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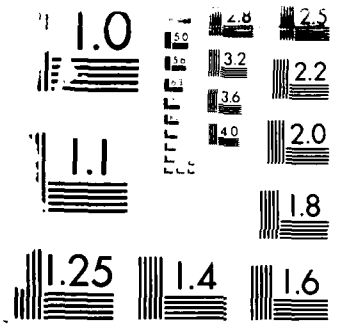
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Studies of the Biology of Phleboviruses in Sandflies

Annual Report

Robert B. Tesh, M.D.

February 1, 1986

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The mechanism of phlebovirus maintenance in nature was studied using a Toscana virus - Phlebotomus perniciosus model. This is a natural virus-vector combination. A laboratory colony of Ph. perniciosus, chronically infected with Toscana virus, was established. Toscana virus was maintained in this sandfly colony by transovarial (vertical) transmission for 13 consecutive generations (23 months). Although the virus transovarial transmission rates tended to decrease in each subsequent generation, the results of this study demonstrate one method by which phleboviruses may be maintained in nature. Transovarial virus infection appeared to have no effect on the developing larvae or pupae. Likewise, the animal pathogenicity of the virus did not change after repeated vertical transmission in the sandfly colony. Transovarially infected Ph. perniciosus females were able to transmit Toscana virus by bite to susceptible animals.

Studies were also done in Lutzomyia gomezi with Arboledas virus, a new phlebovirus from
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19. ABSTRACT - continued

Colombia. Lu. gomezi females feeding on a viremic opossum were readily infected with Arboledas virus, and virus replication occurred in the insects. Arboledas virus was also transovarially transmitted by experimentally infected Lu. gomezi to their F_1 progeny.

Five new colonies of phlebotomine sandflies were established: Phlebotomus colabaensis, Lu. longipalpis (Colombia), Lu. gomezi, Lu. spinicrassa and Lu. abonnenci.

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SUMMARY

The mechanism of phlebovirus maintenance in nature was studied using a Toscana virus - Phlebotomus perniciosus model. This is a natural virus-vector combination. A laboratory colony of Ph. perniciosus, chronically infected with Toscana virus, was established. Toscana virus was maintained in this sandfly colony by transovarial (vertical) transmission for 13 consecutive generations (23 months). Although the virus transovarial transmission rates tended to decrease in each subsequent generation, the results of this study demonstrate one method by which phleboviruses may be maintained in nature. Transovarial virus infection appeared to have no effect on the developing larvae or pupae. Likewise, the animal pathogenicity of the virus did not change after repeated vertical transmission in the sandfly colony. Transovarially infected Ph. perniciosus females were able to transmit Toscana virus by bite to susceptible animals.

Studies were also done in Lutzomyia gomezi with Arboledas virus, a new phlebovirus from Colombia. Lu. gomezi females feeding on a viremic opossum were readily infected with Arboledas virus, and virus replication occurred in the insects. Arboledas virus was also transovarially transmitted by experimentally infected Lu. gomezi to their F_1 progeny.

Five new colonies of phlebotomine sandflies were established: Phlebotomus colabaensis, Lu. longipalpis (Colombia), Lu. gomezi, Lu. spinicrassa and Lu. abonnenci.

A. Brief History of the Project

This research project on the biology of phleboviruses in sandflies began initially on 1 September 1980. During the intervening 5½ year period, the project has been funded by three different contracts and one grant (listed below). However, it should be emphasized that while the sources of funding have changed almost annually, the overall objectives of the project and the personnel working on it have remained the same.

