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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, Aug.-Dec. 1969

3. AUTHOR(S)
(101) Ill. Univ. Dept. of Zoology

4. DOCUMENT DATE 1969	5. NUMBER OF PAGES 20p.	6. ARC NUMBER ARC
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7. REFERENCE ORGANIZATION NAME AND ADDRESS
Ill.

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

9. ABSTRACT

10. CONTROL NUMBER PN-RAB-694	11. PRICE OF DOCUMENT
12. DESCRIPTORS Immunization Malaria	13. PROJECT NUMBER
	14. CONTRACT NUMBER CSD-1432 Res
	15. TYPE OF DOCUMENT

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CONTRACT NO. AID/csd-1432

UNIVERSITY OF ILLINOIS

DEPARTMENT OF ZOOLOGY

URBANA, ILLINOIS

PROGRESS REPORT

AUGUST 1969 - DECEMBER 1969

P. H. Silverman, Director

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SUMMARY AND OUTLOOK

PERSONNEL

Since the last six-monthly report (July, 1969) the senior staff has remained the same. It consists of Dr. Nelda E. Alger, Assistant Professor, Dr. Lawrence D'Antonio, Research Assistant Professor, Dr. Maria C. R. Ronquillo, Research Associate and Dr. Birute P. Jakstys, Research Associate. In addition, 8 Research Assistants (with B. S. degrees), 4 Laboratory Assistants, 2 Laboratory Helpers, 1 Animal Caretaker, 1 Secretary, 1 Clerk-Typist and assorted part-time student help are employed.

Dr. J. Vavra, who was a visiting professor from the Czechoslovakia Academy of Sciences, has returned to Prague. We have had numerous domestic and foreign visitors including scientists from Australia, England, South America and Europe.

During the past six months members of this research group have attended several scientific meetings. These include: "Symposium on the evolution of the immune response", October 20-22, held at the Argonne National Laboratory, Illinois; the Joint Meeting of the American Society of Tropical Medicine and Hygiene and The American Society of Parasitologists, November 3 - 7th, Washington, D. C.; The Society of Protozoologists, December 27-29, Boston, Massachusetts. The group presented four papers at the Washington meeting and two at the Boston meeting.

RESEARCH PROGRESS

Malarial Lines

As each mosquito-transmission of Plasmodium herqhoi is made, we continue to freeze blood samples (-90° C or below) from each new passage to

maintain viable lines for future studies. We previously reported that two lines derived by mosquito passage were found to be less virulent than the original NK65 strain obtained from New York University. Thus, a pattern of avirulence has been established as being transmissible through mosquitoes.

All virulent lines examined, which stem from the original point of divergence into virulent and avirulent, have been found to retain the original pattern of virulence. We now have frozen 6 lines of avirulent as well as numerous virulent and untested lines. A manuscript is in preparation on this section of the work.

It was postulated that avirulence was due either to genetic recombination in the mosquito or to population selection. On this latter basis, we attempted to attenuate the avirulent NK65C line further by blood passing the line every 30 days, rather than every 7 days after infection. Also, an experiment is underway comparing mice infected with the 3rd blood passage of NK65C with others infected with the 40th passage.

A comparison of cell preference and of the resulting anemia of the virulent and avirulent lines is required to understand the differences in virulence. This is emphasized by observations which have been made on an apparent change in virulence of the NYU-2 strain of P. berghei which has long been regarded as highly stable and unvarying. This particular strain, in our laboratory, normally develops a fulminating fatal infection characterized by infection of normocytes and resulting in death after 5 - 7 days. The recently isolated "avirulent" NYU-2 line, on the other hand, limits itself primarily to reticulocytes with animals surviving from 10 - 21 days.

"Attenuated" Line as a Vaccine

Our previous report described the challenge of adult mice recovered from infection with avirulent NK65C. Adult mice were allowed to recover from NK65C and recrudescence several times. Recovered animals were divided into two groups, one of which was rechallenged with NK65C and the other with the virulent NK65E. The repetition of the experiment on larger groups of animals (35-38/group) gave the same result as before. It seems clear that NK65C gives better protection against NK65C than against NK65E.

These results have important implications since both lines were originally derived from the same animal. A manuscript is now in preparation describing these experiments.

