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DORA S. RANE

For the period of August 1, 1973 to December 31, 1974

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Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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Under Contract No. DAMD 17-74-C4019, activated August 1, 1973, a feasibility study was initiated having as its objective: the development of a sporozoite-induced rodent malaria screening procedure, based on the mortality of negative controls, that would provide quantitative evaluations of classes of chemical compounds indicating prophylactic usefulness in Plasmodium berghei infections. These agents would include: (1) compounds structurally related to chemicals of known value in the prophylaxis of P. berghei infections; (2) compounds structurally unrelated to chemicals known to have value in the prophylaxis of P. berghei infections and; (3) structural analogues of compounds demonstrating prophylactic activity and representing novel chemical groups.

From the beginning it seemed that the ideal procedure would follow the pattern successfully established in our screening procedure using Aedes aegypti sporozoite-induced Plasmodium gallinaceum malaria in chicks. In that procedure the parasite was maintained in a slowly evolved donor line by a bite-to-bite routine (mosquito-to-chick-to-mosquito-to-chick, etc.) and produced infections resulting in the death of 100 percent of the negative controls. Controls and test animals were infected with suspensions of sporozoites obtained by grinding whole Aedes aegypti infected with P. gallinaceum. Prophylactic values were then assessed by comparing the maximum survival time of treated sporozoite-infected chicks and the survival time of untreated sporozoite-infected controls. A compound was considered to have potential prophylactic value if it produced a minimum increase of 100 percent in the survival time of the untreated controls.

*Originally set up by D. Rane.

Using the above as a model, the present study was designed to examine in a systematic fashion:

1. the parasite;
2. the animal host;
3. the mosquito vector;
4. the final integration of these into a reliable and precise system, based on the death of all negative controls, for screening compounds for prophylactic antimalarial activity.

The parasite and the animal host introduced problems that required careful consideration but most difficulties in the past have centered around the mosquito vector and these have been the subject of the most intensive investigation.

The parasite

After consultation with the WRAIR staff, Dr. J. M. Bafort (Inst. Trop. Med., Antwerpen, Belgium), Dr. Harry Most (NYU) and Dr. Richard Beaudoin (Naval Medical Research Institute, Bethesda, MD) several organisms were obtained for testing. Of these the NK65 strain (P. berghei) obtained from Dr. Beaudoin was selected to determine its acceptability for our study.

The NK65 strain was first isolated from Anopheles durenii in Katanga in 1965. It was initially maintained in our laboratory by mouse-to-mouse blood passes, each animal receiving 0.5 cc of a 1:50 dilution of heparinized heart's blood drawn from a donor early on the fourth day. All donors infected thus were dead by the end of day 4. Subsequent testing has shown that the NK65 strain can be successfully adapted to the testing regimen desired.

The animal host

Since a uniformly bred highly susceptible host would be an essential part of our test system, we believed that it might be necessary to initiate a systematic examination of a number of strains of mice and possibly several strains of rats. A survey was made of the work and findings reported by others. Tables I and II indicate the parasite susceptibility of various strains of mice. However, since the ICR/HA mouse strain of our own breeding colony has proved to be a highly desirable host in our blood-induced KBG 173 Plasmodium berghei antimalarial screening system, it seemed to be expedient and logical to investigate first how useful this mouse strain might be in developing a sporozoite-induced NK65 P. berghei malaria procedure. To this end mice from our ICR/HA colony were tested for their suitability as the mammalian host in our model. Results have indicated that this mouse strain is highly desirable as the mammalian host in our proposed screening model. Testing included measures to determine variations in response to infection when animals of different weight, age and sex were used. Presently we are using mice of both sexes, 3-5 weeks of age, weighing 10-12 grams, 15-17 grams or 18-20 grams. However, in any given test all mice are of one sex and approximately the same age and weight.

