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Malaria vaccine

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Summary. Among infectious diseases caused by protozoa, malaria is still the greatest killer of children. Mortality in adults living in endemic areas is significantly lower because they frequently acquire partial or complete immunity to the major pathogen, *Plasmodium falciparum*. This natural protection indicates that vaccination may be possible, and the first candidate antigens were cloned with the use of human immune sera as probes. Genetic and biochemical analysis of the parasite proteins revealed that they are polymorphic, and frequently gene sequences were discovered which were specific for a particular parasite isolate, which eliminated most antigens for purposes of vaccine development. The most promising candidate antigens today are the major surface proteins of sporozoites and blood stage parasites. However, the immune response against those is not sufficient for complete protection, and additional, intensive research is necessary to identify new molecules to be included in a vaccine cocktail against malaria. The current spread of the disease due to increasing drug resistance of parasites and mosquito vectors emphasizes the urgent need for a vaccine.
Key words. Malaria vaccine; recombinant DNA; surface proteins; antigen polymorphism.

Malaria as a major health problem

Among the infectious diseases caused by protozoa, malaria is still the greatest killer of children, and it is one of the most incapacitating diseases of adults living in tropical and subtropical countries. The number of cases reported to the WHO in 1984 was over 100 million worldwide, and this number is still increasing. Ten Italian tourists who returned from Africa died from malaria in June 1989, which alarmed the health authorities in Europe. Symptoms such as relapsing fever or hepatomegaly appear shortly after infection with *Plasmodia*. The parasites are naturally transmitted through the bite of an infected *Anopheles* mosquito (fig. 1) but also, like other infectious agents, malaria parasites may be spread by blood transfusions or through the exchange of needles among drug addicts. Malaria tropica caused by *Plasmodium falciparum* is the most severe form of the disease, because it may lead to intravascular hemolysis and plugging of cerebral arteries. Infection by *P. vivax, P. ovale* or *P. malariae* is less deleterious and relapsing fever is frequently the sole symptom. After a fever episode, most of the patients recover and clear the parasite from their blood stream. Various eradication strategies have had only limited success, and today, the spread of the disease is increasing.

Attempts to control insect vectors

Physical protection from insect bites through nets or repellants, together with the use of effective insecticides, have significantly lowered the transmission rate of malaria. Drainage of natural or man-made mosquito breeding-places, together with a modern hygiene system and public information programs, essentially eliminated malaria from the Northern hemisphere in the first half of this century. The presence of huge natural mosquito-breeding reservoirs such as rice fields cannot be eliminated in tropical or subtropical countries for practical and economic reasons. Insecticides and drugs have failed to control the spread of the disease, owing to the development of either drug-resistant *P. falciparum* strains or insecticide-resistant *Anopheles* vectors. For example, in Brazil a DDT (di-chloro-diphenyl-trichloro-ethane) insecticide spraying program against malaria, carried out in 1969, markedly reduced the parasite transmission rate, and the average parasitemia in the population dropped in the

