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Current Status of Malaria and Potential for Control

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INTRODUCTION

"Malaria disaster in Africa" heads the letter from Kevin Marsh to the *Lancet* in September 1998 (107), a disaster, he states, which is not just on its way but is already happening. This warning was prompted by a report from Trape and colleagues (188) on changing mortality patterns in three areas of Senegal over an 11-year period, areas representing three different transmission levels. In two areas, high-transmission and low-to-moderate-transmission areas, the risk of death had more than doubled, a significant rise in itself, but in the third area, of moderate transmission, the risk of deaths in children less than 5 years of age, the group at most risk, had risen by eightfold. The principal reason for this dramatic increase in deaths from malaria in this part of Senegal is attributed to the spread to West Africa of resistance to the first-line antimalarial chloroquine.

The deteriorating situation in Senegal is repeated in other malarious areas. More people are dying each year from malaria than 30 years ago, and malaria is returning to areas from which it had been eradicated and entering new areas such as Eastern Europe and Central Asia (Malaria Foundation International [http://www.malaria.org]). It is perceived as the world's worst health problem, but as the areas of the world which suffer the greatest burden of mortality in early childhood and clinical disease have the least developed systems of health reporting, the (repeatedly) quoted figures for annual deaths and clinical cases are best guesses (177). Thus, global figures for deaths from malaria range from 1.5 to 2.7 million each year, most of whom are children under 5 years of age and pregnant women (160, 180, 212). Almost all these deaths are caused by *Plasmodium falciparum*, one of the four species of malaria parasites in humans. The others are *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. A child is estimated to die from malaria every 12 or so seconds. This burden of mortality is not equally shared, falling most heavily on sub-Saharan Africa, where >90% of these deaths occur and 5% of children die from the disease before reaching 5 years; among the newborns of Africa an estimated 3 million suffer complications from low birth weight, including death, arising from malaria infection during pregnancy. Malaria is responsible globally for 500 million cases of clinical disease and presents a public health problem for 2.4 billion people, representing over 40% of the world's population in over 90 countries (Malaria Foundation International). Almost 10% of the world's population will suffer a clinical attack of malaria each year. Fortunately, most will survive after an illness lasting 10 to 20 days, but during the clinical illness, they will be unable to attend school or work, diminishing educational attainment and productivity. Why malaria is severe and life threatening in only a small proportion of cases is considered by Greenwood, Marsh, and Snow (66) and is thought to be determined by the interaction of a number of factors: these include the size of the infective dose of sporozoites, nutritional status of the host, level of acquired immunity, host genetic factors such as the presence of sickle cell hemoglobin, parasite features such as growth rate and drug resistance, and socioeconomic factors as basic as the availability of health care and education. It has been calculated that 76% more productive life years are lost from malaria than from all cancers in all economically developed countries, while funding of cancer research can be 10- to 50-fold greater (Malaria Foundation International).

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The several factors which contribute to this gloomy picture are briefly summarized to set the scene and will be expanded later in the review. Since malaria is transmitted by the female anopheline mosquito (see below), a major strategy of control is to attack the vector with insecticides. Extended use, however, has led and continues to lead to the emergence of insecticide-resistant mosquitoes. The cost of control programs has forced their reduction or total abandonment in some regions. New breeding sites for mosquitoes are created by road building, deforestation, mining (especially open cast mining), irrigation projects, and new agricultural practices, all environmental changes which might be expected to be of economic benefit. Brazil provides a good illustration (27). In the 1950s in Brazil, control programs had reduced the 5 million malaria cases annually by 100-fold. Twenty years later, there were major but largely unplanned developments in the Amazon region; cattlemen, laborers, prospectors, and others migrated into the area, many of them having no immunity to malaria. In 1991, over 700,000 cases were reported (44). The steep rises in population in some malarious areas, together with migration from rural to more densely populated urban areas, have led to higher rates of transmission: as a consequence, some people with little or no immunity will be exposed to higher rates of infection, for example, in Karachi (Pakistan), Bombay (India), Lagos (Nigeria), Kinshasa (Democratic Republic of Congo), and Dar-es-Salaam (Tanzania) (21). Immigration of people from malarious areas to areas freed from the disease can lead to serious reintroductions. For example, development of gem mines in Cambodia brought large concentrations of miners into a jungle location with few medical services, and malaria extracted a heavy price for the gems in a fatality rate for the miners working for 2 to 4 weeks of 10 to 15%. Worse, miners returning from the mines to Thailand and Burma introduced malaria into areas free of malaria. Drug resistance, alluded to above in the context of chloroquine, a cheap and safe drug and the drug of choice in times past for treating acute malaria, is a spreading and growing problem in South America, Asia, and Africa. Indeed, in some parts of Asia there are parasites resistant to all the principal antimalarial drugs, creating major clinical challenges. For the major pharmaceutical companies, developing new antimalarials does not make economic sense—it is said that not a single major Western pharmaceutical company is currently developing new antimalarials. So the picture is not good, but nonetheless, there are a number of initiatives aimed at preventing the situation from becoming worse and then at improving it. These will be reviewed.

BACKGROUND

In 1998, malariologists celebrated the centenary of the demonstration that the female mosquito transmits malaria. Ronald Ross reported in August 1897 that the parasite could infect the female mosquito. The following year, he showed that the parasite completed its developmental cycle in the mosquito and, when that mosquito took another blood meal, it passed on the parasite (see reference 133 for a summary of the discovery of the life cycle of the malaria parasite). He later wrote that "the discovery was, perhaps, as really important as the discovery of America" (152). His discovery allowed a line of attack on the disease other than using quinine, an antimalarial. Notwithstanding the huge strides in biology and medicine in the 100 years after his discovery, which Ross could never have predicted, he might be surprised if alive today to discover how serious a problem malaria remains.

Briefly, the life cycle is as follows (Fig. 1). Sporozoites, thought to be less than 100 on each occasion (151), are released from the female mosquito's salivary glands, in her saliva, into the circulating blood of the host and within 30 to 45 min have entered hepatocytes. It is not clear how sporozoites squeeze through the sinusoid lining into the space of Disse (or, as might be the case, pass through Kupffer cells on the walls of the sinusoids [193]) to reach the hepatocytes or the precise nature of the sporozoite-hepatocyte ligand-receptor interaction which enables the parasite to recognize the host cell. Peptides forming part of the major surface protein on the sporozoite, the circumsporozoite protein (CSP), have been suggested to interact with receptors on the hepatocyte (29, 167). Growth and division in the liver for the human malaria parasites take from approximately 6 to 15 days depending on the species: approximately 6, 10, and 15 days for *P. falciparum*, *P. vivax*, and *P. ovale* and *P. malariae*, respectively. At the end of the preerythrocytic cycle, thousands of merozoites are released into the blood flowing through the sinusoids and, within 15 to 20 s, attach to and invade erythrocytes. Recognition and attachment are via a receptor-ligand interaction, and at least for *P. vivax* and *P. falciparum*, the host and parasite molecules involved are different (reviewed in reference 60). In *P. vivax* and *P. ovale*, some of the sporozoites appear to develop for about 24 h before becoming dormant as a hypnozoite stage; this form can remain as such for months and even years until reactivated to complete the liver cycle, releasing merozoites into the blood to precipitate a relapse infection.

The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cell after 48 or 72 h for the human malaria parasites, depending on the species, and which then immediately invade additional erythrocytes. The asexual cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in the case of *P. falciparum*). Most malaria parasites developing in the host's red blood cells grow in synchrony with one another, for at least some animal species apparently tuning into the host's circadian rhythms (73). There is no compelling evidence as yet that this is the case for human malaria parasites (73). Consequently, they complete schizogony together at the end of the asexual cycle, releasing pyrogenic materials which induce the characteristic fever spike and clinical symptoms. The morbidity and mortality associated with malaria are derived solely from the erythrocytic stages. A full description of all aspects of severe malaria is given in reference 210.

After invading red blood cells, eventually some merozoites differentiate into sexual forms (gametocytes) and, following ingestion by another female mosquito, will mature to male and female gametes in the blood meal. After fertilization, the resulting zygote matures within 24 h to the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the hemocoel from the midgut. Within the developing oocysts, there are many mitotic divisions resulting in oocysts full of sporozoites. Rupture of the oocysts releases the sporozoites, which migrate through the hemocoel to the salivary glands to complete the

