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Strain Theory of Malaria: The First 50 Years

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Abstract

From the 1920s to the 1970s, a large body of principles and evidence accumulated about the existence and character of 'strains' among the *Plasmodium* species responsible for human malaria. An extensive research literature examined the degree to which strains were autonomous, stable biological entities, distinguishable by clinical, epidemiological or other features, and how this knowledge could be used to benefit medical and public health practice. Strain theory in this era was based largely on parasite phenotypes related to clinical virulence, reactions to anti-malarial drugs, infectivity to mosquitoes, antigenic properties and host immunity, latency and relapse. Here we review the search for a definition of 'strain', suggest how the data and discussion shaped current understandings of many aspects of malaria and sketch a number of specific connections with perspectives from the past 30 years.

1. INTRODUCTION

In the early 1920s, as debates about the number and nature of species that cause human malaria were receding, the idea emerged that each of these species consists of 'varieties, strains or races'. Over the next 50 years, a complex set of ideas developed about such entities—whether they were discrete, independent, mutable; what traits distinguished them; how they affected clinical and epidemiological observations and interventions. By the late 1970s, cloning and cultivation of *P. falciparum* were possible, serologic and molecular techniques were developing rapidly, and concepts emerging from the rise of laboratory-based studies were accompanied by a shift in language: 'strain' and 'race' were largely displaced by the new terms 'clone' and 'isolate'. In the mid-1990s, the concept of a 'strain' re-emerged in anticipation of a vaccine protective against a subset of circulating parasites. This 'strain theory' assumed that malaria comprised discrete, independently transmitted, immutable entities, and concluded that 'control of malaria through vaccination may be far easier than previously assumed' (Gupta and Day, 1994a). Related theory was invoked to explain immunity to clinical malaria (Gupta and Day, 1994b), and, more recently, to describe immunity to *var* gene products (Gatton and Cheng, 2004). Early investigations of 'strains' in malaria were devoted to understanding parasite phenotypes, but, as rapid and reliable molecular techniques for determining parasite genotypes were developed, it seemed clear that 'strains' could be distinguished with unprecedented

precision, either directly or by mapping genotypes to some smaller set of phenotypes. To do so, however, would require at least a provisional definition of ‘strain’.

Here we review the initial search for a definition of ‘strain’, as the theory developed in interplay with practice in the 1920s–1970s. That search is not only of historical interest, but is also relevant as contemporary studies begin to revisit and rediscover important aspects of parasite phenotypes. This historical review points to important themes for contemporary malariology, and reminds us that sorting and classifying the objects of study critically determines how research is framed and pursued. Theories do not simply catalogue observations: they formulate general principles explaining many specific observations in terms of relatively few underlying entities, forces and relationships, so the evidence accumulating for or against a theory often informs a wider field. Thus the pursuit of ‘strains’ was integral to the understanding developed in the 1920s–1970s of many aspects of malaria, notably its immunology—the now-familiar distinction between clinical and parasitological aspects of response, for instance. Here, we quote from the original literature as much as possible, to emphasize the richness of the resource and the degree to which some key points have been echoed and others neglected in recent literature. In a box at the end of each main section, we sketch several such connections between historical perspectives and current knowledge.

2. BACKGROUND

‘Shall we not be obliged to say that tertian and quartan agues are divine too, for nothing can be more regular than the process of their recurrence? But all such phenomena call for rational explanation’ (Cicero, 45BC).

For millennia, the ‘intermittent’ fevers associated with chills (‘agues’) were distinguished and classified on the basis of periodicity—quotidian (daily), tertian (every other day) or quartan (every third day). In 1880, Laveran described crescent-shaped parasites in the blood of a soldier suffering the fevers of ‘paludism’ (malaria). He later distinguished an additional 46 parasite forms, and over the next 40 years maintained that ‘the different forms in which the haematozoa of paludism present themselves belong to one and the same polymorphic parasite’ (Laveran, 1893).

In the late 1880s, Golgi linked periodicity in malaria fevers to parasite replication cycles, showed that morphologically distinct parasites were responsible for tertian and quartan fevers, attributed quotidian fevers to the presence of ‘different parasite generations reaching complete development at a day's interval’ and, while noting that ‘the numerous varieties of intermittent malarial fevers reported are, in the very *great majority* simple varieties or combinations of the two fundamental types (tertian and quartan fevers)’, posited a third distinct type, a tertian parasite characterized by crescent forms, quinine resistance and ‘irregular’ fevers (Golgi, 1889).

In the 1890s, Marchiafava, Bignami, Celli and their colleagues argued that ‘there is no group of fevers which are naturally and per se irregular, but fevers of every class may *become* irregular, and in different ways’ (Marchiafava and Bignami, 1894). Like Golgi, and Laveran (with respect to ‘parasitic elements’), they recognized that many problems in interpretation arose from concurrent infections by different parasite species and ‘colonies’ (Golgi's ‘generations’) within a species, given that fevers in some way followed the relative densities, transitions or transformations of parasites: ‘difficulty is met with in the study of the mixed malarial infections ... it is easy to predict the possibility of a very large series of combinations, caused by the number of the parasitic colonies, by the way in which their life cycles are, so to say, interwoven, &c.; as a resultant therefrom, the fever takes the most different courses’ (Marchiafava and Bignami, 1894). But they insisted on the existence of four distinct

entities, including a ‘true’ quotidian parasite. They differentiated springtime tertians and quartans from ‘malignant’ aestivo-autumnal (summer-fall) crescent producers, and in the latter distinguished tertians and quotidians: ‘The spring tertian and quartan parasites give rise to the mild forms; the aestivo-autumnal tertian and, more rarely, aestivo-autumnal quotidian ... give rise to the severe forms’ (Celli, 1901).

In 1910, Ross who had demonstrated that malaria parasites are transmitted by mosquitoes, wrote of ‘three different types of fever, the quartan, the tertian, and the irregular or malignant type ... Since the time of Golgi, all observers admit ... that these three types are different species ... Some authors consider that there are two if not three varieties (or ? species) of malignant parasites. I am inclined to agree with them, but have not yet satisfied myself sufficiently on the point to admit it in my classification’ (Ross, 1910). He too noted that some patients had concurrent infections with different parasite species, each of which might consist of ‘two or three sets sporulating [i.e. completing schizogony] on different days ... The rule generally accepted is that each set of parasites continues its own evolution independently of other sets which may be present. But much more precise work requires to be done on this point’.

The ‘mild’ tertian and quartan parasites are now known as *Plasmodium vivax* and *Plasmodium malariae*, respectively, the ‘malignant’ tertians as *Plasmodium falciparum* (‘falciform’ means ‘crescent-shaped’, a reference to the distinctive gametocytes—the forms transmissible to mosquitoes—of *P. falciparum*). Quotidian and irregular fevers are rarely mentioned in recent literature, but would be attributed to double tertian, triple quartan or other combinations of infections.

The last of the four species known to cause malaria infections in humans in nature, the ‘tertian’ *Plasmodium ovale*, was fully described in 1922 (Stephens, 1922). In that same year, citing several observations in the literature—notably on the low gametocyte production in *P. falciparum* infections on the coast of West Africa, compared to those in Italy and Macedonia, and on the development of clinical malaria in ‘resistant’ West Africans, who moved from the coast inland, or between countries—Marchoux made a speculative leap: ‘There are not only 3 species or varieties of *Plasmodium*, but 3 groups within each of which there exist a considerable number of varieties’ (Marchoux, 1922).

Laveran, Golgi, Marchiafava, Bignami, Celli, Ross and their contemporaries were familiar with experimental transfers of malaria parasites by blood inoculation, and had noted, for instance, that ‘one species of parasite often disappears from the blood upon inoculation with a different species’ (Deaderick, 1909). Wagner-Jauregg observed that patients with severe syphilis were sometimes ‘healed through intercurrent infectious diseases’, which led him to ‘intentionally imitate this experiment of nature’ through blood inoculations of malaria (Wagner-Jauregg, 1922), which in turn led to his 1927 Nobel Prize. Before the advent of effective antibiotics, tens of thousands of neurosyphilis patients were treated in malariatherapy clinics in the United Kingdom, the United States, continental Europe and elsewhere. A 1984 review concluded that ‘malariatherapy was less expensive and produced clinical improvements more frequently and more rapidly than did the best drug treatments’ (Chernin, 1984). Contemporaneous descriptions of procedures are given in Badenski (1936), de Rudolf (1927), Kupper (1939) and Lomholt (1944). A recent, independent, explicit analysis of relevant ethical issues accompanies extensive information about patient participation and treatment, and descriptions of procedures used in two of the major US facilities, in Collins and Jeffery (1999).

Parasites were transferred to patients by mosquito bite or by blood inoculation. Because most experts thought that the probability of the neurosyphilis cure was related in some way to the number and intensity of fevers, clinic staff often gave subcurative doses of anti-malarial drugs

to manage infections accordingly, and then full therapeutic doses at the end of the treatment regimen. Patients not cured of syphilis by initial infections were sometimes re-infected, typically with some variation in material or procedure. As clinics tried new methods and combinations, their results appeared in research papers and at conferences; much of our current knowledge of malaria is founded on these observations. With respect to Marchoux's 'varieties' and their classification, the practice of malariatherapy meant that 'now strains of widely diverse origins can be brought together and inoculated into patients lying side by side in the same hospital' (Hackett, 1937).

In the 1920s and 1930s, the results reported from malariatherapy, in conjunction with several influential malaria experiments in birds and non-human primates, and field observations in malaria-endemic countries, produced 'general agreement that within each species of malaria parasite there are races or strains which can be recognised as being distinct by their clinical virulence, their infectivity, their reaction to antimalarial remedies and their antigenic properties' (James and Ciuca, 1938). This characterization of 'races' or 'strains' had a strong practical bent: 'clinical virulence' and 'reaction to antimalarial remedies' were important because therapeutic malaria infections should produce fevers and other symptoms at sufficient but not excessive levels, and should be manageable; 'infectivity' and 'antigenic properties' were important because parasite transfer and patient re-infection should be reliable and predictable. It soon became clear that 'the latency and relapse patterns of malarial infections vary with the type and geographic origin of the specific parasites' (Schwartz *et al.*, 1950), and that this fifth distinguishing characteristic had a similar practical importance. In this paper, we use these five characteristics—clinical virulence, reaction to anti-malarial remedies, infectivity, antigenic properties, and latency and relapse—as a framework for reviewing the development of strain theory from the 1920s to 1970s.

