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Protective Antigens of Bloodstage Plasmodium knowlesi Parasites [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1984 **307**, 159-169
doi: 10.1098/rstb.1984.0116

Phil. Trans. R. Soc. Lond. B **307**, 159–169 (1984) [159]
Printed in Great Britain

Protective antigens of bloodstage *Plasmodium knowlesi* parasites

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The simian malaria *Plasmodium knowlesi* provides many favourable features as an experimental model; it can be grown *in vivo* or *in vitro*. Parasites of defined variant specificity and stage of development are readily obtained and both the natural host and a highly susceptible host are available for experimental infection and vaccination trials.

Proteins synthesized by erythrocytic *P. knowlesi* parasites are characteristic of the developmental stage, as are the alterations that the parasite induces in the red cell surface. Erythrocytic merozoites are anatomically and biochemically complex, their surface alone is covered by at least eight distinct polypeptides. Immune serum from merozoite-immunized rhesus recognizes many parasite components, especially those synthesized by schizonts. All of the merozoite surface components and some of the schizont-infected red cell surface antigens are recognized by such immune sera. Rhesus monkeys rendered immune by repeated infection may by contrast recognize comparatively few antigens; a positive correlation was established for these 'naturally' immunized monkeys between protection and antibody directed against a 74000 molecular mass antigen. Immunization with this purified antigen confers partial protection. Other putative protective antigens have been identified by monoclonal antibodies that inhibit merozoite invasion of red cells *in vitro*. The antigens recognized by inhibitory monoclonal antibodies are synthesized exclusively by schizonts and are processed, at the time of schizont rupture and merozoite release, to smaller molecules that are present on the merozoite surface. The multiplicity of protective antigens is clearly demonstrated by the fact that seven distinct merozoite surface antigens are recognized by three different inhibitory monoclonals. None of the protective antigens identified are variant or strain specific.

The clinical manifestations of malaria are associated with the asexual erythrocytic cycle of parasite proliferation. In the case of the primate malaria, *Plasmodium knowlesi*, this cycle occupies approximately 24 h: the shortest periodicity for any primate malaria; others have a 48 or 72 h cycle. *P. knowlesi* parasites develop synchronously, so that judicious sampling yields parasites of predominantly a single developmental stage. *P. knowlesi* is the only malaria species in which the phenomenon of antigenic variation can be clearly demonstrated on the basis of a simple agglutination test that detects variable antigens present on the surface of schizont-infected erythrocytes. The natural host of *P. knowlesi* is the kra monkey (*Macaca fascicularis*) from Malaysia and the Philippines; these animals, when infected, suffer a mild chronic relapsing parasitaemia. Experimental infections have been induced in a wide variety of simian species, but the rhesus monkey (*Macaca mulatta*), which suffers a fulminating, usually lethal, infection, has been the most widely used experimental host. *P. knowlesi* will also infect man and was employed therapeutically from the 1930s to early 1950s for the treatment of cerebral syphilis. The course of *P. knowlesi* infection in man is generally mild, with a tendency towards spontaneous recovery. The *in vitro* cultivation of *P. knowlesi* through a single cycle of

development was first achieved in 1971 by Butcher & Cohen and has since been refined so that the parasite can be maintained in continuous culture over several months (Wickham *et al.* 1980).

P. knowlesi thus provides many valuable features as a plasmodial species for experimental study. It can be grown *in vivo* or *in vitro*, parasites of defined variant specificity and stage of development are readily obtained and both the natural host (the kra) and a highly susceptible host (the rhesus) are available for experimental infection and vaccination trials.

Polypeptides of *P. knowlesi*

The bloodstage parasite assumes very different morphological forms during the course of the asexual cycle. These are associated with cytoplasmic growth, nuclear division and the formation and release of merozoites which are capable of invading fresh red cells. Biochemical and antigenic changes occur concomitantly with these morphological alterations. A detailed study of polypeptide biosynthesis in bloodstage *P. knowlesi* involved metabolic radiolabelling of synchronous parasite preparations during short, sequential, non-overlapping intervals. Analysis of these preparations by SDS polyacrylamide gel electrophoresis demonstrated the complexity of the parasite and established the stage-specific synthesis of plasmodial polypeptides (figure 1). Proteins synthesized by rings and trophozoites are broadly similar, but at the onset of schizogony, ring and trophozoite specific proteins cease to be synthesized and additional proteins emerge. Schizont-specific proteins are generally of higher molecular mass than ring stage proteins. Pulse-chase experiments revealed minimal changes in the major parasite proteins following synthesis (Deans *et al.* 1983).

