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REPORT SUMMARY

CSD-3689 Rec.
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1973/1974

A. Project Title and Contract Number: Malaria Immunity and Vaccination
AID/csd-3689

Principal Investigator: Paul H. Silverman

Contractor: University of New Mexico

Mailing Address: Albuquerque, New Mexico 87131

Contract Period: June 30, 1972 to July 1, 1975

Period Covered by Report: July 1, 1973 to June 30, 1974

Total A. I. D. funding of contract to date: $1,328,774

Total expenditures and obligations through previous contract year: $423,837.22

Total expenditures and obligations for current contract year: (to follow)

B. Narrative Summary of Accomplishments and Utilization

This project under A. I. D. sponsorship since 1966 has demonstrated the feasibility of vaccinating both primates and rodents against malaria. The work done in rodents has involved vaccination against both the sporozoite and blood stages of malaria, while the work done in primates has involved vaccination against blood stages. Recent work has indicated that lyophilized antigen suitable for storage in tropical areas is an effective vaccine. As a prelude to large-scale vaccination it is imperative that *in vitro* methods of cultivation be developed so that malarial antigens can be produced in large quantity. Work is now proceeding along this line. It is now possible to obtain formation *in vitro* of early (ookinete) mosquito stages of malaria comparable to that found *in vivo*. Forms which have the appearance of oocysts have also been obtained. Work is also continuing on *in vitro* cultivation of the mammalian blood stages of the parasite. It appears from recent work that bone marrow may offer a suitable substrate for growth of these stages.

SEMI-ANNUAL RESEARCH REPORT

A. General Background

It is well known that malaria is still an extremely serious problem worldwide, especially in many underdeveloped countries. Drug treatment and vector eradication have been somewhat successful in controlling malaria in certain parts of the world, but it is now apparent that they are not the final solution to the malaria problem. Strains of malaria have arisen which are drug-resistant, and vector control requires constant surveillance. When surveillance breaks down through complacency, lack of funds, or a miriad of other reasons, the number of cases of malaria may rise rapidly to pre-surveillance levels. The problem of malaria control is further exacerbated by the development of insecticide resistance by the mosquito vector.

It was thus felt that vaccination against malaria might be a significant contribution to control of the disease. We have demonstrated that it is feasible to vaccinate against malaria, and as a result of work done in our and other laboratories the question no longer seems to be whether there will be a malaria vaccine, but when will there be a malaria vaccine. It is hoped that vaccination of children against malaria will provide life-long protection against the disease.

B. Statement of Project Objectives as Stated in the Contract

The objectives of this contract are (1) to develop methods for the large-scale production of malaria antigens, and (2) to ascertain efficient means of immunizing mammals, utilizing rodent and primate testing systems. The effort to be undertaken under this contract is based upon the accomplishments of Contract No. AID/csd-1432 which demonstrated that it is possible to induce

resistance in mammals (rodents and primates) to prevent malaria infection and also to enhance the suppression of malaria in an infected animal. The highly significant demonstration that it is feasible to vaccinate against malaria, now focuses attention on whether it is a practical reality. All the vaccine materials utilized by this group have been obtained by (1) a laborious manual dissection procedure to recover infective sporozoite stages from malaria infected mosquitoes, and (2) extracting malaria antigens from the infected blobd of rodents or primates. Neither of these in vivo methods appear to offer the possibility of developing the large-scale production of a malaria vaccine.

It is generally agreed that the only promising, practical way to achieve large-scale production is by the development of in vitro culture techniques. The main focus of effort under this contract will be on the need to develop practical methods for vaccine production. Relevant portions of the in vivo sporozoite work and the rodent model test systems performed previously at the University of Illinois (under subcontract, now terminated) have been picked up at the University of New Mexico.

C. Continued Relevance of Objectives

Our research to date does not indicate a need for modification of the project objectives. We remain convinced that vaccination is, indeed, feasible and our main goal now is the production, in large quantities,of material which can be used for vaccination.

D. Accomplishments to date

IN VITRO CULTIVATION AND ANTIGEN PRODUCTION

Conventional Mosquito Rearing

Adult mosquitoes are being produced in high enough numbers to meet the demands of all ongoing experiments in tissue culture and other purposes.

Aseptic Mosquito Rearing

Am improved diet for aseptically reared mosquitoes has been established. Increased amounts of folic acid, (1.37-20.0 mg/liter) cholesterol (10-20 mg/liter) and high protein content (500 mg liver extract and 500 mg bacto-liver/liter) yield approximately 80% pupation and 100% emergence of pupae into adults. Sporogonic development of the malarial parasite occurs in 60% of the aseptically reared females fed on infected hamsters. It is difficult to raise large numbers of aseptic mosquitoes and methods to increase their yield are being investigated. The ability to obtain bacteria free sporozoites from these mosquitoes might prove of value in expediting the in vitro development of exoerythrocytic stages of the parasite.