Source of funding	Title	Duration
DAMD17-80-C-0178	Studies on the Transovarial Transmission of Phlebotomus Fever Viruses in Sandflies	1 Sept.1980-30 Sept.1982
DAMD17-83-C-3002	Studies on the Biology of Phleboviruses in Sandflies	1 Oct.1982-30 Sept.1983
DAMD17-83-G-9561	Studies on the Biology of Phleboviruses in Sandflies	1 Oct.1983-30 Sept.1984
DAMD17-85-C-5023	Studies on the Biology of Phleboviruses in Sandflies	10 Dec.1984-18 May 1987

B. Sandfly Colonization

Since the last annual report, five new colonies of phlebotomine sandflies have been established: Phlebotomus colabaensis, Lutzomyia longipalpis (Colombia), Lutzomyia gomezi, Lu. spinicrassa and Lu. abonnenci. An updated list of our current sandfly colonies is given below:

Phlebotomus papatasi (India
Phlebotomus papatasi (Israel)
Phlebotomus papatasi (Egypt)
Phlebotomus argentipes (India)
Phlebotomus perniciosus (Italy)
Phlebotomus colabaensis (India)

Lutzomyia longipalpis (Brazil)
Lutzomyia longipalpis (Colombia)
Lutzomyia gomezi (Colombia)
Lutzomyia spinicrassa (Colombia)
Lutzomyia abonnenci (Panama)

All of the above colonies are now well established. In addition, we have tried repeatedly to establish laboratory colonies of Lu. trapidoi (an important vector species in Central and western South America); but to date, our efforts have failed. Thousands of Lu. trapidoi eggs have been obtained from Dr. Byron Chaniotis, U. S. Army Medical Department Activity, Panama and from Dr. Alberto Morales, Instituto Nacional de Salud, Bogota, Colombia; but we have been unable to maintain this species for more than 1 or 2 generations in the laboratory.

During the last two years, sandfly eggs, larvae and/or adults from our colonies have been supplied to researchers at the University of Florida, Gainesville; Harvard University, Boston; Youngstown State University, Youngstown, Ohio; University of California, Berkeley; and U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland.

C. Experimental Infection of Sandflies

(1) Toscana virus

One of the objectives of this project is to determine the mechanism(s) by which phleboviruses are maintained in nature. Since results of both field and laboratory studies indicate that some phleboviruses are transovarially (vertically) transmitted in their sandfly vectors, we have concentrated on this potential mechanism. A Toscana virus - Phlebotomus perniciosus model was selected for study. This virus-vector combination was chosen for the following reasons: (a) many isolates of Toscana virus have been made from field-collected Ph. perniciosus, indicating that it is a natural virus-vector combination; (b) a number of Toscana isolates have been obtained from male Ph. perniciosus, suggesting that transovarial transmission (TOT) of the virus occurs under natural conditions; (c) experimental TOT of Toscana virus has been demonstrated in Ph. perniciosus in the laboratory; (d) we have a vigorous laboratory colony of Ph. perniciosus; and (e) Toscana virus is relatively easy to assay, since it kills mice, makes good CPE in Vero cells in liquid medium and produces sharp plaques under agar.

In earlier studies (Table 1), we demonstrated that Ph. perniciosus could be infected with Toscana virus by either the oral or parenteral route. It was also shown that Toscana virus could be transovarially transmitted by parenterally infected Ph. perniciosus females to their F_1 offspring. Therefore, it was decided to see how long the virus could be maintained in this sandfly species by TOT alone.

The results are shown in Table 2. Initially, 100 Ph. perniciosus females were inoculated with Toscana virus. Five days post-inoculation, the infected sandflies were fed on a clean hamster and their eggs from the first ovarian cycle were collected. After hatching, the F_1 larvae were reared to adults, which were allowed to feed on another clean hamster, and their eggs (F_2 generation) were collected. This process was repeated for 13 consecutive generations. A sample of adults (males and females) from each generation as tested individually by plaque assay in Vero cells for the presence of virus in order to determine the infection rates in each subsequent generation.

Three important facts should be noted about Table 2. (a) At the F_2, F_4 and F_{12} generations, individual females was segregated and tested for virus; only eggs from infected sandflies were used to start the next generation. In all other generations, eggs from all females (infected and non-infected) were pooled to start the next generation. (b) Only the parental generation was experimentally infected by inoculation; in all subsequent generations, infection resulted from transovarial virus transmission. (c) The time period from the initial infection of the parental generation to the emergence of the F_{13} adults was 23 months; each Ph. perniciosus generation took approximately 40 days.

