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Reflections on Early Malaria Vaccine Studies, the First Successful Human Malaria Vaccination, and Beyond☆

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Abstract

Advances towards protective vaccines against malaria were made feasible by the development of a rodent model of mammalian malaria that allowed production of all stages of the malaria parasite for study. Investigations with sporozoites (the stage transmitted by mosquitoes in their saliva) demonstrated that immunization with radiation-attenuated sporozoites could produce a solid, sterile immunity, first shown in studies with mice and later with human volunteers. Protective immune mechanisms involve anti-sporozoite antibodies that immobilize sporozoites injected into the skin by mosquitoes, followed by CD4+ and CD8+ T-cells acting against liver stage parasites produced by sporozoites that have escaped antibody-based immunity and invaded hepatocytes. Two alternative approaches now being used in human trials are immunization with intact, attenuated sporozoites vs. immunization with "sub-unit" vaccines based on immunogenic components of sporozoites or liver stage parasites. In addition to immunization against these pre-erythrocytic stages, encouraging progress is being made on immunization against blood stage parasites and on immunization for production of transmission-blocking antibodies. There is reason to be optimistic that one or more of the approaches will work on a large scale, and that a multi-stage vaccine may be able to combine several of these approaches in a sequential immunological assault against the malaria parasite as it progresses through its stages.

Keywords

Vaccines; malaria; sporozoite; history

1. Introduction

A recent review on vaccination against malaria in "Vaccine" [1] described the disease burden posed by malaria and the need for a vaccine that can protect against this devastating disease. It concluded that the most advanced and well-documented vaccine candidates rely on protective immunity induced by the circumsporozoite protein (CSP) found at the surface of the sporozoite, the stage injected by the mosquito to initiate the disease. One approach to vaccination has been the use of intact, attenuated sporozoites to induce a sterile immunity generated by protective anti-CSP antibodies acting against mosquito-injected sporozoite challenges [2], together with

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CD4+ and CD8+ T-cells that recognize and act against hepatocytes invaded by sporozoites [3]. This attenuated sporozoite approach was first successfully carried out by injection of X-irradiated sporozoites of the rodent malaria parasite *Plasmodium berghei* into mice [4] and soon followed by allowing X-irradiated mosquitoes to inject sporozoites of the human malaria parasite *P. falciparum* into human volunteers [5]. A compendium of human vaccination trials with this approach has shown > 90% of volunteers to be completely protected against challenge by bite of infected mosquitoes [6]. Plans by this group are underway to attempt to vaccinate large numbers of humans by syringe injection of purified, irradiated *P. falciparum* sporozoites (7].

A second approach attempts to use “sub-unit” vaccines based on immunogenic components of sporozoites or liver stage parasites. The review [1] noted that there are multiple such vaccine candidates and concluded that the most advanced candidate is RTS,S. This includes a polypeptide corresponding to amino acids 207 to 395 of the CSP from the human malaria parasite, *P. falciparum*, fused to the hepatitis B surface antigen and expressed in the form of virus-like particles in yeast cells [8]. A recent trial testing this vaccine on infants in a highly endemic area of Mozambique concluded that the adjusted vaccine efficacy was 65.9% [9], although this interpretation of the actual degree of efficacy remains controversial [10].

Thus, in view of the great interest in vaccines that appear to act against CSP on the surface of the sporozoite and on the infected hepatocyte, it is constructive to review the history of how this immunogen came to be identified. As a co-investigator in the initial successful immunization study with mice [4] and the sole survivor of the group that conducted the first successful vaccination study with humans [5], here is a highly personal and selective history of what led up to these studies and how they have since advanced.

2. Early history

Isaac Newton, in a letter to Robert Hooke wrote: “If I have seen further it is by standing on the shoulders of giants.” All of us who have been privileged to work on developing a vaccine against malaria are part of a lineage of researchers, and can trace our debt to those upon whose shoulders we stand. We, in turn, can hope to make contributions that will enable others to reach a shared goal: a practical, protective vaccine for malaria.

