The role of *Plasmodium knowlesi* in the history of malaria research

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SUMMARY

In recent years, a malaria infection of humans in South East Asia, originally diagnosed as a known human-infecting species, *Plasmodium malariae*, has been identified as a simian parasite, *Plasmodium knowlesi*. This species had been subject to considerable investigation in monkeys since the 1930s. With the development of continuous culture of the erythrocytic stages of the human malarial parasite, *Plasmodium falciparum* in 1976, the emphasis in research shifted away from knowlesi. However, its importance as a human pathogen has provoked a renewed interest in *P. knowlesi*, not least because it too can be maintained in continuous culture and thus provides an experimental model. In fact, this parasite species has a long history in malaria research, and the purpose of this chapter is to outline approximately the first 50 years of this history.

Key words: malaria, plasmodium, knowlesi, history, monkey, macaque, immunity, pathology, culture.

> "A *P. knowlesi* infection in a human ….would probably be diagnosed as *P. malariae*" Garnham (1966)

INTRODUCTION

In his book 'Man's Mastery over Malaria', the eminent malariologist Paul Russell quoted a remark by Ronald Ross: 'an historical introduction is always necessary to give coherence to ideas'. If for no other reason, this seems to justify an account of the history of *Plasmodium knowlesi* in malaria research (Russell, 1955). Despite the estimated annual numbers of 250 million cases and 2·5 million deaths due to malaria in Russell's time, he was optimistic concerning the ultimate victory over this disease. The number of cases has fallen dramatically since 1955, and there is renewed optimism that malaria will eventually be eliminated. However, malaria always seems to have a surprise for us, and, in this case, it is that *P. knowlesi*, traditionally considered an infection of monkeys, is now known to be endemic in humans in Southeast Asia.

The parasite is widespread, clinically relevant and potentially lethal (Cox-Singh and Singh, 2008; Cox-Singh *et al*. 2008). This should not have been entirely unexpected, as *P. knowlesi* was known to infect humans (shown by Knowles and Das Gupta, 1932) when it was first formally described and systematically named by Sinton and Mulligan (1932) and subsequently had been widely used to treat patients with neurosyphilis by induced pyrexia (e.g. van Rooyen and Pile, 1935; Ciuca *et al*. 1937). Work of this kind in patients (and experiments in volunteers) led to some insights into the variability of infection in man (see 'Pathology of *P. knowlesi* infection' section below). Interestingly, the likelihood that naturally occurring human infections of this parasite would be found was discussed by Garnham in his classic volume on malaria parasites (Garnham, 1966). In fact, the first demonstrably natural zoonotic infection of man in peninsular Malaysia had already come to academic attention, having been acquired by a man working at night in the bush in 1965 (Chin *et al*. 1965). Garnham also correctly predicted that *P. knowlesi* would be misdiagnosed in a routine laboratory as *P. malariae* because of the similarity of the gametocytes of the two species, or alternatively as *P. falciparum* if only ring forms were present. The newly appreciated zoonotic importance of the species has now contributed to renewed interest in the malaria parasites of macaque monkeys and their potential as experimental models.

STRAINS AND SUBSPECIES OF *P. KNOWLESI*

The importance of human *P. knowlesi* is beginning to lead to molecular identification and clarification of differences amongst isolates. Whilst formally described and named in 1932 (loc cit), the species had almost certainly been seen but not formally described before this in macaques (perhaps first by Franchini, 1927; cited by Garnham, 1966; Coatney *et al*. 1971), and other workers who were not (in the words of the latter) 'taxonomic addicts' and left

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this nomenclatural work to 'the brave'. Apart from the five early monkey isolations of Sinton and Mulligan from *Macaca fascicularis* – K1, K2, K3, K4 and C, differentiated by levels of cross-immunity (Mulligan and Sinton, 1933) – numerous other isolations of the type species have been used in the laboratory, notably 'Nuri' from Negeri Sembilan in peninsular Malaysia (Edeson and Davey, 1953), 'Malaysian' (Collins *et al*. 1967) and 'Phillipines' (Lambrecht *et al*. 1961), which were all isolated from monkeys, whist 'H' was isolated from the human case mentioned above (Chin *et al*. 1965). 'Hackeri' was isolated directly from a vector mosquito, *Anopheles hackeri* (Wharton and Eyles, 1961) in Malaya. No significant differences have been noted in blood stage morphologies or pathology, but in the early years of genetic analysis of parasites a difference was found to exist in the circumsporozoite protein gene repeat sequence between H and Nuri. This involved both the number of repeat sequences and the sequence of amino acids in each repeat (Sharma *et al*. 1985). It may be that such a difference is not taxonomically important (Mun *et al*. 2015), but full genomic data are not yet apparently available for Nuri.

