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UNIVERSITY OF ILLINOIS

DEPARTMENT OF ZOOLOGY

URBANA, ILLINOIS

PROGRESS REPORT

January, 1969 - July, 1969

N. E. Alger, Acting Project Director

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SUMMARY AND OUTLOOK

This report emphasizes the events during the six month period from January to June, 1969.

PERSONNEL

Dr. Paul Silverman is on a six month sabbatical leave covering the period from February through August, 1969. Dr. Nelda Alger has assumed responsibility as acting project director during his absence. During Dr. Silverman's travels, he is contacting scientists in the various countries he visits to discuss mutual problems and exchange ideas.

Dr. D. T. Spira will complete his visit with us and return to Israel at the end of the summer; Dr. Virginia Killby, having received her Ph.D., will be leaving to take a position at Yale University. She will be replaced by Dr. Birute Jakstys who has worked closely with Dr. Killby. In addition, we have secured the services of Dr. Maria Ronquillo to take up the work of insect tissue culture beginning in September.

Dr. L. D'Antonio has resigned, effective October 31. The search for replacements of senior personnel is under way and negotiations with the best available prospects are in progress.

Senior personnel have visited Walter Reed Army Institute of Research and Rockefeller University for collaborative research and scientific discussion, and New York University and Naval Research Institute for a discussion of mutual problems and ideas. Two of our group attended the Miles Laboratory Symposium on protein synthesis in New York.

EQUIPMENT

As previously reported, all major equipment items have been purchased. One rack of semi-isolation type mouse cages and a fraction collector for the mosquito stomach antigen work have been ordered but not yet received.

Consideration is being given to the best way to implement primate work. If the University of Illinois campus should be chosen, primate cages will be required, as well as housing, since space in our building would be insufficient for such large animals.

RESEARCH PROGRESS

Malarial Lines

As mosquito passages of Plasmodium berghei are made, blood from each new passage is frozen (at -90°C) to maintain viability for future studies.

As time permits, these lines are examined for biological characteristics. We previously reported that two lines were found to be less virulent than expected. Both lines had been derived by two separate mosquito passages from the same parent line, which also showed some loss of virulence as compared to the original line. Additionally, a third line also derived from the parent line has been found less virulent.

"Attenuated" Line as a Vaccine

The most recent infection of 6 month A/J mice by one of the less virulent lines resulted in 80 of 100 animals recovering. These mice will be ready for challenge by a virulent line in about 2 weeks. These data indicate 30% more recoveries than found in our experiments in the last report. We are continuing attempts to increase attenuation to an even greater extent by the same procedure

of very late passage. Additionally, we are attempting to elucidate the method of attenuation by repeating the same series of manipulations of the line starting from the frozen blood.

Mosquito Colony and Microsporidia

The microsporidian parasite (Nosema illinia) remains under good control in our insectary, and Dr. J. Vavra (Visiting Professor from Czechoslovakia) has identified it as a new species. A paper on the control of Nosema illinia has been accepted for publication by Invertebrate Pathology.

We plan to try to infect Anopheles stephensi tissue cultures with this parasite which might be used for biological control if sufficiently large numbers can be grown easily.

Observations indicate that Nosema illinia-infected larvae are highly susceptible to insecticides. A collaborative study will be undertaken with Dr. Robert Metcalf to relate infection rate to insecticide susceptibility.

Mosquito Antigen

The previously reported rabbit experiments are being repeated in hamsters but no results are available as yet. Another rabbit was immunized by using much larger injections of antigen. Mosquito feeding tests are presently in progress. A paper on the rabbit system has been submitted to the Royal Society of Tropical Medicine and Hygiene for publication.

Gamma globulin from the previously immunized rabbits has been concentrated and will be injected into hamsters infected with malaria. Mosquitoes will be fed on these hamsters to determine interference with transmission. We anticipate the necessity of repeating the experiment several times to collect sufficient data on which to base a judgment.

Antigen fractions from mosquito stomachs and whole mosquitoes are being prepared by column chromatography and studied by double diffusion methods.

Sporozoite Production

Two small groups of sporozoite-treated animals have resulted in only one infection in the vaccinated animals and an infection in all but two of the normal control animals. Although the initial trials look very promising, the small number of animals used does not allow us to rule out the possibility that the immunized animals may have been challenged with too few sporozoites. Further collection of data should clarify this point. Three other groups of animals are in various stages of vaccination and will subsequently be challenged.

