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PROGRESS REPORT

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MALARIA IMMUNITY AND VACCINATION

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## Duration of Protection With and Without Serotonin

Occasionally, during the course of this work, we have had animals die in the first 24 hour period after injection. In some cases the cause was very clearly bacterial. As work progressed and our antigens were largely free of bacteria, those animals which died were seen to have a more uniform response which appeared to be anaphylactic shock. Mouse anaphylaxis is primarily caused by a combination of histamine and serotonin and can be blocked by the injection of serotonin. Preliminary experiments indicated that the injection of serotonin did not interfer with the sporozoites' ability to infect the animal. Table I is the compiled data on the duration of protection with and without serotonin.

These results indicate quite clearly that hypersensitivity is active as a host protective mechanism in both the sporozoite mechanism and salivary gland control vaccinated animals. In salivary gland vaccinated animals none remained protected when treated with serotonin while those not treated with serotonin showed relatively good protection. Of those animals vaccinated with sporozoites (and contaminating salivary glands) the mice treated with serotonin showed a poorer protective response than those not treated. This seems to indicate at least a dual mechanism of protection probably in response to different antigens. Roughly, 30-40% of protection appears due to hypersensitivity.

It is well known that insect tissue (in particular, hemolymph) may cause hypersensitive reactions. Whether sporozoites injected by the mosquito itself will also elicit this reaction will be determined in a later experiment.

The protection of sporozoite vaccinated animals, in spite of serotonin treatment, is probably due to a more direct sporozoite-antibody type of reaction.

## Route of Injection

Two experiments to compare the efficacy of the subcutaneous (Sub. Q.), intramuscular (IM) and intraperitoneal (IP) routes of vaccination were completed during this period. Table II shows these results.

These results indicate that the IM route of injection is not as satisfactory as the IP route of injection. This is in agreement with the results reported last period. The Sub. Q.route of injection appears more satisfactory than the IM route but not as good as the IP route. This differential may possibly be equalized by the use of adjuvant in subsequent tests. However, since there is considerable variation from test to test, this may represent variation.

In order to challenge by a different route than the oute of vaccination injected we attempted to utilize the oral route of challenge as described by Yoeli. Too many animals in each group died of drowning to draw any conclusions other than that this method will not be used again.

## Adjuvant

Freund's adjuvant is known to enhance both cellular and humoral immunity. Freund's alone has been reported to protect against sporozoite infection (Nussenzweig). This adjuvant was used in conjunction with other experiments to determine whether it alone would affect later experiments of a similar type (Table III). The IM route was used because of the pronounced inflammatory response produced.

Freund's adjuvant under the conditions used here does not affect the outcome of the infection whether by sporozoite or red cell challenge. Any non-specific protection induced by Freund's appears to be absent by the third week following the final injection. Thus Freund's can be used IM for experimental purposes without inducing protection of itself when challenge is made three weeks from the last injection.

## Various Treatment of Sporozoites

Two experiments (with the Paar Bomb method of breaking up the sporozoites) were completed during this period. In the first experiment the supernate of the broken cells was used as antigen, and in the second the whole material was used (Table IV).

These experiments further substantiate the suggestion that the active antigenic portion is probably particulate (possibly cell membrane). This method does not appear to be better than the heated or freeze-thawed preparations. Since more antigenic material is lost in this type of preparation than in heated or freeze-thawed, we do not propose to pursue this method of treatment for sporozoites at present.

The next method of treatment examined was 0.1% formalin as successfully used by Richards in fowl malarial experiments (Table V).

The results of this experiment indicate that this treatment is not as good as the heat treatment method. This treatment combined with the IV route of injection might prove successful and further experiments may be pursued after baselines are established with the IV injected heat treated material as compared to the IV injected irradiated material.

## Schedule of Injection

Two experiments with differing schedules of injection were completed during this period (Table VI).

These experiments seem to indicate that an initial injection of a large amount of antigen (Exp. II) followed by two injections of smaller amounts may be as satisfactory as 7 equal doses. Experiment I seems to indicate that larger amounts of immunogen may give better results. In this instance, the total immunogen given to each group was equal. This experiment should be enlarged to examine the parameters of total immunogen vs. time. To date there has been considerable variation in protection. This may relate to the route of injection or the schedule of injection.

## Normal Mosquito Tissue

The following experiment was carried out to determine whether active antigenic material could be recovered from whole mosquitoes or whether protective activity was lost by the introduction of so many antigens. A fly control was used but 8-10 animals per group were killed by the first injection in spite of the use of penicillin-streptomycin. Deaths were due to bacterial infection. The usual 7 injections were used and serotonin was administered on challenge at 16 weeks following the last injection (Table VII).

The whole mosquito contains considerable numbers of bacteria while the head and salivary gland material have relatively few bacteria. The 40% protection obtained in spite of the use of serotonin suggests that the head is a good source of antigen and that protection is afforded in spite of the blocking serotonin release. Furthermore, 40% protection after 16 weeks or about 1/6 of the life span of the mouse is very promising.

