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The ultimate objectives of the work under Contract AID/csd-3689 to the Department of Biology, University of New Mexico 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 current effort is based on the previous demonstration by this group (under contract No. AID/csd-1432 to the University of Illinois) 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.

The initiation of work at the University of New Mexico was undertaken during July, 1972. The accomplishments of the group at both the Universities of New Mexico and Illinois during the past six months have met all the objectives outlined in the Specific Work Plan of the contract.

IN VITRO ANTIGEN PRODUCTION

INVERTEBRATE MATERIALS

Conventional Mosquito Rearing

Construction of an environmentally controlled insectary that will supply the needs of the malaria project has been completed. The entire windowless, low-ceiling insectary occupies 354 square feet with 2,500 cubic feet of controlled air space, and zoned into four areas: (1) Room A, 204 sq. ft., 27°C, 78% RH for the conventional rearing area with a capacity of 500 pans, 200 cages, or 100,000 mosquitoes per week; (2) Room B, 50 sq. ft., comfort temperature and humidity, housing a sterile air flow hood (HEPA) for the preparation of experiments to be carried out in rooms C and D; (3) Room C,

50 sq. ft., 27°C, 78% RH, aseptic rearing room; (4) Room D, 50 sq. ft., 21°C, 78% RH, with a capacity of about 10,000 female mosquitoes per week. A photoperiod of 14 hours of light and 10 hours of darkness is maintained daily. The new insectary, as a whole, is unique in that it was designed around the particular needs and experiences of the group.

Eggs of Anopheles stephensi from the University of Illinois were maintained in ice (approximately 4°C) during transport to the University of New Mexico. A small scale production of mosquito population was set up in a temporary insectary provided by a University of New Mexico faculty member during the time the new insectary was being constructed.

It is imperative that a rigid feeding schedule be followed and the type and amount of food be standardized to insure uniform and consistent results with Anopheles stephensi. A new diet (Tetramin, fish meal) has been described for the mass rearing of A. stephensi and it was compared with our regular rat chow diet. The Tetramin diet was found to yield 99% pupation within 4 days with 87% emergence into adults. The rat chow diet yielded an average of 89% pupation over a period of 6 days with 74% emerging into adults.

It is well known that living bacteria supports normal growth of mosquito larvae. Differences in the micro-organisms growing in the medium from rat chow diet and Tetramin diet may account for the differences in the life-span of larvae, per cent pupation and per cent adult emergence. Therefore, as a collateral to this, a study of the bacterial content in the rearing medium in the larvae pans were taken and plated on TSA (trypticase soy bacto-agar plate). Subsequent colonies were subcultured and are being stained for morphological and gram stain reaction.

Aseptic Mosquito Rearing

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 (paper accepted for publication, Ann. Entomol. Soc. Am.). Efforts to increase production of germ-free adults is currently in progress. Of the seven different experimental runs using quantity differences of both the basic requirements (yeast, carbon and nitrogen base yeast, isoelectric casein, sterol) and supplementary requirements (sugar, folic acid), the experimental run employing yeast and casein as the basic food with an increased quantity of sugar and folic acid, was by far the best. The larvae pupated on the 7th day and 95% of the pupae emerged as healthy, active adults on the 9th day.

Experimentation will continue along the same pattern until an aseptic diet is formulated that will yield a large number of adults comparable to that obtained in the conventional rearing employing a Tetramin diet.

A plexiglass apparatus specially designed for the aseptic feeding of adult *Anopheles stephensi* on surface-sterilized hamsters infected with malarial parasites has proved to be very efficient.

Primary Tissue Cell Cultures

Primary cell cultures are regularly established using material obtained by microdissection of first instar larvae (as previously described). The procedure has been sufficiently routinized so that primary cell cultures ranging in age from 4 to 60 days are available.