3. CLINICAL VIRULENCE

By the 1920s, it was 'well known that infections vary greatly in severity, and that in some localities nearly every infection is pernicious, while in others pernicious symptoms do not develop, although the same plasmodium is presumably the cause of both' (Craig, 1926). In India, for instance, 'malarial fevers are usually most severe and persistent in low-lying coast districts' (Hehir, 1927). If differences between parasites were responsible for 'the common observation that in some parts of the world malignant malaria is more malignant than in others' (James *et al.*, 1932), then, for malariatherapy, 'pure tested strains, if available for inoculation, are far and away preferable, since not all strains are equally suitable. Some cause only quite mild reactions, whilst others produce severe reactions with more pronounced clinical symptoms' (Nocht and Mayer, 1937). It was also thought 'possible that different strains of *Plasmodium vivax* give different clinical types of malaria' (Grant, 1923), and that 'the tendency also of *P. falciparum* to select certain organs—the intestinal wall, the omentum, the brain—for localization has been explained by tropisms developed by certain strains' (Hackett, 1937). Though differences in virulence were often said to be dramatic, the differences were seldom specified in terms of symptoms, and, when specified—for example, 'the strains under review differed in regard to manner of onset, character of the fever and highest pulse-rates' (de Rudolf, 1924)—were seldom quantified. The clinical differences most often specified and quantified between strains related to fevers—'a very marked difference between the two with respect to the height of temperature produced', for example (Bunker and Kirby, 1925). Later, in the 1940s, the periodicity of fevers with the Baltimore, St. Elizabeth and New Hebrides strains of *P. vivax* was reported as 41.5, 43.4 and 45.8 h, respectively, which 'suggests that each strain might have a characteristic periodicity ... [which] will be a valuable point in distinguishing strains' (Young, 1944).

Strains were typically identified by their geographic site or clinic of origin, the former sometimes assumed rather than known. For instance, Horton Hospital, in England, most often used the ‘Madagascar’ strain of *P. vivax*, obtained in 1925 from an Indian seaman at an English port and named for the putative source of his infection. Compared to a ‘Holland’ strain of *P. vivax* judged inappropriate for malariatherapy, the Madagascar strain had a shorter incubation period (interval between inoculation and first fever), a lower frequency of spontaneous recovery and higher frequencies of quotidian fever and fevers above 106.4°F (James and Ciuca, 1938). With *P. falciparum*, Horton Hospital staff found that ‘the Italian and Sardinian strains are more severe than any we have obtained from India and Africa’, based largely on their observation that ‘severe clinical symptoms’ developed much more rapidly after inoculation. Roumanian strains of *P. falciparum* were found to be less severe than the Italian-Sardinian, but more severe than the Indian–African (Shute and Maryon, 1954).

Another ‘means of comparing the severity of the cases’ at Horton Hospital was the ‘longest period of fever without a fall to the normal temperature’, which averaged 74 h with the *P. falciparum* Rome I and Sardinia strains, 37 h with the Indian I and Indian II strains. Further evidence of these ‘striking clinical differences’ was that the Italian strains required eight times as much quinine for treatment as did the Indian strains, on average, and ‘continued to relapse for a much longer time’ (James *et al.*, 1932).

The application of these studies was seen as urgent and direct: ‘physicians in all malarious parts of the world should endeavour as soon as possible to add to existing information on the clinical virulence of the particular strains of *P. falciparum* prevalent in the countries where they work. Are all the strains in India as mild as those reported in the paper and are all the Italian strains as virulent? If the patient is able to say in what place he contracted the infection, shall we have at hand information indicating what will be the probable course of his illness?’ (James *et al.*, 1932). As the comparisons based on quinine and ‘relapses’ suggest, however, studies of differing virulence were entangled with questions about the nature of parasite drug response and latency, the role of the host and many other factors under investigation, including ‘whether the terms “immunity”, “virulence”, “infectivity”, “epidemic strains”, etc. current in work on immunology as it relates to bacterial infections should be used in work on the immunology of malaria’ (James and Ciuca, 1938).

This succession of quotes from the Horton Hospital points to the interesting context of their use of the term ‘virulence’ during the 1930s. They maintained that ‘within the species there are various geographical races which ... can be recognized as being distinct by their clinical virulence’ (James *et al.*, 1932), and that ‘we have no evidence that the inherent virulence of a particular strain can be increased or diminished’ (James *et al.*, 1936). However, they also revealed that ‘at Horton between 1925 and 1930 we succeeded in increasing the physical vigour and activity of an endemic strain of *P. vivax* from Madagascar to the degree in which it caused this severe “epidemic type” of the disease in 80% of our cases’ (James *et al.*, 1936). The procedure was not described (see Shute, 1951 for a schematic), but ‘careful selection of donors and recipients’ (James and Ciuca, 1938) had produced ‘a strain which multiplies freely and vigorously’ and thereby increased severity because, all else equal, ‘the number of parasites govern the severity of the case’ (James *et al.*, 1936). This increase in severity was ‘not an increase in virulence’, however, given ‘that “virulence” implied the possession or elaboration of a poisonous or venomous active principle (“toxin”). If so, it was not a good word to apply to the malaria parasite in which ... no “toxin” had been found’ (James *et al.*, 1936). Furthermore, it had become ‘evident that “infectivity”, “virulence”, and so on are relative terms depending as much on the receptivity of the host as on the biological properties of the parasite’ (James and Ciuca, 1938). Thus, at Horton Hospital, it appeared that ‘virulence’ might refer to an interaction between a particular host and a fixed property of a strain, in the form of a toxin, but

that the clinical severity of a malaria infection might be determined by malleable as well as 'inherent' properties of the infecting strain.

The contention that clinical features of a strain could change was not unique to Horton Hospital; however, the claim that 'virulence tends to be increased with each successive transmission' (Macbride, 1924) had been supported (Bunker and Kirby, 1925; Grant, 1923), contradicted (Fiertz, 1926; Yorke and Macfie, 1924) and confounded by mixed results (Lilly, 1925) in the mid-1920s. Furthermore, Wagner-Jauregg not only held the unnatural transmission [via blood inoculation] in malariatherapy responsible for 'mildness' and drug sensitivity, but seemed to associate the two traits: 'inoculated malaria showed itself much more sensitive toward quinine than the natural [mosquito-transmitted] malaria ... The mildness of this inoculation malaria may be explained thus, that the plasmodia which always reproduce themselves only in the asexual way are less capable of resistance' (Wagner-Jauregg, 1922). Thus—despite early speculation 'that a race of malarial parasites that is immune to quinine may be developed [as] fresh water amoebae may be gradually habituated to salt water'. (Leslie, 1910)—in the 1930s parasite response to a therapeutic drug was often taken as a token or reflection of 'virulence' as well as a marker of strain identity: 'The virulence of any strain of parasite may be manifested by the toxic symptoms it produces in the host, and this influences the degree of ease or difficulty with which a clinical cure can be produced ... but it may also be manifest by the power of the parasite to resist such means and to survive in the host' (Sinton, 1931).

At Horton Hospital, and elsewhere, the importance of distinguishing between clinical-severity and drug-response traits emerged gradually through the 1940s: 'Failure of a strain of parasite to respond to a given drug is not, however, in itself evidence of virulence; it may be that the strain, though resistant to this particular drug, is highly susceptible to others. The virulence or toxicity of a strain can best be seen by its effect on the host in the absence of drug treatment' (Shute and Maryon, 1954).

The search for a definition of virulence and the underlying causes continues, but 'virulence' has been used to describe so many different aspects of malaria that a specific operational definition remains elusive. The same historical difficulties and ambiguities dominate the current search, including the mutability of 'virulence' and the role of host factors in determining disease presentation. Because it is now considered unethical to allow experimental infections to progress to severe clinical symptoms, and because natural infections are difficult to observe, definitions of virulence have moved away from detailed clinical observations to focus on severe manifestations such as cerebral malaria and life-threatening anaemia. Although, selection studies in a laboratory rodent-malaria model imply that virulence (as indicated by host weight loss) is a heritable trait and may involve parasite replication rate (Mackinnon and Read, 1999), there are still no robust molecular markers of parasite 'virulence'. Genetic association studies have failed to find any reproducible markers of parasites causing cerebral malaria or severe anaemia (A-Elbasit *et al.*, 2007; Dobano *et al.*, 2007), although some differences in the distribution of *var* gene alleles have been reported between severe malaria cases and controls (Bull *et al.*, 2005; Kyriacou *et al.*, 2006). Parasites sequestering in placenta and giving rise to pregnancy-associated malaria do share a common phenotype (adherence of infected red blood cells to chondroitin sulphate A via PfEMP-1) and some candidate *var* genes responsible for this phenotype have been identified (Francis *et al.*, 2007; Salanti *et al.*, 2004; Viebig *et al.*, 2005).

4. REACTION TO ANTI-MALARIAL REMEDIES

Uncertainty about drug resistance as a stable strain characteristic was evident in the extensive studies of anti-malarial drugs conducted during World War II: 'there are two main types of strain—one relatively insusceptible to atebrin [mepacrine] and the other normally susceptible ... [but] variation in the degree of susceptibility to atebrin amongst the relatively insusceptible strains ... suggests that there may have been several strains of *P. falciparum* occurring naturally in the Aitape-Wewak area or that the phenomenon of atebrin insusceptibility was not so much an inherent characteristic of the strains as one which had been, or was, in the process of being acquired' (Fairley, 1946). The initial description of the Chesson strain of *P. vivax*, also from New Guinea, also implies doubt about relying on drug response alone for strain definition: it 'reacted differently to certain drugs than did the St. Elizabeth strain ... This and other characteristics suggest that it might be a strain distinct from some of the American malarias' (Ehrman *et al.*, 1945).

In 1949, using a strain of *P. vivax* from Hong Kong and a strain of *P. falciparum* from West Africa, 'resistance to proguanil was produced ... by giving small doses of the drug to consecutive patients in a series in whom the strain was being maintained by blood-inoculation' (Seaton and Adams, 1949; Seaton and Lourie, 1949). Nonetheless, 'the accidental production in the field of proguanil-resistant strains ... is thought unlikely', because high-level resistance had taken 20 months to arise in *P. vivax* and proguanil had a 'sterilizing effect' on *P. falciparum* gametocytes. At exactly the same time, proguanil resistance was detected in the field, in Malaya: 'the widespread use of proguanil in the Tampin district for the past two years ... we believe, is related to the emergence of resistant strains of *P. falciparum*' (Edeson and Field, 1950). The authors noted that Rollo *et al.* (1948), based on avian malaria experiments, had offered the 'likely explanation ... that resistant parasites occur spontaneously as rare mutants in a normally sensitive strain; and, as a result of selective survival of these resistant mutants when the parasites are exposed to the drug, a stable resistant strain emerges'.

One of the proguanil-resistant strains of *P. falciparum* from Malaya was later reported to be cross-resistant to pyrimethamine (Robertson *et al.*, 1952), and another not (Davey and Robertson, 1957). The appearance and spread of pyrimethamine resistance in *P. falciparum* was demonstrated in field experiments in the mid-1950s in East Africa—'during the course of the treatments the resistant varieties spread, replacing sensitive strains; upon cessation of the treatments resistant varieties regressed and became submerged' (Clyde and Shute, 1954)—but whether their rise and fall occurred through competitive interactions or through 'conjugation with other strains ... could not readily be determined'. Macdonald's summary of the situation, in 1957, was that 'the reactions of strains of parasite are unpredictable and in consequence drugs enjoy very different reputations in various parts of the world' (Macdonald, 1957).