Three additional experiments were completed during this period using different treatments of the salivary gland material (Table VIII).

The ultraviolet light did destroy all bacteria since none could be grown when material was innoculated into growth medium. This treatment appears to have destroyed the activity of the antigen, however. The second and third experiments substantiate the idea that protective activity is centered in the particulate components of the mosquito material (probably the cell membranes).

## Comments

Our early experiments required 70 (average) salivary glands to obtain enough sporozoites for injections. Later experiments, where fewer salivary glands were used, did not yield as good protection. We thus use 70 salivary glands as a basis for our investigation of the mosquito antigens as such.

Table I

Duration of Protection

| No. Weeks to Challenge | No. Injections | No. sporozoites per Injection | Serotonin | Protected/Total | | |
|---|---|---|---|---|---|---|
| | | | | Sporozoites | Salivary Glands | Normal |
| 22 | 7 | 25,000 | + | 0/9 | 0/9 | 0/7 |
| 16 | 7 | 25,000 | + | 18/30 | 0/27 | 0/9 |
| 16 | 7 | (Freund's only) | + | --- | --- | 0/8 |
| 7 | 7 | 25,000 | + | 2/10 | 0/10 | 0/10 |
| 3 | 7 | 25,000 | + | 2/35 | 0/35 | 0/29 |
| 14 | 7 | 25,000 | - | 8/8* | 3/8 | 0/10 |
| 12 | 7 | 25,000 | - | 9/10 | 4/10 | 0/10 |
| 3 | 7 | 25,000 | - | 16/18 | 10/18 | 1/16 |

* 2 animals died of shock

Table II

Comparison of Routes of Injection

| | No. Injections | No. Sporozoites Per Injection | Route of Injection | Serotonin | Protected/Total | | |
|---|---|---|---|---|---|---|---|
| | | | | | Sporozoite | Salivary Gland | Normal Control |
| Exp. I | 7 | 25,000 | IM | + | 0/15 | 0/15 | 0/10 |
| | 7 | 25,000 | IP | + | 5/8 | 0/10 | |
| Exp. II | 7 | 25,000 | Sub.Q. | None | 8/14 | 6/13 | 1/10 |
| | 7 | 25,000 | IP | None | 8/8 | 5/8 | |

Table III

Effect of Freund's Adjuvant on Challenge

| Material Injected | Route of Injection | No. Injections | Weeks to Challenge | Serotonin | Protected/ Total |
|---|---|---|---|---|---|
| EXP. I | | | | | |
| Freund's Complete<br>Freund's Incomplete | IM<br>IM | 1)<br>6) 7 | 16 | + | 0/8 |
| Sporozoites | IP | 7 | 16 | + | 5/10 |
| Salivary Glands | IP | 7 | 16 | + | 0/9 |
| Normal Control | - | - | - | - | 0/9 |
| EXP. II | | | | | |
| Freund's Complete<br>Freund's Incomplete | IM<br>IM | 1)<br>6) 7 | 3 | None | 0/15 |
| Sporozoites | IP | 7 | 3 | None | 8/10 |
| Salivary Glands | IP | 7 | 3 | None | 5/10 |
| Normal Control | - | - | - | - | 0/6 |

All above animals challenged with 2,000 sporozoites

| | | | | | |
|---|---|---|---|---|---|
| Freund's Complete<br>Freund's Incomplete | IM<br>IM | 1)<br>6) 7 | 3 | None | 0/15 |
| Normal Control | - | - | - | | 0/5 |

Challenged with $10^6$ infected red cells.

Table IV

Paar Bomb Treatment

| No. Injections | | Sporozoite Protein | Weeks to Challenge | Serotonin | Protected/Total Exp. | Salivary Gland | Normal Control |
|---|---|---|---|---|---|---|---|
| Exp. I. | 7 | 0.5 mg/animal | 2 | - | 0/8* | | 0/10 |
| Exp. II. | 7 | 0.5 mg/animal | 3 | + | 4/10 | 0/10 | 0/10 |

*Two died of shock

Table V

Formalin Treatment

| No. Injections | No. Sporozoites | Treatment | Route | Serotonin | Exp. | Salivary Gland | Normal Contro |
|---|---|---|---|---|---|---|---|
| 7 | 25,000 | 0.1% formalin | IP | + | 1/10 | 0/9 | 0/9 |
| 7 | 25,000 | Heat | IP | + | 5/9 | 0/8 | |

Table VI

Effect of Schedule of Injection

| No. Injections | | No. Heated Sporozoites | Route | Serotonin | Sporozoite | Salivary Gland | Normal Control |
|---|---|---|---|---|---|---|---|
| Exp. I. | 7 | 450,000 Inj. I 25,000 Inj. 2-7 | IP | + | 4/9 | 2/10 | 0/10 |
| Exp. II. | 7 | 25,000 | IP | + | 2/10 | 0/10 | 0/10 |
| | 3* | 165,000 Inj. I 15,000 Inj. 2 & 3 | IP | + | 2/10 | 0/9 | |
| | 3* | 59,000 | IP | + | 1/10 | 0/10 | |

*Given at the same time as injections 1,4,and 7 of above experiment.