Historically three sub-species divergent from the type have been recognized, for instance by Garnham (1966): *P.k.edesoni*, *P.k.sintoni*, and *P.k. arimai*. These were isolated in Malaysia, Java and Taiwan (infecting *Macaca cyclopis*), respectively. Their distinguishing features were in blood-film morphology and colouration on staining, and in varying propensities to sequester schizonts (Garnham, 1966). Like the type species, all are quotidian; *P. knowlesi* is the only primate malaria with a 24 h asexual cycle in the blood. *P. k.edesoni* was found by Coatney *et al*. (1971) to be much less pathogenic in rhesus than the type, but they report the sub-species as lost. They also record that Yokogawa was reported by Hsieh (1960) as stating that the Taiwanese parasite, *P. k. arimai*, would not infect man. It is not clear how these sub-species relate to populations studied more recently, where a number of clinical isolates have seemed to fall into two distinguishable genomic clusters (Ahmed *et al*. 2014; Pinheiro *et al*. 2015). Similarly lines derived from isolates used in laboratories have been compared genomically with clinical isolates and with the prototype genome (Pain *et al*. 2008) and confirm two current 'clinical' genomic clusters together with a group formed by several laboratory isolates (Assefa *et al*. 2015) Unfortunately it is now essentially certain that the material for the first knowlesi genome did not derive from 'H' strain as believed by the authors (Pain *et al*. 2008), but, as a result of a misunderstanding several decades ago, came from a distinct Malaysian isolate (Collins *et al*. 1967) (Collins, 1978, personal communication; Barnwell, 2016, personal communication; Barnwell, MS in preparation). This mislabelling was strongly suspected by Assefa *et al*. (2015) from their comparative results. Both H and Malaysian are geographically from peninsular Malaysia. The latter however has had a protracted history of rhesus monkey passage (e.g. Barnwell *et al*. 1983; Howard *et al*. 1983), and parasites derived from the same isolation and used by the present authors, but referred to as W strain, had, for instance, ceased to produce observable numbers of gametocytes. As found with cultured *P. falciparum*, artificial passage may have profound effects; possibly such are reflected in the 'laboratory cluster' seen by Assefa *et al*. (2015).

HOSTS AND THE *P. KNOWLESI*-RHESUS MODEL

The definitive (mosquito) hosts of the species were not historically well understood. *Anopheles hackeri* was clearly one (Wharton and Eyles 1961), and *Anopheles balabacensis* was found to be a vector in early survey work (Cheong *et al*. 1965) but it has taken molecular methods to establish the importance of *A. balabacensis*, a far more anthropophilic species than hackeri, as crucial in the bionomics of this malaria as a zoonosis in Sabah (Wong *et al*. 2015) as well as having been a reliable laboratory vector in work on experimental transmission (see Coatney *et al*. 1971). Numerous other *Anopheles* spp. are also known to be vectors, of which *A. cracens* was found biting both man and monkeys in a recent survey in peninsular Malaysia (Jiram *et al*. 2012) and *Anopheles latens* was the principal vector found for *P knowlesi* in the Kapit District of Sarawak, Malaysian Borneo (Tan *et al*. 2008). Vectors have been reviewed recently by Collins (2012).

The most frequent natural intermediate host of *P. knowlesi* is the kra, crab-eater or long-tailed macaque, *M. fascicularis* (previously *Silenus irus* = *M. irus*) but knowlesi has also been found in *M. nemestrina*, *M. cyclopis* and *Presbytis melalophus* (Garnham, 1966). A very full summary of the definitive and vertebrate hosts capable of supporting the parasite is given by Coatney *et al*. (1971). These authors also review the sporogonic and exoerythrocytic development in great depth. The tissue cycle is some 120–140 h, and sporogony takes 12–15 days.

The crab-eating macaque is found in Northern Thailand, through the Malay peninsula and throughout Indonesia and in the Phillipines (Wheatley, 1980). Although *M. fascicularis* may be simultaneously infected with *P. coatneyi*, *P. fragile* and *Plasmodium cynomolgi*, none of these parasites causes their hosts any severe symptoms (Butcher, 1996). In early survey work by several authors across this monkey's range, summarized by Aberle (1945), some 26% of *M. fascicularis* were found to carry malaria parasites of various species. In contrast to the kra, the closely related rhesus monkey (*Macca mulatta*) is extraordinarily sensitive to *P. knowlesi*

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and can even be infected with only ten parasitized erythrocytes (Butcher, 1970). The infection continues unabated until almost all red cells are infected and the animal dies with shock and anaemia, usually about a week or so after infection. There are some rhesus, which may be more resistant, since where these two closely related monkey species are sympatric in Northern Thailand there is some interbreeding (Wheatley, 1980; Butcher, 1996).

PLASMODIUM KNOWLESI IN VITRO: STUDYING THE PARASITE OUTSIDE ITS HOST

From the early years of the 20th century there was interest in maintaining malaria parasites *in vitro*. This was driven in part by a desire to understand the biochemistry of the organism *per se*, for instance, distinguishing essential from inessential compounds, but also to remove the parasite from the host immune response and other variables of its physiology. Once cyclical development and multiplication was achieved *in vitro*, antibody activity and other immune effectors could be titrated.

The importance of being able to study a parasite outside its host could not be over rated. The observation that *P. falciparum* would develop in human blood, when incubated in tubes to which glucose had been added (Bass and Johns, 1912) suggested long term *in vitro* culture was feasible. During World War II the US Army began a programme to define the conditions required for maintaining malaria parasites *in vitro*, with a major focus on *P. knowlesi*. The culture medium devised by the US Army workers in 1946 was based on an analysis of monkey plasma and became known as the 'Harvard Medium' (Anfinsen *et al*. 1946; Geiman *et al*. 1946; McKee and Geiman, 1946; Ball *et al*. 1948). In addition, these authors also tested a wide variety of culture vessels, determined the optimum levels of oxygen in the gas phase and measured glucose uptake and lactic acid production. Because of the rapid growth of *P. knowlesi* (glycolysis is 25 times faster in parasitized red cells compared with uninfected erythrocytes; Bertagna *et al*. 1972) the medium required considerable buffering capacity to keep the pH between 7·2 and 7·5. Initially this was supplied by N-glycylglycine, later replaced by HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Nevertheless, parasite yield was still limited by cell concentration and the frequency or rate at which used medium was removed and fresh medium added: hence the importance attached to the design of culture vessels to achieve optimum growth.

