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Autor(en): **Schlesinger, R. Walter / Stollar, Victor / Guild, Gregory M.**

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Department of Microbiology, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, N.J.

THE SIGNIFICANCE AND NATURE OF DEFECTIVE INTERFERING VIRUSES

R. WALTER SCHLESINGER, VICTOR STOLLAR, GREGORY M. GUILD, AKIRA IGARASHI, THOMAS E. SHENK and JOSEF PELEG

Summary

Deletions in viral genomes appear to be a common occurrence in the replication of all DNA and RNA viruses which have been adequately studied. Such defective genomes can replicate in the presence in the same cell of a helper virus as long as the deletion does not involve the initiation site for genome replication. Coinfection of a cell with defective and "normal" infectious virus leads to reduction in the yield of the latter. The nature of DI viruses and genomes found in Sindbis virus-infected vertebrate cells during "undiluted passage" series is discussed. This procedure leads to the accumulation of progressively shorter viral RNA genomes with internal deletions. The enrichment is limited to genome lengths which are integral fractions (1/2, 1/3, 1/4, etc.) of the complete genome, and these are also found in viral particles released at the corresponding passage levels. It is believed that the selective accumulation of these fragments is governed by constraints of assembly which demand that one full genome equivalent be packaged in a released particle. In contrast to vertebrate cells, cultured mosquito cells do not seem to produce or "recognize" DI particles. Possible implications for the epidemiology of arthropod-transmitted alphaviruses are presented.

Zusammenfassung

Verluste (Deletionen) von Teilen des Virusgenoms scheinen häufige Vorkommnisse bei der Vermehrung aller in dieser Hinsicht adäquat untersuchter DNS- und RNS-Viren zu sein. Solange die Deletion nicht die Initiationsstelle für die Genomreplikation umfasst, können solche Teilgenome sich vermehren, wenn die Zelle gleichzeitig mit einem Helfervirus infiziert ist. Doppelinfektion einer Zelle mit einem defektiven und einem "normalen" infektiösen

Virus führt zu einer Verminderung in der Ausbeute des letzteren. Das Wesen defektiver interferierender (DI) Viruspartikel und Genome im Verlauf "unverdünnter Passageserien" wird diskutiert. Dieses Verfahren führt zur Anhäufung immer kürzerer Virusgenome mit internen Deletionen. Angereichert werden nur Genome, deren Länge einen ganzzahligen Bruchteil (1/2, 1/3, 1/4 usw.) der Länge des vollständigen Genoms umfasst, und solche Genome werden auch in den bei der entsprechenden Passage freigesetzten Viruspartikeln gefunden. Wir vermuten, dass Genomfragmente nur unter der Voraussetzung, dass sie zusammen das Aequivalent eines Normalgenoms bilden, in Viruspartikel verpackt werden können. Im Gegensatz zu Wirbeltierzellen scheinen Moskito-Zellen DI-Partikel weder zu produzieren noch zu "erkennen". Die daraus resultierenden möglichen Folgen für die Epidemiologie arthropodenübertragener Alphaviren werden beschrieben.

Throughout his career, K.F. Meyer was particularly interested in the problem of latent infections and the cyclic nature of epidemic and epizootic diseases, especially those involving multiple host and vector species. STEELE (33) reminds us that "his first article on filterable viruses appeared in 1914, marking the beginning of his research on the cause of equine encephalitis." Although the virus he recovered at that time from a horse dead of encephalitis was lost on passage, the subsequent isolation of Western equine encephalitis virus in 1930 by MEYER, HARING and HOWITT (21) marked the first discovery of a group A arbovirus (now classified as a member of the alphavirus genus of the togavirus family).
The arboviruses, through continuous bidirectional crossing of phylogenetic barriers in their arthropod-vertebrate-arthropod ... transmission cycle, epitomize the complexities of selective pressures to which an animal virus may be exposed in nature (27). Usually, the various transmission patterns of different arboviruses are presented in epidemiological flow charts which do not tell us whether replication in vertebrate and invertebrate hosts subjects the virus to similar or dissimilar modifying effects. Nor do they indicate where or how the virus is maintained during interepidemic seasons, especially in temperate climates.
Schematic drawings of transmission cycles also do not convey the biologically important fact that infection of vertebrates is characteristically a self-limiting episode with or without pathological consequences, while arthropods, once infected via bloodmeal, remain so for the rest of their life span, generally without showing any pathological effects. What are the cellular or organismal factors that determine this significant difference?
In view of K.F. Meyer's contributions to vector-transmitted infections and specifically to arbovirology, it seems appropriate on this occasion to examine these questions in terms of

