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Antigenic variation of bloodstage malaria parasites

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An antigen on the surface of erythrocytes infected with mature asexual malaria parasites has been shown to undergo antigenic variation in two malaria species. *Plasmodium falciparum*-infected erythrocytes from squirrel monkeys express a new antigen that is identified by reactivity with antibody from infected animals in an indirect immunofluorescence assay. Cloned *P. knowlesi* parasites in rhesus monkeys undergo antigenic variation of an erythrocyte surface antigen as defined by antibody-mediated cell agglutination (the SICA test) and indirect immunofluorescence. This variant antigen is a malarial protein that changes both in size (M_r 185000–225000) and antigenicity in cloned parasites derived by antigenic variation *in vivo*. Antigenic variation on the erythrocyte surface probably contributes to the capacity of malaria parasites to establish chronic infections with multiple recrudescences and to the finding that individuals can be repeatedly reinfected. The fundamental reasons for expression of these highly immunogenic antigens on the erythrocyte membrane remain obscure. Other major questions remain to be explored: the repertoire of variant antigens; the genetic basis of antigenic variation and the structural basis for the antigenic uniqueness of each variant antigen. Some properties of malarial antigenic variation indicate that control of antigenic variation in plasmodia will be quite different to antigenic variation in the African trypanosomes. The host spleen is required both for variant antigen expression and antigenic variation, and variant-specific antibody appears to induce antigenic variation rather than select pre-existing variants.

Introduction

Antigenic variation and antigenic diversity

Asexual bloodstage malaria parasites exhibit a remarkable capacity to survive in the immunized host. A single inoculation with asexual parasites may initiate several weeks or months of chronic low-grade parasitaemia. Furthermore, a host that has recovered from several malaria infections is usually susceptible to reinfection, even with the same parasite isolate. The complex interaction of immunized hosts and asexual parasites appears to involve both the evasion of immunity by the malaria parasite and the ability of the host to limit parasite growth below life-threatening levels. This review concerns only the first aspect of this interaction: other papers in this symposium address the nature of parasite-destructive immune responses and the nature of antigens that elicit these responses (L. Miller, J. Deans, N. Holder, this symposium).

Although numerous explanations have been advanced for the chronicity of malaria and the capacity of asexual parasites to reinfect the host (see Brown 1969), extensive serological and immunochemical evidence points to the capacity of asexual malaria parasites to undergo antigenic variation as of paramount importance (Brown & Brown 1965; Brown *et al.* 1968; Voller & Rossan 1969*a*; Brown *et al.* 1970*a, b*; Brown 1971; Butcher & Cohen 1972).

Variant antigens have been identified on the surface of several pathogenic organisms that

are extracellular or free-living for all or part of their existence. For example, the variant glycoprotein on the plasma membrane of salivarian trypanosomes (Cross 1978; Cross, Turner, this symposium), variant antigens on the surface of *Borrelia* spirochetes (Barbour *et al.* 1982; Stoenner *et al.* 1982), variant antigens on the flagellum of *Salmonella* species (Iino 1977; Silverman *et al.* 1979) and on the fimbriae of *Escherichia coli* (Brinton 1959; Eisenstein 1981). Antigenic variation is a property of cloned organisms such that the progeny of a single organism can express alternative antigenic forms of a functionally similar molecule. Variation in antigenic structure of these molecules allows the organism to evade immune responses directed against its outer membrane. The malarial variant antigens are distinguished from these other examples by being expressed on the surface of an infected host cell by an organism that is predominantly intracellular.

Malaria parasites exhibit both antigenic variation and antigenic diversity. Antigenic diversity is the expression of antigenically different forms of an antigen by different malaria isolates. Parasites exhibiting antigenic diversity may be derived from different geographical locations, different individuals at the same location, or different malaria episodes in the same individual. Antigenic diversity may also be identified in parasites derived at different times from a non-cloned isolate cultured *in vitro*. Under such conditions it cannot be assumed that the phenotypically different parasites are derived one from another. Antigens that exhibit diversity may or may not confer a selective immunological advantage to the parasite. Antibody-dependent selection of different phenotypes is not implied.

Numerous malarial antigens exhibit antigenic diversity in different *P. falciparum* isolates – for example, the major glycoprotein ($M_r \approx 200\,000$) on the surface of mature asexual parasites (McBride *et al.* 1982) and the S-antigen released into plasma during rupture of schizont-infected cells (Wilson 1980). With the limited information available it is generally assumed that the antigenic diversity of such antigens reflects the expression of different allelic forms of a single gene. The diverse forms are generated either by accumulation of mutations or genetic rearrangements during meiosis. In contrast, antigenic variation reflects the differential expression of a particular antigenic phenotype from a repertoire of genes capable of expressing alternative phenotypes in progeny organisms. However, antigenic diversity and antigenic variation are not necessarily mutually exclusive properties of a malarial antigen. An antigen that exhibits antigenic diversity could also undergo antigenic variation.

The first part of this review summarizes the serological and immunochemical evidence for antigenic variation in malaria. All of the early evidence for antigenic variation used uncloned isolates of *P. knowlesi*. Thus, new antigenic forms could have represented minor subpopulations of parasites of *independent* genetic origin which escaped immune responses developed against the phenotype of the majority population. Despite this caveat, none of the conclusions reached previously with uncloned organisms have been contradicted by studies with clones. The earlier experiments with uncloned *P. knowlesi* have been summarized elsewhere (Brown 1976; Brown 1977).

Serological characterization of malarial variant antigens

Variant antigens of Plasmodium knowlesi

Mature trophozoite and schizont-infected erythrocytes of *P. knowlesi* from rhesus monkeys are agglutinated by antimalarial antibody (Eaton 1938). Uninfected erythrocytes and cells containing ring stages and young trophozoites are not agglutinated. These observations

identified a new antigen on the erythrocyte membrane (figure 1). Subsequent studies with relapse populations of parasites from a recrudescing non-cloned *P. knowlesi* infection revealed that this new antigen was different with different recrudescences (Brown *et al.* 1968; Brown *et al.* 1970 *a*, *b*). The schizont-infected cell agglutination test (or SICA test) showed that each variant population of parasites stimulated specific agglutinating antibodies in the infected monkey.
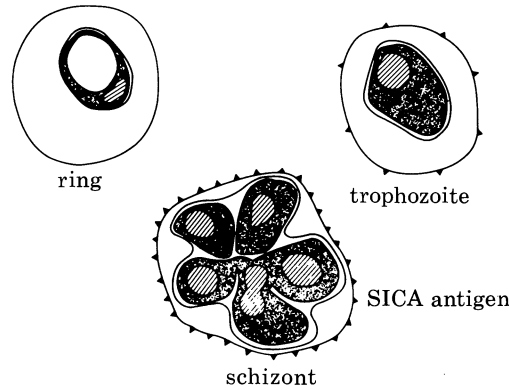

FIGURE 1. Expression of the SICA or variant antigen on the surface of rhesus monkey erythrocytes infected with mature trophozoites and schizonts of *Plasmodium knowlesi*. Indirect immunofluorescence reveals the SICA antigen (▲) on the surface of mature trophozoite and schizont-infected erythrocytes.

Transfer of blood from a chronically infected animal to naive recipients demonstrated the appearance of new variants of this surface antigen, even when the infection was subpatent. Variant parasites collected at this stage remained fully virulent in nonimmune animals (Brown *et al.* 1968). The new surface antigen responsible for antibody-mediated agglutination of infected erythrocytes was called the SICA or variant antigen (Brown *et al.* 1968) (figure 1).