The ability of established primary tissue cultures of *A. stephensi* to support *P. berghei* ookinete and oocyst development *in vitro* has been tested and the results are currently being evaluated by microscopic examination. Primary tissue cultures of varying ages (10, 15, 20, 25 and 30 days old) were prepared. 36 Leighton tubes for each age group of primary cell cultures were

set up (total of 108 in all) and maintained at 27°C. At a specific age (10-days old, 15-days old, 20-days old, 25-days old, and 30-days old) the cultures were pre-conditioned at 21°C five hours before employment. Each pre-conditioned tube was then inoculated with a drop of heparinized infected blood (blood was exposed to varying percentage of CO_2, O_2 and atmospheric air). In each age group, 3 tubes were terminated daily for a period of 3 days. Cover slips were fixed and stained (1-Giemsa, 1-Sudan colorant and 1-Feulgen). Likewise, the supernatant from each tube was smeared on slides and stained.

The fact that Anopheles stephensi mosquito tissues contain materials which are capable of stimulating resistance to infection against malarial infection has previously been established. Efforts will be directed towards identifying the antigenic material. Because of the laborious procedure of hand dissection required to prepare the large amount of mosquito tissues for analysis, a micro system employing mosquito tissue extracts (as little as 1-2 μg of antigenic materials) is presently being set up. Equipment (such as micropippette, loading cassette, specific stains and a special micro-electrophoretic apparatus) have been ordered.

Materials that will be analyzed are: salivary glands (male and female); gut (male and female); sporogonic forms of malarial parasites and cells in culture of varying ages.

Electrophoretic separation of antigenic materials will be scanned using a Helena Quick Scan densitometer, through the courtesy of Dr. Ulrich, Bernalillo County Medical Center, U.N.M., and accurate determination of the amount of material (total protein, lipoprotein, etc.) will be computed using the Biology Department terminal of the U.N.M. Computer Center. Presently,

thin-layered polyacrylamide gel and agarose gel as supporting medium for analysis are being prepared. Sephadex G-200 and Sepharose 6B fractionation will also be investigated.

Precipitation, double diffusion, and immunoelectrophoresis techniques will be used to demonstrate the antigenic property of each fraction using antisera prepared in rabbits.

VERTEBRATE MATERIALS

The future practicality of a malaria vaccine depends in large measure on major technological breakthroughs in the development of *in vitro* cultivation procedures for trophozoite and sporozoite antigens. Towards this end various mammalian cells which have already been adapted to *in vitro* culture procedures are being tested for their ability to host the various intracellular stages of malaria. Cell lines being established for additional experimentation are bone marrow, liver, spleen and kidney of hamster and mouse origin. Also in hand are human lung (WA-38) and Fat-Head Minnow cells. The latter cell line (FHM) has the advantage of growing at a wide temperature range.

Working relations have been established with tissue culturists in the UNM Medical School, as well as with the laboratory of Dr. Datus M. Hammond of Utah State University, Logan. Dr. Hammond's laboratory has been one of the world leaders in the *in vitro* cultivation of coccidia in mammalian tissue cultures. The coccidia, which are related to malaria (both are Sporozoa), are obligate intracellular parasites and seem to have an immunological relationship with their hosts very similar to malaria. Dr. E. Cabrera spent a week in Hammond's laboratory and has brought back several techniques and other information which should accelerate our work.

Cell Fusion Experiments

In addition to the screening of various cell lines as *in vitro* hosts for malaria parasites, the use of artificial means of hybridization of parasitized and non-parasitized cells has been investigated with some success. It is postulated that hybridization of an infected cell with an established non-infected cell line might result in a viable *in vitro* system capable of large-scale production.

Cell fusion, which may occur spontaneously between a small percentage of different cell types mixed in culture, can be greatly enhanced by the use of certain chemicals or viruses. Lysolecithin (LL) is one of the chemicals which when incubated with nucleated cells produce changes in the cell membrane that eventually lead to the formation of multinucleated cells.

