Archiv für die gesamte Virusforschung 40, 375—381 (1973)

The Persistence of Aleutian Disease Virus in the Mosquito Aedes fitchii

By

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With 1 Figure

Received July 14, 1972

Summary

Observations showed that the mosquito, *Aedes fitchii* (Felt and Young), readily feeds on mink. Injection of homogenized mosquitoes, held for various periods after an infectious blood meal, into susceptible mink revealed that this mosquito retained Aleutian disease (AD) virus for 35 days. The virus in ground *A. fitchii* was titrated immediately and 15 days after infective feeding. The titer decreased from $10^4 ID_{50}$/ml to $10^2 ID_{50}$/ml during this time. Conversely, AD virus did not persist for six days in *Aedes aegypti* (Linnaeus). Homogenized pools of heads combined with salivary glands of *A. fitchii* 5, 10, and 15 days after infective feeding were injected into susceptible mink, viral infectivity was demonstrated in this region at 10 and 15 days after infective feeding but not at 5 days. This study suggests that replication of AD virus may occur in *A. fitchii* and a true vector-pathogen relationship exists between AD virus and certain arthropods.

1. Introduction

Aleutian disease (AD) is a slow viral disease which causes the death of mink homozygous recessive for the Aleutian gene α. The course of the disease may be as short as two months in Aleutian genotypes but as long as several years in non-Aleutian genotypes ($A\alpha$ or AA). The etiological agent is considered to be a virus (1—6) and in crude suspensions it is highly resistant to chemical and physical treatments (7, 8). The significant features of the disease are plasmacytosis, hypergammaglobulinemia, vasculitis, and glomerulitis with formation of immune complexes (9—16). Viremia occurs about one week after infection and persists in all Aleutian mink and some non-Aleutian mink until death (8, 17).

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While vertical (mother to offspring) and horizontal (between families) transmitting mechanisms have been documented (18, 19), there have been cases in which the source of epizootics was unknown. Because cases can occur in other than obvious contiguous contacts, and because of the high prevalence of flying blood sucking insects frequenting many mink farms, arthropod transmission has been considered.

Observations on a ranch in eastern Washington showed that *Aedes fitchii* (Felt and Young) is highly attracted to and will readily feed on mink. In the current study we have investigated the behavior of AD virus in this mosquito.

2. Materials and Methods

2.1. Mink

Aleutian mink (the most responsive and sensitive mutation to AD virus) were used. The mink were obtained from an AD-free ranch. Frequent examinations revealed that mink from this source were free from AD and other mink diseases. Prior to the trials, serum gamma globulin levels were established using cellulose acetate electrophoresis and the mink were isolated for one month. The serum gamma globulin levels were then determined again to detect any increase during the isolation period. Only those mink with a gamma globulin level below 14% of total serum proteins were employed in the experiments. The mink were housed individually in cages with a solid metal barrier between cages to avoid direct contact. Special care was taken when feeding, watering, and cleaning to prevent cross infection. The mink were 8—12 months old when used.

2.2. Virus and Donors

To provide virus donors, four mink were inoculated with the 15th passage of the Pullman isolate of the AD virus. This isolate was obtained in 1961 and maintained since that time by periodic transfer in mink and storage at —60° C. The titer of the agent was 10^5 ID_{50} per ml in Aleutian mink.

2.3. Vectors

Since *Aedes fitchii* cannot be reared in the laboratory, adult females were collected by aspiration in the field as they attempted to feed on collectors. Collections were made in areas where mink were not raised. Mosquitoes were placed in gallon ice-cream cartons with moist tissue paper and sucrose solution for transport to the laboratory (20). Upon arrival, approximately 600—800 mosquitoes were transferred into a single mosquito cage (13 × 19 × 13 inches) and maintained in a constant temperature chamber at 20° C with a relative humidity of 75—80% and a 16-hours daily photoperiod. A 10% sucrose solution was employed as maintenance food. These conditions were standard throughout the experiments. For purposes of comparison, similar conditions were employed with *Aedes aegypti* (Linnaeus) except that the colony was laboratory reared and maintained at 28° C under constant light. In one experiment, the maintenance temperature for *A. aegypti* was lowered to 20° C after infective feeding to determine if temperature affected the persistence of AD virus.

2.4. Infective Feeding of Mosquitoes

About 12 hours before blood feeding, the sucrose solution was removed. An AD-infected mink was restrained by first placing it in a wire mesh tube. Then the tube with the mink was placed into a mosquito cage containing 600—800 mosquitoes. Within 8 hours or when about 95% of the mosquitoes were engorged, the mink was removed. Mosquitoes, which had not fed, were removed by an aspirator and the sucrose solution was replaced.

2.5. Persistence of AD Virus in Mosquitoes

After the mosquitoes had fed on infective mink, groups of 20 mosquitoes were removed from the large pen at varying time periods and stored at −60° C in a mechanical deep freezer. The virus was allowed to incubate in the mosquitoes for the following periods: for *A. fitchii*, Experiment I: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 23 days; Experiment II: 0, 5, 10, 15, 20, 25, and 30 days; Experiment III: 30 and 35 days; for *A. aegypti*, 0, 6, and 10 days. Then each incubated group was homogenized in 5 ml of Hanks's solution containing 200 units/ml of penicillin and 250 μg/ml of streptomycin and was injected intraperitoneally into two susceptible mink in equal amounts (2.5 ml each). Mosquitoes not fed on mink were used as controls.

