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UNIVERSITY OF ILLINOIS

DEPARTMENT OF ZOOLOGY

URBANA, ILLINOIS

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PROGRESS REPORT

January 1, 1971 - June 30, 1971

The occasion of this report marks the end of the first five years of work at the University of Illinois under AID contract 1432 which was initiated 1 July 1966. It is appropriate at this time to provide a brief overview resumé of our accomplishments as they compare with the goals outlined in the original STATEMENT OF WORK as well as a progress report.

ARTICLE I - STATEMENT OF WORK

A. General

The general objective of this contract shall be the development of a vaccine for use in the prevention and treatment of human malaria The contractor shall investigate and develop techniques for the preparation and testing of antigens of mammalian malaria using rodent malaria parasites, anopheline mosquito vectors and rodent hosts as an experimental system. The extension of this work to primate malaria will be undertaken as soon as justified by progress.

B. Specific Performance

In carrying out the objectives set forth in Article I-A next above, the Contractor shall:

1. Develop techniques for preparation of antigens from the following:

   a. Sporozoites (infective forms inoculated by the mosquito)

   b. trophozoites (infective forms found in the blood of the mammalian host)

   c. extractions of mosquito tissues

2. Compare the immunogenicity of the sporozoite and trophozoite antigen preparations.

3. Observe effects on vector efficiency by blood meals containing mosquito antibodies, to test the postulate that mosquito antibodies in the blood of the mammalian host will inhibit the development of the malaria parasite in the mosquito

4. Characterize all antigens by serologic and biochemical methods

5. Investigate means to control and standardize antigen production from all sources.

C. Provided that encouraging protectivity is induced in laboratory rodents by some of the immunizing procedures, and upon the mutual agreement of the parties hereto primate hosts will be investigated in similar manner.

Perhaps not clearly stated in the above, but certainly intended, was the need to answer the question of whether it is biologically feasible to vaccinate against malaria using non-viable antigens, i.e. a dead vaccine. In brief summary, the work at the University of Illinois has contributed substantially in providing an unequivocal affirmative answer. It has been shown that it is possible to induce resistance in mammals to prevent malaria infection and also to enhance the suppression of malaria in an infected animal. Most of the results which support the conclusion that it is biologically feasible to vaccinate against malaria have been obtained from a mouse model system originally developed at the University of Illinois. As previously reported, this system is a sensitive test for the presence of protective antigens. A single injection of blood-stage antigen regularly immunizes more than 90% of the mice to resist an otherwise 100% fatal infection derived from the injection of infected red-blood cells. In a similar manner, the mouse model system has been used to demonstrate that antigens from the infective mosquito stages can induce 90% protection against infection with sporozoites. The results obtained with the mouse model system have been used to design tests with trophozoite antigens in primates and from the results reported below it appears possible to translate from the rodent to the primate with some confidence.

BLOOD STAGE ANTIGENS

Primate Immunization Studies

As previously reported, Rhesus monkeys have been successfully vaccinated against *Plasmodium knowlesi* malaria by injection with partially purified

P. knowlesi antigens and Freund's Complete Adjuvant. To date, 5 out of 9 monkeys treated in the above manner were protected. Protection was characterized by low level parasitemias and mild anemias. Maximum parasitemia, in one animal only, was 4.4%. The parasitemias in all other successfully protected animals rarely reached the 1% level. Protected animals apparently completely sterilized themselves of the parasite following recovery. This is substantiated by the subinoculation of the blood of three of these animals, following recovery, into a susceptible animal. The subinoculated blood failed to induce infection. One Freund's adjuvant-antigen treated animal has been held unchallenged as part of a study of the duration of vaccination effect. This animal will be challenged 10 to 12 months following his last antigen injection.