the possible role of defective virus and other aberrant products of viral replication. We have carried out extensive studies on the replication of Sindbis virus in cultured mosquito and vertebrate cells (7, 8, 9, 15, 16, 22, 29, 30, 31, 35, 36, 37, 38). The findings have suggested an interesting interplay between temperature-sensitive (ts) variants and defective-interfering (DI) virions or genomes in these two model systems. After summarizing some of the findings, we shall speculate on their possible implications for an understanding of the natural life cycle of togaviruses.

First, however, and notwithstanding the fact that we are here to honor the memory of a native son of Basel, we cannot refrain from mentioning the key contributions to the study of viral latency and interference by an adopted Basler, ROBERT DOERR (Fig. 1), for many years Professor of Bacteriology and Hygiene here and undisputed master of the Stachelschützenhaus.

Meyer and Doerr were linked not only by Basel or by being contemporaries in the emerging science of virology but - more important - by their pioneering studies of infectious diseases and by an abiding interest in the problem of latency. After Doerr's death, it was only natural that his disciple, Professor Hallauer, teamed up with K.F. Meyer to continue the editorship of Doerr's creations, the "Handbuch der Virusforschung" and the "Archiv für die gesamte Virusforschung".

It was FLAVIANO MAGRASSI, then in Doerr's laboratory, who in 1935 first described the "Konkurrenzphänomen" in which non-encephalitogenic strains of herpes simplex virus blocked the development of encephalitis in rabbits subsequently challenged with an encephalitogenic strain of the same virus (20). This system, subsequently shown by DOERR and coworkers (3, 4) and by HALLAUER (10) to involve "Schienenimmunität" along neuronal pathways, was an early precursor of numerous experimental model systems demonstrating interference between two homologous virus strains differing in virulence markers. The importance of these early experiments lay in the unequivocal demonstration that resistance to pathogenic viruses could be mediated by processes clearly separable from classical humoral immunity. They suggested that infection with an avirulent virus could so alter the milieu of susceptible cells that they no longer supported the replication of the virulent analogue.

In the intervening years, this phenomenon has, of course, been extended to many homologous and heterologous pairs of viruses. Recognition of the role of interferons (18) as mediators of interference temporarily pushed the exploration of other mechanisms into the background.

In recent years, however, the intrinsic interfering capacity of mutant (specifically temperature-sensitive (ts)) and defective viruses or their genomes has been reaffirmed in many diffe-

Figure 1. Robert Doerr (1871-1951), lecturing in 1936. (From the lecture notes of R.W.S.).

rent systems. We are beginning to understand these processes in molecular terms. At the same time, their possible relevance to mechanisms operating in natural infections and in their arrest and to the problems of persistent infection and latency can be appreciated. By extension, ts and defective viruses may play a role in the causation of chronic degenerative diseases. This burgeoning field has been extensively reviewed, especially by HUANG and BALTIMORE (12, 13, 14).

Defective-interfering virus particles are characterized as follows: 1. they are virions containing a partially deleted viral genome but generally normal viral proteins and structural features, though sometimes in particles of reduced size; 2. they are taken up by cells but cannot carry out their own replication; 3. they do, however, replicate in cells coinfected with viral particles which are not deleted in the same function(s) and can therefore complement the defective genome ("helper virus"); 4. in cells dually infected with DI and "normal" standard particles, the replicating DI particles (or genomes) interfere with replication of the helper, thus leading to a reduced yield of infectious progeny virions.

All DNA and RNA containing animal viruses which have been critically examined for generation of DI particles have been shown to produce them under appropriate experimental conditions (this includes, with more or less complete evidence, papova-, adeno-, herpes-, myxo-, picorna-, toga-, rhabdo-, arena-, reo-, and retroviruses (14)). The universality of this phenomenon suggests that errors in the form of deletions are probably common occurrences in the transcription process turning out many genome copies. If such deletions involve the sequence encoding the viral replicase, the replicase produced by the coinfecting helper virus can copy the defective genomes, provided the initiation site is preserved.