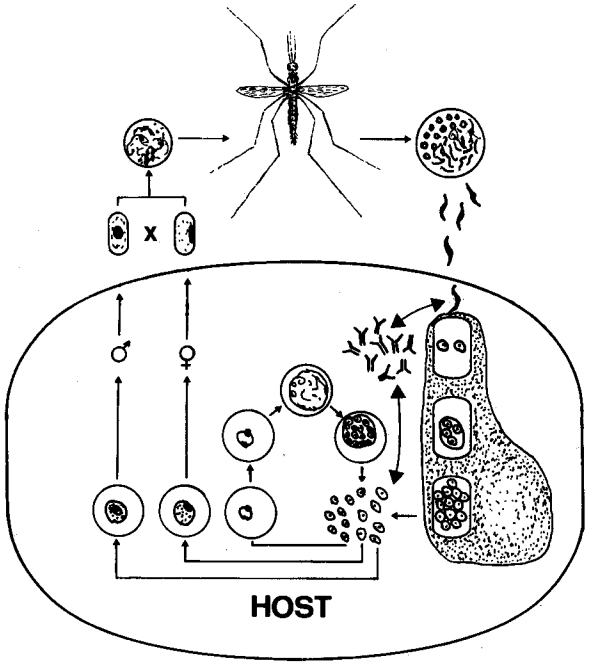

Figure 1. Life cycle of *Plasmodia*. Through the bite of an infected mosquito, sporozoites enter the blood stream, invade hepatocytes and multiply intracellularly. This process ends with the development of a liver schizont, which releases after maturation up to 10,000 merozoites in the blood stream. These invade erythrocytes and can undergo differential development into male and female gametocytes or continue to develop asexually into ring, trophozoite and schizont stages. After this first cycle of replication, parasite multiplication is logarithmic and parasitemias of 4%–6% (2×10^{10} parasites/animal) are reached in experimental animals one week after infection. Circulating gametocytes are ingested by the mosquito, rid themselves of the blood cell membrane in the gut, mate and develop further into oocysts, where development of infectious sporozoites closes the cycle. Only 5 min after infection the sporozoites have invaded hepatocytes, and invasion of erythrocytes by merozoites is even faster. The time available for antibody attack is therefore very short.

following years. But only six years later, DDT-resistant *Anopheles* mosquitoes appeared and as a result both transmission and infection increased, ending with the revival of malaria. Extensive insecticide spraying can also cause indirect side effects. For example, the number of bird species living around Rio de Janeiro has been markedly reduced.

Drugs against malaria

Since the 15th century extracts from the bark of the Cinchona tree have been used to treat patients with malaria symptoms, and in 1930 quinine was discovered as the active ingredient. Since then, a large number of quinine-based drugs, and also sulfonamides and analogues of folic acid, have been used for malaria chemotherapy. In the last decade, extensive use of chloroquine for prophylaxis and therapy has probably selected for multi-drug resistant parasites in essentially all parts of the world where malaria is endemic. The molecular basis appears to be the amplification of the multi-drug-resistance (MDR) locus in *Plasmodium falciparum*, which probably results in overexpression of the gene product inactivating the drug[13]. The increasing spread of drug-resistant parasites explains the urgent need for either novel anti-malarial drugs or a vaccine before time runs out.

Molecular design of malaria vaccine

Parasite biology and the life cycle of *Plasmodium falciparum* (fig. 1) indicate that antibody attack must be limited to the two extraerythrocytic, developmental stages of the parasite. A safe vaccine must contain epitopes from the surface of both sporozoites and merozoites, because the immune escape of a single sporozoite could initiate the infectious cycle. Vaccination of susceptible monkeys and human volunteers with irradiated sporozoites or merozoites induced partial immunity against malaria, and this result initiated intensive worldwide research on vaccines[8,31]. A number of virtually pure merozoite antigens isolated from the parasite induced a marked degree of protection in monkeys[6,21,22,25]. Most of the genes encoding these protective polypeptides have now been cloned, and parts are expressed in bacteria or other microorganisms. This allows the production of large amounts of recombinant antigen, which is necessary because a worldwide vaccination program would require roughly 800 kg of protein. The analysis of the cloned genes and their products at the molecular level, however, revealed properties that make them problematic for vaccine development. Most of the antigens analyzed so far are polymorphic, and full protection may thus be limited to a small subset of parasite variants. It is therefore essential to identify invariable epitopes on the merozoite and sporozoite coats in order to obtain full protection and prevent immune escape[14]. In addition, a vaccine should be strongly immunogenic, and the identification of B- and T-cell epitopes is as important as gene cloning[26,27]. A T-cell epitope present in the major surface protein of sporozoites is recognized by all humans independently of the genetic haplotype, and genetic engineering allowing the addition of this sequence to vaccines would eliminate the problem of MHC-restricted antigen recognition[27].

Malaria sporozoite vaccine development

After a host is bitten by an infected mosquito, *Plasmodium falciparum* sporozoites invade hepatocytes exclusively. In spite of the speed with which infection occurs (sporozoites can be found inside hepatic cells within minutes after infection), protective immunity has been achieved with inactivated sporozoites[8]. Serum of protected individuals neutralizes the infectivity of sporozoites which suggests that the protection is at least in part antibody-mediated[31]. The absence of a strict correlation between antibody titers and protection against a challenge indicates that T-cells and/or lymphokines contribute to the immunity.