Seventeen unsuccessfully protected and control animals all experienced severe parasitemias, anemias and death within 5 days of onset of patency after challenge. These included animals receiving:

1. Antigen + alum (2)
2. Antigen + alginate (2)
3. Normal red cell antigen + Freund's Complete Adjuvant (5)
4. Antigen alone (2)
5. Non-injected controls (6)

Successful vaccination of the Rhesus monkeys against Plasmodium knowlesi malaria confirms the vaccine character of the partially purified plasmodial blood stage antigens and the biological feasibility of malaria vaccination in higher animals. The protection induced by the vaccination procedure appears all the more remarkable when one considers the near 100% mortality and severe rapid progress of P. knowlesi infection in unprotected Rhesus monkeys. In

addition, the sterile immunity to P. knowlesi resulting in challenged vaccinated monkeys is in contrast to the partial immunity operative in animals carrying chronic infections induced by drug suppression. This suggests that vaccination in human populations may serve to prevent the sequelae of chronic disease which often accompany untreated or inadequately treated human malaria.

Purification Studies

The blood-stage antigen(s) used in these studies is essentially similar to that prepared by D'Antonio et al. in 1966. Attempts to isolate and characterize the active components have been frustrated by numerous difficulties associated with the nature of the material. Recent progress using sucrose density gradients seems to offer a means by which qualitative and quantitative characteristics may be determined.

Plasmodial antigens from both Plasmodium berghei and Plasmodium knowlesi have been further fractionated on sucrose density gradients. It has been determined that the distribution of the vaccine antigen material in the sucrose gradient is related to the mode of antigen preparation and subsequent handling. Sucrose density gradient centrifugation of the partially purified plasmodial vaccine fraction, appearing in the void volume eluate of Sephadex G-200, results in the appearance of two separate fractions (peaks). The first peak, appearing in the denser portion of the gradient, is straw colored and turbid. The second peak, which appears in the less dense portion of the gradient, is colorless and at most only faintly turbid. It is presently speculated that the straw colored first peak contains the parasite hematin fraction as well as larger antigen particles or antigen aggregates, or both. The second peak material is thought to consist of small non-aggregated particles. Recent studies on iron content of each peak suggest that hematin

is present in the first but not the second peak. The above results were obtained using antigen preparations frozen for storage and thawed at the time of use. It has been observed that freeze-thawing or prolonged storage at 4°C often results in the formation of a heavy precipitate. This is especially apparent in concentrated antigen preparations and is consistent with the suggestion that at least one portion of the sucrose first peak material represents particle aggregates. Antigens studied to date have been prepared by French Pressure Cell disintegration of isolated plasmodial materials at pressures between 5000 and 10,000 psi. One antigen preparation of P. knowlesi obtained at 17,000 psi resulted in a greatly increased discrete turbid second peak with a reduced straw colored broad first peak on sucrose gradient. Experiments designed to clarify the above situations are in preparation.

A/J mice injected with each of the antigen peaks obtained by sucrose gradient centrifugation were protected by both first and second peak materials derived from freeze-thawed preparations. The greater protectivity appeared related to the first sucrose peak. Experiments along these lines are continuing.

A preliminary experiment has been carried out using the isoelectric focusing technique to fractionate further the partially purified vaccine fraction. The results are encouraging and a systematic study is planned.

It has also been found that the addition to antigen preparations of a 20% final concentration of dextrose will stabilize against precipitation of antigen on freeze-thawing. If borne out by additional studies, this could offer a simple method for storing antigen at low temperatures.

MOSQUITO STAGE ANTIGENS

Rodent Immunization Studies

The following experiments have been successfully concluded since the last report, firmly establishing the feasibility of a sporozoite vaccine. In all experiments, 4-5 week old A/J inbred female mice were vaccinated and challenged by the intraperitoneal (IP) route of injection. Challenge infection was made with 2,000 sporozoites. Both vaccination and challenge injections were made with a virulent deme of NK65. Each group of vaccinated animals had two control groups set aside from the same batch of mice at the beginning of the experiment. One group was injected IP with the same number of salivary glands as were required to collect the necessary number of sporozoites for each vaccinating injection. The third group in each experiment was made up of untreated mice.

