Case Study B.—The Development of a Malaria Vaccine

Lydia Woods Schindler
Darnestown, MD

Introduction

The recognition that malaria stimulates natural immunity gave rise to the hope that a protective immune response could be reproduced artificially. Experiments in animals and humans have shown that this is indeed feasible. Malaria vaccine research today is directed at identifying the immunity-stimulating portions of the parasite or its products, producing them in quantity, and introducing them into the human body in such a way that they stimulate immunity without causing disease.

The undertaking is an ambitious one. A parasite vaccine is difficult to engineer simply because a parasite is so much larger and more complicated than a virus or bacterium (targets of all familiar vaccines), and carries a multiplicity of antigens. The problem is complicated by the fact that antigens on the malaria parasite vary according to the species of *Plasmodium* involved and the stage of the parasite's development. Vaccines are now being developed against the various types of malaria in all stages of the parasite's life cycle, The ultimate vaccine will probably combine antigens to various stages. The main target species is ***P.*** *falciparum* because it is so often lethal and the stakes in preventing it are highest, though eventually a vaccine might protect against several species.

Although natural immunity to malaria develops slowly, over a long period of time, and requires repeated contacts with the parasite, vaccine researchers are encouraged by the prospect that an artificial vaccine may be able to improve upon nature. By using only the immunity-producing antigens—and not the many proteins and contaminants carried by a whole parasite, or in less pure vaccine preparations—they expect to be able to sidestep the multiplicity of immune reactions that are triggered by the intact parasite, many of which may actually favor the parasite's survival.

Each of the three main life stages of the malaria parasite—the sporozoite, the merozoite, and the gamete—has now been shown, under certain conditions, to produce immunity in birds, rodents, and monkeys. Irradiated sporozoites, in an ingenious experimental system (mosquitoes do the inoculating) have also succeeded in immunizing a few human volunteers.

The farthest advanced work centers on the sporozoite, the infectious form transmitted to people by mosquito bite. The immunogenic sporozoite antigen has been isolated and characterized for several parasite species. The gene that codes for the sporozoite antigen of a monkey malaria has been identified, and the antigen reproduced through genetic engineering. Moreover, because this antigen has proved to have a relatively simple structure, it has been possible to synthesize it, and the synthetic antigen has been used to immunize rodents and monkeys. A dramatic announcement in the summer of 1984 revealed success in cloning and elucidating the structure of the antigenic sporozoite protein of the first human malaria, ***P.*** *falciparum*.

Although sporozoite research has captured the lead, most malaria vaccine programs are concentrating on other forms of the parasite. Progress has been impeded by the fact that blood stage parasites carry many antigens, most of which do not elicit a protective immune response. Moreover, blood stage parasite antigens vary not only between stages and between species, but also from one geographic strain to another. Once the antigens are identified, the strategy for producing a vaccine is similar to the strategy for sporozoites. One set of blood stage antigens from a human malaria has been cloned, and these antigens are being studied to see if some of them are candidates for a vaccine.

A vaccine against gametes, sexual forms that occur in the mosquito, would not prevent disease symptoms but wouId block disease spread. Gamete antigens that can elicit antibodies capable of preventing parasite fertilization have recently been identified and are under study.

Several government and international organizations, whose support has financed vaccine research, have already met to make preliminary plans for field trials of a sporozoite preparation; such trials are expected to get under way by *1986* or 1987, although the details have not yet been worked out.

The eventual large-scale production of a malaria vaccine or vaccines, complicated by patent issues, and the difficulties of delivering a vaccine to large numbers of people, many of whom inhabit remote and impoverished areas, will require thoughtful attention and cooperation from the international community.

A first-generation sporozoite malaria vaccine is on the threshold of becoming a reality. Scientists are convinced that they have at their fingertips the capability of identifying and producing those elements of the ma-

laria parasite that evoke an immune response, and public health planners anticipate the start of clinical trials in 1986 or 1987.

This is not to say that work is complete. This first "vaccine," even if it proves safe and effective, would probably constitute no more than one component of the ultimate vaccine. Many formidable hurdles—biological, immunologic, and chemical—are yet to be met, and the logistical problems of field testing, mass producing, and delivering a vaccine to target populations are enormous. Nevertheless, the prevailing mood is one of great optimism.

The development of a malaria vaccine has been propelled by two distinct currents. One is the failure of an international effort to eradicate malaria, due in large part to the emergence of mosquitoes resistant to pesticides and malaria parasites resistant to antimalarial drugs. The other is the recent explosion in biotechnology, which has provided scientists with tools that have swept away obstacles that seemed insurmountable less than a decade ago.

A program to rid the world of malaria, which at the time was estimated to affect *250* million persons annually, with 2.5 million deaths each year (5), was launched by the World Health Organization (WHO) in 1957. Through a combination of large-scale spraying of insecticides and medical treatment, the program succeeded in eliminating malaria or greatly reducing it in about 80 percent of the target areas by the mid-1960s. Just a few years later, however, the picture had begun to change dramatically. In Sri Lanka, where the number of cases had shrunk from 3 million to only 18 reported cases, an epidemic of malaria broke out, and more than 1 million people were affected (128). Although malaria remained vanquished in temperate regions of the world, the disease was making a strong comeback in many tropical areas.

To some extent, the resurgence reflected the difficulties of carrying out such an ambitious and complex program in countries that lack a strong, central, public health program *(63,95,96).* More fundamental, however, was the fact that more and more malaria-carrying ***Anophels*** mosquitoes were becoming resistant to DDT (dichloro-diphenyl-trichloroethane) and other insecticides. By 1981, insecticide resistance had appeared in 51 species of *Anophels* mosquitoes *(96).* Because alternative insecticides, most of them petroleum products, are often too expensive for Third World countries to use, spraying efforts have been curtailed (128).

The first reports that the malaria parasite could resist the effects of chloroquine appeared almost simultaneously in several countries of Southeast Asia and South America around 1960. Resistant strains of P. *falciparum,* the species that causes the most severe disease, have now surfaced in more than two dozen countries in Latin America, Asia, and Africa *(128). New* drugs are being developed, but early reports indicate that the parasite can become resistant to them, too.

In short, despite extensive research efforts, there is little affordable on the shelf to fend off a disease for which one-third of the world's population is at risk, which strikes an estimated *150* million persons a year, and which causes at least 1 million deaths each year.

While efforts to conquer malaria through drugs and insecticides have been faltering, research into the immunology of malaria, with a view to developing a vaccine that could prevent its symptoms and its spread, has been surging ahead. One major breakthrough came in 1976, when it first became possible to grow P. ***falciparum*** in continuous culture, in the laboratory. This ready source of raw material opened the way for a stream of studies on the parasite and its ability to stimulate immunity. The pace accelerated again with the advent of the new biotechnologies. Since 1980, malaria researchers have been using monoclinal antibodies (MAbs) to identify those precise parts of the parasite that produce an immune reaction. They then produce this protective antigen in quantity through recombinant DNA technology, cloning those parasite genes that code for the protective antigens. Alternatively, once the antigen structure has been fully spelled out, scientists can synthesize the antigens chemically.

The Malaria Parasite

Malaria is caused by single-celled protozoan parasites of the genus *Plasmodium.* More than 100 different species are known to cause disease in a variety of animals *(24),* Four species naturally infect humans: ***P. falciparum, P. vivax, P. malariae,*** and ***P. ovale.*** Each of the four has a distinctive appearance and life cycle; each produces a somewhat different clinical effect (72). The most dangerous is ***P. falciparum,*** which can cause severe anemia, kidney failure, and brain damage; it is often fatal, especially among children. In ***P. vivax*** infection, the typical symptoms—cycles of chills, high fever, and sweating with headache, muscle aches, and nausea—are less severe. Although the disease is not often fatal, relapses can occur periodically for up to 3 years. P. *malariae* infections can persist in the blood, without producing symptoms, for life; chronic infections in children can lead to kidney damage.

Other species of ***Plasmodium*** cause malaria in a variety of vertebrates—reptiles, birds, rodents, rabbits and monkeys. Several of these have been used as experimental models in vaccine research: ***P.*** *gallinaceum*

and P. *lophurae* in chickens and ducks; P. *berghei, P. yoelli, P. vinckei,* and P. *chabaudi* in rats and mice; and ***P. knowlesi*** in the rhesus monkey. In addition, human malarias can infect certain higher apes and New World monkeys. An important experimental model is P. *falciparum* in the South American owl monkey.

All malaria parasites have a complex life cycle, alternating between vertebrate host and mosquitoes. In vertebrates they reproduce asexually, first in the liver and then, repeatedly, in the red blood cells (erythrocytes). In the mosquito, they reproduce sexually.

Human infection begins with the bite of an infected female *Anopheles* mosquito. As she ingests a blood meal to nourish her eggs, she simultaneously injects a stream of saliva that can contain plasmodial *sporozoites,* which have been clustered in her salivary glands. The motile, threadlike sporozoites quickly leave the bloodstream and lodge in the cells of the liver *(hepatocytes).* Within about an hour, all sporozoites have disappeared from blood circulation.

Over the next week ***(P.*** *falciparum* and ***P. vivax)*** or 2 weeks ***(P.*** *malariae),* each sporozoite that has invaded a liver cell becomes a *schizont,* a developmental structure that contains thousands of *merozoites.* Liver stage parasites are known as "exoerythrocytic" forms, to distinguish them from blood stage, or "erythrocytic, " parasites. When the schizont is mature, it ruptures out of the infected liver cell and discharges thousands of merozoites into the bloodstream. In ***P. vivax*** and *P. ovale* malaria, some sporozoites become *hypnozoites,* forms that remain dormant in the liver for months or years before they start to proliferate (62).

Merozoites, released into the bloodstream, invade erythrocytes. The invasion process, which can **be** observed by microscopy, takes about *20* seconds. ***P. vivax*** and ***P. ovale*** parasites invade young erythrocytes, while ***P.*** *malariae* preferentially infect mature erythrocytes; ***P.*** *falciparum* invades old and 'young cells alike—one reason why the concentration of parasites in the blood reaches dangerously high levels in ***P.*** *falciparum (82).*

Most of the parasites that enter erythrocytes undergo a second round of asexual reproduction, similar to but quicker and less prolific than that in the liver cells. In 2 or 3 days, depending on the species, the intraerythrocytic parasite has developed from young *ring forms* to *trophozoites* to the dividing form, again known as a *schizont.* Red blood cells infected with ***P. falciparum*** develop "knobs," small, sticky, protrusions of parasite origin that allow the infected erythrocyte to adhere to the lining of small blood vessels while the parasite matures (70,117). The infected cell is thereby prevented from circulating through the spleen, where it could be destroyed.

Depending on the species, each schizont contains 10 to *20* erythrocytic *merozoites.* When the schizont is mature, these merozoites burst out of the erythrocytes and invade yet other red blood cells, thus perpetuating the cycle of infection. It is at this point, when the red blood cells rupture, that clinical symptoms appear; because the cycle can repeat every *48* hours ***(P. vivax, P.*** *falciparum,* and ***P. ovale)*** or *72* hours ***(P. malariae),*** attacks of fever can occur every 2 or 3 days.

Plasmodial parasites thus continue to recycle untiI they are brought under control through drug therapy or through the host's immune defenses, or until the host dies. If reinfection does not occur, ***P.*** *falciparum* infections will generally clear in 1 to 2 years; *P. vivax* and ***P.*** *mahriae* may last 3 years. ***P.*** *malariae,* if untreated, can persist as an asymptomatic infection for decades.

Some of the merozoites that invade red blood cells, instead of developing asexually, differentiate into sexual forms, male and female *gametocytes.* Mature gametocytes, enclosed within the erythrocyte membrane, circulate in the blood, available to feeding *Anopheles* mosquitoes.