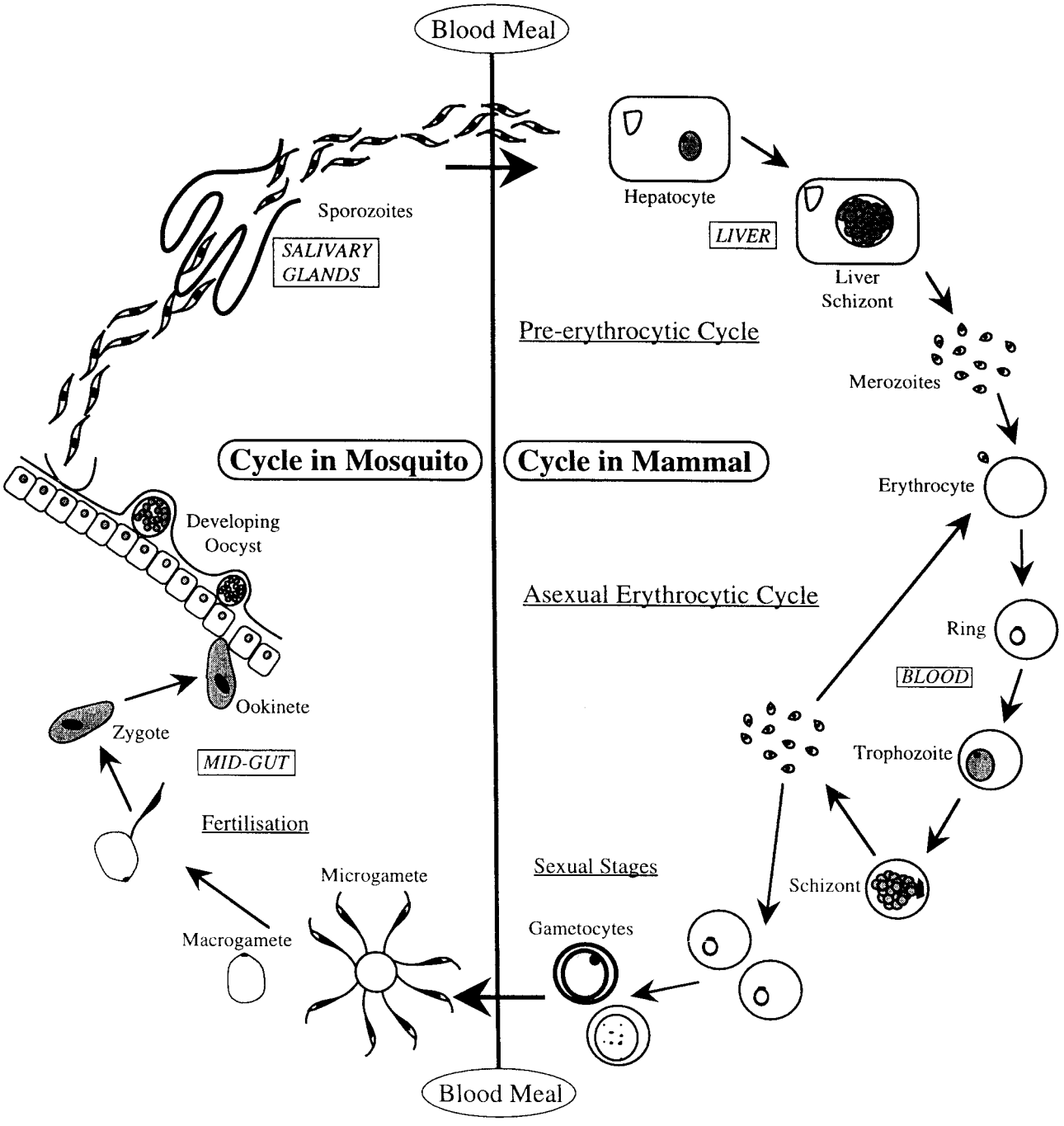

FIG. 1. The life cycle of the malaria parasite in mammals.

cycle approximately 7 to 18 days after gametocyte ingestion, depending on host-parasite combination and external environmental conditions. All stages in the life cycle are through to be haploid, apart from the diploid zygote, which immediately after fertilization undergoes a two-step meiotic division, the resulting cell containing a nucleus with four haploid genomes (14). The sexual process and meiotic division following fertilization allow genetic recombination to occur, which is reflected in the genetic makeup of the sporozoites and together with mutations provides the raw material upon which selective pressures such as antimalarial drugs can work.

DIAGNOSIS

Conventional diagnosis still uses the skilled but laborious and time-consuming microscopic examination of thin and thick blood films stained with Giemsa's or Field's stain. Newly developed tests include the quantitative buffy coat method (Becton Dickinson, Sparks, Md.) for the fluorescent staining of parasites after an enrichment step for the infected erythrocyte (reported to be as good as thick films for *P. falciparum* but not for the other species [6]); the *Para*Sight F (Becton Dickinson) (157) and the Malaquick tests (91) (ICT Diagnostics, Sydney,

Australia), based on the immunological capture of the *P. falciparum* histidine-rich protein 2 in whole blood; and the OptiMal (Flow Laboratories, Portland, Oreg.) assay, which is antibody-based detection of parasite lactate dehydrogenase (102). These antibody-based dipstick tests are still being evaluated. PCR-based diagnostic tests for human malarias have been developed (118), but these are more applicable to large-scale surveys than to clinical diagnosis. PCR has been especially effective at detecting submicroscopic levels of parasitemia. (See references 101 and 208 for an evaluation of these methods.)

MALARIA CONTROL AND PREVENTION

The world's malaria situations can be divided into three categories (211). First, there are areas where malaria transmission is intense and which to date have largely been unaffected by vector control programs, such as tropical Africa. The second category represents the malaria situation in most malarious countries in Asia and the Americas, where large-scale vector control programs are or have been operating—interruption of these programs or a gradual breakdown in the commitment to them leads to outbreaks of the disease. Into the third category are placed areas of rapid economic development and countries seriously affected by social disruption, both of which can lead to environmental disturbances, population movements, and the absence of health care infrastructure.

Malaria is a focal disease which differs in its characteristics from country to country and even within the same country. No single strategy is applicable for all situations, and implementing any of these may be problematic in an area: there has to be a regular assessment of each country's malaria situation. There are a variety of factors to take into account, including (i) the biological, anthropological, cultural, and social characteristics of the population; (ii) the intensity and periodicity of malaria transmission; (iii) the species of malaria parasites and their sensitivity to antimalarial drugs; (iv) the species of the mosquito vector, their behavior, and their susceptibility to insecticides (nearly 70 different species of mosquitoes are thought to be capable of malaria transmission, each with its own ecological requirements and behavioral characteristics); (v) the presence of social and ecological change; and (vi) the characteristics of the existing health services.

Before considering what is new in malaria control, it is worth briefly reviewing the long-established and much-used practices which form the backbone of antimalarial measures (24, 162). History shows that, given proper organization, sufficient funds, and the proper ecological context, e.g., Boston and Rome versus Democratic Republic of Congo, these can give good results in malaria control. The World Health Organization (209) suggests that there are three essential elements of malaria control. First is the selective application of vector control by, for example, reduction of the numbers of vector mosquitoes either by eliminating, where feasible, or reducing mosquito breeding sites; destroying larval, pupal, and adult mosquitoes; and reducing human-mosquito contact. Second are early diagnosis and effective and prompt treatment of malarial disease (which also reduce a source of parasites for infection of mosquitoes as well as reducing morbidity and mortality), in all areas where people are at risk, whatever the economic and social circumstances. The third element is early detection or forecasting of epidemics and rapid application of control measures.

While the value of environmental management in the form of filling in ditches; covering water containers; flushing irrigation channels; clearing ponds of weed growth, which allows the introduction into ponds of fish which eat mosquito eggs and larvae; and other measures to reduce breeding sites and mosquito breeding success is recognized, at the same time there might be major environmental changes which run counter to these efforts. Mining, logging, and land clearance for planting are three such operations which can have a rapid impact on the tropical environment. Extensive borrow pits which hold water are dug alongside new roads constructed for access to these kinds of developments, and the new roads often obstruct existing drainage, causing water to accumulate. Open cast mining by the nature of the operation creates new water catchment areas. Clear felling exposes thin fragile topsoil, which quickly erodes, creating water catchment sites, and in addition, the eroded topsoil can cause siltation in local rivers, leading to shallower river margins. Thus, new mosquito breeding sites emerge, and at the same time a workforce may be introduced from an area where malaria may be absent or infrequent and hence may lack an effective level of acquired immunity. In addition, the increased level of malaria transmission in the locality brought about by the changes can impact on the indigenous population. This is well illustrated by the 70-fold increase in the incidence of malaria in the Yanomami Indians in the Amazon with the introduction of logging and mining into their traditional home region.

Chemicals continue as the mainstay of mosquito control and broadly fall into five groups. Petroleum oils and derivatives sprayed onto water, forming a film, prevent larvae and pupae from breathing through the surface of the water. Introduced in 1921, Paris green (copper acetoarsenite) is another larvicide (24) which, as very small particles, is filtered out by the feeding larvae, which are poisoned. Natural constituents of the flowers of pyrethrum, pyrethrins, and later synthetic derivatives, the pyrethroids, form another group. Organochlorines, which include dichlorodiphenyltrichloroethane (DDT) and dieldrin; the organophosphates, such as malathion and temephos; and carbamates, such as propoxur, constitute the three remaining groups. The last two groups are relatively dangerous in handling, requiring specialist equipment for their use (24). The rationale for use of these chemicals is described in detail elsewhere (209), but in short the basis is indoor spraying with a persistent insecticide, i.e., it remains active on the sprayed surface for weeks or even months to kill or at least repel the adult female mosquito. The problems of resistance are well documented (24, 209) and have increased with time and use, from 2 species of mosquito being resistant to DDT in 1946 to 55 or more species being resistant to one or more insecticides 50 years later; some very important malaria vectors are included among this list. It is worth remembering that, where mosquitoes are resistant, they frequently still find DDT an irritant when they land on it, a sufficient irritant to deflect them from biting a person.

Not only can exposure to insecticides lead to resistant mosquitoes, but it may also result in modifications in mosquito behavior whereby mosquitoes and insecticide have fleeting if

any contact (105, 207). Malaria control in the Amazon region is predominantly based on insecticide use inside dwellings (mainly of DDT) together with active searching for and treatment of cases with antimalarials (chloroquine, Fansidar, and quinine) (see below), but the results have been less than expected (105). Drug-resistant strains of malaria parasites seem to be widespread, and the normally endophilic (resting inside the dwelling after taking a blood meal) *Anopheles darlingi* is exophilic and rests inside for only a few minutes before and after feeding. Endophilic mosquitoes are thereby exposed to the insecticide by resting habits whereas exophilic mosquitoes are not exposed. In the coastal regions of Venezuela and Surinam, *A. darlingi* remains endophilic and DDT is effective (105). DDT remains in use as a residual spray in homes (it is not used for outdoor spraying) in Africa, Asia, and the Americas. Undoubtedly, DDT has saved hundreds of thousands of lives, but 120 countries are currently negotiating a treaty to ban the production and use of DDT by 2007. This ban is felt by some to be unjustified where there are inadequate alternative malaria control methods available at an affordable cost to poor tropical countries (see the discussion compiled by Taverne [186]).