A further factor-limiting parasite replication *in vitro* was the condition of the normal uninfected red cells taken from an infected monkey but which deteriorated rapidly at 37 °C, as judged by reduced susceptibility to merozoite invasion (Trigg and Shakespeare, 1976). It was thought this may have been due to the presence of malarial toxins affecting the red cell membranes, as Dunn (1969) reported alterations in sodium concentrations, and De Zeeuw *et al*. (1972) observed changes in the phospholipid composition of uninfected cells from parasite donors. This suggested that when starting cultures parasite development might be improved with high dilutions of blood from uninfected donors.

Despite great care in following published methods, variability between laboratories of the biological components of the culture milieu impaired comparability. Poor parasite growth gave diminished multiplication. Efforts to improve matters by the addition of antioxidants in the form of vitamin C and vitamin E were unsuccessful. However, when monkeys were put on an ascorbic rich diet, their red cells supported better parasite development (McKee and Geiman, 1946; Butcher and Cohen, 1971). Interestingly, on a relatively low vitamin C diet, cultured red cells were abnormal in shape (acanthocytic) resembling those of patients lacking vitamin E (abetalipoprotinaemia; Butcher, 1970). [As a temporary measure, whilst the dietary situation was being clarified, horse serum enabled some cultures to yield reasonable results (Butcher, 1970; Butcher, 1979)]. More recently, these vitamins have been shown to play an important role in protecting murine parasitized erythrocytes from oxidative stress (Stocker *et al*. 1986). The nutritional status of the cell donors is thus important, as is the status of serum donors and when possible selection of screened, regular donors was highly desirable. As culture techniques improved, a primary aim of culturing knowlesi was to devise an entirely synthetic medium: that is one without serum. Whilst it was claimed that stearic acid could substitute for serum (Siddiqui *et al*. 1969), it was eventually concluded that good growth and multiplication could not be obtained in the absence of serum (see Bertagna *et al*. 1972). Modern substitutes for serum are however greatly improved, and in routine use.

By eliminating individual medium components, those that were essential to *P. knowlesi in vitro* became clear. These included p-aminobenzoic acid, isoleucine and methionine [at very low concentrations in monkey haemoglobin (Butcher, 1970; Siddiqui and Schnell, 1972) biotin, pantothenate, pyrimidines, and a source of lipid, most effectively supplied by serum (Geiman *et al*. 1966; Siddiqui and Schnell, 1972).

The requirements for *in vitro* studies on the erythrocytic stage of *P. knowlesi* and other *Plasmodium* spp. were outlined in a detailed review by Bertagna *et al*. (1972). Advances in culture techniques from the 1970s to the mid-1980s were reviewed again in 1985 (Trigg, 1985), the salient difference between these two reviews being the development of continuous culture, initially of

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P. falciparum (Haynes *et al*. 1976; Trager and Jensen, 1976) followed closely by *P. knowlesi* (Butcher, 1979; Wickham *et al*. 1980). The successful maintenance of parasites in continuous culture was probably due to a number of factors including the use of modern proprietary media (Trigg, 1969) (especially RPMI1640), without antibiotics or with only neomycin or gentamycin, careful attention to cell concentrations, medium replacement, the use of HEPES buffer, low oxygen concentration and recognizing that different isolates, as observed with *P falciparum*, could be quite variable in their requirements (Butcher, 1982).

Plasmodium knowlesi continues to be of value as an experimental model *in vitro*, now adapted to human red cells (Moon *et al*. 2013). Further developments included attempts to culture parasites in the absence of red cells (axenic culture, Nillni *et al*. 1985) and improved methods for *in vitro* culture of exoerythrocytic stages (Millet *et al*. 1988) Latterly, culture has come to be seen as a means of parasite production (minimizing host exploitation), a means of studying precise moments in developmental cycles (such as egress from rupturing schizonts, and the specificity and characteristics of merozoite invasion of red cells), and finally as one of the enabling technologies for the organism's genetic manipulation. Aspects of *in vitro* work are covered in the following sections, and also in the companion paper by Bannister in this volume.

CHARACTERISTICS OF THE *P. KNOWLESI*–RHESUS MONKEY MODEL WHICH PROMOTED ITS USE AS AN EXPERIMENTAL TOOL

A number of characteristics of the knowlesi–rhesus combination were attractive to researchers over many years and were regarded as potentially more suitable for the research envisaged than avian or, later, rodent species. They are summarized here as background to many of the research reports which follow:

(1) The quotidian asexual cycle of *P. knowlesi* is clearly more convenient than using species with a 48 or 72 h cycle.
(2) In the rhesus monkey the parasites are highly synchronous: in the authors' experience (Cohen *et al*. 1969) the vast majority of parasites were early schizonts in the morning, they then multiplied over mid-day to give an almost pure preparation of ring forms in the afternoon. However, synchronicity was only retained for up to six to eight passages through monkeys (using infected blood); after that it became necessary to start with a new frozen stabilate. Multiplication rates were variable depending on the progress of the infection, but could be as high as 10-fold (Garnham, 1966).
(3) As parasitaemias in the rhesus increased it became possible to separate schizonts from uninfected cells and rings by simple centrifugation (Brown and Brown, 1965; Mitchell *et al*. 1973). An upper layer of cells, clearly visible as a chocolate brown colour due to the haemozoin, consisted almost 100% of mature or developing schizonts, which were then used in a variety of experiments, including disruption by various means to provide a source of antigen.
(4) Schizont preparations could be incubated in a specially designed cell sieve (Dennis *et al*. 1975) from which almost pure preparations of merozoites were obtained. The merozoites of *P. knowlesi* are larger than those of falciparum and could be counted readily on a haemocytometer and observed in the presence of red cells of different hosts (Butcher *et al*. 1973) to determine those susceptible to invasion.
(5) Although the rhesus monkey is highly susceptible to knowlesi, if carefully monitored the infection can be controlled with antimalarial drug treatment (Coggeshall and Kumm, 1937; Collins *et al*. 1967; Butcher and Cohen, 1972). Eventually, the monkeys become immune and able to withstand high challenge doses of parasites (up to 10^9); these animals were then a source of immune sera obtained by repeated bleeds without killing the monkey donor.

As noted above, as well as macaques and man, *P. knowlesi* will infect many old and new world primate species, including for instance the common marmoset (*Callithrix jacchus*), a particularly tractable laboratory host, but one in which relatively little work has been reported (Cruz and De mello, 1947; Langhorne *et al*. 1979).

PATHOLOGY OF *P. KNOWLESI* INFECTION

The extreme severity and almost uniform lethality of *P. knowlesi* in naive and untreated rhesus macaques led to investigation of the pathological processes involved. These seem to differ in detail from those found in lethal cases of *P. falciparum*, rendering the model less useful than would otherwise be the case, but features such as anaemia, and splenic and hepatic hyperplasia are in common (Menon, 1939). The terminal events were characterized by Maegraith *et al*. as shock-like, with acute circulatory failure (Maegraith *et al*. 1959). Napier and Campbell (1932) reported the tendency of the (then un-named) parasite to produce haemoglobinurea in macaques, and its fulminating nature in rhesus. Ground breaking work on mechanisms included *in vivo* microscopy, showing the 'sludging' of the circulation in the terminally ill monkey, with adhesion of blood cells to vascular endothelium (Knisely *et al*. 1941). The mechanism was proposed as fibrin-like

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deposition on infected cells, initially being opsonic, but terminally associated with increased viscosity, reduction in capillary flow, and a tendency for leucocyte adherence to 'sticky' endothelium. Knowledge of adhesion molecules has modified the interpretation, but not detracted from the observations! The early work on pathology is reviewed by Clark and Tomlinson (1949).

Pathogenesis in human cases was studied in those being treated for syphilitic general paresis, and also in 'volunteer' prisoners in the US NIH Penitentiary facility, Atlanta. In an early series, of 12 infected patients and several rhesus monkeys in Scotland, patients were observed at two hourly intervals, showing pyrexia exceeding 104 °F (above 40 °C) and sometimes 20-fold multiplication over two schizogonic cycles, having become patent about a week after receiving, typically, 3 mL of infected rhesus blood (van Rooyen and Pile, 1935). Unfortunately the authors omit absolute parasitaemias. These workers showed monkey blood remained infective for up to 16 days if stored in an ice chest after defibrination, that patients' blood could infect a second and third case by passage, and that it remained infective to rhesus. Difficulty was found in infecting patients who had previously been treated with *P. vivax*. Their knowlesi patients' infections were controlled and terminated with atebrin (less effective in man than monkey) and quinine, and given supportive cardiac therapy with digitalis whilst suffering rigours. Ciuca *et al*. (1937) obtained knowlesi from Horton Hospital in the UK (where it had replaced vivax for malariotherapy) because of the difficulty of using such therapy in the then malaria-endemic Romania. In a first series of experiments they showed that prior exposure to *P. vivax, P falciparum* or *P malariae* all reduced the chance of successful infection with the simian parasite. When successfully induced, disease was sometimes severe with pyrexia above 40 °C, but extremely variable parasitaemia (unfortunately given as parasites per field) and sometimes profound anaemia. Interestingly gametocytes were rarely seen, but the line had been maintained solely by blood passage. Of 13 patients re-challenged with the same line after 1, 2, 3 or 4 months, nine remained negative. Of the four re-infected, only one could be infected a third time (2 weeks later); immunity in two was solid for 5 and 11 months, respectively. In the very large group of patients treated over many years by Ciuca *et al*. (1955), the virulence of parasites increased over multiple blood passages (some 170) and its use was subsequently abandoned. In a smaller series, Jolly *et al*. (1937) had characterized the disease as mild and self-limiting in man.

Experimental sporozoite induced infection was carried out with the H strain in penitentiary volunteers by Chin *et al*. (1965, 1968), and compared with blood passage as a route of infection. There was remarkable similarity, with peak parasitaemias often between 10^3 and 10^4 mm^{-3}, around day 8 of patency, which in turn often lasted less than 20 days. Following mosquito bite, patency developed after 9–12 days, and was successfully achieved in man–mosquito–man and man–mosquito–monkey situations. Intriguingly, in the light of subsequent work by Miller's group (e.g. Mason *et al*. 1977), no difference was found in the susceptibility of white and Afro-Americans. With the notable exception of H, the lines used by the workers mentioned here are almost certainly no longer available, but it seems clear that differing isolates of *P knowlesi* vary in human pathogenicity.