This is a personal history; thus, permit me to name some personal “giants”. My own odyssey did not end in Ithaca (as did the classic Greek tale) but rather began in a different Ithaca as a graduate student in Medical Entomology at Cornell University. I attended the 1960 national meeting of the Entomological Society of America in Miami to present my PhD thesis work and had a personal revelation when I wandered into a session on malaria and listened to a talk by Ian McGregor on the successful passive transfer of immunity by transfer of immunoglobulin to children at risk in Africa [11]. Sir Ian's work built upon previous work that had been done with simian malaria. This “proof of concept” made it clear that vaccination against malaria might ultimately be carried out. Listening to this “giant”, upon whose shoulders so many malariologists stand, convinced me that this was an endeavor that I desired to join.

3. The search for research “models” of malaria

Human malaria does not lend itself easily, however, to basic research in immunology. A laboratory model for in vivo study of mammalian malaria (preferably in rodents) had long been a dream of malariologists. Up through the 1940s, several avian models were available. I must confess that my favorite has always been *P. lophurae*, both because this became William Trager's parasite of choice (and his work with it over many years led inexorably to his ultimate success in culturing of *P. falciparum*) but also because it was discovered in the wilds of the Bronx in New York City, not far from where I was born and grew up [12]. Lowell Coggeshall,

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working at the Rockefeller Foundation Laboratories in New York City, was searching for a new species of avian malaria with which to screen anti-malarial drug candidates, and reasoned it would be cheaper and more efficient to examine birds that had been imported into the United States than to mount expeditions to exotic locales. So, in cooperation with an ornithologist and a pathologist at the Bronx Zoo, he examined the blood of birds from Borneo and Ceylon. In June, 1937, a malaria parasite was observed in the blood of a Borneo fireback pheasant, *Lophura igniti igniti*. It was infectious to chickens and ducklings. During WW II, this parasite in ducklings served as a screening model for discovery and testing of new antimalarial drugs. Unfortunately, this line soon lost its gametocytes, thus making sporozoite transmission studies impossible. In spite of collecting expeditions mounted to Borneo, this species of malaria has never been identified in the wild.

For early malaria vaccination studies, Jules Freund and his associates attempted immunization of ducklings with killed *P. lophurae* injected together with what is now referred to as Freund's Complete Adjuvant [13] and achieved partial protection but with associated adjuvant-induced pathology. This group did similar studies with the simian malaria parasite *P. knowlesi* in rhesus monkeys [14]. Nevertheless, the avian immunization studies proved to be a poor substitute for studies with mammalian malaria, while the expense and logistic problems associated with working with simian malaria are so daunting that relatively few laboratories could afford to venture into such studies. Those who desired to do experimental studies with the human malarias faced even more severe obstacles. A considerable amount of research on human malaria was made possible by the introduction of malaria “fever therapy” to treat patients who suffered from general paralysis associated with tertiary syphilis [15]. For the first time, it became ethically justifiable to deliberately infect humans with a disease (malaria) with the intention of treating a more serious disease (advanced syphilis), a therapy for which Julius Wagner-Jauregg received the Nobel Prize in Medicine in 1927. This made possible multiple observations on malaria with patients so treated [16]. At least one unsuccessful attempt was made to use formalinized *P. vivax* parasites to immunize a group of these patients, who were referred to as “volunteers” [17]. Because such attempts at experimental immunization could not possibly confer any benefit on these severely impaired patients, who were unable to give informed consent, it constituted an unethical application of “fever therapy”, whose justification was to attempt to alleviate illness. An analysis of abstracts of publications on “fever therapy” found in *Index Medicus* shows that they reached a peak during the early 1930s but by the 1940s had been largely abandoned due to the introduction of penicillin.