Culture Supernatant as a Vaccine

Aliquots of blood from an infected animal were incubated at 25°C for periods of 24, 48 and 72 hours. The supernatant was then used as a vaccine. All animals were 4-5 weeks old and received three injections. Control groups were either (1) untreated or (2) injected with normal plasma prepared from normal incubated blood. All groups vaccinated showed a prolonged life as compared to control groups; however, the 24 hour supernatant gave the best results.

A similar experiment was carried out using Waymouth's medium during incubation. Prolongation of life was increased as the incubation time was increased, with controls dying between 8 and 16 days and vaccinated animals dying 18 to 38 days in the 72 hour group. Repetition of these results has been started with the addition of a group injected with the supernatant before incubation.

Preparation and Fractionation of Plasmodial Antigen

Isolation of Plasmodium from host components has been studied utilizing the principle of differential selective pressure disintegration of erythrocyte stroma in the French pressure cell. Currently used pressures appear somewhat in excess for maximum preservation of parasites and parasite morphology. Though parasites and parasite materials isolated at these pressures (1000-1200 p.s.i.) have proven adequate for serological and immunoprotective effects, yields of material utilizing P. berghei are less than those that were obtained in other systems such as P. knowlesi and P. falciparum. Accordingly, separation of P. berghei has been carried out at pressures below 1000 p.s.i. The yield at these pressures is greatly increased, as is the number of large schizonts. Morphology as judged by light microscopy appears excellently preserved. In addition, recycling at these lower pressures results in almost complete disappearance of undisintegrated host contaminants, without excessive loss of parasites. As soon as the motor-driven hydraulic press can be fitted with a special gauge to more accurately measure these lower pressures, a more definitive study will be made for preparation of a publication describing this approach.

Fractionation of disintegrated parasitized blood and isolated parasites continues. It has been determined that the first fractionation peak representing the void volume of Sephadex G-200 can be further separated into two major fraction peaks utilizing Sepharose 4B: void volume, and the material immediately after the void volume. Since the void volume material from Sepharose 4B is at least 2×10^6 molecular weight, it would appear that the parasite components in this fraction represent extremely large molecules and/or parasite "particles." Studies are now under way to test the immunogenicity of the solubilized and particulate materials present in this fraction. Additional gel filtration techniques are also being evaluated.

Electrophoretic, physiochemical and serological techniques are employed for the purpose of analyzing purification procedures and localizing active materials. Because the bioassay of active materials as represented by our model mouse vaccination system takes so long to complete, a more rapid physiochemical or biological test for locating the active components is being sought.

Stability of the protective materials and tolerance to different conditions are being investigated. Initial biological evaluation of antigens prepared at very high pressures (20,000 p.s.i.) or exposed to freezing temperatures (frozen at -20° or lower) indicates that immunogenicity is seriously impaired under such conditions.

Protection Studies

Protection studies utilizing both normal and infected blood are under way. Blood disintegrated at 4000 p.s.i., centrifuged free of large particulate material and fractionated on Sepharose 4B has resulted in a number of fractions. Equivalent fractions from both normal and infected blood were injected into A/J mice. Challenge of these animals with P. berghei some 11 weeks later revealed that only the material appearing in the void volume fraction of Sepharose 4B was protective. Animals injected with this fraction had mild infections followed by rapid resolution of disease. All other animals had severe disease and otherwise failed to demonstrate resistance to morbidity or mortality.

Initial results from a study testing the protective effects of a highly purified plasmodial fraction obtained in the void volume eluate of Sephadex G-200 clearly indicate this eluate as containing the protective components.

Similar experiments further evaluating Sephadex G-200 fractions from whole blood and isolated parasites are now under way. A publication describing these results will be prepared upon completion of these studies.

Cross protection studies of plasmodial species are also under way utilizing P. gallinacium-derived materials in A/J mice. In collaboration with the University of Illinois School of Veterinary Medicine, chickens are also being evaluated for the induction of protective effects utilizing P. gallinacium materials.

