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Progress in Malaria Research: Vaccines and Drug Resistance

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This brief review describes the work of The Walter and Eliza Hall Institute, and of a strong contingent of Australian collaborators, on the development of a malaria vaccine directed against the blood stages of the parasite. Most attention has been paid to genetically engineered versions of certain molecules on the surface of the merozoites of *Plasmodium falciparum* where a new molecule, merozoite surface antigen-2 (MSA-2) shows particular promise; and on a series of molecules that appear to help in the act of parasite invasion of red cells, where two molecules, RESA (ring erythrocyte surface antigen) and AMA-1 (apical membrane antigen-1) work as vaccine candidates in model situations and require to be tested in a field situation. A second major interest of the Institute is in the field of drug resistance in malaria. The world faces a major problem with respect to parasites becoming resistant to our best and cheapest drugs. Coming to grips with the molecular and genetic mechanisms which underlie resistance to anti-folates or cinchona alkaloid derivatives is a first step towards designing drugs which beat the problem.

Key Words: ***Plasmodium falciparum*; Merozoite; Adjuvent; Antifolate drugs; Chloroquine**

Introduction

My own research career has been a 37-year long love affair with antibody formation and with the related field of immunological tolerance. The temptation was very strong, therefore, to use this lecture to expostulate on all the recent findings related to B cells, germinal centres, somatic V gene hypermutation and related specialised topics that are exciting to my own small group within The Walter and Eliza Hall Institute Nevertheless, I have chosen a different subject for my lecture, one very close to my heart, but one in which I have done no original work whatever! The choice was made for two reasons, my second great love affair, namely that with The Walter and Eliza Hall Institute; and the extraordinary importance of the field I want to cover for India and indeed for many developing countries. More than a decade ago, we at the Hall Institute decided to embark on a large malaria programme, aimed in the long term at the production of a malaria vaccine. This decision followed a breakthrough in the molecular cloning of *Plasmodium falciparum* antigens through the use of an expression cloning system and human anti-malarial antisera as the screening tool (Stahl et al. 1994). When the group responsible for this work, led by R F Anders and D J Kemp, made this discovery in 1983, the biggest single bottleneck towards the production of a recombinant DNA-based vaccine appeared

to be broken, and a major effort seemed warranted. While we certainly did not anticipate all or even most of the problems that would come up, it seems appropriate to summarise where we are a decade later. Arising out of the molecular and genetic analysis of *P. falciparum* which this work entailed, a secondary interest in drug resistance in malaria has also grown up, which shall be briefly summarised.

The Problem of Malaria

In most of the developing countries, there are large numbers of children and a visit to most villages reveals plenty of smiling, apparently happy youngsters. What such a quick look does not reveal is the fact that, in many countries, these children are the survivors of a very cruel immunological lottery. The simple act of palpating the spleen will frequently reveal that this organ is enlarged and that, thus, the child's energy is still being sapped by chronic malaria, to which the child has developed a reasonable degree of immunity. The survivors are the lucky ones-many from the same cohort died before the age of 5 years, frequently an agonising death from cerebral malaria. The World Health Organization estimates that, in sub-Sahara Africa alone, there are over one million such deaths per year, though, of course, no-one knows the exact figure. The total world population living in areas where malaria is a risk is well over 2,200 million and the cases of clinical illness are over 100 million per year. One estimate puts the annual cost of malaria in Africa at around (US)$180 million per annum. For most of the developing countries, the cost of malaria control programmes has become unsustainable, and in many countries the problem is essentially out of control. Two further extremely serious problems are the progressively greater resistance of parasites to our best and cheapest drugs; and the resistance of mosquitoes to the best and cheapest insecticides. Add to this the increasing environmental concerns about some inseciticides and we have a problem of truly monumental dimensions. Clearly, a vaccine would be the logical way out of this dilemma. A successful vaccine should give at least the same degree of protection to inhabitants of endemic areas as the experience of living in the area for a considerable period, but without the price of prior illness and death. Furthermore, widespread deployment of a vaccine could be expected gradually to diminish the parasite load in a population and thus to lower transmission rates, making the classical control measures more effective.