From the data shown in Table 2, it is apparent that Toscana virus was maintained in the infected Ph. perniciosus line for 13 consecutive generations by TOT alone. However, it can also be seen that the percentage of transovarially infected flies did not remain constant but tended to decrease in

each subsequent generation. For example, from the F_5 to the F_{12} generation, the percentage of transovarially infected insects steadily dropped from 86.0 to 5.8%. Only after selection (pooling eggs from virus-positive females) at the F_2, F_4 and F_{12} generations did infection rates significantly increase.

This phenomenon can be shown another way. Table 3 gives the filial infection rates among F_1 generation offspring from individual Ph. perniciosus females inoculated with Toscana virus. Among this selected group of females, the filial infection rates varied from 37.5 to 88.9%, but in no case did they reach 100%. We still do not understand why some of the offspring from a given female are infected, while others of their siblings in the same ovarian cycle are not. Nonetheless, this does seem to explain why the transovarial infection rate droped in each subsequent generation. If the same phenomenon occurs in nature, then it implies that Toscana virus could not be maintained indefinitely by TOT alone.

We also determined what effect Toscana virus infection might have on developing Ph. perniciosus larvae and pupae. In this study, the F_7 generation of the chronically infected line was used. As shown in Table 4, adult flies of this generation emerged over a 24-day period. Each day as adults appeared, they were removed from the rearing container and frozen for subsequent virus assay. Each specimen was later tested individually by plaque assay in Vero cells for evidence of Toscana virus infection. Overall, 62.9% of the flies were infected. The infection rate among emerging adults remained fairly constant during the 24-day period, suggesting that Toscana virus had no effect on the insects' development. This is in contrast to work with several flaviviruses, which suggested that infection delays larval and pupal development.

We also examined what effect, if any, continual TOT in sandflies had on the virus. For example, some of the F_7 generation females from the chronically infected Ph. perniciosus line (Table 2) were allowed to feed on 4 newborn mice. Three of the mice became sick or died, four days after exposure to the flies, and Toscana virus was recovered from their brains. This indicated that the transovarially infected flies were still able to transmit the virus by bite and that the virus was still virulent for newborn mice.

In another experiment, the animal pathogenicity of the original Toscana virus pool, used to infect the parental generation (Table 2), was compared to that of a virus suspension prepared from a positive F_{13} generation female sandfly. Both virus stocks were titrated simultaneously in newborn mice and in Vero cell cultures. Virus titers in the two assay systems were as follows: Parental virus, $10^{5.8}$ PFU/mL and $10^{6.5}$ suckling mouse ID_{50}; F_{13} virus, $10^{3.9}$ PFU/mL and $10^{3.9}$ $SMID_{50}$, respectively. These data again suggest that animal pathogenicity of the virus did not change during prolonged (23 months) transovarial passage in sandflies.

An attempt was also made to see if the efficiency of TOT of the virus changed after prolonged passage in insects. In this experiment, Ph. perniciosus females from an uninfected laboratory colony were divided into two groups: one group was inoculated with the original virus pool used to inoculate the parental generation (Figure 2) and the other was inoculated with

a suspension of virus prepared by homogenizing an F_9 generation positive female sandfly. The percentage of infected progeny from the two groups was 37.7% (29/77) and 69.7% (67/96), respectively. The transovarial transmission rate was approximately double in the TOT-passaged virus.

Dr. Stuart Nichol, Department of Microbiology, University of Nevada School of Medicine, is currently examining the RNA genome of Toscana virus isolates from different sandfly generations in the infected Ph. pernicious line (Table 2) by T1 ribonuclease fingerprinting to see if significant genetic changes have occurred in the virus during the 23 month period. Since this is presumedly a natural virus-vector combination, the results of this biochemical study may provide information on the evolution of phleboviruses under field conditions. The results of this study also have implications for the use of phlebovirus vaccines and diagnostic probes.