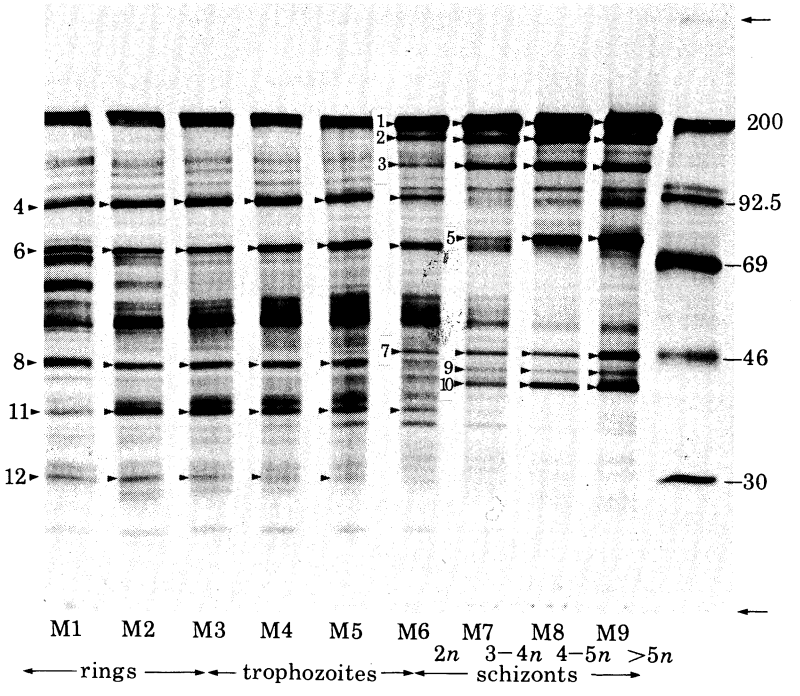

Figure 1. Stage specificity of polypeptide synthesis. SDS p.a.g.e. analysis of *P. knowlesi* parasites metabolically radiolabelled during sequential non-overlapping intervals (M1–M9) from young rings to mature schizonts. Arrow heads identify major bands which are synthesized predominantly by rings and trophozoites or by schizonts and molecular mass markers are shown to the right of the gel (from Deans *et al.* 1983).

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P. knowlesi induces a number of alterations in its host red cell; parasite-synthesized proteins are inserted in the membrane and erythrocyte membrane components are modified by the parasite. Changes in the schizont-infected red blood cell membrane have been demonstrated by surface radioiodination: eight new ^{125}I-proteins were shown to be parasite-dependent components of schizont-infected red cell membranes (Howard *et al.* 1982).

Merozoites have a structural organization that equips them for attachment to and invasion of fresh red cells. However, they are extremely labile and remain invasive for only about 30 min following release from schizont infected erythrocytes (Dennis *et al.* 1975). SDS polyacrylamide gel electrophoretic analysis of merozoite proteins, which had been metabolically labelled with [^{3}H]isoleucine during development from mature schizonts to merozoites, identified a large number of components from 20000 to more than 200000 molecular mass (Johnson *et al.* 1981). The merozoite surface, although covered by an apparently uniform coat of short filaments (Bannister *et al.* 1975) is also biochemically complex. Surface radioiodination of intact merozoites by the lactoperoxidase technique results in the radiolabelling of eight major bands ranging in apparent molecular mass from 22000 to 150000. The components found on the merozoite surface are parasite-specific and do not represent adsorbed serum constituents; they are also unrelated to those present on the surface of normal or schizont infected red cells. Trypsin treatment of viable merozoites blocks their ability to invade erythrocytes by preventing attachment to the red cell. Exposure of intact merozoites to trypsin results in the loss of the two highest molecular mass proteins (150000 and 105000) and the appearance of two smaller ones (molecular mass 70000 and 62000). These high molecular mass proteins are considered to be involved in the attachment of merozoites to erythrocytes (Johnson *et al.* 1981).

Protective role of anti-*P. knowlesi* antibody

Passive immunization of infants with severe *P. falciparum* malaria using IgG from immune adults suppresses the disease and reduces parasitaemia to very low levels (Cohen *et al.* 1961); passive transfer of immune rhesus monkey serum also confers protection against *P. knowlesi* infection, albeit less consistently (Coggeshall & Kumm 1937). Antibody from rhesus monkeys immune to *P. knowlesi* will inhibit the cyclical proliferation of the parasite *in vitro* (Cohen *et al.* 1969); there are several mechanisms whereby this may be brought about: antibodies may interfere with the intraerythrocytic maturation of the parasite, prevent merozoite dispersal, cause agglutination of merozoites, block attachment of merozoites to erythrocytes or inhibit the invasion process *per se*. The inhibition of invasion achieved is not only dependent upon the concentration of antibody included in the assay, but also on the concentration of cells in the suspension (see figure 2) and the starting parasitaemia. The potency of antibody in the inhibition of invasion assay is inversely related to the density of the cell suspension; the lower the cell concentration, the greater the time taken by merozoites to encounter target red cells and the longer the exposure to antibody. The higher the starting parasitaemia, the lower the antibody-to-target ratio and the lower the percentage inhibition of invasion. The demonstrated role of antibody in immunity to bloodstage malaria provides the rationale for using sera from immune hosts to characterize antigens and identify those with putative protective activity.