Clones of *P. knowlesi* that exhibit serologically distinct SICA phenotypes have been derived one from the other by antigenic variation *in vivo* (Barnwell *et al.* 1983 *a*). The steps involved are summarized in figure 2. Schizont-infected erythrocytes were cloned by micromanipulation
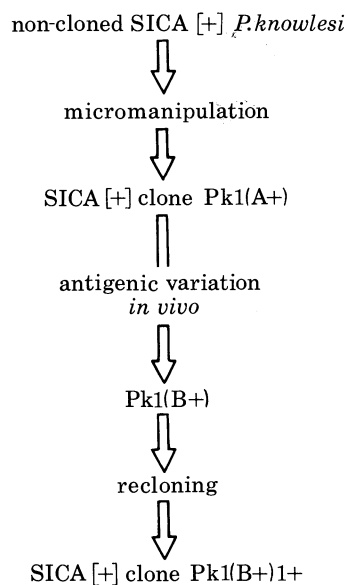

FIGURE 2. Derivation of cloned *P. knowlesi* parasites of different SICA phenotype by antigenic variation *in vivo* (from Barnwell *et al.* 1983 *a*).

and used to infect individual rhesus monkeys. The infection in one animal was drug-cured after cryopreserving the expanded clonally derived parasite population Pk1(A+). Several days later, the serum of this animal showed variant-specific agglutinating antibody to the original inoculum. The animal was then reinoculated with the initial inoculum. A population of bloodstage parasites, Pk1(B+), appeared after several days. These schizont-infected cells were not agglutinated by the animal's serum and were recloned by micromanipulation and expansion in a naive animal. In this way we produced agglutinating serum to the second clone (Pk1(B+)1+). Each clone expresses a SICA or variant antigen and can be specifically agglutinated by homologous serum (table 1). It was possible to produce variant-specific agglutination of schizont-infected erythrocytes from each clone by using sera from immunized rabbits as well as immunized monkeys (table 1). Sera from monkeys chronically infected with non-cloned parasites of the same strain agglutinated infected cells from both clones.

TABLE 1. ANTIGENIC VARIATION IDENTIFIED BY VARIANT-SPECIFIC AGGLUTINATION OF CLONED PARASITES WITH MONKEY AND RABBIT ANTISERA

antiserum		reciprocal agglutination titre	
donor†	immunogen	Pk1(A+)	Pk1(B+)1+
normal M	none	below 10	below 10
infected and immunized M	Pk1(A+)	10240–40960	below 10
infected and immunized M	Pk1(B+)1+	below 10	40960–81920
chronic infected M	non-cloned, SICA[+]	2560–5120	5120–20480
normal R	none	below 10	below 10
immunized R	Pk1(A+)	10240	below 10
immunized R	Pk1(B+)1+	below 10	above 20480

† Rhesus monkeys (M) and rabbits (R) were infected and immunized as described elsewhere (Howard *et al.* 1983 *a*).

Indirect immunofluorescence studies have provided additional evidence for antigenic variation by cloned *P. knowlesi* parasites. Fluorescein-conjugated secondary antibody can identify variant-specific antibody bound to the surface of *P. knowlesi*-infected cells (Hommell & David 1981). This assay demonstrated variant-specific reactivity of different antisera with the cell surface of clones Pk1(A+) and Pk1(B+) (Barnwell *et al.* 1983 *a*). Purified erythrocyte membranes from schizont-infected erythrocytes also react specifically by agglutination and indirect immunofluorescence with homologous variant-specific antisera (Aley *et al.* 1984). The SICA phenotype of the cloned parasite populations Pk1(A+) and Pk1(B+)1+ was homogeneous in each case. There was no evidence for a change in SICA phenotype when either population was expanded in naive animals.

Variant antigens of Plasmodium falciparum

Erythrocytes infected with mature asexual *P. falciparum* express new antigens on the erythrocyte surface (Kilejian *et al.* 1977; Langreth & Reese 1979; David *et al.* 1982; Celada *et al.* 1982; Udeinya *et al.* 1983). Immunoelectromicroscopy has identified new antigens at the knob protrusions characteristic of *P. falciparum*-infected cells (Kilejian *et al.* 1977; Langreth & Reese 1979). Most significantly, new antigens that appear to differ antigenically between parasite isolates have been identified on the erythrocyte surface by binding of antibody-coated

cells to Protein A Sepharose and indirect immunofluorescence (Hommel *et al.* 1982; Hommel *et al.* 1983). With mature parasitized erythrocytes of Palo Alto, FCR-3 and Indochina-1 strains, homologous convalescent sera from infected squirrel monkeys gave a specific, but low titre, surface immunofluorescence reaction. Some hyperimmune sera gave higher titres and remained specific for the homologous strain, others increased in titre but reacted with more than one strain. Treatment of infected erythrocytes with trypsin or chymotrypsin abolished surface immunofluorescent activity, suggesting that the new strain-specific antigen is partly protein in nature. We do not yet know whether the new antigens detected by indirect immunofluorescence are localized at knobs or distributed over the entire erythrocyte surface. With one of the non-cloned strains examined in this study, rechallenge of squirrel monkeys with the initial inoculum or with the first parasite peak produced recrudescent parasite populations (Hommel *et al.* 1983). These recrudescent populations could then be used to reinfect and challenge naive animals, and thereby obtain monkey antiserum against each population. Serum from convalescent animals always reacted specifically with the cells of the inoculated population. Each recrudescent population of *P. falciparum* derived from a single isolate can therefore be identified by a characteristic new antigen on the infected cells' surface. Two explanations may account for the data. (i) Antigenic diversity in recrudescent parasites could be generated if the original non-cloned inoculum contained several serotypes. Antibody generated against the predominant serotype would select for minor serotypes. (ii) The parasite has the genetic capacity to switch from one serotype to another within the time span of challenge and appearance of a patent recrudescence of new serotype. The second explanation is antigenic variation in the sense that is now apparent for *P. knowlesi*.

There are obvious parallels between these results with *P. falciparum*, earlier data of similar nature with non-cloned *P. knowlesi* (Brown *et al.* 1968, 1970a) and more recent results with cloned *P. knowlesi* (Barnwell *et al.* 1983a).

Cloned *P. falciparum* have been shown to undergo modulation of antigenic phenotype at the surface of infected erythrocytes (Hommel *et al.* 1983). This switch in antigenic phenotype occurs during a change in host environment (between nonsplenectomized and splenectomized monkeys). Until this change can be shown to represent the expression of variant forms of an homologous antigen it cannot be described as antigenic variation in the sense that is appropriate to *T. brucei*, *P. knowlesi*, *Borrelia* and other species. This phenotypic switch is discussed in a later section.

Evidence for antigenic variation with other plasmodia

There is no direct serological or biochemical evidence for antigenic variation with plasmodia other than *P. knowlesi* and *P. falciparum*. Nevertheless, the results of numerous biological studies are consistent with the existence of antigenic variation in several rodent and simian malarias. Infections of mice with the rodent malaria, *P. berghei*, can be suppressed by drug treatment. Repeated recrudescences can be isolated from such animals and the properties of relapse and initial inocula compared in previously infected and naive animals. Studies of this type (Cox 1957, 1959, 1962) suggested that parent and drug-induced relapse strains differ in virulence and immunogenicity. Antibody to *P. berghei* has also been used in an attempt to identify antigenic variants (Briggs *et al.* 1966, 1968). Passive transfer of antiserum from rats infected with *P. berghei* suppresses infections of this parasite in mice. The suppressive effect is transient

since a surviving population of parasites subsequently expands and persists in antiserum-treated mice, suggesting the selection of variant. The original and serum-resistant strain of *P. berghei* had the same virulence in naive mice (Briggs *et al.* 1968).

