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AN INCREASE IN DEATH RATE OF *ANOPHELES STEPHENSI* FED ON RABBITS IMMUNIZED WITH MOSQUITO ANTIGEN *

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Trager (1939) presented the first experimental evidence on the development of acquired resistance to a blood sucking arthropod. In his work based on the studies of the American dog tick, Dermacentor variabilis Say, he found that guinea pigs and rabbits which were infected once with larval ticks showed an increased degree of resistance to the successful feeding of the ticks. The mechanism of enhanced resistance was suggested to be the blocking of the blood flow to the sites of engorgement of subsequent batches of larvae. In another set of experiments, Trager (1939) was able to induce resistance against the ticks by guinea pigs, which were injected intracutaneously with larval extracts from the same species of ticks.

On the other hand, Brumpt and Chabaud (1947) and Chabaud (1950) after investigating several host-tick systems, concluded that only some of the individual hosts acquired resistance.

Riek (1962) has shown a great spectrum of variability among cattle in regard to the response against the tick Boophilus microplus. He also described a state of hypersensitivity in some of these vertebrates.

More recently, Roberts (1968) has shown that there is an acquired resistance in cattle against the tick Boophilus microplus. He concluded that the resistance exhibited is the result of an immune response by the host.

The experiments reported in this paper are a study of the reaction against the mosquito Anopheles stephensi, one of the insect vectors of malarial parasites. No previous work has been reported on this relationship.

MATERIALS AND METHODS

Ten 3-4 month old New Zealand rabbits maintained at about 26°C and fed on Purina rabbit chow ad libitum were used. Anopheles stephensi were maintained at

26 ± 1°C and at 81-82% relative humidity. The larvae were maintained at 26 ± 1°C in 30 x 25 x 6.5 cm. enamel pans were fed with Kellogg's Concentrate, yeast and wheat germ (1:1:1:). Larvae of the mosquitoes used for feedings 1,2, and 3 were kept in crowded conditions (about 300 per pan) while the larvae of the adults used for all other feedings were kept only 100-150 per pan.

Antigens

Three different types of antigen were prepared from A. stephensi.

1. Supernatant Whole Mosquito Fraction: (SWM) The first of these antigens was prepared by grinding female mosquitoes (approximately 25) in a tissue grinder which was kept in an ice bath during the procedure. The mosquitoes were ground in 1 ml of 0.85% NaCl. After 10 minutes of slow grinding, the mixture was transferred to a conical 15 ml centrifuge tube and centrifuged at 1063 g for 10 minutes. The supernatant was carefully transferred to a 5 ml screw capped vial with a disposable Pasteur pipette.

2. Sedimented Whole Mosquito Fraction: (IWM) The insoluble pellet packed in the bottom of the conical centrifuge tube from the above procedure was resuspended in 1 ml of 0.85% NaCl to become the sedimented antigen fraction. This pellet was not washed.

3. Mid-gut Antigen: (S) The third antigen was obtained by dissecting the midgut of approximately 100 female mosquitoes and grinding them in a tissue grinder with 1 ml of 0.85% NaCl. The tissue grinder was kept in an ice bath during this procedure. After grinding was finished, the mixture was transferred to a 5 ml. screw cap vial with a disposable Pasteur pipette. Most of the dissected mid-guts also included pieces of Malpighian tubules and some parts of the fore and hind guts.

The mosquitoes used in all preparations of antigens were approximately the same age (7 to 10 days from emergence). Only 10% dextrose was supplied as

nutrient for the period from the day of emergence to the day of antigen preparation.

Antigen Standardization

In order to standardize the antigens for the different injections, the amount of nitrogen in the final antigenic mixture was determined by the use of the Micro Kjeldahl method as described by Lang (1958). Each of the injections made, regardless of the type of material injected, contained from 2.00 to 3.42 mg of protein as calculated from the amount of nitrogen in the samples.

Injections

First injections were given with Freunds complete adjuvant (1:1) in the foot pads and muscles. Injections 2-7 were given with Freunds incomplete adjuvant (1:1) in rabbits 3 and 4. No adjuvant was given in subsequent injections of other rabbits. Injections were given at 7-17 day intervals and mosquitoes were fed on the rabbits 7-19 days post-injection depending on the availability of mosquitoes.

Bleeding

Prior to every injection, but on the same day, each rabbit was bled from the marginal vein of the ear. Sera were stored at -96°C in 2 ml aliquots until needed.

