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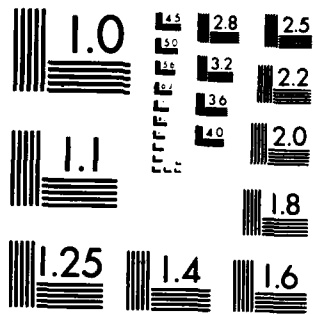
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Analysis of Flavivirus Replication



Annual Report
Final Report

September, 1981

Margo A. Brinton, Ph.D.

Supported by

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The Wistar Institute of Anatomy & Biology
Philadelphia, Pennsylvania 19104

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Summary:

Even though flaviviruses cause significant human morbidity and mortality in many geographical areas of the world, surprisingly little is yet known about the replication of these viruses. The long range objective of our studies is the characterization of the mode of transcription and translation of flavivirus genome RNA. Comparative studies of the replication of West Nile virus (WNV) in cell cultures from genetically resistant C3H/RV and genetically susceptible C3H/HE cells have indicated that resistant cells synthesize less 40S genome RNA than comparative susceptible cell cultures. However, a mutant of WNV isolated from long term persistently infected cultures of resistant C3H/RV cells was found to be able to efficiently replicate 40 genome RNA in resistant cells. Our data also indicate that virus particles containing subgenomic flavivirus RNAs can be generated during WNV replication in mouse cells. We have carried out a preliminary analysis of the RNAs contained in these defective interfering (DI) particles. Further study of the mode of generation of the deleted RNA species and characterization of the WNV mutant should yield insights into the normal mode of transcription of the flavivirus genome.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

Personnel:

Margo Brinton - Principal Investigator
Albert Reinhardt, postdoctoral fellow; 2/15/80 to 9/15/80.
(Dr. Reinhardt left research to take a job in biological quality control at National Drug Company.)
Douglas Schaeffer, Technician - 6/1/80 to present

Progress has been made in each area covered by the contract which began 9/1/80 and continued to 9/1/81. The specific aims of that contract were as follows:

1. To determine whether or not flavivirus defective interfering (DI) particles are generated by serial undiluted passage and, if so, to characterize the deletion in their RNAs

West Nile virus (WNV), strain E101 was used as a representative model flavivirus in our studies. The replication of WNV was studied in embryofibroblast cultures prepared from genetically resistant C3H/RV and genetically susceptible C3H/HE cells. Resistant cells produce lower yields of infectious WNV and were found to incorporate less [^3H]uridine into intracellular 40S RNA than did susceptible cells. Analysis of [^3H]uridine labeled extracellular virus from WNV-infected RV and HE cells indicated that 10 to 20 times less [^3H]uridine was incorporated into particles in RV cell fluids than HE ones. RNA extracted from virions pelleted from culture fluids harvested from infected RV cells contained about 1/100 of the amount of uridine incorporated into 40S RNA found in comparable HE-culture fluids. The majority of the labeled RNA in culture fluids from infected resistant cells sedimented in the top and middle portions of the gradient (Brinton, 1981). It seems unlikely that these RNAs were generated by cell nuclease digestion during the extraction procedure since vanadyl-ribonucleoside complex was added to the samples before virus disruption.

Although flavivirus defective interfering (DI) particles have not yet been fully characterized. Their production by WNV-infected cells had been previously suggested by several types of observations: 1) infection of cells with a high multiplicity of virus produced a lower yield than infection with a lower multiplicity; 2) infectivity titers cycled during serial undiluted passage of the virus; and, 3) homologous interference had been demonstrated. In each case, the effect attributed to the DI particle interference was more pronounced in flavivirus resistant C3H/RV cells, indicating that WNV DI particles may be formed more rapidly and abundantly in resistant cells and/or that DI particle interference with standard virus replication may be more effective in resistant cells (Darnell and Koprowski, 1974). Our recent data indicates that resistant cells do produce less virus containing full size genome RNA and proportionally more particles containing smaller species of RNA than do susceptible cells (Brinton, 1981; Fig. 1).