The mosquito

Although several species of mosquitoes have been used as laboratory vectors of rodent parasites, Anopheles stephensi has generally been found to be the most suitable for various strains of P. berghei and was the mosquito vector chosen for this study. Our A. stephensi colony was

TABLE 1

Mosquito transmission of *P. berghei* (1948-1963)

Authors	Year	Strain	Temperature Celsius	Mosquito	Oocysts	Sporoz. stomach	Sporoz. sal. gl.	Cyclical transmission	Comments
Box, et al	1953	Kasapa	21° 26°	<u>A. quadrimaculatus</u> <u>A. quadrimaculatus</u>	+	+	-	-	100% infected 100% infected
Bray (*)	1954	sp 28	(27°) (**)	<u>A. stephensi</u> <u>A. quadrimaculatus</u> <u>A. gambiae</u> <u>A. l. atroparvus</u>	+	+	+	-	Oocysts mature on 7th day Heavy gut infection Heavy gut infection Heavy gut infection
Celaya, et al	1956	Kasapa	26°	<u>A. quadrimaculatus</u> <u>A. freeborni</u> <u>A. bradleyi</u> <u>C. quinquefasciatus</u>	+	+	-	-	Range size oocysts 30-58 μ 100% infected 85% infected 83% infected
Michiels	1963	sp 11	(28°)	<u>A. stephensi</u> <u>A. gambiae</u> <u>A. quadrimaculatus</u>	+	+	+	-	
Perez-Reyes	1953	Mukata	24° 28° 28°	<u>A. aztecus</u> <u>A. quadrimaculatus</u> <u>A. albimanus</u>	+	+	+	+	Heavy gut infection 14th day sal. gl.

TABLE 11

Mosquito transmission of *P. berghei* (1948-1963)

Authors	Year	Strain	Temperature Celsius	Mosquito	Oocysts	Sporoz. stomach	Sporoz. sal. gl.	Cyclical transmission	Comments
Raffaele & Baldi	1950	?	?	<i>A. aegypti</i> <i>C. pipiens</i>	-	-	-	-	
Ramakrishnan & Prakash	1950	(Paris)?	24-29.5°	<i>A. stephensi</i>	+	+	+	-	
Ramakrishnan, et al (*)	1953	New strain	25-30.5°	<i>A. annularis</i>	-	-	-	-	Attempts with 8 other species of <i>Anopheles</i> unsuccessful
Rodhain & Vincke	1952	sp 28	23-24°	<i>A. l. atroparvus</i>	+	+	-	-	
Rodhain, et al	1955	3 newly isolated strains	(23-24°)	<i>A. l. atroparvus</i> <i>A. stephensi</i> <i>A. gambiae</i>	+	+	+	-	45 pos. sal. gl.
Rodhain, et al	1955	3 newly isolated strains	(23-24°)	<i>A. l. atroparvus</i> <i>A. stephensi</i> <i>A. gambiae</i>	-	-	-	-	
Vincke	1954	New strain	23-24°	<i>A. l. atroparvus</i>	+	-	-	-	
Yoeli & Wall	1951	sp 28	26-27°	<i>A. stephensi</i> <i>A. quadrimaculatus</i> <i>A. l. atroparvus</i> <i>A. gambiae</i>	+	+	+	+	62% infection
Yoeli & Most	1960	Kas. 7307	26-27°	<i>A. aztecus</i> <i>A. quadrimaculatus</i>	+	+	-	+	81-90% inf. oocysts mature on 8th day

(*) Only very brief summary.

(**) No exact record of temperature, but fully mature oocysts on D + 7, therefore temperature probably 27° C. (Yoeli & Most, 1960).

started with eggs received from Dr. Ronald Ward of WRAIR whose mosquitoes were obtained from Mr. P. G. Shute of the Malaria Reference Laboratory, Horton Hospital, Epsom, England. Mr. Shute's supply of A. stephensi originated from material collected in the vicinity of Delhi, India. Techniques for the laboratory rearing and handling of A. stephensi have been reported (5-20) and are used. However, early in this study it was recognized that A. stephensi does not conform easily to laboratory discipline, and a sporozoite-induced screening procedure acceptable to us would require an invertebrate vector that was highly uniform as well as readily susceptible to the parasite and compatible with the vertebrate host. Consequently, modifications and substitutions were made in the known methods of rearing and handling non-infected and infected A. stephensi in order to permit the regulation of the developmental stages of the mosquitoes and through these the infections that would eventually produce the death of all negative controls.