A monoclonal antibody (3D11) raised against sporozoites had similar immunological properties to total serum from immunized individuals, which suggested that both sera recognize a single immunodominant epitope [30]. This epitope is localized on the CS (circum sporozoite) protein, which is the major surface protein of sporozoites. It has unusual biochemical and immunological properties [11]. Depending on the *Plasmodium* species, one-third to one-half of the molecule consists of identical, tandem oligopeptide repeats which form the immunogenic B-cell epitope recognized by serum from infected humans. The amino acid sequences of the repeats differ markedly between different species of *Plasmodia*. The length of the repeat unit and its amino acid composition appear to be conserved within a species. The tetrapeptide repeat unit in *Plasmodium falciparum*, for example, has the sequence Asn-Ala-Asn-Pro (NANP), and in *Plasmodium vivax* the sequence is Pro-Ala-Gly-Asp-Arg-Ala-Asp/Ala-Gly-Ser (PAGDRAD/AGS). The number of amino acids forming CS protein repeats is limited to a subset of 10 amino acids. This could explain why antibodies to *P. falciparum* CS repeats cross-react with the CS protein of the rodent malaria parasite *P. berghei* [1]. There is hope that a protective immune response against the CS protein of *P. falciparum* may protect from infection with other malaria parasites.

Three consecutive repeat units $(NANP)_3$ of the *Plasmodium falciparum* CS protein constitute the immunodominant epitope recognized by semi-immune individuals living in endemic areas. The sequence is present in all isolates of *P. falciparum*. This potential vaccine candidate was therefore chemically synthesized for protection trials in human volunteers [2]. All individuals sero-converted and the appearance of merozoites in the blood was delayed in this group compared to non-immunized controls. This study indicated that this epitope alone cannot confer sterile immunity, but it is possible that addition of other CS protein epitopes could improve the performance of the vaccine. The CS protein of *P. vivax*, for example, was successfully expressed in yeast, and antibodies to the recombinant polypeptide inhibited parasite invasion in vitro [3].

Natural mutations in the NANP repeat unit of *Plasmodium falciparum* CS protein have not yet been discovered, but they could occur as a result of immuno-selection after vaccination unless the maintenance of the sequence is necessary for functional reasons. The CS protein is the only sporozoite surface protein identified to date, and intensive research should therefore be concentrated on the identification of additional sporozoite surface components which could enhance immunity against sporozoites. A receptor-ligand interaction may exist for hepatocyte recognition, and the molecules involved in such a reaction could be attractive candidates as vaccines. Antibodies could block the recognition site and prevent invasion. The CS protein is probably not involved in recognition. Specific interaction of the CS protein with hepatocytes has not yet been demonstrated, and the fact that it is shed from the surface prior to invasion makes its participation in this process unlikely.

Blood-stage vaccine development

Recent progress in the field of development of vaccines against malaria blood-stages is mainly due to the availability of in vitro culturing methods for *P. falciparum* [29] and of an animal model for initial vaccine testing. The owl monkey *Aotus trivigatus griseimembra* and the squirrel monkey *Saimiri* are susceptible to *P. falciparum* infection, and clinical malaria symptoms similar to those in man develop in non-immune animals. A number of antigens isolated from cultured parasites were tested in this animal model, and a number of successful experiments have been reported [6, 7, 21, 22, 25].

Antigens for vaccination studies are commonly selected with sera from semi-immune humans living in endemic areas, with the assumption that antibodies against these antigens confer or at least contribute to protective immunity. This approach led to the identification of the precursor of one of the major merozoite surface proteins, termed p190, which is the most promising blood-stage vaccine candidate today. It is a glycoprotein with a molecular weight of 190–220 kDa [20] and after extensive processing it gives rise to the major components of the merozoite surface [17, 18]. Immunization of *Aotus* monkeys with essentially pure natural p190 protein induced, for the first time, sterile immunity against an otherwise lethal *P. falciparum* infection. This made cloning of the p190 gene a worldwide research challenge, and the first partial gene sequence appeared in 1984 followed by two complete sequences in 1985 [15, 16, 19].