A new phase of studies with human malaria was initiated at prison facilities with prisoner volunteers who agreed to become infected with malaria. The most prominent research on sporozoite-induced malaria at such facilities was carried out at the U.S. Penitentiary in Atlanta, Georgia [18], and at the Maryland House of Correction in Jessup, Maryland [5], as well as at the Illinois State Penitentiary, in Joliet, Illinois [19-20]. Most of these studies focused on the testing of antimalarial drugs in humans. Although studies were generally conducted with a high regard for the safety and humane care of the volunteers, a controversy developed in the 1970s over the ethics of such research in prison facilities, and all the studies were eventually terminated. But even at the height of human studies with “fever therapy” or with prison volunteers, few facilities could cope with the expenses involved and with the logistic and ethical requirements that inescapably restricted the kinds of research that could be done with humans. Yet, memories of the limitations inherent in developing and testing new anti-malarial drugs with inadequate avian malaria models during World War II made researchers cognizant of the need for a new and simple laboratory model of mammalian malaria.

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4. The emergence of rodent malaria as a research model

The issue was resolved with the description of a rodent malaria parasite, *P. berghei*, in central Africa by a Belgian physician (Ignace Vinke) and entomologist (Marcel Lips) in 1948 [21]. As often occurs with important discoveries, these workers had not been searching for what they ultimately found. Vincke spent the years of World War II doing malaria surveys in the former Belgian Congo (now the Democratic Republic of the Congo). In 1942 he observed sporozoites in the salivary glands of the mosquito *Anopheles dureni*, collected near a major mining center, Elisabethville (now Lubumbashi). Precipitin tests on the bloodmeal contents of the mosquitoes' midguts indicated that they had fed on rodents or insectivores. When Vincke and Lips examined the blood of a local tree rat, *Thamnomys* in 1948, they discovered a new species of malaria. They postulated that the mosquito salivary gland infection with sporozoites observed years earlier and the newly described blood infection were the same species. Vincke named the parasite in honor of his close friend, Louis van den Berghe, of the Prince Leopold Institute of Tropical Medicine in Antwerp. However, it was not until 1950 that Vincke was able to show that sporozoites collected from these mosquitoes produced a typical *P. berghei* infection when injected into laboratory mice [22]. The life cycle had been completed!

An interesting sidelight to this discovery was the speed with which the authors felt they had to make the announcement. An American expedition to Sudan in 1948 led by Harry Hoogstral sent back information that they had discovered a new malaria parasite in the elephant shrew *Elephantulus* [23] and a shipment of live shrews was sent back to the United States. The news was widely reported in the American press but as sometimes happens, some American newspapers had garbled the information and reported that malaria had been discovered in elephants! In retrospect, elephants were not shown to be hosts of *Plasmodium*, although a new malaria parasite had been discovered in a small mammal. Vincke and Lips were justifiably concerned about being “scooped” and they searched for a means of rapid publication. By luck, the Fourth International Congress on Tropical Medicine and Malaria was convening in Washington in May 1948. It was already too late to add a Vincke and Lips paper to the agenda but Louis van den Berghe, who already had a paper scheduled on research in the tropics [24], was able to append to his paper an announcement and description of this new rodent malaria parasite. In all respects, this was a landmark International Congress. It was the first one ever held in the Western Hemisphere and it had been 10 years since the previous one in Amsterdam, the planned intervening International Congresses having been canceled during World War II. In attendance were many scientists and physicians who had spent World War II working on malaria and malaria control projects and then were continuing with malaria studies when they returned to academic life. A further elevation of excitement within the community of malaria researchers earlier in this *annus mirabilis*, 1948, was attributable to the announcement of the discovery of the exo-erythrocytic stages of mammalian malaria for simian [25-27] and then human [28] malaria. Van den Berghe's announcement of the availability of a rodent malaria parasite at that Congress was a seed that fell on very fertile ground. Due to the graciousness of the Belgian workers, *P. berghei* was soon widely distributed throughout the world and a new era in malaria research had begun.

