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BIBLIOGRAPHIC INPUT SHEET

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Batch 72

1. SUBJECT CLASSIFICATION

A. PRIMARY
Serials

Y-NS00-0000-0000

B. SECONDARY
Health--Tropical diseases

2. TITLE AND SUBTITLE

Malaria immunity and vaccination; progress report,July-Dec.1971

3. AUTHOR(S)

(101) Ill.Univ.Dept.of Zoology

4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1971	18p.	ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS

Ill.

8. SUPPLEMENTARY NOTES *(Sponsoring Organization, Publishers, Availability)*

(Research summary)

9. ABSTRACT

10. CONTROL NUMBER

PN-RAB-698

11. PRICE OF DOCUMENT

12. DESCRIPTORS

Immunization
Malaria

13. PROJECT NUMBER

14. CONTRACT NUMBER

CSD-1432 Res.

~~CSD-1432 Res.~~

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

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CONTRACT AID/csd-1432

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PROGRESS REPORT

JULY 1, 1971 - DECEMBER 31, 1971

The activities of the Illinois group over the past 6 months, which are briefly summarized in this report, represents the beginning of a different emphasis in our research thrust. With the successful establishment of model vaccine systems in mice and primates for blood stage antigens, and a rodent model system for the study of mosquito stage malarial antigens, new opportunities have been opened to investigate the nature of the antigens, the mechanism of resistance and to determine optimal conditions for prophylactic immunization.

In addition to exploiting the opportunities presented by the availability of the vaccine model systems, the need for the production of substantial quantities of malarial antigen becomes acute. The solution of this largely technological problem will determine in large measure the successful application of our vaccine feasibility studies to man. It is clear that increasing attention and effort will have to be applied in this area. With continued support from USAID, substantial progress in the *in vitro* production of malarial antigens might be accomplished.

BLOOD STAGE ANTIGENS

Immunization Studies

The successful vaccination of Rhesus monkeys against the virulent primate malaria *Plasmodium knowlesi* utilizing partially purified plasmodial blood stage antigens as a vaccine has been detailed in our previous reports. The protection achieved was evidenced by survival of vaccinated animals and

by the appearance of only a slight transient parasitemia. The protection was found to occur in only those monkeys which were vaccinated with antigen mixed with Freund's complete adjuvant (FCA). Antigen or FCA alone were non-protective, as were antigen preparations mixed with aluminum hydroxide or sodium alginate which are recognized adjuvants for human use with some antigens.

The initial successful primate trial utilized a lengthy immunizizing schedule and multiple routes of injection, as well as a lengthy delay after immunization and challenge. These conditions, although useful in order to establish the feasibility of vaccination, would result in an unwieldy primate model system. To determine whether a simpler and less time-consuming immunizing schedule could be applied, an experiment was performed in which monkeys were given only two injections of Freund's adjuvanted malaria antigen at a 4 week interval and challenged 3 weeks later.

Three out of 5 vaccinated monkeys survived a normally lethal challenge infection with P. knowlesi. Two of the monkeys never showed any parasites in their blood, and their hematocrit and RBC counts remained normal. One monkey demonstrated a peak parasitemia of 2.6%. Although its RBC and hematocrit dropped to approximately 20% of normal, the animal survived and now shows a normal blood picture with no parasites in the blood. Interestingly, the parasitemia fluctuated between 1-2% for 5 days, before parasites could no longer be seen in the blood. This is highly unusual in a P. knowlesi infection, which normally kills the animal within 3-5 days after the appearance of parasites. In addition, the parasitemia always rises rapidly, after the first appearance of parasites, and it is not unusual to observe a peak parasitemia in excess of 90%.

Of the two vaccinated monkeys that died, one showed a peak parasitemia of only 5% and apparently cleared the infection since no parasites were seen in the blood 2 days before it died. In spite of the low malaria parasitemia, the red blood cell count and hematocrit dropped to 20% of normal and on the day of death a blood slide revealed a very heavy bacterial infection which was thought to be the primary cause of death. The other monkey which died had been vaccinated with heat-treated antigen and the vaccine appears to have been inactivated by the treatment. The second monkey died shortly after the untreated control animal.