Additional evidence for antigenic differences between *P. berghei* derived under the selective presure of homologous immunity has come from experiments with the Anka line and clones of this parasite. Mice were multiply immunized by repeated infection and drug cure. After infection with a cloned parasite, successive waves of parasites with distinct intervening subpatent periods were observed (Wery *et al.* 1979). These authors suggested that the multiple recrudescences represented antigenic variants. Indirect support for this contention came from experiments in which naive mice were immunized with particular recrudescent populations and challenged with the same or different recrudescent parasites (Wery *et al.* 1979; Wery & Timperman 1979). Challenge with the homologous parasite recrudescence gave lower parasitaemias than challenge with an heterologous recrudescence derived from the same clone.

Antigenic variation has also been suggested to account for the characteristic recrudescence of asexual parasites seen with *P. chabaudi* infections of mice. Infection with cloned *P. chabaudi* initiates two patent parasitaemias; the initial parasite peak disappears by day 17, a recrudescent parasite peak becomes patent by day 25–30 (McLean *et al.* 1982). Serum collected from mice after the first parasitaemia transferred a higher level of protective activity against the initial population than against parasites collected from the recrudescence (McLean *et al.* 1982).

Experiments with *P. cynomologi bastianelli* in rhesus monkeys compared initial and relapse parasites after a single sporozoite-induced infection (Voller & Rossan 1969b). After spontaneous decline of the initial asexual parasite population, the animals were drug cured to remove all asexual blood parasites. Several relapses of *P. c. bastianelli* were observed, each separated by a subpatent period of several days. Naive animals were then infected by blood passage of the initial parasites or one of the relapse parasite populations, and chloroquine treated after spontaneous termination of the primary infection. The course of parasitaemia after challenge with the homologous parasites was compared with heterologous challenge with a different relapse peak. From the course of parasitaemia it appeared that late relapse parasites were immunologically different, both to the initial parasitaemia and to an early relapse population. Furthermore, immunity after infection was, to some degree, specific for the homologous parasite peak.

BIOCHEMICAL IDENTIFICATION OF MALARIAL VARIANT ANTIGENS

The experimental strategy

Identification of new antigens on the surface of erythrocytes infected with mature asexual parasites is complicated by several special properties of these cells. Serological assays indicate that the variant antigens are expressed maximally on schizont-infected erythrocytes (Eaton 1938; Brown *et al.* 1968). These cells are notoriously fragile and some may rupture during routine *in vitro* manipulation (Howard *et al.* 1982). Thus, labelling methods that are surface-membrane-specific with uninfected erythrocytes may also label intracellular components of schizont-infected cells. The increased permeability of the erythrocyte outer membrane to small anions and neutral compounds (Kutner *et al.* 1983) in erythrocytes infected with mature parasites complicates analyses by surface labelling methods. Finally, there are no monoclonal antibodies against variant antigens on infected erythrocytes. The variant-specific sera produced

against the *P. knowlesi* variant antigens and against new *P. falciparum* surface antigens are polyspecific – they react with intracellular malarial antigens as well as new antigens on the erythrocyte surface. Consequently, reactivity of a labelled antigen with antibody *per se* cannot be used to localize that antigen to the cell surface with this type of anti-serum.

As yet, only the *P. knowlesi* variant antigens have been identified (Howard *et al.* 1983*a*). One- and two-dimensional gel analysis of malarial proteins from clones Pk1(A+) and Pk1(B+)1+ of different variant antigen phenotypes has confirmed the phenotypic similarity of these parasites with respect to over 500 malarial proteins other than the variant antigens (Howard *et al.* 1983*b*, *c*). We sought to identify a molecule that would be immunoprecipitated from detergent extracts of each clone only by sera that agglutinate that clone.

Specific immunoprecipitation of radioiodinated antigens by agglutinating antisera

Clone Pk1(A+) has a pair of [^{125}I] protein antigens of M_r 210000 and 190000 that are only immunoprecipitated from SDS extracts of this clone by sera that agglutinate infected cells of this clone (Howard *et al.* 1983*a*). A different pair of [^{125}I] antigens were identified with clone Pk1(B+)1+, M_r 205000 and 200000, that were only immunoprecipitated by antisera that agglutinate this clone (figure 3*a* and Howard *et al.* 1983*a*). Parallel immunoprecipitations of SDS extracts from radiolabelled uninfected erythrocytes fail to show [^{125}I] antigens in this M_r range.
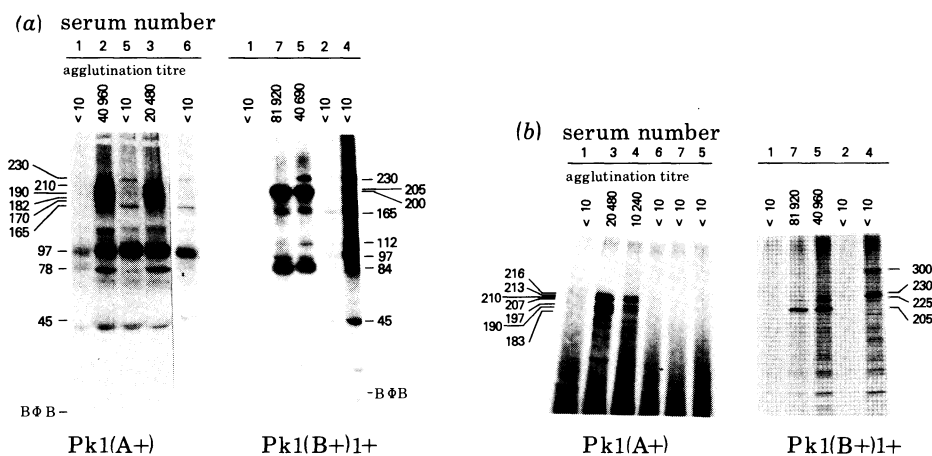

FIGURE 3. Biochemical identification of the *P. knowlesi* variant antigen. Immunoprecipitation of lactoperoxidase-labelled [^{125}I] antigens (*a*) or [^{35}S]methionine-labelled antigens (*b*) from extracts of SI-RBC of clones Pk1(A+) and Pk1(B+) using monkey antisera of defined agglutinability. (*a*) SI-RBC were radioiodinated by the lactoperoxidase method and extracted in 1 % SDS. Immunoprecipitation was performed after addition of Triton X-100 to 1.5 % and dilution of the extract to 0.5 % SDS. Antigens detected by radioautography. (*b*) SI-RBC were labelled by biosynthetic incorporation of [^{35}S]methionine during parasite growth *in vitro*. Purified intact SI-RBC were incubated with various sera, washed and antigen–antibody complexes extracted with 1 % Triton X-100. Antigens detected by fluorography. Gel lanes are identified by serum number and its reciprocal agglutination titre with SI-RBC of the same clone used for immunoprecipitation. The M_r of major antigens are given in kilodaltons. BϕB, Bromphenol blue (from Howard *et al.* 1983*a*). SI-RBC: schizont-infected red blood cells.

