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3. AUTHOR(S)
Alger, N.E.; Branton, Marilyn; Harant, Joyce; Silverman, P.H.

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Plasmodium berghei NK65 in the inbred A/J mouse:
Variations in virulence of demes of the NK65 strain of P. berghei*

Nelda E. Alger, Marilyn Branton, Joyce Harant and Paul H. Silverman
Department of Zoology, University of Illinois
Urbana, Illinois 61801 U. S. A.

Running title: Variations in virulence

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SYNOPSIS

*Plasmodium berghei* NK65 was passed through *Anophelus stophonisi*, golden hamsters and mice. The percent cumulative mortality was compared after each mosquito passage in early (7-13) blood passages. The strain was found to have divided into demes (populations) retaining original virulence and demes which were less virulent. The separation of virulent and less virulent demes was traced to its origin. Both virulent and less virulent demes can be passed through mosquitoes and hamsters. No concomitant organism has been identified.
Variations in the virulence of different strains within a species of Plasmodium have been reported by many investigators. Although some factors contributing to the relative virulence of plasmodial strains for their hosts are known (15,16), elucidation of virulence is still incomplete.

Old laboratory strains of Plasmodium which have maintained a characteristic virulence for many years have been known to increase suddenly in virulence. Hartley (6) observed this in Plasmodium cynomolgi bastianelli in Rhesus monkeys and attributed the change to the decrease in time of blood transfer from 30 to 5 or 10 days. Coatney et al. (2) recorded an increase in virulence of P. cynomolgi for man after the original transfer from monkey to man. The increased virulence was then maintained by either blood or mosquito transfer.

Sergent and Poncet (17) also reported an increase in virulence of a strain of Plasmodium berghei in mice. The day of death decreased from 19 to 9 days with an enhanced parasitemia. Galli and Brambilla (3) reported similar findings in a different strain of P. berghei. Isfan (8), too, observed the variability in virulence among strains of P. berghei and also commented on the great variability within a single strain. His plasmodial studies included observations on several parasite strains in 5 strains of mice.

Carescia and Arcolea (1) noticed the loss of virulence in a blood passed line of P. berghei. Their strain of the parasite normally killed mice in 6-10 days, but after gradually losing virulence, killed in 15-20 days. Seventy-nine 3-day blood passages of the strain in mice restored the original virulence. The more virulent infection was characterized by the invasion of mature erythrocytes, while the less virulent infection was characterized by invasion of basophilic macrocytes.
Observations of differences in survival time in inbred mice and their hybrids infected with *P. berghei* have been made by several investigators (4, 5, 12).

Mehlitz and Schindler (11) concluded that the variations in virulence of *P. berghei* were attributable to, a. resistance in mice, b. virulence of the particular parasite strain, c. number of infecting parasites, d. diet, and e. the role of contamination with concomitant infections. The effect of various concomitant infections has been examined by several investigators (7, 9, 13, 14, 18).

In our laboratory in 1968, a serially blood passed deme (population) of NK65, termed NK65D, was found to be relatively avirulent when compared to the original NK65. Since NK65D had been derived in our laboratory from NK65 by mosquito passage, we undertook an examination of this and other NK65 mosquito-passed demes for the purpose of studying comparative virulence. Demes were traced backwards from our records at this time so that the alphabet does not appear sequentially in our terminology of demes. Comparison was based on cumulative death rate and percent recovery.

**MATERIALS AND METHODS**

General Procedures: The NK65 strain of *Plasmodium berghei* (19) was used in these experiments; no other gametocyte strains of *P. berghei* were maintained in our laboratories. Mosquito passages were made in *Anopheles stephensi* at 20-21°C and 80 ± 2% relative humidity. The larvae were maintained at 26 ± 1°C and fed on Kellog's concentrate, brewer's yeast and wheat germ 1:1:1. Adults were offered 10% dextrose on wicks of cotton dental roll between blood feedings.
Golden hamsters, *Mesocricetus auratus*, infected by blood passage, were offered to the adult female mosquitoes on the third and fourth days of infection. Mosquitoes were dissected in 1:1 serum-saline on the 16th day after a blood meal from an infected hamster. Sporozoites were harvested and injected intraperitoneally in 4-5 week old hamsters. Infection from these hamsters was passed into 3-4 week old CF₁ mice by intraperitoneal injection, then collected and stored in liquid nitrogen (-270 C) or at -70 C in a mechanical freezer, unless immediately used for experimental work.