It was necessary to find a concentration of lysolecithin capable of producing good fusion between the nucleated cells which did not cause harmful changes on the RBC or the parasite. Two well established cell lines were used as nucleated cells. The L-cell line (from mouse fibroblasts) and the BHK-21 cell line (from newborn hamster kidney). These two cell lines were treated with different concentrations of LL and for various time intervals. It was found that a time of approximately 60 seconds and a concentration of 800 μg/ml of LL was best for both cell lines.

On the other hand, when the same treatment was given to infected RBC's rapid lysis and parasite death occurred. Therefore, the dose was lowered to 400 μg/ml. This dose still produced fusion in the nucleated cells while the deleterious effect on infected RBC's and parasites was slight.

Fusion experiments between infected RBC's and L-cells were carried out

under the above mentioned conditions. Samples of the cultured cells were taken (on coverslips) at different time intervals after seeding the cells. After May-Grunwald-Geimsa staining, the coverslips were examined under oil immersion. It was found that fusion had occurred between the L-cells, but no conclusive evidence of parasites within L-cells was observed.

In another series of experiments BHK-21 cells were used instead of the L-cells. This time cytoplasmic inclusions were seen in the BHK-21 cells. These inclusions, in some cases, resembled merozoites of P. berghei. Furthermore, these inclusions were absent from control cultures (BHK-21 + RBC + LL).

This line of research will be pursued further. The use of another hybridizing agent might be looked into. Deactivated Sendai virus, which is known to enhance cell fusion but not to be as harmful as LL, might be a likely candidate.

FHM Cell Line for Oocyst Development

It has been speculated that cell lines other than the normal host (cell culture other than mosquito cells) might serve as an efficient substrate for cultivation of the sporogonic form of the malarial parasite. A FHM (fat head minnow) cell line was obtained from the Department of Microbiology (UNM). The cells proliferate over a wide temperature range (0-36°C) and were grown in MEM (minimum essential medium) without antibiotics or serum in Leighton tubes. Cultures were maintained at 27°C and pre-conditioned at 21°C for 24 hours before use. Three drops of sterile infected blood (87% macrogametocytes or 261 macrogametocytes/3 drops) was then added into each tube. After 24 hours and 44 hours the medium and blood was spun down at 500 rpm (30g) for 4 minutes. Smears were made of the supernatant and pellet and stained with Giemsa. The pellet from the 23 hour tube revealed approximately 242 young ookinetes and

approximately the same number of matured ookinetes at 44 hours. Phase contrast microscopy revealed healthy, viable ookinetes throughout the 44 hour cultivation period. Presently the FHM cell line is being subcultured for further investigation.

ANTIGEN TESTING

PRIMATE MALARIA SYSTEM

To date, over 100 primates have been utilized to establish a vaccine model which might be applied to human malaria.

The work has demonstrated that a nonviable partially purified *P. knowlesi* antigen mixed with Freund's complete adjuvant stimulates resistance against infection and death. The early trials utilized a lengthy immunizing schedule and multiple injections by various routes as well as a lengthy delay after immunization and before inoculation with the challenge infection. Subsequently, it was found that monkeys were protected which were given only two injections at a 4 week interval of Freund's adjuvanted trophozoite antigen and challenged 3 weeks later. Using this simpler and less time-consuming immunizing schedule, a series of 5 monkey experiments have been completed. The aims of these latter experiments have been (1) to find an acceptable substitute for Freund's adjuvant, (2) to determine the effect of lyophilization on the protectivity of the antigen, and (3) to test the efficacy of two antigen fractions prepared by sucrose gradient sedimentation. In addition, serum has been collected for serologic analysis and, because cell-mediated immunity appears to be an important aspect of the immune response, skin testing for immediate and delayed hypersensitivity reactions to malaria antigen before and after vaccination was carried out.

Other parameters which are being examined that had not previously been assessed include blood urea nitrogen, lactic dehydrogenase, bilirubin, serum electrophoresis and possibly bone marrow examinations. These tests are being carried out in collaboration with the UNM Medical School pathology laboratory and should provide useful indices of the effect of vaccination and the nature of the disease process in vaccinated and non-vaccinated monkeys. Post mortems are being performed on all monkeys which die and include preparation of relevant tissues for both electron and light microscopy. This information will be essential for the planning of any vaccine trials in human subjects.