Cultivation of the Sporogonic Forms of the Malarial Parasite

Successful cultivation of the early stages of the malarial parasite including exflagellation of microgametes, fertilization of macrogametocytes, and zygote formation into mature, viable, motile ookinetes can be demonstrated in vitro using both vector and non-vector cells substrates. These substrates include primary cell culture, anopheline cell line, ecdysone treated primary cell culture and fat head minnow (FHM) epithelial cell line. The ookinete morphology and behavioral activity in vitro approximate that observed in vivo.

Primary cell cultures are regularly established using newly hatched larvae as a source of outgrowths. Primary cell cultures ranging in age from 8 to 30 days support ookinete development. Addition of aseptic whole mosquito gut as a supplement to the primary cell culture appears to enhance ookinete formation. Development beyond the ookinete has not yet been observed in this substrate. The anopheline cell line is also able to support ookinete formation.

Primary cell culture treated with the hormone ecdysone give rise to healthy pulsatile myoblast cells. This substrate also supports ookinete development. Ookinete motility lasts for as long as 24 hrs, and the ecdysone, or the cells stimulated by it, appear to enhance the growth of ookinetes.

At this time the fat head minnow epithelial cell line (FHM) appears to be the most promising cultivation substrate. Ookinetes are found intracellularly 24 hrs into the cultivation period and by the eighth day of cultivation, oocyst-like structures can be observed. Ultrastructural studies are currently being undertaken to determine whether oocyst formation has actually occurred, and to assess whether the oocysts are morphologically normal.

In addition to cell culture the use of organ culture has been investigated as a supporting substrate for sporogonic development. Aseptically reared mosquitoes are allowed to feed on infected hamsters and the guts are immediately dissected and placed in culture media. Eight days after the start of cultivation young oocysts can be observed in some of the viable guts. No development beyond the young oocyst has been observed. By changing the culture medium it may be possible to obtain a medium which is capable of supporting the organ as well as further sporogonic development.

Histochemical Analysis of the Cultivation Substrate and in vivo and in vitro Sporogonic Stages of the Malarial Parasite

Chemical characterization of the culture substrates and sporogonic stages has been routinely established. The presence of lipids, mucopolysaccharide, glycogen and nucleic acid have been demonstrated in the

different cell cultures that support *in vitro* growth. Histochemical characterization of the *in vitro* and *in vivo* obtained stages of the parasite reveal identical chemical make up so far as can be assessed.

An adequate knowledge of the chemical nature of the parasite and its environment is necessary so that culture conditions can be altered to maximize the yield of parasites and stimulate development from the oocyst into later stages of the parasite life cycle.

Cultivation of the Parasite in Mammalian Cells

Several different mammalian cell types were used in initial attempts to obtain *in vitro* growth of the erythrocytic stages of *Plasmodium berghei*. Among those studied were embryonic bovine bone marrow, kidney and liver syrian hamster kidney cells (BHK-21) and primary mouse embryo cells. None of these cell types offered a suitable substrate for parasite growth. After the addition of infected red blood cells to the cultures little if any evidence of the presence of intracellular organisms was observed. Attention was therefore directed towards the selection of a cell(s) which might be more suitable for entry or penetration of the parasite, as well as being chemically and metabolically more attractive.

Studies were begun with cultures of mouse and hamster bone marrow which contain phagocytic as well as erythropoietic stem cells. Experiments were conducted with bone marrow obtained from the tibiae and femura of mice and hamsters. The mouse cells grew better than did those of the hamsters and thus most experiments were run in these cells. Individual cell suspensions, as well as primary mouse bone marrow aggregates of up to 200 cells were studied. In all cultures, after the addition of infected red blood cells, many cells showed numerous vacuoles which contained the

infected RBC's as well as various stages of the parasite. In several experiments the number of intracellular parasites appeared to decrease slightly up to 3 days, whereas at 4 to 7 days a considerable increase in the number of intracellular stages were present. More stages were seen at day 7 than on any other day. Parasite numbers progressively decreased from days 8 to 11, and no intact parasites were seen from 12 to 21 days. The majority of the stages were rings, trophozoites and merozoites. Stages similar to gametocytes were rarely seen. The progressive increase in the number of parasites suggested that they were undergoing asexual reproduction.