Recently, evidence has been gathered to show that malaria parasites play an important role in regulating the feeding behavior of their mosquito hosts to the enhancement of transmission (20, 147). For example, in mosquitoes infected with the bird malaria parasite, *Plasmodium gallinaceum*, duration of probing for blood was increased and their biting rate on a host doubled (153). In Papua New Guinea, *Anopheles punctulatus* mosquitoes infected with *P. vivax* or *P. falciparum* were more tenacious in their blood feeding behavior, were less likely to respond to disturbance, and had increased multiple feedings (89, 90). As biting rate is one of the principal entomological variables determining the rate of transmission (53), such change in mosquito behavior can impact on the epidemiology of the disease. Further, it has been found that mosquitoes located blood faster in mice infected with *Plasmodium berghei* than in control mice (154).

Antimalarial drugs form an important element in control programs in treating cases to remove a source of infection for feeding mosquitoes. Antimalarials are also used prophylactically. Drug resistance has emerged and is spreading, not least the resistance to chloroquine, the mainstay for treatment of *P. falciparum*. I shall deal with the current position with antimalarials below.

WHAT'S NEW IN MALARIA CONTROL?

Notwithstanding that the situation is very serious, some positive aspects can be identified in malaria control over recent years (24, 68, 174). According to the work of Greenwood (68), the consensus is that the most efficient malaria control comes from a team effort where malaria control is just one of the public health activities which make up primary health care programs within a region or country. The concern with insecticide resistance and the threat of the withdrawal of DDT have emphasized the need for the search for other control measures (174). Biological control of mosquito larvae with naturally occurring bacteria that synthesize potent larvicidal toxins has received less attention than might be expected, given that these bacteria (*Bacillus sphaericus* and *Bacillus thuringiensis*) have been used safely in the field for many years (140, 144). During sporulation, the bacteria produce as crystals protoxins which are toxic to some insect larvae after ingestion as food. The protoxins are solubilized in the alkaline pH of the larval midgut, where they are proteolytically activated and bind to specific receptors on epithelial cells, leading to cell lysis, and the larva stops feeding and dies. These are safe for animals and the environment. A good example of their use is the very successful control of the larvae of blackflies, the vector of the filarial parasite *Onchocerca volvulus*, which causes river blindness, with *B. thuringiensis* subsp. *israelensis* (81, 144). Although *B. sphaericus* and *B. thuringiensis* have the potential for controlling the mosquitoes *Culex quinquefasciatus*, *Culex pipiens*, and *Aedes aegypti*, their toxicity to *Anopheles* has been found to be marginal and variable (144). Currently, these mosquitocidal toxins have not had very widespread commercial use for a variety of reasons. First, the spores of the bacilli sediment rapidly from the larval feeding zones, a particular problem in the case of *Anopheles* larvae, which feed at the surface. Second, the spore crystal is sensitive to UV light. Third, the rate of killing with spores is relatively low compared with that by chemical insecticides. Finally, the toxins can be relatively specific to mosquito species, but most importantly, the formulations are up to three times more expensive than are chemical insecticides. The cloning of mosquitocidal toxin genes from *B. thuringiensis* and *B. sphaericus* has allowed the re-expression of a combination of toxins from both species in one recombinant cell in an attempt to broaden the target range of mosquitoes of the toxins and possibly produce a synergistic effect (96). Given the current public resistance to use of genetically modified organisms, obtaining permission for release of such modified bacteria into the environment may not be easy, notwithstanding the worthiness of the objectives.

As noted above, control programs have a central structure of antimosquito measures combined with identification and treatment of infected individuals. Treatment has to be readily available and accessible. Such an approach has been very successful in Southeast Asia in reducing the incidence of severe disease, but less so in Africa (209). Almost all antimalarials are primarily targeted at the asexual erythrocytic stages, which cause the morbidity and mortality of malaria. Sexual stages, gametocytes, if they are also present, will not be affected by some antimalarials, and hence transmission from these patients might occur for some weeks after treatment. For example, where treatment is in the form of a combination of chloroquine and Fansidar (pyrimethamine-sulfadoxine), which have little action on gametocytes, transmission may not be much affected. A new class of antimalarials (see below), the artemisinin family, not only controls the asexual erythrocytic stages which cause pathology but also significantly reduces the numbers of gametocytes and hence also reduces opportunities for transmission from the patients (143, 174). A trial in Thailand in which the prevalence of gametocytemia (prevalence of gametocytes in the peripheral blood) was assessed in children after treatment with either mefloquine, halofantrine, or artemesinin showed an average duration of gametocytemia to be 34.1 weeks with mefloquine, 16.7 weeks with halofantrine, and 3.9 weeks with artemisinin.

Protection of the individual from mosquitoes can be achieved through a number of methods including mosquito

repellents, protective clothing, insect screens, siting of dwellings away from breeding areas, improvement of the design and construction of dwellings, and bed nets. One of the most encouraging recent developments in malaria control has been the finding that impregnation of bed nets and curtains with insecticides can significantly reduce morbidity and mortality (94). Choi et al. (34) concluded that studies in Asia and Africa in different epidemiological situations show that insecticide-impregnated bed nets reduce the incidence of clinical attacks by 50%. A trial in the Gambia (2) showed that insecticide-impregnated bed nets (the pyrethroid permethrin was used) reduced overall deaths by >50%. Further trials in other countries after the successful study in the Gambia did not show such a beneficial effect on mortality rates, but nonetheless, there was a reduction in overall child mortality of 17 to 33% (120, 176). Beneficial features of bed nets are that they are readily accepted by the population and that they have the high usage rate of 86%, even in the absence of expensive promotional programs. There is an argument that governments willing to invest money in residual insecticide spraying might be willing to redirect some of this expenditure into bed nets (120). Successful trials were conducted with free nets and insecticide at the village level (2), while other studies have shown that, where nets and insecticide are not free, the cost of reimpregnating the nets—an essential requirement—or even repair of nets, never mind replacing worn or torn nets, is often beyond the means of the population (30). In the Gambia, when a charge of $0.50 for insecticide was introduced, usage declined from >70 to <20% (30). In some communities, the concept of paying for a health delivery system may be new and not readily accepted, and therefore a sustained and monitored bed net program will require some level of subsidy (30). If the nets and/or insecticide is not manufactured locally, purchase uses valuable foreign exchange.

The long-term impact of bed net programs is a matter of some debate (189). Undoubtedly, the reduced level of exposure through bed net intervention is likely to have an adverse effect on the acquisition of immunity (see below), and any beneficial outcome is likely to be transitory in areas of high transmission (98). Greenwood has summarized the arguments (67, 68). The incidence of malaria rises with an increase in entomological inoculation rate (EIR), i.e., the number of bites from an infective mosquito per person per year, but only to a maximum rate, after which incidence levels off or even falls even though the EIR may continue to rise. Thus, vector control may have a measurable effect on morbidity and mortality only where the curve of EIR against mortality is rising, but if EIR is at the plateau, even a substantial reduction of EIR may not measurably reduce malaria. Trape and Rogier (189) conclude that it is premature to invest so much expectation in bed net programs in tropical Africa. In this region, where the EIR is high, it is unlikely that a sufficient reduction in EIR can be maintained long enough to reduce the burden of malaria on the whole community. On the other hand, Greenwood (67, 68) believes that it is premature to draw such pessimistic conclusions, arguing that there should be immediate beneficial effects and that this may be a long enough window for other control measures to be developed and introduced.

Another concern about the use of impregnated bed nets has been their impact on the development of acquired immunity in the population. Acquired immunity (see below) against clinical malaria is thought to develop gradually with time and to be a function of the frequency of infections. There might, therefore, be a concern that the period during which a child is at risk from clinical malaria might increase where bed nets are used (173, 189). If a child was protected by bed nets but later these were no longer provided or were not used, there might be a rebound effect of clinical disease when the child was exposed to highly infectious mosquitoes. There is some evidence that a reduced risk from clinical malaria is associated with the presence of multiple concurrent infections with *P. falciparum* of different genotypes (12). Thus, bed nets might reduce exposure and consequently the multiplicity of infections with a corresponding delay of acquired immunity. A study in Tanzania (58, 185) compared children sleeping with and those sleeping without impregnated bed nets. Although bed nets reduced anemia, the multiplicity of genotypes was not changed between the with-bed net and without-bed net groups; this suggested that the development of acquired immunity in these particular epidemiological conditions was not compromised by the nets.

It has been known for many years that mosquito numbers depend on climate (62, 63) and that monitoring meteorological variables may be helpful in predicting malaria epidemics. Through a better understanding of how the variables of rainfall, temperature, and humidity can influence mosquito populations, it is possible to make better-informed predictions of transmission rates (190). In addition, many mosquito species lay their eggs in specific aquatic habitats which often are characterized by an easily identifiable plant community (119, 149). Remote sensing techniques, such as local aerial photography and satellite-borne sensors to identify mosquito breeding sites, have considerable potential for bringing together climatic and vegetative variables for making predictions about future malaria epidemics and, in time, for antimalarial measures to be planned and implemented to good effect (74). The impact of global warming on malaria transmission is another area for concern (97). Small increases in temperature in temperate and subtropical areas where malaria is unstable or currently absent might markedly increase transmission, whereas stable-transmission areas might be little affected by a temperature rise.

TRANSGENIC MOSQUITOES

There is a developing interest in the possibility of using molecular genetics to manipulate the genomes of insect vectors such that they are no longer capable of acting as a vector. A principal focus of this approach is *Anopheles gambiae*, together with *Anopheles arabiensis* and *Anopheles funestus*, three of the most efficient malaria vectors in the world. The ambitious aim is to replace natural vector populations of mosquitoes with populations which are unable to support complete development of the malaria parasite. The strategy depends on progress in three areas (38): the identification of parasite-inhibiting genes, the availability of techniques for introducing such genes into the mosquito genome, and strategies for spreading these genes through the natural populations of the vectors. Inbred strains of *A. gambiae* that are refractory to infection by malaria parasites have been selected. Several different mechanisms whereby the mosquito inhibits establishment or development

of the malaria parasite have been detected. There are good prospects for identifying and cloning genes controlling these antiparasite processes. In addition, a number of mobile genetic elements which could act as gene vectors have been identified and isolated (126). However, the response of the general public to the release of genetically modified mosquitoes into the environment might be adverse.