Among the recipients of this rodent malaria parasite was Harry Most, who had returned to the United States from the U.S. Army to become Chairman of the Department of Preventive Medicine at New York University (NYU) School of Medicine. During the early 1960s, Dr. Most also served as Chairman of the Armed Forces Epidemiological Board and Director of its Commission on Malaria; these were civilian advisory panels that no longer exist. With the financial support of these groups, he initiated a project at NYU on biology of rodent malaria. He was joined by Meier Yoeli, who had come from Israel by way of William Trager's laboratory at Rockefeller University. The major shortcoming associated with research on *P. berghei* was that no one had succeeded in identifying the conditions permitting a complete parasite cycle

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that included transmission of the sporozoite stage of the parasite by mosquitoes. Laboratory research was restricted to the erythrocytic stages of the parasite and its transfer by blood inoculation between rodents. With the eventual goal of developing a prophylactic vaccine against the pre-erythrocytic stages of the parasite, NYU studies centered on completing the life cycle of this parasite to permit mosquito transmission under laboratory conditions.

As luck would have it, I was in the right place at the right time. I was a post-doctoral fellow at Johns Hopkins University from 1961 to 1963, working on insect physiology, and also studying mosquito transmission of *P. gallinaceum* in the laboratory of Lloyd Rozeboom. During a trip to New York City in May 1963, I visited with William Trager, whom I had met on several previous occasions. (During the war, he had been a friend and associate of my former research advisor at Cornell.) When I asked Trager whether he was aware of any positions open in malaria research, he explained that on the previous evening he had sat next to Harry Most at the annual dinner of the New York Society of Tropical Medicine and Most had asked *him* if he knew of a medical entomologist, trained in insect physiology, who was looking for a job. I contacted Dr. Most, who then interviewed me, and before he even had an opportunity to advertise the position, I was hired to engage in these efforts! So, preparatory to my starting date in September 1963, I immersed myself in what was known about the biology of *P. berghei* in Africa.

The natural mammalian host of *P. berghei* is the tree rat, *Grammomys* (*Thamnomys) surdaster* found within remnants of ancient upland forests (forest galleries) that run alongside rivers in central Africa. The parasite is transmitted by the mosquito *A. dureni*. Vincke, the discoverer of this parasite, had described in his papers that the environmental temperatures were 18° to 21°C during the transmission season [22]. I was struck that this relatively cool microenvironment was not what one normally associates with tropical Africa. These low temperatures were confirmed by my NYU colleague, Meir Yoeli, during a trip to this region in December 1963. Accordingly, I set the NYU insectary temperature to 21°C, even before Yoeli returned. The reward: complete sporogonic development and production of infectious sporozoites in *A. quadrimaculatus* that had fed on gametocyte-carrying hamsters. We reported this in 1964 at the International Colloquium at the Prince Léopold Institute in Antwerp, organized to commemorate the discovery of *P. berghei* 16 years earlier [29]. I also had the privilege at this Conference of meeting Ignace Vincke, the discoverer of this parasite. After further analysis of optimal temperature conditions for sporogonic development [30], our described procedure soon became the standard protocol used for mosquito transmission of rodent malaria.

During the next several years the explosion of research on mosquito transmission of malaria continued with *P. berghei* and other rodent malaria species subsequently discovered in central Africa. It is worthy of note that, by chance, the first of the rodent malaria parasites discovered was *P. berghei*, simply because it is enzootic in the region where Vincke and Lips were working. That this parasite had such unique and restrictive temperature requirements for development in mosquitoes was responsible for delaying exploitation of laboratory research on mosquito transmission of rodent malaria for more than a decade. If another central African parasite such as *P. yoelii* had been discovered first, the much more relaxed temperature requirements for infecting mosquitoes would have allowed sporogonic development to be regularly achieved in the laboratory many years sooner.

During the next couple of years, research was focused on working out the parameters for sporozoite transmission of *P. berghei* to laboratory rodents and the characterization of these mosquito-induced infections in laboratory rodents [31-36]. In 1965, the NYU research group was joined by Robert Herman, an immunologist from Rutgers University and later by Ruth Nussenzweig, an immunologist from Brazil. The next few years continued with biological studies [37-38].