The use of SDS for extraction of radioiodinated infected cells was critical for identification of the variant antigens by immunoprecipitation. Earlier studies using Triton X-100 extracts failed to immunoprecipitate these proteins (for example, Howard *et al.* 1982). Triton X-100 was added before antibody addition to SDS extracts to adjust the extract to 0.3–0.5 % SDS and 1.5–2 % Triton X-100. Several detergents have been compared for their efficacy in

extraction of the [125I] variant antigens (Howard & Barnwell 1983, 1984). SDS is most efficient; sodium deoxycholate can also be used; several neutral, anionic and zwitterionic detergents are ineffective.

Several results confirm a cell surface localization of these variant antigens. (i) Analysis of radioactivity in haemoglobin and in a protein of M_r 230000 known to be localized on the surface membrane of mature intracellular parasites (David *et al.* 1984) indicates that the [125I] variant antigens can be radioiodinated under conditions of predominantly cell surface labelling; (ii) the [125I] variant antigens are cleaved by trypsin treatment (10 µg ml^{-1}, 5 min at 23 °C) of 'intact' radiolabelled cells (Howard *et al.* 1983c). These experiments also showed that trypsin treatment produced [125I]-labelled cleavage fragments of the variant antigen that remained associated with washed cells and were still specifically immunoprecipitated only by agglutinating antiserum. (iii) Variant specific antibody can form an immune complex with [125I] variant antigens during 30 min incubation with 'intact' labelled cells. Triton X-100 (1 %) could solubilize the immune complexes (Howard & Barnwell 1984), suggesting that binding of antibody to the variant antigen in the erythrocyte membrane may affect its interaction with other membrane components such that it is more efficiently extracted by Triton X-100.

Synthesis of variant antigens by the malaria parasite

The origin of new antigens that can be demonstrated serologically on the surface of malaria-infected erythrocytes has aroused considerable discussion and experimentation (see Gruenberg & Sherman 1983). The theoretical possibilities include the synthesis of antigens by the intraerythrocytic parasite or the alteration of host erythrocyte membrane components by some parasite-dependent process to create new antigenic determinants on host-derived molecules. Biosynthetic radiolabelling experiments with the *P. knowlesi* variant antigen have allowed us to prove that this molecule is a product of malarial protein synthesis, rather than an altered host protein.

Infected erythrocytes from each clone were cultured as synchronous populations from the ring stage to late trophozoite–early schizont stage in the presence of [35S]methionine. Various sera of defined agglutinability were added to *intact* biosynthetically labelled cells of each clone and after 30 min incubation the cells washed to remove unbound antibody. Immune complexes were solubilized with 1 % Triton X-100 and antigens analysed after using Protein A Sepharose for immune complex purification. With both clones, major [35S]-labelled proteins were identified that reacted only with agglutinating antisera (figure 3b) (Howard *et al.* 1983a). The M_r of these proteins and immunoprecipitation specificities with the panel of antisera were identical to the [125I] variant antigens for each clone.

The same technique revealed a complex group of variant-specific minor [35S] antigens. Bands of M_r 216000, 213000, 207000, 197000 and 183000 were immunoprecipitated from Pk1(A+) and a minor [35S]-labelled band of M_r 225000 was specifically immunoprecipitated from Pk1(B+)1+.

Biosynthetically labelled variant antigens have also been identified by membrane purification (Aley *et al.* 1984). A membrane fraction isolated from *P. knowlesi* schizont-infected cells was shown to include the outer erythrocyte membrane and an unknown proportion of the parasitophorous vacuole membrane but not parasite membranes. The number of malarial proteins recovered in this fraction after biosynthetic uptake of [3H]isoleucine or [35S]methionine is small (10–15 labelled bands). When labelled membranes from Pk1(A+) and Pk1(B+)1)

were solubilized directly in SDS-sample buffer and electrophoresed, the biosynthetically labelled variant antigens were apparent as the only proteins that differed in M_r between the clones. These radiolabelled proteins were also specifically immunoprecipitated from SDS-extracts of purified membranes only by antisera capable of agglutinating the particular clone (Aley *et al.* 1984).

Methods for antigen identification that employ radiolabelled amino acids to label the variant antigen biosynthetically and which lack specificity for components of the erythrocyte outer membrane have failed to identify the *P. knowlesi* variant antigens. No significant differences were detected in the radiolabelled proteins of clones of different SICA phenotype when compared by one-dimensional electrophoresis of solubilized infected cells (Howard *et al.* 1983*c*). We conclude that the variant antigens represent an extremely small proportion of the total malarial proteins. This low abundance and the presence of invariant antigens of similar size would also account for the failure to detect the biosynthetically labelled variant antigens when labelled infected cells are solubilized *directly* in SDS and the extract immunoprecipitated with a panel of sera of defined agglutinability (Howard *et al.* 1983*c*). There is no information on the structural basis for antigenic variation of the malarial variant antigen. We do not know if the malarial variant antigens bear a carbohydrate component. Attempts to label the *P. knowlesi* variant antigens by biosynthetic uptake of [³H]glucosamine or [³H]mannose have failed (R. J. Howard, unpublished).

The malarial variant antigens are tightly associated with the erythrocyte membrane. The washing of purified membranes in hypotonic solution does not release the *P. knowlesi* variant antigen (Vincent & Wilson 1980; Aley *et al.* 1984). A battery of neutral and zwitterionic detergents that remove lipids and many other proteins fail to dissociate the variant antigen from other insoluble membrane components (Howard & Barnwell 1983, 1984).

Another important question raised by the location of this malarial protein in the host-cell membrane concerns its disposition with respect to the inner and outer faces of the lipid bilayer. Does the antigen penetrate the membrane bilayer to interact with the submembrane cytoskeleton, or is it entirely associated with peripheral proteins and lipids on the external membrane face? There is no direct information on this question. The variant antigen is large enough ($M_r \approx 200\,000$) to traverse the lipid bilayer more than once, as has been shown for band 3 ($M_r \approx 97\,000$) in the normal erythrocyte membrane (see Steck *et al.* 1978). There is circumstantial evidence that the *P. knowlesi* variant antigen is associated with the submembrane erythrocyte cytoskeleton as well as the lipid bilayer. The variant antigen is not solubilized by neutral detergents under conditions that solubilize the bulk of erythrocyte membrane lipids and a large proportion of band 3 and other integral membrane proteins (Howard & Barnwell 1984). Neutral detergents also fail to solubilize the cytoskeletal proteins (Yu *et al.* 1973). Sodium dodecyl sulphate does solubilize the variant antigen and concomitantly solubilizes the erythrocyte cytoskeleton (Howard *et al.* 1983*a*). These detergent solubility properties are quite different to those of the major malarial glycoprotein of the parasite plasma membrane. The latter protein is completely solubilized by Triton X-100 treatment (R. J. Howard, unpublished), suggesting that it is a typical integral membrane protein associated with the lipid bilayer (Yu *et al.* 1973). We have also attempted to release the malarial variant antigen from the membrane by specifically disrupting and solubilizing the erythrocyte cytoskeleton, using methods first developed for this purpose with normal human erythrocytes (Steck & Yu 1973). Protein perturbants such as lithium diiodosalicylate and *p*-chloromercuribenzoate selectively solubilize

a proportion of the membrane spectrin, without solubilizing membrane lipids, integral membrane proteins or the variant antigen. Sodium hydroxide solubilizes a proportion of the variant antigen and practically all spectrin. This treatment irreversibly destroys some immune-reactivity of the variant antigen. These results led us to expect that the variant antigen might interact with both the lipid phase and the cytoskeleton. It might then require sequential treatments with neutral detergents and protein perturbants to solubilize it completely. Such treatments failed to solubilize more than 20–30 % of the total variant antigen (R. J. Howard and M. Dayan, unpublished).