The tentative conclusions (which require confirmation because of the small groups of monkeys with which each experiment has had to contend) concerning the three essential aims outlined above, are as follows:

1. It appears that aluminum hydroxide may be an effective adjuvant for malaria antigen. Additional work confirming our preliminary results is required and further studies to determine the optimal conditions for its use with malaria antigen will be carried out (aluminum hydroxide is widely used as an adjuvant in a variety of human vaccines).

2. Lyophilized malaria antigen continues to retain resistance stimulating qualities. This highly significant finding, if confirmed, opens the way for antigen standardization and characterization. It will enable the long term storage of vaccine and permit biochemical fractionation, purification and quantification to be undertaken. The primary restraint on progress in this area is the number of monkeys and the rate at which protection tests can be implemented.

3. Preliminary results indicate that fractionation of the antigen by sucrose gradient sedimentation results in two fractions, both of which retain

some protective antigenic activity. The continued use of this fractionation method will continue. The effect of the purification treatment on antigen and the interaction with adjuvant make it difficult to predict protectivity or to interpret partial protection results. Much experimentation, some of an emperical nature, remains to be done.

Since it is felt that the protective antigen is very likely closely associated with or part of a membrane fragment, we have been attempting solubilization procedures which have been found to be useful in other membrane systems. Biochemical purification of the antigen will be greatly facilitated if the material can be solubilized. To date, we have been most successful with Triton X-100, a non-ionic detergent. Upon addition of Triton, the previously turbid antigen immediately becomes clear, At present, we are working on a means of removing the detergent which is believed to be absorbed to protein. This can be done by means of acidified N_1N - Dimethyl formamide. Once the Triton is removed it should be relatively easy to purify further the solubilized antigen by column chromatography or gel electrophoresis. We have also been experimenting with the chelating agent EDTA which has been shown to solubilize red blood cell membranes, in some systems, by the selective removal of cations.

RODENT-SPOROZOITE SYSTEM

It has been demonstrated that non-infective sporozoites and certain related mosquito antigens when used as a vaccine are capable of stimulating resistance in mice to an infection with viable P. berghei sporozoites. Work at the University of Illinois has been concerned during the past six months

with investigating various aspects of the immunization procedure. The factors studied include (1) the effect of age of the mice at the time of initiation of the lengthy 14 week injection schedule, (2) the route of injection, (3) the effect of sodium alginate as an adjuvant, (4) the effect of varying quantities of sporozoite antigen, (5) the effect of the rodent anti-hypersensitivity agent (serotonin), and (6) the effect of certain non-specific agents as inducers of protection.

The results of the work to date are reported below. The clarity of interpretation of results is somewhat clouded by the variation in protected groups (40-95%) and because both the immunizing injections and the challenge inoculations are given intraperitoneally. It is hoped that the anticipated future use of the intravenous route will eliminate some variation.

1. The age of mice at the initiation of the immunization procedure (3 to 12 weeks) was not found to have any influence on the degree to which they became resistant to infection. Nor, was there found to be any difference in susceptibility to infection at challenge.

2. Immunization procedures with, or without, sodium alginate as an adjuvant by the intramuscular route (with sporozoite antigen) resulted in no evidence of protection being conferred on the treated mice. In contrast, mice which were immunized at the same time (with sporozoite antigen) by the intraperitoneal route yielded evidence of resistance to infection.

3. The use of sodium alginate together with sporozoite antigen by either the intramuscular or intraperitoneal route of injection was not found to enhance the level or consistency of protection achieved in the absence of sodium alginate.

4. Increasing the number of sporozoites given for each of the 7 immunizing injections to 50,000, 200,000 or 450,000 was not found to enhance

the level of protection achieved with 25,000 sporozoites.

