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PROGRESS REPORT

July 1, 1970 - December 31, 1970

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PERSONNEL

The senior staff remains the same as reported in our last progress report of June 1970. Dr. Nelda Alger has again assumed the responsibility of Acting Project Director during Dr. Paul Silverman's one year leave of absence.

During the period from the last progress report members of the group attended the Second International Congress of Parasitology in Washington, D. C. and the American Society for Tropical Medicine and Hygiene in San Francisco, California.

RESEARCH PROGRESS

Malarial Lines (demes)

Demes of *Plasmodium berghei* which are not currently in use are stored in liquid nitrogen for future study.

Our studies on the basic mechanism of virulence changes of demes of NK65 *P. berghei* are still under way. In our latest "attenuation" experiment we obtained 44% recovery in young A/J mice and 95% in retired breeders. Recovered animals are protected against severe illness on rechallenge. Attempts to reinfect these animals result in no visible parasitemia or illness to, at worst, a fleeting parasitemia with no apparent illness or anemia. Following the same procedures used in this experiment we are attempting to "attenuate" the population still further. We suspect that the mechanism is a population selection. If this experiment is as successful as anticipated we will attempt to duplicate it starting from a virulent deme. We have no way of knowing to what extent "attenuation" is possible under these conditions since the mechanism would be "built into the genome" of the particular parasite. Such information, however, helps to explain variations in virulence found in various lines isolated from

the field and subsequently leads to a better understanding of the relationships between host and parasite.

If "attenuation" can be carried far enough a live vaccine may be possible. The criterion would be infection without real illness. Continuous blood passage will eliminate the gametocyte so that the live "attenuated" vaccine deme could not be transmitted. Three papers concerning the initial work in this area have been submitted for publication. We feel that these very encouraging results must be followed up.

Work on the cell preference of virulent and less virulent demes indicates that there is no difference in the cell preference. Data are not complete on experiments comparing segmenter merozoite numbers of the two demes. In experiments where the two demes are mixed together the less virulent deme appears to inhibit the more virulent deme. We have been unable to rule out a viral infection to date; however, further work will be carried out in this area.

We feel that the most important area of this work to be pursued is continued attenuation. Until we reach an end point beyond which we cannot attenuate further and assess that deme for stability and pathogenicity, we will not know if "attenuation" is a feasible method of producing a live vaccine.

Sporozoite Antigen

As previously reported the sporozoite crude antigen has proved effective as a vaccine. Sporozoite protection is virtually an all or none phenomena in any one animal. That is, the animal will show no parasitemia if protected or nearly a normal course of infection if unprotected. Increases in the number of animals protected is currently being investigated with the crude heat treated antigen. There is evidence from experiments with irradiated sporozoites which indicates that exposure to antigen over a longer period of time is important in establishing protection. In accordance with this

information we have started protection tests in which 6 or 7 injections will be administered rather than the previous 3 or 4.

It is possible that the number of sporozoites injected is not as important as the time span over which they are administered. We are currently testing injections of 25,000 to 450,000 per mouse injection. In addition, we are using freeze thawed sporozoites and two different temperature treatments. Challenges of some of these groups will begin in about two weeks. New immunization schedules will be developed after analysis of these tests with the aim of transfer to the primate system at as early a date as possible.

Mosquito Antigen

The use of normal mosquito stomachs as a substitute for sporozoite antigen was previously reported. Using the same rational in relation to injection number as that for sporozoites we are currently injecting animals over a longer period of time. In the last report we reported that rabbit anti-normal-mosquito-stomach antiserum reacted with mosquito stomach, roach and sporozoite antigen in double diffusion tests, but not with any dilution of bacterial antigen (bacteria cultured from mosquitoes). Protection tests with roach, mosquito stomach and sporozoite antigens indicate that a similar, if not identical, antigen appears to be present in all these crude antigens. Our present vaccination program includes the roach as an alternate to the mosquito stomach. Six injections will be given, rather than the previous 3 or 4.

