Anderson et al., 1965). In trypanosomiasis, repeated parasitaemias in the presence of lytic antibodies are produced by a succession of antigenic variants. Each variant stimulates a specific trypanocidal antibody and at the same time remains unaffected by antibodies to other variants; over 20 variants have been recorded from one strain (Lourie & O'Connor, 1937). In the Brucell group of trypanosomes the variants appear to differ in the antigenic constitution of at least two groups of soluble proteins (Brown, 1963; Brown & Williamson, 1964; Williamson & Brown, 1964).

There is indirect evidence that some antigenic variation can occur in rodent malaria (Cox, 1959), but repeated antigenic changes, of an order which might account for chronic simian or human malaria, have not previously been demonstrated. Alternative explanations of chronicity in malaria include the suggestions that the parasite is only poorly immunogenic, or that being intracellular, it is not accessible to antibodies (Taliaferro, 1949). On the other hand, it is unlikely that such a complex foreign organism would be poor in antigens, and the intracellular schizonts at least

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are known to react with antibodies (Eaton, 1938). These considerations suggest that frequent antigenic variation, similar to that occurring in trypanosomes, is the most likely explanation of chronic malaria.\footnote{The authors refer to manifestations of malaria infection subsequent to the primary attack. The limitations of the term "chronic malaria" are indicated in the "Terminology of malaria and of malaria eradication" (WHO, 1963) \textit{Editor's remark}.} Using a parasite agglutination test (Eaton \textit{op cit}), direct evidence has now been obtained for repeated antigenic variation in \textit{Plasmodium knowlesi} infections of monkeys. These experiments, and some of their implications for the immunology of malaria, are summarized here; full details will be published elsewhere.

\section*{METHODS}

A syringe-passed derivative of the Nuri strain of \textit{P. knowlesi} was used (Jawant Singh et al., 1953), which is highly virulent in rhesus monkeys (\textit{Rhesus mulatta}); death occurred within 4 to 6 days of patency, up to 90\% of the erythrocytes being infected. Subcurative chemotherapy produced a chronic relapsing infection with parasitaemias rarely rising above 1\%. A frozen stablilate of this strain (Lumsden \& Hardy, 1965), designated K1, was isolated by freezing citrated blood of a monkey 6 days after infection. Stabilates of four relapses, K1A, K1B, K19 and K21 were also isolated. K1A and K1B were successive relapses occurring 10 and 21 days after subcurative proguanil treatment of a K1-infected monkey. Stabilate K19 was collected from a monkey infected with K1A, cured with sulphadiazine when the infection was patent, and then reinfected with K1A. Reinfection produced without further drug treatment a series of spontaneously terminating parasitaemias of which stablilate K19 was the fourth; it appeared 34 days after reinfection. Another stabilate (K21) was collected at the fourth patency in a second chronically infected monkey. When nearly all the stock of a stablilate had been used, a direct derivative was isolated by inoculating the stablilate into a monkey from which further blood samples were frozen down soon after patency. These secondary isolations were designated K1A2 and K1D2.

Antisera to parasites derived from specific stablilates were obtained from monkeys cured with sulphadiazine, or sulphadiazine plus proguanil, when the infection had become patent following inoculation of the stablilate; sera were taken before infection and 7 to 14 days after drug treatment. Serum samples were also collected at intervals from several chronically infected monkeys. All the sera were stored at $-20^\circ C$, and inactivated at $56^\circ C$ before use.
For the agglutination test, schizont- or trophozoite-infected erythrocytes were separated by centrifuging from heavily parasitized blood taken 6 to 7 days after infection with a stabilate. Fivefold dilutions of the serum in diluent (1% normal monkey serum in saline) were set out in WHO haemagglutination trays, and equal volumes (0.4 ml) of a saline suspension of either schizont- or trophozoite-infected cells containing approximately $4 \times 10^7$ cells per ml, were added to each well. The trays were incubated at $20 - 22^\circ$C for at least 3 hours and then examined macro- and microscopically for agglutination. One unit of antibody was arbitrarily taken as the maximum dilution producing agglutination; serum titres were recorded in units/ml (Humphrey & White 1963).