Soon after chloroquine resistance was detected in the early 1960s, in Colombia (Moore and Lanier, 1961) and Thailand (Young *et al.*, 1963), a series of studies began to compare and differentiate 'strains of *Plasmodium falciparum* resistant not only to chloroquine but to other groups of synthetic anti-malarial drugs' on the basis of cross-resistance patterns, determining, for instance, that 'the Malayan I strain differs from the Thailand strain in its susceptibility to pyrimethamine; the Malayan III strain differs in its susceptibility to mepacrine' (Contacos *et al.*, 1963). Though all strains appeared susceptible to quinine, an experimental transfer of a Malayan chloroquine-resistant strain between quinine-treated prison volunteers gave 'strong evidence indicating the emergence of a variant strain characterized by lessened sensitivity to quinine compared to that of the parent strain' (McNamara *et al.*, 1967), an 'exceptional' result cited in support of the hypothesis that 'new strains may continually be emerging in the endemic area and adapting to the intermittent and irregular drug pressure encountered there' (Clyde *et al.*, 1969). The World Health Organization's conclusion, in 1969, was that 'most of the drugs

commonly used to treat malaria have shown variations in effect that are related to the strain of parasite ... [but] changes in sensitivity may be transient or permanent. ... The problem is particularly complex if one attempts to distinguish between the inherent differences in the response to drugs of various strains of the same species and the changing pattern of response induced by previous contact with drugs' (WHO, 1969).

In the 1930s, differences in drug response had already suggested that 'each strain of tertian or of aestivo-autumnal malaria is a problem in therapeutics by itself' (Hackett, 1937), and that 'isolation, selection, and other factors known to bring about change in other organisms would act upon malarial parasites ... [though] malariologists have been slow in acknowledging this possibility' (Huff, 1938). By the 1980s, the challenge had become that 'isolates that exhibit multiple drug resistance may, in fact, be mixed infections of parasites exhibiting resistance to each drug separately' (Rosario, 1981), and the role of drug response in characterizing 'strains' had become less clear.

The determination of the molecular/genetic basis of resistance to several anti-malarial drugs (Gregson and Plowe, 2005; Wellems and Plowe, 2001) has allowed many of these hypotheses to be tested. For example, a single point mutation (pfcr K76T) provides high-level chloroquine resistance in *P. falciparum* infections, but the mutation is found with nine other mutations. Discrete point mutations in a single bi-functional molecule confer resistance to pyrimethamine and to sulphadoxine. These mutations occur with relatively high frequency but impose real fitness costs on the parasite that are observed when selection pressure is relaxed. Following the switch away from sulphadoxine-pyrimethamine as a first-line anti-malarial in East Africa, the regression of resistant varieties first described by Clyde and Shute (1954) and the re-emergence of sensitive strains has been documented (Hastings and Donnelly, 2005), confirming the partial loss of fitness associated with drug-resistance mutations. In Malawi, the loss of chloroquine resistance has been dramatic (Kublin *et al.*, 2003). As postulated by Rollo *et al.* (1948), mutant parasites are selected and increase in frequency by spreading among hosts in populations exposed to drugs (Anderson and Roper, 2005; Pearce *et al.*, 2005; Mita *et al.*, 2006), but 'conjugation with other strains' (Clyde and Shute, 1954), that is, recombination, allows highly- or multiply-drug-resistant strains (carrying multiple point mutations) to emerge and allows spread into many different parasite genetic backgrounds. Thus, in the case of resistance to anti-malarial drug resistance, there appears to be a simple map between genotype and phenotype, and there is a clear basis for defining resistant strains.

5. INFECTIVITY

Whatever their other characteristics, parasite strains in the field and in most malariatherapy clinics had to be transmissible from humans to mosquitoes (and back to humans) to persist. Gametocytemia was taken as a marker of transmissibility to mosquitoes, and gametocyte production seemed to vary between strains. At the Horton Hospital, for instance, the Roumanian *P. vivax* Apostol 'strain was unsatisfactory because it did not produce a sufficient number of gametocytes', in contrast to the 'large number of gametocytes which are usually produced by this [Madagascar] strain' (Shute, 1937).

Dramatic changes in gametocyte production were associated with transfer solely by blood inoculation: 'malaria strains when inoculated from patient to patient more or less rapidly lose the capacity of producing gametes, and thus of infecting mosquitoes' (Pijper and Russell, 1926), and, though such 'gametocyteless' strains were sometimes propagated (Jeffery, 1951), clinics were cautioned to 'keep patients undergoing malariatherapy in screened rooms' because 'one can never be sure that such loss of power to produce sexual forms is permanent, and strains

of malaria are known which retain the ability to infect mosquitoes more or less indefinitely' (Russell *et al.*, 1946). While studies increasingly indicated that 'gametocyte density is not a reliable guide to the probably resulting qualitative infection of mosquitoes' (Boyd, 1942a), it was the most reliable gametocyte producers that became the strains commonly used in most clinics.

As for the actual infection of mosquitoes, *Anopheles* species often showed differing susceptibilities not only to different *Plasmodium* species (Boyd and Stratman-Thomas, 1934; Boyd *et al.*, 1935), but also to different strains within each species: 'we failed entirely to infect our English maculopennis with the Indian strains of *P. falciparum* [but] with the European strains from Rome and Sardinia we succeeded ... the capacity of each strain to infect a named species of anopheles must be separately worked out' (James *et al.*, 1932). Further studies with 'English maculopennis' at Horton Hospital reported successes with *P. falciparum* strains from Roumania, and failures with strains from East and West Africa (Shute, 1940). European vectors transported to Africa gave a similar result: 'experiments in Garki, Nigeria, with *A. atroparvus* from Italy have shown a refractoriness to infection with the local strain of *P. falciparum*' (Ramsdale and Coluzzi, 1975). However, '*A. gambiae*, originating from the Nyanza province of Kenya, was able to transmit the Malayan strains of *P. falciparum* with no more difficulty than African strains' (Davey and Robertson, 1957). Accordingly, even the broadest of summaries mentioned exceptions: 'European strains of *P. falciparum* require a European mosquito to transmit them, and tropical strains tropical mosquitoes, but in the latter case it may be noted that the Indian *A. stephensi* is a good vector of the far distant West African *P. falciparum*' (Garnham, 1966).

In the United States, it appeared that *A. quadrimaculatus* and *A. punctipennis* 'vary widely in their susceptibility to different strains of *P. falciparum* [but] are approximately equally susceptible to both strains of *P. vivax*', and, as one result of such findings, the Rockefeller Foundation clinic 'abandoned the propagation of the Coker strain' but continued the Holzendorf, Long and Manuel strains of *P. falciparum* (Boyd and Kitchen, 1936a). As results of vector-parasite transmission tests accumulated, it appeared that 'local strains of parasites may or may not show a high degree of adaptation to anophelines which are coindigenous to their own faunal regions, and ... anophelines may or may not show a high degree of susceptibility to exotic strains of parasite' (Boyd, 1940a), and the practical implications of strain infectivity were seen to extend well beyond malariatherapy clinics. At Horton Hospital, 'results indicate that persons carrying gametocytes of *P. falciparum* of tropical origin would be unlikely to cause any outbreak of fresh cases of malaria in this country through the agency of our English maculopennis. On the other hand, our four English anophelines become infected when fed on tropical strains of *P. vivax* and our *A. maculopennis* is a very efficient carrier of *P. ovale*' (Shute, 1940). In the United States, extensive transmission experiments with indigenous species of *Anopheles* and 'exotic' strains of *P. vivax* and *P. falciparum* were undertaken during World War II (e.g. Boyd, 1949) in part because 'studying foreign malaria imported by returning servicemen, it has been shown that these strains are infective to and can be transmitted by our native malaria vectors' (Young *et al.*, 1947). A particular concern was that 'returning soldiers with such infections may be responsible for the establishment in this country of epidemic or endemic foci for imported vivax strains' (Watson, 1945).

Extensive studies of *P. vivax* infections from the 'Solomon Islands, New Hebrides islands, New Guinea, Tunisia, Liberia, Trinidad, and the China-Burma-India theater' with the major malaria vectors of the Western and Eastern United States found that, based on the fraction of fed mosquitoes infected, '*A.m. freeborni* is more susceptible to these foreign malarias than *A. quadrimaculatus*' (Young and Burgess, 1948), the same result as with 'a domestic strain of *P. vivax*', St. Elizabeth (Burgess and Young, 1950). *P. vivax* strains from the Mediterranean and India, however, infected a lower percentage of *A. freeborni* than *A. quadrimaculatus* (Young

et al., 1949). Similar studies with *P. falciparum* showed that the Malayan IV strain was much more infective, and the domestic McLendon strain slightly more infective to *A. freeborni* than to *A. quadrimaculatus* (Coatney *et al.*, 1971), that *A. albimanus* was highly susceptible to the Panama strain, but almost refractory to the Thailand strain (Collins *et al.*, 1963), and so forth, thus that ‘the infectivity of isolates of *P. falciparum* to different anophelines is dependent to some extent on the geographical origin of either the parasite or the mosquito’ (Coatney *et al.*, 1971).

The usual inference was that, while ‘there may exist, between particular strains of parasites and their definitive hosts, a very high degree of local adaptation, which under certain conditions may conceivably be a natural barrier to the extension of the range of a given strain of the parasite ... the human intermediate host is not likely to be a factor in limiting the extension of the range of strains of these parasites’ (Boyd *et al.*, 1938a). In what was apparently the only published study of its kind, the results suggested that ‘anophelines infected on a person concurrently infected with two strains of a single species (*P. vivax*) may either: (a) become infected with but one of the strains, or (b) may possibly become infected with both of the strains and simultaneously propagate both’ (Boyd *et al.*, 1941). Thus, it was plausible that ‘the happy adjustment of parasite to vector in any area has come about through a weeding-out process, in which ill-adapted strains (and species) have failed to be transferred at proper intervals and have consequently become extinct’ (Hackett, 1937).

If there was any correlation between infectivity and gametocytemia, however, it seemed certain that ‘the accidental introduction of a strain of parasite producing large numbers of gametocytes would lead to an increase in incidence’ (Bishop, 1955), and, since ‘gametocyte output is considerably greater in European strains than in Indian or African strains’ of *P. falciparum* (Shute and Maryon, 1954), for instance, it was not clear what, other than strict local matching of vectors and parasites, might constrain such introductions, or selection for increased gametocyte production. This question led directly to another: ‘are the male gametocytes of one strain of a species of parasite able to fertilize the females of a different strain? ... inability to do so would be essential if strains are to retain their identity where more than one occurs in a given locality’ (Shute and Maryon, 1954). If cross-fertilization were possible, “How long would an imported strain retain its identity when it was introduced into an endemic area?” And the answer, we think, would be: “not very long providing the indigenous anopheles were susceptible to infection”. ... [This] helps to explain why it is fairly obvious that there are separate geographical strains, but so very much less obvious that there are separate ‘locality strains’ (Shute and Maryon, 1954).