Animals injected with cell suspensions obtained from primary mouse bone marrow culture which had been inoculated up to 3 days earlier with P. berghei infected cells developed a parasitemia whereas those animals injected with cells from 4 to 12 day old cultures did not develop a patent parasitemia. The importance of this finding has yet to be determined.

Examination by light and electron microscopy of bone marrow cultures to which infected red blood cells had been added showed that various asexual stages of P. berghei occurred in proerythroblasts, in early intermediate and late normoblasts, in macrophages and in small to intermediate sized epithelioid cells similar in appearance to reticular (= stem) cells. Attempts to clone those cells, which appear similar to stem cells, are currently being conducted. Mouse bone marrow is now being grown as a secondary cell line and cultures of these cells will be inoculated with the parasite.

Attempts to initiate bone marrow cultures from Rhesus monkeys have been made. The monkeys were relatively old, however, which made culturing of the cells extremely difficult. Younger monkeys will be used in future

attempts. Once monkey bone marrow cells are established, it will be possible to investigate whether these cells are able to support growth of the primate malarial parasite, P. knowlesi.

Investigations are also currently underway to determine whether the malarial parasite can grow in established cell lines. Cell lines which have been selected and which are currently being maintained in our laboratory include: Chang liver (human origin, epithelial like), green monkey kidney (fibroblast), three different human sternal marrow cell types (all epithelial), HeLa (from human cervical carcinomal, epithelial), embryonic human intestine (epithelial), Rhesus monkey kidney (epithelial), mouse liver (epithelial) and a lymphoid neoplasm (fibroblast). Epithelial cells in culture are believed by some investigators to retain more of the characteristics of the cells from which they were originally obtained than do fibroblast cells.

Two cell types of the human sternal marrow (Detroit 6 and Detroit 98) have been studied for their ability to support growth of P. berghei. Relatively few intracellular stages, including trophozoites, schizonts and merozoites have been seen in these cultures 1 to 9 days after inoculation with P. berghei infected red cells. Cultures will be inoculated with P. knowlesi to determine whether it can grow in these cell lines.

Experiments with P. vinckei vinckei using primary cultures of bone marrow, liver and cells from whole mouse embryos as well as the available established cell lines are just beginning.

Cultivation of Exoerythrocytic Stages

Sporozoites of P. berghei, obtained from Anopheles stephensi mosquitoes, have been inoculated into human sternal marrow cell cultures in attempts to obtain development of exoerythrocytic stages. No intracellular stages of the parasite have been observed. Before progress can be made in this area, techniques for freeing the sporozoites from mosquito tissue and debris must be refined. Techniques must also be improved for inhibiting the growth of contaminating microorganisms without affecting the viability of the sporozoites.

ANTIGEN TESTING AND IMMUNOLOGICAL STUDIES

Antigen Testing in Primate System

It has been demonstrated conclusively that primates can be vaccinated with lyophilized antigen. This finding is important in that it provides the potential for easing shipment and storage of material in the field when a human malarial vaccine becomes a reality. It has also been demonstrated that protection can be induced in monkeys with relatively small quantities of antigen.

Twenty-two female Rhesus monkeys were utilized to determine quantitative antigenic thresholds. Fourteen were vaccinated with antigen emulsified in adjuvant, 4 received adjuvant alone and 4 served as uninjected controls. A two injection schedule was utilized as in previous experiments. Of the 14 vaccinated monkeys, 10 received lyophilized antigen in doses of 250 μg/ injection, 500 μg/injection, or 1500 μg/ injection, as measured by protein determination. Four monkeys received the frozen preparation of antigen utilized in previous experiments.

All of the monkeys which were not vaccinated died of a fulminating infection after challenge with 2.5×10^5 P. knowlesi infected red cells,

which is uniformly lethal in untreated Rhesus monkeys. The surviving monkeys all showed transient parasites in their peripheral blood indicating that they had become infected. The average peak parasitemia in these monkeys (2.4%) was significantly lower than in the control monkeys (26.3%). The prepatent period (period prior to the first detection of parasites in the peripheral blood) was significantly shorter in control animals than in vaccinated animals. Although 6 of 14 vaccinated monkeys died after challenge, they died significantly later than did the control animals indicating that even those vaccinated animals that died showed some evidence of protection as a result of sub-threshold quantities of antigen. From this experiment it was concluded that as little as 250-500 μg of malarial protein antigen is capable of inducing immunity.