Earlier observations made with the use of monoclonal antibodies showed that certain epitopes in p190 are specific for particular parasite isolates [20, 23, 24]. Similarly to the CS protein and other malaria antigens, the p190 protein contains immunodominant repeat units [4, 9, 10]. A comparison of p190 gene sequences revealed that the repeat sequences and their number are allele-specific, which eliminated them as potential vaccine components. The remainder of the p190 sequence can be divided into conserved, semi-conserved and variable segments [28]. Polymorphism in this part of the gene is mainly generated by multiple, intragenic mitotic chromosome crossover events of two parental alleles in the sexual, diploid stage of the life cycle (fig. 2). The large number of isolate-specific, non-repetitive sequences can thus be explained at the genetic level. This mechanism demonstrates the almost unlimited potential for the generation of new sequence patterns in p190, assuming that large parts of the molecule are non-essential for parasite function.

Besides these rather polymorphic sequences highly conserved, non-variable amino acid stretches were also detected at the genetic and protein levels [14]. The DNA encoding the constant parts of the p190 was fused

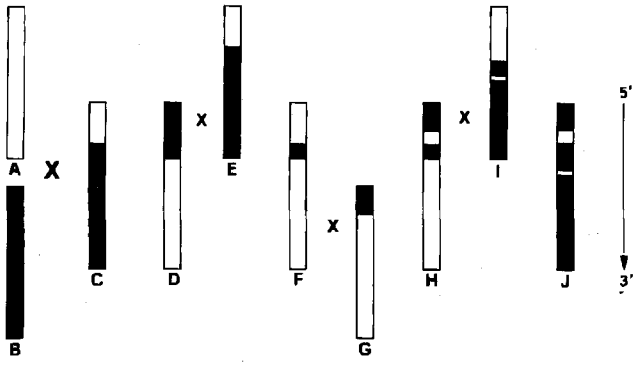

Figure 2. Generation of p190 mutants by intragenic chromosome crossover. A crossover event of two parental p190 alleles (A and B) gives rise, for example, to two new rearranged genes (C and D). Gametocytes carrying D are ingested by a mosquito from one donor and a new, already-rearranged allele (E) is taken up from a second individual by the same mosquito. The result is the allele F, which now carries only a small segment of the parental allele B. Multiple additional crossover events of already-rearranged alleles finally give rise to J, where two sequence stretches of A are inserted into the B background. These insertions can be as short as two amino acids[4]. This demonstrates the almost unlimited potential for creating new sequence combinations. So far, recombination appears to be limited to the amino-terminal third of the p190 gene. The 5'-end of the gene is at the top and the 3' end is at the bottom.

genetically and expressed in bacteria. The purified recombinant protein (190 N) was used to immunize *Aotus* monkeys. Two out of five monkeys controlled the challenge infection without chemotherapy, which suggests that this recombinant protein can induce partial immunity, but additional B- or T-cell epitopes may help to achieve complete protection against malaria (fig. 3).

A similar degree of protection was obtained with recombinant proteins derived from the RESA (ring-infected-erythrocyte-surface-antigen) in *Aotus* monkeys[7]. The best protection, however, was induced by a highly repetitive sequence which is subject to antigenic variation and therefore of limited use. More important, cultured parasites were discovered that had abolished expression of the RESA gene as a result of a chromosomal deletion. This may occur in nature and it is therefore evident that vaccine candidates against malaria cannot be selected on the basis of a protective immune response since their efficiency may only last for a short period of time owing to the genetic flexibility of *P. falciparum*.