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5. Initiation of immunization studies with rodent malaria

By 1967, our immunization studies had begun. By immunizing mice via intravenous injection of sporozoites obtained from dissected-out mosquito salivary glands and then attenuated by X-irradiation, almost total protection against subsequent challenge with viable sporozoites was achieved [39]. Over the next two years the fundamental characteristics of this protection were established, including such things as its species and stage-specificity [40], the humoral component of its action [41-42] and the effects of radiation on sporozoites [43]. Parenthetically, I had obtained the original idea for sporozoite attenuation by X-irradiation from similar studies that were then being done on immunization against schistosomiasis with X-irradiated cercariae [44-45] by Elvio Sadun at the Walter Reed Army Institute of Research. It was only later that earlier studies on attenuation of avian malaria sporozoites by in vitro maintenance [46], or by drying or UV treatment of the sporozoites [rev in 47] came to our attention. In the studies with immunized chicks, none that were challenged had been protected from developing a patent malaria infection, but a reduced mortality rate compared with non-immunized controls was observed.

As previously noted, most of the currently active candidates for vaccination against pre-erythrocytic malaria include components of the CSP of the sporozoite stage, either from intact, irradiated sporozoites [48] or intact, genetically attenuated sporozoites [49-51] or within sub-unit vaccines that include a peptide component of CSP, such as the RTS,S vaccine [9]. An important additional approach is aimed at development of a vaccine that would elicit a cell-mediated immune response able to interfere with the intra-hepatocytic multiplication cycle of the parasites by killing the parasite-infected hepatocytes [1]. Because of the promising use of CSP as an immunogen, either in intact, attenuated sporozoites or in sub-unit vaccines, it seems useful to present some personal recollections of the early efforts that first identified this protein as a candidate for vaccination. Among the most helpful findings was my astonishment at observing that upon in vitro exposure to serum from immunized mice, an antibody-mediated precipitant reaction was formed around sporozoites, and projected from one end [42]. This reaction, termed the circumsporozoite precipitation (CSP) reaction, now permitted an in vitro correlate to immunity. The terminology for the CSP reaction was suggested to me by the similar appearing circumoval precipitation (COP) reaction around schistosome eggs incubated in immune serum [52]. The abbreviation, CSP, originally referred to the circumsporozoite precipitation (CSP) reaction but has come, instead, to refer to the circumsporozoite protein itself. Because of the striking way in which serum from immune animals deformed sporozoites, this was postulated to be the basis for a humoral component of protective immunity against sporozoites. Forty years afterward, I still believe this to be the case. I later showed that sporozoite motility could be initiated by exposure to albumin [53] and demonstrated that this motility was rapidly inhibited by anti-CSP antibodies both in vitro [53] and in vivo [2], thus giving a rational basis for how anti-CSP antibodies may function in protective immunity against sporozoites.

6. The first human trial

After the initial successes with immunization of mice against sporozoites, as well as with simians (squirrel monkeys) [54], it seemed timely to move toward trial studies with human volunteers. Sporozoites obtained directly from dissected-out mosquito salivary glands, however, could not ethically be injected IV into humans, as had been done with rodents. An alternative approach was suggested from studies on mosquito-borne viruses. Serological surveys, done after epidemics of infections with these viruses, consistently showed that only a very small percentage of seropositive individuals had actually experienced signs or symptoms of disease. Thus, from an epidemiological standpoint, mosquitoes could be considered to be more important as vehicles of immunization than as vectors of disease. Accordingly, a trial

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was conducted with rodent malaria, and used infected, irradiated mosquitoes as substitute "hypodermic syringes" to deliver sporozoites. Our argument, presented in 1970, was as follows [55]:

> "The technique that we presently use for immunization involves the intravenous injection of infected mosquito salivary glands which have been dissected out, ground up and irradiated. However, this preparation contains considerably more extraneous mosquito debris than sporozoites, and the injection of such material into humans would possibly pose medical risks of embolisms and sensitization. Until sporozoite preparations can be purified it would seem prudent to avoid this. A more reasonable approach for the present would be to X-irradiate infected mosquitoes and then let them feed on volunteers, thus allowing the mosquitoes to inject the sporozoites in a relatively uncontaminated condition. Such a technique would have limited practicality, but it has the advantage of being performable at the present time. If protective immunity could be demonstrated under such circumstances, it might encourage further work on attempts to establish purification procedures for sporozoite homogenates. The injection of irradiated sporozoites by mosquitoes should thus be viewed as an attempt to test the feasibility of vaccination in humans, which if successful could lead to trials using more practical techniques".