ANTIMALARIAL DRUGS AND RESISTANCE

Reviews of antimalarials, their history, their mode of action, and the development of resistance have been provided by a number of authors (56, 130, 145). In general, malaria is a curable disease, and if everyone has access to early treatment, nobody should die from it. For the past 50 years, there have been two main classes of antimalarial agents in use, the antifolates and the cinchona alkaloids or the quinoline-containing drugs. In most cases, these agents are targeted at the asexual erythrocytic stage of the parasite. The antifolates include the diaminopyrimidines, such as pyrimethamine and trimethoprim; the biguanides, represented by proguanil (cycloguanil) and chlorproguanil; and the sulfa drugs, including the sulfonamides and the sulfones. In the 1960s, it was discovered that the latter two drug types have synergistic activity with pyrimethamine or proguanil, the most frequently used combinations being pyrimethamine-sulfadoxine (Fansidar) and pyrimethamine-dapsone (Maloprim) (145). The quinoline-containing drugs include the cinchona alkaloids, quinine and quinidine, and the aminoalcohol quinine analogues mefloquine (203) (a 4-quinoline methanol) and halofantrine (a 9-phenathrene methanol), which are recent introductions. There are also the 8-aminoquinoline primaquine, which is used for its gametocidal effect and its action on the liver stage of *P. vivax*, and the 4-aminoquinolines, chloroquine and its relative amodiaquine.

In addition to mefloquine and halofantrine, newer therapies in development include antibiotics such as tetracyclines, doxycycline, and azithromycin, which is closely related to erythromycin; atovaquone, which is a hydroxynaphthoquinone (11); pyronaridine, which was developed in China 20 years ago as a derivative of mepacrine; and derivatives (59) of artemisinin (qinghaosu), the active antimalarial principle in the leaves of the wormwood plant which was isolated in 1972 (26). Artemisinin has been used in traditional Chinese medicine for centuries for the treatment of falciparum and vivax malaria. Artemisinin itself is poorly absorbed, and therefore a number of derivatives have been prepared and evaluated: three semisynthetic derivatives are in use, water-soluble artesunate and two oil-soluble compounds, artemether and arteether. These compounds are widely used in China and Southeast Asia. Artemisinin derivatives are the fastest-acting antimalarials available (202). When they are used by themselves, there is a relatively high recrudescence rate, but in combination with other antimalarials, particularly mefloquine, complete cures have been recorded (205). The main concern with the lipid-soluble derivatives is related to their toxicity in animals (22), but similar toxicity has not become an issue with humans.

With the possible exception of artemisinin derivatives, resistance to all antimalarials has been recorded (201, 204) but has developed at different rates for different compounds. Quinine, the active ingredient from the cinchona bark, introduced into Europe in the 17th century from South America and isolated by the French chemists Caventou and Pellitier in 1820, has had the longest period of effective use, but resistance has been reported (201). Nonetheless, it remains an important member of the antimalarial armory. Chloroquine, originally synthesized by German chemists in the mid-1930s, after the Second World War rapidly became the drug of choice both for treating acute falciparum malaria and for prophylactic purposes. It is cheap, at around $0.08 per treatment, and in 1994 was said to be the second or third most widely consumed drug in the world after aspirin and paracetamol (57). Chloroquine too has had a reasonable period of effective deployment, albeit the first reports of chloroquine resistance appeared in 1957 and more significant reports appeared in 1961 (199). Initially spreading through South America and Southeast Asia, chloroquine resistance by *P. falciparum* reached East Africa, from the east, in 1978 and had crossed the African continent by 1985 (15). Chloroquine resistance has also been found in *P. vivax* (159). Undoubtedly, the low cost of chloroquine and its ready availability contributed to the development of resistance (see below). Resistance to the antifolates (dihydrofolate reductase [DHFR] inhibitors) developed more quickly after their introduction, although the combination of pyrimethamine with sulfa drugs delayed the spread of resistance to pyrimethamine. Resistance to mefloquine was reported even before the drug was brought into routine use in Southeast Asia, and its usefulness in this area is now severely compromised.

The mechanisms of resistance in the antifolates such as pyrimethamine and the sulfa drugs are well known. Malaria parasites meet their folate needs by de novo synthesis. The two main targets of the antifolates in the folate synthesis pathway are the enzymes DHFR and dihydropteroate synthase (56). Pyrimethamine and proguanil target plasmodial DHFR, while the sulfur drugs target dihydropteroate synthase, hence their synergistic activity when used in combination. Resistance to the antifolate drugs is the result of point mutations in the substrate binding site of the target enzymes (42, 139). At the present time, there are seven identified point mutations occurring in the DHFR gene which are associated with reduced drug binding capacity in resistant DHFR strains of *P. falciparum*. For example, a serine-to-asparagine change at codon 108 is the critical mutation found in all pyrimethamine-resistant isolates (41, 131).

Mechanisms of resistance to chloroquine have been much more difficult to unravel, and indeed the mode of action of this antimalarial is not beyond debate. Chloroquine is thought to accumulate to high levels in the food vacuole of the asexual erythrocytic malaria parasite, where it interferes with the polymerization of heme. The parasite derives a substantial part of its nutrition from the digestion of the host cell's globin in hemoglobin, leaving heme as a by-product. Since heme is toxic, the parasite polymerizes it to the harmless hemozoin or malaria pigment. Wellems and colleagues (181), examining a cross between a chloroquine-sensitive and a resistant strain of *P. falciparum*, have found two genes on chromosome 7, *cg1* and *cg2*, which have a complex polymorphic mutation pattern when the chloroquine-sensitive (HB3) and the chloroquine-resistant (Dd2) clones of *P. falciparum* are compared. *cg2* codes for a transmembrane protein. The chloroquine-resistant Dd2-type polymorphisms in *cg1* and especially in *cg2* were significantly

associated with chloroquine resistance when tested against chloroquine-resistant parasites from Africa and Asia but not when tested against chloroquine-sensitive parasites from these two continents. Immunoelectron microscopy indicates that the localization of CG2 protein is not inconsistent with its being a transporter protein. It would not be unreasonable to speculate that chloroquine resistance is associated with a transporter protein which prevents the antimalarial from reaching lethal concentrations in the cytoplasm or food vacuole of the parasite. For a full discussion on the possible mechanisms of chloroquine action and resistance, see the work of Ginsburg and colleagues (64).

The widespread and in some cases rapid development of drug resistance requires that measures be taken to prevent such being the fate of the remaining effective compounds and any new compounds which might be produced in the future. White and Olliaro (204) review the scenarios which might promote or delay or prevent emergence of drug resistance. Ideally, drugs should become available only when needed and should be given to a patient who is compliant with respect to the treatment regimen. A number of factors have been identified as playing a role in the emergence of drug resistance. The pharmacodynamic and pharmacokinetic properties of the antimalarials are important (196). Drugs which have a long elimination half-life are potentially more likely to select for drug resistance than those which are rapidly eliminated (196). In the former situation, some parasites are likely to be exposed to the drug at suboptimal levels for extended periods of time; these suboptimal levels are sufficient to inhibit but not to kill the parasite, thereby providing conditions which may promote selection of resistant parasites. Ironically, antimalarial drugs with long half-lives were seen as desirable because they could be used prophylactically and taken on a weekly basis rather than daily and when used for treatment could be given in a single dose (196). Most of the commonly used drugs such as chloroquine, pyrimethamine, the sulfur compounds, and mefloquine fall into this category. Drugs which are rapidly eliminated (and therefore require multiple treatments over a period of time) will exert minimal selection pressures, as they remain at subtherapeutic antimalarial levels for a short period of time (196); these are conditions where resistance will develop more slowly.

Another factor determining the rate at which resistance may develop is the nature of the mechanism of resistance. Where resistance is conferred by a single mutation, as was noted for resistance to the antifolate drugs, then resistance may develop very suddenly. For example, resistance of *P. falciparum* in Thailand to the synergistic combination of pyrimethamine and sulfadoxine took only 5 years to develop (132). Frequency of reinfection, i.e., intensity of transmission, can influence the rate at which resistance emerges (204). Thus, if a patient is reinfected after recently being successfully treated, but when subcurative levels of the drug remain in the blood, conditions prevail which can exert selective pressure on the reinfection parasites. Mutations resulting in resistance are at low frequency, and therefore the chance that a resistant parasite will emerge from the reinfection will generally be higher where the parasite load is higher.

With this information, how can the life of existing antimalarials be extended? In the choice of drug combinations, the drugs should have similar pharmacokinetics and pharmacodynamics and not interact in any adverse way such that additional problems of toxicity might occur. It is preferable that known drug resistance mechanisms in each of the drugs being combined are not linked. A number of drug combinations have been explored (200). The combination of artemisinin derivatives with mefloquine has been very successful in southeast Asia (84, 142). Artemisinins are so potent that a single dose can reduce the parasite load by a factor of 10^4, and over a 3-day course the parasite numbers can be reduced by 10^8, leaving an average of 10^3 and a maximum of 10^5 parasites. Consequently, there will be a relatively low parasite load remaining for the second drug, mefloquine, to deal with when at its maximum therapeutic level. These conditions reduce the chances that a drug-resistant mutant will escape. In Thailand, the use of this combination therapy against *P. falciparum* has not only helped with the serious problem of mefloquine resistance in the parasite but has apparently led to a drop in the level of mefloquine resistance in the area (N. J. White, personal communication).