Natural proteins isolated from parasite cultures give the best protection observed up to now in the monkey animal model. This could be directly related to the biochemical nature of the antigens, or to other factors such as purity. Post-translational modifications such as glycosylation or acylation of certain epitopes may be important for a protective immune response. Sterile immunity of monkeys against malaria was only obtained by immunization

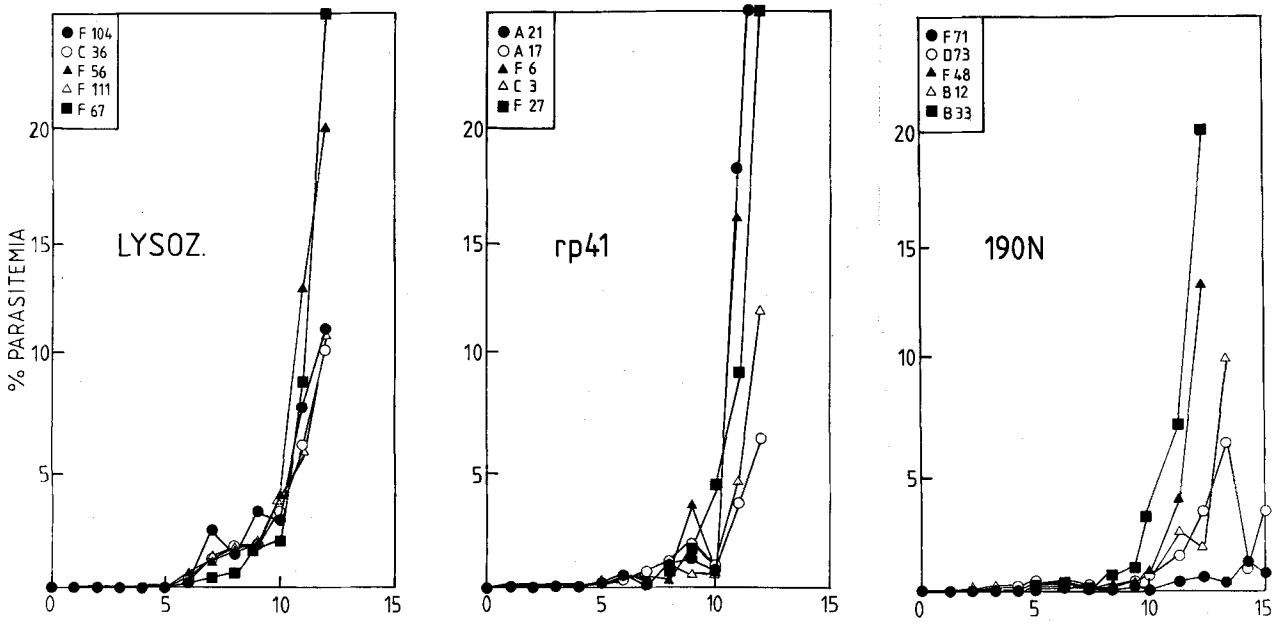

Figure 3. Example of a malaria protection trial in *Aotus* monkeys. Three groups of five monkeys were immunized with lysozyme (control group), recombinant p41 *P. falciparum* aldolase[5], or 190N[14], a recombinant protein in which the constant regions of the p190 surface protein are jointly expressed in *E. coli*. As expected, all monkeys in the control group were infected and developed high parasitemias by day 10. The recombinant aldolase, in contrast to the natural antigen, gave no protection, and the parasitemia after 10 days was almost identical to that in the control group. Two animals in the 190N group recovered from the infection and cleared the parasites. Three animals in that group required chloroquine treatment. All animals had similar levels of antibody against the antigens used. In this experiment the highly virulent FVO isolate of *P. falciparum* was used for challenge. The numbers on the left-hand top corner are the animal codes, and the days after challenge are shown on the X-axis. The Y-axis shows the parasitemia developed after challenge.

with p190 protein prepared from parasite cultures [25]. For parasite challenge and protein purification the same strain of *P. falciparum* was used. The structure of the antigenic epitopes can therefore be expected to be identical, at least at the sequence level. Such a situation does not exist in nature, and reports about positive vaccination trials in man or animals require critical evaluation with respect to immune escape. The unusual nature of *P. falciparum* proteins and genes may contribute largely to the difficulty of expressing full-length vaccine candidate molecules, such as p190, in microorganisms. This is currently the limiting step in vaccine development against malaria blood-stages, rather than the number of cloned antigens available.