Blood ingested by the female *Anopheles* carries the gametocytes into the mosquito's stomach. There, perhaps triggered by changes in temperature and pH, they shed the red blood cell envelope. Male gametocytes rapidly transform into motile, spermlike structures and fertilize the larger, egglike female gametes, forming zygotes.

Forms that develop from zygotes in about a day, called ookinetes, burrow into the mosquito's stomach wall, where they form oocysts; 9 to 14 days later the oocysts rupture and release the motile, threadlike sporozoites. The sporozoites infect the mosquito's salivary gland. One cycle is complete.

The malaria parasite's many life stages present a variety of possibilities for interrupting the infectious cycle by immunization. Potential targets include:

- sporozoites before they enter the liver cells;
- . infected liver cells;
- merozoites before they enter the red blood cells;
- red blood cells carrying infectious schizonts; and
- gametes before fertilization occurs.

Each system has its advantages and disadvantages. The ultimate vaccine is likely to combine antigens against several stages of the parasite. A sporozoite component would provide high immunogenicity; a merozoite component would act as a backup, preventing disease should even one sporozoite escape the antisporozoite defenses. A gamete component, even though it offers no direct benefit to the individual being vaccinated, would help to prevent disease spread by interrupting the transmission of parasites from the mosquito to a new vertebrate host.

Malaria and Immunity

It is clear from the course of natural infections that the *Plasmodium* parasite, in its various manifestations, can stimulate immunity. Persons who live in areas where malaria is endemic, and who are frequently exposed to infected mosquitoes, gradually develop some immunity. But the development of immunity usually takes repeated infections, over a period of years (82). Vaccines are designed to mimic the natural process, stimulating protection without producing any of the adverse effects that accompany natural infection.

The Immune System

The immune system has evolved to protect an individual from invasion by "foreign" substances, including micro-organisms such as viruses, bacteria, and parasites. Components of the immune system, including white blood cells called lymphocytes, recognize substances as being foreign by features of their chemical makeup that are unlike "self. " The chemical entities recognized as such are called "antigens. "

Once the immune system recognizes an antigen, it can set in motion a variety of responses designed to rid the body of the invader. One is the production of antibody by a family of white blood cells known as B-lymphocytes, or B-cells. Antibody is a substance that "matches" the invading antigen and can inactivate it or speed its uptake by scavenger cells. Another set of responses involves T-lymphocytes, or T-cells. Some subsets of T-lymphocytes work in collaboration with B-lymphocytes, helping either to induce or suppress the production of antibodies. Other T-lymphocytes produce potent chemicals (one example is interferon) that call into play yet other cells, and other responses. Other types of immune cells, including "macrophages" and "monocytes," are scavengers equipped to take up and digest foreign molecules and micro-organisms; natural killer cells attack tumor cells and perhaps aid in the elimination of parasites.

Some cells of the immune system become "memory" cells. After the host's initial encounter with a specific antigen, the body's defenses are primed to attack it quickly: the individual acquires immunity.

Natural Immunity

P. falciparum typically takes its greatest toll among children. Infants are protected temporarily by virtue of antibodies they receive from their mothers in breast milk (92). But by the second year of life, children who live in highly endemic areas become victims of severe and recurrent attacks. Those who survive gradually acquire immunity; by 5 to 10 years of age they show few or no further symptoms of disease. Immune adults rarely experience acute attacks. However, such immunity is generally not complete. Even though the individual develops no symptoms, small numbers of parasites may continue to cycle through the red blood cells. The combination of clinical immunity and continued low-grade infection, a condition known as "premunition," reflects a balance between parasite survival and host resistance. Importantly, the person with asymptomatic low-grade parasitemia remains a reservoir for transmitting the disease.

If a person, once recovered, is not re-exposed to infection, immunity gradually wanes. Symptoms may also flare up in otherwise immune individuals when the immune system is disturbed by events such as surgery or pregnancy. Pregnant women in endemic areas, especially those pregnant for the first time, are much more likely than their nonpregnant counterparts to contract acute ***P.*** *falciparum* malaria.

Innate Resistance

Some people inherit traits that make them naturally resistant to malaria. In general, these involve some peculiarity of the red blood cell that makes it inhospitable to *Plasmodium*. Because they favor human survival where malaria is endemic, these traits have become prevalent in such areas.

One example is sickle-cell hemoglobin, which is common in areas of west Africa where malaria is widespread. Under conditions of low oxygen tension—which prevail in the small blood vessels where parasite-infected red blood cells sequester—parasites within cells containing sickle-cell hemoglobin die (33). Although children with sickle-cell hemoglobin develop ***P.*** *falciparum* malaria, the disease is much less likely to be fatal than in children with normal hemoglobin.

Another trait that confers resistance to malaria involves a receptor(s) on the surface of red blood cells; the receptor is a prerequisite for parasite invasion. It has long been known that most west Africans and many American blacks are completely resistant to infection with *P. vivax*. These persons are also known to be "Duffy blood-group negative": their red blood cells lack genetically determined surface markers known as Duffy antigens A and B. Studies with parallel infections using ***P. knowlesi,*** a monkey infection similar to ***P. vivax,*** indicate that these Duffy antigens are closely associated with, if not identical to, the specific receptors that merozoites recognize. Cells lacking these receptors are unable to form a junction with the parasite (74).

Malaria Antigens

Malaria parasite antigens are remarkable for their diversity. Each species and stage of the parasite carries its own characteristic surface structures that mark it as immunologically distinct. Antigens of blood stage parasites, but apparently not sporozoites (132), also differ from one strain to another (67).

To offer protection, the immune system must tailor a response to fit each variation. A person who is immune to ***P.*** *falciparurn,* for instance, can be susceptible to infection with P. *vivax,* and vice versa. Persons immune to ***P.*** *falciparum* in one country—or in one part of a country—may become infected with a different strain of falciparum when they travel. Even within a given area, several strains may coexist; what were once thought to be "relapses" may possibly represent a series of infections with different strains. This phenomenon may also explain the slow development of natural immunity, over the course of many infections (19,53).

In addition, some malaria parasites exhibit antigenic variation. In response to changes in their environment (for instance, the host's deployment of effective immune defenses), the parasite changes surface antigens (53,54).

Immune Responses

A malaria infection stimulates a spectrum of immune responses, not all of them beneficial. These include the production of antibody by B-cells and the participation of various sets of T-cells, as well as the activation of a variety of nonspecific responses, including macrophages, monocytes, and natural killer cells.

Antibodies are clearly important to immunity against malaria. Persons living in endemic areas develop increasing levels of serum antibodies to sporozoites as well as to blood stage parasites as they build up immunity (80). However, much of the antibody produced in response to malaria infection is nonspecific. Other antibodies, though specifically matched to certain parasite antigens, are not protective.

Some antibodies, however, are both specific and protective, and their role has been established in several ways. For one thing, immunity can be transferred passively, by taking serum from immune individuals or experimental animals and injecting it into nonimmune individuals. In a dramatic clinical demonstration, serum from immune adults living in west Africa was given to 12 infants with severe malaria; it cured their symptoms and sharply reduced the levels of asexual blood stage parasites, though the protection lasted only a short time (17). However, it did not affect levels of circulating gametocytes—an early indication that the asexual and sexual stages carry different antigens.

It is also possible to induce protective antibodies by vaccinating animals with antigens from defined stages of the malaria parasite. Like immune serum, MAbs to sporozoites, merozoites, and gametocytes can be used to transfer passive immunity (97) and to inhibit the growth of parasites in vitro (129).

Antibody appears to prevent sporozoites and merozoites from entering their target cells; it can cause them to agglutinate; it may coat them so they become attractive targets for macrophages; it can prevent gametes from forming zygotes.

T-cells are essential for the development of immunity in malaria (56,110). In addition to their major role, which is assisting B-cells to produce antibody, they appear to secrete mediators that recruit and activate other immune cells, including macrophages and monocytes. They may possibly also exert a direct toxic effect on parasites (36).

Several types of nonspecific mechanisms (that is, those which do not depend on the recognition of a particular malaria antigen) can affect the malaria parasite. For one, general potentiators of the immune system, which carry no malaria antigens, can trigger general cell-killing activity. Malaria-infected animals also have increased numbers of macrophages in the spleen, liver, and bone marrow. These activated macrophages are probably responsible for the high levels of interferon, a natural immunopotentiator, seen in such animals. The macrophages can also secrete other soluble substances, monokines, that can trigger immune activity (lo).

Natural killer cells are another nonspecific immune defense. Although their known main target is tumor cells, they can also attack virus-infected cells. Levels of natural killer cells increase in people recovering from ***P.*** *falciparum* malaria. Also, strains of mice that have high levels of natural killer cells are more resistant to plasmodial infection than mice with low levels, When mice are immunized with irradiated sporozoites, levels of both natural killer cells and interferon rise (91).

Adverse Immune Responses

Although the overall effect of the immune system's activity is to curb the parasite's growth and gradually eliminate it, some of the immune responses elicited by plasmodial infections work to the host's detriment. To begin with, malaria leads to a general suppression of the immune system, impairing the host's ability to cope with other, nonmalarial antigens, as well as the malaria infection itself. Children with malaria, as well as animals infected experimentally, may be unable to mount an effective response when they encounter an

antigen for the first time. They may, for instance, respond poorly to vaccination against other diseases such as typhus, meningitis, or measles. Children with malaria are also prone to more, and more severe, viral diseases, including measles, respiratory tract infections, and gastroenteritis.

The immune response to malaria has been described as "hyperactive and at the same time highly inefficient" (126). While some aspects are suppressed, others are overactive. Among the excess of antibodies produced in response to malaria infection are autoantibodies directed against a variety of the body's own tissues. Other antibodies may form potentially damaging immune complexes by combining with soluble parasite antigens. (Soluble antigens are generally considered detrimental to the host, a sort of decoy for the parasite.) Immune complexes are believed to initiate the kidney damage that occurs in malaria; the damage is then thought to be sustained by autoantibodies.

A Malaria Vaccine

Early Vaccine Studies

The earliest reported attempt to vaccinate against malaria dates back to 1910. Two Algerian brothers, Etienne and Edmond Sergent, during the course of "a lifetime of experimental work on malaria control by every means" (41), inoculated birds with killed sporozoites (103).

These experiments came just three decades after Alphonse Laveran, looking through his microscope at blood smears from malaria patients, discovered "elements that seemed to me to be parasites" and identified them as the principal cause of malaria. Only in 1898 did Ronald Ross put an end to theories incriminating bad air (the Italians called it *mal' - aria) (41),* decay, or filth, by demonstrating that the source of malarial infection was the mosquito.

Vaccination was not attempted again for several decades. By that time a variety of studies had established that antiparasite antibodies existed, could be detected, and could be used to transfer immunity passively in monkeys (14).

During the 1930s and 1940s, it became clear that vaccination was feasible. Birds, rodents, and eventually primates were immunized against malaria, using one of two relatively accessible forms of the parasite, either sporozoites inoculated by feeding mosquitoes, or the blood stages contained within infected erythrocytes. The usual strategy was to inactivate or kill the parasites, inject them into an experimental animal, wait for immunity to develop, and then challenge the animals by exposing them to infection with virulent parasites.

The first successful vaccine used P. *gallinaceum* sporozoites to immunize fowls. Fifty percent of the birds were able to survive a subsequent challenge *(79).*

In a series of experiments in the 1940s, Freund and his colleagues studied killed blood stage parasites. They used mature schizonts, derived from red blood cells and inactivated by formalin, with and without adjuvants, to immunize ducks (114). With adjuvants, they succeeded in protecting rhesus monkeys from an invariably fatal infection with P. knowlesi (32).