The parameters examined were:

- (1) methods of handling eggs, larvae and pupae;
- (2) the nutritional requirements and diets of larvae, pupae, and adult mosquitoes;
- (3) types of blood meals.

Presently, Anopheles stephensi mosquitoes are being satisfactorily raised in an insectary which has the temperature maintained at $80^{\circ} \pm 2^{\circ}$ F and relative humidity fixed at $64\% \pm 2\%$. Except for occasional short periods during the work day the light in the insectary is constantly dim. Larvae and pupae are fed a 2.5% liver solution; emerging mosquitoes are

fed a 10% sugar solution. Mosquitoes emerge within 8-12 days from the time of egg seeding.

Egg laying cages remain in the insectary at all times. To control the age of the mosquitoes, those of each egg-laying cage are limited to 5-7 blood meals. All blood meals are provided by mice with a microscopically demonstrable parasitemia.

Test cages and cages intended for the biting of normal mice get their first infected blood meal in the insectary. They are then moved to a "cold room", kept at $68^{\circ} \pm 2^{\circ}$ F and 62% humidity. Eight days later they receive a second infected blood meal in the cold room. These mosquitoes remain in the cold room until they are used in a test or to bite mice used to maintain the bite-to-bite donor line of parasite.

Integration of the participants

Early in this investigation we reasoned that the successful development of the desired test hinged upon the quality of the mosquitoes. Furthermore, we concluded that mosquito quantity, uniformity and viability might very well depend upon the type of blood meal received by developing mosquitoes.

We found while developing the avian-Aedes aegypti system that the quantity, uniformity and viability of the mosquitoes could be greatly enhanced by providing egg laying mosquitoes parasite infected blood meals rather than the conventionally used uninfected blood meals (2). We, therefore, initiated a similar program of providing infected blood meals. These were first provided by mice used to maintain the parasite by blood passes.

Then prompted by evidence presented by Bafort regarding the role of the spleen we tried a new approach. Some mice used to maintain the parasite by blood passes were killed by exsanguination. Spleens from these were removed, minced, suspended in isotonic saline solution, adjusted to a spleen cell-saline suspension of 1:50. Intraperitoneal injection of 0.5 ml of this suspension produced infections that caused death of all mice within 5 days.

With this demonstration that a spleen cell suspension from mice with a blood induced infection was almost as effective as a blood induced infection from similarly infected mice, we reasoned that we might be able to carry this procedure one step further.

Rather than using spleen cells from mice with a blood induced infection we chose to harvest spleen cells from mice which 5 days earlier had been given a sporozoite induced infection. If this sporozoite induced for blood induced substitution was successful in producing high parasitemias and death by Day 5 in mice given infected spleen cells, we believed that it would be a major step forward. Not only would this provide heavily parasitized blood meals preferable for egg laying mosquitoes but it should progressively increase (with subsequent cycles) the responsiveness of new generations of mosquitoes to the NK65 parasite. Thus, this attempted substitution procedure was undertaken.

Initially it was found that spleen cells from mice with the sporozoite induced infection, if injected in the manner and dilution previously described, produced acceptable parasitemia levels and death of injected mice in 7-12 days after infection. Although the spleen cell suspension prepared from mice with this sporozoite induced infection initially

was not as effective as the blood induced infection in producing a rapid parasitemia and subsequent death it has now been developed to that point. This increase in virulence of the sporozoite induced infection was accomplished by first inducing the sporozoite infection, then monitoring the blood for gametocytes 4-6 days later. Each time gametocytes were seen, mice in which they were seen were killed, exsanguinated, and a spleen suspension prepared and injected into normal uninfected mice as previously described. This blood monitoring - exsanguinating - spleen cell passing cycle (called spleen passes) was repeated until all mice died on day 5 after receiving the spleen cell induced infection. In the beginning 3 or 4 spleen passes were required to cause death 5 days after infection. At the present time the virulence has been increased to the point that only a single spleen pass is required. Moreover, the extensive parasitemia developing after the single spleen pass can be controlled to provide selected blood meals on specific days to mosquitoes in the egg production cages.