As noted above, since it was possible to induce acquired immunity in the rhesus by sub-curative drug treatment in the face of its intense pathology, the nature and extent of this host's immune response became the focus of a great deal of research.

IMMUNITY TO *P. KNOWLESI*

The past few decades of the 19th century and the early years of the 20th century witnessed the beginnings of immunology. However, right at the start investigators tended to divide into two camps: those who believed in the overriding importance of cellular mechanisms in resistance to pathogens and those who regarded humoral immunity as the more important (Porter, 1997). Inevitably these attitudes influenced ideas about immunity to malaria from the earliest studies, as recounted in an extremely detailed and comprehensive review of work published up to 1949 (Taliaferro, 1949) The debate has continued right up to modern times, and results from experimentation on the knowlesi–rhesus model have been of major significance in this context. For the sake of clarity, the work on humoral immunity will be summarized first.

Antibody-mediated immunity

Many attempts were made with avian and occasionally human *Plasmodium* spp. to demonstrate a role for humoral immunity in malaria by the passive transfer of serum, following methods used for bacteria and bacterial toxins, but these were unsuccessful (summarized in Taliaferro, 1949). The success of passive transfer of immunity by sera from immune rhesus, immunized by repeated infection and cure, with known numbers of *P. knowlesi*, provided the first secure evidence for the role of antibody in malaria (Coggeshall and Kumm, 1937) though in some experiments quite large volumes of immune sera were required. Pre-incubation of parasites with immune sera, subsequently injected together into monkeys, resulted in best protection but attempts to induce immunity by different treatments of infected erythrocytes – formol or heat treatment

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and so forth – were unsuccessful (Coggeshall, 1943). Complement fixation was also demonstrated (Coggeshall and Eaton, 1938) as well as the agglutination of schizont-infected red cells by immune sera.

Definitive evidence for antibody-mediated immunity in human malaria came from the success of Cohen and McGregor, in The Gambia, reducing parasitaemias of *P. falciparum* in young children given immune immunoglobulin from adults (Cohen *et al*. 1961). Timing of the reduction in parasitaemias suggested that the schizonts were the main target of antibody and encouraged the suggestion that a vaccine against malaria might be a possibility.

In an attempt to reproduce the Gambian results in a laboratory animal, it was hoped that by using minimal infective doses of *P. knowlesi* in rhesus monkeys simultaneously injected with immune IgG, the animals would be protected and this would provide a method of testing potential protective antigens. Unfortunately, such large doses of IgG were required to demonstrate any protective effect that this approach was clearly impractical (Butcher *et al*. 1970; Butcher and Cohen, 1971). The failure of the knowlesi experiment in contrast to the success of the human experiments may have been due to a number of factors. First, *P. falciparum* parasites incubated in samples of the immune human immunoglobulin absorbed a 5-fold greater concentration of antibody than *P. knowlesi* incubated in immune rhesus IgG (Cohen and Butcher, 1969). Further, immunity generated by infection and cure in rhesus monkeys caused no increase in immunoglobulin levels, in contrast to the higher production and turnover in humans (Cohen *et al*. 1961). Lastly, the infected Gambian children who received the immune IgG would have been experiencing greatly enhanced phagocytosis in their spleens, liver and bone marrow (see below) unlike the uninfected rhesus given antibody and parasites together. Interestingly, Mulligan *et al*. (1940) reported that immune sera were more effective against *P. knowlesi* if the monkeys were also infected with *P. cynomolgi*.

The *in vivo* approach was therefore abandoned in favour of *in vitro* culture using labelled amino acids to monitor parasite growth in order to assess the effect of antibody. Cultures of *P. knowlesi* from infected non-immune rhesus initiated with early schizonts supported development through one cycle, with multiplication rates of 3–6-fold, but in the presence of immune sera or IgG (to a lesser extent IgM) parasite multiplication was completely inhibited (Cohen and Butcher, 1969; Cohen *et al*. 1969) although there was no effect on parasite development within red cells. Microscopic observation of individual schizonts rupturing on a warm stage revealed agglutination of merozoites by immune sera, and their reduced ability to attach to red cells compared with parasites incubated in normal serum, visibly confirming that a major target of immune antibody was the merozoite (Butcher and Cohen, 1970), a conclusion supported by other groups working with *P. knowlesi* (Miller *et al*. 1975) and subsequently in *P. falciparum* (Mitchell *et al*. 1976).

The high degree of species and strain specificity of immunity between the simian malarias (Voller and Rossan, 1969*a*, *b*) and between human *Plasmodium* spp. (Taliaferro, 1949; Cohen *et al*. 1974) is consistent with the concept that once established, immunity is predominantly dependent on antibody.