Feeding of Mosquitoes

Jars containing 200-300 pupae collected on a single day were placed in 15 x 15 x 15 cm. wire cages supplied with 10% dextrose. The number of cages used for each experiment depended upon the number of available pupae and on the number of rabbits to be used. Six to 9 days after the pupae were placed in cages, the dextrose was removed, and 24 hours later, the mosquitoes were allowed to feed on the rabbit's ears.

After feeding, the mosquitoes were transferred to a plastic cage designed

by Alger (1968).The transfer was accomplished by introducing a small wire cage into a large plastic one and opening the small cage, allowing most of the mosquitoes to fly out. The ones that did not do so were gently blown out so that they were not unnecessarily damaged. The unfed females were then removed and the dextrose replaced.

If the mosquitoes did not feed immediately, the rabbit's ears were moistened with a 1:60 dilution of armpit sweat in distilled water as proposed by Brown, et. al. (1951).In 7 out of 8 feedings reported here, 95-100% of the female mosquitoes had engorged within 40 minutes. In the only poor feeding (Feeding 2), the rabbits were exposed to the mosquitoes for 4 hours and, even after this period of time, the mosquitoes had not engorged normally.

Counts of the dead female mosquitoes were made every day for at least 3 weeks. At the end of the counting period, the remaining females were counted and added to the number of dead ones to obtain the total.

The general information concerning all the feedings done is summarized in Table I.

Calculations

After counts were tabulated, the cumulative per cent of dead mosquitoes was computed for each day. A smooth curve was obtained by calculating the "smoothed value" of each day using the method of Roberts (1968). The smoothed daily value was defined by Roberts as the per cent of mosquitoes dead up to a particular day, plus the per cent of dead the day before and the day after, plus 1/2 the per cent of the dead for the day two days before and for the day two days after, divided by 4.

Statistical tests (Steel, 1960) were applied using as a parameter the difference in the cumulative death rate between the experimental and the respective control animals. This difference was compared with the difference between an injected

control and an uninjected control and with the difference between 2 uninjected animals. Analysis of variance at the 2.5% level of significance was used in order to examine the data. When a significant difference was found by the use of this test, Duncan's new multiple range test was used to locate the source of this difference.

Double Diffusion Test

A 1% Oxoid Agar-Agar 3* solution in physiological saline was heated until the agar dissolved. To this solution, 0.1% sodium azide was added as a bactericidal agent. 3 x 2 inch glass slides were cleaned in 95% alcohol and placed on a horizontal surface. Hot, freshly-melted agar solution was poured on the slide to a depth of approximately 3 mm. The agar was hardened at room temperature and the prepared slides were stored at 4°C in petri dishes which had a moist piece of tissue paper in the bottom.

A hexagonal pattern of wells 8 mm in diameter and approximately 12 mm from the center of the center well was drilled in the agar by using a number 4 cork borer and a plexiglass pattern.

The antigen was usually placed in the well drilled in the center of the hexagonal pattern and antisera were placed in the surrounding 6 wells.

The plates were incubated at 4°C and examined in a viewbox every 24 hours for 5 to 7 days (Ouchterlony, O. 1949; Oudin, J. 1946).

Micro Complerent Fixation Test

The serum collected prior to each feeding was titrated by using the micro complement fixation test suggested by Levine (1967).

RESULTS

Figures 1-8 represent the smoothed curves of the different feeding experiments. The results were examined statistically as described under methods. Differences in death rates between mosquitoes fed on rabbits injected with mid-gut and

mosquitoes fed on control rabbits were found to be significant in feedings 1,3,4,5, and 7. Differences in death rates between the mosquitoes fed on rabbits injected with the supernatant whole mosquito antigen and those fed on control rabbits were significant only in feeding 1. In feeding 6 (Fig. 6) there is a significant difference between mosquitoes fed on the control animal and those fed on the experimental animal injected with sedimented whole mosquito antigen, (IWM) but this difference is in the opposite direction from the ones mentioned above, i.e. the mosquitoes which fed on the rabbit injected with IWM had a lower death rate than those which fed on the control animal. This same feeding gave no significant difference between death rates for mosquitoes fed on the mid-gut injected rabbit and those fed on control rabbit. In feeding 2 (Fig. 2), there was no significant difference between death rates of mosquitoes fed on any of the rabbits used.