2.6. Titration of AD Virus in *A. fitchii* at Initial Infective Feeding and 15 Days After

Infective feeding and homogenization of mosquitoes were completed in the same manner as described above. The homogenate was centrifuged for 15 minutes at 1000 r.p.m. The supernatant was considered as a 10° suspension, then 10-fold serial dilutions were prepared in Hanks's solution in 4.5 ml amounts. Each dilution was injected intraperitoneally into two susceptible mink in equal amounts (2.5 ml each).

2.7. Determining the Presence of AD Virus in the Salivary Glands, the Midgut and the Ovaries of Infected *A. fitchii*

After mosquitoes were fed on infected mink, groups of 30 adults were dissected following 5-, 10-, and 15-day holding periods. The head with the salivary glands (without the proboscis), the midgut, and the ovaries were removed. Each organ group was collected in a common container, washed three times with Hanks's solution containing antibiotics, and stored at −60° C. Then each organ group was homogenized in 5 ml of Hanks's solution and injected intraperitoneally into two susceptible mink in equal amounts (2.5 ml each).

2.8. Criteria of AD Infection in Mink

In all instances, infection was determined by gross examination of the mink and serum gamma globulin levels. In doubtful instances, it was determined by the morphologic evaluation of paraffin-embedded hematoxylin- and eosin-stained sections of liver, kidney, spleen and prescapular lymph nodes. The criteria of disease were lesions of AD as described previously (21), coupled with gamma globulin levels elevated to 20% or more of the total serum proteins.

3. Results

Figure 1 shows that AD virus persisted in *A. fitchii* up to 35 days after infective feeding. AD infections occurred in 38 of 42 mink inoculated with mosquitoes held from 0 to 35 days after infective feeding. The four mink that did not become infected included one mink in each group inoculated with mosquitoes held for 6, 16, 23, and 30 days. Serum gamma globulin began to rise in most mink between 3—5 weeks postinoculation (p.i.) and hypergammaglobulinemia was clearly demonstrated by 7 weeks p.i. No infections occurred in control mink inoculated with *A. fitchii* which had not fed on infected mink. All mink inoculated with *A. fitchii*, frozen immediately after feeding on infected mink, developed AD.

Conversely, AD virus did not persist in *A. aegypti* (Table 1). Infections only occurred in mink inoculated with A. *aegypti* immediately after infective feeding (0 day). Lowering the incubation temperature of the mosquito from 28° to 20° C did not affect the persistence of the virus.

Table 2 shows the results of titrating AD virus in whole ground *A. fitchii* immediately after feeding on AD-infected mink and again after a 15-day holding period. The titer was 10^4 ID_{50}/ml immediately after the infective blood meal and was 10^2 ID_{50}/ml when the mosquitoes were held for 15 days. Thus, the virus titer decreased 100-fold during the 15-day holding period.

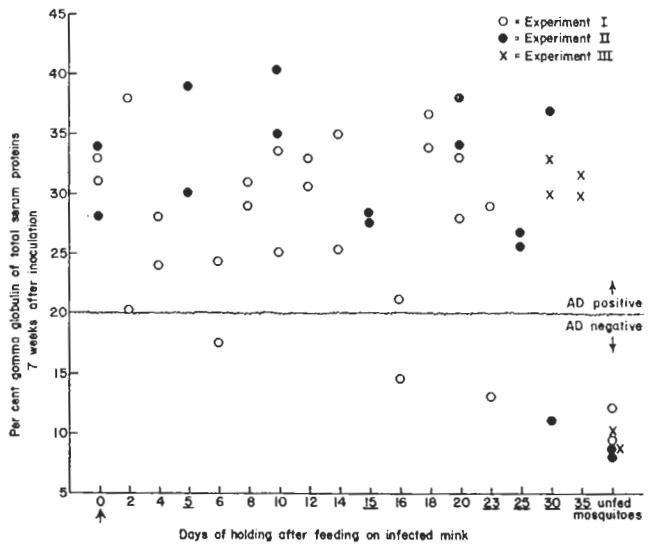

Fig. 1. The persistence of Aleutian disease (AD) virus in *Aedes fitchii*. Virus was detected by injecting homogenized mosquitoes into susceptible mink following various holding periods after feeding on AD infected mink. Twenty mosquitoes were injected into two mink in equal amounts. Infection was indicated by hypergammaglobulinemia; gamma globulin levels elevated to 20% or more of the total serum proteins. The gamma globulin level of all mink was less than 14% of total serum proteins prior to injection. Each dot or cross represents one mink

Table 1. *Differential Survival of Aleutian Disease Virus in Aedes fitchii and Aedes aegypti*[a]

Day after infective feeding	*A. fitchii*	*A. aegypti*	
	Incubation temperature		
	20° C	28° C	20° C
0	4/4[b]	4/4	2/2
6	4/4	0/2	0/2
10	4/4	0/2	0/2

[a] Virus was detected by injecting ground mosquitoes into susceptible mink. Twenty mosquitoes were injected into two mink in equal amounts.