Persistent WNV infections have been established in resistant and susceptible cells without the addition of antiviral antibody or interferon (Brinton, 1982). Plaque size and ts mutants have been selected from fluids obtained from these cultures (Brinton, 1983). Persistently-infected cultures that had stopped producing plaquing virus could not subsequently be stimulated to produce detectable amounts of virus by treatment with antibody to interferon, temperature shifts, or cocultivation with BHK cells (Brinton, 1982). However, these cultures were resistant to superinfection with WNV, but not with unrelated viruses. Although these cultures did not produce plaquing virus, they continued to produce non-plaquing virus-specific particles which could be detected in culture fluids by [^3H]uridine or [^{35}S]methionine labeling. These particles contained predominantly RNA species

smaller than the normal 40S genome (Brinton, 1982). That these small RNAs are capable of interfering with the replication of standard WNV was indicated by the observed reduction in infectivity titer as well as in the amount of intracellular 40S RNA synthesized during coinfection of BHK cells with concentrated preparations of non-plaquing particles from persistently infected cells and standard WNV (Brinton, 1982).

That defective interfering virus particles may also be generated in animals has been indicated by serial undiluted passage of WNV in resistant and susceptible adult mouse brains. Brains were harvested when animals showed the first signs of illness. Under conditions where a high dose of standard WNV is administered by the intracerebral route, RV animals do become sick but the time from infection to the onset of disease symptoms is twice as long as that observed in HE mice. After the 4th serial passage in resistant mouse brains, the resulting virus brain pool caused no sickness when injected undiluted into resistant animals. Virus passaged in susceptible mice did not show this change. Virus particles produced by these mouse-passaged pools in cultures of resistant and susceptible cells are currently being analyzed.

2. To further analyze the structure and makeup of the flavivirus genome

Although the mechanism of generation of the subgenomic RNAs is not yet known, several distinct sizes are generated. These have been separated by gel electrophoresis under denaturing conditions (Brinton, 1982). These RNAs were also found to be of plus strand polarity since they hybridized to a single-stranded DNA complementary to the WNV 40S virion RNA (Brinton, 1982).

A non-ts mutant of WNV, designated WNV-RV, has been isolated from culture fluid harvested from a persistently infected culture of RV cells. WNV-RV was found to produce higher yields of infectious virus in all types of cells than the parental WNV used to initiate the persistent infection (Brinton, 1981). When intracellular RNA synthesis was analyzed in WNV-RV-infected resistant cells, the amount of [³H]uridine incorporated into 40S RNA was found to be markedly increased as compared to infections of RV cells with the standard strain of WNV. 40S RNA also represented a larger proportion of the total RNA within a given labeling period in HE EFBs and BHK cells infected with WNV-RV. However, the increase in 40S synthesis was most dramatic in RV cells.

Although 40S RNA was found to be the major species of RNA present in pelleted extracellular virus particles produced by both resistant and susceptible cells infected with WNV-RV, other species of smaller-sized RNAs were also observed to be associated with the pelleted particles (Fig. 1). A similar heterogeneous pattern of RNA species was not observed from particles produced by susceptible cells infected with standard WNV even when virus was not pelleted from culture fluids but viral RNA was analyzed after addition of SDS to unconcentrated culture fluid. Viruses that are resistant to interference by defective interfering RNAs, but that allow the synthesis of these RNAs have been reported in several systems (Adachi and Lazzarini, 1978; Horodyski and Holland, 1980; Jacobson and Pfau, 1980). It is possible that the WNV-RV mutant is such a virus. This hypothesis is supported by our recent observation that the WNV-RV mutant is able to efficiently superinfect WNV-persistently-infected cultures that are resistant to super-infection with standard WNV (Brinton, 1982).

3. To study the synthesis of viral-specific flavivirus RNA in resistant (RV) cells

Analysis of intracellular viral RNA synthesis indicated that the incorporation of [^3H]uridine into 40S WNV genome RNA was markedly reduced in RV cells (Fig. 2). After the latent period this RNA is normally the major intracellular species of virus-specific RNA in flavivirus-infected cells. In contrast, the incorporation of uridine into alphavirus 26S and 42S RNA was equivalent in Sindbis-infected RV and HE cells indicating that there was probably little difference in the availability of labeled uridine in the cytoplasm of the RV and HE cells. In WNV-infected BHK cells and HE-EFBs, distinct peaks of intracellular RNAs were observed with sedimentation coefficients of 20 and 26S. In contrast, RNA patterns from WNV-infected RV-EFBs did not show distinct peaks in these regions indicating that RV cells may synthesize a more heterogeneous population of RNA as well as less 40S RNA. Experiments in which WNV-infected RV and HE cell lysates were mixed before extraction indicated that nuclease activity was not higher in the cytoplasm of RV cells.