Particularly in the context of chloroquine resistance in *P. falciparum*, the spread of resistance from Southeast Asia to the eastern seaboard of Africa and then its remorseless westward move to the west coast of the African continent have been described elsewhere (199). Spread occurs via the mosquito vector; two aspects of this should be briefly mentioned. First, most patients are infected with mixtures of genetically distinct clones in areas where malaria is highly endemic. When mosquitoes take blood from such patients, there is a strong possibility that cross-mating will occur between such clones. The process has been demonstrated both in crossing experiments in the laboratory and in naturally infected wild-caught mosquitoes in the field (3, 4, 194). Following such cross-mating, recombination generates parasites with genotypes different from those of the parental populations. Thus, because there is frequent recombination in nature, parasites with the potential to exhibit a range of responses to different drugs will be generated.

The second aspect to consider is whether resistance to antimalarial drugs might bring with it other changes in the biology of the parasite which might promote or diminish its survival in the host and transmission to another host. Resistance to antimalarials was low before their widespread use, suggesting that resistant mutants were usually eliminated in the absence of the selection pressure of antimalarials, i.e., they were at a selective disadvantage compared with the drug-sensitive parasites. This suggestion is supported by the observation that, in a few cases where drug pressure in the field has decreased, there is some evidence that drug resistance has also decreased 88). These observations could indicate that resistant parasites are less well transmitted than are sensitive parasites (88). Laboratory studies, however, with rodent malaria parasites, such as *Plasmodium yoelii nigeriensis* (82), and recent field studies have found that infectiousness to mosquitoes was higher in chloroquine-resistant parasites than in sensitive parasites (70). Increased infectiousness of gametocytes to a mosquito does not automatically mean that the parasite will have an increased chance of being transmitted to another host. In order to be transmitted to another host, the mosquito must survive long enough for the parasite to complete its life cycle and produce viable sporozoites. There is, however, evidence that malaria parasites increase the mortality of mosquitoes and that the

increase is commensurate with the number of malaria parasites (32). Therefore, drug resistance may increase infectiousness to the vector but this infection may also lead to premature death of some mosquitoes before they can transmit. Which of these conflicting pressures is more important and determines whether drug resistance promotes transmission of the parasite compared with drug-sensitive strains is not certain, but the fact that, when drug pressure is withdrawn, the incidence of drug resistance can diminish suggests that drug resistance carries a cost rather than a benefit (see the work of Koella [88]).

Notwithstanding the major problems with drug resistance briefly discussed above, and the continued importance of malaria across the tropics, the fact is that no major pharmaceutical company deems it worth their while to invest in the development of new antimalarial compounds. The potential market is, of course, very large, comprising almost half the world's population living in areas of endemicity together with a sizable market of 20 to 30 million people living in Europe and North America who visit malarious areas on business or for pleasure (57). As already noted, chloroquine, which can be used both for treatment, as it is rapidly acting, and prophylactically, has the advantage of being cheap. Any replacement compound has to be equally cheap, otherwise it will be limited to use for travelers or as a second-line drug in a limited number of hospitals. The relatively recent introductions, such as mefloquine and halofantrine, are too expensive. For example, mefloquine costs about 10 times as much as chloroquine, and halofantrine costs 20 to 30 times as much. Regrettably, the major stimulus for development of new antimalarials has been war in tropical or subtropical regions of the world, in particular the Second World War and the conflict in Vietnam.

VACCINES AGAINST MALARIA

The foregoing gloomy picture of malaria and the armory of methods to combat its ravages, mainly in indigenous populations, has led some commentators to conclude that vaccination against *P. falciparum* and *P. vivax* is the method of intervention with the greatest potential to reduce the morbidity and mortality associated with severe malaria in areas of intense transmission (112) and even contribute to eradicating the disease, which is not an impossibility (67): the success of early vector control programs suggests that an optimistic view is not unreasonable. As with antimalarials, there is little commercial incentive for industry to invest in development of vaccines against any human parasite, including malaria.

A malaria vaccine represents a major challenge, even with unlimited resources to devote to the task. In this section, I will outline the nature of this challenge, the steps forward (and backward) toward meeting the challenge, and some of the current endeavors and favored strategies. In this discussion, I will be referring to *P. falciparum* unless stated otherwise, because almost all of the effort is directed to this, the most dangerous of human malaria parasites. As previously mentioned, most residents living in areas of endemicity and exposed to *P. falciparum* malaria on a regular basis do develop an acquired immunity to malaria, and with few exceptions, the process of acquisition of immunity starts in babies and infants and is sustained into later life. Most of these young people survive infection with *P. falciparum*, but in about 1 to 2% of infections, severe malaria develops and can be fatal (66). It would be a very tall order to produce a vaccine against a parasite for which natural exposure does not stimulate a protective immune response. Although protective immunity does develop against *P. falciparum*, such protection is not easily won (136). In areas of endemicity, the pattern is for babies and infants up to the age of about 5 years to be at risk of dying from the disease, but parasite prevalence, parasite density, and the number of clinical episodes do decline progressively with increasing age (69, 109, 110). Even in adulthood, however, immunity is never complete (109, 110), and during the transmission season, it is not unusual for the occasional clinical episode to occur and for parasites to be detectable in the blood even when the patient is clinically well. It might be that, after first infections in adulthood, an effective immunity develops more quickly (5, 7, 83). As a result of government policy of economic development in Indonesia, there has been a massive movement of people from heavily populated areas, often free of malaria, to the outer islands, where malaria is endemic. Many of these transplanted peoples have set up agricultural schemes involving irrigation, creating ideal breeding sites for mosquitoes. Consequently, malaria exploded among these peoples, for whom it was observed that among the adults there were no fatalities after 2 years of residence, suggesting that lifesaving immunity developed in 2 years in adults compared with 5 years in small children (5, 83). Current thinking is that natural acquired immunity is normally specific for a species of malaria parasite. People who move out of an area of endemicity for an extended period appear to lose their immunity, suggesting that repeated exposure is necessary to maintain resistance (110). Immunity is apparently, therefore, invariably incomplete, and to think in terms of a vaccine which gives total protection, we are looking for a level of immunity never achieved by natural exposure. If natural immunity could be mimicked, then vaccination would prevent severe malaria and malaria-related deaths but would not give complete protection.

A vaccine to provide immunity comparable to that naturally acquired in areas of endemicity would have to be targeted at the asexual intraerythrocytic stage of the infection, because it is this stage which is responsible for the pathology and clinical symptoms of the disease: prolonged protection by this vaccine would depend on regular boosting by reinfection. (In general, there is a correlation between high parasitemia and disease severity.) Such a vaccine may be of little value to the traveler to a malarious area. He or she would ideally require complete protection, and that would be obtained by inducing protection which prevented the parasite from either gaining entry to the hepatocyte or completing its development in the liver. Nonetheless, a partially effective preerythrocytic vaccine may be of some value to residents of areas of endemicity insofar as a reduced number of merozoites emanating from the liver will extend the period before the asexual parasites in the blood build up to numbers causing pathology, giving the host an additional period in which to mobilize the immune response. Thus, there are two principal targets for a malarial vaccine—the preerythrocytic stage (sporozoite and hepatic stages) and the asexual erythrocytic parasites. There is a third target which some investigators are pursuing, and that is the sexual or trans-

mission stages, with the aim of producing a vaccine which would prevent sexual stages taken up by a feeding female mosquito from completing their development in the mosquito and thereby prevent the parasite being transmitted to another host, limiting the spread of the parasite (109). Since this transmission-blocking vaccine is an unusual vaccine in that it does not directly protect the vaccinee, it has been called an altruistic vaccine.

The search for vaccine formulations has, to some extent, gone hand in hand with attempts to understand better the mechanisms of protective immunity induced by infection. Such knowledge might indicate those protective mechanisms which a vaccine might preferentially induce and also indicate which are the major antigens for consideration as vaccine candidates. Further, it is clear that some of the pathology associated with acute malaria has an immunological basis, and therefore a vaccine should not induce pathological reactions directly during the vaccination process or leave the vaccinee at a greater risk from such harmful responses if a natural challenge infection follows vaccination. A difficulty of investigating immune mechanisms in human malaria is that the human parasites are largely host specific and will infect only people and a few nonhuman primates and monkeys. The availability, expense, and ethical considerations of using nonhuman primates require that much of our knowledge comes from rodent malarias. A look at the life cycle of the parasite might suggest particularly vulnerable points for immune attack. Extracellular stages would be more accessible to immune attack than would intracellular stages: these are the sporozoites introduced into the blood by the feeding mosquito and which circulate for up to 30 to 40 min, the merozoites released from hepatocytes and erythrocytes at the end of the preerythrocytic and asexual erythrocytic cycles but thought to be extracellular for only 15 to 20 s before attaching to and invading erythrocytes, and the male and female gametes which are released when the gametocytes mature in the midgut of the mosquito after a blood meal. Changes to host cell surfaces as a result of parasitization could make them recognizable by the host's immune response (109). Little is known about changes to the surface of hepatocytes following parasitization, but there is some evidence that parasite-derived peptides might be located in or on the cell surface. For example, cytotoxic T cells have been identified which recognize a peptide from the CSP on malaria-infected hepatocytes (see "Preerythrocytic Vaccines" below) (198). CSP is a major protein on the surface of sporozoites.

Changes to the red blood cell surface after parasitization have been reported previously (13, 25). For example, in *P. falciparum* and a few other species, in the last third of each asexual cycle the infected erythrocytes bind (cytoadhere) to endothelial cells lining postcapillary venules and as a result stop circulating in the peripheral blood. This process is known as sequestration. The infected erythrocyte remains bound until schizogony is completed, whereupon the released merozoites rapidly invade more red blood cells. A major parasite-derived molecule or antigen becomes detectable on the surface of the infected erythrocyte as the asexual parasite develops, and this molecule mediates binding of the infected erythrocyte to receptors on the endothelial cells (13). (There are likely to be other cytoadherent molecules as yet not characterized.) In *P. falciparum*, the cytoadherent molecule is known as PfEMP1 (*P. falciparum* erythrocyte membrane protein 1). There are a number of host cell receptors involved (intercellular adhesion molecule 1, CD36, thrombospondin, vascular cell adhesion molecule 1 [reviewed in reference 13]), and different populations of *P. falciparum* may use different combinations of these receptors. The immune response to PfEMP1 is discussed below. Gametocytes of this species also are sequestered during their extended period of development of 9 to 10 days, and this too appears to be mediated by PfEMP1 (150). A surface antigen is also exposed on the red cell surface in other species, for example, in the monkey malaria parasite *Plasmodium knowlesi*, where sequestration does not occur; the function of such a molecule in these is not known (1). Other prospective targets of immune attack would be molecules involved in the recognition of, attachment to, and penetration of the host cell.

