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UNIVERSITY OF ILLINOIS

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PROGRESS REPORT

January 1, 1970 - June 30, 1970

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PUBLICATIONS LIST SINCE JANUARY, 1970

PERSONNEL

No changes in senior staff have taken place since our last report of January 1970; as indicated in the project work plan of March 1970, appointment of an additional Research Associate to work in the insect tissue culture unit is anticipated for September, 1970. A full complement of Research Assistants is now employed, as well as other supporting staff.

Members of the group have attended several scientific meetings and have been invited to give lectures and seminars. These include:

- "Symposium on Arthropod Cell Cultures", held at the National Naval Medical Center, Bethesda, Md., March 17-19.
- "21st Annual Tissue Culture Association Meeting", Shoreham Hotel, Washington, D. C., June 17-18.
- "Federation of American Societies for Experimental Biology-American Association of Immunologists", Atlantic City, New Jersey, April 12-17.
- "Congreso Nacional de Medicina Veterinaria Zootecnia", Lima, Peru, South America, May 17-23.
- "Universidad de San Marcos, Facultad Veterinaria", Lima, Peru, South America, May 24-26.
- "Pathogenesis and Immunology of Plasmodial, Babesial and Trypanosomal Infection - A symposium", Ohio State University, Columbus, Ohio, June 2-3.
- "The biological effects of polynucleotides", Miles Laboratory, Americana Hotel, New York, New York, June 4-5.
- "International Symposium on Ecology and Physiology of Parasites", University of Toronto, Toronto, Canada, February 19-20.
- "The Meeting of The Regional Parasitology Club", University of Toronto, Toronto, Canada, February 21.

RESEARCH PROGRESS

MALARIAL LINES

Each mosquito-passed line of Plasmodium berghei is stored in liquid nitrogen to maintain viable lines for future study as previously described.

As reported before, virulence studies established that a line had become relatively less virulent and that this loss of virulence could be mosquito-passed to derive other less virulent lines. Since the last report, we have discovered that one of the less virulent lines has reverted to a more virulent line after a mosquito passage. The reversion to the more virulent form suggests that a population selection has taken place.

Although considerable literature exists on factors affecting virulence, the mechanisms of virulence are still not clear. Our studies standardize factors known to contribute to variations such as parasite inoculum, diet, heat, light, sex, age, genetic strain of host and handling. Nevertheless, there is considerable difference in the date of death. These virulent and less virulent lines appear to offer excellent material to study the contribution made by intrinsic and extrinsic factors on variation.

Because of increase in virulence with successive blood passages has been reported for various species of malaria, we compared the 18th and 40th blood passages of one of our less virulent lines. The 40th passage was found to be slightly more virulent than the 18th passage but it was still unmistakably less virulent than the normal virulent lines. Papers summarizing these findings are almost completed.

We also have under way a comparison of the virulent and less virulent lines as to cell preference and resulting anemia. Additionally, we are starting an experiment comparing the merozoite numbers formed by schizonts

of a virulent line and a less virulent line.

Results on an experiment mixing the two lines in various proportions (groups of mice infected with 100% virulent, 75%:25, 50:50, 25:75 and 100% less virulent) are not quite complete at this date. Initial incomplete data support the idea that our less virulent line and the virulence reversal could have been due to a population selection, although we are unable to rule out the accidental acquisition and subsequent loss of a concomitant virus.

Knowledge of the mechanisms of virulence will help to shed light on the problems involved in immunization with different parasitic lines.

ANTI MALARIAL - RIFAMPICIN

The drug Rifampicin of the rifamycin group has been found to be active against some viruses. Since the difference between the virulent and less virulent lines of P. berghei might be caused by a concomitant virus infection, mice infected with both lines of malaria were treated with Rifampicin. No significant depression of parasitemia was observed but the length of life of the mice was prolonged. The data suggested that Rifampicin may be of value in the treatment of malaria. A paper discussing these findings was accepted for publication by Nature.

A subsequent test at higher dosage levels has shown depression of parasitemia, as well as considerable prolongation of life. Lines of malaria isolated after a 3 day exposure to Rifampicin will be tested for virulence. One treatment with the antibiotic should not "drug select" a line of the parasite; but, if a concomitant virus susceptible to Rifampicin was present,

the drug may well have eliminated it, leading to a change in virulence.

MOSQUITO ANTIGEN STUDIES

Repetition in mice of a previously reported experiment, in which we immunized rabbits with large doses of mosquito stomach antigens, and then fed mosquitoes on them, was attempted. The Anopheles stephensi mosquitoes were reluctant to feed on mice and could only be induced to do so after 48-72 hours of starvation. Apparently, starvation weakened them so much that the majority of mosquitoes died within a few minutes after their blood meal. The remaining mosquitoes were too few to yield sufficient data to be of value in assessing the effects of mouse-antimosquito antibody on A. stephensi adults.