The yield of infectious extracellular virus produced as well as the amount of [^3H]uridine incorporated into intracellular virus-specific RNA within a given time period after infection was observed to be increased in both RV and HE cells that had been incubated with antibody to mouse interferon (AIF) for 24 hr prior to WNV infection. However, the effect of the AIF was not sufficient to increase the level of 40S viral RNA synthesis in RV cells to a level equal to that observed in HE mouse cells. Infected HE culture fluids were consistently observed to contain 8 to 10 times higher levels of interferon than RV culture fluids. In addition, no dramatic increase in the brain titer of WNV produced in AIF-treated flavivirus-resistant mice was observed (Brinton et al., 1982). These data suggest that interferon does not play a specific role in bringing about the reduced yields of infectious WNV observed from flavivirus resistant cells. In contrast, interferon has been found to be a necessary component for the expression of resistance to orthomyxoviruses in A2G mice (Haller et al., 1979).

4. To study the mechanism of protein synthesis and to study viral-specific protein synthesis in RV cells

Analysis of intracellular virus-specific proteins in WNV-infected cells revealed that incorporation of [^{35}S]methionine into viral-specific proteins was much more efficient in HE than in RV cells (Fig. 3). The level of incorporation of [^{35}S]methionine into viral proteins was markedly increased in resistant cells infected with the mutant, WNV-RV, as compared to standard WNV-infected resistant cells (Brinton, 1981).

We have also prepared an antisera in rabbits immunized with WNV-infected BHK cells. This antisera reacts with structural and non-structural WNV proteins and is currently being used to immunoprecipitate viral proteins from the two types of mouse cells.

Other Progress: Persistently infected cultures and temperature sensitive mutants

SV-40 transformed lines of resistant and susceptible cells were infected with either uncloned WNV-100 or three times plaque purified WNV-103 at a moi of 10. Susceptible cultures displayed only a partial CPE, while resistant cultures showed no CPE. Infected cultures were subcultured at a ratio of 1:5 96 hr after infection. Subsequently, these cells were subcultured at weekly intervals at a ratio of 1:10. For the first six passages, all cultures were maintained at 37°C. Resistant cultures continued to shed low yields of virus, while virus titers from susceptible cultures were observed to cycle. After passage 6, duplicate cultures were maintained at both 32°C and 37°C. Increased virus yields from cultures shifted to 32°C were observed. Starting at passage 9 plaques were picked from titration plates at every fourth passage and stored frozen. Both large and small plaque variants were picked. Several times persistently infected cell lines went through a period of crisis, but this was not always correlated with an increased virus yield. In each instance, some cells survived and re-established the culture. Aliquots of culture fluids from persistently-infected resistant and susceptible cell cultures maintained at 37°C and 32°C were frozen at each subculture. These were subsequently titered at 32°C. To date, about 450 plaques have been picked from titration plates and tested for plaquing efficiency at 32°C and 40°C. One ts mutant was obtained from the sixth passage of a 32°C susceptible culture and one from the 21st passage of a 32°C resistant culture. Both of these mutants show 40°/32°C ratios of about 3×10^{-6} . By the 29th passage virtually all plaques picked from 32°C resistant cultures showed 40°/32°C ratios of $4-6 \times 10^{-6}$. All the ts mutants isolated so far are RNA minus, but they differ from each other in their ability to make protein at the non-permissive temperature.