Acquired Immunity

A brief resume of protective immune mechanisms to the malaria parasite following infection and reinfection is as follows. There is considerable uncertainty about mechanisms in vivo in humans.

Ironically, understanding of the immune mechanisms directed against the preerythrocytic stages has come from vaccination experiments, initially with animals and later with humans (125), rather than from observations of natural or experimental infections. Very strong resistance to a challenge with viable sporozoites is induced with irradiated sporozoites of *P. berghei* (124) and *P. yoelii* (197) in mice and of *P. falciparum* (36) and *P. vivax* (36) in humans. The irradiated sporozoites are able to invade hepatocytes but do not complete their development in the liver (146), and it is thought that this period, albeit shortened, of metabolic activity is important for mobilizing protective immunity. In these murine systems, the degree of immunogenicity of the irradiated sporozoites varied with the inbred strain of mouse and species of rodent malaria agent (reviewed in reference 183). The protection mechanisms induced by irradiated sporozoites were investigated, and initially the focus was on the role of protective antibody. Serum from immunized mice passively protected mice against a sporozoite challenge, probably by blocking invasion of the hepatocytes by the sporozoites (141). Subsequently, it was found that mice lacking B cells but not T cells (33) could be immunized with irradiated *P. berghei* sporozoites, which demonstrated that the antibody-independent (cellular) as well as antibody-dependent immune mechanisms are induced by the irradiated sporozoites. Much work has gone into investigating the nature of the antibody-independent responses. It has been found that primarily $CD8^+$ T cells but also $CD4^+$ T cells mediated the protection, by cytotoxic activity directed at the infected hepatocyte, and by secreting gamma interferon which, in turn, induces nitric oxide-dependent killing of the parasite within the hepatocyte (111, 121).

It is very uncertain to what extent the mechanisms of protection against the preerythrocytic stages induced by vaccination with irradiated sporozoites are induced by natural infection. In areas of endemicity, an antibody to sporozoites is detectable in residents from about 10 years of age (122), and it is reported that serum containing such antibodies can inhibit invasion of hepatoma cells by sporozoites (121). Similarly, us-

ing in vitro systems there is evidence of cytotoxic $CD8^+$ T cells (50, 76) in the peripheral blood of immune adults.

Immunity to the asexual erythrocytic stages following infection is multifactorial. It is uncertain to what extent the wealth of information derived from animal models and in vitro assays can be applied to malarias in people. Antibody has an important role. Numerous experiments with animal models showed that passive protection with serum was possible. Cohen, McGregor, and Carrington (37) and later Druihle and colleagues (17, 18) controlled a patent *P. falciparum* parasitemia by passive transfer of immunoglobulin G from immune adults. The question is how is the protective antibody exerting its protective effect? In different host-parasite combinations and at different times after infection or reinfection, the mechanism might be different. Thus, roles reported include blocking invasion of red cells by merozoites, opsonization of merozoites and infected erythrocytes promoting their uptake by phagocytic cells, antibody-dependent cellular cytotoxicity, agglutination of merozoites, preventing sequestration of *P. falciparum*, and neutralizing malaria toxins (100, 136). As some of these proposals are based solely on in vitro observations, it cannot be assumed that they have a similar role in vivo.

Cellular antibody-independent mechanisms of immunity to the asexual erythrocytic parasites are also reported elsewhere (95). Cytokines such as tumor necrosis factor alpha and most importantly gamma interferon play a major role. In a rodent model, there is a switch from antibody-independent immune mechanisms during the acute blood parasitemia to antibody-dependent mechanisms as the infection becomes chronic (95, 134, 187, 192), but it is not clear if this progression occurs in human infections. Exactly how the parasite is affected by the immune response, either within the red cell or as the merozoite passes from one red cell to another, again is not certain.

Finally, protective immunity to the sexual stages is thought to be induced by infection; again, there is evidence from different host-parasite combinations that both antibody-dependent and antibody-independent mechanisms can play a role (76). These anti-sexual-stage protective factors come into effect in the blood meal in the mosquito midgut and may prevent fertilization from occurring.

The foregoing summary indicates the complexity of the immune response to malaria parasites. There are a number of other features of malaria parasites which make vaccine development particularly difficult. Unlike, for example, most viral vaccines, malaria vaccines almost certainly must be subunit vaccines because, although it is possible to grow all stages of the malaria life cycle in vitro, it is not feasible presently to envisage growing any stage of the life cycle on a mass scale to provide material for a whole-parasite vaccine. Protozoa are relatively complex, and in malaria parasites, it is estimated that there are 5,000 to 7,000 proteins. Immunity to each stage of the life cycle is largely specific, probably because each stage produces its own novel antigens. Therefore, selecting targets for vaccine-induced immunity and the corresponding peptides with which to induce that immunity has been extremely difficult. Many of the immunodominant antigens in natural infections have not been shown to be targets of protective immunity (79). Many antigens recognized by the immune response show allelic polymorphisms (136). Antigenic diversity is compounded by the fact that at least one antigen, PfEMP1, and its possible homologue or analogue in a small number of other species such as *P. knowlesi* and *Plasmodium chabaudi* undergo clonal variation during the course of infection and contribute to the parasite's immune evasion mechanisms (135, 156, 172). Thus, where the antigen included in the subunit vaccine shows allelic polymorphisms or clonal variation, the conserved regions will be selected as being parts of the molecule concerned with a vital biological function and where variation would be unlikely. It is assumed that the optimally effective vaccine will induce appropriate humoral and/or cellular responses against several key parasite antigens expressed during various stages of the life cycle. There are two important additional complicating factors which hinder progress. The first is that animal models are not ideal for evaluating vaccine candidates to be used in humans, albeit valuable information has come from animal models. Second, there is no suitable in vitro assay for measuring levels of protective immunity in vivo (113), for example, being able to relate levels of antibody to a specific antigen to the level of protection in vivo. Therefore, the only way of determining, at present, the efficacy of a vaccine candidate is to set up human trials, involving natural or experimental challenge, which are very expensive and take a long time to complete and evaluate.

It is worth repeating that severe malaria, predominantly manifest as anemia and cerebral malaria, can have an immunological component, something which a vaccine should not induce (114). The pattern of disease in African children, usually in children under 5 years of age, is linked to the levels of transmission, as well as genetic factors and probably virulence of the infecting populations of *P. falciparum* (106, 175). In areas where transmission rates are low (<15 bites/individual per year), severe anemia occurs in children around 2 years old and cerebral malaria occurs in children 3 to 4 years old. This suggests that there might be a developmental process of susceptibility to cerebral malaria, linked to low transmission rates and a corresponding slow buildup of immunity. In high-transmission areas (>100 bites/individual per year), severe anemia occurs earlier and cerebral malaria is very infrequent (106, 112), possibly because the children become immune before the age at which cerebral malaria occurs. Therefore, assumptions that a vaccine will limit the incidence of severe malaria by limiting parasitemia may be incorrect—some complications might be worsened by vaccination. Malaria vaccines, like other malaria control measures, have the potential for upsetting a balance between the malaria parasite and its host for the worse.

Once the target antigens have been identified, with the coding genes being cloned and sequenced, safe and potent delivery systems will be required. Adjuvants for use in humans are an issue. In experimental animals, very powerful antimalarial immunity has been induced, for example, with crude or purified blood-stage antigens with complete Freund's adjuvant (23, 115). Complete Freund's adjuvant is not acceptable for human use: the only licensed adjuvant for use in disease control is alum, and to date this, in the context of candidate malarial vaccines, has not induced the level of response thought to be required for consistent protection. A number of novel adjuvants and delivery systems are under investigation (54). There has been considerable promising progress in nucleic acid vaccines using plasmid DNA encoding various malaria genes (54).

Preerythrocytic Vaccines

The first effective preerythrocytic vaccines used radiation-attenuated sporozoites. Introduced by allowing the infected and irradiated mosquitoes to feed on volunteers, irradiated sporozoites of *P. vivax* and *P. falciparum* induced complete protection (36). The protection was species specific but appeared to be independent of the parasite strain (36). This vaccine would be ideal for travelers to malarious areas because it completely prevents disease. It is completely impractical, however, because vaccinating each individual requires thousands of infected mosquitoes. From human vaccination studies and similar animal studies (125), a protein on the surface of the sporozoite was identified as a target for vaccine-induced immunity. The coding gene in *P. falciparum* was cloned and sequenced in 1984 (46). The protein, known as the CSP, is the dominant protein on the sporozoite and contains, in the center portion of the molecule in *P. falciparum*, the 4-amino-acid sequence NANP (Asn-Ala-Asn-Pro) repeated more than 40 times. The repetitive region is conserved within species but varies among species. Synthetic and recombinant vaccines based on the B-cell epitope NANP have been tested in phase I and II trials with limited success (54). In the early trials, however, with alum as adjuvant, two individuals with the highest antibody responses were completely protected, indicating CSP to be a viable vaccine candidate. Increasing the complexity of the immunogen combined with novel adjuvants protected six of seven volunteers against sporozoite challenge (179). The antigen, RTS, S, is a hybrid containing the central NANP repeats and most of the C terminus of CSP fused to the hepatitis B virus surface (S) antigen in a complex adjuvant mixture based on monophosphoryl lipid A and QS21 (SBAS4). Field trials with this combination in the Gambia have shown that the formulation gives good short-term protection (G. A. T. Targett, personal communication). A vaccine targeting the immune response to the sporozoite ligand which binds to a receptor on the hepatocytes is being pursued. CSPs across different *Plasmodium* species have two stretches of amino acids which are highly conserved, named region I and region II (168, 169), and hence these might be predicted to be possible parasite ligands for binding to hepatocytes. Some evidence suggests that this is the case and that they might be vaccine targets (168). A synthetic peptide representing region I bound specifically to hepatoma cells in vitro, and antibodies to the region inhibited invasion of hepatoma cells by sporozoites (167, 169). Thus, a sequence in the CSP has been identified as a binding ligand and is being examined along with a second sporozoite surface protein (SSP2) which has sequences homologous to region II in CSP. Region II contains a well-known cell-adhesive motif, and peptides representing region II inhibited both CSP binding to hepatoma cells and sporozoite invasion.