Successful sporozoite vaccination prevents infection so that no parasites appear in the blood. If the vaccination is unsuccessful, the blood stage is established and the infection runs its normal course. In mice which become infected the length of the prepatent period apparently corresponds to the number of infective sporozoites. No adjuvants were used with any antigen. All animals were challenged 2 weeks after the final injection. Eight to ten mice were used in each group.

Expt. No.	Sporozoites per Injection	Sporozoite Treatment	No. Injections	Percent Uninfected After Challenge		
				Normal Controls	Normal Salivary Gland Controls	Sporozoite Vaccinated
1	450,000	Freeze-thawed 3X	6	0%	70%	90%
2	25,000	Heated @ 40-42° 45 min.	7	12.5%	50%	90%
3	25,000	x-irradiated	5	5%	-	88%
4	450,000	Heated 42°C 60 min.	6	0%	70%	77%
4a	Rechallenge at 12 weeks of mice recovered from Expt. 4		-	0%	43%	83%

Spleen Weights as a % of Total Body Weight
(A/J Female Mice of the Same Age)

		No. Mice	Spleen Weights*
Group A	Sporozoite vaccinated, challenged, protected	11	0.33%
Group B	Normal salivary gland injected, challenged protected	5	0.35%
Group C	Normal untreated (unchallenged)	6	0.32%

* No significant differences between groups

The results of experiments 1-4a establish that freeze-thawed and heat-treated sporozoites are highly effective (77-90%) against sporozoite infection. The observations on the spleen weights do not suggest that the protective mechanism is due to a nonspecific macrophage-type reaction. It is particularly encouraging to note that there are no significant differences in spleen weights between the groups injected with the specific (sporozoite) antigens and the group injected with nonspecific (mosquito tissue) antigens.

Further data which support the view that uninfected *Anopheles stephensi* mosquito tissue contains antigens which are capable of stimulating resistance to infection are summarized below. The mice were challenged with 2,000 *Plasmodium berghei* sporozoites.

Expt. No.	No. Injections	Mosquito Tissue	mg. Protein/inj.	% Survival Untreated	% Survival Injected	Time After Last Injection (weeks)
1	7	Midgut	2.7	10	42	2
2	6	Salivary Gland	0.5	0	33	19

The laborious procedure of hand microdissection required to prepare suitable mosquito tissue antigens limits the rate at which this promising

ine of work can be pursued. Material from the *in vitro* culture of mosquito tissues is being accumulated and stored at low temperatures in liquid nitrogen. Further immunization studies will be undertaken as soon as sufficient material is collected.

Purification Studies

Efforts to isolate the active antigens from sporozoites and related mosquito tissue antigens, as previously reported, continues. Three major fractions have been obtained after chromatographic separation of sporozoite material through columns of Sepharose 6B. Immunization procedures in mice have begun although the amount of protein in each of the antigen fractions is considered to be less than the minimum amount required to stimulate an immune response. Efforts are being concentrated on the isolation of adequate amounts of material which will be needed to exceed the minimum threshold required to induce protection.

Chromatographic fractionation of normal plasmodium-free mosquito extract is also being carried on. Interestingly, the same three major fractions are found in whole uninfected mosquito extracts, midgut and sporozoite preparations. Preparations from the three fractions are being collected for protection tests.

IN VITRO CULTIVATION

The need is urgent to produce adequate amounts of trophozoite and sporozoite related antigens. Ideally, the best means for antigen production would be an *in vitro* system that can be closely controlled and scaled up to produce large quantities of quality antigen. Indeed, the translation of the demonstrated biological feasibility of malaria vaccination into a practical reality depends on antigen production. The problems involved, in establishing

an *in vitro* culture method capable of accomplishing these aims, are great with few guidelines available. Although, in principle, the *in vitro* cultivation of the blood and insect stages of malaria parasites is feasible, very few workers have reported attempts to accomplish the task. Of those who have worked on the problem, only a few have reported some partial success in maintaining various stages *in vitro* for short periods of time or obtaining limited development through one or two stages.