Feeding 8 (Fig. 8), showed no significant difference between any of the rabbits used. The purpose of feeding 8 was to determine the extent of variation between death rates of mosquitoes fed on normal rabbits (uninjected controls) and those fed on injected controls. There was no significant difference in death rates between mosquitoes fed on any of the rabbits used in this feeding.

Results from micro complement fixation tests were positive but the tests had no relation to the death rates.

Serum from control rabbits gave no precipitin lines with any antigen in double diffusion tests. Whole mid-gut antigen gives at least four precipitin lines with its homologus anti serum (R7) while giving only 3 lines with SWM antiserum and 1 with IWM antiserum. Most of the lines are rather diffuse and suggest that lines have precipitated so close together that we are not distinguishing them. Intersections of bands between wells are, for the most part, not clear.

Extract of whole Musca domestica and gut antigen were also examined. There

was no reaction between the rabbit anti S serum (R7) and the gut of the fly nor did this same antiserum react with extract of whole fly. Anti SWM (R9) did react with extract of fly body, however, demonstrating either a cross reaction or a common antigen.

DISCUSSION

Significant differences between death rates of groups of mosquitoes (Feedings 1,3,4,5 and 7) fed on rabbits injected with mid-gut and groups fed on controls or other antigens indicate that these injections have induced some difference in the blood meal. In the only feeding (2) that showed no differences between groups of mosquitoes, the mosquitoes had not fed normally even after four hours. No significant difference in death rate was found between mosquitoes fed on any injected or uninjected control animal (Feeding 8).

Under conditions used in our laboratory, crowding of larvae results in slower development and weaker adult mosquitoes with a shorter life span. Thus, in feedings 1,2 and 3 (Fig 1,2 and 3) the results reflect the shorter life span of the insects as well as greater differences in death rates between mosquitoes fed on experimental and control rabbits.

Serological tests indicate that there is a good response to the injected antigens. It is not yet clear which, if any, of these antigens is related to the death of the mosquito. However, if several antigens are injected into an animal at one time (as whole mosquito) each antigen may interfere with the rabbit's response to the other antigen even though the individual antigen is a good antigen if used alone.

The increased mortality rate of the mosquitoes feeding on animals immunized with mid-gut antigen (S) (Feeding 1,3,4,5,7) is probably related to antibodies produced by rabbits. The specific antigens in the mosquito's gut which elicit the production of these antibodies is not known at the present time.

The mode of action of the antibodies could be one or a combination of the following: (1) mechanical damage of cells in the mosquito digestive tract; (2) the inhibition of a protease or other enzyme by its specific antibody; (3) mechanical blockage by binding together of microvilli by antibody, thus reducing considerbly the absorption surface of the mosquito's gut or (4) death of the bacterial flora of the gut. The use of purified or partially purified antigen (2) may give a better response in the animal resulting in even higher death rates in the mosquitoes if the antibody formed is active against mosquito tissue (mid-gut).

SUMMARY

Five 3-4 month old New Zealand rabbits were injected with Freund's complete adjuvant and (1) supernatant of whole ground mosquitoes, (2) unwashed pellet from whole ground mosquitoes (3) ground mosquito mid-gut. Successive injections were given at 7-15 day intervals. Mosquitoes were fed on the above rabbits on normal control rabbits and on adjuvant control rabbits. Death rates of mosquitoes which fed on rabbits injected with mid-gut antigen was higher than the death rate of mosquitoes which fed on the control rabbits or those injected with whole mosquito antigens. Suggested mechanisms to explain this phenomenon are presented. Serological data cannot be quantitatively correlated with the death rates as yet.

REFERENCES CITED

Alger, N. E. (1968). Individually humidified closed plastic cages for rearing Anopheline mosquitoes. Mosquito News, 28, 121.

Brown, A. W. A., Sarkara, D. S. and Thomson, R. P. (1951). Studies on the responses of the female Aedes mosquito. Part I. The search for attractant vapours, Bull. Ent. Res., 42, 105.

Brumpt, E. and Chaubaud, A. G. (1947). L'infestation par des ixodinés provoque-t-elle une immunité chez l'hôte? I. Note preliminaire. Ann. Parasit., 22, 348.

Chabaud, A. G. (1950). L'infestation par des ixodinés provoque-t-elle une immunité chez l'hôte? (2 une note). Ann. Parasit. 25, 474.