In the laboratory we can accentuate the accumulation of defective genomes by infecting host systems at a very high input multiplicity, i.e., under conditions leading to infection of each cell with many infectious standard virus particles. Further amplification can be forced by "serial undiluted passage" (40), i.e., the rapid serial transfer of the total viral yield from one to the next generation of host organisms or cell cultures.

Production of DI particles in Sindbis virus-infected vertebrate cells. The latter procedure, first used for Sindbis virus by S. SCHLESINGER (28) and INGLOT et al. (17), is illustrated in Table 1 (7). Plaque-purified SV was used to initiate an undiluted passage series in chick embryo fibroblast (CEF) cultures. Initially, the typical high yield of infectious virus was obtained, but after a few passages there was a dramatic drop in titer, followed by a return to high yields and repetition of the progressive decrease. This type of cyclic fluctuation may continue for several passages but such a series usually becomes equilibrated at a level of virus production 10- to 100-fold lower than that of a first passage initiated with standard virus. Contrary to some other viruses which, under similar conditions, produce DI particles that are smaller and can be physically separated from standard virions, no such physical heterogeneity is exhibited by SV preparations producing low yields (e.g., chick embryo passage 6 (CP-6) in Table 1). There are, however, two ways in which the presence of DI particles in such stocks can be demonstrated: these are 1. reconstruction experiments, 2. analysis of intracellular and virion-associated RNA species.

Table 1. Serial undiluted passage of Sindbis virus in chick embryo fibroblast cultures.[a]

Passage No. CP[b]	Yield at 16 hrs. (log_{10} PFU/ml)	Passage No. CP[b]	Yield at 16 hrs. (log_{10} PFU/ml)
1	9.5	13	7.1
2	9.1	14	8.0
3	8.0	15	8.5
4	5.8	16	7.6
5	7.3	17	7.5
6	8.8	18	7.3
7	5.5	19	7.7
8	7.2	20	7.4
9	7.3	21	8.0
10	8.5	22	7.2
11	7.2	23	7.9
12	7.0	24	7.5

(a) For details see (7).
(b) CP = chick embryo fibroblast passage

1. In reconstruction experiments, standard virus stocks, by themselves producing high yields of infectious virus, are mixed in appropriate proportions with low yielding stocks such as CP-6. The yields of infectious virus are greatly reduced compared with those from companion cultures infected with standard virus alone. From this we can infer that CP-6 contains DI particles. Moreover, we can explain the cyclic variations in infectious titers in the undiluted passage series itself: as the amount of infectious virus decreases to a level at which only a minority of the cells in the next passage receive both DI and helper particles, the total yield from these few cells will not contain enough DI particles to saturate all cells in the following passage. This provides an opportunity for cells to be infected only with "normal" virus particles which will multiply unhindered to high titer.

2. RNA species associated with SV replication.

a) Standard virus: The "normal" (standard) Sindbis virion is a roughly spherical particle, 55-60 nm in diameter, which is made up of an icosahedral nucleocapsid surrounded by an envelope. The latter is a typical lipid bilayer membrane with projections containing equimolar amounts of 2 virus-specified glycoproteins E_1 and E_2 (25). A third smaller glycoprotein (E_3), which contains 45 % carbohydrate, has been shown to be associated with Semliki forest virus (6) but not yet unequivocally with Sindbis. The nucleocapsid is made up of a lysine-rich core protein, MW 33,000 daltons (39,6), and the genome, a linear single-stranded RNA molecule with a sedimentation coefficient of 42S and MW 4.3×10^6 daltons (Reviewed in (9)).

The virion contains no RNA replicase and the isolated RNA is infectious. Therefore, by definition (1), it is of (+) (or messenger) polarity. The genome contains polyadenylate at its 3' end and can serve as a template for in vitro protein synthesis (2). Its 5' end is capped and has the sequence m^7G(5')pppApUpGp ... (11). Of special interest is the inference from various evidence (see 9) that the 5' and 3' ends carry inverted complementary sequences, each about 250 nucleotides long, which can form a double-stranded "handle" and thus circularize the rest of the molecule. Whether or not this type of circularization plays a role in RNA replication remains to be determined.

By genetic analysis and by cell-free translation, it has been established that the structural polypeptides are encoded in the one-third of the genome representing approximately 4600 nucleotides in from the 3' end, leaving about 8000 nucleotides in the 5' two-thirds for nonstructural proteins. The latter include the gene(s) for viral RNA replicase(s).