Notwithstanding the inhibitory action of antibody outlined above, it was well known that parasites persist in the blood of hosts that are clearly immune [termed premunition; for summary and comparison of premunition and immunity see Garnham (1966)]; a situation which puzzled researchers over many years. Brown and Brown (1965) took samples of infected blood from rhesus chronically infected with *P. knowlesi*, when there were sudden minor peaks in parasitaemia. These parasite samples were subsequently tested in an agglutination assay (SICA–schizont-infected cell agglutination assay) against sera from the same monkey which had been taken either before or after the parasites were isolated. They observed a pattern of changes in agglutination by sera before and after each peak which suggested that at each peak, a new surface antigen appeared on the schizont-infected red cell, termed antigenic variation. This demonstration of antigenic variation has proved pivotal in malaria research. Howard *et al*. (1983) showed that the antigen was a parasite product, and not modified host material, and by lactoperoxidase iodination were able to demonstrate molecular masses of the new proteins and the specificity of SICA antisera in immunoprecipitating them. This paved the way for understanding the variant gene family (SICAvar), and later the nature of PfEMP1 in *P. falciparum*. Despite the quite distant relationship between knowlesi and falciparum, there are significant homologies in the two families of variant antigens (Korir and Galinski, 2006).

By constantly changing an antigen on the surface of the infected erythrocyte, parasites could evade opsonizing antibody, thus at least partly explaining premunition. However, in a series of experiments in which monkeys were repeatedly infected and cured, despite the appearance of new SICA variants, the monkeys showed increased levels of protection, which correlated with rising levels of anti-merozoite antibody (Butcher and Cohen, 1972). This result further encouraged the belief that a vaccine against malaria was possible and that protective antigens might be detected and characterized from the merozoite (see below). In an extraordinary twist, it was later shown that if the parasites were passaged in splenectomized monkeys, expression of SICA antigens was lost, and such parasites showed reduced

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virulence when used to infect intact rhesus monkeys (Barnwell *et al*. 1983).

Immunity in the natural host (*M. fascicularis*) has received almost no attention from researchers. Since most research on *P. knowlesi* used adult wild-caught monkeys, it was difficult to know to what degree immunity might vary between adults and juvenile animals, although it was well established that mature kra monkeys experienced no ill effects and kept parasite numbers at a low level, often barely detectable. A small study of knowlesi in young kra monkeys born in the UK and then infected with *P. knowlesi* parasites confirmed the view that the infection was easily controlled (maximum parasitaemia was 0·3%). Although inhibition of parasite growth *in vitro* by kra sera revealed a more rapid response than the rhesus, maximum levels of inhibition were much lower that observed with sera from immune rhesus (Butcher *et al*. 2010). The immune mechanisms, which operate in the kra to keep parasites from overwhelming the host, remain a mystery. The same applies to the other simian malarias in their natural hosts (Butcher, 1992, 1996). Whatever these mechanisms might be, they require an intact spleen, but they could be overwhelmed by the injection of high parasite doses (Butcher, unpublished data).

Cell-mediated immunity

It was clear from numerous histological examinations of sections of organs of many malaria-infected hosts that phagocytic activity in the spleen, liver and bone marrow were greatly heightened (Taliaferro, 1949). Non-parasitized and parasitized red cells as well as free pigment were taken up by macrophages and this was thought to be important in protection, though it was not quantifiable. However, in South American *Cebus capucinus* monkeys infected with *P. brasilianum*, in addition to the phagocytic activity, Taliaferro and colleagues observed damaged intracellular parasites and schizonts with few merozoites (Taliaferro and Taliaferro, 1944). These were termed 'crisis forms' by analogy with the crisis in tuberculosis. In contrast, experienced malariologists such as Sir Ian McGregor FRS, who spent many years in Africa, never saw crisis forms in human infections, and did not know what they were (McGregor, personal communication). It was not surprising, therefore, that this aspect of immune responses remained largely ignored (see WHO, 1975) despite the Taliaferros' publications, until the 1970s.

The demonstration by M.W. Chase that immune responsiveness, characterized by delayed hypersensitivity reactions, could be transferred by lymphoid cells, as opposed to serum, provided the basis for investigations in cell-mediated immunity (see Daniel, 2010). In rodent malaria, Phillips (1970) showed that specific immunity to *P. berghei* in rats could be transferred by lymphocytes. Clearly, experiments of this type could not be done in outbred monkeys, but other experiments to assess their immune status were undertaken.

Plasmodium knowlesi antigens injected intradermally into chronically-infected immune rhesus caused an immediate hypersensitivity response, confirming the importance of immune antibody, but delayed hypersensitivity reactions only occurred in animals immunized with schizonts in the presence of an adjuvant [Freund's complete adjuvant (FCA)], of which 50% survived challenge infections (Phillips *et al*. 1970). Attempts to demonstrate cytotoxic activity with immune spleen cells using ^{51}Cr labelled parasitized erythrocytes gave a negative result (Brown *et al*. 1970*a*, *b*); Butcher *et al*. did similar experiments and obtained the same result (unpublished data and Langhorne *et al*. 1979).

Cell-mediated immune cytotoxicity was demonstrated in knowlesi merozoite-immunized rhesus monkeys (see below) infected with *P. cynomolgi* challenged with high doses of *P. knowlesi* such that parasites were immediately patent (Butcher *et al*. 1978). Damaged intracellular *P. cynomolgi* parasites were observed, but nevertheless the infection remained chronic. Interestingly, rhesus monkeys which had received an inadequate vaccine and were then challenged with *P. knowlesi* exhibited crisis forms but died later than usual, with very low parasitaemias, possibly reflecting an uncontrolled cell-mediated response (Mitchell, 1975; also see below). Lastly, although crisis form parasites were detected in chronically infected kra monkeys, the infection was not cleared (Butcher *et al*. 2010).