An experiment was run to determine if monkeys vaccinated with blood stage antigen are protected against sporozoite challenge. All previous challenges have been done with infected red blood cells and it is important to determine if monkeys vaccinated by the current method are protected against a natural infection. The results so far obtained although suggestive that this is the case are statistically inconclusive. Although 3 of 4 vaccinated monkeys survived after being vaccinated, 4 of 10 control monkeys also survived after being bitten by infected mosquitoes. The survival of unvaccinated monkeys after challenge with P. knowlesi is rare and leaves the unanswered question of why such a high percentage of the control animals survived. There were, however, differences in the course of the infection between control and vaccinated animals. In a parallel experiment run at the same time, in which the animals were vaccinated in a similar manner to the above animals, but were challenged with infected red blood cells, 3 of 4 vaccinated animals survived whereas all 10 of the control animals succumbed to the infection.

To date the vaccinating material has been able to immunize successfully only when combined with Freund's Complete Adjuvant which is not suitable for use in man. The problem of obtaining an adjuvant to replace Freund's is currently being investigated. In an experiment now being run, animals have been vaccinated with Adjuvant 65 and Adjuvant 65 + BCG in combination with antigen. Adjuvant 65 is a peanut oil based adjuvant which is in the process of being licensed for use in the influenza vaccine, while BCG is commonly used to vaccinate against tuberculosis and has also been demonstrated to stimulate nonspecifically the cell mediated immune system. As we feel that cell mediated immunity is an important component of the response to plasmodial infection it seems critical to use an adjuvant which stimulates cell mediated immunity and is also safe for human use. It is hoped that Adjuvant 65 + BCG will meet these criteria.

Immunological Studies in Primates

Skin testing of monkeys was performed in the primate vaccination experiments previously described, in an attempt to develop an in vivo test that would enable us to predict, prior to challenge, the degree of protection afforded immunized animals. The antigen used for skin testing was the same antigen employed in the vaccination of the animals. The results were very encouraging.

All animals when skin tested prior to vaccination gave a negative response. In the first experiment described, using lyophilized antigen, the results were markedly different when the same animals were tested after vaccination, but prior to challenge. Unvaccinated controls and monkeys treated with adjuvant alone gave a negative reaction to the antigen. Those vaccinated monkeys that later survived challenge with

the P. knowlesi parasitized blood gave a very pronounced reaction at 24 hours (mean diameter, 11.88 mm) prior to challenge. Examination of histological sections of skin biopsies taken at the reaction site indicated that the reaction is similar to the typical delayed type hypersensitivity reaction. This is to be expected in view of the delayed onset of the reaction. Although some of the vaccinated monkeys which died after challenge also gave a positive skin response, it was possible to predict correctly 86% of the time, based on skin test response whether or not a monkey could be expected to be protected.

The monkeys were also tested with P. berghei antigen and the reaction, in many cases, was similar to that observed at the P. knowlesi test site. Although the correlation with protection was not as good as with P. knowlesi, there was a high enough correlation to suggest that P. knowlesi and P. berghei share common antigens which may be related to protection.

Skin testing of the monkeys in the mosquito challenge experiment was also carried out one week prior to the first vaccination. Neither the two different doses of skin testing antigen (15 μg and 30 μg) which were tested nor 30 μg of BSA gave positive results.

At the conclusion of the vaccination schedule and one week prior to challenge, the animals were skin tested again with the same materials. The diameter of induration and the double thickness of skin were used as measures of reactivity. Reaction sites were examined and measured at 6, 24, 48 and 72 hours. None of the controls gave any signs of positive reactions. The eight vaccinated animals showed a definite reaction at the sites tested with antigen. At 24 hours the average diameter of the

indurated area was 7.69 mm ± 3.24 at the 15 μg site and 9.05 ± 4.32 at the 30 μg site. There was also a significant increase in the double thickness of skin.

Some degree of protection was observed in all of the vaccinated monkeys. The group that was challenged with infected red blood cells showed a 75% survival (3/4). The animal in this group that died showed a much delayed day of death when compared to the controls.

The three survivors of the mosquito challenged group showed a significantly lower parasitemia than did the surviving controls. The vaccinated monkey that died did so on day 25 post challenge while the controls died between days 9 and 16.

In summary in the blood challenge group all of the monkeys giving a negative skin test (8/8) died, while of the 4 monkeys giving a positive sking test 3 survived and one died much later than the controls. In the mosquito challenged groups, none of the controls showed a positive skin test and 5 of 8 died. Of the 4 vaccinated monkeys, one gave a negative reaction and survived, two gave positive reactions and survived, and one gave a positive reaction and died although death was significantly later than in control monkeys.

In addition to skin testing the ideal situation would be to have several other *in vitro* immunological assays which correlate with protection. Two such assays which are being studied in depth are the lymphocyte transformation test, a correlate of cell mediated immunity, and the radioimmunoassay, which measures antibody production. Encouraging results have been obtained with both of these assays.