The results showed that mice so immunized were completely protected from sporozoite challenges that caused parasitemia in 100% of non-immunized control mice. With the success of this approach in mice, it seemed appropriate to move towards human trials. Others were similarly motivated. Shortly after our presentation of our rationale and results at the 1970 national meeting of the American Society of Parasitologists, Karl Rieckmann, who was doing collaborative work with investigators at the Illinois State Penitentiary, telephoned me to request information about the details of our technique and I shared this information with him.

Dr. Most and I set up a collaborative arrangement with David Clyde, the director of a program studying anti-malarial drugs against sporozoite-induced malaria in human volunteers at the Maryland House of Correction in Jessup and associated with the University of Maryland School of Medicine. David Clyde had had a long and distinguished career working with the human malarias, both in the field and with volunteers. I had met him while I was doing my post-doctoral fellowship in Baltimore; Vince McCarthy, who worked with Clyde as a medical entomologist, had been trained in our department at NYU. This collaboration, for which I traveled regularly to Maryland over the course of a year to do the immunizations and challenges together with Vince McCarthy, resulted in the first successful protective immunization of a human against malaria [5]. It was a long and sometimes frustrating effort because the first series of immunizations used X-ray doses that we had previously used on sporozoites in our rodent studies. The first series of immunizations of volunteers resulted in some breakthrough parasitemias during the attempted immunizations, so several months were lost while we retooled and began again with several new volunteers and with higher doses of radiation against the infected mosquitoes. This time there were no breakthroughs during the immunizations. The vaccinated individuals along with unvaccinated control volunteers were then challenged by bites of mosquitoes whose numbers and degree of infection were sufficient to have induced parasitemia in every single volunteer that had ever taken part in prior trials conducted by David Clyde and his associates. Upon challenge, all of the non-vaccinated volunteers developed parasitemia, as expected, whereas one of three vaccinated volunteers was found to be totally protected. Immediately after the challenges, I traveled back to New York with sporozoite-infected mosquitoes and with serum from the challenged volunteers. The sporozoites exhibited strong CSP reactions induced by the serum of one of the vaccinated individuals (GZ). I immediately telephoned Clyde and predicted that GZ would be protected. We were all delighted when this turned out to be true. It is perhaps relevant that GZ had taken part in the first vaccination trial, although he was not one of the vaccinees who had experienced a breakthrough

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parasitemia. So he continued into the second series of vaccinations and thus received an especially extended schedule of immunizations. This fits the important later conclusion, based on a meta-analysis of multiple studies, that a relatively high dose of attenuated sporozoites is necessary to attain sterile immunity, [6].

One robin does not a spring make, or one successful vaccinee a vaccine. Yet, while indicating the limitations of this single success and the difficulties that lay beyond, we concluded that this first successful vaccination was *"an encouraging step towards the goal of immunizing man against malaria"*. Our limited success served to establish what had been hoped for, namely, a clear "proof of concept" demonstrating that production of sterile immunization in humans might be biologically feasible and was deserving of further efforts. Not everyone appreciated the value of this first modest step. Some seemed to expect a fully-developed, functioning vaccine on its first try in humans [56]. To the contrary, we were gratified that even one of the volunteers had been completely protected. We felt that we had fulfilled our previously stated goal that *"The injection of irradiated sporozoites by mosquitoes should thus be viewed as an attempt to test the feasibility of vaccination in humans, which if successful could lead to trials using more practical techniques"*. Indeed, subsequent studies extended this initial modest result [57-61] and further analysis has shown that when sufficient numbers of irradiated sporozoites were used for immunization, there was total protection in 94% of challenges (33/35) with protection lasting for at least 10 months, and with effectiveness against multiple strains of *Plasmodium falciparum* [6].