Molecular Targets for a Vaccine Against Malaria

Three sets of molecular targets have been identified for a vaccine against malaria. Picking up the life cycle of the parasite at the stage where the female anophelene mosquito bites the patient, the life-form introduced into the body is termed a sporozoite, and clearly a vaccine which would cause the neutralisation, phagocytosis or destruction of the sporozoite before it could reach its target, namely the hepatocyte, would represent effective protection. A great deal of work has been done on the major circumsporozoite antigen. We have chosen, however, to concentrate on the erythrocytic cycle which is initiated when merozoites emerge from the liver and enter erythrocytes. Our work has been on the asexual stage of multiplication, during which the merozoite develops inside the red cell into a ring stage, then a trophozoite and finally into a schizont, prior to the red cell rupturing and further merozoites being released. Finally, also within the erythrocytic cycle, gametocytes are produced which are taken up by the mosquito during a blood meal and which mature inside the mosquito into gametes. The sexual stages would also represent an interesting target for a vaccine, because if people made antibodies

destructive of gametes, these would be taken up by the mosquito, and either in the host or in the mosquito, the sexual stages would be neutralised and transmission would be reduced or prevented.

The Australian Malaria Joint Venture

Following reasonably prolonged negotiations with the Australian Government, an incorporated joint venture was formed to pursue the blood stage vaccine most actively. This joint venture was incorporated as Saramane Pty Ltd. and the partners were The Walter and Eliza Hall Insititute of Medical Research, the Queensland Institute of Medical Research, the Commonwealth Serum Laboratories and Biotechnology Australia Pty Ltd, the latter two being commercial entities with considerable research and development expertise. The Australian Industry Development Corporation, at that time an arm of Government and now partially privatised, was a fifth partner and provided a substantial proportion of the funds. Since 1990, the Swiss multinational pharmaceutical house, F Hoffmann La Roche, has joined the partnership.

The partners have examined a large number of antigens as potential vaccine candidates, and of these two main types have emerged as showing considerable promise. The first are antigens on the surface of the merozoite, and the second antigens which are somehow involved in merozoite penetration into the red blood cell.

It is logical to consider antigens assoicated with the merozoite surface as vaccine candidates because, even though the time between rupture of the parasitised erythrocyte and entry of the merozoite into the next cell is relatively short, for that brief period merozoite surface antigens should be accessible to antibody. Holder and Freeman (1984) in the United Kingdom have identified and done considerable work on an antigen, MSA-1, which is fragmented from an initially large molecule of molecular weight around 200,000 into at least three smaller pieces which become associated with the merozoite surface through a GPI anchor. Antibodies against MSA-1 inhibit merozoite replication in cultures dependent on human erythrocytes, and animal trials show that *P. falciparum* MSA-1 can act as an effective vaccine in monkeys provided Freund's complete adjuvant is used.

The Australian group has identified a second merozoite surface antigen, MSA-2 (Smythe et al. 1988). An excellent summary concerning this and the other vaccine candidates identified by the Australian group, and giving detailed references to many other candidate antigens, has been presented in a recent review by Anders and Saul (1994). MSA-2 is also a GPI anchored protein, but is not cleaved into fragments and is much smaller in molecular weight (molecular weight predicted from the gene sequence being about 28,000). Two main families of MSA-2 alleles have been identified, although there are more than two types of tandem repeats (so characteristic of malarial antigens). Antibodies to MSA-2 inhibit the erythrocytic cycle *in vitro* and peptides from the invariant portions of *P. falciparum* MSA-2 protected mice against the murine *Plasmodium chabaudi* (Saul et al. 1992).