Age Resistance in A/J Mice

Groups of 17-20 four-week-old and six-month-old A/J mice were compared for age resistance. Two lines, one virulent and one avirulent, were used for primary infection. The evidence of age resistance in older A/J mice seems quite clear. 11/17 six-month-old mice and only 1/20 four-week-old mice recovered when infected with avirulent NK65C. Likewise, on day 22 after challenge with virulent NK65E, 65% of six-month-old A/J mice and 10% of four-week-old A/J mice still survived. These results emphasize the need to consider age resistance when older mice are used in the vaccination procedure. A manuscript on this work is in preparation.

Insectary

A production schedule demanding a high level population of adult mosquitoes is a basic need in our project, particularly in the area

concerning the mosquito antigen and sporozoite vaccination. However, quantitative analysis of adult population tends to show peaks of abundance during summer and a recession during winter. An investigation of the problems and means of alleviating seasonal fluctuations were considered. The objective of the investigation is to store stocks of eggs which can be hatched at any desired period and therefore replenish the population during the winter period. Records of mosquito production during the past year show that the population has increased from an average of 500-600 per day during January to March 1969, to a maximum of 4,942 per day during August and September. This approximate level of production has been maintained.

From several completed experiments and some still in progress, it has been established that hatching of A. stephensi eggs can be delayed for up to 26 days to yield 30% viable embryos. Experimental eggs were held on a damp surface at constant low (5, 10 and 15° C) temperatures or a combination of low temperatures. Controls (batches of eggs obtained at the same time as those used in the experiments) were maintained at 26° C and 78% R. H. and hatched normally, i.e. after 72 hours.

A series of papers will be submitted under the heading, "Laboratory studies on the bionomics of Anopheles stephensi Liston (Diptera, Culicidae)".

Part I. Hatch rate of eggs maintained at various low temperature.

Part II. Survival rate of the developmental stages obtained from low temperature treated eggs.

Part III. Reproductive capacity and longevity of adults
reared from low-temperature treated eggs.

Microsporidia

Two descriptive papers on Microsporidia have been accepted for publication, one on Nosema algerae as a new species and a second on its control (see publications). No further work will be pursued in this area under this contract.

Mosquito Antigen Studies

An experiment described in our last report on transfer of anti-mosquito rabbit gamma globulin from immunized rabbits to normal hamsters was completed. The experiment as carried out was not successful. Preliminary tests indicated that rabbit gamma globulin was rapidly destroyed in the hamster and reached very low levels only 15 minutes after injection. In our experiment, mosquitoes had not completed their feeding in this short length of time. Although transmission of sporozoites was achieved to the same degree in both the control and experimental animals, we feel that the short feeding time during which the rabbit antibody could be picked up by A. stephensi did not enable the mosquito to ingest enough antibody for it to be effective.

A somewhat similar experiment with mice is in progress. We are presently completing mosquito mortality rates as previously described for mosquitoes fed on rabbits. Groups of mosquitoes are necessarily smaller for mice than for rabbits, since too many mosquito's bites could exsanguinate a mouse. We hope to find correlation between the mosquito mortality

and sporozoite transmission when mosquitoes are fed on the infected immunized mice.

In the event that the inhibitory activity of the antiserum could somehow be due to bacteria associated with the mosquito, we have isolated, but not attempted to identify, bacteria from mosquito mid-guts. These bacteria have now been prepared for studies with the original rabbit antisera and for injection into mice for (1) further study on death rates of mosquitoes, (2) interference with sporozoite transfer and (3) double diffusion comparison.

Double diffusion tests with the original rabbit antisera are being studied with concentrated mosquito antigens. Preliminary serologic data indicate that either the mid-guts have a unique antigen or the remainder of the mosquito has very small amounts of a particular antigen which does not precipitate in double diffusion tests.

A group of 9 dd/s mice were each immunized with approximately 120 mosquito mid-guts, i.e. about twice the usual number used for sporozoite immunization experiment controls. Ten mice were injected with an equivalent amount of protein extracted from cockroaches and 10 were used as unimmunized controls. All of the untreated controls became infected by sporozoite challenge and died; 10% of the cockroach immunized group and 33 1/3% of the mosquito immunized groups survived and remained negative. Both parasitemia and mortality results look very encouraging and the experiment will be repeated with larger numbers of mosquito mid-guts. The data suggest the possibility of common antigens existing between sporozoites and the mosquito host.