By the 1980s, *in vitro* cultivation of *P. falciparum* made it possible to distinguish between a ‘clone’ (‘genetically identical organisms derived from a single cell by asexual division’; Walliker, 1983) and an ‘isolate’ (‘a sample of parasites, not necessarily genetically homogeneous, collected from a naturally infected host on a single occasion’; Walliker, 1983). Studies soon demonstrated that gametocyte production and infectivity might differ between clones grown from a single isolate (Burkot *et al.*, 1984; Graves *et al.*, 1984a), and that ‘for those strains that appear to lose gametocyte formation in culture this is a result of selection operating on a mixed population’ (Bhasin and Trager, 1984). Indeed, inability of cultured asexual forms to produce gametocytes was found to be due to complete and irreversible loss of large segments of genetic material (Day *et al.*, 1993). Host immunity that reduces gametocyte densities and that blocks infection in mosquitoes suggests a role for the host in determining infectivity. High gametocyte production in response to sulphadoxine–pyrimethamine suggests that gametocyte production can respond to some cues within the host, and is not a fixed trait (Barnes and White, 2005). Incompatibility between parasite strains and various species and strains of *Anopheles* is now believed to reflect an interplay between polymorphic innate immune response genes of the mosquito

that promote or retard parasite killing (melanization) and unknown melanization resistance genes of the parasite (Vlachou and Kafatos, 2005), hence current discussions often invoke evolutionary costs (Lambrechts *et al.*, 2005; Sinden *et al.*, 2004). Simultaneous propagation of multiple strains of malaria by a single mosquito has been confirmed by molecular genetic studies (Babiker *et al.*, 1994; Huber *et al.*, 1998; Ranford-Cartwright *et al.*, 1993), but the high frequency of recombination during the sexual phase of the parasite life-cycle (confirming that ‘male gametocytes of one strain’ can indeed ‘fertilize the females of a different strain’) may mean that outcrossing, between strains, is as common as ‘selfing’ and thus, as predicted by Shute and Maryon (1954), that strains rapidly lose their identity in endemic areas. Hence, as with drug response, the role of infectivity in maintaining and characterizing strains began to appear highly complex.

6. ANTIGENIC PROPERTIES

6.1. Homologous and heterologous response

In 1898, an army doctor observed that ‘the contrast between West Indians and the natives composing the Sierra Leone Frontier Police when serving in the field in the same column was most marked: the former much troubled by fever, the latter having none’, and inferred ‘that the malarial organism of the West Coast is of a different variety to the plasmodium of the West Indies, and that immunity acquired to the first mentioned confers no protection from the second’ (Smith, 1898). In India, ‘there is always a certain amount of malaria of local origin in most towns, but this affects mainly children and new-comers; it is probable that adults sometimes acquire a certain measure of immunity to local infection, but are not immune to the more virulent type found at certain places in the interior of the districts’ (Kenrick, 1910). By the 1920s, it was recognized that ‘immunity developed against one species of *Plasmodium* does not confer a similar protection against the other species’ (Yorke and Macfie, 1924) and, retrospectively, with respect to strains, that in the ‘intermingling of men coming from malarial regions’ during and soon after World War I, ‘inhabitants more or less resistant to local strains showed little or no immunity to foreign strains and the immigrants had no resistance against the local virus [parasite]’ (League of Nations Health Organisation Malaria Commission, 1934). With such intermingling, and ‘numerous bites from innumerable mosquitoes ... the chance of a person receiving an infection with one or more strains of parasite of varying degrees of virulence was considerable [and] with infection by each new strain the sufferer would be liable to a recurrence of clinical manifestations of greater or lesser intensity’ (Sinton, 1931). Thus, it began to appear that ‘persons can easily be immunized against a particular strain ... but that the resistance breaks down if they are inoculated ... even with a different strain of the same species’ (Thomson, 1931).

Deciphering the nature of this ‘immunity’ or ‘resistance’ required more precise information about how it developed, and how it might affect ‘virulence’, drug response and transmission. Such studies might explain pronounced differences in the initial responses of malariatherapy patients: ‘the patients of our series were not of a homogeneous susceptibility to the strains ... some clearly possessed a pristine susceptibility, others exhibited evidence of previous autochthonous infection, varying from partial immunity to complete refractoriness, the latter interpreted as an immunity homologous to the strain we employed’ (Boyd, 1942b). Similarly, the results might help to explain differences in responses to re-infections, in that ‘the usual criterion of adequate malariatherapy is the actual number of paroxysms reaching a certain febrile height ... [so] heterologous immunity exists when reinfection of a patient with a strain of *P. vivax* differing in geographic origin from the original strain results in definite clinical activity sufficient for the completion of a course of malaria therapy’ (Kaplan *et al.*, 1946).

Many of the early re-infection studies led to straightforward conclusions: 're-inoculation of a patient who has recovered from a *P. falciparum* attack with the same strain of the parasite will not result in a second clinical attack ... Re-inoculation of a patient who has recovered from a *P. falciparum* attack with a strain of the parasite different from that which caused the primary attack, will result in a second clinical attack which may be as severe as the first' (Boyd *et al.*, 1936). Thus, homologous and heterologous responses were seen to reveal underlying similarities and dissimilarities between strains: a patient 'exhibits a resistance when reinoculated homologously with the same line of parasites, but if reinoculated with parasites of the same species from a different source and presumably of a different (heterologous) line, he acquires an infection. From this it is inferred that the parasites of the second inoculation represent a different race or strain' (Brumpt, 1949). Accordingly, cross-inoculation became a common means of strain differentiation: 'the derivation of two lines of parasites from presumably unrelated patients cannot be taken as a basis for their characterization as different strains ... [especially] when applied to lines of parasites of obviously local indigenous origin. Their antigenic dissimilarity must be proven by the cross inoculation of convalescents' (Boyd, 1940a). Cross-inoculation studies determined not only that 'the White, Wilson Dam, and Mayo strains are immunologically distinct from the McCoy strain ... [and] the Cuban, Mexican, and Panamanian strains are different from the Long strain' (Boyd, 1940a), for instance, but, by comparing responses of patients from different regions of the United States, that 'West Florida and contiguous Alabama are indicated as the indigenous habitat of the McCoy strain' (Boyd and Kitchen, 1936b). Further afield, 'little heterologous immunity was shown between vivax infections from the South Pacific, China-Burma-India theatres and from the United States. In fact, one infection originating from New Guinea exhibited little immunity to another infection from the same area, suggesting multiplicity of strains in small areas' (Young *et al.*, 1949).

Other studies introduced complications, however. Some patients still developed fever at a fourth and parasitemia at a tenth infection with a homologous strain of *P. falciparum* (Ciuca *et al.*, 1934). One possible explanation was that 'immunity to a malarial infection exists when the subject is cured and then challenged to a reinfection with the homologous organism ... this residual humoral immunity, however, is rapidly lost ... [with] no evidence of cross immunity with a heterologous strain ... [so] it is more than apparent that there is little reason to hope for an effective vaccine for malaria' (Yount and Coggeshall, 1949). However, if this 'rapid loss' existed, the rate of loss was uncertain—for example, 'homologous immunity to superinfection persists for as long as a year and may last seven years' (Boyd, 1949)—and might differ with different methods of parasite transfer, for example, as 'a temporary immunity to reinfection by the homologous organism, lasting up to two months with trophozoite inoculation and up to 13½ months with sporozoite inoculation' (Schwartz *et al.*, 1950). Furthermore, it began to appear that 'convalescents from artificially induced falciparum infections usually exhibit a distinct clinical tolerance when artificially reinoculated with a heterologous strain of this parasite, manifested by a shortened period of clinical activity ... [thus] the immunity developed during convalescence from a falciparum infection has an appreciable heterologous value' (Boyd and Kitchen, 1945). Thus, residents of malaria-endemic countries might experience within a 'period of time a series of episodes successively involving different strains. With a progressive series of inoculations, while the original clinical reaction might be characteristic of complete susceptibility, later episodes would exhibit characteristics of heterologous-strain immunity until the individual had acquired experience with all of the locally prevalent strains. Any inoculation subsequent to this period would result in a homologous-strain reaction' (Kitchen, 1949).

Another set of difficulties arose with 'premunity' (Sargent *et al.*, 1924, 1925), the doctrine that 'resistance to a malaria infection is dependent upon an existing infection, either active or subclinical' (Yount and Coggeshall, 1949): that is, some responses to seeming re-infection might actually be responses to superinfection. One implication of latency or sub-detectable

parasitemia (see below) was that, at least with respect to homologous immunity, ‘we never know when any one is cured. Our only proof is a renewed susceptibility to reinfection by the same strain of parasite’ (Hackett, 1937). Moreover, conclusions about homologous and heterologous responses with one species of *Plasmodium* might not be directly applicable to other species: ‘Superinfection with the same strain of *P. vivax* rarely, if ever, takes place [whereas] with *P. falciparum*, superinfection is apparently possible, but the course of the second infection is very greatly modified’ (Earle *et al.*, 1939) and ‘there is less indication that acquired immunity to *P. falciparum* has heterologous value than in the case of acquired immunity to *P. vivax*’ (Boyd *et al.*, 1936). Perhaps ‘the immunity acquired to the three species is of a different order in each case ... and immunity to *P. falciparum* is the least complete. The alternative explanation is that there exists a greater variety of strains of *P. falciparum*, and that, while immunity to one strain is reaching a high titre, infection with a new strain, or with an older strain to which immunity has already largely disappeared, intervenes ... [though] such a multiplicity of strains in one area seems improbable’ (Wilson, 1936).

Wilson (1936) would likely be astounded by the level of genetic diversity that is now known to occur within *P. falciparum*. Diversity is linked to levels of malaria endemicity, such that parasites from unrelated patients in Africa or New Guinea are likely to represent different strains whereas parasites from unrelated patients in South America have a higher chance of belonging to the same strain. Species-specific and strain-specific immunity is believed (although far from being formally demonstrated) to be due to (allelic) polymorphism of genes encoding the major surface proteins of each stage of the parasite, such that antibodies raised to one form of the protein bind less efficiently to heterologous forms present in other parasite species or strains. That allelic diversity is greater among *P. falciparum* compared to other human malarias, predicted by Wilson (1936), is being confirmed by molecular genotyping but only partially explains the lack of homologous immunity. The ability of a single strain of *P. falciparum* to cause numerous bouts of fever or parasitemia in a single patient (Ciuca *et al.*, 1934) and the shorter duration of immunity following trophozoite inoculation compared with sporozoite inoculation (Schwartz *et al.*, 1950), are likely explained by clonal antigenic variation (Dzikowski *et al.*, 2006; Kraemer and Smith, 2006). Sequential expression among genetically identical blood stage parasites of heterologous *var* genes that encode for PfEMP1 expressed on the surface of infected red blood cells may enable a single parasite strain to escape effects of the developing immune response; strains with inherently high switching rates (allowing rapid sequential expression of novel antigens) would be expected to be able to cause repeated infections in the same patient. The gradual reduction in severity of disease with successive reinfections by the same strain might be explained by increasing immunity to non-variant antigens or gradual exhaustion of the repertoire of clonally variant antigens.