5. The use of serotonin to inhibit the mouse hypersensitivity response to the immunizing antigens was not found to affect the level of protection achieved when similar antigens were used in the absence of serotonin.

6. Uninfected salivary gland antigen by itself stimulates resistance to to sporozoite infection almost to the same levels achieved with inactivated sporozoite antigen. It was found that about 70 salivary glands per injection are required to induce protection. While these results with salivary gland material confirms earlier reports, an additional new finding is that hamster serum alone is capable of inducing resistance to infection. In one experiment in which groups of mice were injected 7X with 0.025 ml of hamster or rat serum, 4/18 mice injected with hamster serum were protected whereas 0/20 mice injected with rat serum were protected. When the amount of serum was increased to 0.05 ml per injection, 9/19 mice which received the hamster serum were protected. The significance of these findings remains to be explored.

Efforts to prepare and isolate the active antigenic fraction from disintegrated whole mosquitoes by chromatographic separation of the extract have been unsuccessful. Another approach involving high speed centrifugation separation on polyacrylamide gel is being pursued.

Continued selection of a mosquito colony which can utilize blood meals from mice is in hand in order to determine transmission interference by antimosquito antibody. Some sporozoite transmission via a mouse-mosquito-mouse cycle has been achieved.

PUBLICATIONS

1. Vaccination of Rhesus monkeys (*Macaca mulatta*) against *Plasmodium knowlesi* by use of nonviable antigen. *Bulletin of the World Health Organiztion*. (in press). Robert H. Schenkel, Gary L. Simpson and Paul H. Silverman.
2. Aseptic rearing of *Anopheles stephensi*. *Annals of Entomological Society* (in press) M. C. Rosales-Ronquillo, R. W. Simons and P. H. Silverman.
3. Adoptive transfer of immunity to *Plasmodium berghei* by spleen and lymph node cells from young and old mice. *Journal of Protozoology* (in press). Lloyd Kasper and Nelda E. Alger.

OTHER RELATED ACTIVITIES

Members of the group from both New Mexico and Illinois attended the combined meeting of the American Society of Parasitologists and the American Society of Tropical Medicine and Hygiene held in Miami, Florida, November 3-5, 1972. A paper on the primate malaria vaccine model was presented by Dr. R. H. Schenkel and was well received. An expanded version of this paper has been accepted for publication in the Bulletin of the World Health Organization.

As part of the trip to the Miami meeting, part of the group visited the Primate Malaria Unit, Laboratory of Parasitic Diseases of the Center for Disease Control in Chamblee, Georgia. Full cooperation has been offered by Dr. Collins and his colleagues in the anticipated need to examine additional primate-malaria systems. This relationship will be particularly valuable in the development of primate sporozoite transmission cycles.

Enquiries have been received from Dr. Paul E. Carson, Rush Medical College, Chicago, Illinois who is director of the Human Malaria Project at Statesville Prison at Joliet. He has indicated a willingness to begin exploring vaccination in human volunteers using antigen derived from non-human primates. This type of study will be an essential preliminary to any field trials. Plans have been made for planning meetings sometime during the spring.

Contact with the Institute of Tropical Veterinary Medicine of Texas A & M University has been established. Closer liason and exchange of information is being planned. Both groups are funded by AID and have very similar goals. Currently under study is a proposal for a workshop involving the Texas, New Mexico and CIAT laboratories in Columbia.

During a visit to the Biomedical Division of the Los Alamos Laboratories,

several offers of cooperation were received. The facility has available large-scale equipment for *in vitro* cultivation and apparatus for a wide variety of molecular and cellular experimental procedures. Available for use, among other equipment, is a multiparameter cell sorting system. The system depends upon the flourescence resulting from staining with vital stain levels of acridine orange. In one preliminary run with infected *Plasmodium berghei* blood, 95% of the infected red blood cells were effectively separated from the whole blood. This elegant technique (which requires a laser) offers the possibility of research with small populations of a pure type of cell.