Gametocyte vaccines and transmission blocking

P. falciparum goes through sexual multiplication during a part of its life cycle [12]. Male and female gametocytes are ingested by the mosquito vector, rid themselves of the erythrocyte membrane, and develop into mature gametocytes in the gut lumen. Antibodies against gametocyte antigens ingested with the blood meal could therefore block the mating process in the mosquito. A transmission-blocking vaccine could therefore inhibit the process of antigen variation, by eliminating crossover events at the diploid stage. Even if it proved to be successful under experimental conditions, vaccination against gametocytes would be of limited practical use. Firstly, the immune system of humans is never exposed to gametocytes during a malaria episode and a natural infection will not boost the immune response against a vaccine. Secondly, travellers immunized with a transmission-blocking vaccine alone would not be protected against a primary infection with sporozoites.

Immune escape

The extremely high degree of flexibility of repetitive *P. falciparum* sequences, together with the high immunogenicity, are probably part of the parasite's immune escape strategy [1]. A protective immune response against repetitive epitopes would only inactivate one parasite 'type' and could not prevent the multiple infections which occur in nature. In addition, the immunodominance of repetitive epitopes may prevent a response to more vulnerable epitopes of functional proteins. Antibodies against repetitive epitopes cross-react with repeat sequences contained in unrelated antigens. It is proposed that these cross-reactions interfere with the normal maturation of a high-affinity antibody response in malaria, by causing an abnormally high proportion of somatically-mutated B-cells to be preserved during clonal expansion. The high degree of antigen variation in *Plasmodium falciparum*, the immune suppression caused by malaria, and the mainly intracellular development of the parasite, are apparently sufficient to enable the parasite to escape from an ongoing immune attack in the vertebrate host.

Vaccine-independent immunity

Our current understanding of acquired immunity to malaria is still incomplete. Protection apparently requires first the induction of high serum levels of neutralizing antibodies, secondly the boosting of antibody levels by the parasite, and thirdly the stimulation of cytotoxic T-cells capable of killing infected cells. A protective immune response in the course of a primary infection has to occur rapidly in order to maintain the parasitemia below critical levels. An average adult, for example, has about 4×10^{13} red blood cells. At a low parasitemia of 0.01%, 100 million red blood cells are already infected but malaria symptoms may still not occur. However, the next round of parasite replication may already be lethal.

Monkeys protected from malaria after immunization with a single antigen contain a large spectrum of antibodies against the invading parasite after challenge. It is therefore experimentally difficult to distinguish whether the initial immune response induced by the vaccine, or the secondary response against the invading parasite, prevented infection. The fact that natural immunity to malaria requires multiple natural infections is in favor of the latter possibility, thus demonstrating that immunity can be acquired without passive vaccination. For malaria control, especially in neonates, it may be sufficient to discover agents which reduce the growth rate of *P. falciparum* after infection, thus allowing the immune system to respond. This would to a large extent eliminate the geographical and ecological difficulties of passive immunization against malaria and – more importantly – the difficulty of finding protective antigens.

Outlook

The results reviewed above concentrate mainly on two malaria vaccine candidates, although other antigens have been successfully tested in monkeys or even man. A mixture of synthetic peptides made according to sequences of the p190 protein, and another less characterized merozoite antigen, protected monkeys and human volunteers to a certain degree from malaria. The technical limitations to producing large amounts of the vaccine by peptide synthesis strongly reduce its practical value. Other antigens successfully tested belong to a group of highly polymorphic proteins, which makes them less useful in the long run.

Recombinant DNA technology has proved to be essential for malaria vaccine development. First, technical know-how today allows a more-or-less automated production of pure recombinant proteins such as vaccines from bacterial cultures. More important, cloned genes were successfully used to obtain an insight into parasite genetics, which provided clues about the mechanisms by

which antigen diversity is created in *P. falciparum*. More recently, the possibility of separating parasite chromosomes on gels has revealed that not only the genes but also the chromosomes of malaria parasites are variable in size. This reflects the high genetic flexibility of this organism, which is required for successful immune escape. In fact, most of the candidate antigens were cloned with human semi-immune sera as a probe, because these sera inhibit parasite growth in vitro. However, most of the proteins cloned by this approach had variable, repetitive sequence motifs. They can therefore not give universal protection, and an immune response against variable sequences could in fact be part of the parasite's immune escape strategy.