RESULTS

The results of testing schizont-infected erythrocytes against antisera to homologous and heterologous parasites are summarized in Table 1, which shows both the high specificity of the reaction produced by each relapse type, and the titre of agglutinins detected. The antigenic specificity of each relapse indicated by these results was further emphasized when K19-derived parasites, which had failed to react with the antisera to other relapses, were tested against sera from the chronically infected monkey from which it was isolated. Two serum samples taken during the infection, but before the K19 relapse had no effect on K19 parasites at 1/50 dilution, although the first sample agglutinated K1A, and the second sample agglutinated both K1A and K1B, at titres greater than 1250 units/ml. In contrast, K19 parasites were agglutinated by serum collected 6 days after the isolation of K19 at a titre of 6250, and by a sample collected 67 days later at 31 250 units/ml. A similar result was obtained with sera and the stabilate K21 obtained from the other chronically infected monkey. These K21 parasites appeared to be antigenically related to K19 (see Table 1).

Schizonts derived from K1A2 and K1B2 (secondary isolations of K1A and K1B) were not appreciably less specific than K1A and K1B, but parasites at the second monkey passage of K1A reacted with both K1A2 and K1B2 sera at higher titre, indicating that an increase in antigenic heterogeneity had occurred with passage.
Later relapses in the chronically infected monkeys rarely showed more than one parasite per hundred erythrocytes. The stabilates (K19 and K21) derived from late relapses produced a lethal infection when inoculated into normal monkeys, indicating that the low parasitaemias in the chronically infected animals were due to immune suppression of the parasites and not to a loss of virulence by these later variants.

DISCUSSION

As judged by the titres of agglutinins detected in our tests, *P. knowlesi* and presumably therefore other species of malaria, are quite strongly immunogenic. Natural antibodies cannot be involved in these reactions since sera collected before infection were without effect. In some instances, artificial dissociation of the parasites by antimalarials may have enhanced the immune response, but comparable titres of agglutinins were also detected after the spontaneous disappearance of the K19 and K21 relapses. Parasites derived from one relapse stabilate agglutinated at high titres only with antisera obtained from monkeys infected with the same stabilate, while sera from monkeys cured of infection with parasites from other relapses had little or no effect. It is conceivable that the difference shown by the K1A and K1B stabilates (i.e. of successive relapses) were drug-induced, but this cannot apply to the other relapses, so that the specificity of the reaction seems explicable only on the basis of antigenic variation analogous to that occurring in trypanosomes. Also in trypanosomiasis drug and antibody-induced relapse variants are antigenically indistinguishable (KNB - unpublished). Eaton (1938) has shown that trophozoite-infected erythrocytes are not agglutinated, and we have found this to be so with antisera from monkeys infected with both homologous and heterologous stabilates.

In a comparison of schizont- and trophozoite-infected cells isolated at the same time from a K1-infected monkey, the schizont-infected cells gave titres of >1000 units/ml for anti-K1 serum, but the trophozoite-infected cells were not agglutinated by this serum or by antiserum to any other relapse. The absence of agglutination with homologous antiserum implies that either the trophozoites and schizonts are antigenically different, or that the trophozoites, unlike the schizonts, are protected from antibody by the containing erythrocyte. In exoerythrocytic *P. gallinaceum* the host
cell becomes progressively damaged as the parasite matures (Meyer & Musacchio 1965), and similar damage by the mature schizont to erythrocytes may render the schizonts more accessible to antibodies.

So far, the present experiments have shown only relapse-specific agglutinins and not the protective antibodies of similar specificity which are implied by the relapsing nature of chronic malaria. The appearance of aberrant schizonts and the decrease in the proportion of mature to immature schizonts which occur at crisis (Taliaferro & Taliaferro, 1934; Taliaferro, 1949), indicate the possible protective role of antibodies reacting with schizont-infected cells, but it is not certain whether the cells are destroyed by lysis or by phagocytosis following agglutination or opsonization.

