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1. SUBJECT CLASSIFICATION

A. PRIMARY
Public Health

B. SECONDARY
General Public Health

2. TITLE AND SUBTITLE
Malaria immunity and vaccination; semi-annual research report, July-Dec. 1973

3. AUTHOR(S)
New Mexico Univsity, Dept. of Biology

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Department of Biology, New Mexico University, Albuquerque, New Mexico 87131

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

9. ABSTRACT

The objectives of this AID-sponsored project are to develop methods for the large-scale production of malaria antigens, and ascertain efficient means of immunizing mammals, using rodent and primate testing systems. Project work has demonstrated the feasibility of vaccinating primates and rodents against malaria. The work done with rodents has involved vaccination against both the sporozoite and blood stages of malaria, while the work done with primates has involved vaccination against blood stages. As a prelude to large-scale vaccination it is imperative that in vitro methods of cultivation be developed so that large quantities of malarial antigens can be produced. Work is now proceeding along this line. It is not possible to obtain formation in vitro of early (ookinete) mosquito stages of malaria comparable to that found in vivo. 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.

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A N D

V A C C I N A T I O N

Semi-Annual Report July - December, 1973

University of New Mexico

Albuquerque, New Mexico 87131

PN-AAC-105

REPORT SUMMARY

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 December 31, 1973

Total A.I.D. funding of contract to date: $650,227.12

Total expenditures and obligations through previous contract year: $326,196.47

Total expenditures and obligations for first six months of current year: $324,030.65

Estimated expenditures for next six months: $380,428.

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. 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*. 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.

Malaria Immunity and Vaccination

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 signifi 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 blood 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.

Aseptic Mosquito Rearing

Efforts to improve the diet to raise germ-free adults have been successful. Both basic requirements (either yeast or casein), and supplementary requirements (increased amounts of folic acid 1.37 mg ⟶ 20 mg; Cholesterol 10 mg ⟶ 20 mg; and a combination of 500 mg liver extract + 500 mg of bacto liver) yielded a 95% rate of emergence into healthy adults. The step-wise transfer of the mosquito developmental stages into different containers makes the production of large numbers of aseptic adults difficult. Possible ways to increase production of aseptic adults are currently being investigated.

Cultivation of the Sporogonic Forms of the Malarial Parasite

Successful cultivation to the ookinete stage was reported in the preceding progress report. Appraisal of different ages of mosquito primary cell culture which may be used to support development of the parasite beyond the ookinete stage is currently in progress. Experiments using graded concentrations of the insect hormone ecdysone added to the culture substrate are continuing. These are being done as a result of earlier studies which indicated that myoblast-like cells in culture may offer a preferential site for parasite development. Ecdysone appears to stimulate the development of these myoblast-like cells in culture. Histochemical analysis of primary tissue culture employed in the cultivation of the parasite is also in progress.

A new approach to the cultivation of the mosquito phase of the parasite has been initiated. Guts taken from aseptically reared mosquitoes when incubated in minimum essential medium (MEM) at 21 C remain viable for 15 days without loss of morphological and behavioral integrity. This organ culture may serve as a suitable substrate for parasite development.

Ookinetes have been observed attempting to penetrate red blood cells and fat head minnow cells (FHM). Actual entry into the cells was not observed, although in some stained preparations ookinetes appear to be located intracellularly in thin cytoplasmic-like spaces. An electron microscope study currently in progress will confirm whether or not ookinetes are present in the cells.

Cultivation of the Parasite in Mammalian Cells

Several different mammalian cell types were utilized in attempts to obtain *in vitro* growth of the erythrocytic stages of *Plasmodium berghei*. Embryonic bovine bone marrow, kidney, and liver appeared to be unsuitable for the development of *P. berghei*. When cultures of these cell types were inoculated with 2 to 8 million *P. berghei*-infected red blood cells intracellular inclusions similar in appearance to stages of the malarial organism were not seen.

Cultures of liver from 5-day-old mice,which had been fed on 24 hours earlier by mosquitoes infected with *P. berghei* sporozoites, were not a suitable culture medium for the parasite either. These cultures became contaminated with bacteria, whereas control cultures of livers from uninfected mice did not. Apparently, bacteria are injected when the mosquito feeds upon the host; these bacteria may then accumulate in the liver and later cause contamination of the cultures.