All available mosquitoes are being used for both sporozoite experiments and the above work on antigenic "mimicry".

Sporozoite Immunization

The first unequivocal demonstration of the protective effect in mammals of sporozoite immunization appears to have been completed. Of four dd/s mice immunized with heat treated (42-44° C for 30 min.) sporozoites, all four survived, while none of the controls, injected with mosquito stomach or left untreated survived when infected with sporozoites.

Of A/J mice, we currently have 4 groups varying in size from 6 to 15 animals each immunized with heat treated sporozoites and ready for challenge infection in the near future. It should be emphasized that successful sporozoite infection depends on the viability and vigor of the sporozoites and this is still somewhat unpredictable. It is difficult to ensure 100% infection in the controls and numerous replications and large numbers of animals are needed.

Ookinete Cultivation

Results of previous experiments suggest that the dying plasmodia and red cells in the culture may be toxic to the ookinetes. Two methods of separation of viable parasites are under investigation: (1) gradient separation on albumin and renografin either before cultivation to collect gametocytes or after cultivation to collect ookinetes, and (2) cultivation in a membrane cell surrounded by a large volume of medium. It is hoped that in the second method the large volume of medium will carry off the toxic products, or that the ookinete will pass through the membrane, thereby separating itself.

The few trials of gradient separation have not so far been successful. Further cultivation experiments depend on delivery of awaited chemicals and time to carry out the procedures. The development of more standard and reproducible culture techniques will also enable further investigation of the culture supernate as an immunogen as previously reported.

Insect Tissue Culture

Insect tissue culture technique has been incorporated as a new approach to accelerate some promising leads developed in this research unit. This involves the use of a mosquito cell line in a defined medium as a substrate to cultivate oocysts and sporozoites for immunological studies. Performance to date regarding this phase are: (1) completion of tissue culture facilities, (2) training of personnel, and (3) several experiments, in progress, on the aseptic rearing of Anopheles stephensi as a source of mosquito cell line.

Trophozoite Antigens

Preparation of parasites free from host cells. Investigation of new techniques for the isolation of blood stage plasmodia has continued. The motor driven hydraulic press has been fitted with a low pressure gauge for operations with the French pressure cell (FPC) below 1000 psi. P. berchei freed at pressures between 600 to 800 psi remain morphologically unaltered with preservation of large numbers in different stages of development. Yields of intact free parasites have been greatly increased by this technique. In addition, it has been found possible to remove unbroken erythrocytes and plasmodia still contaminated with stromal material with

phyto-hemagglutinin (PHA). PHA readily agglutinates stroma containing elements which are then easily removed at very low centrifugal speeds. P. gallinacium has similarly been freed from infected chicken cell nuclei and red cell stroma.

Additional fractionation of solubilized free parasites has been carried out using sucrose density gradients. By this technique it has been possible to separate the solubilized materials into soluble, particulate and hematin components. These studies are being extended to determine optimum conditions for further isolation and characterization of active materials.

Also underway is an ultrastructural evaluation of plasmodial products obtained during each step of isolation, washing and final antigen preparation.

Protection Studies. These studies have concentrated on evaluation of variously prepared plasmodial material. The protective properties of heat inactivated plasmodially infected blood has been extensively investigated in various strains of mice. Inactivation of P. berghei has been obtained at temperatures as low as 41° for 2 hours and as high as 56° for one hour.

A/J, Carworth and dd/s mice have been effectively protected by heat inactivated materials. The A/J and dd/s strains show a high level of resistance following a single injection of such materials. Approximately 20% of Carworth mice demonstrate protection following a single injection and 80% to 100% following 2 to 6 injections. Our results now clearly

indicate that each strain of mouse has its own minimum dose requirements for optimum protection. This finding is of special importance in evaluating vaccination requirements in different species of animals and malarias.