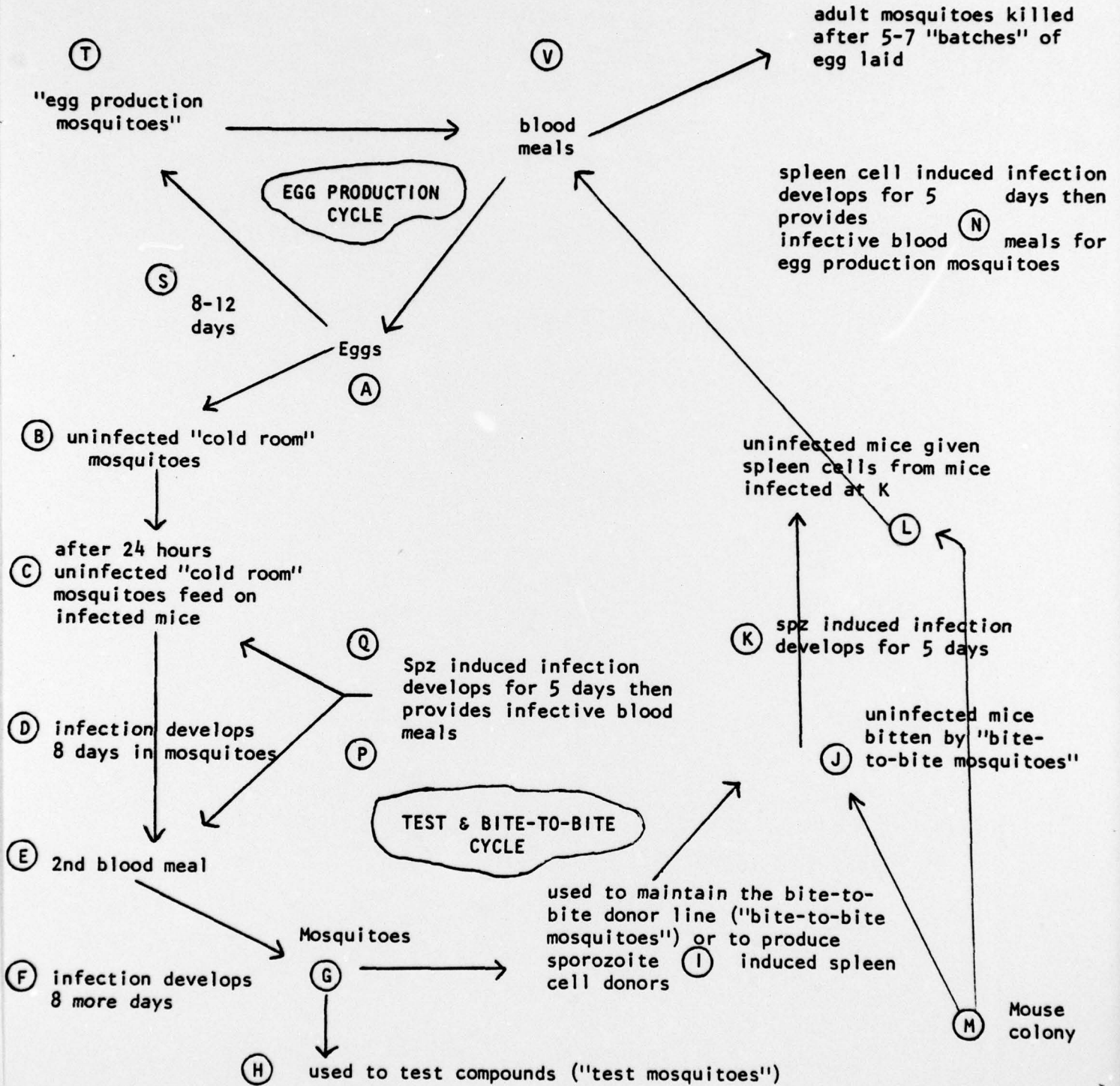
The laborious time consuming procedure described above has paid off handsomely in terms of healthy, viable mosquitoes that we believe are now very responsive to infections with the NK65 strain of parasite. The latter will be more fully discussed subsequently. Egg harvests are now as much as 50% greater than in the beginning. Larvae and pupae are conspicuously larger and appear to be stronger. Whereas in the beginning infected mosquitoes available for testing weighed 150-300 mg per cage, at this time we are doing tests with mosquitoes weighing as much as 1600 mgs per cage.