In 1946, Heidelberger attempted to vaccinate human beings. Using formalin-killed blood stage P. *vivax* parasites, he inoculated a series of patients and volunteers. The effort was not successful (44). According to current opinion, "perhaps the most remarkable result of this trial was that eight injections of antigen given subcutaneously, intracutaneously, and intravenously over a period of 2 weeks did not cause marked reactions in the volunteers" (25).

During the 1950s, while dreams of eradicating malaria through vector control and drug therapy flourished, research on malaria vaccines was relatively quiescent. Lacking any ready source of malaria parasites, and thus malaria antigens, researchers focused on transferring immunity passively, by means of antibody-containing serum from immune individuals, or to newborn animals through maternal milk. These studies led, in the early 1960s, to the demonstration that passive immunity could be effective in humans—not preventing malaria infection but sharply reducing the severity of the disease (17).

The pace did not pick up until the latter half of the 1960s. By that time, the success of the WHO malaria eradication program was in doubt, and several laboratories began to make some headway in malaria immunology.

The U.S. Agency for International Development (AID) began funding research toward a vaccine, an effort that has been sustained and has been at least partially responsible for much progress in this area. The first all-encompassing AID contract—to grow both sporozoite and blood forms in culture, and to isolate their protective antigens—was awarded to researchers at the University of Illinois in 1966; by 1972, the program had expanded into a seven-site network and was still growing (29).

Vaccination With Sporozoites

Most of the immune response to a natural infection with malaria is elicited by blood stage parasite antigens. Sporozoites, however, are also highly immunogenic. Despite the facts that only a relatively small number of sporozoites are inoculated by a mosquito bite and that they spend a very brief time in the host's

bloodstream, sporozoites trigger antibody production (81). Furthermore, experimental sporozoite vaccination—without the use of adjuvants—completely blocks infection, but only for a few months.

For a sporozoite vaccine to be effective, it must kill ***all*** sporozoites. If a single sporozoite escapes, it can infect a liver cell and eventually give rise to up to *40,000* merozoites (in *P. falciparum)* that can then infect red blood cells, creating a fullblown attack of malaria.

A drawback to sporozoite research has been supply. Sporozoites live in the salivary gland of an infected female mosquito. To produce sporozoites, mosquitoes must be raised, then infected by feeding them gametocytes. After the appropriate interval, laboratory workers must dissect the mosquitoes' salivary glands. Even though a single salivary gland teems with sporozoites, dissection is a painstaking, labor-intensive process that is obviously unsuited to large-scale production. Furthermore, the sporozoites must then be purified of mosquito saliva and other contaminants.

The good news is that recombinant DNA technology promises to significantly ease sporozoite research. The protective sporozoite antigen, which is distributed uniformly over the surface of the sporozoite, consists largely of a single antigen and short marker or epitope, repeated several times. This antigen has now been identified, using MAbs, and produced by recombinant DNA technology. Moreover, the repeating epitope of the ***P. knowlesi*** sporozoite has been synthesized, and used to immunize monkeys (34).

Research in the *1960s* **and** 1970s.—In the late 1960s, Richards revived Mulligan's studies of a quarter century earlier by showing that killed *P. gallinaceum* sporozoites protected birds from challenge with sporozoites, but not blood forms, of the malaria parasite *(98).* Richards subsequently used the same technique to protect mice against infection with *P. berghei* and ***P.*** *chabaudi (99).*

Meanwhile, Nussenzweig and her coworkers at New York University (NYU) had begun what would become a major contribution to the development of a sporozoite vaccine. NYU parasitologists had just succeeded in growing the rodent parasite, P. *berghei,* through the mosquito cycle. Taking advantage of this steady supply of sporozoites, Nussenzweig showed that vaccination was effective. Repeated intravenous injections of X-irradiated ***P.*** *berghei* sporozoites protected more than *90* percent of the mice against an otherwise lethal challenge; immunity lasted about 2 months (87).

Subsequent studies elucidated other features of the immune response to vaccination with intact sporozoites:

- Sporozoites are more immunogenic if they are injected intravenously than if they are administered intramuscularly, subcutaneously, or orally (109).
- Not all sporozoites are immunologically equal: only "mature" sporozoites obtained from mosquito salivary glands *17* to 18 days after the mosquito becomes infected are protective; younger sporozoites collected from the mosquito stomach wall are not effective because they lack an immunogenic surface antigen *(121).*
- Antisporozoite immunity is species and stage specific. Mice immunized with *P. berghei* sporozoites could be infected with erythrocytes *(87)* or schizonts (123). They were also susceptible to avian and simian malarias (86). However, mice protected with one species of rodent malaria were protected against all other rodent species tested (89).

To get around the problem of injecting material that was contaminated with a relatively large proportion of mosquito salivary gland tissue, the NYU researchers turned to the mosquito for help. Infected female *Anopheles* were exposed to enough X-irradiation to render the parasites they were carrying noninfectious, then they were allowed to bite experimental animals. Mosquito inoculation met with most of the known criteria: mature sporozoites, inactivated by X-irradiation, delivered intravenously, via multiple inoculations. Mice repeatedly bitten by the infected, irradiated mosquitoes developed both circumsporozoite protein antibodies and protection against sporozoite challenge *(122).*

The technique was soon applied to humans. For *84* days, three volunteers were exposed to a total of *397* mosquitoes that had been infected with ***P.*** *falciparum* and then irradiated. On day *98,* the volunteers were challenged by exposure to heavily infected but nonirradiated mosquitoes. Two of the three men became infected, but the third resisted infection. The third man subsequently resisted challenge with other strains of ***P.*** *falciparum,* but not ***P. vivax*** (12). It is thought that the two volunteers who were not protected had probably been inoculated with too few sporozoites during the 84-day immunization period.

The same technique was used to immunize another volunteer in 1974 (100). The following year, a volunteer (the researcher himself) was protected sequentially against ***P.*** *falciparum* and ***P. vivax;*** the former immunity lasted 3 months, the latter, *6* months (11).

Early attempts to immunize rhesus monkeys using ***P.*** *cynomolgi* were not very successful (18,125). Results were better using multiple injections of large num-

bers of P. *knowlesi* sporozoites over a period of several months (39).

Recent Sporozoite Vaccine Research.—The search for purified malaria antigens surged ahead when biotechnology made it possible to produce MAbs —that is, antibodies that are secreted from clones of a single, hybrid parent cell and which are thus identical, all equipped to recognize and link to one single, specific antigen. In the case of the malaria sporozoite, MAbs were derived by fusing nonsecreting but long-lived cells from a plasmacytoma (a plasma cell tumor) with antibody-secreting spleen cells from mice that had been immunized with *P. berghei* sporozoites.

The resultant hybridoma secreted a MAb that singled out the immunogenic sporozoite antigen. This antigen, which has a molecular weight of 44,000 daltons (a measure of mass), and is known as "Pb44" (129), is distributed uniformly over the surface of mature sporozoites, but it is not found on the other stages of P. *berghei* (except for the very early stages within the liver). Additional studies identified two more antigens, Pb54 and Pb52 (130), which are recognized by the same MAb; these have proved to be intracellular precursors of Pb44.

The importance of this MAb in immune protection has been demonstrated in several types of laboratory tests (81,97,129). The antibody also prevents sporozoites from invading target liver cells in vitro (51). In addition, the passive transfer of very small amounts (10 Kg) completely protects mice against sporozoite challenge. Similar MAbs were produced against *P. falciparum* and ***P. vivax*** sporozoites (81) as well as against *P. knowlesi* (13).

Work using these MAbs has identified one surface antigen and one intracellular antigen on each of two human malaria sporozoites, Pf58 and Pf67 in *P. falciparum,* and Pv45 and Pv5l in ***P. vivax.*** One surface antigen, Pk42, and two intracellular precursors, Pk52 and Pk50, have been identified for *P. knowlesi.*

With the availability of genetic engineering, it became only a matter of time until a potentially protective sporozoite antigen was cloned. The first success was reported in 1983 (27). Researchers at NYU extracted messenger RNA from mosquitoes infected with *P. knowlesi,* converted the messenger RNA into complementary DNA, and inserted the complementary DNA into a plasmid. The plasmid containing the genetic material from the ***P.*** *knowlesi* was then introduced into an *Escherichia coli* bacterium. Using MAbs to identify the many proteins being produced by the various E. coli colonies, they isolated three clones that produced the sporozoite surface antigen.

Analysis of the DNA sequences in these clones showed that they code for an epitope of 12 amino acids, which is repeated 12 times in tandem (35). Because the shortest of the three DNA sequences cloned contains nothing but the repeating unit, it was possible to deduce the amino acid sequence that the DNA codes for. Then they corroborated their deduction by chemically synthesizing it, and showing that it and the native ***P.*** *knowlesi* circumsporozoite protein behave identically (35), This peptide was then used to immunize rabbits and monkeys (34).

The announcement that the circumsporozoite protein gene of *P. falciparum* had been cloned, inevitable but still a cause for excitement, came in August 1984 (21). As predicted, a repeating sequence of nucleotides makes up a large portion of the molecule. The surprise is that the repeat is shorter than the corresponding section in ***P.*** *knowlesi,* consisting of only four amino acids, with some slight variation. The simplicity of this antigenic protein should be an advantage in the vaccine development work that now is proceeding.

Other avenues of research are yielding ingenious approaches to vaccine delivery and testing. In a twist of genetic engineering, the gene encoding for the circumsporozoite protein of ***P.*** *knowlesi* has been incorporated into the genetic material of the vaccinia virus, the agent of smallpox vaccine. When this recombinant vaccinia virus is introduced into a cell, the infected cell produces not only protective vaccinia proteins but also the protective circumsporozoite protein.

The recombinant vaccinia virus system has already been used to vaccinate animals against hepatitis, influenza, and herpes (108). Now rabbits have been vaccinated with a recombinant *Plasmodium-vaccinia* virus, and have responded by developing antisporozoite antibodies (107). Because the vaccinia virus has a large capacity for foreign DNA, it might eventually be possible to incorporate genetic material for antigens to several of the malaria parasite's life stages (107),

A test to measure a sporozoite vaccine's effectiveness—a pivotal concern of field testing—has evolved from studies of the parasite's cycle within the liver. The new test, called the inhibition of sporozoite invasion assay, measures the ability of *P. falciparum* and *P. vivax* sporozoites to invade cultured human hepatocytes. Such invasion is blocked not only by monoclinal antibodies to the circumsporozoite protein, but also by serum from immunized volunteers and serum from persons living in endemic areas. Once the test has been adapted for use in the field, it should be possible to evaluate levels of preexisting immunity

in a broad population, and to monitor the effects of a sporozoite vaccine in clinical trials (51).

Vaccination With Blood Stage Parasites

As the form that induces disease as well as natural immunity, blood stage parasites—either mature schizonts in red blood cells or free merozoites—are good candidates for a vaccine, and blood stage antigens are the object of most of the malaria vaccine research in the world today. Although a blood stage vaccine would not block either sporozoite or liver stage infection, those stages produce no symptoms. An effective blood stage vaccine would prevent disease and interrupt further transmission.

Vaccines to blood stage parasites have succeeded in producing immunity in several animal models, including P. knowlesi and P. *fragile* in the rhesus monkey and the human malaria P. *falciparum* in the *Aotus* monkey. However, the degree of protection depends on the nature of the antigen used and the way it is administered. Moreover, it is typically necessary to combine the parasite component with an adjuvant, which independently and nonspecifically boosts the immune response; most adjuvants used in animals are unsuitable for use in humans because of adverse side effects.