These results, which confirm our earlier trials with partially purified P. knowlesi antigen, indicate that successful immunization can be established with only two injections and immunity capable of resisting a fatal infection is present by the seventh week after the initial immunizing injection. This "model" reduces substantially the time span required to test active immunizing substances.

An additional advance appears in hand as judged from the results of a small pilot experiment in which the malaria antigen was adjuvanted with BCG (T.B. vaccine) instead of Freund's adjuvant. The BCG vaccine which is safe for human use was found to be as effective as Freund's in enhancing resistance to P. knowlesi infection. Further trials with BCG are under way, as well as with other adjuvants of equal acceptibility. (Interestingly, we failed in attempts to vaccinate mice against P. berghei using BCG as an adjuvant to shorten the long delay (7-8 weeks) now required before challenge).

As indicated above, work with the rodent model system continues. Means of shortening the currently required 3 month test period are being pursued.

These include multiple injections, adjuvants and alternate routes of injection. In addition, the effects of lyophilization and further purification in the antigen will be continued.

Purification Studies

The method for preparation of the partially purified blood stage malarial antigen is currently being systematically re-evaluated to establish a standard procedure for use by both the Chicago and Urbana laboratories. In addition to determining rigid conditions for each stage of antigen preparation, attempts to establish biochemical and serological criteria are also being pursued. One change already introduced is the replacement of Sephadex G-200 with Biogel 1.5 as the chromatographic column packing. Molecular filtration through Biogel is many times more rapid, the material offers greater use in handling and the degree of molecular separation appears as effective as that achieved with G-200.

MOSQUITO STAGE ANTIGENS

Mouse Immunization Studies

Data have been presented in our previous reports that establish that freeze-thawed or heat-inactivated sporozoites act as an effective vaccine against *P. berghei* sporozoite infection in the A/J mouse. We have also reported that normal (nonplasmodial infected) *Anopheles stephensi* mosquito tissues contain antigen(s) which are capable of stimulating resistance to infection with *P. berghei* sporozoites. Recent ultrastructural studies suggest the reason for this since it appears that the sporozoite is

invested with an outer membrane derived from the host-mosquito. This is discussed further in the section on ultrastructure. The electron microscopic studies also reveal that the mosquito antigens, used in the immunization studies, contain very little bacterial contamination. This observation, together with other evidence, argues against bacteria being involved in the protective immune cross-reaction between sporozoites and mosquito antigens.

Work during the past six months has been concerned with, (1) the duration of protection induced by vaccination with either sporozoites or mosquito tissues, (2) the effect of route of injection on vaccination efficacy, and (3) the effect of various injection schedules and varying quantities of sporozoites and mosquito antigens.

The following table summarizes the results from a study designed to determine the duration of immunity induced by vaccination with either sporozoites or mosquito antigens. All mice were vaccinated with 7 injections of 25,000 heat inactivated sporozoites or the estimated equivalent of mosquito antigens. The challenge inoculation consisted of 2,000 sporozoites and like the vaccination injections was given intraperitoneally.

DURATION OF PROTECTION

	Protected/Total Vaccinated		
	Vaccine Treatment		
No. weeks from final injection to challenge	Sporozoite injected	Mosquito salivary gland antigens	Uninjected control
12	9/10	4/10	0/10
14	8/8	3/8	0/10
16	6/10	0/8	0/9
16	7/10	0/10	0/9
16	5/10	0/9	0/9
22	0/9	0/9	0/7

In the sporozoite injected groups the protection appears to begin to drop after the 14th week and is insufficient to protect any animals by the 22nd week. The normal mosquito tissue control has already lost some of its protective ability by week 12 and, in this set of experiments, has reached zero by the 16th week. In a former experiment, however, some protection was still present at 19 weeks.

Route of injection: Two experiments to compare the relative efficacy of the intraperitoneal and intramuscular routes of vaccination were hampered because the challenge sporozoites proved to be of poor viability. Some protection appears to have been induced by the intramuscular route but less than that achieved by intraperitoneal vaccination. The work is being repeated.