Transcription by the viral RNA replicase proceeds from an initiation site near the 3' terminus. The resulting (-) strand includes the transcript of the 250 bases at the 5' end which are thought to be complementary to those at the 3' end of the parental genome. Therefore this portion of the (-) copy would be identical with the corresponding sequence at the parental 3' end. If the initiation site for transcription is located in that sequence, the same RNA transcriptase as that involved in the first (+) (-) transcription can presumably also carry out the transcription in the reverse order, i.e., the production of complete progeny (+) strands (42S) and the formation of double-stranded replicative forms (RF, 22S). However, there is an alternative, internal, initiation site which is located at or near the region separating the genes for nonstructural from those for structural viral polypeptides. The result of this internal initiation is the transcription, in great excess, of a mRNA of sedimentation coefficient 26S which is translated into the structural (virion) polypeptides. The 26S mRNA has a capped 5' end identical to that of the complete 42S genome (5). The transcription of the two mRNA's (42S and 26S) must be tightly regulated, by mechanisms which remain to be elucidated. This unique transcription sequence is typically reflected in the appearance, in cells infected with standard virus, of three major RNA species, viz., 42S single-stranded (ss) progeny genomes, 26S ss mRNA (the predominant species), and 22S double-stranded (ds) RF RNA representing base-paired (-) and (+) strands.

b) DI virus and genomes. The pattern just described for cells infected with standard virus changes drastically in the course of an "undiluted passage" series: there is progressive shortening of the predominant ds and ssRNA species. This process is summarized in Table 2 for chick embryo cells (7, 8, 9). Electron microscopic length measurements of dsRNA molecules

Table 2. Estimated molecular weights of intracellular viral RNA species generated during undiluted serial passage of Sindbis virus in chick embryo fibroblasts (CP).

Passage No.	ds RNA Species	ds RNA MW(a)	ss RNA Species	ss RNA MW(b)	Genome Equivalent (%)
-(c)	22S	8.7×10^6	42S	4.3×10^6	100
CP 6	18S	4.4×10^6	33S	2.2×10^6	50
CP 15	15S	2.8×10^6	24S	1.4×10^6	33
CP 24	12S	2.0×10^6	18-22S	$0{,}75\text{-}1.0 \times 10^6$	20-25

(a) Based on electron microscopic length measurements.
(b) Based on migration rate in polyacrylamide gel electrophoresis.
(c) Standard virus passage.
For experimental details, see (8) and (9).

and determination of the rate of electrophoretic migration of ssRNA species in polyacrylamide gel has revealed the startling fact that the molecular weights of intracellular viral RNA's evolve as progressive integral divisions of the whole viral genome and its ds equivalent (Table 2) (8, 9).

Moreover, the virion populations harvested at the various passage levels contain, in addition to the standard 42S genomes, smaller species of the same size as the intracellular ssRNA's just described. Since, as already mentioned, the DI particles (which presumably contain these small genomes) cannot by physically separated from standard virions, it has been proposed (9, 19) that the defectives contain a total of 4.3×10^6 daltons of RNA (the normal genome equivalent). Thus, DI particles would contain two 33S, three 24S, four 22S, five or six of the smaller molecules, linked in some as yet unknown fashion. This hypothesis would imply that the constraints of packaging in a preformed capsid would exert the selective pressure that forces the enrichment of certain integral fractions of the genome. Such a mechanism would subject the occurrence of deletions, presumably a randomized process, to some regulation of size order predominance.

Finally, exhaustive hybridization (9) and oligonucleotide (19) analyses have led to the conclusion that all rescuable (i.e., viable) DI genomes have retained some 1000 nucleotides from the 3' and variable sequence lengths from the 5' end. Thus, all such deletions involve the mid-portion of the genome - presumably reflecting the need, for viability, to preserve the initiation site for RNA replication in both, the (+) and (-) copies.

It is not known whether, or to what extent, the various defective genomes differ with regard to their interfering capacity. The observation that, as the undiluted passage series reaches

a relatively stable level of infectious yields (Table 1), the predominant DI genomes have lost 75-80 % of the standard genome, suggests relatively limited interfering capacity of these maximally deleted remnants.

Failure of mosquito cell cultures to produce or "recognize" DI particles. When cultured Aedes albopictus cells (32) are infected with standard Sindbis virus, they support efficient virus replication without ordinarily showing recognizable cytopathic effects (34). The initially high yield of infectious virus gives way to the indefinite continued release of infectious virus at relatively low levels. Such persistently infected cultures are normal in appearance and growth rate. However, the released virus eventually becomes temperature-sensitive (ts) and produces smaller plaques (sp) than the standard virus.