[b] Number of mink affected with Aleutian disease / number of mink inoculated.

Table 3 contains data obtained by injecting pools of homogenized head and salivary glands, midgut, and ovaries, which were dissected from *A. fitchii* 5, 10, and 15 days after infective feeding. AD virus was not detected in the head and salivary glands 5 days after infective feeding, but was demonstrated at 10 and

15 days. The virus was present in the midgut 5 days after infective feeding, but was not found at 10 and 15 days. No AD virus was detected in any of the pools of ovaries.

Table 2. *Titration of Aleutian Disease Virus in Aedes fitchii Immediately after and 15 Days after Infective Feeding*[a]

Day after infective feeding	Dilutions inoculated								
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0	2/2[b]	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2
15	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2

[a] Virus was detected by injecting each dilution into two susceptible mink in equal amounts. Thirty mosquitoes were used for each titration.

[b] Number of mink affected with Aleutian disease/number of mink inoculated; based on hypergammaglobulinemia two months after inoculation.

Table 3. *Detecting Aleutian Disease Virus in the Head and Salivary Glands, the Midgut, and the Ovaries of Aedes fitchii Following Various Holding Periods after Feeding on Infected Mink*[a]

Organ pool	Days after feeding on infected mink	No. of mink affected / No. of mink inoculated
Head and salivary glands	5	0/2
	10	2/2
	15	1/2
Midgut	5	2/2
	10	0/2
	15	0/2
Ovaries	5	0/2
	10	0/2

[a] Virus was detected by injecting pools of structures into susceptible mink. Each pool consisted of structures dissected from thirty mosquitoes and was injected into two susceptible mink in equal amounts.

4. Discussion

Aedes fitchii (22) is widely distributed in the northern U.S. and southern Canada and has as single generation a year. Eggs are laid at the edge of receding grassy pools of water in wooded areas where the larvae develop in the spring. The adults are found at the edge of forest areas surrounding mountain meadows. In the mink ranch studied, the adults reached a seasonal population peak in early June, which correlated well with the time when newborn mink usually venture from their nest boxes and are exposed to mosquito attack. However, the lack of an accurate correlation between a high mosquito population density and its prevalence of AD may be due to the prolonged incubation period and chronicity of the disease.

Since infected Aleutian mink exhibit a minimum AD virus titer of 10^4 ID_{50}/ml in the serum which persists until death (17), the demonstration of a consistent titer in *A. fitchii* immediately after infective feeding indicates that infected mink

can serve as an infectious source during blood feeding. The longevity of *A. fitchii* adult females in nature is not known, but since they have been maintained for at least 35 days in our laboratory, the species probably has an adult life span sufficient for transmission. The frequency of blood feeding in nature is also unknown, but we observed only a small percentage of refeeding. However, this does not imply that this mosquito only requires one blood meal in nature. It is a common phenomenon that when insects are brought into the laboratory from the field, they may show a behavior change due to the artificial environment. The long persistence of AD virus in one species of mosquito, but not in another strongly suggests that a true vector-virus relationship exists in this system.

In general, the persistence of virus in mosquitoes may be explained by highly efficient stabilization of virus by the insect tissues or by a continuous replication equal to, or slightly exceeding virus die-off rate (23). Therefore, the possibility of AD virus replication in *A. fitchii* cannot be eliminated, even though the titer of the virus in this mosquito decreased 100-fold during the first 15 days of the holding period. If the decrease in titer in 15 days represented the die-off rate of the virus in this mosquito and if this event occurred uniformly, the mosquitoes should have little or no detectable virus by the end of a 35-day holding period. On the other hand, our data revealed that the virus persisted for at least 35 days.

The possibility of *A. fitchii* serving as a vector of the AD agent in nature is speculative because we are unable to achieve transmission by refeeding or interrupted feeding. However, the long persistence of the agent in *A. fitchii* but not in *A. aegypti* and its apparent migration from the midgut to the head and salivary glands is reminiscent of other viruses which replicate in mosquitoes and are transmitted by feeding on subsequent susceptible hosts. That there might have been higher titers demonstrable after a more prolonged incubation period, *i.e.*, beyond the 15 day holding period is a possibility. Aleutian disease virus, being one of the slow viruses, may have unique characteristics and might not replicate as rapidly in mosquitoes as the more conventional arthropod-borne viruses.

The lack of an *in vitro* system for detecting the agent, the long incubation period of AD in mink, and the inability to establish a laboratory colony of *A. fitchii* have made extensive studies difficult and time consuming. However, further studies are underway, especially to ascertain whether AD virus replicates in this mosquito.

Acknowledgements

This investigation was supported in part by a National Institute of Health, General Medical Science predoctoral fellowship (No. 5-F01-GM-40171-02) and in part by NIH Grants AI06477, AI06591, and RR00515.

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Printed in Austria