The highly specific host-cell recognition of malaria parasites almost certainly requires some sort of ligand-receptor interaction, and consequently the process is a potential target for immune attack. The molecules involved may be designed not to be immunogenic in man, and as a result they have escaped detection by gene cloning with human antibody probes. In addition, only highly immunogenic proteins can be expected to give a strong immune response in humans, since merozoites and sporozoites are only exposed to the host's immune system for a few minutes. The processes of parasite maturation and replication occur inside cells, and only macrophages or cytotoxic T-cells can be expected to destroy intracellular parasites. In the future it will be necessary to identify additional surface molecules with a defined biological function for the parasite. The use of functional molecules for vaccination would greatly reduce the risk of immune escape by antigen variation; mutations in such proteins would be expected to be lethal. The current situation with regard to malaria vaccines is not encouraging, and we still have a long way to go. A more complete picture of parasite biology and genetics, together with an efficient expression system for malaria antigens, may help in the development of agents which can at least restrict the current spread of malaria.

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Molecular biology and parasites

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Parasites, the central theme of the articles in this series, all have one thing in common, despite their biological differences – the uniformly negative connotation which they carry in our minds, as well as in our everyday language. Despised, feared, cursed, they are eternal threats to human well-being, destroyers of cultures and lethal enemies to development and prosperity in much of today's world. Consequently, all of the endeavours reviewed in this issue are directed towards the speedy elimination of the fiendish organisms from afflicted individuals and populations, as well as from their whole environment. Despite this emphasis of the current series of articles, let us not forget that this essentially negative view of parasites, however well-founded it may be because of the afflictions and the suffering which they bring upon us, is highly anthropocentric. In the wider context of biological evolution, parasites have always played an essential role as selective agents of evolution (Lively[14], Endler and Lyles[6], Pomiankowski[18], Hamilton and Zuk[10]), and therefore they are our partners, in a biological sense, as much as our foes. Also, much as we may deplore the ill-effects of parasitism on man and his domestic animals, parasitism is the way of life of the majority of organisms on our earth (Price[19]). Thus, if we are inclined to take a gloomy view of life we, the hosts, may consider ourselves as endangered minorities which survive on this planet merely to serve as substrates for the parasitic majority.

Be that as it may, the term 'parasite' itself has different connotations even in different biologists' minds. Traditionally, the term is applied to protozoa and multicellular eukaryotes. However, in a biological sense, many bacteria and all viruses have parasitic lifestyles, and the viruses in fact represent the ultimate parasites. This wider definition of parasitism has helped tremendously to overcome traditional barriers between the once widely separated fields of bacteriology, virology and parasitology. Nevertheless, the present collection of articles focuses on parasites in the more traditional sense (protozoa and worms), and even within this narrow framework it is necessarily incomplete. Its emphasis lies, in terms of organisms, on human disease agents, and in terms of technical approaches, on basic biology, diagnosis, epidemiology and vaccine development. Veterinary parasites are dealt with only briefly (Hide and Tait) and plant parasites (Camargo et al.[3]) are not considered at all. These omissions are due to space constraints only and not to an underestimation of the importance of these disease agents. They are at least as great a threat to human well-being as are the parasites that cause human disease, because they deprive man of essential resources such as food, energy, materials and tradeable goods. Another topic not addressed in the current issue is the important technical approach of drug development, but several reviews covering this topic have recently appeared (Fairlamb[7], Lacey[12], Gutteridge[9]).

Parasitology as a science has long been the exclusive domain of organismally oriented biologists, veterinarians and medical doctors. Within the last decade or so, some parts of this field have been rapidly and dramatically transformed (as have many other domains of biology and medicine) by the advent of molecular biology and concomitant developments in neighbouring sciences such as protein chemistry, computer science, immunology and instrumentation. The current collection of articles is intended to illustrate a few facets of these rapid and exciting developments, and to indicate where the usefulness of new technologies lies, not only for the laboratory scientist but also for the field epidemiologist and medical