The approaches used to identify the *P. knowlesi* variant antigen have also been used to identify a malarial protein of *P. falciparum* expressed on the outer membrane of late-trophozoite and schizont-infected erythrocytes (Leech *et al.* 1984). This is the first malarial protein identified on the surface of mature *P. falciparum*-infected erythrocytes. This protein exhibits antigenic diversity in comparison of non-cloned isolates of different geographic origin. It may therefore be responsible for the phenomenon of antigenic diversity of erythrocyte surface phenotype seen in recrudescent *P. falciparum* parasites by the indirect immunofluorescence assay (Hommel *et al.* 1983). Several properties of this molecule are shared by the *P. knowlesi* variant antigen (table 2).

TABLE 2. COMPARISON OF *P. FALCIPARUM* AND *P. KNOWLESI* ANTIGENS IDENTIFIED ON INFECTED ERYTHROCYTES

	P. knowlesi variant antigen	*P. falciparum* antigen†
lactoperoxidase-catalysed radioiodination	+	+
M_r	185000–225000	260000–300000
parasite protein (metabolic labelling)	+	+
accessible to specific antibody on erythrocyte surface	+	+
trypsin sensitivity	+	+
detergent solubility:		
Triton X-100	−	−
SDS	+	+
antigenic diversity and M_r diversity	+	+
variation in clones derived one from the other	+	?
function	unknown	cytoadherence?

† Summarized from results of Leech *et al.* (1984).

With different non-cloned isolates of *P. falciparum* this protein exhibited diversity in M_r after lactoperoxidase-catalysed radioiodination or metabolic labelling with [³H]amino acids or [³⁵S]methionine (M_r 260000–300000). Like the *P. knowlesi* variant antigen, this protein was not solubilized by Triton X-100 but was extracted with SDS (Leech *et al.* 1984). The M_r 260000–300000 *P. falciparum* antigen is also of interest for the correlation of some of its properties with those of the moiety on infected cells that mediates cytoadherence to human endothelial cells (Udeinya *et al.* 1981) or amelanotic myeloma cells (Schmidt *et al.* 1982). Cytoadherence of infected erythrocytes is exquisitely sensitive to trypsin treatment. The [¹²⁵I]-labelled protein on infected cells is cleaved by the same conditions (Leech *et al.* 1984). Most importantly, cytoadherence is blocked by *Aotus* monkey antisera raised against the same parasite strain, but not by antisera raised against other strains (Udeinya *et al.* 1984). The

specificity of different sera for immunoreactivity with the [³H]- or [¹²⁵I]-labelled protein on intact infected cells is the same as their specificity for blockade of cytoadherence (Leech *et al.* 1984).

The spleen and malarial antigenic variation

The spleen is a major organ of host defence in malaria (reviewed by Wyler 1983). Recent studies have identified a hitherto unknown role of the spleen in malaria that raises many new questions on the dynamic interplay of host and malaria parasite. The presence or absence of the host spleen has been shown to affect the expression of at least four parasite-induced phenotypic alterations of the infected erythrocyte membrane: one example with *P. knowlesi* and three with *P. falciparum*.

SICA[+] and SICA[−] P. knowlesi

Passage of cloned *P. knowlesi* parasites that express a variant or SICA antigen (that is, SICA[+]) in splenectomized rhesus monkeys leads to loss of expression of variant antigen on the erythrocyte surface (that is, SICA[−]) (Barnwell *et al.* 1982). The parasites obtained from passage in splenectomized hosts cannot be agglutinated by any rhesus monkey or rabbit antisera – including sera raised by immunization or infection with the same parasites. Indirect immunofluorescent tests for variant antigen expression are negative with all sera (Barnwell *et al.* 1983*a*) and surface radioiodination experiments followed by SDS-extraction and immunoprecipitation fail to identify any new proteins in the M_r range of the variant antigens (R. J. Howard and J. W. Barnwell, unpublished). It was concluded that the host spleen was required for expression of the *P. knowlesi* variant antigen on the erythrocyte surface. This phenotypic change was not due to unusual properties of erythrocytes from splenectomized animals since SICA[−] (that is, non-agglutinable) parasites often remained SICA[−] on passage to naive intact animals (Barnwell *et al.* 1983*a*). The loss of SICA antigen expression was not due to loss of genetic material. In some cases when SICA[−] parasites were inoculated into intact hosts the parasite population detected after several days had switched back to expression of variant antigen (that is, to SICA[+]). An experiment was also performed with cloned SICA[+] parasites inoculated into a splenectomized animal, to test whether the SICA[−] population which appeared was derived by switching of SICA[+] parasites or selection of a small number of pre-existing SICA[−] parasites (Barnwell *et al.* 1983*a, b*). By indirect immunofluorescence testing with antiserum specific for the cloned SICA[+] inoculum it was possible to assay the surface antigen phenotype of individual infected cells in the emergent population. On first passage in a splenectomized host the agglutination titres of infected cells fell 4- to 16-fold but the percentage of fluorescence reactive cells remained the same as in the inoculum (more than 99%). The switch from SICA[+] to SICA[−] in splenectomized animals is therefore an alteration of expression by the total SICA[+] population, characterized by a decrease in the quantity of SICA antigen expressed in the erythrocyte membrane.

The biochemical basis of the SICA[−] phenotype is not known. The absence of a variant antigen on the erythrocyte membrane could reflect a spleen-dependent alteration of variant gene expression, altered translation of variant antigen mRNA or altered intracellular transport and insertion of variant antigen into the host cell membrane. Methods for identification of the variant antigen at cellular locations other than at the erythrocyte membrane have not been

developed. Immunization studies suggest that SICA[−] parasites do not contain the variant antigen. Hyperimmune rabbit or monkey antisera raised against various SICA[−] parasites fail to agglutinate SICA[+] parasites. However, this does not exclude the possibility that SICA[−] parasites synthesize a rare variant type but do not express it on the erythrocyte membrane. The SICA[−] phenotype affords a valuable biological probe for investigation of the functional roles of the *P. knowlesi* variant antigen.

In addition to this effect of the host spleen on variant antigen expression *per se*, the spleen has also been shown to be required for conversion of one antigenic phenotype to another in the presence of homologous antibody (Barnwell *et al.* 1983*a*, *b*). Studies with non-cloned (Brown 1973; Brown & Hills 1974) and cloned (Barnwell *et al.* 1983*a*, *b*) *P. knowlesi* parasites suggests that antigenic variation is an antibody-induced variation in gene expression. Reinoculation of intact monkeys with the same cloned SICA[+] parasite induces a change to another variant phenotype. This change is dependent on the presence of variant-specific agglutinating antibody since reinoculation of animals with a different SICA[+] clone does not induce a change in phenotype. Splenectomized rhesus monkeys with circulating variant-specific agglutinating antibodies have been used to examine the parasite's capacity for antigenic variation in this host environment (Barnwell *et al.* 1983*a*, *b*). Parasites did not switch their variant antigen phenotype despite the presence of variant-specific agglutinating antibody. Thus, antigenic variation requires the presence of both the spleen and variant-specific agglutinating antibodies.