On the basis of previous data which suggested the possibility of common antigens between sporozoites and the mosquito host, groups of mice were immunized with homologous A. stephensi stomach antigens and heterologous antigens from house flies, cockroaches and their associated bacteria. Numerous problems were encountered during immunization and challenge with sporozoites. Nevertheless, preliminary results continue to support the suggestion of antigenic mimicry between sporozoites and mosquito antigens which may be useful in the stimulation of immunity. The following data is combined from several experiments:

<u>Groups</u>	<u>No. of Mice</u>	<u>% Infected on Challenge</u>
Untreated Controls	28	93
Immunized with heterologous antigens (fly, roach or bacteria)	31	97
Immunized with <u>A. stephensi</u> stomach antigens	20	75

These encouraging results warrant further work which will include additional immunizing injections and partially purified mosquito antigens as described below.

It is of interest to note that in double diffusion studies, house fly-stomach antigen did not react with rabbit anti-mosquito-stomach antiserum (MSA) but that the cockroach (whole body) antigen did react. None of the bacteria isolated from the mosquito stomach reacted at any dilution. The sporozoite antigen reacted to MSA with at least 2 precipitin lines and appeared to show identity with substances from whole mosquito and mosquito stomach. These serologic results support the possibility of recovering a non-specific functional antigen from the mosquitoes.

PURIFICATION OF MOSQUITO ANTIGEN

Since it is well known that one antigen may interfere with the reaction to a second antigen in a mixture, it is possible the preceding immunizations may be much more successful if the material is in a more purified form. Initial purification trials were performed with agar gel electrophoresis and column chromatography. Fractionation by column chromatography appeared to offer the best method. Fractionation of Sepharose 6B columns yields 3 major peaks, one of which is in the void volume. Fraction 1 (void volume) has been further fractionated into 2 major peaks on Sepharose 4B. Fractions 2 and 3 were separately concentrated and run through Sephadex 200. Fraction 2 yielded 3 subfractions after separation on Sephadex 200 and fraction 3 gave 2 subfractions after separation on Sephadex 200.

The three major peaks from Sepharose 6B all show precipitin lines with rabbit-anti-mosquito-serum in double diffusion plates. The sub-fractions have not been collected in sufficient quantity to be tested by double diffusion.

Methods of collecting reproducible material from the Sepharose 6B columns are good, but it will be some time before sufficient material is available for immunization tests from Sepharose 4B and Sephadex 200.

OOKINETE CULTIVATION

During this period our efforts have been directed toward separating the developed ookinetes from the dying RBC's and blood stage parasites. Although some RBC's are still mixed with ookinetes, a fairly successful method for concentration and separation has been developed using renografin-albumin. The ookinetes do not appear to be damaged and will live an additional 72 hours in the renografin-albumin. Attempts will be undertaken to find conditions for conversion from ookinete to oocysts, exploring the possibility of conversion to oocyst in the absence of mosquito tissue first and, when the mosquito tissue cultures are well enough established, in the presence of insect tissue.

TROPHOZOITE ANTIGENS

Immunization

Immunization studies against the blood stage of plasmodia have involved three principal systems. These are: Plasmodium berghei-Rodent; P. knowlesi-Rhesus monkey; and P. gallinaceum-chicken.

The P. berghei-rodent model system using different mouse strains has been actively pursued. Because of its high level of sensitivity to blood stage plasmodial antigen, the A/J mouse has continued to be used for various studies involving antigen preparations, stability and immunological mechanism. The dd/s mouse has also been found to be highly responsive to vaccination and is presently being bred in our laboratories for use along with the A/J model. The Carworth mouse, an outbred strain, has been successfully immunized by increasing the amount and number of antigen injections.

All three strains of mice have been immunized, utilizing heat-killed parasite preparations or partially purified materials. Our present studies indicate that, in any case, the level of protection increases with the amount and number of injections.

Each strain of mouse offers a particular set of circumstances for study of immunization procedures. The A/J, as mentioned, is highly sensitive and, in addition, has a certain level of age resistance to P. berghei NK65D strain presently in use. The dd/s mouse is highly sensitive but has thus far failed to demonstrate age resistance or any form of survival without vaccination. The Carworth mouse is less sensitive, has no age resistance and appears to respond more readily to more than one route of vaccine administration.