It is in the above context that the accomplishments of the *in vitro* culture unit at the University of Illinois should be viewed. For the first time, the aseptic (bacteria-free) rearing of *Anopheles stephensi* from egg, through the juvenile stages to egg laying adults has been established. These aseptic mosquitoes have provided a source of insect tissues and organs for the establishment of antibiotic-free cultures. Several organ-explants and cell cultures have been established and maintained in an active growing, dividing and metabolizing condition for up to 60 days without change of the nutrient medium. Subculturing has been successfully accomplished. The *in vitro* maintenance and development of ookinetes to the young oocyst stage containing sporozoites has been achieved. Numerous modifications of the culture technique and the nutrient medium are currently being tested. Preliminary attempts to cultivate the blood stages have been encouraging and suggest that the culture conditions initially used may lead, with modifications, to a suitable cultivation system for trophozoite antigen production. These aims are very large and will require an effort of major proportions to accomplish.

SUPPORTING ACTIVITIES

Ultrastructural Studies

The electron microscope has been a valuable tool during the past four

years that it has been available to the project. It has been used to evaluate the various methods of preparation of trophozoite antigen and, more recently, it has been used to determine the efficiency and purity of various preparations of sporozoite antigen. Both of these monitoring activities must be carried out on a regular ongoing basis. The studies on the ultrastructural state of the plasmodial stages during various treatment processes provide information not otherwise available.

A recent ultrastructural study on the effect of a blood meal on mosquitoes fed on rabbits previously immunized with preparations of mosquito stomach antigens revealed certain striking morphological changes in the mosquito stomach epithelium. Since these observations were made on mosquitoes from a single experiment, the results will have to be substantiated by repetition of the original experiment. Analysis and comparison of the electron micrographs obtained from mosquitoes fed on a normal rabbit and one that had been immunized with mosquito stomach preparations revealed a structural alteration of the endoplasmic reticulum in the epithelial cells of mosquitoes fed on the immunized rabbit. The usual arrangement of the endoplasmic reticulum in the mosquito stomach epithelium is in the form of numerous complex whorls which open up shortly after a blood meal. Approximately 50 hours post feeding the whorls reform. This sequence of events appears to have taken place in mosquitoes fed on the control rabbit. The endoplasmic reticulum, however, failed to reorganize in the cells of four of the five mosquitoes examined which had been fed on the immunized animal. The inability of the endoplasmic reticulum to resume its pre-blood meal state has many implications as to the importance of its function in digestion which are too complicated to be dealt with at this time. Those results suggest that anti-mosquito

antibody may act on this subcellular organelle and its effects may account for the increased mortality observed in mosquitoes fed on immunized rabbits as compared to those fed on normal rabbits.

The electron microscope will also be used to evaluate the *in vitro* cultivation developments. Analysis at the ultrastructural level of cell and organ cultures and the sporozoite and trophozoite stages may yield guidelines as to the effects of various cultivation conditions.

Immunobiologic Studies

A matter of great urgency is an appropriate *in vitro* test which can indicate when an animal is adequately immunized to malaria. The only way, currently, to ascertain protectivity is to challenge the animal with infective sporozoite or trophozoite stages of malaria. Obviously, this places several constraints on the rate of progress and is an inapplicable method to be used on humans.

As previously reported, a variety of serologic studies were undertaken (e.g. complement fixation, hemagglutination, precipitin and lymphocyte transformation tests.) Currently, efforts are being exerted to work out the macrophage migration inhibition test (MIF) with correlation as to cell mediated immunity in the rodent-malaria system. To date, only crude malaria blood stage antigen preparations have been tested and the results appear to be promising. Due to certain methodology changes and improvements in technique employed in the test much of the work will have to be repeated. Eventually the partially purified blood stage antigens will be tested.