Schedule of injection: Three groups of mice each consisting of three sub-groups of 9 mice each, were injected at varying intervals with antigens as indicated below during an 84 day immunizing period. Heat-inactivated sporozoites or the equivalent in mosquito salivary gland tissue was used in the antigen-treated sub-groups.

			Protected/Total Vaccinated		
			Vaccine Treatment		
Group	No. injections at day interval	No. sporozoites per injection	Sporozoites	Salivary gland antigen	Uninjected control
I	7 @ 14 days	25,000	8/8	3/8	0/9
II	3 @ 42 days	25,000	3/8	1/6	0/9
III	13 @ 7 days	5,000	2/8	0/5	0/9

Although the total number of sporozoites is different in each group, earlier experiments indicated that the timing of injections was of greater

significance than the quantity of antigen in inducing a maximum immune response. The data presented here suggest that both factors are important and the above experiment is being repeated with each group receiving equal numbers of total sporozoites. Work will continue to attempt to determine the minimum amount of sporozoite antigen capable of stimulating a good protection.

Additional work underway with sporozoite or mosquito antigens includes the effects of formalin, heat, ultraviolet irradiation, centrifugation and ultrafiltration on various antigen preparations. It is hoped by some of these means to learn more about the nature of the active immunogen. Antigen recovered after gel filtration is still being collected and the various chromatographic fractions are soon expected to be available in sufficient quantities for immunization tests.

IN VITRO CULTIVATION

Since the last report the *in vitro* tissue culture unit has been relocated to a new laboratory which has approximately doubled the amount of space allocated to this activity. Additional increases in personnel and equipment have been made. A controlled temperature room for aseptic rearing of *Anopheles stephensi* has also been assigned and units for the control of humidity, photoperiod, etc., are being installed.

The work during the past six months has concentrated on three main activities: (1) *in vitro* cultivation of sporogonic forms of *P. berghei*, (2) aseptic rearing of adult *A. stephensi* from surface-sterilized eggs, and (3) primary cell cultures as a potential source of sporozoite-like antigens.

Sporogonic Cultivation

The attempts to cultivate sporogonic forms of P. berghei were initiated with infected heparinized hamster blood collected under aseptic conditions. After exposure to CO_2 for 30 seconds at a flow rate of 33 cc/min, the blood was inoculated into four different culture media at 20°C. To assess the degree of ookinete development, 2 smears were made every hour over a 48 hour period and stained either with Giemsa or a fluorochrome dye. Medium A, a modified Schneider-Grace formulation, was found to be the best of the four media tested but it is not yet clear whether optimal conditions have been attained.

P. berghei, unlike other mammalian malarias, does not clearly demonstrate cyclic peaks of gametocytes in infected rodents. However, Tandon and Baattacharya (1970, Bull. Wld. Hlth. Org. 43:344-347) reported that treatment with an immunodepressant induces a high gametocytemia. Two experimental trials with dexamethesone 21-phosphate have confirmed their observations. Although the latent period of infection with P. berghei was lengthened, the gametogenesis level was increased by 460%. Exflaggelation was excellent and there is hope that this method will enhance the sporogonic culture attempts by increasing substantially the number of available ookinetes.

Aseptic Rearing

Efforts to increase the production of germ-free A. stephensi adult mosquitoes continues to receive a high priority. After surface sterlization, the eggs were placed in three different diets formulated by Akov (1960), Trager (1938) and Johnson (1970) for Aedes. The best results

which have been obtained are with a modified Johnson's diet. It is hoped to pursue this phase of the work vigorously to provide an aseptic source of mosquito antigens for use in the immunization studies.

Primary Cell Cultures

Cell cultures are regularly established using material obtained by microdissection of first instar larvae. The procedure has now been sufficiently routinized so that primary cell cultures ranging in age from 4 to 60 days are regularly available. Those cell cultures not used for experimentation are collected and stored at -70°C for future use as antigen.

As a preliminary to selecting appropriate cultures for antigen production, methods for identifying the various types of cells need to be established, as well as identifying serologically the antigenic character of the various cells and their relation to the antigens active in stimulating immunity to sporozoite infection.