Lang, C. A. (1958). Simple micro-determination of Kjeldahl N_2 in biological materials. Anal. Chem., 30, 1962.

Levine, L. (1967). In, Methods in Immunology, Chapt. 20, ed. by D. M. Weir, F. A. Davis Co., Philadelphia.

Ouchterlony, O. (1949). Antigen-antibody reactions in gel. Arch. Kemi. Mineral Geol., B26, 1.

Oudin, J. (1946). Methode d'analyse immunochemique par precipitation specifique en milieu gelifie. Comp. Rend. Acad. Sci., 222, 115.

Riek, R. F. (1962). Studies in the reaction of animals to infestations with ticks. VI. Resistance of cattle to infestation with the tick Boophilus microplus (Canestrini). Austral. J. Agri. Res., 13, 532.

Roberts, J. A. (1968). Acquisition by the host of resistance to cattle tick Boophilus microplus (Canestrini). J. Parasit., 54, 657.

Roberts, J.A. (1968). Resistance of cattle to the tick Boophilus microplus (Canestrini). I. Development of ticks on Bos taurus. J. Parasit., 54. 663.

Steel, R.G.D. and Torrie, J.H. (1960). Principles and Procedures of Statistics McGraw-Hill, New York.

Trager, W. (1939). Acquired immunity to ticks. J. Parasit., 25, 57.

Trager, W. (1939). Further observations on acquired immunity to the tick Dermacentor variablis Say. J. Parasit., 25, 137.

Table I

MOSQUITO FEEDING EXPERIMENT DATA

FEEDING NUMBER	RABBITS ON WHICH MOSQUITOES FED	NUMBER OF INJECTIONS BEFORE FEEDING	NUMBER OF FEMALE MOSQUITOES FED	DAY ON WHICH FEEDING TOOK PLACE*
1	R-2 (No Injection)	0	100	65
	R-3 (SWM)	6	80	
	R-4 (St)	6	80	
2	R-2 (No Injection)	0	165	75
	R-3 (SWM)	7	143	
	R-4 (St)	7	163	
3	R-2 (No Injection)	0	136	85
	R-3 (SWM)	7	133	
	R-4 (St)	7	136	
4	R-7 (St)	3	55	42
	R-9 (SWM)	3	60	
	R-11 (Sed. WM)	3	58	
	R-12 (Injected Control)	3	57	
5	R-7 (St)	5	60	63
	R-12 (Injected Control)	5	60	
6	R-7 (St)	6	135	78
	R-9 (SWM)	6	107	
	R-11 (Sed. WM)	6	104	
	R-12 (Injected Control)	6	111	
7	R-7 (St)	8	165	94
	R-9 (SWM)	8	44	
	R-12 (Injected Control)	8	143	
8	R-12 (Injected Control)	8	169	108
	R-14 (No Injection)	0	197	
	R-15 (No Injection)	0	143	
	R-16 (No Injection)	0	184	