P. falciparum surface antigens and the spleen

Experiments with *P. falciparum* in intact and splenectomized owl monkeys (*Aotus trivergatus griseimembra*) and squirrel monkeys (*Saimirei sciureus*) have shown that the spleen can modulate expression of knob protrusions on the outer erythrocyte membrane (Barnwell *et al.* 1983*b*) and the capacity of infected erythrocytes to bind to endothelial cells *in vivo* or *in vitro* (Barnwell *et al.* 1983*b*; David *et al.* 1983). Passage in splenectomized hosts can lead to loss of cytoadherence properties without loss of knob expression or to loss of knob expression and loss of cytoadherence. These phenotypic alterations of the erythrocyte membrane have not been linked to antigenic variation.

The spleen has also been shown to modulate expression of the new surface antigens on *P. falciparum*-infected cells that are detected by indirect immunofluorescence with monkey antisera (Hommell *et al.* 1983). Parasites of several *P. falciparum* strains have been maintained by continuous passage in intact (S+ lines) or splenectomized animals (S− lines). Different antigens are expressed on erythrocytes infected with the S+ and S− parasites. The evidence suggesting that the antigens detected by indirect immunofluorescence of S+ parasites in recrudescent populations are variant antigens was discussed earlier.

A clone (B-11), derived by limiting dilution of line PLF-3/S−, has been used to show that the antigenic difference between S+ and S− parasites represents a phenotypic switch modulated by the host spleen (Hommel *et al.* 1983). Clone B-11 was transferred from a splenectomized to an intact animal and the surface antigen phenotype assayed by indirect immunofluorescence using antisera to S+ and S− parasites. The surface antigens progressively switched from the S− to the S+ specificity over a six day period. A partial switch back to the S− specificity was observed when clone B-11/S+ was transferred into a splenectomized animal. Thus, the antigenic difference between S+ and S− lines is not due to a difference in erythrocyte properties between intact and splenectomized animals.

Hommel *et al.* (1983) have also shown that antibody to S+ parasites can select against this phenotype. When an intact monkey infected with the non-cloned line PLF-3/S+ was treated with anti-S+ serum the parasitaemia was dramatically suppressed. After several days of subpatency, a recrudescent peak was observed that proved to be reactive with anti-S− serum. Parasites of the S− phenotype may have been derived from S+ parasites, or may have been a minority population in the initial inoculum.

I have reviewed these experiments with S+ and S− surface antigen phenotypes at some length in view of the authors' contention that this spleen-modulated antigenic switch is a process of antigenic variation (Hommel *et al.* 1983). The term antigenic variation as applied to *T. brucei*, *Borrellia* sp. and *P. knowlesi*, refers to the expression of alternative antigenic forms of a particular antigen. Functional homology and some degree of structural homology of the alternative antigenic forms is implied. We do not know if the antigen or antigens detected by anti-S+ sera with S+ infected cells are in any way functionally or structurally homologous with those detected on S− infected cells with anti-S− sera. One antigen could be switched off on passage to a splenectomized host (possibly the variant antigen) and another antigen or set of antigens induced for expression. This situation would resemble the expression (on and off) of individual differentiation antigens under particular environmental conditions. At this stage I prefer to use the term 'antigenic switch' to describe the change in phenotype between S+ and S−-infected cells. The change undergone by *P. knowlesi* in splenectomized animals from SICA[+] parasites to SICA[−] parasites, is similarly an 'antigenic switch'. An obvious difference from *P. knowlesi* is that the parasites derived from splenectomized passage are serologically non-reactive at the surface of infected cells.

FUNCTION OF MALARIAL VARIANT ANTIGENS

Antigenic variation is clearly related to the evasion of immune responses directed against pre-existing variants. This avoidance phenomenon has been reviewed in detail by other workers (Brown & Brown 1965; Brown 1971; Voller & Rossan 1969a; Butcher & Cohen 1972). These considerations alone do not answer the more fundamental question of the functions of each individual variant antigen. Why has the intraerythrocytic malaria parasite advertised its presence to the immune system by expressing a highly immunogenic molecule at the erythrocyte surface that it must vary to escape an antibody response? In the case of *T. brucei*, the capacity to express alternative antigenic forms of a molecule that completely covers the plasma membrane of a parasite that is extracellular is a distinct evasive advantage (see Cross 1978). I can only suggest speculative possibilities for the functions of the malarial variant antigen.

The only relevant experimental evidence on this question comes from comparative studies on the biological properties of SICA[+] and SICA[−] parasites of *P. knowlesi* (Barnwell *et al.* 1983a, b). The growth rates of these clones, and of other SICA[+] and SICA[−] parasites, are indistinguishable in the early stages of infection (up to 1 % parasitaemia) in intact monkeys (Barnwell *et al.* 1983a). In addition, SICA[−] parasites uniformly reach high parasitaemias in splenectomized animals. We conclude that expression of variant antigen on the erythrocyte surface is not necessary for optimal growth.

The most intriguing observation from these studies was the greater virulence of SICA[+] parasites over SICA[−] parasites in intact (that is, non-splenectomized) hosts (Barnwell *et al.* 1983a and figure 4). Inoculations with SICA[−] parasites which remain SICA[−] are less

virulent than SICA[−] inoculations which switch to the SICA[+] phenotype, or inoculations with SICA[+] parasites (figure 4). Expression of the variant antigen *per se* appears to favour parasite survival in an immunologically competent (that is, intact) host. Variation of this antigen may be an *additional* mechanism to allow survival in the face of a variant-specific immune response. We have suggested (Barnwell *et al.* 1983*a*, *b*) that the variant antigen may depress a spleen-dependent response to another target of parasiticidal immunity. Such a target could be variant or strain-transcending. Alternatively, the variant antigen may somehow prevent access of the immune response to the parasite. These experiments must be repeated in the natural host of *P. knowlesi*, the kra monkey (*Macaca fascicularis*), as the virulence of SICA[+] parasites in the unnatural host (*M. mulatta*) is clearly abnormal, all rhesus monkeys being lethally susceptible to SICA[+] parasites.
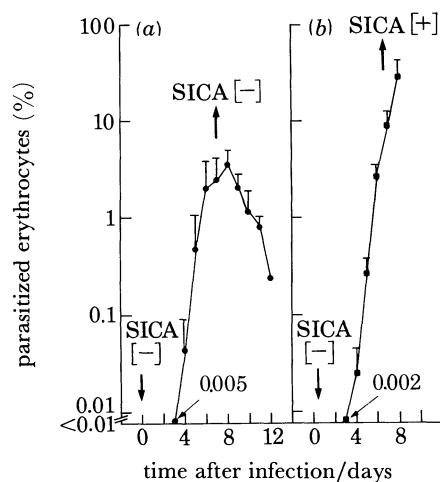

FIGURE 4. Evidence for a relation between expression of the variant antigen on *P. knowlesi*-infected erythrocytes and parasite virulence. Non-splenectomized rhesus monkeys were inoculated with cloned SICA[−] *P. knowlesi* and the mean asexual blood parasitaemias determined every 24 h. (*a*) Four monkeys in which the parasites remained SICA[−] during infection. The parasites in the fourth monkey were not SICA typed. (*b*) Four monkeys in which parasites reconverted to SICA[+] at some time during the infection. In each case parasite growth was not controlled. The mean prepatent periods for groups (*a*) and (*b*) were 3.75 ± 1.0 days and 4.25 ± 0.5 days respectively. (Results of Barnwell *et al.* 1983*b*.)