Sera from rhesus monkeys previously infected with *P. knowlesi* or vaccinated, have been employed for antigenic analysis. The number of components recognized by sera of vaccinated rhesus depends on the composition of the vaccine, its mode of presentation and the individual

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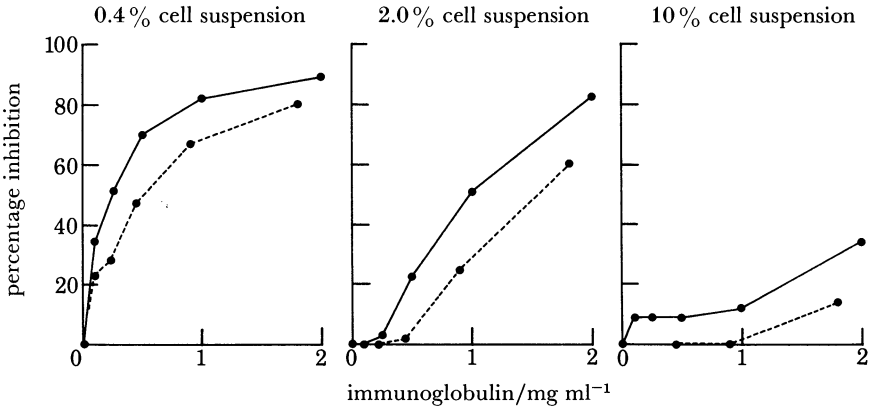

FIGURE 2. *In vitro* inhibition of merozoite invasion of red cells by antibody. Normal and *P. knowlesi* schizont-infected erythrocytes were mixed to yield a starting parasitaemia of 5%. The cells (at a final suspension density of 10%, 2% or 0.4%) were incubated with monoclonal antibodies R3/1C2 (——) and R3/2C3 (– – –) (see Deans *et al.* 1982) at a range of concentrations. Percentage inhibition of invasion was calculated by comparison with control cultures grown without antibody.

animal. Although not all the parasite constituents recognized by such sera are necessarily important for the induction of immunity, some certainly have functional significance.

TOTAL ANTIGENS OF *P. KNOWLESI*

A pool of serum from merozoite-immunized rhesus monkeys was used in a crossed immunoelectrophoretic analysis of rings, trophozoites and schizonts of *P. knowlesi*. This study identified qualitative and quantitative differences in antigens present at the different stages of development (Deans *et al.* 1978). Eleven major *P. knowlesi* antigens were recognized by this serum. Nine were present at all stages of development and two were found only in schizonts or trophozoites and schizonts respectively. This observation that ring-stage antigens are also present in schizonts (albeit at a relatively reduced concentration) was later confirmed by pulse-chase studies which revealed minimal levels of protein turnover during the development of bloodstage parasites (Deans *et al.* 1983).

Immunoabsorption with IgG from merozoite-vaccinated rhesus monkeys, of synchronous metabolically radiolabelled *P. knowlesi* preparations, demonstrates that only a small proportion of parasite polypeptides are antigenic. The immune IgG preferentially recognizes late stage antigens: in particular those synthesized by mature schizonts (figure 3).

The intracellular parasite induces biochemical alterations in the red cell membrane that cause the parasitized erythrocyte to become immunogenic to its host. Crossed immunoelectrophoretic analysis of surface labelled schizonts revealed that four of the 11 antigens recognized by serum from merozoite-immunized rhesus are exposed on the surface of schizont-infected erythrocytes (Deans & Cohen 1979). Howard *et al.* (1982) demonstrated that all the parasite-dependent proteins exposed on the outside of the red cell (demonstrated by surface radioiodination) are recognized by sera from infected monkeys.

Variant antigens appear on the surface of *P. knowlesi* trophozoite- and schizont-infected erythrocytes and are detected by antibody-mediated schizont infected cell agglutination (SICA). The variant antigen is synthesized by the parasite and becomes closely integrated with

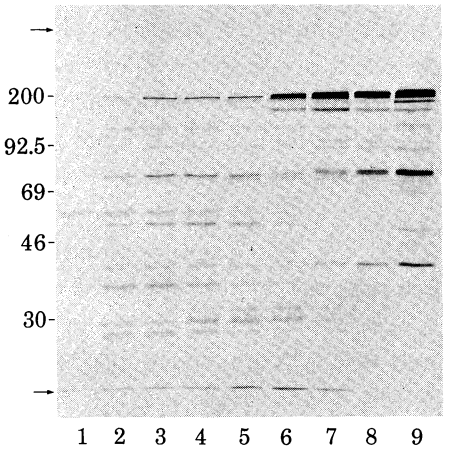

FIGURE 3. Stage-specific antigens recognized by immune rhesus IgG. Equal quantities of proteinaceous radioactivity from metabolically labelled cultures M1–M9 (see figure 1) were immunoabsorbed with IgG from merozoite vaccinated rhesus monkeys; proteins were analysed by SDS p.a.g.e. and fluorography. Tracks 1–9 represent antigens immunoabsorbed from preparations M1–M9 respectively.