6.2. Clinical and parasitological response

Given this accumulation of studies suggesting that ‘homologous’ protection was not necessarily immediate, absolute or permanent, that ‘heterologous’ responses might include partial protection, and so forth, more nuanced interpretations began to emerge, for example, that ‘a considerable degree of resistance to reinfection and superinfection with a homologous strain of malaria parasite, may be acquired after a single infection with such a plasmodium, and that, in some instances at least, this resistance may be increased by successive inoculations with the same strain of parasite’ (Sinton, 1940). Homologous resistance was increasingly described in terms of clinical severity and duration: ‘If a patient has an acute attack with a certain strain prevalent in one area and recovers from it, it is found that inoculation again with that strain shows an immunity. The attack is not so acute and he recovers more rapidly. Now if he is inoculated with a different strain brought from an area several hundred miles away, he

has another acute attack similar to the first one. In other words he has some homologous immunity but no heterologous immunity' (Bispham, 1944). By the late 1940s, it appeared that cross-inoculation might not always distinguish between heterologous strains, at least not unambiguously, without careful elaboration of the terms of reference: 'reinoculation ... after the chronic infection is no longer microscopically patent, with the same species and strain involved in the previous infection, is not likely to result in more than a transitory subclinical parasitemia. ... (Boyd, 1949) Successive reinfections ... with the same strain ... result in progressively milder infections than the initial infection, i.e. they progressively enhance the homologous immunity ... [and] a number of races or strains ... may be immunologically similar. Others may not only vary in virulence and respond differently to treatment, but also fail to protect against another strain ... An immunity may exist between strains of the same species although to a less extent than against the homologous strain ... partial heterologous immunity between some strains and not between other strains in man has been substantiated'.

One critical refinement in the terms of reference built on the observation that there were two major kinds of effects of this 'immunity', providing 'some protective mechanism against both the multiplication of the parasites and their pathogenic effects. The rate of development, the efficacy, and the duration of this "immunity" appear to vary with (a) the species or strain of *Plasmodium* responsible for the infection and (b) the degree of resistance or susceptibility possessed at the time by the host' (Sinton, 1939). There seemed to be an incomplete correspondence between these effects on symptoms and parasitemia in re-infection: 'upon recovery from an attack of malaria ... the patient possesses a very potent homologous immunity to that strain ... [which] manifests itself by two characteristics: (a) the acquirement of a tolerance to densities of the parasite that in a susceptible person would produce a clinical reaction, and (b) the acquirement by the body of an ability to destroy and remove the parasites. As immunity becomes established the former characteristic is first acquired; the latter develops more slowly' (Boyd *et al.*, 1938b). Thus, responses to homologous and heterologous re-infection were seen as more sharply distinct in clinical than in parasitological terms, in both magnitude and duration: with 'the reaction of "heterologous (strain) immunity" ... the latent state ... is attained as a rule in 3 to 7 days' after the peak parasitemia, and the attacks 'are relatively brief, usually not exceeding 14–16 days in duration of clinical activity'; with 'the "homologous (strain) immunity" reaction ... the infection provokes no febrile reaction ... [and] parasitemia is usually brief' (Kitchen 1949). If patients could acquire 'an immunity to fever but not to parasites' (James and Ciuca, 1938), there might be 'two independent kinds of immunity, one to the parasite itself, leading to its corporal destruction, and one neutralizing the pathological effects of its growth and activity upon the host' (Hackett, 1937).

In the 1930s, as the more complicated results from re-infection studies were beginning to suggest new interpretations, some authors began to use the term 'tolerance' specifically to refer to reduced or absent clinical response: 'inhabitants of an area may become tolerant to the local strain of parasite, yet at the same time be susceptible to the pathogenic effects of strains present in other areas' (Sinton, 1931). And, while the details would require investigation, 'that the time taken to produce this tolerance is prolonged may possibly be explained if it might be assumed that there are numerous strains of parasites of malaria specifically differing in their antigenic properties' (Thomson, 1933). Not surprisingly, premunition and other emerging issues often obscured the distinction: 'during an attack of malaria a person acquires a tolerance to the parasite of the strain harbored, which renders him refractory to reinoculation with the same strain' (Boyd and Stratman-Thomas, 1933a), but, while 'a patient with a latent benign tertian infection [*P. vivax*] does not possess a heterologous tolerance to other strains' (Boyd and Stratman-Thomas, 1933b), 'following superinfection by a heterologous strain ... we have found the attack caused by the superinfection to be less severe, indicating the actual existence of some degree of heterologous tolerance' (Boyd and Stratman-Thomas, 1933c). However, as distinctions between clinical and parasitological aspects of response were further elaborated

—for example, ‘heterologous immunity to malaria is rarely strong enough to prevent infection but it may so modify a second attack that it closely resembles a relapse’ (Russell *et al.*, 1946) —some authors began to use the term ‘immunity’ strictly to refer to anti-parasite response, and, by the late 1940s, studies of homologous and heterologous re-infections were investigating the two aspects of response in parallel, in corresponding terms: given ‘less reaction by the host to a given bulk of infection—tolerance ... [and] an increased ability of the host to limit the bulk of infection developed—immunity ... [we found] that the development of tolerance preceded the development of immunity and that immunity was strain specific ... [while] tolerance was not strictly strain specific’ (Blackburn, 1948).

With respect to the ‘antigenic properties’ of parasite strains reflected in these responses, ‘the only explanation would seem to be that each of these strains contains immunological elements in common with each of the others’ (Hackett, 1937) and ‘that the antigenic constitution of the malaria parasites is analogous to that of some pathogenic bacteria ... [i.e.] in all specimens of a particular strain of a malarial parasite there are parasites of two types, one containing specific antigen, the other group antigens ... [and] that the antigens may be subdivided into 1o group antigens which are common to all members of the group, 2o group antigens which are common only to certain members of the group, 3o specific antigens peculiar to each type’ (James and Ciuca, 1938). Thus, in an attempt to integrate the evidence, the observation that ‘differences between the results of homologous and heterologous reinoculations lie mainly in the number of febrile infections produced ... suggests that possibly the common antigenic factor in these strains may be related more to the antiparasitic element than to the anti-toxic one ... [while the] more rapid decline of the percentage of cases showing febrile reactions after successive reinoculations than of the recorded parasitic infections ... might suggest that the antitoxic immunity factor was developed more rapidly than the anti-parasitic one’ (Sinton, 1940). Then, ‘in the condition of premunition, while both defensive factors are in operation, the antitoxic element is probably the more efficient ... [but] the duration of the efficacy of the anti-parasitic element persists for a longer time than does the anti-toxic one’ (Sinton, 1940).

As with ‘virulence’, and drug response, several authors asked whether antigenic features of a strain might change with time. Most concluded that, since observations ‘do not suggest that extensive sexual reproduction has altered the antigenic composition of one strain ... the antigenic characteristics of the parasites upon which immunological differentiation of strains is based, are evidently firmly fixed and retained through an indefinite number of passages through the definitive and intermediate hosts’ (Boyd, 1940a). Huff, however, argued that with ‘parasites which are distinguishable only on immunological grounds ... whether they constitute separate races, possibly incapable of cross-breeding or whether they are simply manifestations of variations within a variety or species due to sexual reproduction can only be guessed at the present time ... Individuals in endemic areas probably build up an immunity to one strain of malaria only to suffer from another immunologically different strain. And since there is the possibility that strains of malaria may change genetically in immunological characteristics, man in these areas is being subjected to reinfection by a multiplicity of genetic stocks of parasites. If, on the other hand, these strains of malaria have evolved far enough that they no longer cross breed it ought to be possible for individuals living in a given region to develop eventually an immunity to superinfection to all of the strains, providing the number of these strains is not unreasonably large’ (Huff, 1938).

The molecular basis for the two types of anti-malarial immunity—immunity against the parasite itself and tolerance to the pathology it causes—is still somewhat unclear, although the distinction is still widely appreciated (Schofield and Grau, 2005) and the former is still regarded to be somewhat more strain-specific but longer-lasting than the latter. One theory, that tolerance is provided by short-lived, T-cell-independent antibodies to non-polymorphic glycopospholipid ‘toxins’, is consistent with these observations as is the notion that

immunity to parasites is provided by longer-lived and boostable antibodies to polymorphic protein antigens. Of these, conserved or relatively conserved proteins such as the circumsporozoite protein would fit the James and Ciuca (1938) definition of 1o group antigens, proteins such as merozoite surface protein 1 (MSP-1) and MSP-2 which exist as a small number of allelic families defined by conserved family-specific sequences would meet the definition of 2o group antigens, whilst the individual variants within the MSP-1 and MSP-2 families—or indeed the clonally variant PfEMP-1 proteins—could be classified as 3o group antigens. Whilst diversity within the MSP-1 and MSP-2 families seems to have arisen by a combination of both point mutation and intragenic recombination (Ferreira and Hartl, 2007; Ferreira *et al.*, 2003), thus refuting the notion of Boyd (1940a) that antigenic characteristics are firmly fixed, it does still seem to be the case that the antigenic properties of a strain do appear to be rather more durable than—for example—drug sensitivity, suggesting perhaps that the selective forces imposed by the immune system are rather less than those imposed by chemotherapy. Regardless of the mechanism, mathematical models of epidemiological data support the view that immunity to clinical disease [tolerance] develops earlier in life than does anti-parasite immunity (Filipe *et al.*, 2007) and that immunity to severe malaria is acquired quite rapidly (Gupta *et al.*, 1999).

6.3. Superinfection

Thus, again, there arose the question of distinguishing between re-infection and superinfection, now complicated by questions about the stability of antigens that might give rise to immunity. Using the available clinical, parasitological and immunological methods, it would be 'necessary to know whether the presence of one parasite influences the course of infection with the others. Further, we must know if superinfection can take place and if so, at what stage of the previous infection this is possible. Finally we will need to know how many strains of each parasite there are ... In nature either the number of strains or the times that they will infect in the presence of other parasites must be limited. Otherwise in endemic areas adults would have difficulty in developing immunity to all strains that existed' (Earle *et al.*, 1939). Sequential, single-strain infections might be just one among many sets of possibilities to be considered in interpreting responses, whatever the number of strains present. Re-infections following concurrent infections with multiple strains suggested that 'the homologous immunity to either of two strains of *P. vivax* which follows simultaneous inoculation with the two strains is not so effective as that acquired after inoculation by a single strain. The immunity is characterized by the heterologous rather than the homologous properties ... [i.e.] the simultaneous presence of two strains delays the development of an adequate homologous immunity to either'. (Boyd *et al.*, 1938b) In World War II, it was 'recognized that Pacific vivax malaria represents a complex situation in that each patient frequently harbors multiple strains which are not synchronized ... the clinical features of this group of cases should be interpreted as the characteristics of multiple strain infections and possibly superposed infections of homologous strains ... the complexity of the presence of multiple strains is such that the features of this study are only significant in a clinical light, and cannot be applied to the duration or study of immunity of a single strain infection' (Hill and Amatuzio, 1949).