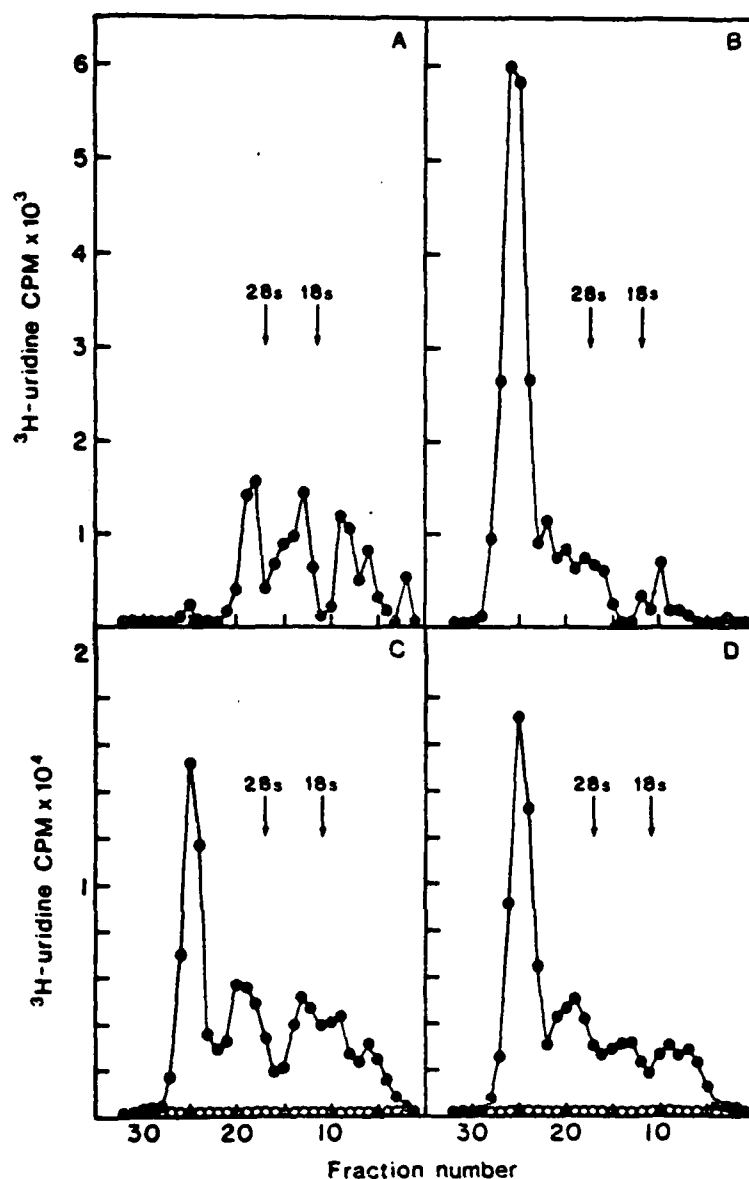


Fig. 1. Rate zonal sedimentation analysis of [^3H]uridine-labeled RNA in culture fluids from WNV-103-infected (A) resistant (RV) and (B) susceptible (HE) embryofibroblast and RE-WNV-infected (C) RV and (D) HE cultures. Cultures were incubated with [^3H]uridine (20 $\mu\text{Ci/ml}$) from 4 to 29 h after infection. Virions in harvested culture fluids were pelleted, extracted and sedimented through 15 to 30% (wt/vol) sucrose gradients. Unlabeled BHD rRNAs were used as markers. Symbols: ●, infected; ○, uninfected control.