Experiments are presently in progress to test the protective properties of partially purified blood stage Plasmodium knowlesi antigen in Rhesus monkeys. These are being conducted at the University of Illinois Medical

Research Center in Chicago. Partially purified antigens are being prepared by the method of D'Antonio et al. and being injected into various groups of animals with different schedules, routes and adjuvants. Blood counts, smears and sera are being evaluated during the course of immunization. The first series of immunizations have been carried out and the second series is now in progress. Evaluation of the vaccination effectiveness will be carried out sometime in late August or September.

A project for immunization of chickens utilizing partially purified P. gallinaceum antigens has been carried out in cooperation with the University of Illinois School of Veterinary Medicine. Present results indicate protection is obtained by the use of plasmodial antigen, as well as normal red cell components! This differs from the mammalian system in which normal cell components have been found non-protective. This occurrence in the chicken offers a clear cut opportunity to study some of the other factors involved in malaria immunity and could well contribute to better understanding of the mammalian system. In addition, because of their anatomical localizations, it is possible to separate clearly and thus manipulate the humoral and cell mediated response systems in the chicken. This offers an opportunity to study each aspect of malaria immunity separately.

Antigen Stability, Purification and Characterization Studies

A series of experiments has been set up to test the activity of partially purified trophozoite antigens following low temperature storage, freeze-thawing, various elevated temperatures and lyophilization. Early indications

are that freezing and freeze-thawing have no adverse effect. Additional studies on the effects of various enzymes, pH alteration and different methods of storage will be included. One study, testing the stability of alum adsorbed antigen, indicates retention of activity.

Further purification of the antigen is proceeding. As mentioned in our previous report; ultracentrifugation has resulted in sedimentation of a particulate component which possesses vaccination properties. This was the subject of an abstract presented in April at the meeting of the Federation of Societies of Experimental Biologists. This study is being pursued, utilizing different centrifugal, electrophoretic and chromatographic techniques for separation of particulate materials.

The nature of the protective antigens is not yet clear. They do not appear to be polysaccharides or related to nucleic acids. Present indications are that the protective antigens may be lipo-protein material. It is now felt that biochemical characterization of the active antigen or antigens is essential in furthering our understanding of blood stage immunity as well as opening the way for alternate, more direct and efficient methods of antigen purification.

Electron microscopic studies of various stages of preparations are being made in order to characterize further, as well as to determine levels of host cell contamination.

A paper describing various protective antigen preparations was presented at the Ohio State University in Columbus, Ohio on June 2 and 3.

Mechanisms of Immunity

It has been found that antigen injected at the same time as anti-thymocyte serum fails to vaccinate. On the other hand, anti-thymus serum fails to break vaccination once it is established. In addition, spleen cells from mice recovered from P. berghei malaria has imparted protection to a certain number of non-protected mice. The above argues strongly in favor of a cell-mediated response as one of the principal mechanisms in blood stage vaccine immunity as presently carried out by us.

Preliminary experiments, utilizing in vitro lymphocyte cultures from recovered rats in conjunction with specific plasmodial antigens indicates lymphocytic stimulation. This, if confirmed, could become a valuable tool in the detection and evaluation of antigens, evaluation of host immunity and harvesting of specifically sensitized cells. These studies will continue.

CELLULAR IMMUNITY

As a preliminary step in a series of experiments concerned with the mechanisms of cellular immunity in malaria, effects of the routes of inoculation of transferred cells were investigated.

Spleen cells (10×10^7) of Lewis rats recovered from P. berghei infection were injected into normal animals of the same inbred strain. Three routes of injection were used: [1] Intravenous (IV) in the tail vein; [2] Intraperitoneal (IP); and, [3] Intramuscular (IM). The successful use of the IV route of injection has been reported previously.

As anticipated, the intravenous injection of the spleen cells from recovered animals conferred a high degree of immunity against infection on

the recipient animals when challenged IP with 10×10^6 infected RBC's. Control groups of 6 animals each were: [1] injected with normal spleen cells, [2] injected with Hank's BSS (suspending medium); and, [3] untreated. Statistical analysis showed that the experimental IV injected group was significantly different from any of the control groups. The difference among the control groups was not statistically significant.

Animals injected IP with immune spleen cells, whether challenged IP or IV, showed a significant degree of protection as compared to control animals. On the other hand, animals injected IM with immune spleen cells showed no significant protection when compared to controls.

These preliminary experiments showed that the IP route of immune cell transfer, although not as effective as the IV route, conferred significant protection to animals so treated. The use of the IP route in cell transfer experiments will be of value for several reasons. Intravenous injections in the tails of rats are sometimes fatal, and frequently inaccurate, since in some cases not all the material is introduced in the vein. The IP route will avoid the dangers of death from an injected embolus and will insure accuracy in the number of cells injected.