* Day 1 = Day Injection One was Given

Fig. 1 R= Rabbit

SWM= Supernatant whole mosquito

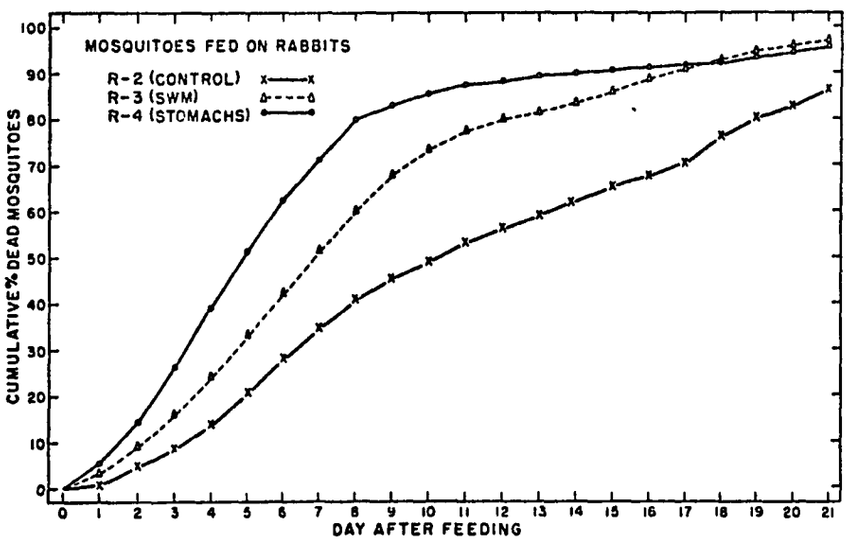

Fig. I

Feeding I

Fig. 2 R=Rabbit

SWM= Supernatant whole mosquito

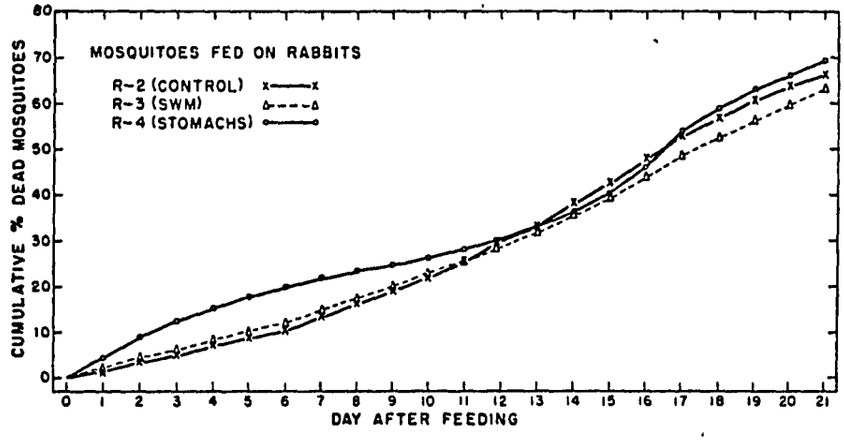

Fig. 2

Feeding 2

Fig. 3 R= Rabbit

SWM= Supernatant whole mosquito

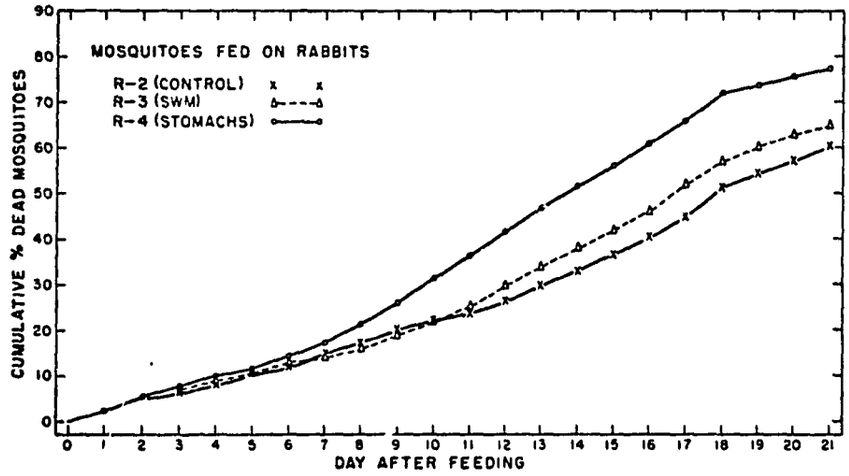

Fig. 3

Feeding' 3

Fig. 4 R= Rabbit

SWM= Supernatant whole mosquito

IWM= Insoluable pellet whole mosquito