the red cell cytoskeleton from which it is poorly extracted by Triton X-100 but more efficiently dissociated by sodium dodecyl sulphate. Part of the variant antigen is exposed on the outside of the schizont-infected red cell where it can be labelled by lactoperoxidase catalysed radioiodination and mediate the schizont-infected red cell agglutination reaction. The size of the SICA antigen is characteristic for the variant and is approximately 200000 molecular mass (Howard *et al.* 1983; Howard, this symposium). The variant antigen is only expressed on schizont-infected red cells from intact (non-splenectomized) rhesus monkeys, suggesting that the spleen has a modulating effect on antigenic expression in *P. knowlesi*. Expression of the variant antigen appears to influence parasite virulence (Barnwell *et al.* 1983). However rhesus monkeys with high titre SICA antisera are unable to control an infection, indicating that the variant antigen *per se* does not constitute an effective protective antigen.

Merozoites are antigenically complex: a comparative crossed immunoelectrophoretic analysis of merozoites and schizonts against immune rhesus serum revealed that they both contain the same range of antigens, albeit in different proportions. The two antigens found only in schizonts, or trophozoites and schizonts respectively are major merozoite antigens; both contain lipid moieties and are expressed on the surface of schizont-infected red cells where they are accessible to lactoperoxidase catalysed iodination (Deans & Cohen 1979). Surface labelling of merozoites identifies a restricted range of polypeptides, all of which are recognized by serum from immunized rhesus (J. A. Deans, unpublished).

PROTECTIVE ANTIGENS OF *P. KNOWLESI*

The outcome of *P. knowlesi* infection in the natural host, the kra monkey, and the laboratory host, the rhesus monkey, is very different. *P. knowlesi* infection follows a relatively similar course in the kra and rhesus during the first few days, but by the end of the first week the kra controls but does not eliminate the infection, whereas parasitaemia is progressive and fatal in the rhesus. Antibody capable of inhibiting merozoite invasion of red blood cells by several distinct variants

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of *P. knowlesi* is found in the serum of kra monkeys during the second week of infection (Cohen *et al.* 1977). An analysis of the early antibody response of kra monkeys to infection with *P. knowlesi* revealed antibody to schizont antigens of approximately 70000 and 150000 to 200000 molecular mass in resistant kra sera by day 10; further antigens were recognized by sera taken 2–3 weeks after initial infection (Johnson & Cohen 1984). The ability of the kra to control its infection suggests that antigens recognized early are likely to be important for the induction of immunity.

Rhesus monkeys can be successfully vaccinated against blood stage *P. knowlesi* by immunization with a variety of preparations containing defined stages of the parasite (Cohen & Mitchell 1978). The antibody specificities generated by immunized and functionally immune rhesus vary with the type of vaccine used, and the individual animal (Deans *et al.* 1978). The number of antigens recognized by immune sera is increased and the *P. knowlesi*-specific antibody titre elevated by repeated challenge. The identification of individual protective antigens from the wide spectrum of components recognized by sera of vaccinated rhesus is impossible.

Sera from rhesus monkeys rendered immune by repeated infection and drug cure may, by contrast, react with a comparatively restricted number of plasmodial antigens (Schmidt-Ullrich *et al.* 1979). Only two plasmodium-specific components (designated 1 and 13) were recognized by two 'natural immune' sera; these antigens are present in schizonts and membranes of schizont-infected erythrocytes of both Malaysian and Phillipine strains of *P. knowlesi*. Lactoperoxidase-catalysed radioiodination of schizont infected erythrocytes revealed that both are exposed on the surface of parasitized red cells. A longitudinal study revealed that the antibody titre against component 1 declined after the last challenge infection whereas that against component 13 did not and the animals remained clinically immune; component 13 was therefore considered to be a significant protective antigen. A survey of 27 rhesus monkeys revealed a positive correlation between anti-component 13 antibody and protection in animals rendered immune by repeated infection but not in monkeys protected by vaccination, indicating that an alternative immune mechanism must exist in the latter animals (Schmidt-Ullrich *et al.* 1981).