Attempts continue to understand the mechanism(s) responsible for the immune resistant response to malaria. In view of previous work by this

laboratory, as well as others, which indicates that malaria immunity is of the cell mediated type, studies on the methodology associated with the use of immunocompetent cells have been pursued.

By using a discontinuous albumin gradient method, six populations of cells have been isolated. Preliminary differential counts of these populations indicate that small lymphocytes are concentrated in great numbers in the top fractions. Large lymphocytes, although present in smaller numbers, are also concentrated in the top fractions. Plasma cells and macrophages are not found in the top layers, but appear in the pellet and in the layer just above the pellet.

Experiments to determine the immunocompetence of these populations of cells in rats are being prepared.

Other experiments with serial doses of small numbers of parasites have established that premunition (immunity due to the presence of a small number of parasites) is not adequate to protect rats against a challenging dose of *P. berghei*. It is important to ascertain this point since it eliminates the question of whether immunity is transferred by the injected immune cells or by a small number of undetected parasites.

Additional studies have been undertaken to determine the role of immunological mechanisms in relation to parasite virulence. Anti-thymocyte serum (ATS) of rabbit origin was administered to Carworth CF_1 mice subsequently infected with *Plasmodium berghei yoelii*. *P. berghei yoelii* is normally a moderate non-lethal disease in CF_1 mice. The ATS treated mice developed severe disease and very high mortality rates. Similar results were obtained with mice first treated with the immunosuppressive drug, Lukeran. These

results suggest that parasite virulence is in some yet unknown way associated with the hosts' immunological defenses. The situation here is reminiscent of the mild character of more virulent P. berghei malaria which develops in mice first vaccinated and suggests a dynamic relationship between parasite and the diseased host. A practical suggestion which emerges is that ordinarily non-virulent strains of malaria in conjunction with even weak vaccination may offer a good means of inducing sterile immunity against virulent disease.

A study of the effect of rifimpicin on the course of P. berghei in mice was undertaken to determine whether or not avirulence could be induced by chemical means. The reported inhibition of rodent malaria by this compound coupled with its unique mechanism or action involving the synthesis of cellular nucleic acids served to encourage this view (Alger et al., 1970).

In over one hundred animals studied a slight delay in the death rate was observed,using NYU-2 P. berghei and no apparent differences were noted using NY65D P. berghei. The slight shift in death rate noted above seemed to occur mainly in older mice. The present conclusion is that the drug very likely acts by some secondary effect involving the host and not directly on the parasite itself. Only further study might clarify this point.

RESUMÉ SUMMARY

A retrospective assessment of the general and specific objectives of the contract reveal that all the goals have been met and, in some cases, exceeded. Vaccine model systems utilizing both rodents and primates have been established and have been used to demonstrate the efficacy of trophozoite and sporozoite antigens as immunizing agents.

Additional dividends, not anticipated in 1966, have emerged in the form of studies on malaria virulence variation and the possible use of plasmodia of low virulence as contributing to vaccine methodology.

Ultrastructural studies, in addition to their importance as a monitoring device, have revealed the possible existence of a stage in the life-cycle of malaria not previously described.

Insights into the nature of the mechanism of malaria have been obtained. The evidence that immune resistance is of the cell-mediated type has implications of general interest as well as importance for vaccination.

An important contribution to mosquito maintenance has been made by developing a method to control microsporidian parasitism of mosquito larvae.

Important progress has been made towards culturing insect tissue *in vitro*. *Anopheles stephensi*, the mosquito vector of *Plasmodium berghei* and *P. knowlesi*, is being reared under bacteria-free conditions. Organs, tissues and cells from *A. stephensi* have been cultured and subcultured in antibiotic-free medium.

Finally, there has been established at the University of Illinois a facility and personnel to concentrate its efforts on the complex problem of malaria immunity. The group is uniquely holistic in its multifaceted approach and is in a good position to contribute substantially to the development of an effective vaccine against malaria.

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