Two sets of organelles within the merozoite, namely the rhoptries and the micronemes, play an important role in merozoite invasion of the erythrocyte. Both the group of Peter Perlmann et al. (1984) in Sweden and the Australian group (Cowman et al. 1984) have done a great deal of work with an antigen synthesised within the micronemes which Perlmann terms Pf-155 and the Australian group terms RESA (for ring-infected erythrocyte surface antigen). Immuno-electronmicroscopy indeed does define RESA as being closely associated with

the membrane of erythrocytes containing ring-stage parasites, but it turns out that this molecule is not actually available on the outside of the erythrocyte membrane, but rather becomes associated with spectrin on the inner side of the red cell membrane (Foley et al. 1991). While relatively little is known about how RESA is involved in parasite invasion of red cells, it has been clear for some time that anti-RESA antibodies inhibited the process (Wahlin et al. 1984). A further and important characteristic of RESA making it of interest to the would-be vaccine developer is the virtual absence of antigenic diversity.

An interesting more recent candidate is an antigen which moves from the neck of the rhoptries to the surface of the merozoite, and is known as apical membrane antigen-1, or AMA-1 (Peterson et al. 1989). This turns out to be homologous to an antigen of the monkey parasite, *P. knowlesi*, which was actively investigated by Sydney Cohen's group (Deans et al. 1984) and found to be a protective antigen in the monkey model. AMA-1 is unusual in that it does not display tandem repeats like most other malarial antigens. Again anti-AMA-1 antibodies inhibit merozoite invasion *in vitro* and animal vaccine trials appear promising.

A variety of other antigens derived not from the neck but from the body of the rhoptries, including rhoptry antigenic proteins (RAP-1 and RAP-2) are also looking promising (Schofield et al. 1986, Crewther et al. 1990, Saul et al. 1992) but as yet none of those lost three antigens (AMA-1, RAP-1 and RAP-2) has progressed to the stage where large amounts are available for clinical trial.

It would also be desirable to have one or more vaccine candidates representing molecules specific to invaded red cells. Of particular interest may be antigens involved in cytoadherence, which is the phenomenon that causes red cells to adhere to the endothelium of capillary vessels, including importantly small cerebral vessels. It is, in fact, the impedence to blood flow occasioned by these red cells sludging out in the brain capillaries which lies behind cerebral malaria, the frequently fatal complication of *P. falciparum* malaria. The Australian group is pressing on vigorously with research into cytoadherence, as, of course, are many other groups.

The Need for Adjuvants

It is striking that in many of the animal trials of recombinant or peptide vaccines, Freund's adjuvant was required to achieve impressive protection. It is now a matter of the utmost urgency to progress research into various adjuvants suitable for human use. A number of adjuvants are progressing towards clinical trials. These include both water-in-oil and oil-in-water emulsions. They may or may not include immunostimulatory chemicals such as muramyl dipeptide or its derivatives, non-ionic block polymers, or bacterial derivatives such as monophosphoryl lipid A. Furthermore, the nature of the emulsifying agent itself strongly influences immunogenicity. For example, Quil A and its purified derivative QS-21 have intrinsic adjuvant properties. Rendering adjuvants particulate may be part of the overall strategy as with the immune stimulating complexes (ISCOMS) or with micro-encapsulation or liposome technologies. Genetic constructs which include hepatitis B surface antigen can spontaneously self-assemble to create an immunogenic particulate structure. Of special promise is the emerging field of live vectors for antigen genes, which may be viruses (e.g. vaccinia or its relatives such as canary-pox) or bacteria such as *Salmonella* and BCG. Malarial genes coupled to pox viruses are under active investigation in a number of models.

In 1992, the Australian group in association with Hoffmann La Roche found that recombinant circumsporozoite protein

and MSA-2 could be given together adsorbed onto alum to non-immune adult male volunteers, and the trial vaccine was safe but proved to be of relatively low immunogenicity. In 1994, safety and immunogenicity trials in non-immune adults will be conducted in Brisbane for a combination of MSA-1, MSA-2 and RESA. It is hoped at later stages to perform extended safety and immunogenicity trials in non-immune adults and later older children in Papua New Guinea. Only if all of this work progresses without untoward effects will efficacy trials in young children (perhaps in Papua New Guinea) be progressed. While progress has, of necessity, been somewhat slow, the Australian group remains hopeful that an effective vaccine will eventually be developed.