A criticism by others relates to allegations that conduct of research with volunteers within prisons is inherently unethical. This was addressed in our paper [5], which noted that the study was conducted in strict accordance with regulations established by medical ethics committees of the University of Maryland and the supporting agency, and conforms to the guiding principles of the Declaration of Helsinki [62]. The operating principle comes from the Nuremberg Code of 1947, enacted after the war crimes trials of Nazi doctors, and which declares, *"The voluntary consent of the human subject is absolutely essential"*. In 1974 the American Civil Liberties Union (ACLU) filed suit to prevent volunteer studies in all prisons, including the Prison Volunteer Research Unit at the University of Maryland, where our work had been conducted. The ACLU lost the case in federal court because it was unable to prove any instance in which the volunteers had been coerced. The presiding judge praised the studies and agreed that the highest ethical standards had been maintained [63]. Nevertheless, the University of Maryland program closed in 1974 because of ongoing, opposing legal actions on behalf of prisoner volunteers. David Clyde said at the time that problems arose when he moved from *P. falciparum* to *P. vivax* vaccination studies. Because the black prisoners were Duffy-negative, and thus innately resistant to vivax malaria, they were necessarily excluded from these studies; however, these prisoners alleged that this exclusion was discriminatory towards them. At the same time, according to Clyde, white prisoners complained that they were being used as "guinea pigs" in a project from which black prisoners had been excused. Because of these highly charged issues, vaccination studies were discontinued within prisons and later continued with non-prisoner volunteers [6].

7. Continued approaches to immunization

7.1. Attempts at in vitro production of sporozoites

The next question to be faced was how to go from the obviously impractical approach of mosquito injection of irradiated sporozoites to a more practical large-scale vaccination protocol with purified sporozoites. In the mid-1970s, armed with ignorance, the answer seemed to be to try to develop culture procedures to raise large numbers of sporozoites without mosquitoes. An important stimulus was the encouragement provided by the 1976 success in continuous culturing of *P. falciparum* blood stages [64], a product of many years of effort by William

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Trager. Our approaches included attempts to characterize the milieu of the mosquito hemolymph, which nourishes sporogonic development [65-67], studies on in vitro formation and culture of ookinetes [68-70], and studies on growth and differentiation of sporozoites in vivo [71]. The overall results showed some modest successes [72-73] but by and large, early attempts by us and others to use culture techniques to produce large numbers of sporozoites for immunization proved impractical. Where to go from there?

7.2. The promise of subunit vaccines

By 1980, the maturation of molecular biology as a scientific endeavor suggested an entirely new approach, namely the development of a synthetic vaccine based on peptide constituents of CSP. At about this time, Victor Nussenzweig became involved in sporozoite studies and joined Ruth Nussenzweig. A team led by the two Nussenzweigs began to characterize the sporozoite surface antigens against which the CSP antibody acted. An early step was the use of monoclonal antibodies to isolate CSP [74]. The participation of Nigel Godson, Chair of Biochemistry at NYU, soon made possible the successful cloning of the CSP genes of several species of the malaria parasite in rapid succession at NYU and elsewhere [75-79]. These and similar studies opened the door to identification and subsequent synthesis of antigenic components of the CSP, especially the immunodominant repeat regions of CSP and the use of these as components of vaccines. As noted at the onset of this review, the most prominent of these sub-unit vaccines in current trials is RTS, S [8-9].

7.3. Back to the future? Large scale production of sporozoites in mosquitoes

In contrast to this approach, one group (www.Sanaria.com) has returned to the possibility of large-scale vaccination with live, radiation-attenuated, whole sporozoites. This approach has become conceivable due to the successes reported by Sanaria's Chief Scientific Officer, Steve Hoffman, on its ability to resolve difficult technological problems, such as mass production of sporozoites in mosquitoes, collecting and purifying sporozoites sufficiently so that it will be safe for them to be injected into humans, and being able to freeze-preserve these sporozoites. It should be noted that these production and preservation techniques are also applicable to intact, genetically attenuated sporozoites [47-49].