These two distinguishing markers can be used to show that the (ts, sp) variants interfere efficiently with replication of standard virus in mosquito as well as vertebrate cell cultures. This interference is limited to the homologous virus; another alphavirus (EEEV) is not similarly inhibited, in accordance with the reported lack of interferon production by mosquito cells (35). It is important to emphasize that the (ts, sp) virus recovered from persistently infected A. albopictus cell cultures, though capable of interfering, retains the capacity to replicate at permissive temperature in both normal mosquito and vertebrate cells. Therefore it is not, by definition, defective.

Indeed, IGARASHI and STOLLAR (15) have reported that 48 serial undiluted passages of Sindbis virus in A. albopictus cells failed to produce convincing evidence for accumulation of DI particles either in terms of infectious yield or of changing RNA patterns. Moreover, when normal A. albopictus cells were infected with virus stocks from undiluted series in BHK21 or chick cells containing large amounts of DI particles, they produced the same yields of infectious progeny as cells infected with standard virus alone. In contrast to similarly infected BHK21 cells, the mosquito cells did not produce detectable 12S dsRNA.

Two additional observations should be noted:

1. While the data just summarized suggest that mosquito cells fail to make or "recognize" DI particles, persistently infected cultures, after several weeks of continuous culture, do accumulate large amounts of intracellular 12-15S dsRNA (16). The nature of this species remains to be studied further, as does its possible role in regulating virus replication in these cultures.

2. Cloned ts viral variants recovered from persistently infected A. albopictus cultures were shown (31) to have a low spontaneous reversion rate to (ts+). However, when such clones were subjected to a few undiluted serial passages in BHK21 cells, even at 34°C, a remarkab-

ly rapid accumulation of particles of non-ts phenotype occurred (with retention of the small plaque marker in all cases). This finding suggests that, in contrast to mosquito cells, these vertebrate cells provide a milieu favoring the selective growth of particles with wild-type response to temperature shifts.

Defectiveness in the natural life cycle of a hypothetical arthropod-borne alphavirus. In attempting to speculate on the possible relevance of the various observations summarized above to the natural life cycle of a Sindbis-like virus, we recognize that Nature does not operate under the selective constraints that we can impose on experimental manipulations. In the laboratory, we can reasonably well separate ts mutants, DI particles, and interferons although operationally we measure their activities in the same way, i.e., in terms of their depressing effects on yields of infectious progeny virus. In the experimentally or naturally infected animal host, these factors operate together and are further modulated by the various components of the immune response.

Nevertheless, increasing evidence from a number of animal studies suggests the generation of DI virions (or genomes) may play a major role in limiting the spread of acute virus infections (reviewed in (14)). On the other hand, temperature sensitivity of many different viruses is generally associated with greatly reduced virulence for susceptible vertebrate hosts. Arguments can be advanced in favor of the hypothesis that arthropod-transmitted togaviruses have their evolutionary origin and are maintained in the arthropod vectors rather than in vertebrate hosts (26), and current studies on the structure of togavirus and insect mRNA's tend to support this view (D.T. DUBIN, pers. communication). From a teleological point of view, it would make no sense for a virus depending on interepidemic maintenance in a vector to give rise to DI particles which would effectively minimize the transmission of fully infectious yields.

The idea that "cold selection" of ts viral variants in cultured mosquito cells has its parallel in naturally hibernating mosquitoes is supported by the studies of REEVES et al. (23) on wild-caught mosquitoes infected with WEE virus. ROZEBOOM and KASSIRA (24) reported on the ability of an attenuated (ts?) strain of West Nile virus to interfere with replication of a virulent strain of the same virus administered to mosquitoes by the same parenteral route 7 days later.