If the malarial variant antigens can eventually be shown to possess structurally invariant domains, another type of function can be invoked. Invariant domains could confer new properties to the membrane of infected erythrocytes that may be absolutely essential for parasite survival. For example, an alteration of metabolite flux. The fundamental role of the variant antigen could then be seen as the function of the invariant portion of the molecule.

Finally, the malarial variant antigen may be inserted into the host cell membrane to induce variant-specific killing and thereby ensure that both host and parasites survive. A chronic low-grade infection is of advantage to the parasite provided the levels of gametocytes remain sufficiently high for mosquito transmission. Evidence for variant-specific parasiticidal immune responses has been obtained for *P. knowlesi* infection in rhesus monkeys (Brown *et al.* 1970*a*, *b*; Brown 1971; Butcher & Cohen 1972; Brown & Hills 1974).

Genetic basis and control of malarial antigenic variation

Recent experiments proved that a cloned malaria parasite has the genetic capacity to express at least two different variant antigen phenotypes (Barnwell *et al.* 1983*a*). The size of this genetic repertoire is unknown but probably large. Serotyping of the *P. knowlesi* variant population Pk1(B+) that was derived by antigenic variation from clone Pk1(A+) *in vivo* (see figure 2) suggested that these parasites expressed several SICA phenotypes other than the phenotype of the recloned parasite Pk1(B+) (J. W. Barnwell, unpublished).

Antigenic variation could be generated by the differential expression of a particular variant antigen gene from a repertoire of variant antigen genes, or by a process of concomitant mutation. This question generated considerable argument with the *T. brucei* variant antigens. It was finally resolved only when structural information on the amino acid sequence of variant antigens expressed by progeny of a single organism showed that the extent of structural differences was too large to have been generated by concomitant mutation (see Cross 1978).

Expression and variation of the variant antigen of *P. knowlesi* requires the presence of the host spleen. There is no information on how the host spleen determines parasite phenotype. Perhaps spleen-derived factors (steroids, peptides) are required for some aspects of plasmodial gene regulation and expression. The sensitization and homologous challenge experiments with cloned (Barnwell *et al.* 1983*a, b*) and noncloned *P. knowlesi* (Brown *et al.* 1975) proved that antigenic variation only occurs in the presence of homologous agglutinating antibody. As variation was shown to occur without extensive parasite destruction, the evidence indicates that antigenic variation is actually *induced* by homologous antibody.

Antigenic variation: relevance to immunity and natural infections

Variant, strain and species-specific immunity

Immunity to asexual *P. knowlesi* infections in rhesus monkeys has been shown to exhibit three levels of specificity that can be induced by different infection regimes or immunization: variant-specific immunity, strain-specific immunity and species-specific immunity (Brown *et al.* 1970*a, b*; Brown *et al.* 1968; Butcher & Cohen 1972). Since these results were reviewed last (Brown 1976, 1977) there have been no major advances in understanding the molecular basis for this specificity, other than identification of the variant antigens.

Variant-specific agglutinating antibody does not appear to be protective (Brown *et al.* 1970*a, b*). Sensitized animals that produce high titres of agglutinating antibodies are fully susceptible to challenge with the same variant, owing to the parasites' capacity to undergo antigenic variation. Agglutinating antibody exerts an inductive effect on antigenic variation, since antigenic variation occurs without extensive parasite destruction. However, the capacity of infected monkeys to restrict the growth of new antigenic variants as they arise during chronic infection points to the existence of variant-transcending immune responses, which must destroy a large proportion of parasites. Immunization experiments using parasites of a particular variant phenotype and complete or incomplete Freund's adjuvant proved that an animal could induce immune responses that protect against challenge with other variants of the same strain but not against variants of another strain (that is, strain-specific), or protect against challenge with other strains of *P. knowlesi* but not against other simian malaria species (that is, species-specific) (Brown *et al.* 1970*a, b*).

Other experiments provided evidence for variant-specific parasiticidal responses that appear to be different to variant-specific agglutinating antibody. Variant-specific antibodies that mediate specific opsonization of schizont-infected cells have been demonstrated (Brown *et al.* 1970*b*; Brown & Hills 1974). The kinetics of appearance of these opsonizing antibodies can be different to the agglutinating antibodies. Where variation-inducing (agglutinating) antibodies appear in advance of parasiticidal (opsonizing) antibodies, the parasitaemia remains high even though it may fluctuate. When variant-specific agglutinating and opsonizing antibodies develop simultaneously during infection, the parasitaemia remains low. Furthermore, the rate of opsonizing antibody synthesis increases with the duration of infection, relative to the rate of agglutinating antibody, and both responses occur more rapidly to late variants than to early ones (Brown *et al.* 1968; Brown & Hills 1974). The molecular basis for the different properties of agglutinating and opsonizing variant-specific antibodies has not been elucidated.

Variant-specific parasiticidal responses that inhibit parasite multiplication at the terminal stage of intracellular development or merozoite attachment and invasion have also been identified (Butcher & Cohen 1972). The location of the variant-specific antigenic target in this inhibition assay was suggested to be on the merozoite surface.