The plasmodial deme derived from each mosquito passage was recorded by the addition of a letter or letters to NK65 (e.g., NK65E). Letters were not used in sequence and double letters were resorted to when the alphabet was exhausted. Each blood passage subsequent to a mosquito passage was recorded as a subscript number (e.g., NK65E₁₀). A history of the demes investigated is given in Fig. 1.

Inoculum: Inoculum of individual demes was prepared in mice for each experiment by 3 blood passages at 3 day intervals (e.g. blood passage on Monday, Thursday, Sunday and into experimental animals on Wednesday. Inoculum in separate experiments varied from 5 x 10⁵ to 1 x 10⁶ infected erythrocytes. The number of blood passages at the time of comparison of the demes varied from 7-13.

Experimental Protocol: All mice used for comparison of demes were 3-4 week old inbred A/J females randomized by weight and maintained under the same conditions of heat, light, diet and handling. Blood smears were made from the tail every second or third day and stained with giemsa. Percent parasitemia and day of death were recorded for each animal. Examination of slides for signs of concomitant bacterial infections was negative. Ten to 20 animals were used per group.
Splenectomy: Hemobartonella and Eperythrozoon coccoides have both been recorded (see Introduction) to affect the course of infection with P. berghei. Examination of blood smears normally taken at 2-4 day intervals on all animals over a 4 1/2 year period has never demonstrated the presence of either organism. During the past 3 1/2 years each deme has retained the virulence stated in the results under the conditions stated.

Twenty-four 5 week old CF₁ and 25 five week old A/J randomly selected mice were splenectomized. Daily blood smears were made beginning 24 hours after splenectomy, continued for 6 days after challenge and subsequently every other day. Eight animals were challenged on day three, after splenectomy, with \( 1 \times 10^6 \) NK65₁ and eight with \( 1 \times 10^6 \) NK65₁₀. Four intact mice of each breed were challenged with the same inoculum. No Hemobartonella or E. coccoides were found on any blood smear. The course of infection followed the usual course for each deme in intact animals.

Serum Transfer: Since it was thought possible that a virus might be affecting our results the following experiment was performed to transfer virus from the less virulent deme to the virulent deme. Serum from rats infected with NK65C was filtered through 0.45μ millipore filter and injected into mice. Each of 5 mice received 0.4 ml of serum followed by NK65E infected blood. Control animals received normal serum plus the same number of infected cells from the same inoculum.

RESULTS

Representative cumulative percent mortalities of some of the demes are compared in Figs. 2 and 3. In Fig. 1, all those demes of NK65 to the left
of the broken line (with the exception of EE) were found to have cumulative
death curves similar to those of D, II, BB or T as shown in Figs. 2 and 3.
Demes of NK65 to the right of the broken line in Fig. 1 showed death rates
similar to the original NK65 or to QQ, RR and A in Fig. 2. Demes of NK65
to the right of the broken line we have called virulent (100% mortality),
and to the left less virulent (some recoveries). In the less virulent
demes we have found from 0-20% recovery in any single experiment with 3-7
week old A/J mice (Table I). The virulent demes averaged 13% parasitemia
on day 6, while the less virulent lines still averaged less than 1% of the
red cells infected. Those animals which did recover ran parasitemias as
high as 70% for up to 40 days before recovery. After recovery very few
animals failed to show recrudescence and most animals have had several
recrudescences. Lines G and CC were lost during storage due to mechanical
failure of the freezer.

Sera transferred from animals infected with the NK65C deme into animals
inoculated with NK65E had no effect on the normal course of infection,
suggesting that we had not transferred a filterable virus.

DISCUSSION

Differences between cumulative percent mortality on days 12+ for demes
we called virulent and those we called less virulent are so clear as to leave
no doubt that there is a real dissimilarity.

The loss of virulence has been transmitted by both blood and mosquito
passage (Figs. 1, 2 and 3, Table I, and unpublished data), yet, at the same
time, the original virulence is maintained in other demes apparently handled
in the same way.
Peters (15) attempted to correlate drug sensitivity to virulence. If such a correlation does exist in our demes, it could not be due to drug pressure since no drugs have been used in these animals.