Initial experiments with the microlymphocyte transformation test did not give consistent results. The conventional lymphocyte transformation test therefore was standardized and is currently being used. The first large scale experiment was performed on monkeys being used in the Adjuvant 65 + BCG experiment. The lymphocyte transformation was performed one week prior to challenge on 12 of 24 monkeys in the experiment. A pure lymphocyte preparation was obtained after drawing 10-12 ml of peripheral blood from each monkey. The lymphocytes were washed and resuspended to a concentration of 10^6 lymphocytes/ml in supplemented medium 199. Three sets of 5 tubes from each monkey were treated with 20 µg PPD/ml, 10 µg *P. knowlesi* antigen/ml, or were left without being stimulated. All tubes were incubated at 37° for 5 days after which time 2 µCi of ^{3}H-thymidine (S. A. 2 Ci/mmole) were added and the tubes were incubated an additional 18 hrs. T-lymphocytes which are sensitized to the antigen will divide and this division is indicated by an increased uptake of radioactive thymidine, which can be measured in a scintillation counter. A blastogenic index which is calculated from this data indicates that while PPD does not cause increased uptake of tritiated thymidine, *P. knowlesi* antigen when incubated with lymphocytes from vaccinated animals causes a marked uptake in the radioactive label. For those animals vaccinated with antigen + Freund's adjuvant the blastogenic index is 6 times higher than in controls. The animals receiving antigen in combination with BCG + Adjuvant 65 show a two fold increase in the blastogenic index.

Although it is not possible yet to determine how well these results correlate with protection it is obvious that the lymphocytes of the vaccinated monkeys have been stimulated by the *P. knowlesi* antigen.

In addition to having the potential for predicting the success of vaccination this assay may be an extremely useful tool in assessing the biological activity of different antigen fractions.

The radioimmunoassay is the second *in vitro* assay which has been studied in depth. The ultimate goal of this assay is to be able to predict whether antibody titers after vaccination correlate with the level of protection which is obtained. Much of the work to date has involved standardization of methodology.

Experimentation with various methods of separating ^{125}I-labeled proteins from the free isotope remaining in the reaction mixture resulted in development of a fast and efficient filtration method using Sephadex G-25. This along with quantitative comparison of protein-bound to unbound radioactivity allowed the preparation and use of antibody tracer reagents of much higher purity and specific activity.

It was also found that the malaria antigen did not iodinate well or separate from free iodine easily. It was thought that solubilization with Triton X-100 might help, however, triton was found to interfere with the basic processes of the assay, preventing protein binding to plastic and removing coatings already bound. The antigen, moreover, was not able to coat plastic tubes with the same efficacy as antibody. These factors ruled out the use of the antigen as an initial coat or as a labeled tracer, either of which would have permitted the use of fairly simple RIA sandwich systems.

It was decided a 4-layer solid phase RIA, as has been tried with other particulate antigens, such as viruses, would be most practical. With this method, rabbit IgG made against malarial Ag is first coated

onto the test tube as a type of immuno-absorbant to hold the next layer of antigen in place.

Before attempting the new design, intensive investigation was made into the mechanism of protein binding to plastic, incubation time, concentration, buffers, surface area, and level of radioactivity. The data on time and concentration of protein confirmed that which is found in the literature. This allowed rapid running of the first step and repeated use of antibody solutions.

The variety of buffers suggested by others for dilutions and washing was unnecessary and in instances was detrimental to the immune reactions desired. The standard PBS used in the antigen preparation was found to be the most convenient and best buffer throughout the procedure. Lower radiation levels than generally quoted were found as efficient in this application.

The first runs of the complete assay showed some promise but useful data was almost obscured by nonspecific variation or "noise" levels within sets of tubes for each test serum. In later experiments, however, random variation was reduced to a tolerable level. With the availability of more antigen, the surface area of each protein coat can be increased, reducing variation further.

Successful assays have been run recently on serum samples from 4 monkeys vaccinated with lyophilized antigen (2 survivors and 2 non-survivors) and 2 non-vaccinated control monkeys. The sera represent 4 baseline weeks before vaccination and 3 separate weeks after injection. Antibody levels detected show only minor fluctuations during the baseline weeks. Levels in vaccinated monkeys climbed significantly after the first injection and still higher in 3 out of four after the second

injection. This data is significant to 99% confidence with computor statistical tes' :. . There seems to be a correlation between antigen dose and antibody reaction as well as between skin testing and the RIA, although the sample is too small to draw any definite conclusions. Changes in antibody levels of the controls are insignificant in comparison with the vaccinated animals.

It has been confirmed that a dilution effect for test sera exists in the assay. This makes it likely that the results can be quantitated and some assessment made as to how antibody levels correlate with protection.