(2). Arboledas virus

Studies were also done on the behavior of Arboledas virus in Lu. gomezi. To date, we have obtained 5 isolates of this agent from sandflies collected in the Municipio of Arboledas, Norte de Santander, Colombia. Serological studies indicate that Arboledas virus is a new member of the phlebotomus fever serogroup (Bunyaviridae: Phlebovirus). In examining human and animal sera from the collection site, it was found that only about 4% of the human residents had neutralizing antibodies to Arboledas virus, but that 31% of opossums (Didelpohis marsupialis) did. Several Didelphis were subsequently inoculated with Arboledas virus, and these animals developed detectable viremia which lasted 4 days.

Although our research to date with sandflies indicates that these insects are relatively refractory to phlebovirus infection by the oral route, we nonetheless did an experiment to test the oral susceptibility of Lu. gomezi to Arboledas virus infection (Table 5). To our surprise, almost all of the female flies feeding on a viremic opossum (blood titer $10^{5.6}$ PFU/mL) because infected and virus replication in the insects was clearly demonstrated. Eggs were collected from some of the orally infected flies, and the emerging F_1 offspring were reared to adults and were subsequently tested for virus. None of the offspring coming from eggs in the first ovarian cycle were infected with Toscana virus; however, this does not prove much, since studies with several California group viruses in mosquitoes have shown that TOT occurs only in the second and subsequent ovarian cycles (Note: We have tried repeatedly with Toscana and Arboledas viruses to get infected sandflies to take a second blood meal and to lay a second batch of eggs; but unfortunately most of the females die during or shortly after oviposition, thus it is very difficult to get a second batch of eggs.

Consequently, another group of Lu. gomezi females was inoculated with Arboledas, held for 5 days at 25OC, and then fed on a clean hamster. Their F_1 progeny were reared to adults and tested individually for virus. Eighty percent (25/31) of a sample of the F_1 progeny were infected with Arboledas virus, thus indicating transovarial virus transmission.

D. Publications Resulting from or Supported by This Project

1. Travassos da Rosa, A.P.A., Tesh, R.B., Travassos da Rosa, J.F., Herve, J.P. and Main, A.J. Carajas and Maraba viruses, two new vesiculoviruses isolated from phlebotomine sand flies in Brazil. Am. J. Trop. Med. Hyg. 33: 99-1006, 1984.

2. Travassos da Rosa, A.P.A., Tesh, R.B., Pinheiro, F.P., Travassos da Rosa, J.F.S., Peralta, P.H. and Knudson, D.L. Characterization of the Changuinola serogroup viruses (Reoviridae: Orbivirus). Intervirology 21: 38-49, 1984.

3. Tesh, R.B. and Modi, G.B. Studies on the biology of phleboviruses in sand flies (Diptera: Psychodidae). 1. Experimental infection of the vector. Am. J. Trop. Med. Hyg. 33: 1007-1016, 1985.

4. Tesh, R.B. Undifferentiated fevers: dengue, phlebotomus fever, Rift Valley fever, West Nile fever and fevers caused by alphaviruses. In: Cecil's Textbook of Medicine, 17th edition. J.B.Wyngaarden, L.H.Smith and F. Plum, editors. W.B. Saunders Co., Philadelphia, pp.1737-1740.

5. Tesh, R.B., Peleg, J., Samina, I., Margalit, J., Bodkin, D.K., Shope, R.E. and Knudson,D.L. Biological and antigenic characterization of Netivot virus, an unusual new Orbivirus recovered from mosquitoes in Israel. Am. J. Trop. Med. Hyg. 35: 418-428, 1986.

6. Tesh, R.B. Phlebotomus fevers. In: Epidemiology of Arthropod-Borne Viral Diseases. T.P. Monath, editor. CRC Press, Boca Raton (in press).