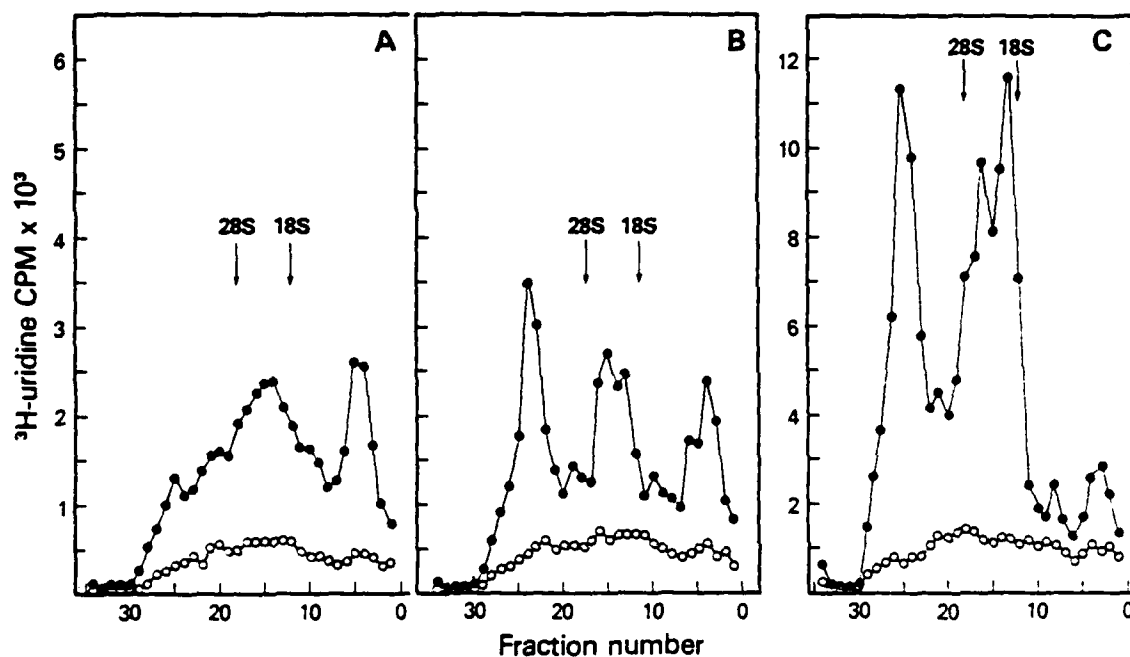


Fig. 2. Intracellular WNV RNA synthesis in (A) resistant (RV) embryofibroblasts, (B) congenic susceptible (HE) embryofibroblasts and (C) BHK 21/WI2 cells. RNA synthesized during 24 and 25.5 h after infection was analyzed by rate-zonal sedimentation in 15 to 30% (wt/vol) sucrose gradients. RNA patterns for WNV-103 infected (●) and uninfected (○) control cultures are shown.

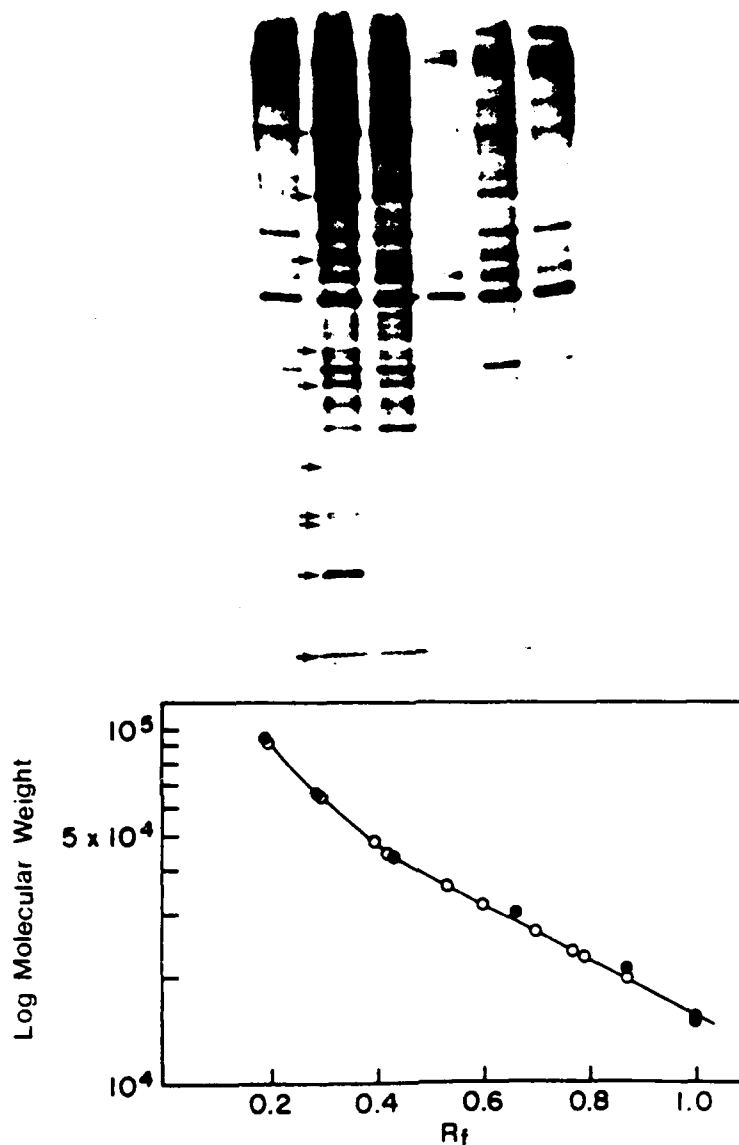


Fig. 3. Viral proteins synthesized in resistant and susceptible cell cultures after infection with WNV-103 or RE-WNV. Cultures were incubated in methionine-free media containing 2 μ g/ml Actinomycin D beginning at 20 h and [35 S]methionine from 24 to 28 h after infection. Cells were lysed by addition of buffer containing 2% SDS and aliquots containing equal amounts of total cell protein were electrophoresed on a 10% polyacrylamide slab gel. Cell extracts applied to the gel tracks were from left to right (1) HE cells uninfected; (2) HE cells infected with RE-WNV; (3) HE cells infected with WNV-103; (4) RV cells uninfected; (5) RV cells infected with RE-WNV; (6) RV cells infected with WNV-103. The autoradiogram exposure time was 7 days.

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