Antigenic variation and human malaria

After release of one population of asexual *P. falciparum* parasites from the liver, the persistence of bloodstages in recrudescences may reflect the parasites' capacity for antigenic variation, as with chronic *P. knowlesi* infections in rhesus monkeys. Antigenic variation may also account for host susceptibility to reinfection. Liver-stage parasites from different mosquito inocula can establish asexual infections in hosts that have already cured multiple infections of the same malaria species. Each new population of asexual parasites released from the liver may express a different variant phenotype, or even change its phenotype, to evade variant-specific immune responses developed against earlier parasite inocula. With malaria species that undergo true relapses of liver stages (that is, release of different populations of asexual parasites from the liver at different times as a result of a single mosquito inoculation, as with *P. vivax*), it is also possible that each relapse population expresses a different variant antigen phenotype. Experiments with *P. cynomologi bastianelli* in monkeys have suggested that such relapse populations are antigenically different (Voller & Rossan 1969*b*), although the molecular basis for this observation remains to be demonstrated. Although it will be extremely difficult to document precisely the relevance of antigenic variation to the course of natural infection in man, this phenomenon is likely to contribute to the chronicity of natural infections and the capacity of malaria parasites to repeatedly reinfect the immunized host.
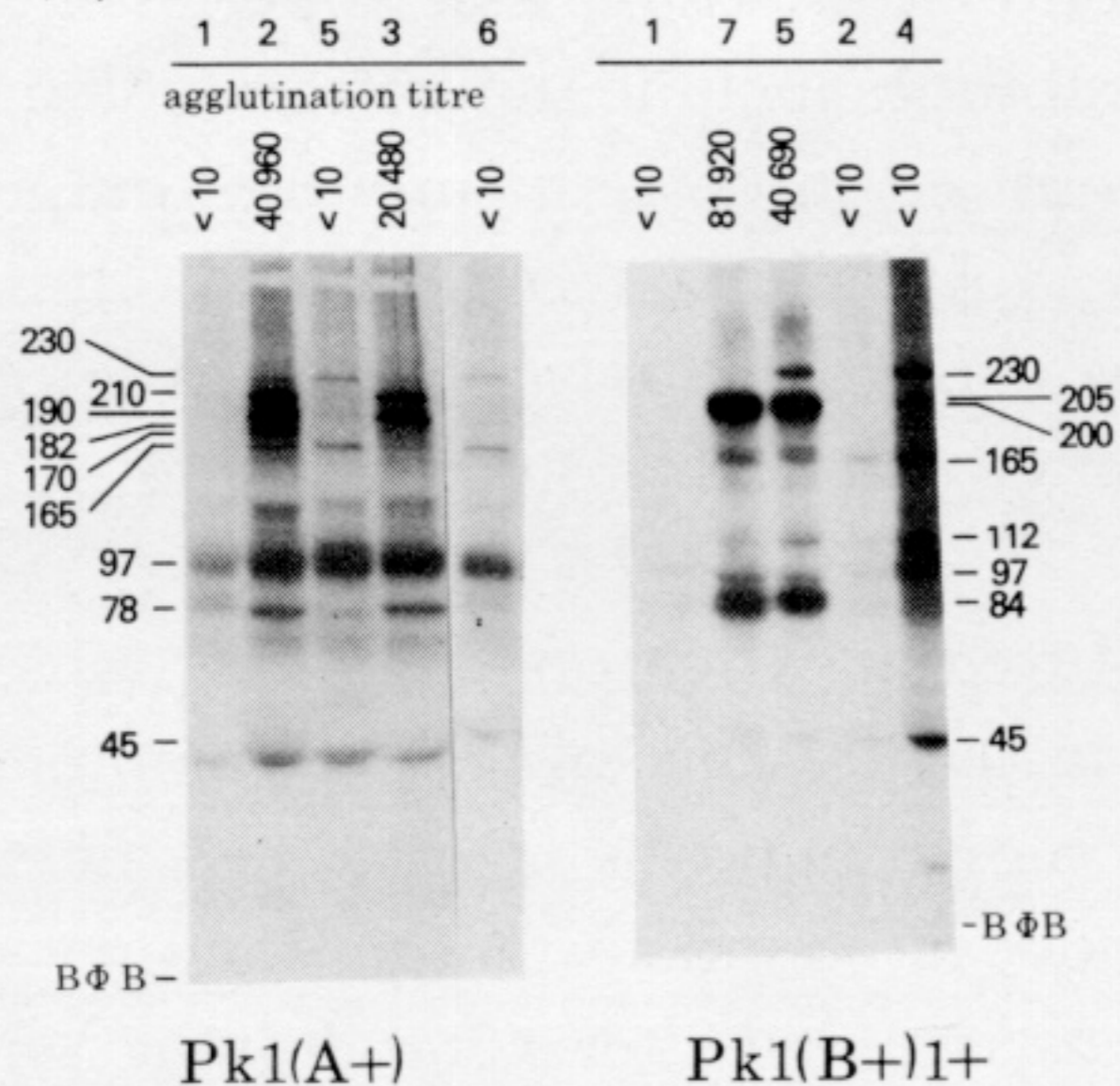
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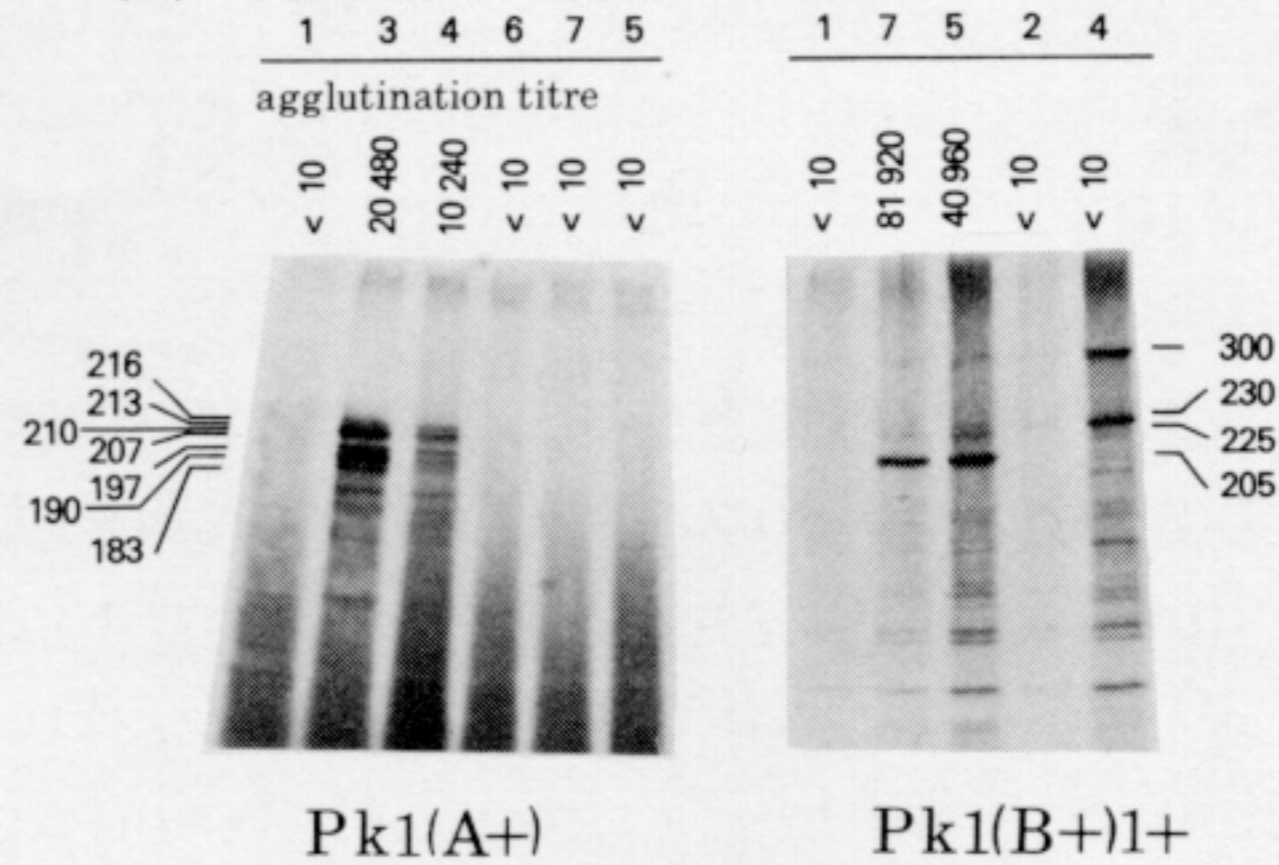
FIGURE 3. Biochemical identification of the *P. knowlesi* variant antigen. Immunoprecipitation of lactoperoxidase-labelled [^{125}I] antigens (*a*) or [^{35}S]methionine-labelled antigens (*b*) from extracts of SI-RBC of clones Pk1(A+) and Pk1(B+) using monkey antisera of defined agglutinability. (*a*) SI-RBC were radioiodinated by the lactoperoxidase method and extracted in 1% SDS. Immunoprecipitation was performed after addition of Triton X-100 to 1.5% and dilution of the extract to 0.5% SDS. Antigens detected by radioautography. (*b*) SI-RBC were labelled by biosynthetic incorporation of [^{35}S]methionine during parasite growth *in vitro*. Purified intact SI-RBC were incubated with various sera, washed and antigen–antibody complexes extracted with 1% Triton X-100. Antigens detected by fluorography. Gel lanes are identified by serum number and its reciprocal agglutination titre with SI-RBC of the same clone used for immunoprecipitation. The M_r of major antigens are given in kilodaltons. BϕB, Bromphenol blue (from Howard *et al.* 1983*a*). SI-RBC: schizont-infected red blood cells.