The work described above on cell transfer of immune cells and the preliminary results obtained from the lymphocyte culture study supports the view that immunity to malaria is of the cell-mediated type, i.e. of the host-versus-graft variety.

INSECT TISSUE CULTURE

We are pleased to report several outstanding accomplishments by Dr. Ronquillo and her colleagues in the insect tissue culture unit. This

work, which has already gained national recognition, has resulted from the intensive development and application of tedious and technically difficult new methods. These methods involve the establishment of axenic (i.e. aseptic and antibiotic-free) cultures of mosquito larvae from eggs and the careful dissection of primordia or anlagen cells, whose origin had been previously determined by embryological studies. These methods contrast with those used by other workers who have utilized macerated insect tissue heavily treated with antibiotics. The surviving cells in previous cultures are of unknown origin and must be insensitive to antibiotics, a factor of some selective importance.

Presently, both primary tissue culture and organ culture of the larval mosquito using Anopheles stephensi Liston have been successfully grown in vitro. Growth of the culture has now been maintained for 3 months. Five different types of tissues have been developed and consist of: (1) fibroblast-like cells; (2) transparent vesicles made up of a single layer of polygonal and round cells; (3) epithelial cells; (4) clumps of cells several layers thick; and, (5) chains of attached globule-like tissues. Organ cultures consist of intact and fragmented gut, which show rhythmical pulsations 4 days after culture and throughout the culture period.

Observations and studies of the culture have been made possible with (1) phase contrast microscopy; (2) time-lapse microcinematography; and, (3) microphotography.

A paper regarding this phase of work currently in preparation, entitled "Growth of mosquito larval gut and tissue in vitro." will be submitted for publication in the near future.

With Phase I of the work (described above) successfully accomplished, Phase II involves the cultivation of the mosquito phase of the parasite for immunological studies, using the established primary tissue culture as a substrate. The technique and methods in this phase of work will place certain preliminary demands upon the experimenter during the research period, so a methodological approach has been outlined for the purpose of acquiring substantial information that will provide meaningful input into the experimenter's technique. The first task is to confirm and validate some preliminary work of other investigators in their attempt to infect normal cultures with parasites. The second task is to study host-parasite relationships in vitro with special reference to types of tissues and organs supporting the growth and propagation of the parasites.

Axenic Rearing of *Anopheles stephensi* Liston

As the program in insect tissue culture progresses, results point up a need for germ-free explants from late larval pupae and adult mosquitoes. Experiments on aseptic rearing of *Anopheles stephensi* Liston has been so successful that axenic sources of other types of tissue and organs taken from late larval, pupae and adult is now possible. An intact intestine from an axenically reared adult *A. stephensi* has now been transferred to an in vitro environment. This is the first time that an *Anopheles* species has been reared under aseptic conditions from egg to adult and the first time that such organ explants have been established.

ELECTRON MICROSCOPY STUDIES

To date, all attempts at studying the ultrastructural characteristics of the erythrocytic and exocrythrocytic stages of *P. berghei* in the rat,

mouse, and hamster spleen, bone marrow, and liver have been complicated by several interpretive problems. At present, a different plan of approach is being considered in localizing the tissue sites of malarial infections.

The efficacy of two different methods of malarial antigen preparation have been investigated. The results of ultrastructural examinations of the isolated erythrocytic stages and the subcellular fractions of P. berghei prepared by the French pressure cell disintegration technique and the Paar-Bomb technique revealed that the two methods are comparable. Based on the appearance of the morphologic characteristics of the malaria parasites, neither of the above two methods appears to be superior to the other. However, considering the physical aspects involved in the process of cellular disintegration by the two methods, the Paar-Bomb may be the preferred technique. It appears that there is less increase in temperature in the material being disintegrated by the Paar-Bomb than by the French pressure cell technique.

During the course of evaluating the efficacy of malarial antigen preparations by the different methods of cell disintegration, interesting observations were made on the different morphological appearances of NK65-P. berghei obtained from peripheral blood of hamsters as compared to the parasites obtained from rats. The malaria parasites obtained from hamsters appeared to be more uniform in their morphological characteristics than those obtained from rats. Also, the hamster P. berghei forms seemed to be less fragile, or more specifically, less susceptible to cellular disintegration than the various P. berghei stages obtained from rats. Only further studies will ascertain whether the observed differences in P. berghei

from hamsters and rats are real or only apparent due to different or perhaps more synchronous reproduction of the parasite in the hamster host.

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