Another problem is that blood stage parasites are antigenically very complex. In contrast to the sporozoite, which has a single immunodominant antigen with a short repeating epitope, blood stage parasites carry a mosaic of antigens, many of which elicit responses that are not protective. These antigens may vary not only between stages and between species, but also from strain to strain and even in the course of one infection.

Evaluating the effectiveness of blood stage vaccines is complicated by the use of experimental systems that yield many different patterns of clinical immunity. The same preparation—for instance, blood parasites attenuated by irradiation—will protect the rat, but give less protection against more virulent infections in mice or monkeys (15). Additionally, different programs have used various doses and immunizing schedules, and different ways of measuring the outcome.

Malaria vaccine research took a tremendous leap forward, when, in 1976, it became possible to grow *P. falciparum* blood stage parasites in continuous culture in the laboratory. Nevertheless, with current techniques in vitro cultivation is not suitable for Iarge-scale production *(24),* nor has it eliminated problems of contamination by cells in which the parasites are grown.

Again, biotechnology holds out the promise of solutions. The major challenge now is to identify the protective antigens and produce them through cloning, or possibly, chemical synthesis. Hybridomas are being used to produce MAbs, which are, in turn, being used to isolate antigens that are protective.

Research in the 1960s **and** 1970s.—Attempts to induce immunity against the erythrocytic forms of malaria parasites have used a variety of approaches. In some cases the animal (mouse, rhesus monkey, *Aotus* monkey) was exposed to a severe or lethal infection (transmitted by parasitized blood), then treated with drugs or dietary manipulation; once cured, the animal was immune. Monkeys given subcurative drug therapy, however, develop chronic recrudescent infections, and each recrudescence is associated with an antigenically different population of parasites (3). Such antigenic variation may contribute to chronic infections and explain the slow development of natural immunity over the course of repeated infections (19,53).

In other immunization experiments, animals were inoculated with parasitized erythrocytes that had in some way been altered: attenuated (weakened by growth in culture), heat-inactivated, killed, or combined with other adjuvants. Sometimes parasite fractions have been used. Such immunization can convert lethal infections to chronic disease, with most animals eventually recovering (P. *berghei* in mice and P. *falciparum* in the *Aotus* monkey), or at least prevent a portion of the animals from dying (P. *knowlesi* in rhesus monkey) (4).

Many blood stage immunization studies have concentrated on the free-living merozoite. Immunity to malaria is at least partly mediated by antibody, and in vitro studies have shown that merozoites are the target of this protective antibody; it prevents their entry into red blood cells (16).

Merozoites have proved to be an effective form of vaccination. When P. knowlesi merozoites, combined with Freund's complete adjuvant were used to vaccinate rhesus monkeys, 100 percent of the animals survived this usually fatal infection (77, 78). Merozoite vaccines, with and without adjuvants, have also succeeded in immunizing *Aotus* monkeys against P. *fa]ciparum* infection, with 100 percent survival (78, 104). Merozoite vaccines have shown varying degrees of effectiveness in birds, chickens, and rodents.

In Vitro *Cultivation.* —Until the mid-197&, merozoites for vaccine studies had to be separated out of suspensions of schizont-infected red blood cells. Scientists had, for more than *60* years, been attempting to develop a steady supply of blood stage parasites by growing them in continuous culture. In 1912, two malariologists, having cultivated the parasite through three cycles (6 days), predicted that continuous culture would be achieved within the year (71).

The approach that finally succeeded in 1976, capping more than 30 years of research by Rockefeller University parasitologist William Trager. Trager, working with James Jensen, devised an apparently simple system that resembles the normal physiological environment. P. ***falciparum*** blood stage parasites are grown in human red blood cells in a culture medium that is supplemented with normal human serum, in an atmosphere reduced in oxygen and enriched with carbon dioxide. As schizonts mature and rupture, extracellular merozoites accumulate in the culture medium (115). A similar system was devised independently by scientists at the Walter Reed Army Research Institute, though the parasites recycled for only 21 days *(43).*

Because the system is easy to reproduce, it was soon in widespread use and was improved upon. In addition to providing material for immunization experiments, the in vitro culture technique proved useful for screening antimalarial drugs, for studying parasite-red blood cell interactions, and for exploring the cellular abnormalities underlying sickle cell anemia (116).

It also benefited research on vaccines against other stages of P. ***falciparum,*** because with further manipulation, asexual blood forms could undergo development into gametocytes. These are proving useful not only for gamete vaccine research, but also—when fed to mosquitoes—as a means of producing sporozoites.

Recent Blood Stage Vaccine Research.-The search for protective blood stage antigens—on the surface of either the merozoite or the infected erythrocyte—has been hindered by their tremendous complexity: ***P. falciparum*** appears to carry about 40 antigens (47,93). Blood stage antigens also change as the parasite develops and matures.

Monoclinal Antibodies.–MAbs made with hybridoma technology have been raised against the blood stages of several species of malaria—P. yoelli (81), ***P.*** knowlesi (23,28), ***P. falciparum (94),*** and P. ***berghei*** (93,94)–but not against parasite antigens on the erythrocyte membrane (73).

Because most MAbs are produced by immunizing mice with the entire schizont-infected erythrocyte, or through mosquito-borne infection, and not by immunization with a pure parasite antigen, the clutch of MAbs that results needs to be sorted out by laboratory screening. Then each MAb is tested for effectiveness: When mixed with merozoites in vitro, will it cause the parasites to agglutinate, or otherwise prevent them from invading red blood cells? When injected into test animals, will it confer passive immunity? If a MAb passes these tests, its corresponding antigen becomes a target for vaccine studies.

Purified Blood Stage Antigens. -Both immune serum and MAbs have been used to identify potentially protective blood stage antigens. In general, such antigens have proved to have relatively high molecular weights, to be synthesized at a late stage in the schizont cycle, and to be processed into smaller, discrete fragments *(46).*

One type of antibody target is antigens on the surface of the merozoite. Antibody to these antigens prevents merozoites from invading red blood cells by agglutinating the merozoites as they rupture from the infected erythrocyte and also, perhaps, by blocking specific receptors on the merozoites that allow them to recognize and forma junction with the erythrocyte.

One merozoite surface antigen of the rodent malaria parasite, ***P. yoelli,*** which has a molecular weight of *230,000* daltons, has been used to immunize mice; (47), and a related MAb inhibited ***P. yoelli*** proliferation in vivo (66). Comparable merozoite surface antigens have been identified in ***P. knowlesi*** and ***P. falciparum*** *(22,28).*

Numerous studies, using human serum and/or MAbs, are examing the structure of these antigens and their role in the immune response *(2,93).*

Second possible point of attack are antigens on the surface of the erythrocyte. Antibody directed against these antigens could destroy intraerythrocytic parasites in a number of ways. By coating the infected erythrocyte, for example, antibody may make it attractive to macrophages. Alternatively, antibody might imperil parasite survival by locking onto "knobs," thus preventing the infected erythrocyte from sequestering in small blood vessels.

One erythrocyte-associated antigen is known as the S-antigen. Although it stimulates antibody formation, the S-antigen varies from one strain of ***P. falciparum*** to another; many distinct types of S-antigen occur even within a restricted geographical area. The S-antigen is thus more likely to serve as a mechanism to help the parasite evade host immune defenses than to help the host destroy the parasite *(19).*

Evidence for another type of erythrocyte membrane antigen comes from studies that show that antibody (or other factors) can damage the ***P. falciparum*** parasite within infected erythrocytes, causing schizonts to degenerate (94), or inhibiting intracellular growth (57,111). Thus, there may be two (or more) groups of antigens expressed on the parasitized erythrocyte surface which elicit antibodies that can block normal parasite metabolism. A purified erythrocyte membrane antigen from ***P. knowJesi*** infected cells has been successfully used to vaccinate rhesus monkeys (101).

Genetic Engineering. –In a novel attempt to study blood stage proteins, Australian investigators manipulated the usual gene-cloning procedure. They extracted messenger RNA from the various blood stage

forms of ***P.*** *falciparum,* copied all of it into complementary DNA, and inserted the complementary DNA into E'. coli. Then, instead of using a MAb to isolate a single piece of DNA that codes for a particular protein, they used human immune serum containing many anti-P. *falciparum* antibodies to identify the many clones manufacturing immunogenic proteins. The result is a library of several hundred clones that express a wide range of so-called "monoclinal antigens, " each of which can be studied in detail. In their ensemble, these clones probably represent a large portion of the antigens carried by ***P. falciparum (59).*** However, it is not yet clear just what antigens they include. The first of these clones to be examined proved to code for a protein with a molecular weight of 220,000 daltons. Although it contains repeated epitopes, like the sporozoite surface protein, it is an S-antigen that varies from strain to strain (19).

Several laboratories are working to streamline recombinant DNA production of malaria antigens. In one approach, developed at the National Institutes of Health (NIH), the genomic DNA itself (as distinct from copies generated through messenger RNA and complementary DNA) is cut into fragments. These gene-carrying fragments are cloned, and the clones screened with MAbs to find which ones are producing the desired antigens (68).

Vaccination With Gametes

Like sporozoites and merozoites, male and female gametes carry antigens that are capable of provoking an immune response. Gamete immunization has prevented parasite fertilization in chicken, mice, and monkey malarias, and blocked subsequent transmission of the disease.

The strategy for gamete vaccination follows a circuitous route. An individual is inoculated with gamete antigens and makes antibodies to them. When a female mosquito feeds on this person, she ingests not only gametocytes, but also antibodies. In the stomach of the mosquito, after the gametes emerge from the erythrocyte casings, they are exposed to these antibodies, which quickly immobilize the male gametes and prevent fertilization.

Studies have shown that chickens immunized against ***P.*** *gallinaceum* gametes produce antibodies that block infectivity in mosquitoes (7,37). Although the chickens were still susceptible to malaria infections, the mosquitoes that fed on those chickens developed no or few oocysts. Subsequently, gamete vaccination against ***P. yoelii*** infection in mice *(69),* as well as ***P. knowlesi*** in the rhesus monkey, totally suppressed gamete infectivity in mosquitoes.

Gamete vaccine research moved ahead when, in 1981, it became possible to culture ***P.*** *falciparum* gametocytes with regularity. Previously, blood stage forms would only sometimes develop into gametocytes in culture. When hypoxanthine, a substance found in many body tissues, was added to the culture, gametocytes were found to predictably develop into mature infectious parasites (55).

Two sets of target antigens have been identified on ***P.*** *falciparum* gametes. The first is a set of three proteins with molecular weights of *250,000, 60,000,* and 55,000 daltons. These antigens occur on both male and female gametes, as well as newly formed zygotes, and are shed shortly after fertilization. Antibodies to these antigens block gamete fertilization and, in the presence of complement, destroy both gametes and zygotes. Unfortunately, these antigens, like some merozoite antigens, may vary within a species *(8).*

The second target of antigamete antibodies is a single 26,000-dalton protein which is synthesized by the zygote and expressed on the zygote surface. Antibody to this antigen prevents zygotes from developing (8).

The gamete vaccine is known as an "altruistic" vaccine because it does not prevent infection or cure disease, or otherwise directly benefit the person being vaccinated. Rather, it benefits the community by drying up the supply of infected parasites, preventing further malaria spread. As a result, it probably will be used only if it is combined with a sporozoite and / or merozoite vaccine. Moreover, its value in the field may be difficult to prove. The effectiveness of a gamete, or transmission-blocking, vaccine would depend on how long immunity lasts, the proportion of the population that is immunized, and the intensity of transmission. In some parts of Africa, the rate of transmission is so high that a single infected individual can lead to the infection of more than *500* others; in such an area a gamete vaccine would hardly make a difference. In other areas, such as India or Sri Lanka, where the transmission is less intense, such a vaccine, possibly combined with other control measures, would have a chance of eliminating malaria (73).