Blood Stage Antigen in P. berghei Rodent System

Reports have appeared in the literature suggesting that ribosomal material extracted from bacteria may, after injection into mice, protect the animals upon subsequent challenge. An attempt was made to see if this was also true of plasmodial ribosomes. Ribosomal material was extracted from P. berghei and was quantitated by protein determination. Mice were injected with either 20 μg or 2 μg of material, with or without adjuvant. Eight days after challenge 62% of the vaccinated mice were alive while only 15% of the controls were alive. By day 16, 36% of the vaccinated mice were still alive, while all of the controls were dead. Thirty-three percent (10/30)of the mice receiving antigen and Freund's adjuvant were alive while 58% (11/19) of those animals receiving ribosomal material without adjuvant were alive. Most reports on the protective properties of ribosomal material suggest that it must be incorporated in an adjuvant. This preliminary study suggests that an adjuvant may not be necessary.

More recent experiments have confirmed the original observations. These results show that 10 μg of ribosomal material, based on protein

determination, elicits significant protection against challenge with *P. berghei*. This is true if the ribosomal material is given alone or in combination with Freund's Incomplete Adjuvant. No protection was observed with yeast RNA given at the same dose level as a nonspecific control. Analysis of the ribosomal preparation shows an RNA:Protein ratio of 0.6:0.8, and transmission electron micrographs of the preparation demonstrate primarily ribosomes. Intact membranes were not observed. Studies are currently being planned utilizing *in vitro* immunological assays to determine whether the protection is caused by the cell mediated or humoral immune system.

Vaccination with specific subcellular fractions of the blood stage parasites should produce information about the location of the protective antigen(s). Such information could lead to increase in the yield of these antigen(s). In addition to the ribosomal fraction, a light microsomal membrane fraction of the parasite was obtained. The results from the first vaccination experiment in mice using this fraction give no indication of protection. The mice were vaccinated once with doses of material ranging from 10 to 100 μg protein emulsified in Freund's Incomplete Adjuvant and were challenged two weeks later with 5×10^4 infected red blood cells.

Since it is not known whether cell mediated or humoral immunity is responsible for protection in malaria, the type of adjuvant used might have a profound effect on whether or not protection is induced. It appears from work done in other laboratories that ribosomal material stimulates cell mediated immunity, and thus Freund's Incomplete Adjuvant can be used. If on the other hand, the light microsomal (membrane fraction) stimulate antibody production it might be necessary to combine it with an adjuvant such as Freund's Complete Adjuvant before protection can be

induced. In subsequent experiments using the membrane fraction, different dose levels of antigen as well as different adjuvants will be examined. Attempts are currently being made to obtain P. berghei merozoites from cultures of infected red blood cells. If these are obtained in sufficient quantity, intact merozoites as well as fractions will be used as vaccinating materials.

Antigenic Analysis

Chemical composition of the P. knowlesi antigen normally used to vaccinate Rhesus monkeys has been determined per milligram of protein. The antigen contains 90-125 µg RNA, 60 µg DNA, 400 µg phospholipid, 200 µg cholesterol and trace amounts of carbohydrate. There is a considerable variation in the quantities of these materials found in individual antigen preparations. This might be a function of the proportion of each parasite stage present in the blood at the time of the antigen harvest.

Vaccination with Sporozoite Material and Isolation of Sporozoites

No vaccination experiments were run using sporozoite material. All sporozoites currently being obtained are placed in cell cultures to attempt to achieve exoerythrocytic development.

Light and Electron Microscopic Studies

As previously mentioned transmission electromicrographs have confirmed the presence of ribosomes in parasites prepared so as to recover this material.

Fine structural studies of the P. knowlesi antigen are currently being undertaken.

The motility of P. berghei ookinetes obtained in culture was studied by light microscopy, and the fine structure of the ookinetes obtained in culture was studied by scanning electron microscopy. Both motility and fine structure of in vitro forms were the same as those observed for in vivo obtained forms.

Tissues from the liver, lung, bone marrow, brain, lymph node, spleen, heart, and kidney were obtained at autopsy from monkeys in the primate experiment previously described, and were prepared for light and transmission electron microscopy (TEM). A comparative ultrastructural study of the pathological aspects of malaria in the vaccinated-protected, vaccinated-unprotected and control animals will be made.

Sections were prepared from cultures of primary mosquito tissue for TEM. The development of the fertilized macrogametocyte (zygote) into the ookinete stage will be followed. In addition, midguts obtained from infected and uninfected aseptically and normally reared mosquitoes have been prepared to compare their ultrastructural aspects.