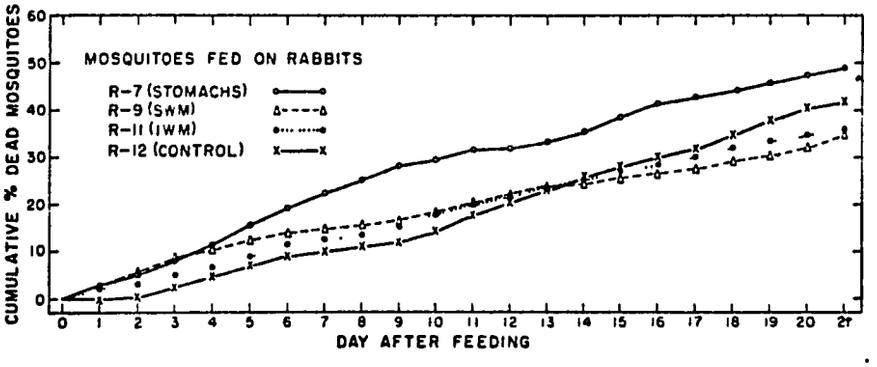

Fig. 4

Feeding 4

Fig. 5 R= Rabbit

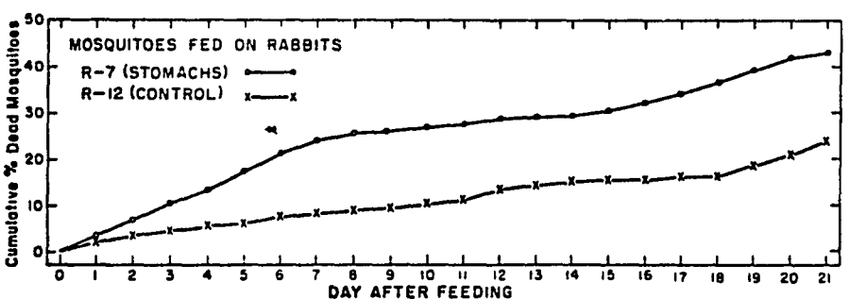

Fig. 5

Feeding 5

Fig. 6 R= Rabbit

SWM= Supernatant whole mosquito

IWM= Insoluable pellet whole mosquito

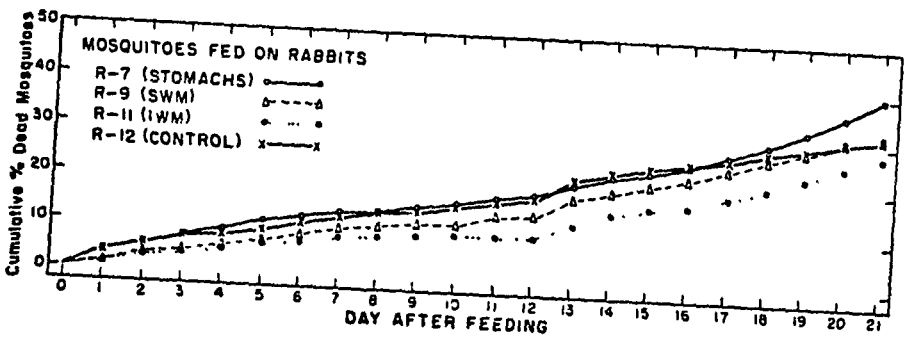

Fig. 6

Feeding 6

Fig. 7 R= Rabbit

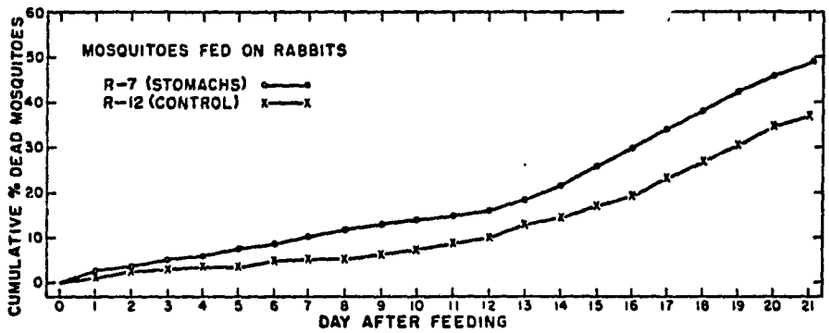

Fig. 7

Feeding 7

Fig. 8 R= Rabbit

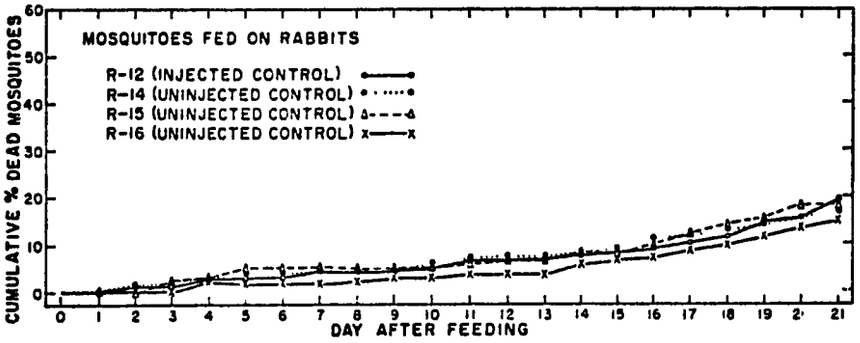

Fig. 8

Feeding 8