One of the crucial reasons why the Sanaria scientists were able to move into large scale production of sporozoites was a consequence of earlier successes in the infection of mosquitoes with *Plasmodium falciparum* gametocytes. The great roadblock to working with volunteers in the 70s and before was obtaining infectious gametocytes. Volunteers had to be maintained safely for long periods of time with low levels of non-threatening parasitemia, yet with sufficiently high gametocytemias to make them infectious to mosquitoes feeding on them. Our own studies in Maryland had to be scrubbed on a regular basis because gametocytes were not being produced or because parasitemias had risen to a threatening level, and safety requirements called for a radical cure of the volunteer. Indeed, in the original study, only 6 of 33 volunteers became successful as gametocyte donors [5]. Thus, far more volunteers had to be infected with malaria to produce gametocytes than the numbers of volunteers actually used in the vaccination trial itself. Furthermore, these gametocyte donors had to remain infected for relatively long periods of time. So, the fact that the Trager-Jensen in vitro system actually produced gametocytes was supremely important. Fortunately, I was nearby, and had heard Trager present his results at a meeting of the New York Society of Tropical Medicine prior to publication, and was the first to attempt to raise gametocytes in culture and then feed them to mosquitoes. Initial attempts were negative but ultimately we were able to develop culture techniques that allowed the gametocytes to fully mature in culture and to produce sporozoites in the salivary glands of mosquitoes that had fed on the cultures [80]. This was followed rapidly by others who demonstrated the infectivity of the sporozoites [81].

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7.4. What next?

What of the future? In addition to CSP, other antigens such as Liver Stage Antigens 1 & 3 and the Thrombospondin Related Anonymous Protein (TRAP) [rev. in 82] have been implicated in protection against pre-erythrocytic stages. Recent publications indicate that still other antigens may also be involved in protection, even in the absence of CSP [83-84]. Identification of such protective antigens could be useful for the rational addition of another antigen to a sub-unit vaccine such as RTS,S. Furthermore, aside from attempts at immunization against pre-erythrocytic stages, upon which this review is focused, encouraging progress continues to be made on immunization against blood stage parasites and on immunization for production of transmission-blocking antibodies [rev. in 85]. There is reason to be optimistic that one or more of the approaches will work on a large scale, and that a multi-stage vaccine may be able to combine several of these approaches in a sequential immunological assault against the malaria parasite as it progresses through its stages. Researchers too numerous to cite, as members of a long line of “giants” standing on one another's shoulders, have played significant roles in the development of these vaccine candidates. Indeed, considering all the remarkable and sophisticated results, one is reminded of Newton's metaphor about “standing on the shoulders of giants”, and I am inclined to put forward its obverse corollary, namely, “If I have not seen as far as others, it is because giants were standing on my shoulders.”!

Until the initial technological hurdles to production of large numbers of sporozoites for an attenuated vaccine had been overcome, some malaria vaccine developers working on sub-unit vaccines were often dismissive of these efforts. Now, with limited amounts of resources available to researchers, some continue to call for granting agencies to focus their investments on just one of these approaches and put their money on a single “horse”. Furthermore, funds for much needed basic research to identify new antigens and new presentations of these antigens are often diverted towards the assumption of a soon to be delivered “finished product”. Personally, I see no advantage in favoring one “horse” at the expense of the other. It seems more sensible to increase one's odds by betting on both contenders rather than placing all money on an assumed favorite. Arbitrarily disqualifying one of the horses before the race has been run carries the risk of having the remaining horse falter, and ending up with no horse at the finish line. The tower of “giants” standing upon one another's shoulders has not only soared but multiplied into several distinct lineages. Until grant support agencies can be certain of which of these piles may lead to a practical vaccine, the importance of the overall effort behooves them to support all reasonable efforts.

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