A study relating the role of adjuvants, route of injection and strain of mouse to induction of malaria resistance is now being completed. Our initial results indicate that in the mouse systems now under study, adjuvants are of no special advantage. Animals receiving Freund's complete adjuvant alone were unprotected. Those receiving protective plasmodial materials alone or with Freund's complete adjuvant appeared equally protected. The intraperitoneal route of antigen administration appeared superior for all mouse strains studied. Carworth farm mice appeared better protected than A/J mice when inoculated by the subcutaneous route. The intramuscular route has not yet been evaluated.

Fractionation of plasmodial antigen. A study has been completed for evaluation of the protective properties of various fractions of the solubilized, isolated plasmodia. This study confirms earlier preliminary results linking the protective properties with the Sephadex G-200 void volume peak. The void volume peaks of bio gel 5M and Sepharose 4B have similarly been protective. All materials after the various void volume peaks, as well as materials obtained from normal erythrocytes alone, have been unprotective. A publication describing the protective effects of the highly purified Sephadex G-200 1st peak material is being prepared.

Preliminary experiments indicate that a fine particulate component accompanying each of the void volume peaks mentioned above is responsible

for the induced protection. Work is being actively pursued along these lines. The above findings will be presented at the meetings of the Federation of American Societies of Experimental Biologists in April of 1970.

Cross-protectivity. Evaluation of the cross-protective properties of P. gallinacium against P. berghei infection in mice has been completed. No clear-cut protection was observed in mice receiving P. gallinacium antigens, further confirming the impression that malarial protection is species specific. This is supported by our experiments utilizing P. gallinacium preparations as protective materials against the same infection in chickens. These studies were carried out in collaboration with Dr. Dean Ferris at the University of Illinois School of Veterinary Medicine.

Cellular Immunity

The work with antithymocyte serum (ATS) and the demonstration of its dramatic effect on abolishing both innate and age resistance to malaria infection has strengthened the view that immunity to malaria is cell-mediated, i.e. of the host-versus-graft variety. A manuscript (copy attached) has been prepared based on these data for submission for publication.

Further work on the effectiveness of transfer of immunity by spleen cells has been carried out. It has been determined that resistance can be transferred by viable spleen cells and by apparently nonviable spleen cells which have been frozen and thawed -- but spleen cells which have been heated to 60° C for 30 minutes cannot transfer immunity. It appears

that the "mesoero" (mexA) alone may be capable of transferring the necessary information to a naive animal if the mesoero is not denatured. A similar phenomenon on induced immunologic sensitivity has been reported by Australian workers using the mixed-lymphocyte reaction in sheep as a test system.

The efficacy of the antilymphocyte serum was further demonstrated by its ability to prevent A/J mice from responding to the antigen in D'Antonio's model vaccination system. The known specific immunosuppressant effect of ALS confirms the immunological basis of the vaccination model.

Electron Microscopy

Preliminary ultrastructural studies on hemopoietic centers have been initiated in order to investigate these tissue sites as they are involved in the processes of infection of red blood cells. It is also hoped that this study will provide better understanding of the role played by the reticular-endothelial system in the resistance response. At present only the spleens and bone marrows of rats at different times of infection (with P. berchei NK65D line) and convalescence have been looked at. Further studies are to include the rat liver as well as the hemopoietic centers of infected mice and hamsters. Results of these experiments are to be compared with centers of infection of animals infected with other malarial strains routinely used in our laboratory.

Material at various stages of antigen preparation by means of the French press has been processed for electron microscopic investigation. These studies are to determine the possible time at which fine-structural damage may occur in the parasites during the process of liberating the

parasites from their host cells. A correlation is to be made from this investigation as to the degree of parasite damage and the amount of host contaminant present in the preparation.

As previously reported the more than 1500 electron micrographs accumulated from several studies by Dr. Killby are still under evaluation and analysis. The results of immunoferritin studies which were undertaken to determine antigenic sites in the trophozoite stages of P. bertrandi have been prepared for publication. It represents pioneer work in the field of malaria and will provide valuable guideposts for other workers in the application of ferritin-labeled antibody techniques.

Assessment of the micrographs appears to confirm the existence of an additional morphological stage which has tentatively been identified as a pre-gameteocyte form. This information, together with other ultrastructural observations will form the basis of another publication.