The protective response following immunization with irradiated sporozoites, as noted above, included antibodies and protective $CD4^+$ and $CD8^+$ T cells exerting their effect as cytotoxic cells and releasing cytokines. Low levels of circulating malaria-specific $CD8^+$ T cells in humans suggest that boosting of these $CD8^+$ T cells with a vaccine might enhance natural immunity (138). Efforts continue to identify the peptides recognized on the infected hepatocytes by the cytotoxic cells as potential vaccine candidates. Results to date indicate that protective T cells may recognize epitopes from the CSP, SSP2, and other liver-stage-specific antigens such as liver-stage antigen 1 (51). Murine models, such as *P. yoelii*, have indicated the potential of liver-stage antigens in a multicomponent vaccine, and the goal will be to develop a vaccine containing sporozoite surface proteins and the proteins expressed early in the development of the liver stage. Naked DNA is being developed as a delivery strategy for this stage vaccine because it might be a particularly useful means of inducing $CD8^+$ T cells (71, 72, 195). For example, immunization of mice with a plasmid carrying DNA coding for the CSP of *P. yoelii* induced extremely high levels of anti-*P. yoelii* CSP antibodies and cytotoxic T lymphocytes (161). This immunity protected up to 83% of mice challenged with *P. yoelii* sporozoites and was abolished by removal of $CD8^+$ T cells, by treatment with antibody to gamma interferon, and by prevention of nitric oxide production. Hill and colleagues have now shown that priming a mouse with DNA coding for a preerythrocytic-stage antigen and boosting with a recombinant poxvirus expressing the same malaria antigen (so-called prime-booster strategy) resulted in much higher levels of cytotoxic T lymphocytes (158). This approach is currently being tested in humans.

Blood-Stage Vaccines

It has long been known that crude blood-stage vaccines can induce strong resistance in animal models; for example, in the monkey malaria parasite *P. knowlesi* in an abnormal and very susceptible host, the rhesus monkey, a crude merozoite vaccine in complete Freund's adjuvant gave very good protection against both homologous and heterologous strain challenges (117). The challenge now, as outlined above, is to identify within these crude complex mixtures those antigens essential for induction of protection. A number of candidate antigens are under evaluation, a proportion of which have been found using monoclonal antibodies (80). Monoclonal antibodies raised to rodent malaria and human malaria parasites, especially *P. falciparum*, were tested for protective activity. In the case of the rodent malaria agents, this was in protection tests of passive transfer of the antibody into naive mice, and in the case of *P. falciparum*, those showing growth-inhibitory activity in vitro were deemed to be protective, but as noted above, antibodies active in vitro may not be so in vivo. The molecules recognized by these protective antibodies were determined, and in a number of cases the coding genes were cloned and sequenced as described below.

One of the front-runners for a blood-stage vaccine is merozoite surface protein 1 (MSP-1), and this was initially described with a protective monoclonal antibody to *P. yoelii* (80). The affinity-purified protein induced some protection in mice. Homologous proteins were identified in other species with molecular masses of between 190 and 230 kDa. The candidacy of this molecule in *P. falciparum* was established when the native protein was shown to protect squirrel monkeys completely (165). MSP-1, the major merozoite surface protein, is synthesized by the intracellular schizont as a 185- to 205-kDa precursor protein. On the released merozoite surface, it is present in the form of a complex of polypeptides following the proteolytic processing of the precursor (16, 40, 49); only the carboxy-terminal 19 kDa ($MSP\text{-}1_{19}$) remains on the surface and is

carried into the newly infected red blood cell (16). This fragment is a conserved region of MSP-1, has two cysteine-rich epidermal growth factor-like domains, and is thought to play a crucial role in the process of invading the red blood cell. A monoclonal antibody which inhibits the growth of *P. falciparum* in vitro targets the first of the epidermal growth factor domains (31). Immunization experiments with mice and *Aotus* monkeys with recombinant proteins from the MSP-1_{19} of *P. yoelii* and *P. falciparum*, respectively, were successful, and the native form was also successful in *Aotus* monkeys (92). Investigations with mice indicate that immunization is successful through the induction of protective antibody (84).

Other leading vaccine candidates (136) for *P. falciparum* include apical membrane antigen 1 (AMA-1), one of the proteins found in the rhoptry organelles which are involved in invasion of the red blood cell; erythrocyte-binding antigen 175 (EBA-175); and serine-rich antigen (SERA). The first evidence that AMA-1 was a vaccine candidate came from studies with the monkey parasite *P. knowlesi* (48). A monoclonal antibody which inhibited invasion of *P. knowlesi* in vitro reacted with a 66-kDa protein located initially in the merozoite rhoptries but which was partially transferred to the merozoite surface at about the time of schizont release. Later, AMA-1 was found in other species including *P. falciparum*. The *P. falciparum* protein has been expressed as a full-length protein and has been used in seroepidemiological studies and immunization experiments (39). There are a number of factors which determine whether a merozoite can invade a red blood cell, and these include the presence of particular molecules of the erythrocyte and the age of the erythrocyte (116). Different species or even clones of species can have different erythrocyte specificities. Binding of parasite ligands to specific receptors on the surface of the erythrocyte is an integral part of the invasion process, and antibodies to these parasite ligands might have neutralizing activity. A concern is that they may be exposed on the merozoite surface only for a very short time. Of those identified to date (155), the best-characterized receptors are glycophorin A for *P. falciparum* and the Duffy blood group antigen for *P. vivax* and *P. knowlesi*. Sialic acid residues are the binding specificity on glycophorin A (166). EBA-175 is a microneme protein (micronemes are part of the organelles making up the apical complex at the anterior end of the merozoite thought to be the invasion apparatus), and binds to sialic acid, and presumably it is released when the merozoite and the erythrocyte come into contact. The gene for EBA-175 has been cloned and sequenced (166), and the erythrocyte-binding domain has been located (47). Antibodies to EBA-175 can inhibit growth of *P. falciparum* in vitro, and hence it is considered an important vaccine candidate. SERA is a soluble protein synthesized by late-erythrocytic-stage parasites and secreted into the parasitophorous vacuole and may be a protease. Evidence that SERA is a vaccine candidate comes from direct immunization experiments with monkeys (129).

The only malaria vaccine to undergo extensive field trials in Latin America, Africa, and Southeast Asia is SPf66 (128), a synthetic peptide cocktail developed in Colombia by Patarroyo and colleagues. Three peptides, which induced some protection in *Aotus* monkeys against a blood-stage challenge, were synthesized as a single 66-amino-acid peptide, polymerized, and bound to aluminum hydroxide (127). In these field trials, the vaccine, under conditions of high as well as low malaria transmission, gave partial protection in some studies (191) and none in others (45). An analysis of all published trials to date in various age groups and epidemiologies concluded that SPf66 had a combined estimate of efficacy of 23% (45, 148).

A major obstacle to developing a malaria vaccine, as noted above, is that of antigenic diversity and immune evasion. In *P. falciparum*, one antigen in which clonal antigenic variation occurs in PfEMP1, and a major advance was the cloning and sequencing of the corresponding gene (9, 170, 182). In fact, there is a family of 50 or more *var* genes encoding PfEMP1 which occur on multiple parasite chromosomes. The importance of this molecule in *P. falciparum* is that it appears to be the major molecule on the surface of the infected erythrocyte, mediating the cytoadherence of infected erythrocytes to the endothelial cells lining the postcapillary venules. Binding leads to sequestration, a process which contributes to pathology. The molecule is exposed on the red cell for about 30 of the 48 h of the asexual cycle. As antibody develops to one antigenic type and parasites are destroyed, new antigenic types are constantly reappearing to replace them. Many field studies have attested to the extreme variability in parasite-induced surface antigens on the infected erythrocyte (93); however, the biological function of PfEMP1 might require a domain within this otherwise very variable molecule which is conserved (184). The serological response to different variants in the field indicates that the conserved adhesive domain (10, 123) for some reason is either weakly immunogenic, cryptic, or unrecognized by the host's immune response. Nonetheless, it may be possible to develop an immunogen of this domain that induces antibody, with an appropriate adjuvant perhaps, to block endothelial attachment.

Transmission-Blocking Vaccines

Currently, there are three target antigens of transmission-blocking immunity (86): (i) prefertilization antigens expressed either completely or predominantly in gametocytes, (ii) postfertilization antigens expressed solely or predominantly on zygotes or ookinetes, and (iii) late-midgut-stage antigens such as parasite-produced chitinase required for the ookinete to penetrate through the peritrophic membrane.

More than half a dozen prefertilization target antigens have been discovered to date. Monoclonal antibodies to a number of them, for example, Pf230, Pf48/45, and Pf11.1, have transmission-blocking activity in vitro, but results of studies to demonstrate whether similar antibodies occur naturally in the field have given mixed results. For example, Graves et al. (65) reported that the presence of anti-Pfs230 antibodies but not anti-Pfs48/45 antibodies in the sera of humans in areas of endemicity in Papua New Guinea was strongly associated with transmission-blocking activity. This and other studies suggest that antibodies to Pfs230 may be complement dependent, and therefore inclusion of such an antigen in a vaccine may require an appropriate adjuvant to induce antibodies of the right isotype. A caveat is that in *P. vivax* transmission-blocking antibodies, produced as a result of natural infection, may enhance transmission when present in low dilution (137, 171).