Vaccination With Liver Stage Parasites

Any possibility of developing a liver stage vaccine was impeded until recently by researchers' inability to grow intrahepatic parasites in culture. Although the liver or exoerythrocytic stages of avian malarial parasites had been grown in continuous culture since 1966, it was not until 1981 that mammalian malarial parasites were induced to grow and develop through a complete cycle, beginning with the entry of a sporozoite into the target cell, through the parasite's development

into a liver schizont, complete with the release of merozoites (49). Subsequently, the parasitic infection was carried full cycle. The liver merozoites, injected into mice, caused red blood cell infection, and some of the blood stage parasites in the infected mice developed into gametocytes. Mosquitoes allowed to feed on these mice developed sporozoites, which, when inoculated into the cell culture system, invaded the target cells and became liver schizonts.

The initial work was performed using ***P.*** *berghei* in cultured human embryonic lung cells. More recently, scientists have managed to grow both ***P. falciparum*** and ***P.*** *vivax* (as well as ***P.*** *berghei)* in a cultured cell line derived from a human liver cancer (48). This advance is particularly significant for ***P. vivax*** research, since it has not yet been possible to grow ***P. vivax*** blood stage parasites in continuous cultures.

Using the ***P.*** *berghei* system, it is possible to watch as sporozoites encounter target cells, enter them, and develop into trophozoites. The process of attachment and entry, which seems to parallel in many ways the invasion of merozoites into red blood cells, appears to be effected by the circumsporozoite protein (Pb44); conversely, MAbs to the circumsporozoite protein will block sporozoite entry into liver cells (50).

The hepatoma culture system is also being used to discover how some *P. vivax* liver stage parasites, instead of promptly developing into schizonts, lay dormant for long periods of time. When these dormant forms, hypnozoites, later reactivate and develop into schizonts that release merozoites, they produce a relapse. Electron microscopy is being used to document the differentiation of liver parasites into hypnozoites during the first few hours of development (48).

Scientists are currently working to isolate, purify, and characterize the cell receptor that permits sporozoites to enter the liver cell. Once the nature of this receptor is better understood, it may be possible to prevent infection by suppressing these receptors with drugs. Alternatively, if parasite antigens can be detected on the surface of infected liver cells, it may be possible to attack infected cells by linking antimalarial drugs to antibodies that will recognize and join up with these antigens.

The culture of liver stage plasmodia has yielded two major spinoffs. One, the inhibition of sporozoite invasion assay described above, can be used to evaluate the effectiveness of a sporozoite vaccine in the field. The other is the adaptation of the liver parasite-hepatoma culture system to test new antimalarial drugs. As a fast and inexpensive alternative to testing candidate antimalarial in costly and scarce primates, the tissue culture system promises to revolutionize drug development. Both the U.S. Department of Defense (DOD) and WHO are exploring its use.

Funding Sources

U.S. Government, international, and philanthropic institutions spent about $20 million in 1984 for malaria vaccine research. The biggest contributors are AID and the National Institute of Allergy and Infectious Diseases (NIAID) of NIH. The next biggest are the U,N. Development Program/World Bank/WHO Special Program for Research and Training in Tropical Disease (TDR), DOD, the Centers for Disease Control (CDC), and the Rockefeller Foundation. In addition, a few pharmaceutical companies are conducting malaria vaccine research.

In 1984, AID spent close to $8 million on the development of a malaria vaccine. The $8 million figure represents nearly a doubling of the Agency's original commitment. In late 1983, sensing that the goal was within reach, AID requested an increase in funds that would bring its outlay for fiscal years 1983 to 1985 from $11.9 to $22.7 million.

Since launching the malaria vaccine program in 1966, AID has spent roughly $35 million, and expects the program effort to cost an additional $15 to $25 million before a ***P.*** *falciparum* vaccine is ready for general use. By way of comparison, AID contributed more than $1 billion (and other countries, an additional $4 to $5 billion) to the WHO malaria eradication campaign since its inception in the 1950s (29). AID funded 14 projects throughout the United States in 1984.

AID also provides a variety of support services. One contractor, in addition to research, is charged with testing and characterizing all materials injected into test monkeys; another produces and supplies selected strains of *P. falciparum,* as well as MAbs, to network laboratories. Looking to the future, AID has hired experts to help contractor structure their research so that it will answer Food and Drug Administration (FDA) requirements, and other experts to counsel on matters of patent rights (30). AID is currently taking the lead in laying the groundwork for clinical trials.

NIAID sponsors both intramural and extramural research on malaria, with a total annual expenditure of close to $4 million (120). Including basic research on topics such as recombinant DNA technology or cell receptors, which NIAID itself does not usually classify as "vaccine research" (90), NIAID spends roughly $2 million for intramural research related to malaria immunology, either in the Laboratory of Parasitic Diseases or the Laboratory of Microbial Immunity. The Institute awards an additional $2 million in grants (not contracts) to about 20 institutions, primarily universities, throughout the United States.

Over the past 15 years, the focus of NIAID's malaria research has shifted heavily in favor of immunology and vaccines, and funding commitments have

shown a steady growth. During the first 4 years of this decade, when the tempo of research was rapidly accelerating, NIAID's outlay doubled. However, it represents only a minute fraction of the NIH budget. In 1981, when the NIH was spending a total of *$3.6* billion, NIAID's share was *$232* million or *6* percent; of this, *$27* million went to tropical disease research, and about one-fifth of that was given to all aspects of malaria.

Even though the payoffs may not always be so immediately visible as those of the closely managed, product-oriented AID program, the type of steady support provided by NIAID assures the continuous growth of a broad expanse of knowledge. Beyond achieving their own breakthroughs, or even beyond satisfying intellectual curiosity, such studies generate a rich resource for more intense development projects.

TDR is sponsored jointly by the U.N. Development Program, the World Bank, and WHO. It is funded by contributions from its cosponsors, as well as from the governments of more than *25* countries and from several businesses and foundations.

From its beginning in 1976 through March 1983, TDR had received more than $117 million in contributions: $15 million from the United States, $22 million from Denmark, and $14 million from Sweden, plus *$9* million from the U.N. Development Program, close to $5 million from the World Bank, and $7 million from WHO *(118).* For the 2 years, 1982 and 1983, TDR had budgeted just over $61 million.

Research on all aspects of malaria (chemotherapy and field applications as well as malaria immunology) accounts for about 30 percent of TDR's research and development budget. In 1981, this amounted to $3.2 million. Of this, $1.36 million went to support 40 projects on malaria immunology and vaccines (see chapter 3). Of these, 24 were in the United States (119). Others were in Great Britain, France, Switzerland, and Australia.

Malaria vaccine research within DOD is focused on preventing disease in American troops stationed abroad. Thus, DOD's malaria vaccine efforts give special emphasis to sporozoite and liver stage vaccines, which have the potential of preventing the initial infection and symptoms. AID or TDR efforts are also interested in blood stage vaccines as a means of curbing symptoms and interrupting the parasite's life cycle in areas where the disease is endemic and transmission is heavy.

Within DOD, the Army is the lead service in malaria research, and its work is headquartered at the Walter Reed Army Institute of Research. Navy activities are carried out by the Naval Medical Research Institute.

Although DOD vaccine studies extend back 15 years, studies began to accelerate around 1980, when the new biotechnology opened the possibility of obtaining antigen in purified form. The Army has collaborated with NIH in the effort to clone and characterize a sporozoite antigen; it is also working toward the chemical synthesis of the sporozoite's antigenic epitopes. The Navy has pioneered work on the liver stages of the parasite.

The Army and Navy malaria vaccine groups, each of which employs about 20 persons, work closely together. Expenditures for each of the groups has been estimated to be close to $1 million a year (1,30,45).

CDC of the U.S. Public Health Service participates in malaria vaccine research at several levels. Its primate resource center contains more than 100 New World monkeys of value for research on P. *falciparum* and P. *vivax;* several aspects of malaria immunology are the subjects of in-house research projects; and CDC serves as a reference center for the many strains of P. *falciparum*. It also serves as a source of materials; as of early 1984, CDC has supplied researchers at NYU with 40 million P. *vivax* sporozoites, and was developing millions more. CDC also runs field units on three continents; these may provide appropriate sites for clinical testing.

CDC'S annual expenditure on malaria in recent years has been estimated to be under $1 million (30). CDC allocates approximately $160,000 to $200,000 a year in direct support of malaria vaccine development (6).

The Rockefeller Foundation, through its Great Neglected Diseases of Mankind program, spends just under *$500,000* a year to fund several projects that are directed toward, but not restricted to, malaria vaccines. The three main projects, which have been funded for each of the last 8 years, are located at Harvard, Oxford, and the Walter and Eliza Hall Institute in Melbourne. The group at NYU has also received grants-in-aid, and is currently receiving funds for work on synthetic vaccine development (125a).

Four industrial organizations are involved in vaccine research. The Burroughs-Wellcome Co. of Great Britain, which funnels profits into research efforts through the Wellcome Trust, has a long-standing commitment to tropical disease research. Since 1979, Wellcome scientists have been working on a malaria vaccine, primarily the genetic engineering of blood stage preparations. Its current outlay is approximately $500,000 a year. In Australia, the recently launched Australian Biotechnical Corp. is now investing an estimated $1 to $2 million annually on malaria vaccine projects. Roche Pharmaceuticals, a leader in the antimalarial drug field, recently initiated a collaboration with the Swiss company Biogen to develop a malaria

vaccine, also through genetic engineering. Support is reported to be in excess of $5 million.

The Future of Malaria Vaccine Research

Scientists have at hand an "antigen preparation" for one stage of the parasite (the sporozoite) for two species of human malaria (P. *falciparum* and P. *vivax),* and they are making plans leading to clinical trials. The path leading from there to the successful control of malaria through vaccination is long and uncharted. The challenges ahead include: developing a polyvalent (multiple component) vaccine, demonstrating that it is safe and effective, conducting large field trials in developing countries, producing the vaccines in quantity, and delivering it to the populations at risk.

Developing a Polyvalent Vaccine

Antigens.—An ideal vaccine would likely combat multiple forms of the parasite—sporozoite, blood stage, and/or gamete, and perhaps liver stage, as well as two or more types of malaria—. ***falciparum*** and P. *vivax,* perhaps with F'. ***malariae*** or P. ***ovale.*** Alternatively, different preparations might be prescribed for different populations. A sporozoite vaccine, for instance, might be appropriate for persons whose exposure is limited, such as tourists, whereas a merozoite vaccine could be given to control disease symptoms in an area where malaria is highly endemic; a gamete preparation could be part of a public health campaign to eliminate the disease.

A protective sporozoite antigen for P. *falciparum* and ***P. vivax*** should soon be ready for testing, but it is likely to take another 2 years or more to isolate and produce pure antigens from the blood stages or gametes. Work on liver stage antigens is still preliminary.

To counter the problems of antigenic variation, researchers am exploring a variety of possibilities. These include presenting a parasite structure that is not normally antigenic to the host (but which is common to all of the strains of a species) in such way that it becomes antigenic. Alternatively, they might be able to identify parasite surface structures that play such an important role (for instance, those that enable merozoites to attach to or invade red blood cells) that they should be the same in every strain.

Fortunately, the protective antigen from the sporozoite does not appear to vary from strain to strain (132). For a blood stage or gamete antigen to be useful for a vaccine, however, one must be found that is common to all parasites of a given species, or at least have limited variability. Current experimental work indicates that although a large number of unique geographically defined antigens exist, other antigens are common to many strains. Researchers are now working to discover such antigens, and determine how they might be manipulated for immunization purposes.