In parallel with the gradual accumulation of data on antibody mediated immunity and merozoite immunization in the 1970s, those who worked on rodent malaria were already familiar with crisis forms, which appeared as the infections peaked and parasitaemias declined, particularly *Plasmodium chabaudi* and *Plasmodium vinckei*. Progress on this aspect of responses to malarial infection has come from further work on rodent and human infections and is summarized by Clark (2009).

THE *P. KNOWLESI* MEROZOITE AS A SOURCE OF IMMUNOGEN

As emphasized above, by 1972, functional humoral immunity in rhesus monkeys which had been rendered resistant to *P. knowlesi* seemed to be largely directed against merozoites, and to limit the progress of disease by reducing or curtailing asexual multiplication at the point of red cell invasion. It was therefore logical to use purified merozoites as immunogen in an experimental vaccine. Despite the discrepant pathology, it was thought that such a demanding model for immunization might yield results applicable to *Plasmodium falciparum* in man.

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Attempts to immunize rhesus had had a previous history: in general schizont infected cells had been used, rendered non-infectious after formol fixation, by Freund *et al*. – one of the first applications of his eponymous adjuvant – (Freund *et al*. 1948) and by Targett and Fulton (1965), who also used immune serum-lysed cells; by Brown *et al*. (1970*a*) after repeated freezing and thawing, and by French press disruption (Schenkel *et al*. 1973). In general, FCA was used by these workers for primary immunization, followed by boosting with the incomplete adjuvant (FIA), and in summary 30–60% of immunized monkeys survived initial challenge infection. Where FIA was used in primary immunization, or where BCG or PolyAU adjuvants were used instead of FCA, protection was not seen (Brown *et al*. 1970*a*, *b*; Schenkel *et al*. 1973). There is little basis for comparison of immunogen doses, but derivation from about 2 mL P.C.V., or approximately 10^{10} schizont infected cells, was representative. The SICA variant used in challenge was usually unknown (summarized in Mitchell *et al*. 1975).

Immunization with preparations of naturally released merozoites (Mitchell *et al*. 1973; Dennis *et al*. 1975) proved to be more successful than schizont-based vaccines. In a number of separate trials, the mean vaccine dose of $1{\cdot}9 \times 10^9$ merozoites was contaminated with less than 0·1% host cells and was generally given at least twice in FCA. Of 21 monkeys thus treated, and challenged by inoculation of infected blood (10^4 parasites) or sporozoite suspension (2×10^3 sporozoites), or by infected mosquito bite, 20 survived. FIA and BCG were ineffective as adjuvants, and formol treatment of merozoites rendered them ineffective as immunogen (Mitchell, 1977). Merozoites could be frozen or freeze-dried and retained protective immunogenicity (Mitchell *et al*. 1977), but no attempt to replace FCA with a potentially clinically acceptable adjuvant was successful (Mitchell *et al*. 1979). Interestingly, the protection which followed merozoite vaccination was broader than would have been suggested by the titres of merozoite inhibitory antibody found in the vaccines, inhibitory antibody was induced when non-protective adjuvants were used, and splenectomy (which did not diminish circulating inhibitory antibody) rendered previously successfully protected monkeys susceptible to challenge (Butcher *et al*. 1978).

Alternative routes to immunization with P. knowlesi

The understanding that transmission blockade could be a significant aspect of an effective vaccination strategy for malaria led to experiments to immunize rhesus monkeys with gamete-enriched and partly gamete-purified preparations of *P. knowlesi*. As well as developing antibodies which blocked fertilization in mosquitoes fed after challenge, the partly purified preparations induced considerable immunity to the asexual infection (Gwadz and Green, 1978). Again, FCA could not be functionally replaced.

The situation with sporozoite immunization differed: intramuscular immunization with FCA emulsified sporozoites was ineffective either for protection or for antibody induction. Both required intravenous inoculation of irradiated sporozoites. High levels of circulating antibody, when induced, correlated with protection against sporozoite challenge, but death from anaphylactic shock was suspected in one of four animals in one trial (Gwadz *et al*. 1979). Whilst sporozoite immunization was not pursued to a great extent in *P. knowlesi*, an epoch-making advance for the field was made with this species: the genetic cloning of the first circumsporozoite protein in 1983 in Godson's laboratory (Ellis *et al*. 1983). This exemplified what was to become the familiar pattern of immunodominant multiple repeat epitopes in this category of surface protein.

EARLY ADVANCES IN THE ANALYSIS OF FUNCTION AND OF PROTECTIVE IMMUNOGENES OF *P. KNOWLESI* MEROZOITES

By the early 1980s, a nexus of interesting problems had emerged with regard to the asexual infection and immunity effective against it, together with the beginnings of the molecular technologies which were eventually to prove capable of commencing their resolution. These problems can be summarized as follows: how did the mechanism of red cell invasion relate to components of the merozoite, which could induce immune responses? The emergent technologies were gene cloning and monoclonal antibody techniques, but biological probes, inhibitors and labelling techniques all played important roles. Despite the advent in 1976 of reliable long-term cultivation and cyclical proliferation *in vitro* of *P. falciparum*, the advantages and existing understanding of *P. knowlesi* kept this species in intense experimental use.