Nussenzweig has reported that antiserum, from animals protected by irradiated sporozoites, precipitates as one end of a sporozoite (circumsporozoite test). Sera from our rabbits and mice injected with normal mosquito stomachs also form this precipitate while sera from uninjected rabbits and mice do not. This test will be carried out on mice injected with normal mosquito

stomachs, sporozoite and roach antigens and the serum titres correlated to protection. This test gives further evidence that a common antigen (or cross-reacting antigen) is found in normal mosquito stomach and sporozoites and substantiates our suggestion that normal mosquito stomach may be substituted for sporozoites.

A group of mice is being immunized with each of three major fractions of normal mosquito stomach from a Sephadex G-200 column. Each of these fractions can be further fractionated as previously reported. Protection tests with the sub-fractions will not be attempted until the results of tests on the major peaks are analyzed. Since purification of an antigen usually enhances its activity we anticipate better protection from fractions than from the crude material. Evaluation of the amount of fraction required for protection may entail several experiments.

Cellular Immunity

Using mice naturally recovered from the less virulent deme of NK65 *P. berghei*, spleen cells and lymph cells of young (17 week) and retired breeder A/J mice were transferred into 4 week old A/J mice. Although the experiment is not quite finished, it is clear that lymph node cells of both young and old mice failed to transfer immunity and that spleen cells of older mice conferred a higher degree of immunity than those of younger mice. This experiment will be repeated to confirm the results, however, we suspect that at least one clue to age immunity in the A/J mouse lies in the spleen cells.

In the Lewis rat system, work is underway attempting to elucidate the mechanisms of cellular immunity. Immune spleen cells heated to 42°C for 45 minutes failed to transfer protection, when compared to unheated control cells, in spite of the fact that they were still alive by the trypan blue exclusion test. This experiment will be repeated with some modifications.

In another experiment immune spleen cells were placed in millipore chambers and inserted in the peritoneum of Lewis rats which were then challenged. No differences were found, however, examination of the cells in chamber indicated that the majority of cells had died and the number of surviving cells would have been insufficient to confer immunity (20,000,000 cells are required to give a detectable difference in protection).

The macrophage inhibition test, thought to be the most definitive test of cellular (graft vs. host) immunity, is, at present, being worked out for the malarial system. It is too early to make any definitive statements since the work has just begun.

A study of the passive transfer of cellular and humoral immunity is planned in the primate model using vaccinated challenged monkeys.

Electron Microscopic Studies

Samples of the Sephadex G-200 partially purified *P. knowlesi* antigen as well as a control sample of normal monkey blood prepared in a similar manner have been analyzed with the electron microscope. Before final conclusions can be drawn samples of antigen from different lot preparations will have to be examined and compared.

Preliminary work with the macrophage migration inhibition test (MIF) appears to show promise for possible application to the malarial system as a test for cell mediated immunity. Should this test prove to be as successful as anticipated, subcellular investigations of the inhibited cells will be pursued with the electron microscope. At present, only normal control cells have been prepared for electron microscopy.

In addition to the above studies, samples of spleen and spleen cell suspensions, prepared from rats demonstrating resistance to *P. berghei*, have been processed for ultrastructural studies. It is expected that this investigation will help elucidate the specific subcellular morphologic alteration in the lymphoid tissue in response to the malarial parasite as

the animal acquires immunity.

Considerable study will be required to fully evaluate this experiment.

Insect Tissue Culture

As reported previously the complete life cycle of *Anopheles stephensi* is maintained axenically as a source of cells and/or organs for tissue culture.

At the time of our last report we had successfully established cell and organ cultures from both egg and juvenile stages of the life cycle. This work has now been extended to the adult stage. The axenic rearing of the life cycle has enabled us to culture organs in a nutrient medium without antibiotics for as long as thirty days. Additionally, cells had been cultured in a defined medium without antibiotics.

The one fact that cells and organs can be cultured without antibiotics is tremendously exciting. Undoubtedly, many insect cells are sensitive to the antibiotics normally used in culture, thus, they are selected against when cell cultures are initiated. In this system we will be able to grow these cells.

We are already in the second sub-culture of one of our cell lines. These sub-cultures are made at 25 day intervals. Other cell lines are being isolated by micromanipulation so that homogenous populations can be propagated for use as a source of mosquito antigen.