Adjuvant.—All experimental malaria vaccine preparations except the sporozoite have required the use of an adjuvant. Unfortunately, the adjuvant that has been most successful in animal studies, Freund's complete adjuvant, produces side effects that make it unacceptable for human use.

Several alternatives are being explored. A bacterial derivative and another substance have both successfully replaced Freund's complete adjuvant in immunizing ***Aotus*** monkeys against ***P. falciparum*** (105,106); however, their effects in humans are not known. Parasite-specific MAbs have also worked as an adjuvant, enhancing the effects of blood stage vaccination of mice *(42).*

Antigenic Variation.—Multiple strains of *P. falciparum,* each with unique antigens, have evolved. Different strains are found not only in different parts of the world, but within given geographic areas. Moreover, some parasite populations appear capable of changing antigens over the course of an infection.

Demonstrating Safety and Efficacy

A likely scenario for the trial of a candidate vaccine begins with several months of testing for toxicity and carcinogenicity in mice and rabbits. Next the vaccine would be tested in primates to see that the preparation produces no discernible adverse effects and that it does stimulate a protective immune response. Assuming that all is going well, the investigators will file an Investigational New Drug application with FDA.

The first clinical tests (which might begin as soon as 1985, under ideal conditions) will use healthy male volunteers, recruited either from a university setting, the military, or industry, and including some of the scientists themselves (29). Again, the first round would be to make sure that the preparation produces no adverse effects. A second round, lasting 18 months, would be designed to answer questions of efficacy: Does it stimulate antibody production? Are these antibodies protective? How long does protection last? In the case of an AID-sponsored vaccine, these volunteer studies would then be replicated in endemic areas, using local volunteers and personnel (29).

Conducting Field Trials

The pilot vaccine will first be tested in healthy males, then healthy nonpregnant females, and then in pregnant females, and children, In addition to the basic

issues of safety and efficacy, new questions will arise in endemic areas. Will persons whose immune responses have been dampened by previous malaria infections respond in the same way as volunteers who have never had the disease? Will nutrition affect the body's ability to respond? What about antimalarial drugs?

Neither sites for field trials nor strategies have been arranged. AID, which will work closely with WHO in setting up, and to some extent funding, clinical trials, had convened two planning meetings by mid-1984 with representatives from DOD, CDC, FDA, NIH, and the Pan American Health Organization, as well as WHO. In March 1985, clinical trials will be the focus of a meeting of the scientific working group of WHO's Malaria Immunology program.

For the results of the trial to be meaningful, epidemiologists will need to have mapped patterns of malaria transmission in the test area, and documented the extent of preexisting immunity. The trials will need to be carefully planned and closely supervised, conducted by skilled personnel working in close cooperation with the test population. Those people who are vaccinated will have to be closely monitored and treated when necessary.

Producing a Vaccine

The research institutions in which vaccines are being developed are not geared to produce large quantities of vaccine material; that formidable task will be the concern of pharmaceutical or genetic engineering companies.

The ensuing interrelationships among scientists/universities, research sponsors such as AID and WHO, and industry lead to a tangle of conflicting interests. Who "owns" the discovery? Who should make a profit from it? What are the incentives for genetic engineering/pharmaceutical companies to get involved? Who will buy a vaccine—AID, WHO, DOD, philanthropic foundations?

The issues took shape in 1981, following the Supreme Court's ruling that biological are patentable. NYU filed patents for the Nussenzweig group's work (presumably involving MAbs used in identifying and cloning the circumsporozoite protein). When NYU entered into negotiations with a genetic engineering firm, Genentech, to produce the circumsporozoite protein, Genentech asked for exclusive license to market the vaccine.

WHO, which had long supported the NYU work, and which represents many developing countries, held fast to its contractual requirements for public access to work that it supports. AID, another Nussenzweig sponsor, holds patent rights in the United States under Federal law; AID asked NYU to submit the requisite "petition for greater rights."

The conflict dissipated in *1983* when NYU and Genentech dropped their plans to work together. Genentech said it was too busy with other projects; NYU developed superior genetic engineering capabilities of its own. The issues raised, however, remain unanswered,

To date, seven patents have reportedly been applied for in the United States by four different laboratories (30). Four involve sporozoite vaccines, and three are related to merozoite vaccines.

In the meantime, AID is in the process of revising the patent language in its contracts. The new U.S. patent law, which took effect in 1981, allows grant recipients to take out patents on Government-sponsored work, providing the Government is allowed royalty-free use of the invention. AID's goal is to make sure that any agreements struck between its contractors and industry will not impede a vaccine's getting to the market, AID would also like the vaccine to be available to Third WorId countries and to the U.S. miLitary on a cost-plus basis (58).

AID has held discussions with six domestic companies possibly interested in producing a vaccine. At this time it *seems* likely that vaccine development in this country will proceed under the Orphan Drug program of FDA (30). Overseas companies, particularly those that have established working relationships in the developing countries where malaria is prevalent, have also expressed some interest. Parke-Davis, the sole U.S. drug company to be involved in malaria vaccine development in the past, and which worked through the AID program, pulled out of the field when the company was sold in the late 1970s,

Delivering a Vaccine

In order for a malaria vaccine to alleviate disease and prevent death, it must reach the people who inhabit those parts of the tropics, often impoverished and remote, where the disease is prevalent. To accomplish this, it will be necessary to raise funds, build an excellent logistical support system, and train personnel. The success of such an effort will depend on close collaboration among international organizations, industry, philanthropic foundations, and national health systems.

The history of the human battle against malaria has been marked by a series of overly optimistic expectations followed by disappointments (41), and the hopes for a vaccine may prove no exception. However, the flood of progress has been so strong, and the possibilities created by the new technologies so vast, that re-

searchers are resorting to phrases like "the most incredible time in the history of malariology. "

Conclusions

Having watched the most pressing problems of the 197&-antigen supply and purity-give way before the wonders of genetic engineering and protein chemistry, scientists are confident that, with ingenuity, they will be able to meet today's challenges. Perhaps it will be possible to boost an antigen's immunogenicity by presenting it to the host in a new way, or to prolong immunity by developing slow-releasing antigens, or to "vaccinate" people by incorporating a parasite gene into bacteria that normally inhabit the gastrointestinal tract. Louis Miller of NIAID likens the search for solutions to standing next to a wall: "Suddenly someone puts a hole in it and beyond are vistas we've never imagined" (73).

The problems of testing, production, and delivery are no less imposing, but again the outlook is optimistic, and planners are pressing ahead. Moreover, the liaisons and lessons of the WHO malaria eradication campaign should stand the vaccine effort in good stead.

Just what form the first vaccine will take, and where and when it will appear cannot be predicted, but, according to Miller, "there is no question that vaccines will be developed against malaria; vaccines have been successful in every animal model tested" (73).

The sentiment is a venerable one in malaria research. In 1897, Ronald Ross, in an attempt to prove that the mosquito was the source of human infection, faced the prospect of dissecting thousands of mosquitoes. Undaunted, he wrote: "The things are there and *must* be found. It is simply a matter of hard work" (41).

Not even its most enthusiastic proponents expect a vaccine, of itself, to subdue malaria. To be successful, a vaccine must be complemented by both improved vector control and better drugs—and fresh, creative approaches to both are being explored. These include mosquito-killing bacteria, mosquito-devouring fish, and mosquito-debilitating micro-organisms, on the one hand, and a Chinese herbal remedy, on the other. A three-pronged attack, combining vaccine(s), drugs, and vector control, provides the best chance yet of bettering the lives of millions.

Case Study B References

1. Beaudoin, R. L., Naval Medical Research Institute, National Naval Medical Center, Bethesda, MD, personal communication, June 1984.
2. Brown, G. V., Anders, R. F., Mitchell, G. F., et al., "Target Antigens of Purified Human Immunoglobulin Which Inhibit Growth of *Plasmodium falciparum* in Vitro," *Nature* 297:591-593, *1982.*
3. Brown, K. N., and Brown, I. N., "Immunity to Malaria: Antigenic Variation in Chronic Infections to *Plasmodium knowlesi*, " *Nature 208: 1286-1288, 1965.*
4. Brown, K. N., Brown, I. N., and Hills, L. A., "Immunity to Malaria. I. Protection Against *Plasmodium knowlesi* Shown by Monkeys Sensitized With Drug-Suppressed Infections or by Dead Parasites in Freund's Adjutant, " *Exp. Parasitol.* 28:304-317, *1970.*
5. Bruce-Chwatt, L., "Malaria: From Eradication to Control, " New *Scientist,* pp. 17-20, Apr. 19, 1984.
6. Campbell, C. C., Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA, personal communication, June 1984.
7. Carter, R., and Chen, D. H., "Malaria Transmission Blocked by Immunization With Gametes of the Malaria Parasite, " *Nature* 263:57-60, *1976.*
8. Carter, R., Miller, L. H., Rener, J., et al., "Target Antigens of Transmission-Blocking Immunity, " *Proc. Roy. Soc.,* in press.
9. Chapin, G., and Wasserstrom, R., "Pesticide Use and Malaria Resurgence in Central America and India, " Soc. Sci. *Med.* 17:270-293, *1984.*
10. Clark, I. A., Virelizier, J.-L., Carswell, E. A., et al., "Possible Importance of Macrophage-Derived Mediators in Acute Malaria, " *Infect. Immun.* 32:1058-1066, *1981.*
11. Clyde, D. F., "Immunisation of Man Against Falciparum and Vivax Malaria by Use of Attenuated Sporozoites, " *Am. J. Trop. Med. Hyg.* 24:397-401, *1975,*
12. Clyde, D. F., Most, H., McCarthy, V., et al., "Immunization of Man Against Sporozoite-Induced Falciparum Malaria, " ***Am. J. Med. Sci.*** 266:166-177, ***1973.***
13. Cochrane, A. H., Santoro, F., Nussenzweig, V., et al., "Monoclinal Antibodies Identify the Protective Antigens of Sporozoites of *Plasm odium knowlesi*," *Proc. Nat. Acad. Sci. (USA)* 79:5651, *1982.*
14. Coggeshall, L. T., and Kumm, H. W., "Effect of Repeated Superinfection Upon Potency of Immune Serum of Monkeys Harboring Chronic Infections of *Plasmodium knowlesi*, " *J. Exp. Med.* 68:17-27, *1938.*
15. Cohen, S., "Progress in Malaria Vaccine Development, " *Br. Med. Bull.* 38:161-165, *1982.*
16. Cohen, S., Butcher, G. A., and Crandall, R. B.,