A collaborative experiment initiated with the Department of Serology, Walter Reed Institute of Research, for the induction of resistance in Rhesus monkeys utilizing P. knowlesi has been completed. Two control monkeys and one "protected" animal developed fulminating infections following challenge, with a fatal outcome resulting in 5-6 days. Another "protected" monkey remained without sign of infection for 5 days following injection of P. knowlesi, but then developed a fulminating parasitemia and fatal outcome 9 days after infection. Though the results here are equivocal, they are possibly suggestive. Certain parameters (such as handling of antigens, time of challenge, protective dose and schedules) are to be carefully considered. The antigens utilized in the above experiment were previously frozen before the apparent inactivating effect of this step was appreciated, which might account for the results obtained. Further studies along these lines must await policy decisions concerning the manner in which primate work will be pursued. It is felt that investigation of this system is extremely important and should be pursued as soon as possible.

Evaluation of heat-inactivated infected blood as a protective material is being pursued. Recent experiments have determined the temperatures and length of exposure which will result in non-infective protective material. Materials thus derived appear to be highly protective in two strains of mice and possibly in rats. It is felt that this approach could result in the production

of materials with enhanced activity, and a large experiment for confirmation of these effects with appropriate controls has been set up. As sufficient data are obtained, a publication describing this effect will be prepared.

Lastly, experiments to determine the role of route of injection, adjuvants, increased and multiple dosage and the earliest age at which protective responses can be elicited in mice are being carried out.

An initial experiment testing the protective effects against sporozoite challenge in blood stage protected animals has been completed. Two protected mice failed to develop disease following intravenous injection of 21,000 viable P. berghei sporozoites. They both showed the characteristic reticulocytosis known to precede the immediate pre-patent period of such infections, and one animal experienced a very light parasitemia noted on one day only. No infection was noted after several weeks of follow-up. A non-protected control animal developed a fulminating infection which terminated fatally 7 days following patency. Pursuit of this phase of the work has been hampered by the low availability of viable sporozoites.

A publication describing induction of protection in mice has been accepted for publication in Nature and is currently in press.

Mechanisms of Protective Response

Our efforts in this period concentrated mainly on the basic aspect of malarial immunity. The question of relative involvement of cellular and humoral protective mechanism has been raised in the previous report. The general approach, namely the use of anti-lymphocyte serum and passive transfer of immunity by serum and cells from immune donors was also outlined in the previous report.

Effects of anti-lymphocyte serum. Anti-lymphocyte serum (ATS) was prepared in rabbits against Lewis rat thymocytes. Lewis rats of two different ages (6 to 7 weeks, weighing about 130 grams, and 9 to 10 weeks weighing about 160 grams) were treated with ATS, normal rabbit (NRS) or normal saline. One ml. of the respective serums was injected IP on days -1, 1 and 3 relative to infection. The serums were absorbed with normal rat erythrocytes to avoid blood loss due to anti-erythrocyte contamination. The parasitemia in ATS-treated rats of both age groups infected with 4×10^6 P. berghei NK65 parasitized rat RBC was greatly enhanced. The infections were fulminating and resulted in deaths of 14/15 rats. Of the 16 NRS or saline-treated young rats, 7 died and the others recovered after high parasitemia. The older rats treated with NRS or saline experienced a low transient parasitemia.

ATS is the most effective immunosuppressant of cellular immunity. In addition to prolonging skin grafts, it enhances infectious agents with known cellular reactions as Vaccina, Raucher Leukemia, lymphocytic choriomeningitis and Mycobacteria, but has no effect on Yellow Fever, Influenza or Staphylococcus. Our conclusion, therefore, is that a significant proportion of malaria resistance is a cell-bound immunity.

The manuscript describing the appearance and course of a serum electroadsorbing factor (SEAF) in P. berghei of rats has been accepted for publication and is to appear in the November, 1969 issue of American Journal of Tropical Medicine and Hygiene.

On a recent visit to Rockefeller University, serums obtained from two infected rats, one of which had first been treated with lymph node-derived anti-lymphocyte serum (ALS) were tested for SEAF levels. The ALS-treated

animal had approximately one half the level found in the non-ALS treated animal, which is highly suggestive that the lymphocyte may be the origin of the SEAF.