Analysis of cell types in culture will be carried out by examination of subcellular structure, as well as by light microscopy.

P. berghei infected hamster blood was added to an established primary culture. The hamster cells showed no toxicity to the mosquito cells. The red cells tended to aggregate around already degenerating mosquito cells. In this initial test merozoites appeared to re-invade red cells which remained viable for 14 days. Red cells in the medium alone were not invaded by the merozoite. Further tests will be attempted with parasites freed from the

host cell.

Considerable effort has been expended toward simplifying the culture medium. Variations and improvements in the media used will be a continuing effort.

Blood Stage Antigen

The study of immunity induced against blood stage plasmodial parasities by vaccination has continued, using three strains of mice. These are A/J, DDS and Carworth. Protection induced in each strain is essentially the same and is characterized by transient low level parasitemias, mild to moderate anemias and high survival rates. Unprotected DDS and Carworth mice develop intense parasitemias with anemias and 100% mortality to the *Plasmodium berghei* strains studied. Unprotected A/J mice similarly experience severe infection, but tend to survive in increasing numbers with increasing age.

Experiments designed to test the effects of non-specific stimulation of blood stage immunity have been carried out utilizing alum, sodium alginate, Freund's Complete Adjuvant and hexadecane. Each administered alone, failed to stimulate protection against challenge infection or to induce resolution of ongoing infections following parenteral administration. There is presently suggestive evidence that sodium alginate may enhance vaccine activity when given in conjunction with malaria blood stage vaccine materials. This effect, as well as potential adjuvant effects of other materials, is being further tested.

Investigation of mechanism of vaccination and immunity are continuing in the mouse model system. It has been possible to transfer immunity from recovered vaccinated DDS mice by injection of spleen cells into recipient susceptible DDS mice. There is also evidence that the vaccine fraction will

stimulate peripheral lymphocytes of immune animals in vitro. This effect, if confirmed, will be of great value in diagnosis as well as in the further evaluation and understanding of malaria immunity. Additional experiments are being designed to further test and confirm the above systems.

Major emphasis in the past several months has been on the testing of the feasibility of vaccinating rhesus monkeys against Plasmodium knowlesi malaria. Monkeys have been divided into four major groups:

Group I-A animals received simultaneous injections of antigen alone intravenously (IV), intramuscularly (IM) and in combination with Freund's Complete Adjuvant (FCA) at a separate IM site. Injections were repeated two additional times at five week intervals. The second and third injections utilized Freund's Incomplete Adjuvant (FICA).

Group I-B animals received a smaller amount of antigen in combination with adjuvant (FCA and FICA as above). (No antigen was free of FCA or FICA).

Group II-A animals received antigen alone in small aliquots 5x a week either IV or IM for approximately fifteen weeks.

Group II-B received antigen with alum IV and IM simultaneously 3 x at five week intervals.

Group II-C received antigen with calcium alginate IV and IM 3x at five week intervals.

Group II-D received antigen with alum and FCA combined, for the first injection, then antigen with alum and FICA combined, for two additional times approximately five weeks apart.

Group III animals received normal monkey red blood cell materials in a manner identical to that in Group I-A animals.

Group IV animals remained uninjected.

Group III acted as normal red cell antigen controls and Group IV acted as uninjected controls for the other groups.

Red blood cell counts, serum collection, hematocrits and stained blood smears were made before and after each periodic immunization and periodically following challenge infection.

All animals were skin tested with the vaccine fraction following completion of immunization. In vitro, lymphocyte transformation studies are being performed on all animals prior to challenge infection. All serum collected will be measured for immunoglobulins IGA; IGE, and IGG. In addition, serum will be evaluated by indirect fluorescent antibody tests. Upon completion of the above studies, serum will also be tested for complement fixation activity with the vaccine fraction. The results will be evaluated for correlations between serological tests, as well as with the presence or absence of active protection against challenge infection. These studies should contribute valuable insights into the effects of vaccination procedures and permit more rational evaluation of future vaccination experiments in both human and monkey malaria.