The concept of multiple concurrent, superinfecting, interacting strains had implications for epidemiology and control as well as for clinical and immunological understanding. For an individual, the presence of 'an unknown number of strains without any cross-immunity to speak of among them [implied that] only by chance is he infected twice in succession with the same kind of parasite, and his individual malaria season draws to a close only when he has solidified anew his resistance to the principal strains which are in the air around him every night. We have no idea how many strains of each parasite there are in any one locality ... [but] any reduction in anopheline density begins by removing layer after layer of these superimposed

infections before it cuts down the amount of malaria, or number of infected persons' (Hackett, 1937). Because 'each new strain, like a new species, finds the host defenceless and initiates a train of events culminating in an acute attack, and a period of gametocyte production ... chronic malaria, then, is due to overlapping infections of different species and heterologous strains of plasmodia. Mixed infections must be the rule and not the exception in localities with even a moderate transmission rate ... [and thus] children can not grow up in a malarious locality of even moderate endemicity without acquiring a representative assortment of all the species and many of the strains of plasmodia with which the local anopheles are infected ... The transmission rate, thus determined, creates a corresponding tolerance which is made up of highly specific reactions to the numerous and immunologically independent strains and species of the parasite. The picture is one of growing multiplicity of mixed infections, resulting in chronic malaria and the attendant phenomenon of mutual parasite antagonisms. The mass effect, however, of group immunity at high levels of intensity is one of powerful protection of the older age groups and the shifting of the struggle to childhood or even infancy' (Hackett, 1941). Rates at which protective responses were acquired with age were uncertain, however, even in hyperendemic regions, as were the rates at which those responses were lost (if they were): 'attacks are less and less frequent until the age of twelve, when, if the child survives, an immunity is produced which lasts the entire life of the native if he does not leave the locality in which he was reared. Leaving the area per se does not lessen the child's immunity, but he would be subject to attacks of other strains of the parasite' (Bispham, 1944) or, 'because of the variety of strains and species, a more or less stable immunity may develop somewhere between 20 and 30 years' (Boyd, 1949).

However, given that 'the greater the number of bites the greater the chance of the introduction of multiple strains of parasite, [which] might account for the presence of both the more virulent and the more cure-resistant features recorded' (Sinton, 1931), and that many protective responses appeared to be strain-specific, strain introductions were invoked to 'explain the numerous observations of sudden, severe, and acute outbreaks of malaria, which can be called "malaria epidemics," in endemic districts' (Nocht and Mayer, 1937). In more general terms, the diversity of strains, clinical and parasitological effects, and frequencies of superinfection and reinfection were all seen to vary with the prevalence of infection and intensity of transmission: 'a single or at most very few strains of parasites would be prevalent where endemicity is at a low level. The acquired immunity will render further clinical activity improbable in the event of homologous re-inoculation ... At higher levels of endemicity the number of strains prevalent may be expected to be greater [and] through repeated reinoculations in the course of time with other strains, the individual's immunity will become polyvalent to all of the species and strains of parasites which are locally prevalent ... A person may be reinoculated one or more times with the same or a different species or strain of parasite, after an interval which should be expected to vary with the endemic or epidemic level prevailing ... [if] with the same species and strain of parasite which caused a previous infection, it may result in a superinfection if homologous immunity is as yet incomplete. If effected with a different species and strain, it is distinguished as a reinfection ... Where endemicity is at low or moderate levels, it is likely that reinoculations would be infrequent within a short interval, so that the first infection would have become latent, or even have been eradicated, before reinoculation. ... Under conditions of high or hyperendemicity, or during epidemics, reinoculation may be experienced at shorter intervals ... [which] produces a protracted or continuous infection and clinical activity' (Boyd, 1949).

While the number of strains at a given site would be expected to correlate with basic entomologic and epidemiologic variables, the same strain might be present at different sites: 'The immunity of persons living in regions where malaria is widespread is due to the fact that ever since birth they have been inoculated by an unpredictable number of strains of the different plasmodia, some of these strains having the same antigenic properties as those which they

might contract in other endemic regions, even far removed from their original surroundings' (Brumpt, 1949). Cross-inoculation experiments in Liberia produced equivocal results: 'we are unable therefore, to support the hypothesis that a number of immunologically differing strains exist in relatively small areas and its commonly held practical consequence that a semi-immune who travels relatively short distances in Africa is particularly liable to a "foreign" malaria infection with symptoms' (Bray *et al.*, 1962). Resistance to a seemingly novel strain might reflect some common antigenic property, or, perhaps, some innate or acquired host factors not yet identified: 'whether the African volunteers were infected with an African strain or a Malayan strain there was no obvious difference in the symptoms or course of the malaria ... [our results] all suggest that if there has been intensive infection throughout childhood a very definite immunity is built up which extends to a strain of *P. falciparum* from several hundred miles away or even from another continent the different response to infection appeared to be much more dependent on the origin, and on the quantitative degree of immunity of the patient, than on the origin of the strains' (Davey and Robertson, 1957).

Summarizing the evidence in the mid-1950s, Macdonald agreed that 'in nature there are probably a number of strains and species of human parasite transmitted at any one moment', but, at least in children in hyperendemic regions, 'the occurrence of one infection with *falciparum* makes no material alteration to the probability of another during its course, and that fresh infections during this time are marked by a fresh onset of parasitemia materially unaffected by the previous one' (Macdonald, 1957). He noted that responses differed between *Plasmodium* species, and that responses to different strains were difficult to distinguish in nature: 'infection with *P. vivax* confers a homologous immunity preventing superinfection or subsequent reinfection with parasites of the same strain though not necessarily with other strains of the same species. A small degree of heterologous immunity to other strains is produced and repeated infection with several strains may ultimately produce a firm heterologous immunity to all. In the case of *P. falciparum* the general picture is somewhat similar, but the degree of immunity conferred is considerably less. The clear-cut effects of infection with particular strains cannot be observed in the field, but only the general effect of inoculation with parasites of two or more species and of an unknown number of strains of each. In such circumstances there can be little doubt that superinfection, that is the imposition of a second infection on a first before it has died out, commonly occurs' (Macdonald, 1957). Soon after, Bruce-Chwatt wrote that 'it is surprising, however, that apparent superinfection should be so easy in African adults who, having been exposed to malaria since childhood could presumably have a considerable "multi-strain" resistance to infection ... [so] until we have the immunological means of distinguishing between two strains of the same species of *Plasmodium* the explanation of such happenings and their follow-up will be difficult' (Bruce-Chwatt, 1963).

Hence, even amidst the technical advances of the 1960s, 'it is unwise to say more than that residual immunity and not premonition [premunition] follows recovery from some infections, that the immunity is strain specific, and lasts for at least 3 years' (Garnham, 1966). The challenge remained that 'immunological differences exist ... among geographically isolated strains of the same plasmodial species; but the extensive available evidence for this is based on the degree of cross-immunity among species and strains rather than on clear-cut antigenic definition' (Zuckerman, 1964), and thus it was 'further hoped that by serological means it will be possible to say which malaria parasites an individual has been infected with in the past, which ones he still carries, and to which strains or species he is immune' (Voller, 1964).

Though experimental infections with rodent malarias indicate within-host competition between parasite clones (Mackinnon *et al.*, 2002), such that one drops to sub-detectable levels when the other is introduced, no data are available on the quality of the immune response induced in such situations. Longitudinal studies in humans suggest that

competition and immunity mainly affect parasite density, not the time to clearance (Sama *et al.*, 2006); apparent loss of particular genotypes is common, as densities drop below detection limits. Molecular genotyping has confirmed the expectations that ‘mixed infections must be the rule ... in localities with even moderate transmission rate’ (Babiker *et al.*, 2000; Hackett, 1941; Peyerl-Hoffmann *et al.*, 2001; Sallenave-Sales *et al.*, 2000), that superinfection occurs more commonly in areas of higher transmission (Arnot, 1998), that novel genotypes infecting otherwise immune children give rise to symptomatic infections (Contamin *et al.*, 1996), and that introductions of new phenotypes can cause epidemics of clinical malaria (Arez *et al.*, 1999; Laserson *et al.*, 1999). There is less evidence from these studies of small-scale variations in genotype frequencies that might lead to exposure to new strains after travelling ‘relatively short distances in Africa’ (Bray *et al.*, 1962), but such variations have been observed in remote South American villages (Machado *et al.*, 2004), suggesting that human population movements can create mosaics of local parasite diversity at various spatial scales. Surprisingly, despite the characterization of vast numbers of antigenic variants, the use of serological methods to determine patterns of prior exposure to different parasite strains is still in its infancy (Gray *et al.*, 2007).

7. LATENCY AND RELAPSE

With the development of malariatherapy, blood samples were sometimes taken before the onset of fever as well as after, and from these it appeared that parasites could usually be detected before the first fever occurred. Clinicians and researchers remained more focused on the incubation period (the interval between parasite inoculation and first fever in the host) than the pre-patent period (the interval between parasite inoculation and first detected parasitemia in the host blood), but noted considerable variation in the duration of each, even more with *P. vivax* than *P. falciparum*. Because the time of inoculation was known, it seemed likely that during pre-patency the parasites were not only present but multiplying at sub-detectable levels, and that the lag to the first fever involved a ‘pyrogenic threshold’. Furthermore, these intervals and levels might reflect properties of strains, arising, for instance, because ‘the number of merozoites formed at schizogony varies with these different strains of *P. vivax*’—referring to an average 17–18 observed with Madagascar, 16 with McCoy, 12–13 with Dutch (Boyd, 1941).

Instances in which the initial latency was protracted for months were particularly striking: thus, based on ‘those very common benign tertian [*P. vivax*] infections of northern Europe in which even the primary attack is suppressed ... this primary latency or prolonged incubation seems a character which belongs particularly to certain strains’ (Hackett, 1937). The pronounced differences between *P. vivax* strains in average incubation period—for example, 13.5 days with Madagascar and McCoy, 16.5 days with St. Elizabeth, 21 days with Dutch, 282 days with Roumanian—typically reflected differing proportions of patients with this protracted initial latency (Boyd, 1941, 1949; Kitchen, 1949). In contrast, there seemed to be relatively little variation between *P. falciparum* strains in average incubation period—for example, 12 days with Roman, Sardinian and West African strains, 13 days with Coker, Costa and Long strains—despite sometimes wide ranges within strains, for example, 6–25 days with Coker (Boyd, 1941; Coatney and Young, 1941; Kitchen, 1949).