Table VII

Whole Mosquito Vs. Salivary Glands

| Antigenic* Material | Ag. Treatment | Protected/Total |
|---|---|---|
| Whole mosquito | Supernate | 2/10 |
| Whole mosquito | Pellet | Killed all animals on 1st injection |
| Sal. Glands | Ground | 3/10 |
| Mosquito heads less sal. glands | Ground | 4/10 |
| Normal Control | -- | 0/10 |

*70 mosquito equivalents were used for each injection IP

Table VIII

Salivary Glands

| Treatment of Salivary Glands | Weeks to Challenge | Serotonin | Protected/Total |
|---|---|---|---|
| EXP. I. (7 injections) | | | |
| UV (to destroy bacteria) | 3 | + | 0/14 |
| Ground | 3 | + | 2/15 |
| Control | | + | 0/10 |
| EXP. II. (6 injections) | | | |
| Millipore Filtered | 3 | - | 0/15 |
| Control | 3 | - | 0/10 |
| EXP. III. (7 injections) | | | |
| Ground | 3 | - | 1/10 |
| Supernate | 3 | - | 0/10 |
| Pellet | 3 | - | 3/9 |
| Millipore Filtered | 0 | - | 0/10 |
| Normal Control | | - | 0/10 |

## ELECTRON MICROSCOPIC STUDIES

### Sporozoites

At the time of the last progress report, due to the peculiar appearance of the cell wall in a small number of heat-treated Plasmodium berghei sporozoites, the question arose as to the true origin of the parasite's cell wall. It was suggested that perhaps one of the cell membranes composing the multilaminated sporozoite pellicle had its origin from the cells of the mosquito salivary glands. Search of the literature revealed that other investigators claim that sporozoites receive their full membrane complement in the midgut cells of the mosquito. This may very well be the case, but at the moment we are still in no position to resolve the question. Suitable sporozoite specimens for this purpose have not yet been encountered. The sporozoites in this study should be untreated samples rather than freeze-thawed or heat-treated as was the previous case. The exposure of the sporozoites to various physical trauma may introduce artifacts which might suggest an erroneous interpretation and conclusion as to the origin of the various membranes composing the sporozoite pellicle.

One problem concerning the sporozoites, however, has been resolved. Previously, structures reminiscent of virus-like particles had been observed. Since, it has been clarified that these structures are not of viral origin, but are transient organelles of the motile stages of the malaria parasite.

### In Vitro Cultures

An in depth ultrastructural analysis of cultured Anopheles stephensi mosquito cells which had undergone the vesicular stage formation resulted in several interesting observations. Two types of vesicles have been observed. One type of vesicle, when observed under the light microscope,

gives the impression of being acellular. In the electron microscope, however, it becomes evident that the wall of the vesicle is composed of a string or a very fine protoplasmic extension of a cell. This suggests that this particular vesicle increases in size by stretching its cytoplasmic contents in a manner similar to the vertebrate fat cell. The other type of vesicle is composed of many cells and is reminiscent of a morula. The vesicle is hollow and it has been suggested that its interior contains the culture medium in which it has been growing. Although those vesicles were obtained from a culture considered to be aged, nevertheless, many of the cells making up the cellular vesicle appeared to be still active. The surface of these cells had a sparce covering of microvilar-like cytoplasmic projections, intact mitochondria, Golgi membranes, rough surfaced endoplasmic reticulum, and a nucleus. Within the cytoplasm were also seen many myelinated profiles and other unidentified multivesicular structures. In Aedes aegypti it has been suggested that originally the membranous structures may be chitin-like and initially arose from cells which had released a substance indentified as melanin. Still another cell type was observed in the cellular vesicle. The cytoplasm of these cells contained numerous round structures of various sizes, subtended by a membrane, the previously mentioned myelinated figures, and many small vesicles. No nuclei were evident, but many mitochondria were seen throughout the cytoplasm between the round structures. Rough surfaced endoplasmic reticulum was also evident. The striking difference between these cells and the aforementioned cells was the complete absence of microvilar-like cytoplasmic projections. The latter cells gave the distinct impression of being highly specialized and perhaps more aged.

Cells grown in monolayers on a nuclepore filter as the supporting surface were also observed with the electron microscope. At present very little can be said about these cells because the apical portion of the cells

appeared to have been lost during the preparation for ultrastructural study. Hopefully, improvement in the handling technique will yield intact cells next time.

## Monkeys

It has been documented in the past that many individuals infected with malaria succumb with renal failure due to glomerular nephritis resulting from the immune response. In the near future we hope to study some of the kidneys from the Rhesus monkeys which had been used in our vaccine studies. A comparison will be made between kidneys obtained from monkeys which had been protected with the *Plasmodium knowlesi* antigens prepared from the blood stages and those which received the antigens, but were not protected.