"Action of Malarial Antibody in Vitro," *Nature* 223:368-371, *1969*.
17. Cohen, S., McGregor, I. A., and Barrington, S. C., "Gamma Globulin and Acquired Immunity to Human Malaria," *Nature 192:733-737, 1961*.
18. Collins, W. E., and Contacos, P. G., "Immunization of Monkeys Against *Plasmodium cynomolgi* by X-Irradiated Sporozoites," *Nature* 236:176-177, *1972*.
19. Coppel, R. L., Cowman, A. F., Lingelbach, K. R., et al., "Isolate-Specific S-Antigen of *Plasmodium falciparum* Contains a Repeated Sequence of Eleven Amino Acids," *Nature* 306:751-756, *1983*.
20. Cross, G., Rockefeller University, New York, personal communication, June *1984*.
21. Dame, J. B., Williams, J. L., McCutchan, T. F., et al., "Structure of the Gene Encoding the Immunodominant Surface Antigen on the Sporozoite of the Human Malaria Parasite *Plasmodium falciparum*," *Science 225(4662):593-599, 1984*.
22. David, P. H., Hadley, T. J., Aikawa, M., et al., "Processing of a Major Surface Glycoprotein Occurs During the Ultimate Stages of Differentiation in *Plasmodium knowlesi* Malaria," *Mol. Biochem. Parasitol.*, in press.
23. Deans, J. A., Alderson, T., Thomas, A. W., et al., "Rat Monoclinal Antibodies Which Inhibit the in Vitro Multiplication of *Plasmodium knowlesi*," *Clin. Exp. Immune].* 49:297-309, *1982*.
24. Deans, J. A., and Cohen, S., "Immunology of Malaria," *Am. Rev. Microbiol.* 37:25-49, *1983*.
25. Desowitz, R. S., and Miller, L. H., "A Perspective on Malaria Vaccines," *Bull. W.H. O. 58:897-908, 1980*.
26. Elliott, V., "The Funding of Tropical Disease Research," preliminary report prepared for the Office of Technology Assessment, U.S. Congress, Washington, DC, March *1984*,
27. Ellis, J., Ozaki, L. S., Gwadz, R. W., et al., "Cloning and Expression in *E. coli* of the Malarial Sporozoite Surface Antigen Gene From *Plasmodium knowlesi*," *Nature* 302:536-538, *1983*.
28. Epstein, N., Miller, L. H., Kaushal, D. C., et al., "Monoclinal Antibodies Against a Specific Surface Determinant on Malaria (*Plasmodium knowlesi*) Merozoites Block Erythrocyte Invasion," *J. Immune].* 127:212-217, 1981.
29. Erickson, J. M., "Malaria Vaccine Development: An Integrated Management and Research Appreach," presentation to National Council for International Health, Department of State, Washington, DC, Apr. *26, 1984*.
30. Erickson, J. M., U.S. Agency for International Development, Washington, DC, personal communication, April 1984.
31. Freeman, R. F., Trejdosieqic, A. J., and Cross, G., "Protective Monoclinal Antibodies Recognizing Stage-Specific Merozoite Antigens of Rodent Malaria Parasites," *Nature* 284:366-370, 1980.
32. Freund, J., Thomson, K. O., Sommer, H. E., et al., "Immunization of Monkeys Against Malaria by Means of Killed Parasites With Adjutants," *Am. J. Trop. Med.* 28:1-22, *1948*.
33. Friedman, M. J., Roth, E. F., Nagel, R. L., et al., "*Plasmodium falciparum*: Physiological Interactions With the Human Sickle Cell," *Exp. Parasitol.* 47:73-80, 1979.
34. Glysin, J., Barnwell, J., Schlesinger, D. H., et al., "Neutralization of the Infectivity of Sporozoites From *Plasmodium knowlesi* by Antibodies to a Synthetic Peptide," *J. Exp. Med.*, in press.
35. Godson, G. N., Ellis, J., Svec, P., et al., "Identification and Chemical Synthesis of a Tandemly Repeated Immunogenic Region of *Plasmodium knowlesi* Circumsporozoite Protein," *Nature* 305:29-33, 1983.
36. Grun, J. L., and Weidanz, W. O., "Immunity to *Plasmodium chabaudi adami* in the T-Cell Deficient Mouse," *Nature* 290:143-145, 1981.
37. Gwadz, R. W., "Malaria: Successful Immunization Against the Sexual Stages of *Plasmodium gallinaceum*," *Science* 193:1150-1151, 1976.
38. Gwadz, R. W., and Green, I., "Malaria Immunization in Rhesus Monkeys: A Vaccine Effective Against Both the Sexual and Asexual Stages of *Plasmodium knowlesi*," *J. Exp. Med.* 148: 1311-1323, 1978.
39. Gwadz, R. W., Cochrane, A. H., Nussenzweig, V., et al., "Preliminary Studies on Vaccination of Rhesus Monkeys With Irradiated Sporozoites of *Plasmodium knowlesi* and Characterization of Surface Antigens of These Parasites," *Bull. W.H. O.* 57(Suppl.1):165-173, 1979,
40. Hadley, T. J., David, P. H., McGinniss, M. H., et al., "Identification of an Erythrocyte Component Carrying the Duffy Blood Group Fy-a Antigen, " *Science* 223:597-599, 1984.
41. Harrison, G., *Mosquitoes, Malaria & Man: A History of the Hostilities Since 1880* (New York: E.P. Dutton, 1978).
42. Harte, P. G., Cooke, A., and Playfair, J. H, L., "Specific Monoclinal IgM Isa Potent Adjuvant in Murine Malaria Vaccination," *Nature* 302:

256-257, 1983.
43. Haynes, J. D., Diggs, C. L., Hines, F. A., et al., "Culture of Human Malaria Parasites *Plasmodium falciparum*," *Nature* 263:767-769, *1976.*
44. Heidelberger, M., Prout, C., Hindle, J. A., et al., "Studies in Human Malaria. II. An Attempt at Vaccination of Paretics Against Blood-Borne Infections With *Plasmodium vivax*," *J. Immunol.* 53:109-112, *1946.*
45. Hockmeyer, W. T., Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC, personal communication, May 1984.
46. Holder, A. A., and Freeman, R. R., "Immunization Against Blood-Stage Rodent Malaria Using Purified Parasite Antigens," *Nature 294:361-364, 1981.*
47. Holder, A. A., and Freeman, R. R., "Biosynthesis and Processing of a *Plasmodium falciparum* Schizont Antigen Recognized by Immune Serum and a Monoclinal Antibody," ***J. Exp. Med.*** 156:1528-1538, 1982.
48. Hollingdale, M. R., Bio-Medical Research Institute, Rockville, MD, personal communication, May 1984.
49. Hollingdale, M, R., Leef, J. L., McCullough, M., et al., "In Vitro Cultivation of the Exoerythrocytic Stage of *Plasmodium berghei* From Sporozoites," ***Science*** 213:1021-1022, 1981.
50. Hollingdale, M. R., Leland, P., Leef, J. L., et al., "Entry of *Plasmodium berghei* Sporozoites Into Cultured Cells, and Their Transformation Into Trophozoites,"*Am. J. Trop. Med. Hyg. 32:685-690, 1983.*
51 Hollingdale, M. R., Nardin, E. H., Tharavanig, S., et al., "Inhibition of Entry of *Plasmodium falciparum* and *P. vivax* Sporozoites Into Cultured Cells; An in Vitro Assay of Protective Antibodies," ***J. Immunol.*** 132:909-913, *1984.*
52. Hommel, M., "Malaria: Immunity and Prospects for Vaccination," *West. J. Med. 135:285-299,* 1981.
53. Hommel, M., David, P. H., and Oligino, L. D., "Surface Alterations of Erythrocytes in *Plasmodium falciparum* Malaria: Antigenic Variation, Antigenic Diversity and the Role of the Spleen," ***J. Exp. Med.*** 157:1137-1148, ***1983.***
54. Hommel, M., David, P. H., Oligino, L. D., et al., "Expression of Strain-Specific Surface Antigens on *Plasmodium falciparum*-Infected Erythrocytes," *Parasite Immunol.* 4:409-414, *1982.*
55, Ifediba, T., and Vandenberg, J. P., "Complete in Vitro Maturation of Falciparum Gametocytes," *Nature* 294:364-366, 1981.
56. Jayawardena, A. N., Targett, G. A. T., Carter, R. L., et al., "The Immunological Response of CBA Mice to *P. yoelii*. I. General Characteristics, the Effects of T-Cell Deprivation and Reconstitution With Thymus Grafts," *Immunology* 32:849-59, *1977.*
57. Jensen, J. B., Boland, M. T., and Akod, M., "Induction of Crisis Forms in Cultured *Plasmodium falciparum* With Human Immune Serum From Sudan," *Science* 216:1230-1233, 1982.
58. Jordan, D., American Institute for Biological Sciences, Arlington, VA, personal communication, May *1984.*
59. Kemp, D. J., Coppel, R. L., Cowman, A. F., et al., "Expression of *Plasmodium falciparum* Blood-Stage Antigens in *Escherichia coli*: Detection With Antibodies From Immune Humans," *Proc. Nat. Acad. Sci. (USA)* 80:3787-3791, 1983.
60. Kilejian, A., "Histidine-Rich Protein as a Model Malaria Vaccine," *Science* 201:922-924, *1978.*
61. Kilejian, A., "Characterization of a Protein Correlated With the Production of Knob-Like Protrusions on Membranes of Erythrocytes Infected With *Plasmodium falciparum*," *Proc. Nat. Acad. Sci. (USA)* 76:4650-4653, 1979.
62. Krotoski, W.A., Garnham, P. C. C., Bray, R. S., et al., "Observations on Early and Late Post-Sporozoite Tissues in Primate Malaria. I. Discovery of a New Latent Form (Hypnozoite), and Failure of the Immunofluorescence Technique To Detect Hepatic Forms Within the First 24 Hours After Infection," *Am. J. Trop. Med. Hyg.* 31:24-35, *1982.*
63. Lancet, "Epitaph for Global Malaria Eradication?" ***Lancet*** *15-16, 1975.*
64. Laveran, C. L. A., "A Newly Discovered Parasite in the Blood of Patients Suffering From Malaria: Parasitic Etiology of Attacks of Malaria (Classics in Infectious Diseases)," Rev. ***Infect. Dis.*** 4:908-911, ***1982.***
65. Lucas, A. O., "New Knowledge is Accumulating Fast" (Interview), *WHO Chronicle* 36:26-36, *1982.*
66. Majarian, W. R., Daly, T. M., Weidanz, W. P., et al., "Passive Immunization Against Murine Malaria With an IgG 3 Monoclinal Antibody," ***J. Immunol.***, in press.
67. McBride, J. S., Walliker, D., and Morgan, G., "Antigenic Diversity in the Human Malaria parasite *Plasmodium falciparum*," *Science 217: 254-257, 1982.*
68. McCutchan, T. F., Hansen, J. L., Dame, J. B., et al., "Mung Bean Nuclease Cleaves *Plasmodium*