PUBLICATIONS

- D'Antonio, L. E., D. T. Spira and P. H. Silverman. 1969. A model system for study of artificially induced resistance to malaria. *Nature*, 223(5205): 507-509.
- D'Antonio, L. E., N. E. Alger and C. Rainor. 1969. The temporal relationship between a serum electroadsorbing fraction and the course of acute Plasmodium berghei malaria in rats. *The American Journal of Tropical Medicine and Hygiene*, 10(6): 866-871.
- Kilby, V. A. A. and P. H. Silverman. 1969. Isolated erythrocytic forms of Plasmodium falciparum: an electron-microscopic study. *American Journal of Tropical Medicine and Hygiene*, 17(5): 836-859.
- Kilby, V. A. A. and P. H. Silverman. 1969. Fine structural observations of the erythrocytic stages of Plasmodium chabaudi Leger, 1969. *Journal of Protozoology*, 16: 354-370.
- Alger, N. E. and A. Undeen. 1970. The control of a microsporidian parasite, Nosoma algerae, in an anopheline colony by an egg rinsing technique. *Journal of Invertebrate Pathology*, 15: (In press).
- Cabrera, E. and N. E. Alger. 1969. An increase in death rate of Anopheles stephensi fed on rabbits immunized with mosquito antigen. Master of Science Research Report, Department of Zoology, University of Illinois.
- Spira, D. T., P. H. Silverman and C. Gaines. 1970. Antithyocyte serum effects on Plasmodium berghei infection in rats. (To be submitted)
- Vavra, Jiri and A. H. Undeen. 1969. Nosoma algerae N. Sp., (Cnidospora, Microsporida) a Pathogen in A Laboratory Colony of Anopheles stephensi Liston (Diptera, Culicidae). 1969. (Accepted for publication in *The Journal of Protozoology*).

D'Antonio, L. F., D. T. Spira, R. C. Fu and D. F. Dagnillo, 1979. Malaria Resistance: artificial infection with a partially purified plasmodial fraction. (To be submitted).

SUMMARY AND OUTLOOK

In a recently submitted Project Work plan for fiscal year 1970, the future direction and emphases of our work was outlined. This plan necessarily reflects some of the important findings and promising leads derived from our research.

Malaria lines: The question of variability in virulence of malaria is a finding of overriding importance not only in the laboratory but as an added dimension to the complex epidemiologic parameters involved in this disease. Although other workers have indirectly hinted at similar observations, it does not appear that previous investigators have undertaken a sustained critical evaluation of this phenomenon. We are convinced of the existence of this variability and place a high priority on elucidating factors affecting it both in terms of population selection and pathophysiology. It should be clarified that our use of the term "avirulence" is a relative one and that this does not imply a non-pathogenic line but simply a relatively less virulent line.

Mosquito antigens: This technically difficult problem has yielded relatively meager results when compared to the research effort invested. Nevertheless two promising leads have emerged and will be pursued. The first approach is the determination of the effects on vector efficiency of prior immunization of infected animals with mosquito antigens. The second approach is based on the use of certain mosquito antigens as vaccines to protect against sporozoite infection. The work will be carried out in mice.

Sporozoite immunization: The success so far achieved with sporozoite immunization has been most encouraging, particularly in view of the numerous technical difficulties which have afflicted this investigation. Other workers (at New York University) appeared to have reached the conclusion that sporozoite immunization in a rodent malaria was not likely to succeed. However, from our studies it is strongly suggested that the quantitative levels of sporozoites used by the N.Y.U. workers were below the threshold required to stimulate resistance. The further pursuit of this venereal, vaccination and biochemical lines will depend to a large degree on the success of our insect tissue culture unit.

Trophozoite antigens: The continuing success of the DiAntonio rodent nasal vaccination system has enabled fractionation, isolation and purification of non-viable resistance-inducing antigens to proceed to an extent not achieved by any other laboratory. Further details of progress are presented in a manuscript which is enclosed with this report. High priority will be given to development of a similar nasal system in primates.

Cellular immunity: A deeper insight into the nature of the host resistance response has been obtained by our work on cell-mediated processes. Some of the details are provided in a copy of a manuscript which is enclosed with this report. Clearly, cell-mediated immunity is substantially involved in rodent malaria. The fact that passive transfer of immunity can be achieved by either intact spleen cells or certain non-viable components suggests several exciting lines for future investigation. We will devote a significant effort to this area of research.