Syrian hamster kidney cells (BHK-21) in BHK-21 medium containing various concentrations of hydrocortisone acetate, were inoculated with 10-15 million *P. berghei*-infected red blood cells. At 3, 5, and 7 days post-inoculation,mice were injected with the medium containing the infected red

blood cells, or with the BHK-21 cells, obtained by trypsinization. None of the injected mice ever showed parasites in their peripheral blood. Upon microscopic examination the cultures showed little if any evidence of the presence of intracellular organisms and only small amounts of hemazoin pigment were present. This suggests that the parasite has difficulty in penetrating these cells. Primary mouse embryo cells did not offer a suitable substrate for parasite growth either. Attention was therefore directed towards the selection of a cell 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. Since there was the chance that parasites engulfed by phagocytes would be digested, hydrocortisone, dexamethasone or cyclic AMP were added to some of the cultures. It has been reported that these substances inhibit the release of enzymes from polymorphonuclear leukocytes *in vitro* apparently by strengthening the integrity of the lysosomal membrane. Experiments were conducted in MEM, Diploid Growth Medium, NCTC 135 and Medium 199, 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 t day 7 than on any other day. Parasite numbers progressively decreased rom days 8 to 11. 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 suggests that they were undergoing asexual reproduction. This encouraging finding is being studied in more detail and experiments are being designed to check the viability of the parasites by injecting them into mice.

Similar results were obtained in the cultures treated with 10^{-7} and 10^{-8} M of cyclic AMP and dexamathasone. Intracellular parasites appeared healthier in these than in the untreated cultures. Higher concentrations of these substances caused disintegration of most of the bone marrow cells. Culture media is normally replaced at 4 day intervals. The duration of the effect of cyclic AMP and dexamethasone in *in vitro* cultures is not well understood and whether it lasts for as long as 4 days is currently under investigation.

It is hoped eventually to be able to obtain a bone marrow culture which does not complete erythropoiesis but rather remains relatively undifferentiated with stem cells capable of reproducing and supporting the parasite.

ANTIGEN TESTING AND IMMUNOLOGICAL STUDIES

Antigen Testing in Primate System

It has been conclusively demonstrated 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. 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 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.

To date all challenges have been done with infected red blood cells. An experiment is currently being set up to insure that vaccinated animals are protected when challenged by the natural route of infection. A. quadrimaculatus infected with P. knowlesi sporozoites will be used. A pilot study recently completed indicated that there will not be any problem involved in infecting our monkeys with mosquitoes obtained from an extraneous source. If protection

is obtained the major areas remaining to be investigated with *in vivo* obtained antigen are the determination of a suitable adjuvant, and whether protection can be obtained across species lines. The answer to the latter problem will be expedited by cooperation with the group being sub-contracted to at Rush/Stateville in Chicago. As soon as they are able to collect *P. falciparum* antigen we should be able to determine if this material protects against *P. knowlesi.* Other similar systems can also be set up. If there is cross species protection the production of a practical malaria vaccine will be greatly simplified.

The problem of obtaining an adjuvant to replace Freund's is being investigated. Hopefully new information being obtained about the nature of the protective material, and the type(s) of immunological responses responsible for protection will suggest more about the nature of the necessary adjuvant. At present adjuvants used commercially with non-viable antigens stimulate antibody production without stimulating the cell mediated immune system. If cell mediated immunity is a necessary component of the immune response for protection against malaria, the development of an adjuvant other than those presently available may be required. Although this may prove to be a rather formidable task, avenues of approach are available such as the addition to the antigen of stimulators of the cellular immune system (i.e. BCG). Experiments utilizing such adjuvant are currently being contemplated.

Immunological Studies in Primates

Skin testing of monkeys was performed in the primate vaccination experiment 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. 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). Examination of histological section 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 expect in view of the delayed onset of the reaction. Although some of the vaccinat animals which died after challenge also gave a positive skin response, it was possible to predict correctly 86% of the time, based on skin test respon whether or not a monkey could be expected to be protected.

The animals were also tested with P. berghei antigen and the reactio 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 and P. berghei antigen, there was a high enough correlation to suggest that P. knowlesi and P. berghei share common antigens which may be related to protection.

In addition to skin testing, the ideal situation would be to have several other immunological assays which correlate with protection. We are presently working on such assays.