Drug Resistance in Malaria

Anti-malarial drugs fall into three main groups, namely anti-folates such as the dihydrofolate reductase inhibitors, pyrimethamine and proguanil, together with the sulphonomides—sulphones: cinchona alkaloid derivatives such as quinine, chloroquine, mefloquine and halofantrine; and miscellaneous other drugs including Quinghausu and tetracyclines. Resistance to the more common and widely used of these drugs is becoming an increasing problem. While research into drug resistance does not form part of the Saramane joint venture, the Hall Institute has nevertheless made a major commitment to the field, driven chiefly by Drs Alan F Cowman and Simon J Foote, who have recently prepared an excellent review on the subject (Foote & Cowman 1994) on which my tew summary remarks have been based. The two subjects under study are resistance to pyrimethamine and proguanil; and resistance to the quinoline-containing drugs.

Dihydrofolate Reductase Inhibitors

The dihydrofolate reductase gene of *P. falciparum*, which has been located within chromosome 4, is found to be mutated in isolates resistant to pyrimethamine and proguanil (Cowman et al. 1988). The critical mutation in pyrimethamine resistance is a point mutation from serine to asparagine at position 108 which alone confers an approximately 10-fold resistance. A further approximate 10-fold increase in resistance can occur if there are other mutations, for example, an asparagine to isoleucine change at position 51. Resistance to cycloguanil, the active form of proguanil, also involves a change at position 108, but on this occasion, the change is from serine to threonine. Interestingly, resistance to both of these two drugs is unusual, and most cycloguanil-resistant parasites are quite sensitive to pyrimethamine and vice versa. Cross resistance does occasionally occur but seems to require at least one further mutation. This raises the question of whether the combined use of pyrimethamine and proguanil would delay the appearance of resistant parasites. No detailed structural information on the DHFR gene of *P. falciparum* is available, but the structure of other DHFR's suggests that anti-folates bind in a hydrophobic groove which contains the active site of the enzyme, and of which position 108 forms an important part.

Chloroquine Resistance

An interesting early finding (Foote et al. 1989) was that several chloroquine-resistant isolates of *P. falciparum* had an amplification of the multi-drug resistance gene *Pfmdr*, leading to the suggestion that chloroquine resistance was essentially similar in nature to multi-drug resistance in cancer cells. This initial hypothesis has had to be modified in a number of respects. First, *P. falciparum* actually manufactures two p-glycoprotein homologues (Cowman et al. 1991) coded by two separate genes though the second, *Pfmdr*-2, does not appear to be amplified in chloroquine-resistant strains. Secondly, not

every case of chloroquine resistance has the amplified *Pfmdr*-1 gene. Mutations have been noted within *Pfmdr*-1 that are linked to chloroquine resistance, and they seem to fall into two main allelic types, one having a single amino acid substitution and the other having four different ones. However, again this is not a consistent feature for all isolates. Furthermore, a genetic cross between a resistant and a sensitive parasite failed to show a linkage to *Pfmdr* gene segregation (Wellems et al. 1990). These considerations show that chloroquine resistance is a much more complex phenomenon still requiring a molecular explanation.

Mefloquine resistance has also been investigated (Cowman et al. 1994). Most chloroquine resistant isolates are still sensitive to mefloquine. If these are now placed under increasing mefloquine pressure in vitro, amplification and over-expression of the *Pfmdr*-1 gene occurs and interestingly this may involve a decrease in the level of chloroquine resistance. Furthermore, the *Pfmdr*-1 gene appears to be amplified and over-expressed in all mefloquine-resistant field isolates. This could lead to an increased rate of expulsion of the drug from the parasitophorous vacuole where it accumulates in the sensitive strain. Calcium channel antagonists would militate against this increased expulsion.

While the research into molecular mechanisms of drug resistance is still at a relatively early stage, it seems clear that further extensions will lead to knowledge which will help in the design of new anti-malarial drugs circumventing the resistance. It is clear that in the years ahead, these will be very sorely needed if this dreadful tropical health scurge is gradually to come under control.

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