Genomic DNA at Sites Before and After Genes," *Science* 225:625-628, 1984.
69. Mendis, K. N., and Targett, G. A. T., "Immunization Against Gametes and Asexual Erythrocytic Stages of a Rodent Malaria Parasite, " *Nature* 277:389-391, *1979*,
70! Miller, L. H., "Distribution of Mature Trophozoites and Schizonts of *Plasmodium falciparum* in the Organs of the *Aotus trivirgatus*, the Night Monkey," *Am. J. Trop. Med. Hyg.* 18:860-865, 1969.
71, Miller, L. H., "Current Prospects and Problems for a Malaria Vaccine," *J. Infect. Dis.* 135:855-864, 1977.
72, Miller, L. H., "Malaria," *Tropical and Geographical Medicine*, K.S. Warren and A.A.F. Mahmoud (eds.) (New York: McGraw-Hill, 1984).
73. Miller, L. H., National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, personal communication, April 1984.
74. Miller, L. H., Aikawa, M., and Dvorak, J. A., "Malaria (*Plasmodium knowlesi*) Merozoites: Immunity and the Surface Coat, " *J. Immunol.* 114:1237-1242, 1975.
75. Miller, L. H., David, P. H., and Hadley, T. J., "Perspectives for Malaria Vaccination, " *Proc. Roy. Soc.*, in press.
76. Miller, L. H., Aikawa, M., Johnson, J. G., et al., "Interaction Between Cytochalasin b-Treated Malarial Parasites and Erythrocytes, " *J. Exp. Med.* 149:172-184, 1979.
77. Mitchell, G. H., Butcher, G. A., and Cohen, S., "A Merozoite Vaccine Against *Plasmodium knowlesi* Malaria," *Nature* 252:311-313, *1974*.
78. Mitchell, G. H., Butcher, G. A., Richard, W. H. G., et al., "Merozoite Vaccination of Douroucouli Monkeys Against Falciparum Malaria, " *Lancet* 1:1335-1338, 1977.
79. Mulligan, H. W., Russell, P. F., and Mohan, B. N., "Active Immunization of Fowls Against *P. gallinaceum* by Injections of Killed Homologous Sporozoites, " *J. Malaria Inst. India 4:25-34*, 1941.
80. Nardin, E. H., Nussenzweig, R. S., McGregor, I. A., et al., "Antibodies to Sporozoites: Their Frequent Occurrence in Individuals Living in Areas of Hyperendemic Malaria, " ***Science*** *206: 597-599, 1979.*
81 Nardin, E. H., Nussenzweig, V., Nussenzweig, R. S., et al., "Circumsporozoite Proteins of Human Malaria Parasites *Plasmodium falciparum* and *Plasmodium vivax*, " *J. Exp. Med.* 156:20-29, 1982.
82. Neva, F. A., "Looking Back for a View of the Future: Observations on Immunity to Induced Malaria," *Am. J. Trop. Med. Hyg.* 26:211-215, 1977.
83. Neva, F. A., National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, personal communication, May 1984.
84. Nussenzweig, R. S., "Progress in Malaria Vaccine Development: Characterization of Protective Antigens, " *Scand. J. Infect. Dis.* (Suppl.) 36:40-45, *1982.*
85. Nussenzweig, R. S., New York University Medical Center, New York, personal communication, May 1984.
86. Nussenzweig, R. S., Vandenberg, J., and Most, H., "Protective Immunity Produced by the Injection of X-Irradiated Sporozoites of *Plasmodium berghei*. IV. Dose Response, Specificity and Humoral Immunity, " *Milit. Med.* 134:1176-1182, 1969.
87. Nussenzweig, R. S., Vandenberg, J., Most, H., et al., "Protective Immunity Produced by the Injection of X-Irradiated Sporozoites of *Plasmodium berghei*, " *Nature* 216:160-162, *1967.*
88. Nussenzweig, R. S., Vandenberg, J., Most, H., et al., "Specificity of Protective Immunity Induced by *Plasmodium berghei* Sporozoites," ***Nature*** 222:488-489, 1969.
89. Nussenzweig, R. S., Vandenberg, J., Spitalny, G., et al., "Sporozoite-Induced Immunity in Mammalian Malaria: A Review," *Am. J. Trop. Med. Hyg.* 21:722-728, 1972.
90. Nutter, J., National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, personal communication, May 1984.
91. Ojo-Amaize, E. A., Vilcek, J., Cochrane, A. H., et al., "*Plasmodium berghei* Sporozoites Are Mitogenic for Murine T-Cells, Induce Interferon, and Activate Natural Killer Cells, " *J. Immunol.* 133:1-5, 1984.
92. Orjih, A. U., Cochrane, A. H., and Nussenzweig, R. S., "Active Immunization and Passive Transfer of Resistance Against Sporozoite-Induced Malaria in Infant Mice, " *Nature* 291: 331-332, 1981.
93. Perrin, L. H., and Dayal, R., "Immunity to Asexual Erythrocyte Stages of *P. falciparum*: Role of Defined Antigens in the Humoral Response," *Immunol. Rev.* 61:245-269, 1982.
94. Perrin, L. H., Ramirez, E., Lambert, P. H., et al., "Inhibition of *P. falciparum* Growth in Human Erythrocytes by Monoclinal Antibodies," *Na-*

ture 289:301-303, *1981.*

95. Peters, W., "Current Concepts in Parasitology: Malaria," N. *Eng. J. Med.* 297:1261-1264, *1977.*
96. Peters, W., "Panel on Malaria: Introduction, " *Scand. J. Infect. Dis. 36* (Suppl.):24-25, *1982.*
97. Potocnjak, P., Yoshida, N., Nussenzweig, R. S., et al., "Monovalent Fragments (Fab) of Monoclonal Antibodies to a Sporozoite Surface Antigen (Pb44) Protect Mice Against Malaria Infection," *J. Exp. Med.* 151:1504-1512, *1980.*
98. Richards, W. H. G., "Active Immunisation of Chicks Against *Plasmodium gallinaceum* by Inactivated Homologous Sporozoites and Erythrocytic Parasites, "Nature 212:1392-1394, 1966.
99. Richards, W. H. G., "Antigenic Studies of the Class Sporozoa With Particular Reference to Species of *Plasmodium*, " Ph.D. thesis, University of London, 1969.
100 Rieckmann, K. H., Carson, P. E., Beaudoin, R. L., et al., "Sporozoite Induced Immunity in Man Against an Ethiopian Strain of *Plasmodium falciparum*," *Trans. Roy. Soc. Trop. Med. Hyg.* 68:258-259, *1974.*
101. Schmidt-Ullrich, R., Lightholder, J., and Monroe, M. T. M., "Protective *Plasmodium knowlesi* Mr *74,000* Antigen in Membranes of Schizont-Infected Rhesus Erythrocytes, " *J. Exp. Med.* 158:146-158, *1983.*
102. Schwartz, A. L., Fridovich, S. E., Knowles, B. B., et al., "Characterization of the Asialoglycoprotein Receptor in a Continuous Hepatoma Line," *J. Biol. Chem.* 256:8878-8881, *1981.*
103. Sergent, E., and Sergent E., "Sur l'immunite clans le paludisme des oiseaux. Conservation in vitro des sporozoites de *Plasmodium relictum*. Immunite relative obtenue par inoculation de ces sporozoites, " *Comptes rendues de l'Academie des Sciences, Paris 151:407-409,* 1910.
104. Siddiqui, W. A., "An Effective Immunization of Experimental Monkeys Against a Human Malaria Parasite, *Plasmodium falciparum*," *Science* 197:388-389, *1977,*
105. Siddiqui, W. A., Kan, S.-C., Kramer, K., et al., "Use of a Synthetic Adjuvant in an Effective Vaccination of Monkeys Against Malaria, "*Nature* 289:64-66, 1981.
106. Siddiqui, W. A., Taylor, D. W., Kan, S.-C., et al., "Vaccination of Experimental Monkeys Against *Plasmodium falciparum:* A Possible Safe Adjuvant," *Science* 210:1237-1239, *1978.*
107. Smith, G, L., Godson, G. N., Nussenzweig, V., et al., "*Plasmodium knowlesi* Sporozoite Antigen: Expression by Infectious Recombinant Vaccinia Virus," *Science* 224:397-399, 1984.
108. Smith, G. L., Mackett, M., and Moss, B., "Infectious Vaccinia Virus Recombinant That Express Hepatitis B Surface Antigen, " *Nature* 302:490-495, *1983.*
109. Spitalny, G. L., and Nussenzweig, R. S., "Effect of Various Routes of Immunization and Methods of Parasite Attenuation on the Development of Protection Against Sporozoite Induced Rodent Malaria," *Proc. Helminth. Soc. Wash. 30* (Special Issue) :506-514, 1972.
110. Spitalny, G. L., Verhave, J. P., Meuswissen, J. H. E. T., et al., "*Plasmodium berghei*: T-Cell Dependence of Sporozoite-Induced Immunit y in Rodents," *Exp. Parasitol.* 42:73-81, *1977.*
111. Stanley, H. A., and Reese, R. T., "In Vitro Inhibition of Intracellular Growth of *Plasmodium falciparum* by Immune Sera, " *Am. J. Trop. Med. Hyg.* 33:12-16, 1984.
112. Tapchaisri, P., Chomcharan, Y., Poonthong, C., et al., "Antisporozoite Antibodies Induced by Natural Infection, " *Am. J. Trop. Med. Hyg.* 32:1203-1208, *1983.*
113. Targett, G. A. T., and Fulton, J. D., "Immunization of Rhesus Monkeys Against *Plasmodium knowlesi* Malaria," *Exp. Parasitol.* 17:180-193, *1965.*
114. Thomson, K. J., Freund, J., Sommer, H. E., et al., "Immunization of Ducks Against Malaria by Means of Killed Parasites With or Without Adjutants," Am. *J. Trop. Med. Hyg.* 27:70-105, *1947.*
115. Trager, W., and Jensen, J. B., "Human Malaria Parasites in Continuous Culture," *Science 193: 673-675, 1976.*
116. Trager, W., and Jensen, J. B., "Cultivation of Malarial Parasites," *Nature* 273:621-622, *1978.*
117. Udeinya, I. J., Miller, L. H., McGregor, I. A., et al., "*Plasmodium falciparum* Strain-Specific Antibody Blocks Binding of Infected Erythrocytes to Amelanotic Melanoma Cells, " *Nature 303: 429-431, 1983.*
118. U.N. Development Program/World Bank/ WHO, Special Programme for Research and Training in Tropical Diseases, *Sixth Programme Report: 1 July 1981-31 December 1982* (Geneva: WHO, *1983).*
119. U.S. Agency for International Development, *A Profile of Selected Biomedical Research Efforts into Diseases of Major Public Health Importance to People of Developing Countries* (Washington, DC: AID, 1982).
120. U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Allergy and Infectious Diseases,

NIAID Awards and Projects, 1967-1983 (Washington, DC: DHHS, 1984).

121. Vandenberg, J. P., Nussenzweig, R. S., and Most, H., "Protective Immunity Produced by the Injection of X-Irradiated Sporozoites of *Plasmodium berghei*. V. In Vitro Effects of Immune Serum on Sporozoites," *Milit. Med.* 134:1183-1190, 1969.
122. Vandenberg, J. P., Nussenzweig, R. S., and Most, H., "Protective Immunity Produced by the Bite of X-Irradiated Mosquitoes Infected With *Plasmodium berghei*," *J. Parasit.* 56:350-351, *1970.*
123. Vandenberg, J. P., Nussenzweig, R. S., Sanabria, Y., et al., "Stage Specificity of Anti-Sporozoite Antibodies in Rodent Malaria and Its Relationship to Protective Immunity," *Proc. Helminth. Soc. Wash.* 39:514-525, *1972.*
124. Vogel, R. J., University of Arizona, Tucson, AZ, personal communication, June 1984.
125. Ward, R. A., and Hayes, D. E., "Attempted Immunization of Rhesus Monkeys Against Cynomolgi Malaria With Irradiated Sporozoites," *Proc. Helminth. Soc. Wash.* 39:525-529, 1972.

125a. Warren, K. S., Rockefeller Foundation, New York, personal communication, 1984.

126. Wigzell, H., "Malaria Immunology," *Scand. J. Infect. Dis.* 36(Suppl.):37-39, *1982.*
127. World Health Organization, "Development of Malaria Vaccines: Memorandum From a USAID/WHO Meeting," *Bull. W.H. O.* 61:81-92, *1983.*
128. Wyler, D. J., "Malaria-Resurgence, Resistance, and Research," *New Eng. J. Med.* 303:875-878, 934-940, 1983.
129. Yoshida, N., Nussenzweig, R. S., Potocnjak, P., et al., "Hybridoma Produces Protective Antibodies Directed Against the Sporozoite Stage of Malaria Parasite," *Science* 207:71-73, 1980.
130. Yoshida, N., Potocnjak, P., Nussenzweig, V., et al., "The Protective Antigen of Sporozoites of *Plasmodium berghei*," *J. Exp. Med.* 154:1225, *1236, 1981.*
131. Zavala, F., Cochrane, A. H., Nardin, A. H., et al., "Circumsporozoite Proteins of Malaria Parasites Contain a Single Immunodominant Region With Two or More Identical Epitomes," *J. Exp. Med.* 157:1947-1957, *1983.*
132. Zavala, R., Masuda, A., and Nussenzweig, R. S., "Species-Specific Epitope Is Identical in Phenotypically Different Circumsporozoite Proteins of Human Malaria," *Fed. Proc.* 43:1808 (Abst.), 1984.