There were complications in interpreting strain differences in incubation period, however, for instance that ‘many more sporozoites of *P. vivax* than of *P. falciparum* are required to ensure the onset of the malarial attack within the usual incubation period’ (James and Ciuca, 1938), and that ‘the duration of the incubation period tends to vary inversely with the dose of sporozoites received [and] the duration of the clinical attack appears to vary directly with the dose of sporozoites’ (Boyd, 1940b). At Horton Hospital, it appeared that though ‘latency in

BT [*P. vivax*] malaria ... occurs more frequently with temperate region strains than it does with tropical strains ... true latency occurs only when the sporozoites injected are too few to set up an immediate attack'; however, with patients seemingly recovered from Madagascar-strain infections, 'if they were infected with a different strain of the same species, some developed fever and parasites but with a protracted incubation period, usually of several months duration' (Shute, 1946). Initial latency might be influenced not only by dose, and parasite strain, but individual host response: 'the time which elapses between the date of being bitten by an infected mosquito and the date when the earliest clinical symptoms are felt by the patient varies with the dose of sporozoites injected, the virulence of the particular strain of parasite, and the different factors which tend to lessen or to increase the patient's resisting power' (James, 1920). Thus it might be, in contrast to those who 'suggest that there is not a constant pyrogenic threshold for all strains ... that varying susceptibility of patients rather than varying virulence of different strains of parasites is chiefly responsible for the variations in density noted at the onset' (Boyd, 1941), and thereby the variations in incubation and pre-patent periods.

When re-infection or superinfection could be excluded as possibilities, it became difficult to interpret the renewed fevers and parasitemia that sometimes followed an initial attack after latent periods of varying lengths. Several types of renewed activity could be differentiated in accord with their timing: 'for our own purposes, and quite arbitrarily, we distinguish between the returns of fever and parasites which may follow recovery from a primary attack, thus: Recrudescence ... Relapse ... Recurrence' (James, 1931), here with the first category applied to renewed activity within 8 weeks after recovery, the second 8–24 weeks after, the third more than 24 weeks after. It gradually emerged that the last of these categories—now considered 'relapse'—might occur with *P. vivax*, but not *P. falciparum*: 'neither latency nor long-term relapses occur in malignant tertian malaria ... based on a study of several geographical strains of both tropical and sub-tropical origin' (Shute, 1946).

Frequencies of relapse appeared to vary dramatically between *P. vivax* strains, for example, 'we have observed renewed clinical activity after cessation of the primary attack approximately ten times more often in patients inoculated with the McCoy strain than in those inoculated with other strains' (Boyd, 1940a). The frequency of protracted initial latency with the Dutch strain was five-fold higher than with the Madagascar strain, and the frequency of relapses eight-fold lower (James and Ciuca, 1938). A geographical pattern emerged: 'strains of *P. vivax* that originate in tropical areas characteristically produce clinical attacks of malaria at frequent intervals throughout the year ... [but] Korean vivax malaria exhibits a bimodal pattern of clinical activity and a period of long-term latency similar to other strains of *P. vivax* originating in temperate climates ... similar in all major respects to the pattern of the St. Elizabeth strain' (Hankey *et al.*, 1953). Thus 'the likelihood of secondary attacks may vary with the strain ... [and] New World strains have exhibited a decidedly less frequent tendency to become reactivated after long intervals of quiescence than have the Old World strains' (Boyd, 1941), a difference that may have arisen because 'in areas where transmission by mosquitoes can occur during only a short period of each year and where the infective inoculum per patient is often small, strains of *P. vivax* which can hibernate for many months within the human host would be much more likely to survive until the next transmission season than would strains which relapse promptly' (Coatney *et al.*, 1950a).

Frequencies of relapse were important in evaluating potential introductions of 'exotic' strains of *P. vivax*, so studies during World War II compared relapse rates and infectivity 'by origin of strains'—sometimes by whether the infections had most likely been acquired in islands 'A, B or C'—reporting, for instance, that patients were most infectious during their 6th–15th relapse, and when asymptomatic (Watson, 1945). One large US clinic reported relapses in 80% of *P. vivax* cases returning from the Pacific, with an average latent period between recurrences of 4.2 months, compared with relapses in 30% of cases from the Mediterranean and 2% with

the US St. Elizabeth strain (Schwartz *et al.*, 1950). An extensive series of studies conducted on *P. vivax* from returning soldiers reported that the average pre-patent and incubation periods were shorter, and parasitemia at first fever was higher, in Mediterranean than Pacific strains, and that 'Mediterranean malarias had a higher gametocyte density and a higher parasite level at clinical relapse than the Pacific malarias. However, the Pacific malarias showed a higher proportion of patients relapsing after treatment and a greater relative prevalence of parasitemia ... [and therefore] the Pacific malarias might be considered as being more virulent in man than the Mediterranean malarias' (Young *et al.*, 1949).

Thus, as with initial latency, it had become 'apparent that the pattern of relapse in *P. vivax* infections is determined by the strain of parasite, as well as by immunity, chemotherapy, and the size of the infective inoculum' (Coatney *et al.*, 1950a), and, again, that several of these effects might be intertwined, for instance through 'a stage of the parasite living in fixed tissue cells which intervenes between the sporozoite and the trophozoite, the development of which may either be retarded, or inhibited or narcotized by a drug' (Boyd, 1941). It appeared that 'the frequency, duration and severity of "relapses" (including recrudescences) depend on the amount of "tolerance" or "immunity" which the patient may have acquired during the primary attack. Patients who are treated with quinine very early in the primary attack acquire little or no tolerance' (James, 1931). One set of studies showed that 'after a single attack of Chesson strain vivax malaria, cured with pentaquine-quinine, there was no appreciable homologous strain immunity. After four to seven attacks, followed by chemotherapeutic cure, homologous strain immunity was present, but was inadequate to prevent an abortive attack following sporozoite inoculation ... [the data] strongly suggest that a large proportion of vivax infections resulting from small numbers of sporozoites will display short courses and few relapses and that they may subside under non-curative therapy without the development of homologous strain immunity' (Coatney *et al.*, 1950b). Another study with the Chesson strain confirmed that 'immunological phenomena have a profound effect on the intervals between attacks and on the frequency of relapses ... the immune cases reinoculated with the homologous parasite ... almost invariably experienced some symptoms early ... and also almost invariably experienced one parasite recurrence, always asymptomatic ... relapses occurred later and less frequently after treatment of extended clinical attacks than was the case in those attacks terminated prior to extensive clinical malaria' (Jeffery, 1956).

Furthermore, 'an important factor in relapsing malaria may be multiple mosquito bites involving perhaps a diversity of strains of the parasite ... [and] the greater the infection dosage the greater the liability to relapse and to do so for a longer period' (Russell *et al.*, 1946). If so, perhaps, 'every second, third or fourth infected *Anopheles* mosquito bite which is prevented means the avoidance of one, two or three relapses later on' (Horing, 1947), with different strains. An attempt to integrate the evidence hypothesized that 'when pre-erythrocytic parasites are discharged from an exo-erythrocytic depot, there may be several strains amongst them but one strain predominates ... [and] immunity develops against the predominating strain, but, since there will be some overlapping of the antigenic patterns, there may also be some, possibly transient, cross-immunity against other strains. When later there is another discharge of mixed strains from the depot, the immunity that has developed against the first strain will prevent the development of the erythrocytic schizogony cycle by that strain, and another strain will predominate; this goes on until immunity has developed against all the strains present ... The concept of multiple strains being in some way responsible for relapses is gaining ground [though] the therapeutic strains hitherto in use were probably mixed (multiple) strains; that it would be possible to initiate an infection from a single sporozoite seems improbable, but it should be possible by a process of dilution to induce a single-strain infection which would, if the above hypothesis is correct, be a nonrelapsing one' (Napier, 1947).

During and just after World War II, studies began using the clinical, parasitological and immunological methods available to investigate these hypotheses about connections between relapses and concurrent multiple strain infections, initially by comparing cross-inoculation responses to parasites (A, B, C, D) taken from different relapses in the same soldier. Given that ‘different strains vary characteristically in the frequency with which secondary episodes occur’ and that the effects of ‘cross inoculations with a heterologous strain’ were recognizable, ‘the results suggest that “Strains” C and D are closely similar antigenically, if not identical, but that “Strain” A substantially differs from “Strains” C and D’ (Boyd and Kitchen, 1948). Another study, having noted that ‘successive attacks of vivax malaria in an individual exposed in a malarious area need not necessarily be relapses caused by the same strain of parasite [and that] it is theoretically possible for the fixed-tissue parasites of two or more strains of *Plasmodium vivax* to coexist in a host and produce malarial attacks independently’, took advantage of ‘the characteristically different relapse patterns of the Chesson and St. Elizabeth strains of *P. vivax* ... [i.e.] the Chesson strain usually produces an infection with several closely-spaced attacks, whereas the St. Elizabeth strain infection exhibits an early primary attack, several months of latency and a series of late attacks in close succession ... Volunteers infected with the Chesson and St. Elizabeth strains of *P. vivax* displayed a relapse pattern consistent with a combination of the relapse patterns which were exhibited by the two strains when present separately’ (Cooper *et al.*, 1950).

Thus, with relapse as with re-infection and superinfection, it seemed reasonable that ‘if several strains were present more attacks of malaria would be required before tolerance and immunity to all strains would develop’ (Cooper *et al.*, 1950), and, accordingly, ‘when many strains are superimposed, in individuals of differing ages and states of nutrition, who are subject to repeated reinfections as occurs in malaria in its natural habitat, there is little wonder that the pattern of relapsing vivax malaria can appear to be hopelessly complex’ (Coatney *et al.*, 1950b).

It is disconcerting to realize how recently the hypnozoite stage of *P. vivax* was discovered (see below) and how little progress has been made since then in understanding how hypnozoite formation and reactivation is controlled; the inability to culture *P. vivax in vitro* has severely limited opportunities for molecular research on this parasite. Nevertheless, the ability to genotype relapsing infections has led to confirmation that relapses show a strong tendency to be clonal and that multiple relapses in a single patient reflect reactivation of different parasite genotypes (Chen *et al.*, 2007; Imwong *et al.*, 2007), in line with the predictions noted above. The notion that the time between being bitten by an infectious mosquito and the onset of either parasitemia or clinical symptoms is dependent, amongst others, on the number of sporozoites inoculated (James, 1920) has been supported by molecular techniques allowing accurate quantification of subpatent parasitemia (Bejon *et al.*, 2005).

8. SUMMARY AND DISCUSSION

“The question is,” said Alice, “whether you can make words mean so many different things.”
 “The question is,” said Humpty Dumpty, “which is to be master—that’s all” (Dodgson, 1872).

The historical study of strain theory provides a basis for framing contemporary debate, but the context of the debate has changed. The genetic bases for resistance to chloroquine and sulphadoxine–pyramethamine are now reasonably well-understood. The questions of infectivity and relapse remain underinvestigated. The goal of distinguishing strains in terms of clinical virulence and immunity remains as elusive as ever, in part because it is now possible

to see more genetic variability than is expressed in the phenotype. The genes for merozoite stage proteins are known to be highly polymorphic, for example, but much of the observed genetic variation is neutral, or nearly so, such that multiple genotypes have essentially the same phenotype. The clinical, immunological and epidemiological relevance of genetic variability remains poorly understood. It is now evident that each *P. falciparum* genotype can express 50–60 different phenotypes of the PfEMP1 protein through *var* gene switching, and that the genes are distributed across all of the *P. falciparum* genome. It is plausible that heterologous/homologous immunity to *P. falciparum* is explained by different *msp* or *var* gene families and their patterns of cross-immunity. Human immune response may also be heterogeneous, in that the state of clinical immunity in different humans could be conferred by immunity to different sets of immunogens. Continual sexual reassortment of the genes for merozoite-stage proteins and the *var* genes during meiosis in the mosquito, and strong disruptive selection provide new variation. What, if anything, is a strain? If a strain could be clearly defined in one parasite generation, it might not exist in the next, and an operational definition of a strain with respect to one trait (e.g. drug resistance) might not be coherent with respect to other traits (e.g. infectivity). Aside from the limited experience of malariatherapy and laboratory experiments, the existence of a strain may be too transient for any definition to be useful. Therefore, it is unclear whether increasingly detailed genetic identification of strains will ever converge with the clinical definition of strains pioneered during the 1920s–1970s.