In our experiments, relapses in chronic infections usually produced low parasitaemias only, but when stabilates of these relapses were inoculated into normal "non-immune" monkeys, virulent fatal infections followed. Thus, there appears to be another partially protective immune response transcending antigenic variation, although purely variant-specific antibodies are probably decisive in terminating each relapse. The nature of the more general immunity is not known but it appears to be effective against all relapse variants, and may be a response to antigens common to all variants (unpublished gel-diffusion experiments have shown that common antigens occur). Alternatively, an adjuvant action by parasite constituents may produce a non-specific heightening of the immune response (Munoz, 1964). Some of the splenomegaly and liver and bone marrow changes characteristic of malaria (Taliaferro, 1949; Clark & Tomlinson, 1949) are perhaps associated with an adjuvant action (Havens, 1959; Munoz, 1964), although in one study, malarious children of three years of age, showed no better response to tetanus toxoid than controls (McGregor & Barn, 1962); auto-immune destruction of erythrocytes may also play a part in reducing parasitaemias (Zuckerman, 1964).

Exoerythrocytic infection was unlikely to have occurred with our blood-induced P. knowlesi infections (Garnham et al., 1957), but in other species, e.g. P. vivax, erythrocyte-infecting relapses may also arise from the exoerythrocytic pool (Garnham, 1951).
There may be separate and distinct immunity to the two phases, erythrocytic and exoerythrocytic (Shortt & Garnham, 1948), and the persistence of exoerythrocytic infection as in *P. vivax* may itself involve antigenic variation. In infections like *P. falciparum*, a variant-transcending immunity in both the liver and blood may limit the duration of the infection.

The implications of antigenic variation for trypanosomiasis have been discussed elsewhere (Brown, 1963), and some of these possibilities may be applicable to malaria. Insect transmission has a stabilizing effect on antigenic variation in trypanosomes, as all variants generally return to a common parent type following development in the vector (Broom & Brown, 1940; Gray, 1962). The possible occurrence of this reversion in malaria is of considerable biological interest, particularly as this parasite is known to have a sexual stage in the vector, and it may also be important in the interpretation of epidemiological and immunological data. The fluorescent-antibody and haemagglutination tests (Stein & Desowitz, 1964; Tobie, 1965), are currently used in malaria epidemiology but these reactions are not species specific and probably normally measure "common" antigens. Nevertheless, when human malarial parasites are used as a source of antigen, possible reactions with the "variable" antigens should be taken into account.

Frequent antigenic variation by the parasite may also be a factor contributory to the persistent macroglobulinaemia which sometimes accompanies malaria (Tobie, 1965), as each successive variant antigen is likely to stimulate the production of a new $1^\circ$ antibody (Haurowitz, 1965) to give the raised IgM levels observed during continuously patent infections and at each relapse; a similar macroglobulinaemia occurs in trypanosomiasis (Mattern et al., 1961).

The simultaneous occurrence of two levels of immunity, one specific for each relapse variant, and the other transcending antigenic variation and only partially inhibiting all relapses, may account for the inconsistent results that have been obtained by artificial immunization (Taliaferro, 1949; Jerusalem, 1965), where the possibility of antigen variability within the strain used has not been allowed for. The development of an antimalarial vaccine, particularly against *P. falciparum*, now seems to depend on the following considerations, (a) the successful in vitro
cultivation of the human parasite as a source of dead antigen or of attenuated live material (Weiss, 1965), (b) the absence of appreciable differences between strains and (c) the ability of humans to develop an immunity transcending antigenic variation. Geographically separated strains may not be as different as once thought (McGregor et al., 1963), and human adults do develop reasonable immunity to \textit{P. falciparum} (McGregor, 1964). Children exposed to endemic malaria however, show high parasitaemias up to 6 to 10 years of age, and this susceptibility may mean that resistance depends on an experience of a wide but finite range of antigenic variants occurring in one locality. Alternatively, children, unlike adults, are perhaps incapable of developing a generalized immunity effective against all variants. Children do appear to become blood positive earlier than adults after prophylactic antimalarial treatment (Kligler & Mer 1933, Clyde 1965), and young rats infected with \textit{P. berghei} relapse more frequently and with higher parasitaemias than older animals (INB unpublished data). Thus the young of some hosts may be constitutionally incapable of developing a generalized immunity, and if this should prove to be so, immunization is unlikely to be effective. Much more evidence is needed on this point.