The lymphocyte transformation test, a test for cellular immunity, has been standardized for use with mouse blood. The test uses whole blood

and not purified lymphocytes, as have been previously used. Attempts to standardize this test for use with monkey blood have not yet been successful. New approaches are being attempted in order to make this test applicable in the primate model system. Another test for cellular immunity, the macrophage migration inhibition assay, is also being investigated. This assay necessitates obtaining sensitized lymphocytes, which are normally harvested in experimental situations from the peritoneal cavity. Since this is not possible when using primates, the assay is presently being modified to permit extraction of these cells from peripheral blood. Preliminary experiments utilizing commercially available Lymphoprep (Nyagaard & Co., Oslo, Norway) indicate that this will be possible.

A fluorescent antibody assay is also being developed to determine if antibodies can be detected after vaccination and if such antibodies correlate with the protection seen on subsequent challenge. A radioimmunoassay which is being worked on will serve a similar purpose if it can be perfected. Some problems have been encountered in this assay which must be worked out. Iodination of the antigen with I^{125} results in low and inconsistent counts. This more than likely is due to the particulate nature of the antigen. Solubilization of the antigen by Triton X-100 seems to offer a reasonable alternative provided that this procedure does not alter the antigenicity of the antigen. Several parameters have been standardized but others have to be worked out before the assay will have pratical application.

Blood Stage Antigen in *P. berghei* Rodent System

Several very interesting findings have been recently made utilizing this 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 animals 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) 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. Experiments are in progress to confirm these results.

Based on the success which had been achieved in the primate model system, it was decided to set up a similar system in rodents utilizing P. berghei. Mice were given two intramuscular injections of antigen in adjuvant at approximately one month intervals and challenged several weeks later. It was found that the animals which had been vaccinated died significantly sooner than did the controls. Twelve days after challenge 17 of 20 (88%) control animals were alive, while only 4 of 30 (13%) vaccinated animals were alive. This suggests that immune tolerance may have been induced. The possibility of this is enhanced by the fact that each mouse received 175 μg/injection, which is at least 300 x larger than the dose given to monkeys, based on body weight. Experiments are currently in progress testing this theory. In one experiment mice have been given several different doses of

antigen on the same schedule as in the above experiment, while in another experiment they were injected on a schedule and with quantities of antigen shown to induce tolerance in other immune systems. Conclusive evidence that the protective response is dose dependent would be extremely important and of great practical significance in vaccination.

Vaccination with Sporozoite Material and Isolation of Sporozoites

Because of problems in obtaining sufficient numbers of sporozoites, no vaccination experiments have been run. An intensive effort is presently being made to obtain pure populations of sporozoites by density gradient separation techniques. Preliminary results indicate that, while it may be possible to obtain pure sporozoites, the numbers obtained may be so small as to preclude the use of this method to obtain large enough numbers of sporozoites. If it is felt that the gradient method can not be modified to meet our needs, it may be necessary to go back to the laborious procedure of manually dissecting out salivary glands from infected mosquitoes.

Antigenic Analysis

Chemical characterization of the protective material is necessary so as to be able to determine what subcellular fraction(s) of the parasite are present. Such information may suggest better ways to prepare the material and, in addition, might give us more information on the parameters to be considered in vaccination

It has been established that both RNA and DNA are present in the antigen. A rough estimate indicates that there is approximately 5-6 μg RNA/mg protein in this material. Quantitation of DNA is presently being done. The exact origin of this RNA is not yet known. It could be host or parasite and

either soluble or ribosomal. It is possible that the antigen used for vaccination could contain polysomes, since these would come off with the antigen in the column chromatography technique that is being used. If polysomes are present, obtaining Svedberg numbers by ultracentrifugation will provide evidence as to whether these are derived from host or parasite.

By the use of several different solvent systems, it has been possible to determine that the antigen contains the normal array of lipids found in a membrane. The lipids will be quantitated to provide information concerning the amount of membrane material present, as well as its origin. Further evidence will come from membrane marker enzymatic assays.

The antigen is also being investigated for the presence of phospholipases, since they might have a direct bearing on the anemia which is observed in malaria. If phospholipases are present in the antigen, they may act on membrane phospholipids to produce phosphatids which are extremely hemolytic.