The most readily available biological probes for invasion-related merozoite ligands were of course red cells. These could show the presence or absence of molecules required for recognition and invasion, and for *P. knowlesi* this had been approached by Butcher *et al*. (1973), using human, simian and pro-simian red cells in comparison with other mammalian red cells. In the same year, Miller *et al*. (1973) showed that receptors for *P. knowlesi* on human and macaque red cells differed by their sensitivity to enzymic removal. Band 3 protein, the red cell anion transporter, was suggested as a receptor. Miller extended this work, first by showing that Duffy blood group negative (FyFy) human r.b.c. were refractory to invasion

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(and by extension implicating this blood group in innate resistance to *P. vivax* seen in West Africa), and by showing that antibody to the Fy^a or Fy^b determinants blocked invasion into cognate r.b.c. (Miller *et al*. 1975). It was also apparent that Duffy (much later characterized as the erythrocyte chemokine receptor (Horuk *et al*. 1993)) was not uniquely necessary for knowlesi invasion, since the enzymic treatment of human Duffy negative cells rendered them susceptible without causing them to be Duffy positive (Mason *et al*. 1977). Despite this, it was clearly valuable to pursue identification of the merozoite binding partner(s) involved.

Differential Interference Contrast videomicroscopy (Dvorak *et al*. 1975) and Electron Microscopy of invading merozoites (Bannister *et al*. 1975) had shown the steps in the invasion process, leading to a hypothesis for the invasion mechanisms, essentially derived from *P. knowlesi*, by Miller (1977). This described merozoite attachment, the induction of red cell membrane upheaval, apical orientation of the merozoite, and its endocytosis followed by re-sealing of the parasitophorous vacuole membrane (PVM) so-formed. Hints of the addition of merozoite material to the developing PVM appeared later: freeze fracture studies suggested new proteins were inserted in the PVM during invasion (McLaren *et al*. 1977), but the addition of lipidic material was not proposed until some years afterwards (Bannister *et al*. 1986).

The initial invasion hypothesis needed modification in other respects, for instance in the light of specific chemical inhibitors of the process. Cytochalasin was shown to block invasion at the point of junction formation between the apical prominence of the merozoite and the red cell, so suggesting an actin dependent component (Miller *et al*. 1979). Protease inhibitors (pepstatin, chymostatin, leupeptin, phosphoramidon and elastatinal) were shown variously to abort or partially inhibit the invasion process (Banyal *et al*. 1981). The release of merozoites from schizonts was unaffected and the use of pretreated r.b.c. was immaterial to invasion, suggesting that the invasion process itself depended in part on protease activity. However, these results were challenged, but also extended, by findings of Hadley *et al*. (1983). Knowlesi invasion inhibition by chymostatin was confirmed (but not that due to pepstatin or leupeptin) and both N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK), and L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) inhibited the process, but at different points: the latter two stopped attachment of merozoites to the red cell whereas chymostatin allowed events as far as junction formation. It was unclear if enzymic alteration of the target red cell, or modification of the merozoite's own surface proteins (or both) was implicated. The junction of the invading merozoite's apex with the red cell membrane had become a focus of special interest. This had been observed by Bannister *et al*. (1975), but was named and described in detail by Aikawa *et al*. (1978). Moreover, Miller *et al*. (1979) had demonstrated that knowlesi invasion into Duffy negative cells failed at the point of junction formation rather than initial attachment. It remained unclear if monoclonal antibody to rhesus r.b.c. Band 3 protein, which blocked invasion, did so at precisely the same point (Miller *et al*. 1983). The value of this approach to merozoite function by red cell receptor studies is implicit in the continuing candidacy of the Duffy receptor protein in vaccination studies.

Monoclonal antibodies were key reagents with which to investigate origin, location and characteristics of merozoite molecules, which had been identified initially by screening the antibodies for inhibition of invasion. Two such merozoite molecules are exemplars: a 66 kD microneme protein, later to be called AMA-1, and a 140 kD merozoite surface protein (Deans *et al*. 1982; Miller *et al*. 1984). In both instances, antibody-affinity purified antigen was used in experimental vaccination of rhesus monkeys, but neither proved totally successful (David *et al*. 1985; Deans *et al*. 1988). AMA-1 nevertheless remains a viable candidate for immunization, partly because of its apparent constancy despite immune pressure (Deans *et al*. 1988). By contrast the 140 kD molecule varied when placed under the selection pressure imposed by the immunized monkeys (David *et al*. 1985). The occurrence of antigenic variation at the merozoite surface was not in itself surprising: it was the hallmark of schizont surfaces in recrudescing infection, and was strongly suggested by studies with merozoite-inhibitory antiserum, as reviewed above. Antigenic variation, thanks to *P. knowlesi*, must always be in the minds of those determined to vaccinate mankind against malaria.

Concluding remarks

This review is centred on the first 50 years of research on *P. knowlesi* (1932–1982) and while much was achieved, interesting questions still remain with regard to the interactions of host and parasite. Not least, why do these organisms not cause their simian natural hosts any apparent ill health, how do they continue to survive in the presence of immune responses, and what is different in the immune mechanisms in the rhesus that makes it so vulnerable compared with other closely related macaques? Clearly, and sadly, mankind is affected in a manner closer to that of *M. mulatta* than *M. fascicularis*.

We hope it is clear that early experimental and observational work on this malaria species was ground-breaking in important respects; we also hope that advantage to mankind still accrues from those studies.

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