Table 1

Growth of Toscana virus in Ph. perniciosus females following ingestion and intrathoracic inoculation

Day post-infection	Virus titers in fed flies	Virus titers in inoculated flies
0	3.2, 3.4, 3.5, 3.6, 3.8*	2.0, 2.0, 2.2, 2.2, 2.7
1	2.9, 3.4, 3.7, 3.7, 3.9	2.0, 2.3, 2.5, 3.0, 3.0
2	1.7, 1.8, 1.9, 2.3, 3.0	2.5, 2.9, 3.0, 3.2, 3.7
3	1.3, 1.5, 2.0, 2.0, 2.8	3.2, 4.0, 4.0, 4.2, 4.2
4	<0.7,<0.7,<0.7, 1.0, 3.0	1.7, 2.8, 3.2, 4.2, 4.7
5	<0.7,<0.7,<0.7, 2.7, 2.8	3.0, 3.3, 3.7, 4.0, 4.2
6	2.8, 2.9, 3.0, 3.2, 3.7	3.8, 4.0, 4.0, 4.3, 4.7
7	<0.7, 3.5, 3.6, 4.0	3.0, 3.8, 3.8, 3.9, 4.0

*Virus titers expressed as $\log_{10}$ of PFU per insect. Five sand flies were sampled each day.

Table 2

Vertical transmission of Toscana virus in chronically infected Ph. perniciosus line

Generation	Percentage infected	(Number /Total) (infected/tested)
Parental	100*	(25/25)
	↓	
F_1	40.2	(107/266)
	↓	
F_2	43.8	(35/80)
	↓	
F_3	82.5	(33/40)
	↓	
F_4	45.4	(54/119)
	↓	
F_5	86.0	(74/86)
	↓	
F_6	72.0	(54/75)
	↓	
F_7	61.8	(285/461)
	↓	
F_8	48.3	(42/87)
	↓	
F_9	33.3	(15/45)
	↓	
F_{10}	NT	
	↓	
F_{11}	14.1	(19/135)
	↓	
F_{12}	5.8	(7/119)
	↓	
F_{13}	57.9	(11/19)

* Female sand flies in the parental generation were experimentally infected by inoculation. In all subsequent generations, infection resulted from transovarial virus transmission.

NT = F_{10} generation sand flies were not tested.

Table 3

Toscana virus filial infection rates among F_1 generation offspring from individual Ph. perniciosus females

Female number	Number of progeny/Total infected /offspring tested	Filial infection rate (%)
1	13/21	61.9
2	7/16	43.7
3	23/27	85.2
4	15/27	55.6
5	23/35	65.7
6	20/27	74.1
7	3/5	60.0
8	12/24	50.0
9	12/22	54.5
10	6/16	37.5
11	8/9	88.9
12	23/27	85.2
13	17/25	68.0
14	12/13	92.3
15	9/19	47.4

Table 4

Toscana virus infection rates among F_7 generation transovarially infected male and female Ph. perniciosus adults

Date of eclosion	Males		Females	
	Number /Total infected/tested	(Percentage infected)	Number /Total infected/tested	(Percentage infected)
March 10-12	10/13	(76.9)	--	--
March 13-15	8/20	(40.0)	12/18	(66.7)
March 16-17	18/24	(75.0)	14/25	(56.0)
March 18-20	17/23	(73.9)	17/25	(68.0)
March 21-22	15/25	(60.0)	18/25	(72.0)
March 23-25	16/25	(64.0)	12/25	(48.0)
March 26-28	9/16	(56.3)	12/20	(60.0)
March 29 - April 2	5/8	(62.5)	10/15	(66.7)
TOTAL	98/154	(63.6)	95/153	(62.1)

Table 5

Growth of Arboledas virus in Lutzomyia gomezi after feeding on infected opossum*

Day post-feeding	Virus titer per insect sampled**
0	1.6, 1.6, 1.6, 1.7, 2.0
1	1.2, 1.3, 1.3, 1.4, 1.5
2	<0.7, 1.7, 2.0, 2.0, 2.0
3	1.6, 2.0, 2.0, 2.6, 3.0
4	2.2, 2.3, 2.7, 3.0, 3.4
5	3.0, 3.2, 3.4, 3.5, 3.6
6	3.9, 4.0, 4.0, 4.2, 4.2
7	4.2, 4.3, 4.4, 4.4

*Flies were fed on an opossum (D. virginiana) with a blood virus titer of $10^{5.6}$ PFU/mL.

**Five female flies were sampled daily. Virus titers expressed as $\log_{10}$ of PFU per insect.

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