The idea that each of the species that cause human malaria consists of ‘varieties, strains or races’ emerged from the observation that malaria infections seemed to differ in severity from place to place and the inference that these differences might arise from biological differences between morphologically identical parasites. When efforts to control and improve responses to malariatherapy made it necessary to distinguish and compare the parasites in clinical use, most such ‘strains’ were named on a geographic basis. The common assumption was that, in nature, ‘in each region, malaria has a character of its own conferred upon it by the peculiarities of the local parasites. No doubt there are a multitude of strains of each species of plasmodium, differing as widely in virulence, response to treatment and tendency to relapse as though they were separate species’ (Hackett, 1937).

If, worldwide, ‘each parasite has many strains ... [but] a strain prevalent in one area is frequently not found in another several hundred miles away’ (Bispham, 1944), then ‘some strains may have a localized habitat or geographical distribution’ (Boyd, 1940a). More drastically, researchers at the Horton Hospital concluded that their three decades of studies had given evidence for several strains each of *P. falciparum* and *P. vivax*, ‘from widely separated geographical areas’, but only one each of *P. malariae* and *P. ovale*, and that ‘while it is fairly certain that there are different geographical strains of malaria parasites it is extremely difficult to detect variations sufficiently well defined to justify the conclusion that different strains can exist within a single circumscribed locality’ (Shute and Maryon, 1954).

The number and geographical distribution of strains—along with their infectivity and relapse characteristics—became topics of wider practical concern with the rising potential for introductions and reintroductions of malaria during World War II. Though the introduction of a particular ‘new’ strain might be probabilistic, its persistence and spread might be constrained by the relative susceptibilities of local mosquitoes or local humans. In humans, presumably, protective responses to endemic strains developed with continued exposure. Thus, ‘among the foreign infections brought into this country, there must be many different and distinct strains. As these are propagated in nature they are added to the various strains already indigenous ... where there is little malaria now, outbreaks due to importation of foreign strains would be fairly obvious ... [but elsewhere] the spread of foreign malaria, except under unusual and rare circumstances, could not be detected ... As these foreign strains are immunologically distinct, it means that no protection is gained by previous infections with native malarias ... [so] we can

expect additional strains to be added to those already present in this country' (Young *et al.*, 1949).

During this same period, concurrent multiple strain infections became accepted as common and significant. Though it was well known that multiple *Plasmodium* species co-existed at almost all malaria-endemic sites, and that co-infecting *Plasmodium* species appeared to interact in suppressing each other's parasitemia (Boyd and Kitchen, 1937, 1938), the idea that strains might similarly co-exist, co-infect and interact took hold slowly and unevenly. Understanding strain co-infections posed complex challenges: sequential infection or superinfection might elicit homologous or heterologous responses, in clinical or parasitological terms, and might influence infectivity, relapse or other features. Thus, 'considering the opportunities for mixed infections of an unknown number of strains, it would be remarkable if any case of malaria resembled another ... and each of these strains in turn may not be pure but may comprise a different assortment of immunological and other elements, part of which it owns in common with other strains of the same region ... We are only at the beginning of these studies ... [and] it is only natural that the imagination of workers in this field has seized upon the existence of such strains to explain all kinds of obscure phenomena in malaria' (Hackett, 1937).

If 'strains' could not be considered independent or immutable, but collections of changeable, exchangeable elements, expressed as antigenic and other properties, how could they be defined? Thus, 'the problem of strains within species is both interesting and important, but it is by no means easy to define what is meant by this term; indeed, we have been compelled to ask ourselves, "What is a strain?" ... if there are no insurmountable barriers which would prevent the spread of the parasite either by man or by the mosquito, it is the persistence of separate strains within a locality which are so difficult to comprehend, especially in hyperendemic areas ... [since] if each strain is to retain its individuality, it must be immune to cross breeding with other strains. If several strains of a species were present in a given locality, presumably it would not be long before a large proportion of the population would be infected with two or more strains ... [so if] a strain retains its identity in a locality where other strains occur ... the gametocytes of one strain must be resistant to fertilization by another strain' (Shute and Maryon, 1954).

In the late 1960s, a WHO expert committee noted that 'a parasite strain has been defined as "a population of common stock descending from a single ancestor or derived from a single source and maintained without intermixture from other sources through a number of generations" [WHO, 1963]. This definition may be appropriate for experimentally selected and maintained laboratory strains, but it is too precise for the present purpose' (WHO, 1969). With 'parasites recovered from natural infections in the field ... the term "strain" is used for a population of parasites, recovered from a source in a given geographical area, that possesses confirmed or suspected distinctive characteristics that may be the results of the pressure of natural selection ... [thus] biological differences in the behaviour of *P. falciparum* may be apparent in (a) the susceptibility to drugs, (b) the pattern of human infection, (c) the immunological response of the host and (d) the ability to infect mosquitos' (WHO, 1969). In addition, it was thought that three types of *P. vivax* strains could be distinguished on the basis of their relapse patterns (WHO, 1969). That is, while a new concern about precision was noted, and discounted, the listing of strain characteristics remained identical to that given in the early 1930s.

In the 1970s and early 1980s, when it became possible to clone and cultivate *P. falciparum* (Rosario, 1981; Trager and Jensen, 1976; Trager *et al.*, 1981), it became clear that virtually every natural 'isolate' contained a mixture of parasite entities, each of which when cloned and cultivated might demonstrate strikingly different phenotypic properties with respect to growth rate, drug susceptibility, gametocyte production, antigen and enzyme variants (Burkot *et al.*,

1984; Graves *et al.*, 1984b). This wide range of ‘clones’ was seen to reflect ‘the extent of the genetic diversity which can exist within a single isolate, or “strain,” of these parasites’; thus, were an isolate cultured in conditions which favored the growth of some clones over others, ‘some “strains” of *P. falciparum* might well undergo changes in such characters as drug resistance or antigenic phenotype’ (Thaithong *et al.*, 1984).

Similarly, the report of a 1981 WHO expert committee on ‘malaria parasite strain characterization’ considered ‘isolate’ and ‘strain’ synonymous, noting that ‘the advent of cloning of asexual blood forms of *P. falciparum* is expected to provide a number of well-characterized uniform strains’ with respect to ‘the available biological markers, including drug sensitivity, isoenzymes, antigenic determinants, plasmodial infectivity to insect vectors, and DNA and other biochemical characteristics’ (WHO, 1981). If an ‘isolate’ or ‘strain’ was a community of parasite entities, however, it was not at all clear what constraints preserved its character from one host to the next. If ‘phenotype’ was a matter of proportions within a mixture, then it might be the uniformity or diversity of hosts and transmission between them that determined phenotypic stability by shaping those proportions: hosts were mixing vessels, sampled by mosquitoes. But, for parasites with an obligate sexual phase in the mosquito, it was not clear how ‘clones’ could be the constituent entities in nature. The questions have since shifted to (multi-locus) geno-types, but they still echo aspects of strain theory from long ago, for example, that ‘partial immunities, partial tolerances might be explained by the loss of certain elements during the passages of composite strains’ (Hackett, 1937).

Much of our current knowledge of malaria derives from investigations into the ‘obscure phenomena’ of strains. This is most obviously so in malaria immunology—for example, in distinguishing between homologous and heterologous, clinical and parasitological aspects of response, or in proposing that responses might differ due to premunition or inherent and acquired differences between hosts. Not until the mid-1970s, for instance, could the insusceptibility of black patients to *P. vivax* malariatherapy, and the rarity of *P. vivax* infections in Sub-Saharan Africa, be attributed to an absence of Duffy receptor (Miller *et al.*, 1976)—a finding counter to the suggestion, noted above, that ‘the human intermediate host is not likely to be a factor in limiting the extension of the range of strains of these parasites’ (Boyd *et al.*, 1938a).

Similarly, over time, the observation that while incubation and pre-patent periods might vary between strains, they were generally shorter with blood than with sporozoite inoculation—that ‘the method of inoculation (natural or artificial) appears to have some influence on subsequent events’ (Stratman-Thomas, 1941)—led to the hypothesis that the natural parasite life-cycle included an intermediate stage in fixed tissue: it ‘may be that by blood inoculation only the forms of the parasite which live in red blood corpuscles are introduced, whilst by the natural method of infection a form of the parasite is introduced which has always lived, not in red blood corpuscles, but in tissue cells ... true (long interval) relapses, and recurrences are not observed (so far as we can ascertain) in inoculated cases, while they occur in 50 per cent of mosquito-infected cases. This difference has led to various suggestions about what happens to sporozoites when they are injected by the mosquito’ (James, 1931). Naturally-induced infections often appeared less responsive to drugs, less infectious to mosquitoes and less resistant to re-infection during the initial latent period than later (James and Ciuca, 1938), but hepatic forms of *P. vivax* and *P. falciparum* were not detected until the late 1940s (Shortt and Garnham, 1948; Shortt *et al.*, 1951), and *P. vivax* hypnozoites not until the 1980s (Krotoski *et al.*, 1982).

Yet it can still be asked, as it was 50 years ago, ‘Is not the word “strain” in regard to malaria used much too loosely?’ (Shute, 1958). Beginning in the 1920s, the theory was that ‘strains’ exist, defined by distinct, observable characteristics. Over the next 50 years, attempts to tighten

'loose' definitions produced important insights into clinical virulence, infectivity, reaction to anti-malarial remedies, antigenic properties, latency and relapse—among them the insights that, upon closer examination, some characteristics that had seemed evident might prove too elusive to be useful in definition. Strain theory had seemed to make testable predictions, but the insights it produced arose from its ramifying ambiguities, and from the recognition of ever more complex confounding variables.

What, if anything, will be said of the second 50 years of strain theory? The first dictionary of the English language defined 'theory' as 'speculation; not practice; scheme; plan or system yet subsisting only in the mind' (Johnson, 1755). Atoms, genes and germs were once 'only in the mind', with definitions that were sometimes 'too loose' or 'too precise', but those terms have persisted as the concepts were refined and explanations tested, with profound practical consequences. Other terms—for example, 'phlogiston' and 'humours'—have been abandoned. If the four species that cause human malaria contain coherent units that behave as 'strains', interventions would likely be improved by understanding them. Progress towards that understanding requires a testable, falsifiable theory of *Plasmodium* 'strains', no less now, than in the 1920s.

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