A limited vaccination trial of rhesus monkeys with purified *P. knowlesi* antigen 13 has been performed (Schmidt-Ullrich *et al.* 1983). *P. knowlesi* component 13 is a 74000 molecular mass protein with an isoelectric point of 5.2 which was described in earlier publications as a 65000 molecular mass glycoprotein. The antigen was isolated from purified membranes of schizont infected erythrocytes (containing four to six nuclei) either by extraction of membranes with Triton X-100 and immune precipitation with rhesus immune IgG or by solubilization of membranes with SDS. By using either of these preparations, the 74000 molecular mass protein was eluted after SDS polyacrylamide gel electrophoretic separation. The yield of 74000 molecular mass protein represented approximately 1% of the membrane protein. Four rhesus monkeys were immunized four consecutive times with 70 μg of purified 74000 molecular mass protein combined first with f.c.a. and then with f.i.a. After challenge, the vaccinated monkeys exhibited a delayed onset of patent parasitaemia which resolved between days 14 to 16 after peak parasitaemias of between 7 and 11%. Control animals injected with adjuvant alone developed parasitaemias of 25 and 39% seven days after an identical *P. knowlesi* challenge and required drug cure. Immunochemical analyses revealed antibody directed only against the 74000 molecular mass protein after the first two immunizations. After repeated antigen injection, however, antibodies appeared which were reactive with additional *P. knowlesi*

components of 102000, 140000 and 230000 molecular mass. These four immune-precipitated proteins exhibited a high degree of tryptic peptide homology and it was surmised that the 230000 molecular mass component represents a precursor protein from which the smaller proteins are derived. The degree of protection afforded in these experiments was only partial, and although the pre-patent period was extended, the maximum parasitaemias were very high. A search for other more potent protective antigens is indicated.

The application of hybridoma technology to malaria research has permitted the probing of individual antigens with reagents of unique specificity. Merozoites constitute the most efficacious form of bloodstage vaccine and this parasite stage has therefore been used to immunize the spleen cell donors. The limitations of using primate hosts has meant that the rodent monoclonal antibodies have not been used in passive transfer experiments. Instead, the ability of the monoclonal antibodies to block merozoite invasion of red cells *in vitro* has been used to identify functionally important antigens on the merozoite surface.

Epstein *et al.* (1981) produced 12 mouse hybridoma cell lines secreting monoclonal antibodies against *P. knowlesi* merozoites. Antibodies from three of the 12 lines agglutinated merozoites and the two most active in this respect blocked merozoite invasion of erythrocytes. Both inhibitory antibodies bound to antigens distributed over the entire merozoite surface and both specifically precipitated a single biosynthetically labelled schizont glycoprotein of apparent molecular mass 250000 present in both Malaysian and Philippine strains of *P. knowlesi*. This antigen, later defined as a 230000 molecular mass glycoprotein, is synthesized throughout schizogony, during the last 4–6 h of the 24 h cycle (David *et al.* 1984). The glycoprotein is a major parasite surface component which is inserted into the membrane of immature schizonts (not the schizont infected erythrocyte membrane). This location is revealed by immunofluorescence and immunoelectronmicroscopy using schizonts released from red cells by saponin lysis and by polyacrylamide gel electrophoresis after surface iodination and metabolic labelling associated with trypsin treatment. When the schizonts reach full maturity and the infected erythrocytes lyse, the 230000 molecular mass glycoprotein is processed to polypeptides of 75000, 57000, 50000 and 43000 molecular mass, which are present on the surface of the merozoite. Other cleavage products of the 230000 molecular mass glycoprotein were polypeptides of 200000, 145000 and 110000 molecular mass: these were not the result of natural *in vivo* processing, but were generated *in vitro* during detergent extraction by proteolytic enzymes active in the parasite during the later stages of schizogony. Whether or not this family of antigens constitute an effective vaccine has not yet been tested.

Johnson *et al.* (1981) identified proteins exposed on the merozoite surface and demonstrated that those of molecular mass 150000 and 105000 were completely removed by trypsin treatment. These molecules were thought to be involved in the attachment of the merozoite to the erythrocyte surface since trypsin treatment of merozoites prevents such attachment. A polyclonal antiserum to the higher molecular mass *P. knowlesi* merozoite surface antigen was raised by immunizing mice with liposomes containing merozoite proteins isolated from the 140000 molecular mass region of polyacrylamide gels (Hudson *et al.* 1983) (the 150000 molecular mass surface protein described by Johnson *et al.* (1981) was recalculated to be 140000 molecular mass). This serum immunoprecipitated only the 140000 molecular mass protein from surface-labelled merozoites, but the major protein immunoprecipitated from metabolically labelled schizonts had a molecular mass of 144000 (later designated 143000 molecular mass). It has since been shown that the 143000 molecular mass protein is converted to the 140000

TABLE 1. PROPERTIES OF (PUTATIVE) PROTECTIVE *P. KNOWLESI* ANTIGENS

reference† ...	(1)	(2)	(3)	(4)
defining Ab	'natural' immune rhesus serum	mouse mab 13C11	mouse mabs e.g. 1019C3	rat mabs R3/1C2 + R3/2C3
in vitro inhibition of invasion by Ab	n.t.	yes	yes	yes
Mz agglutination by Ab	n.t.	yes	yes	no
Ag protects *in vivo*	partial	n.t.	n.t.	n.t.
major or minor Sz Ag	major	major	?major	minor
Ag synthesized by Sz	?230000 glycoprotein	230000 glycoprotein	143000 protein	66000 protein
time of synthesis	?	throughout schizogony (4–6 h)	mid-schizogony	last 1.5–2 h of schizogony > 7*n* Sz only
Ag location in schizont-infected red cell	outer surface of schizont-infected red cell membrane (74000)	schizont membrane (230000)	?	?
processed antigen(s) on Mz surface	n.t. or none?	75000, 57000, 50000, 43000	140000	(66000), 44000, 42000
Mz surface antigens shed	not applicable	no	no	yes