No concomitant bacterial infections were identified which could be responsible for a plasmodial suppression.

Concomitant viral infections cannot be ruled out as a cause of the loss of virulence. However, no viruses were observed in an extensive study of NK65D with the electron microscope (10), nor was any suppression found by our serum transfer experiment in which we attempted to transfer a filterable virus, if any were present and responsible for suppression.

Because all experiments were performed in mice from highly inbred strains of the same age and sex, variability in virulence cannot be ascribed to these factors. Diet and handling (light, temperature, stress) of all groups of mice were also identical.

At present the most reasonable explanations for this loss of virulence appear to be: (1) an unidentified virus which is carried through mosquito, hamster, and mouse; (2) a genetic recombination in the mosquito at the 89 to T passage (BB9 to EE); (3) a mutation; or, (4) population selection. Proof of the absence of a virus is almost impossible, hence, this explanation cannot be ruled out. If the virulence changes are due to a virus, then it is possible that only part of the parasite population is infected with the virus and that the virus can be picked up by the malarial parasite as it passes through virus-infected mosquitoes or mammals. The virus might be lost from a population by injection of very low numbers of virus-free infective sporozoites so that the recipient animal (hamster) may occasionally receive no virus-infected parasites.
Since the change in virulence appeared after mosquito passage in which very few sporozoites were used, either a genetic recombination during the sexual cycle in the mosquito, a mutation, or a population selection at this point in the cycle appears reasonable. The number of infective sporozoites from any inoculum of whatever size is always questionable. If only a few sporozoites survive, then the genetic possibilities are more limited. If sporozoites from only one oocyst were to infect the hamster, the result would be a clonal population. The reversal to normal virulence at BBg-EE argues against the suggestion of a clonal population. Since the number of sporozoites at the Bg-T2 passage was small, the possibility of the selection of a population, predominantly less virulent exists. One might then expect that in a mixed population the more virulent, faster growing deme would overgrow the less virulent, slow growing deme after only a few blood passages. This has not happened after 40 transfers of NK65C, however.

The selective pressure of 3 abnormal hosts might be expected to induce changes in the parasite population. It seems hardly likely, however, that a reversal of virulence could take place if this pressure were the cause of the original change in virulence.

None of these explanations seems entirely satisfactory at the present time.

ACKNOWLEDGMENT

The original stock of Anopheles stephensi was furnished by Dr. H. F. Schoof, Biology/Chemistry Section, National Communicable Disease Center, U. S. Public Health Service, P. O. Box 769, Savannah, Georgia.
REFERENCES


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<th>Number of Inoculated Parasitized Cells</th>
<th>Number and Age of Female A/J Mice</th>
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<th>Percent Recovery</th>
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<tr>
<td>Less Virulent Demes</td>
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<td>Percent Recovery</td>
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<td>H10</td>
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<td>8 7 wk</td>
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* This deme was returned to us by another laboratory in which records of blood passages were not kept. All other demes were kept in our own laboratory.
Fig. 1

History of *Plasmodium berghei* NK65 in our laboratory

NK65 (Received from Dr. Meir Yoeli)

An arrow represents a mosquito passage in *Anopheles stephensi*. The subscript number represents the number of blood passage at the time of mosquito infection.

** Lost in storage due to mechanical failure.

Letters were not used in alphabetical sequence.

* An arrow represents a mosquito passage in *Anopheles stephensi*. The subscript number represents the number of blood passage at the time of mosquito infection.
Fig. 2. Comparison of cumulative death rates of 3 week old A/J female mice infected with different demes (A,F,D,Q,Q,RR) of *Plasmodium berghei*. Twenty mice were used for each group except RR where 15 mice were used. Infective inoculum was $5 \times 10^5$ parasitized cells.
Fig. 3. Comparison of the cumulative death rates of 3 week old A/J female mice infected with different demes (II, BB, T, EE, H) of Plasmodium berghei NK65. 15 mice were used for groups II and BB and were inoculated with $1 \times 10^6$ parasitized cells. Eight mice were used for groups T, EE and H and were inoculated with $5 \times 10^5$ parasitized cells.