**SUMMARY**

Frequent antigenic variation has been demonstrated in blood-induced \textit{P. knowlesi} infections, which implies that it probably occurs in human malaria also. This variation can explain the chronicity of the disease and the inconsistent results obtained with artificial vaccines; it may possibly account for, or contribute to, the persistently raised IgM levels which accompany malaria. Some degree of immunity transcending antigenic variation also occurs, but its potential value as a possible basis for the development of a vaccine is uncertain.
CERTAINS PROTOZOAIRE PARASITES PEUVENT SURVIVRE ET SE MULTIPLIER DANS L'ORGANISME D'ANIMAUX HÔTES CHEZ LESQUELS EXISTE UN TAUX D'ANTICORPS PARASITICIDES ÉLEVÉ RÉSULTANT D'UNE INFECTION. ON POSSÈDE DES PREUVES INDIRECTES QU'UNE CERTAINE VARIATION ANTIGÉNIQUE PEUT SE PRODUIRE DANS LE PALUDISME DES RONGEURS, MAIS L'APPARITION DE VARIATIONS ANTIGÉNIQUES RÉPÉTÉES, À UN DEGRÉ QUI POURRAIT EXPLIQUER LE PALUDISME SIMIEN OU HUMAIN CHRONIQUE, N'A PAS ÉTÉ PRÉCÉDEMMENT DÉMONTRÉES.

On a utilisé un dérivé de la souche Nuri de Plasmodium knowlesi, qui a été injecté par seringue à des singes rhésus soumis à une chimiothérapie insuffisante pour être curative, de manière à produire une infection chronique récidivante. Un stabilat congelé de cette souche a été isolé ainsi que les stabilats de quatre rechutes. Des isolats secondaires ont également été obtenus.

Des sérum antiparasitaires dérivés de stabilats déterminés ont été obtenus par prélèvement sur des singes guéris et des échantillons de sérum ont été recueillis sur des singes chroniquement atteints. Tous les sérum ont été conservés à -20°C et inactivés à 56°C avant d'être utilisés. Pour l'épreuve d'agglutination, des érythrocytes infectés par des schizontes ou par des trophozoïtes ont été séparés par centrifugation de sang fortement parasité prélevé 6 à 7 jours après infection par un stabilat.

Une variation antigénique fréquente a été mise en évidence dans des cas d'infection à P. knowlesi provoqués par injection de sang, ce qui implique qu'elle a probablement lieu également dans le paludisme humain. Cette variation pourrait expliquer la chronicité de la maladie et l'incohérence des résultats obtenus au moyen de vaccins artificiels; elle expliquerait peut-être, ou contribuerait à expliquer, l'élévation constante des taux de IgM qui s'observe dans le paludisme. Un certain degré d'immunité dépassant la variation antigénique a aussi été constaté, mais l'intérêt qu'il pourrait présenter comme base pour la mise au point d'un vaccin a paru douteux.
<table>
<thead>
<tr>
<th>Immunized with stabilate</th>
<th>Serum</th>
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<tbody>
<tr>
<td></td>
<td>K1</td>
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<tr>
<td>Monkey</td>
<td>1</td>
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<tr>
<td><strong>Antigen</strong></td>
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<tr>
<td>K1</td>
<td>&gt;500</td>
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<td>K1A</td>
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<td>K1A2</td>
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<td>K1B</td>
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<td>K1B2</td>
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<tr>
<td>K19</td>
<td>&lt;10</td>
</tr>
<tr>
<td>K21</td>
<td>&lt;10</td>
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</tbody>
</table>

* Serum taken from chronically infected monkey after isolation of the stabilate.
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