Ab, antibody; Mz, merozoite; Sz, schizont; Ag, antigen; n.t., not tested.
† (1) Schmidt-Ullrich *et al.* (1983); (2) David *et al.* (1984); (3) Miller *et al.* (1984); (4) Deans *et al.* (1984).

molecular mass polypeptide at the time of schizont rupture and merozoite release. A panel of 11 hybridomas has been produced, all of which secrete monoclonal antibodies specific for the 140000 molecular mass merozoite surface protein (Miller *et al.* 1984). Four of the 11 monoclonal antibodies partly blocked invasion of erythrocytes by merozoites released from schizont-infected cells: they also caused multiple invasion of individual erythrocytes. However, when these monoclonal antibodies were incubated with free merozoites, they did not block invasion; they therefore appear to act by weakly agglutinating merozoites newly released from infected red cells. The failure to block invasion by free merozoites indicates that the merozoite receptor for red blood cells is probably not the 140000 molecular mass merozoite surface antigen; however it may constitute a significant protective antigen.

A panel of 28 rat monoclonal antibodies has been produced, which reacted with a wide variety of *P. knowlesi* components. Eight of these monoclonals were tested for their ability to inhibit parasite multiplication *in vitro*. Two of the purified antibodies (R3/1C2 and R3/2C3) inhibited multiplication in a dose-dependent manner (Deans *et al.* 1982). Inhibition was not associated with detectable damage to intracellular parasites, indicating that the inhibitory antibodies acted by blocking invasion of red cells by newly released merozoites. No agglutination of merozoites was observed with either antibody, suggesting that they may block attachment of merozoites to red cells, or the invasion process *per se*. Immunofluorescent analysis revealed that both inhibitory antibodies bound to schizonts with an intensity that increased during parasite maturation. Both reacted diffusely with isolated merozoites. Preliminary studies indicated that the two antibodies bound specifically to the same or closely related epitopes on a single 66000 molecular mass parasite protein. This putative protective antigen, which is common to W and Nuri strains of parasite, is synthesized only by schizonts with seven or

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more nuclei during the last 1.5–2 h of the 24 h erythrocytic cycle. The 66000 molecular mass antigen, which constitutes a minor parasite protein, is processed at the time of schizont rupture and merozoite release, to give rise to two smaller molecules of 44000 and 42000 molecular mass. The 44000 and 42000 molecular mass antigens and traces of the 66000 molecular mass antigen are present on the surface of isolated merozoites and the smaller antigens are readily shed (Deans *et al.* 1984). None of these molecules can be detected in newly invaded ring stage parasites, indicating that they are either excluded when the merozoite invades or, if internalized, they rapidly undergo further structural alteration. Vaccination trials with this family of antigens have not been carried out. The very minor proportion of the total parasite proteins that they represent makes direct purification of adequate quantities exceedingly difficult.

From the above studies it is apparent that four *P. knowlesi* antigens thought to have a protective role have been characterized (table 1), but only one has been used in immunization trials (and that with limited success). The antigens identified by workers in different laboratories are apparently all distinct, with the possible exception of the 74000 molecular mass glycoprotein described by Schmidt-Ullrich *et al.* (1983), which might correspond to the 75000 molecular mass fragment derived from the 230000 molecular mass glycoprotein defined by a mouse monoclonal antibody (13C11) (David *et al.* 1984). All the antigens with protective activity are found exclusively in merozoites or schizonts, or both. Those defined by inhibitory monoclonal antibodies are synthesized during schizogony and then cleaved to a smaller molecule, or molecules, at the time of schizont rupture and merozoite release. The smaller antigens are expressed on the merozoite surface. All are common to different variants and strains of the parasite. Whether any one of these antigens is capable of inducing strong immunity against *P. knowlesi* infection remains to be tested. The mode of antigen presentation, the type of adjuvant, the route of administration and the simian species immunized will all influence the results of such a trial.

I am grateful to Professor S. Cohen for his constructive criticisms in the preparation of this manuscript. The work described from this department is supported by grants from The Royal Society, the Medical Research Council and the Malaria Component of the U.N.D.P.–World Bank–W.H.O. Special Programme for Research and Training in Tropical Diseases.