The cell identification techniques being developed include histochemical, vital staining and both transmission and scanning electron microscopy. Histochemical methods already in use include analysis for carbohydrates, lipids and nucleic acids. Transmission electron microscopy has revealed the presence of numerous whorl-like structures suggesting secretory organelles in certain of the primary cell cultures. The scanning electron microscopy has helped to establish the morphological framework of cultured epithelial cells and will be applied to other cells.

Serologic techniques utilized, or being developed, include agar gel diffusion and direct and indirect immunoflourescent methods.

ELECTRON MICROSCOPIC STUDIES

Antimosquito Antibody Effects

As previously reported, preliminary ultrastructural analysis of the effect of a blood meal on mosquito midgut epithelium in mosquitoes fed on rabbits injected with antigens prepared from mosquito stomachs revealed irreversible alteration of the endoplasmic reticulum in the midgut epithelial cells. Subsequent experiments, however, have failed to confirm the original subcellular observations. There may be several explanations for this discrepancy. The problem of sampling a mosquito gut at the ultrastructural level can result in the examination of only a tiny fraction of the gut and an affected portion could have been easily overlooked. Another factor may be that the diet of the mosquitoes has been greatly improved from the one previously used and as a result the mosquitoes may be able to withstand and overcome short exposures to trauma and adverse environmental conditions much more readily than before. How these dietary conditions relate to mosquitoes in the field is unclear at this time.

The experiments reported here involved the exposure of mosquitoes to several blood meals containing anti-mosquito antibody. These observations were made at sequential time intervals, beginning with an undigested blood meal, partially digested, as well as completely digested blood meal. From the sample observations made the endoplasmic reticulum appeared unaffected.

Sporozoite Antigens

Subcellular analysis of various sporozoite antigen preparations was carried out. These included untreated, heated and freeze-thawed sporozoites obtained from salivary glands. The preparations showed a

relative lack of bacterial contamination. However, these antigen preparations did contain an enormous amount of cellular debris of mosquito tissue origin. As expected, some differences were seen between the untreated and heated or freeze-thawed sporozoites. There was no problem in identifying the heated sporozoites, for the cytoplasm of the parasites, in most instances, appeared slightly clumped or coagulated. The freeze-thawed preparation, on the other hand, more often than not, contained highly damaged and barely recognizable sporozoites with most of their contents released and dispersed into the surroundings. Although the disintegration of the sporozoites by these two different methods resulted in two highly diverse subcellular preparations, immunological results do now show much disparity between these two methods of sporozoite antigen preparations. These observations add weight to the suggestion that the active antigen material is closely related to the sporozoite membrane.

Electron microscopic observations were also performed on the cell wall of mature sporozoites obtained from mosquito salivary glands and sporozoites in the process of development still within the oocyst located in the mosquito gut epithelium. Because of the incomplete development of the sporozoite within the oocyst, it is very difficult to determine to what extent the sporozoite cell wall obtains its origin from the ookinete cytoplasmic mass and to what extent, if any, from the mosquito cell. This question arose because of observations made on the cell mass of mature sporozoites which still had some remnants of salivary gland cells surrounding them. At present we are not aware of any published studies on the origin of the sporozoite cell wall. The mature sporozoite has a thick,double cell mass

enveloping it, the outer portion of which appears to be contributed by the mosquito salivary gland cell membrane. This does not seem to be a simple adhesion of the salivary gland cell membrane to the sporozoite because this envelope cannot be removed by physical means. It becomes the outer membrane of the double cell wall of the mature sporozoite. These ultrastructural observations present supporting evidence and a plausible explanation as to the relationship of the immunizing and cross-reacting antigen between the sporozoite and mosquito tissue antigens.

Further ultrastructural studies involving sporozoites produced some highly interesting and important observations not seen before by us, and to the best of our knowledge, by other investigators. In a large number of sporozoites, small organelles resembling viruses in appearance, were noted. These virus-like structures fall within viral measurements, have an electron dense central core surrounded by an electron lucid space subtended by a membrane, and in many instances display an elongated hexagonal shape, the shape being dependent on the plane of section. These virus-like particles have not been seen in the mosquito stomach or salivary gland tissues, nor have they ever been observed in the blood phase of the rodent malaria parasite. Before any conclusive statements can be made as to the significance of these findings, more extensive and systematic studies are needed. These studies most certainly would elucidate as to the possible reasons for the variation in the malarial demes presently isolated in our laboratory.