E. Dissemination and Utilization of Research Results

Drs. Ronquillo, Speer and Barr attended the 26th Annual Meeting of the Society of Protozoologists in Houston, Texas. Dr. Speer presented a paper entitled, "Scanning Electron Microscopy of Primary Mosquito Cell Cultures used in Cultivating Plasmodium berghei." Dr. Ronquillo presented a paper entitled, "Mode of Locomotion of Ookinete Cultivated in vitro." Dr. Barr attended the 4th International Convocation on Immunology in Grand Island, New York. She presented a paper entitled "Vaccination of Mice with Ribosomal Material Extracted from Plasmodium berghei."

Drs. Ronquillo, Speer, Schenkel and Silverman attended the Workshop on Problems Related to an Anti-Malarial Vaccine in Washington, D. C. at the National Academy of Science. See also the list of publications.

F. Statement of Expenditures and Obligations and Contractor Resources

(To follow)

STATEVILLE/JOLIET SUB-CONTRACT

Currently 54 *Aotus* monkeys are in the colony at the Stateville prison, and 15 are infected with an Ethiopian strain of *Plasmodium falciparum*. The human malaria is regularly maintained and transmitted from monkey to monkey.

The necessary equipment (centrifuge, French Press, etc.) have been obtained to prepare the red cell stage antigen for vaccination studies.

Unfortunately, the uncertainties surrounding the future of the malaria project inhibit the initiation of the vaccination studies. A decision about the proposal to move the entire operation to New Mexico is currently under active consideration.

G. Workplan and Budget Forecast for 1974-75

WORKPLAN AND BUDGET FORECAST 1974-5

I. Antigen Production

A. *In vitro* culture

1. Sporozoite and related antigens

a. To continue cultivation of insect cells and non-vector cell lines to be used to achieve further sporogonic development

b. To expand and improve large scale culture methods

c. Depending on success, to vaccinate animals with *in vitro* obtained sporogonic material

2. Blood stage antigens

a. To continue cultivation of blood stages in primary and established mammalian cell lines

b. Depending on success, to vaccinate animals with *in vitro* obtained antigens

B. Maintenance of the parasite *in vivo*

1. Sporozoite and related antigens

a. To continue maintenance of species of anophelines for cyclic transmission of rodent malaria

b. To continue preparation of sporozoites to be used in cell culture to attempt to obtain exoerythrocytic development

2. Blood stage antigens

a. To continue maintenance of rodent and primate malaria

b. To continue investigations on ways to maximize yields of *in vivo* produced antigen

II. Antigen Investigation

A. Rodent Model System

1. Sporogonic antigens

a. To test the immunizing properties of *in vitro* produced antigens if they can be obtained in sufficient quantity

b. To isolate and purify the active immunological components of these antigens

2. Blood stage antigens

a. To continue efforts to identify the active antigenic component(s) of *in vivo* produced blood stage antigens

b. To isolate sub-cellular components of *in vivo* produced antigens and test them for immunizing ability

c. To test the immunizing properties of *in vitro* produced blood stage antigens if these can be produced in sufficient quantity

B. Primate Model System

1. To examine effects of varying immunizing procedures with emphasis on finding an alternative to Freund's Complete Adjuvant

2. To immunize with subcellular components of the malarial parasite

3. To initiate vaccination studies with Aotus monkeys and *P. falciparum*

4. To check cross-protection between different species of *Plasmodia*

C. Immunological Assays

1. To continue skin testing in monkeys
2. To continue work with lymphocyte transformation assay and to determine its correlation with vaccination success
3. To continue work with radioimmunoassay and to determine its correlation with vaccination success

Budget Forecast 7/1/74 thru 6/30/75

Salaries	$249,904
Fringe Benefits	40,476
Consultants	3,875
Travel	13,375
Equipment	34,290
Supplies	231,805
Publications	3,000
Indirect Costs	114,320
Subcontract	100,730
	$791,775

BIBLIOGRAPHY and ABSTRACTS

1. Some qualitative aspects of spleen cell transfer studies on *Plasmodium berghei* infected rats. Journal of Protozoology, 20(3): 449-452 (1973) Edelberto J. Cabrera, Nelda E. Alger and Paul H. Silverman.

Abstract: Adoptive immunity to *Plasmodium berghei* was transferred by intraperitoneal injections into rats never exposed to this parasite of either 2×10^7 or 2×10^8, but not of 2×10^6, spleen cells from syngenic rats which had recovered from a primary *P. berghei* infection. When the spleen cells from the latter animals were kept at 47 C for 45 min they remained alive, but no longer were able to transfer protection. The capacity to transfer adoptive immunity was not found in spleen cells from adult rats capable of age immunity. On the other hand, this capacity was found in spleen cells from rats that had suffered a very transient parasitemia (<1% peak parasitemia).