As a result of the increased and more uniform size we are now able to use a mechanical device to deliver equal numbers of larvae into rearing cages. Too, we are now able to separate males and females by employing slanted wire screens. Being able to employ these simple devices greatly facilitates the growing of mosquitoes in the massive numbers required for a high volume testing procedure.

Having described the method of raising a more viable NK65 strain responsive invertebrate vector it is appropriate to describe how a "bite-to-bite donor line" of NK65 parasites is being established. To simplify this explanation Figure 1 is provided. It should be pointed out that the EGG PRODUCTION CYCLE at the top of the figure (Cycles A, S, T, V) and the spleen pass leg (Points J, K, L, N, V) have been described in the previous paragraphs. The establishment of a bite-to-bite donor line may be more fully explained by referring to Points C, D, E, F, G, P, Q in the lower left portion of Figure 1. The figure depicts how development of the test system is presently progressing. Initially parasites at Points P or Q (Figure 1) were microscopically few or undetectable in the blood of mice infected by mosquitoes. It was known, however, that a parasitemia was likely because (1) sporozoites were consistently recognized in suspensions of infected mosquitoes (Point I) and (2) subinoculation of blood from mosquito infected mice (Point J) consistently produced a microscopically observable parasitemia in mice receiving the parasitized blood. After seven passages (Cycles C, D, E, F, G, I, P, Q) the virulence and/or numbers of the infecting organism has been

FIGURE 1

TEST SYSTEM DEVELOPMENT - RANE
(Mean Survival Time - Mouse - Spz)



greatly increased, perhaps in a manner as described by Coatney et al (4) for P. cynomolgi. Evidence for this is (1) a consistently pronounced parasitemia in mice with a mosquito induced infection as compared to no microscopically detectable blood parasite in earlier runs, (Points P and Q, Figure 1), (2) approximately 10% deaths in these same mice as opposed to no deaths in earlier runs, (3) a greatly shortened survival time in mice sub-inoculated with spleen cells (Point N, Figure 1) as the test developed, (4) greater numbers of sporozoites in mosquitoes infecting the mice (Point I) as development continues, (5) almost 100% deaths in approximately 10 days in mice which more recently have been given a suspension of test mosquitoes (Point H). Regarding item 5, early in test development 50 mg of mosquitoes suspended in saline given to each mouse produced no deaths; at the present this same dosage produces the near total mortality in test animals at 7 to 12 days.

Considerable effort was made in determining optimum "incubation times" for parasites developing in mosquitoes (Points C, D, E, F, G). We found that optimum results were obtained by inducing mosquito bite infections in mice 15 or 16 days after the mosquitoes had received their first infected blood meal (Point C, Figure 1); that a second blood meal after 8 days (Point E) was superior to a single blood meal and that the maximum gametocyte levels occurred on the 5th day after mosquito bite induced infection (See Points P and Q).

Tests have now also been accomplished to determine: (1) the value of single versus multiple infected blood meals and (2) the effectiveness of mosquito suspensions prepared in various media.

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