It has been believed that the protective antigen is parasite membrane or some fraction thereof. The information gained from the biochemical studies should help to determine whether this is actually the case and if it is not should determine what the protective antigen is.

Light and Electron Microscopic Studies

Tissues from the liver, lung, bone marrow, brain, lymph node, spleen, heart, and kidney were obtained at autopsy from all 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*." See also the list of publications.

F. Statement of Expenditures and Obligations and Contractor Resources

	July 1, 1973 thru December 31, 1973
Salaries	$ 96,754.28
Materials & Services	69,406.14
Equipment	19,025.56
Travel	1,519.27
Fringe Benefits	10,383.77
Sub/Contract-U of I	81,853.80
Consultants	424.55
Publications	398.19
Indirect Costs	44,265.09
	324,030.65

G. Workplan and Budget Forecast for 1973-74

Work Plan

ANTIGEN PRODUCTION

In vitro Culture

Sporozoite and related antigens

To continue insect cell line cultures and identification of antigenically active tissues.

To expand and improve large-scale culture methods.

Depending on previous success, to pursue *in vitro* sporozoite production.

Trophozoite antigens

To continue attempts at trophozoite cultivation with ultimate aim of large-scale cultivation.

To begin cultivation of primate trophozoites.

2. *In vivo* Culture

Sporozoite and related antigens

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

To continue limited preparation of sporozoite and related antigens by microdissection.

Trophozoite antigens

To continue maintenance of rodent and primate malaria.

To investigate the conditions conducive to maximum production *in vivo* of simian malaria in various species of monkeys.

ANTIGEN INVESTIGATION

1. Rodent Model System

Sporozoite and related antigens

To continue testing of *in vivo* prepared sporozoite and related mosquito antigens for their immunizing properties.

To examine the effects of varying methods of antigen preparations, adjuvants, etc.

To test cross-protectivity with trophozoite stages.

To continue efforts to isolate and purify the active antigen component(s) of sporozoite and mosquito origin.

To test effects of sporozoite related antigens on cyclic transmission of rodent malaria.

To continue activities from FY1972 with increased emphasis on *in vitro* produced antigens as available.

Trophozoite antigens

To continue efforts to identify the active antigenic component(s) of *in vivo* produced trophozoite stages.

To continue study of the varying methods of immunization on the protective response.

To establish a standardized biological criterion for assessment of antigenic activity.

2. Primate Model System

Trophozoite antigens

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

To study the pathophysiology of immunized, acutely and chronically ill monkeys.

To continue the previous activities utilizing *in vivo* produced antigens.

To emphasize isolation and characterization of active antigens.

SUB-CONTRACT TO RUSH/STATEVILLE

1. Establish Aotus monkey colony.
2. Establish *P. falciparum* in colony.
3. Run vaccination experiments to reproduce work done in Rhesus monkey-*P. knowlesi* system.

Budget Forecast

	UNM	RUSH/STATEVILLE
1. Salaries	$114,425	$20,500
2. Fringe Benefits	15,716	-
3. Consultants	1,842	1,500
4. Travel	5,900	1,000
5. Equipment	22,500	19,950
6. Materials & Supplies	87,500	24,931
7. Publications	650	-
8. Indirect Costs	52,349	11,665
Total	300,882	$ 79,546
	$380,428	

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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 Studies Against Malaria in Rhesus Monkeys. 1. Vaccination against Plasmodium knowlesi with lyophilized antigen. (In preparation). R. H. Schenkel, E. J. Cabrera, C. A. Speer and P. H. Silverman.

6. Vaccination Studies Against Malaria in Rhesus Monkeys. 2. Skin Testing as an Assay for Protection. (In preparation). E. J. Cabrera, C. A. Speer, R. H. Schenkel and P. H. Silverman.

7. *In vitro* Ookinete Development of the Rodent Malarial Parasite *Plasmodium berghei*. (In preparation). M. C. Rosales-Ronquillo and P. H. Silverman.

8. *Plasmodium berghei* Ookinete Formation in a Non-vector Cell Line. (In preparation). M. C. Rosales-Ronquillo, G. Nienaber and P. H. Silverman.

9. Scanning Electron Microscopy of *Plasmodium berghei* Ookinetes in Primary Cell Cultures. (In preparation). C. A. Speer, M. C. Rosales-Ronquillo and P. H. Silverman.