To date, three monkeys in group I-A, one monkey in Group I-B and two monkeys in Group IV have been challenged with 7 to 11 x 10^3 living Plasmodium knowlesi parasites. One animal in Group I-A responded with no apparent infection and two animals with mild transient parasitemias not exceeding 0.55%. These were accompanied by only mild anemias. Subinoculation of a susceptible monkey with 2 ml of blood from each of the resistant animals failed to produce infection, indicating a sterile immunity against the disease in the vaccinated challenged animal. This result, should it be a general one, may add immensely to the value of malaria vaccination in preventing the development of chronic and relapsing stages.

The Groups I-B and IV monkeys developed severe disease and died shortly after patent infection. This result suggests that injection of antigen, in combination with FCA or FICA, may be deleterious to the antigen and that administration of antigen by separate route from the adjuvant may be necessary when using such adjuvants.

The above protective results are quite remarkable when one considers the fulminating, lethal character of P. knowlesi malaria in the rhesus monkey. A situation reminiscent of acute Plasmodium falciparum malaria in man.

The remaining monkeys will be challenged shortly as the basic evaluations and studies noted above are completed.

Future plans call for use of adjuvants more suitable to man in vaccination procedures.

Intensive histological and clinical pathological studies are planned on the experimental animals in anticipation of human vaccine studies.

Purification and Characterization of Blood Stage Antigen

These studies have proceeded along two major lines. These are (1) evaluation of new procedures for isolation of antigens,and (2) refinement of purification procedures for antigens as they are now obtained. The latter studies are directed toward the final complete isolation and characterization of the active immunogenic materials.

The mouse model is being used to detect and evaluate each new antigen fraction as it is obtained. It is anticipated that in vitro lymphocyte transformation may also be used for this purpose in the future should it prove to correlate with actual immunity.

A new method for direct separation of blood stage plasmodial antigens has been explored utilizing the principle of co-precipitation. The active

malarial antigens readily precipitate with calcium alginate from French Pressure Cell disintegrated infected blood. The co-precipitated antigens are readily resuspended by resolubilization of the calcium alginate. Antigens obtained in this manner are protective. Further evaluations for degree of purity are under way.

The partially purified plasmodial antigens obtained by the chromatography of disintegrated plasmodia on Sephadex G-200 are being further purified. It has been determined that separation of material from the above antigen preparations will occur following centrifugation over linear sucrose gradients. Each of the fractions obtained in this manner are being tested for immunogenecity. Normal red cell materials are being similarly fractionated. Present results indicate that the red cell components distribute in a much different manner, in the sucrose gradient, in comparison to the distribution of the plasmodial materials. These results suggest that such gradients may offer a direct means of separating plasmodial antigens from disintegrated infected blood. In addition, the sucrose gradients offer an additional step in obtaining homogenous contaminant-free antigens. Sucrose gradient studies utilizing different type gradients, centrifugation times and gravities will be further explored. Additional purification steps will include isoelectric focusing, electrophoresis, and adsorbtion chromatography.

The active antigens, as well as red blood cell components, have been chemically dissociated and the dissociated fractions separated by polyacrylamide gel electrophoresis. A number of electrophoretic bands have developed. These will be evaluated and compared to determine levels of purity and homogeneity. The dissociated fractions will additionally be tested for immunogenecity.

Both the sucrose gradients and electrophoretic techniques described above promise to be powerful tools in the purification and characterization of the plasmodial antigens of rhesus monkeys against *Plasmodium knowlesi* malaria.

Pathophysiology

A study of the dynamics of *in vivo* red blood cell invasion by malaria parasites has been carried out and was the topic of a masters report by one of our students. Interesting findings concerning the distribution of schizonts in various tissues, such as liver, spleen and bone marrow were made. These findings will be followed up as time and resources permit.

Another study is under way to test the effects of elevated temperatures on the course of *P. berghei* in rats and mice. Preliminary results in rats suggest that maintained elevated body temperatures in rats may prevent infection. These studies also will be pursued as time and resources permit.

PUBLICATIONS LIST SINCE JULY, 1970

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