Postfertilization antigens are antigens not expressed by the parasite when it is in the vertebrate host. The major disadvan-

tage associated with these antigens is that antibody responses would not be boosted after natural infection. On the other hand, because these antigens have not been exposed to the immune response over thousands of years, they will not have been exposed to selection of immune evasion mechanisms. Two postfertilization antigens are being explored for their vaccine potential: P25 (8) (Pfs25 in *P. falciparum* and its analogues in other species) and P28 (52) (Pfs28 in *P. falciparum* and its analogues in other species). Pfs25 was the first malaria sexual-stage antigen gene to be cloned, and hence there is more known about this molecule than about any other sexual-stage constituent (see references 85 and 86). Although detectable in maturing gametocytes, Pfs25 is mainly expressed after release of the gametes and fertilization and is the major surface protein on the zygote and ookinete, where it is continually expressed. In a number of parasite isolates from different malarious areas, Pfs25 showed little antigenic diversity. Recombinant Pfs25 has been prepared in mammalian expression systems and in yeast cells and has the capacity to induce transmission-blocking antibody in mice and monkeys (8, 87). Pfs25 has recently gone into phase I trials (86). Work on P28 is less advanced, but antibodies to Pfs28 and its homologue in the avian malaria parasite *P. gallinaceum* have transmission-blocking activity (52).

In the mosquito vector, the developing parasite spends most of its time associated with the midgut, beginning to develop almost immediately after ingestion, and 1 day later the ookinete reaches the midgut epithelium prior to attaching itself to the hemocoel side of the midgut. The midgut provides the nutrients and signals to support the parasite's development but also exposes the parasite to a number of biochemical and physical barriers (164). Following ingestion of the blood meal into the midgut of the mosquito, the peritrophic membrane-matrix, a chitinous layer, is formed around it, thereby presenting the parasite with a barrier in its passage from the blood meal through the epithelial cells of the midgut wall to the outside of the midgut where the oocyst develops. There is good evidence that the ookinete releases a chitinase from its apical end (possibly activated by host midgut proteases) which allows the ookinete to digest its way through the peritrophic membrane (52). Subsequently, it was found that a chitinase inhibitor blocked both parasite infectivity in the mosquito and transmission (163). The ookinete interacts with the midgut in two distinct steps, recognition of the midgut epithelium followed by adhesion to the midgut surface. Receptors for both events are being sought as potential targets for blocking malarial transmission. Further investigations of this site in the mosquito as a source of antigen(s) for anti-mosquito antigen vaccines, which might block a wide range of vector-borne diseases, are ongoing.

Multistage, Multivalent Vaccines

There is a good case that malaria vaccines should be multistage and multivalent (54). We have already noted that there are antigens specific for each life cycle stage. A vaccine, therefore, directed against one stage, if not fully effective, has no activity against later stages in the life cycle. Hence, a vaccine should be more effective if it contains antigens against more than one stage. The immune response has a genetic element in its control (75). Thus, within a population there will be individuals unable to generate $CD8^+$ or $CD4^+$ T-cell or B-cell responses (in the latter case through lack of T-cell help) to specific epitopes on different parasite populations of different *Plasmodium* spp.: a vaccine with multiple epitopes should reduce or eliminate this difficulty. Further, the potential problems from antigenic diversity and antigenic variation by malaria parasites have been covered above, and this might be alleviated by including a selection of the more frequently occurring allelic variants of the protective polymorphic antigens. NYVAC-Pf7 is a genetically engineered, attenuated vaccinia virus, multistage, multicomponent *P. falciparum* vaccine that includes the transmission-blocking Pfs25; the pre-erythrocytic antigens CSP, SSP2/TRAP, and liver-stage antigen 1; and the asexual blood-stage antigens MSP-1, AMA-1, and SERA. This recombinant vaccine has been through phase I-IIa safety and efficacy studies, but the results in terms of protection were disappointing (178).

MALARIA GENOME PROJECT

It is clear that new targets for drugs and vaccines are essential and that a better understanding of the biology of the parasite is prerequisite, but this is hampered by the complexity of the parasite's life cycle. The sequencing of the *Plasmodium* genome should contribute to our knowledge of the parasite, and a combined effort of several groups, the Malaria Genome Sequencing Consortium (28, 55, 77), is currently sequencing the genome of *P. falciparum*. (Some 16 microbial genomes have been sequenced in the last 3 years: a bacterial genome is approximately the same size as a single *Plasmodium* chromosome.) The genome of *P. falciparum* is approximately 30 Mb, contains 14 chromosomes ranging in size from 0.65 to 3.4 Mb, and has a base composition of 82% A+T. The biased nucleotide composition presents technical difficulties for the sequencing project. Chromosomes from different field isolates show extensive size polymorphisms. In *P. falciparum*, there are also two organellar genomes. The mitochondrial genome is a 5.9-kb tandemly repeated DNA molecule, and there is also a 35-kb circular DNA molecule carrying genes that are normally associated with plastid genomes (206). Chromosome 2, containing about 209 protein-encoding genes, was sequenced in 1998 (61), and chromosome 3, which carries 215 genes, was sequenced in 1999 (19). These two chromosomes together make up approximately 7% of the genome, and it is estimated that the total genome codes for approximately 6,500 genes. Some interesting patterns have emerged from these two chromosomes. Genes encoding proteins produced at similar stages in the parasite's life cycle or proteins with similar functions appear to be grouped in particular chromosomes or chromosomal locations. For example, there are several genes on chromosome 2 encoding membrane-associated proteins and serine repeat antigens and also genes encoding proteins found on the surface of the merozoite. The genes for variable antigens on the surface of the infected erythrocyte, the *var* and *rif* genes, are found close to the telomeres of both chromosome 2 and chromosome 3, each telomere being flanked by one *var* gene and several members of the *rif* family. It is estimated that there are 200 *rif* genes per genome, which makes it the largest gene family in *P. falciparum*. As *var* and *rif* are located close to one another, it is

possible that they are coexpressed and coregulated. Bowman and colleagues (19), in their analysis of chromosome 3, have discovered additional gene families such as the *clag* (cytoadherence-linked asexual gene) and CTP (conserved telomeric protein) families, located in the subtelomeric regions of chromosome 3. The role of the *clag* genes is unknown, but the *clag* gene products might be expressed on the surface of the infected erythrocyte and have a biologically important function.

The Malaria Genome Sequencing Consortium aims to generate almost all of the *P. falciparum* genome sequence in a rough form in the year 2000, although a fully annotated representation of the genome may take another 2 to 3 years. Almost all of the parasite's genes will, therefore, become available for other malaria researchers, and from the genes vaccine and drug targets may be identified. It will be no easy task to screen the thousands of new proteins and then identify those with potential as vaccine candidates. As Hoffman and colleagues (78) note, the current gene-by-gene and protein-by-protein approach will hardly scratch the surface. They propose a scheme for DNA "vaccinomics" which will allow screening of all open reading frames for their potential as coding for proteins relevant to the protective immune response.

The postgenomics agenda for functional analysis of *P. falciparum* has been reviewed previously (43). What are the functions of the thousands of parasite proteins? The recently developed transfection system for *P. falciparum* which allows specific knockout of a gene provides a powerful tool for understanding the biological function of proteins and also their contribution in some cases to the pathophysiology of infection.

CONCLUDING REMARKS

At the start of the new millennium, malaria still poses the greatest threat of all parasites to human health, and one would guess that this represents no change from 100 years ago. There are substantial parts of the world, however, which were malarious 100 years ago but are now malaria free. Thus, there has been substantial progress, although how much of this is due to application of control programs and how much is due to improvements in living conditions in general may be debatable. There must have been optimism in the early years of the 20th century that a knowledge of the parasite's life cycle, methods available for controlling the vector, and an effective antimalarial in the form of quinine might lead to the eradication of the disease, an aim which was boosted in the 1940s by the introduction of DDT and chloroquine. The fact that this has not been achieved should only reinforce the resolve that early in the 21st century this aim should be fulfilled. The recent rapid and spectacular developments in molecular biology and allied biological sciences raise hopes that new antimalarials and antimalarial vaccines will be developed, but the underlying problem remains one of financing, initially the necessary research and development work and secondly the cost of providing vaccines and antimalarials to communities that currently would not have the means to pay for them.

The driving force for malaria control early in the last century was the pursuit of economic and commercial gain, be it the building of the Panama Canal, for example, or the development of agricultural schemes for fruit, rubber, and other crops (99). The discovery of new antimalarials was in part driven by wars. The former incentive applies only on a local basis, and I hope that the latter will no longer be a factor. Hence is seen the importance of the recent initiative of the Roll Back Malaria Programme in which the World Health Organization will lead a partnership of a number of agencies and interested bodies in order to coordinate and focus activities and agree on priorities in the pursuit of the control of malaria and to alleviate the suffering that the disease causes. The international commitment to alleviating the suffering from malaria will be a window on the commitment that the richer nations have to dealing with poverty, the use and abuse of the world's natural resources, and damage to the environment.

This review has necessarily been selective and has not touched in any detail on many exciting areas of malaria research. Thus, the fact that life-threatening malaria can develop so rapidly has prompted detailed investigations into the pathogenic processes (grossly seen as neurological disturbances and respiratory distress), and from this knowledge, it may be possible to plan more effective intervention strategies (35, 104, 108). Research into how mosquitoes find their host and the recognition that this can be a nonrandom choice of host by the mosquito influenced by factors such as the bacterial skin flora of the host have extended the epidemiology of malaria into the microepidemiology of the disease (20). Exciting times in malaria research are ahead, but history has taught us that this excitement has to be tempered with realism and that progression from the laboratory bench to a benefit in the field can be a slow and tortuous path.

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