2. Aseptic rearing of *Anopheles stephensi* (Diptera: Culicidae). Annals of Entomological Society, 66(5): 949-954 (1973). M. C. Rosales-Ronquillo, R. W. Simons, and P. H. Silverman.

Abstract: Germ-free adults of *Anopheles stephensi* Liston have been reared successfully from newly hatched larvae obtained from surface sterilized eggs in a modified aedine diet. The sterile diet included high protein and vitamin sources (liver extract, casein and yeast), cholesterol, RNA, inorganic salts, sucrose, and additional water-soluble vitamins. At 27 ± 1°C, 78% RH, and a 12-hr light:12-hr dark photoperiod, aseptically reared larvae pupated in an average of ca. 9 days, and adults emerged within 3 days.

3. Vaccination of Rhesus Monkeys (Macaca mulatta) Against Plasmodium knowlesi by use of Non-viable Antigen. Bull. W.H.O. (In press). R. H. Schenkel, G. L. Simpson, P. H. Silverman.

Abstract: This study is part of a long term project whose ultimate goal is the development of a malarial vaccine suitable for human use. Rhesus monkeys vaccinated with two injections of an antigen whose preparation is described were protected against a normally lethal challenge of Plasmodium knowlesi when the antigen was given in combination with Freund's adjuvant. Use of the antigen alone or in combination with several other adjuvants was not successful. The fact that monkeys were protected against an infection which is normally lethal, suggests that a similarly prepared antigen might be of use in human malaria systems.

4. Vaccination of Rhesus Monkeys against malaria by use of Sucrose Density Gradient Fractions of Plasmodium knowlesi Antigens. Nature. (in press). G. L. Simpson, R. H. Schenkel and Paul H. Silverman.

Abstract: Plasmodial antigen previously described as being protective, when subjected to sucrose density gradient centrifugation, gives two distinct bands. Each of these bands is capable of imparting protection against P. knowlesi in Rhesus monkeys. It is uncertain at this time what the different bands represent, although it has been hypothosized that one band contains antigen alone, while the other may contain antigen complexed with the malarial pigment hemazoin.

5. Vaccination of Rhesus Monkeys Against *Plasmodium knowlesi* with Lyophilized Antigen. (In preparation). R. H. Schenkel, E. J. Cabrera, C. A. Speer, and P. H. Silverman.

6. Delayed Dermal Hypersensitivity of Rhesus Monkeys Vaccinated Against *Plasmodium knowlesi*. (In preparation). E. J. Cabrera, C. A. Speer, R. H. Schenkel, and P. H. Silverman.

7. Thin-layer Chromatographic Analysis of Phospholipids Isolated from Non-viable Plasmodial Antigen. (In preparation). R. Beckwith, R. H. Schenkel, and P. H. Silverman.

8. Vaccination of Mice with Ribosomal Material Extracted from *Plasmodium berghei*. (In preparation). M. M. Barr, J. Heidrich and P. H. Silverman.

9. Adoptive Transfer of Immunity to *Plasmodium berghei* with Spleen Cells Fractionated by Centrifugation on a Discontinuous Albumin Gradient. (In preparation). E. J. Cabrera, N. E. Alger, and P. H. Silverman.

10. Scanning Electron Microscope Observations of *Plasmodium berghei* Ookinetes in Primary Mosquito Cell Cultures. J. Invert. Path. (In press). C. A. Speer, M. C. Rosales-Ronquillo, and P. H. Silverman.

11. Motility of *Plasmodium berghei* Ookinetes in Mosquito Cell Cultures. (In preparation). C. A. Speer, M. C. Rosales-Ronquillo, and P. H. Silverman.

12. *In vitro* Ookinete Development of the Rodent Malarial Parasite, *Plasmodium berghei*. J. Parasit. (In press). M. C. Rosales-Ronquillo and P. H. Silverman.

13. *Plasmodium berghei* Ookinete Formation in Non-vector Cell Line. J. Parasit. (In press). M. C. Rosales-Ronquillo, G. Nienaber, and P. H. Silverman.

14. Mass Rearing of *Anopheles stephensi* Using a Commercial "Fish Food," TETRAMIN. (In preparation). M. C. Rosales-Ronquillo, G. Nienaber, and P. H. Silverman.

15. Ultrastructural Analysis of *Plasmodium berghei* Sporozoite Antigens Prepared by Freeze-thawing and Heat Inactivation. J. Protozool. (In press). B. P. Jakstys, N. A. Alger, J. A. Harant, and P. H. Silverman.