The ALS serum noted above was evaluated for its effects on the course on P. berghei infection in large normally resistant rats. In contrast to the "breaking" of age resistance in rats following the administration of anti-thymocyte serum, the rats receiving the lymph node-derived serum showed no apparent break in resistance. This finding, if confirmed, could be of great importance in further unraveling the nature and mechanism of natural resistance and deserves further study.

Passive transfer of immunity. Protection against infectious agents can be transferred between inbred hosts, either by serum or cell transfer. In a series of experiments, young Lewis rats were injected with either serum, lymph node cells or spleen cells, all from recovered Lewis rats. The immune spleen cells produced the strongest protection in recipient rats, which strengthens our conclusions from the ATS experiment. To elucidate the nature of this protection, different preparations and treatments of immune spleen cells are currently being tested. Experiments are in progress to elucidate whether the immunity is adoptive-produced by live spleen cells, active-produced by residual antigen or non-specific - produced by stimulation of the lymphoid-macrophage system by non-viable spleen cells and ingested malaria pigment. This experimental system will complement our results obtained with ATS in the study of cellular involvement in malaria immunity.

Immuno/structural Electron Microscopic Studies

Since the last progress report, our manuscript concerning the electron microscopic studies of isolated forms of Plasmodium berghei has been accepted for publication in the November issue of the American Journal of Tropical Medicine and Hygiene.

Emphasis is presently being placed on the reassessment of the remaining 1500 electron micrographs of P. berghei accumulated to date. In these micrographs, certain observations, with only limited further analysis, appear to warrant consideration for publication. For example, the existence of a very dense stage of P. berghei accompanying the more typical trophozoites has been encountered in several thin sections and has not been illustrated before. The identity of this stage is unknown, but more blocks of P. berghei-infected cells are being sectioned in an effort to establish its significance. Furthermore, additional blocks of P. berghei-infected cells are being examined in order to establish more completely the fine structure of the gametocyte stage. An elaborate plasma membrane and microtubules in the cytoplasm are features which have commonly been observed with this stage. We must now consider all data regarding P. berghei gametocytes in terms of the very recent report of Ladda (1969) presented at a May symposium in Washington. He has, for instance, confirmed the presence of microtubules in the gametocyte cytoplasm. However, he claims that the gametocyte is surrounded by only a single plasmalemma.

Blocks containing P. berghei material incubated with ferritin-labeled antibody preparations are also being studied more extensively, and the data are being evaluated for possible publication as a preliminary study on the application of ferritin-conjugation techniques to the investigation of malarial antibodies. An abstract dealing with these pilot experiments has just been submitted for presentation at the American Society of Parasitology meetings in November. The results of these experiments stress the need for rigorous controls, for the preparation of the conjugates free of unbound ferritin and for the careful initial determination of the distribution of native ferritin in the parasites.

Finally, the data obtained regarding the cross-reactivity of the anti-P. berghei antibodies, as ascertained by Ouchterlony tests, continue to be analyzed and, if warranted, will be prepared for press.

SUMMARY AND OUTLOOK

The period from February to July, 1969 might be described partially as a period of confirmation and consolidation. Additionally, significant new discoveries have been made. The discoveries by Dr. Dan Spira that anti-lymphocyte serum (ATS) greatly enhances parasitemia and that transferred immune spleen cells produce protection gives considerable insight into the mechanisms of malarial immunity. Complete elucidation of these mechanisms will undoubtedly lead to a better understanding of the way to enhance immunity.

Continuation of the purification of plasmodial antigen and protection of animals by these products is progressing well, and the end of the summer should give us considerably more confirming data.

An increase of 30% in the recovery rate of mice from the "attenuated" line of malaria is probably due to the selection of an avirulent population, in an abnormal host, by late blood passage. The limit of attenuation is not yet known, and thus the passages continue followed by intermittent examinations for virulence:

Although the barrier from ookinete to oocyst has not yet been broken, new methods are being applied in our laboratories in the hope that this can be done. The anticipated establishment of mosquito tissue culture may help in crossing this barrier. Additionally, we expect to establish a method for growing (1) large amounts of Nosema illinia for biological control tests and (2) stomach cells for collection of antigen.

Continued confirmation of protection from blood stage antigens by Drs. Spira and D'Antonio point up the necessity of establishing the validity of this system in primates. Enquiries are in progress to establish the best ways to undertake large scale primate studies.

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