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Discussion

B. Mach (*Department of Microbiology, University of Geneva Medical School, 64 avenue de la Roseraie, 1205 Geneva, Switzerland*). I would like to summarize work carried out by Michael McGarvey, Luc Perrin and Bernard Mach, at the University of Geneva, and which is very relevant to the points just made by Dr J. Deans concerning the importance of proteins expressed *late* in schizogony.

(i) Antigens important for a protective immune response are produced at the late stages of the asexual cycle (mature schizonts–merozoites). Monoclonal antibodies against specific merozoite proteins are inhibitory in *in vitro* infections with *P. falciparum*. Some of these merozoite proteins have been found to protect Saimiri monkeys *in vivo*. Furthermore, some of the important protective antigens may not be very immunogenic and consequently will not be easily identifiable with human serum.

(ii) By differential hybridization using ring and mature schizont-specific mRNA probes, we have identified, in a cDNA library, clones corresponding to stage-specific genes expressed predominantly or exclusively in merozoites. These clones have been narrowed down to only

12 different genes, which represents a considerable enrichment of stage-specific clones in a total cDNA library (10 000 clones). mRNA studies have confirmed the stage-specific expression of these genes.

(iii) DNA sequencing has shown, in one case, a repeated pattern with a repeat unit of 12 base pairs (four amino acids).

(iv) These merozoite-specific cDNAs can be efficiently expressed in bacterial cells, under the control of an inducible promoter. The *Plasmodium*-specific polypeptide made can represent as much as 5 % of the bacterial proteins. Production of the protein products corresponding to each of the 12 merozoite-specific genes identified is under way.

(v) Some of the merozoite-specific cDNA clones produce a polypeptide that is recognized by monoclonal antibodies specific for the 200 000 and the 41 000 molecular mass merozoite polypeptides, using Eliza and Western blot assays.

The interest of this approach lies in the possibility of identifying the genes expressed late in schizogony, independently of the serological reactivity of their products with particular antisera or antibodies. The production of these merozoite polypeptides in bacteria, on a large scale, will permit them to be tested as protective antigens *in vivo*.