These and other considerations led us on an earlier occasion (27) to propose a model for the seasonal cycling of a hypothetical alphavirus in temperate climates (Fig. 2). It is assumed that the virus, after prolonged replication in hibernating arthropods (or via transovarial transmission or in hibernating animals) becomes progressively more ts (upper semicircle). As

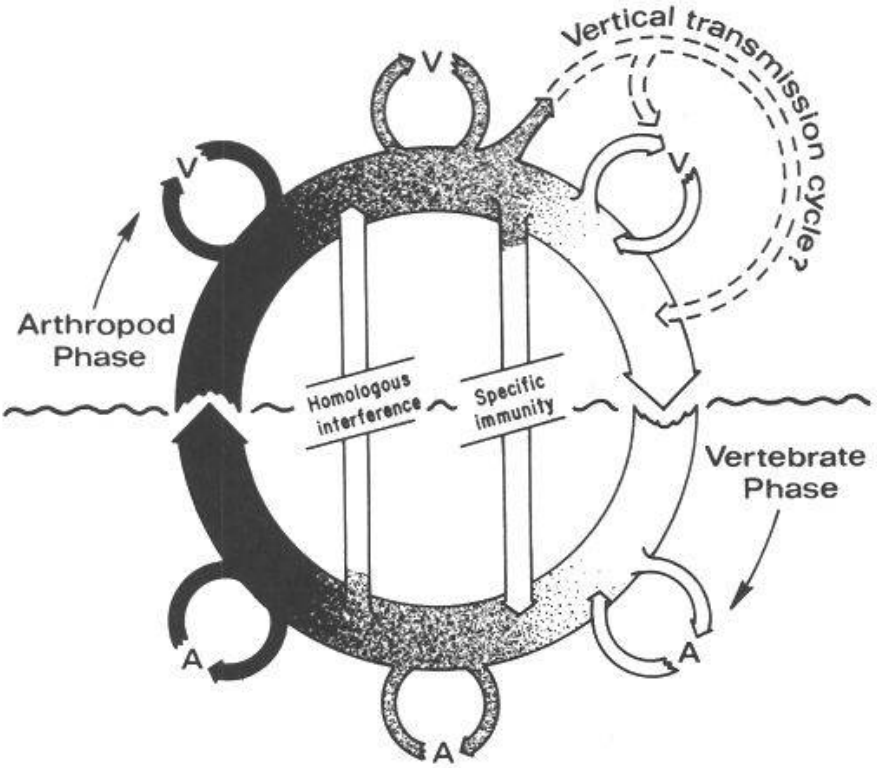

Figure 2. Hypothetical scheme of the seasonal transmission cycle of a potentially pathogenic togavirus in moderate climate. It is assumed that the cycle begins on the right-hand side with ts virus of the kind described in the text as characteristic of persistently infected mosquito cells, which has overwintered in surviving adult arthropods, coldblooded or hibernating animals, or by transovarial transmission. Transmission of this virus to warmblooded vertebrates leads to accumulation of ts^+ (virulent?) virus as in the experimental model described in the text. The large semicircles represent the entire arthropod and vertebrate populations involved, the small ones individual arthropod-vertebrate encounters. The solidly black portion of the diagram would correspond to the epidemic or epizootic disease prevalence. (The time scale is, of course, arbitrary). See text for further details. Shading in "arthropod phase": decreased intensity expresses increasing proportion of ts virus (avirulent?). Shading in "vertebrate phase": increased intensity expresses increasing proportion of ts + virus (virulent?). A = arthropod; V = vertebrate.
From reference (27), with permission of Medical Biology, Helsinki.

the biting season commences, this fully infectious virus is transmitted to warmblooded vertebrates. In these, as in the BHK21 cell cultures experimentally infected with the ts virus from persistently infected A. albopictus cell cultures, selection of non-temperature-sensitive (and, for pathogenic viruses, increasingly virulent?) viral variants is favored (lower semicircle). The pairs of small semicircles, representing individual bidirectional arthropod-vertebrate encounters, reflect in the degree of their shading the kind of virus that is transmitted. The validity of this model for natural and experimental infections of whole arthropod and vertebrate organisms can be tested. It should be noted that a plaque-purified stock of ts, small-plaque Sindbis virus (SV-S) derived from persistently infected A. aegypti cell cultures (22)

replicated in mosquitoes infected via membrane feeding, but transmission by the mosquitoes to suckling mice could not be demonstrated (21a). It remains to be seen whether viral variants arising in whole mosquitoes and not subjected to selection in several cell culture passages would behave similarly. Experiments to this end, employing intrathoracic inoculation of mosquitoes and tests for virulence in suckling and adult mice, have been initiated by Dr. Russell Regnery in our laboratory.

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Address of author: Prof. R. Walter Schlesinger, M.D., Department of Microbiology, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, N.J. 08854 (USA)