In vitro Cultures

Ultrastructural analysis of cultured mosquito cells are already underway. Initial observations were made on an approximately 35-day-old

culture which had undergone the vesicular stage formation and was considered an old culture. The substructure and composition of the vesicle was clearly evident, but specific identification and interpretation of these culture results at this time is too premature until additional sequential culture samples are analyzed.

In addition to the above studies, an electron microscopic survey was also initiated on samples of parasitized hamster blood which had been exposed for approximately 68 hours to the culture medium used for mosquito tissue cultures. Originally it was observed under the light microscope that such prolonged exposure of the parasitized RBC to the medium causes release of the parasites into the medium. Preliminary ultrastructural observations showed that this technique of freeing parasites will have to be improved because of the severe damage to the parasites which rendered them unidentifiable ultrastructurally.

IMMUNOBIOLOGIC STUDIES

Several years ago it was demonstrated in this and other laboratories that the mechanism of resistance to the blood stages of P. berghei involved a large component of "cell mediated immunity". The implication of these findings, which were established by experiments utilizing inbred Lewis or Fisher rats, suggested that humoral factors probably played only a minor role in malarial immunity. Indeed, the results seem to explain the lack of correlation between serologic tests and resistance or susceptibility of mammalian hosts.

Subsequently, a graduate student (working with Dr. Alger) demonstrated that the A/J mouse system could be utilized to study the transfer of cell mediated immunity against the trophozoite stages of P. berghei. He showed that spleen cells, but not lymph node cells, could transfer resistance and that older mice were better donors of immune cells than young mice.

The availability of the A/J mouse system made it feasible to study the effects of cell transfer in sporozoite immunity. In contrast to the results obtained with the trophozoite test system described above, it was found that 120×10^6 spleen cells transferred from mice immune to sporozoite infection failed to confer protection on recipient mice to a challenge of 2,000 sporozoites. These results, if substantiated by further studies now underway, indicate that humoral rather than cell mediated immunity is involved in resistance to the sporozoite stages. These findings underline the complex nature of the mechanism of resistance to malaria and indicate why it is so difficult to apply information from other fields of infectious immunology or even from what is known about the host-parasite relations of one stage of malaria to another stage.

Other studies on cell mediated immunity to trophozoite stages in Lewis rats indicate that immune spleen cells from donor rats which had survived a primary infection as long as 7 months previously, still showed excellent protective qualities. It was also determined that cells from donor rats with very low peak parasitemias (~ 1%) were capable of transferring excellent immunity and suggesting that the immunity acquired by rats to P. berghei is independent of the number of parasites to which they are exposed.

The work on isolating specific immune cells utilizing the discontinuous albumin gradient described in our last report, has resulted in at least one population of cells which is capable of transferring immunity without transferring a secondary antibody response to trophozoite stages.

ADDITIONAL MANUSCRIPTS IN PREPARATION OR PUBLISHED SINCE LAST PROGRESS REPORT

Long-term maintenance in a nutrient medium of adult organs taken from aseptically reared mosquitoes, Anopheles stephensi. (In preparation) R. W. Simons, M. C. R. Ronquillo and P. H. Silverman.

Cell cultures derived from mosquito tissues, Anopheles stephensi. (In preparation). M. C. R. Ronquillo, R. W. Simons and P. H. Silverman.

Sporozoite and normal mosquito gland induced immunity to malaria. (In preparation). N. E. Alger, J. Harant, L. Willis and G. Jorgensen.

Density gradient fractionation of microsporidan spores. J. of Invert. Path., 18:419-420. A. H. Undeen and N. E. Alger.

Plasmodium berghei NK65 in the inbred A/J mouse: I. Variations in virulence of demes of the NK65 strain of P. berghei. J. of Protozool. 18:598-601. N. E. Alger and M. Branton and P. H. Silverman.

Transfer of adoptive immunity to Plasmodium berghei: A comparison of routes of injection. J. of Protozool. 18:596-597. N.E. Alger and E. J. Cabrera.