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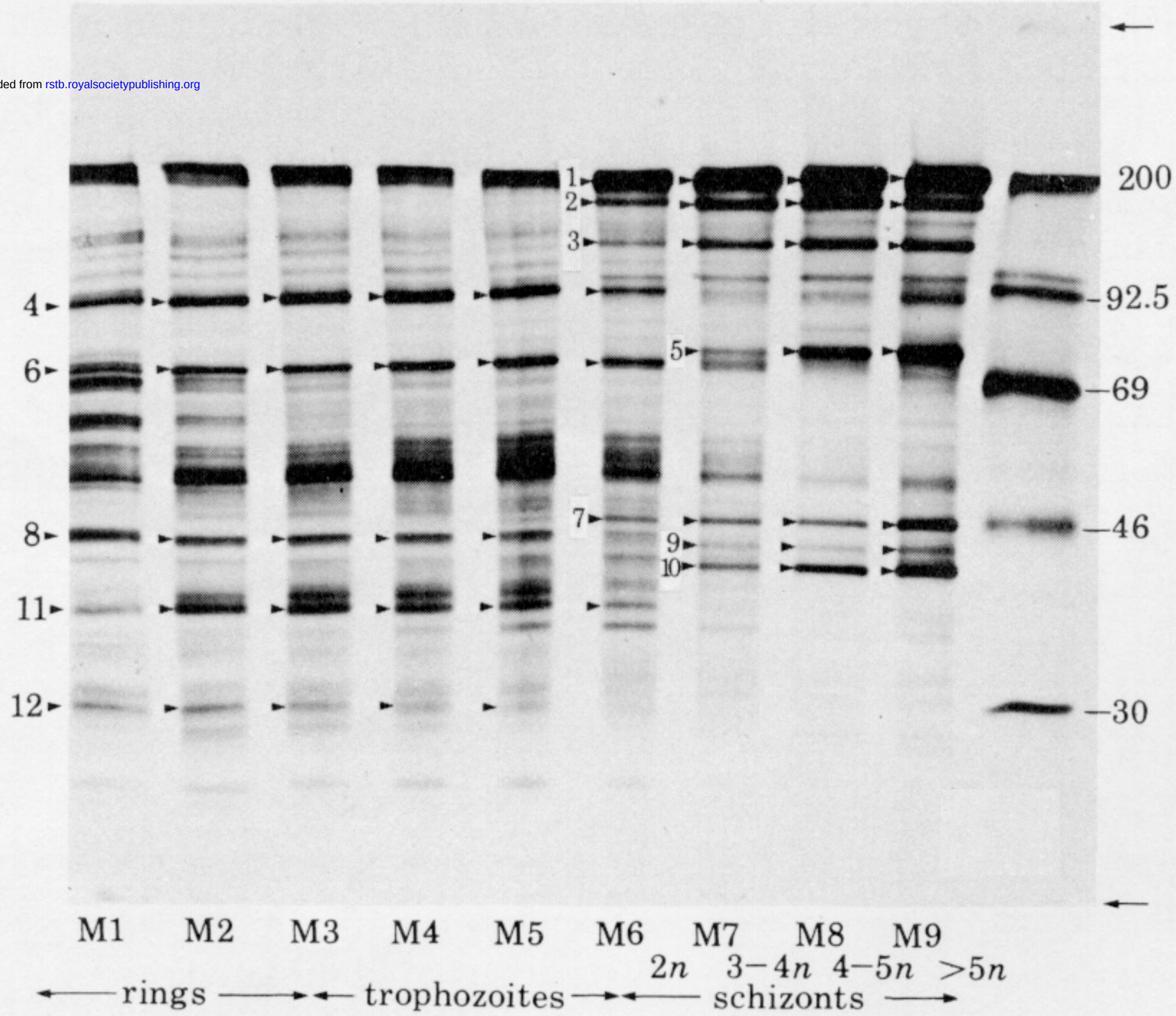
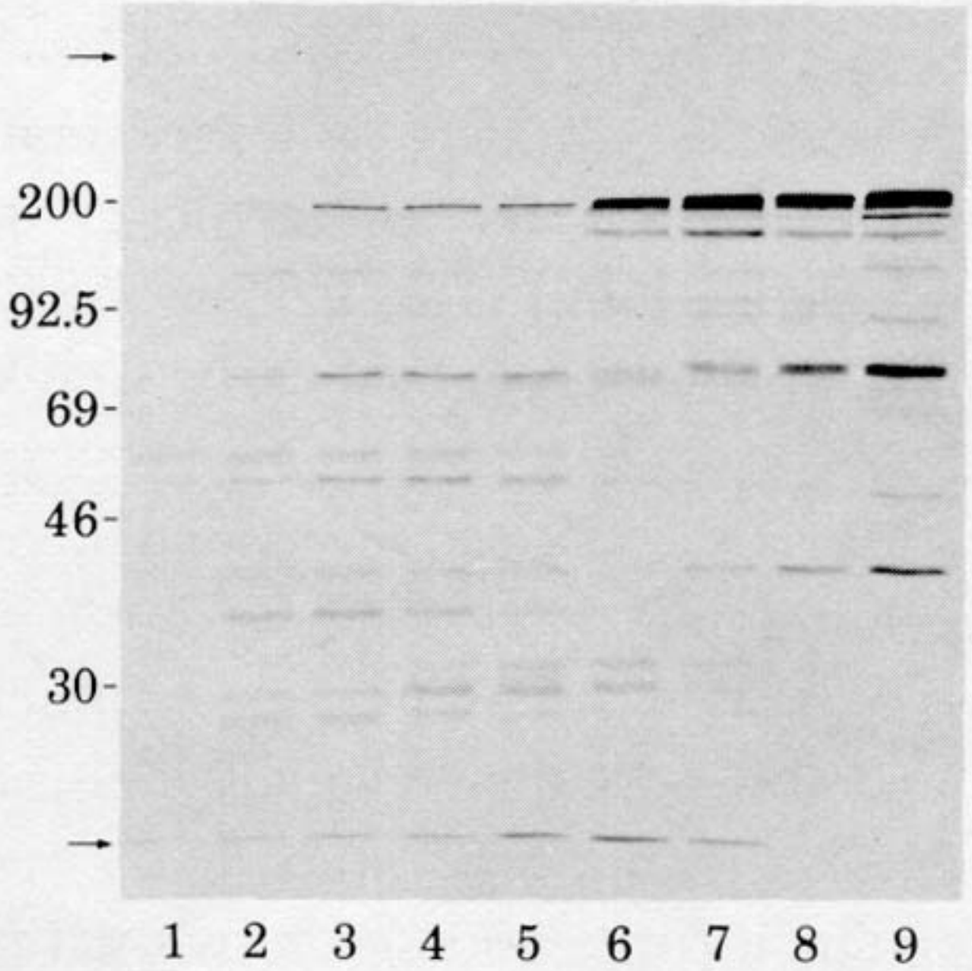


FIGURE 1. Stage specificity of polypeptide synthesis. SDS p.a.g.e. analysis of *P. knowlesi* parasites metabolically radiolabelled during sequential non-overlapping intervals (M1–M9) from young rings to mature schizonts. Arrow heads identify major bands which are synthesized predominantly by rings and trophozoites or by schizonts and molecular mass markers are shown to the right of the gel (from Deans *et al.* 1983).



GURE 3. Stage-specific antigens recognized by immune rhesus IgG. Equal quantities of proteinaceous radioactivity from metabolically labelled cultures M1–M9 (see figure 1) were immunoabsorbed with IgG from merozoite vaccinated rhesus monkeys; proteins were analysed by SDS p.a.g.e. and fluorography. Tracks 1–9 represent antigens immunoabsorbed from preparations M1–M9 respectively.